5-2015

Exploring the roles of host and tumor cell α4 and α7 nicotinic acetylcholine receptors in lung cancer.

John Caleb Greenwell
University of Louisville

EXPLORING THE ROLES OF HOST AND TUMOR CELL α4 AND α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN LUNG CANCER

By

John Caleb Greenwell
B.S., University of Louisville, 2010

A Thesis
Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the Requirements
for the Degree of

Master of Science
in
Pharmacology and Toxicology

Department of Pharmacology and Toxicology
University of Louisville
Louisville, Kentucky

May 2015

EXPLORING THE ROLES OF HOST AND TUMOR CELL α4 AND α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN LUNG CANCER

By

John Caleb Greenwell
B.S., University of Louisville, 2010

A Thesis Approved on

1/30/2015

by the following Thesis Committee:

Jesse Roman, M.D. – Thesis Director

Levi Beverly, Ph.D.

Shirish Barve, Ph.D.

Jun Yan, M.D., Ph.D.

Ramesh Gupta, Ph.D.

ACKNOWLEDGEMENTS

I am indebted to a number of individuals for investing their lives in mine. I owe more than I can give to my beloved wife and sweet children. My wife, Tricia, has strengthened, encouraged, and sharpened me as I have worked on this project. I could not have done it without her. I thank my mother for her sacrifice and patience every day in the classroom. I thank my father for modeling for me and instilling in me what it truly means to be a hard working and disciplined man. I thank my mentor, Dr. Jesse Roman, for allowing me to be a part of his lab. I consider it an honor to have worked with such a great researcher, whose passion for science inspires and motivates everyone around him. I would also like to thank those who were by my side daily, Jeff Ritzenthaler, Edilson Torres-González, and Glenn Vicary. They helped me tremendously in the lab and made every tough day bearable. Lastly, I would like to thank my committee members Dr. Shirish Barve, Dr. Ramesh Gupta, Dr. Levi Beverly, and Dr. Jun Yan, for their comments and guidance.

ABSTRACT

EXPLORING THE ROLES OF HOST AND TUMOR CELL α4 AND α7 NICOTINIC ACETYLCHOLINE RECEPTORS IN LUNG CANCER

John Caleb Greenwell

1/30/2015

Lung cancer is the leading cause of cancer death in men and women worldwide. Tobacco exposure represents the major risk factor. Nicotine, an addictive plant alkaloid found in tobacco, has been demonstrated to stimulate lung carcinoma cells directly via nicotinic acetylcholine receptors (nAChRs) that trigger downstream signals capable of promoting lung cancer growth and progression. Attention has been given to α7 nAChRs, while less is known about α4 nAChRs. However, most studies evaluating these receptors relied on chemical inhibitors notorious for their off-target effects. Consequently, the true role of α4 and α7 nAChRs in lung cancer remains unclear. To address this, we performed *in vitro* and *in vivo* studies using Lewis Lung Carcinoma (LLC) cells silenced with shRNA for α4 or α7 nAChRs. As expected, nicotine stimulated the proliferation of LLC cells *in vitro*. However, tumor cells treated with specific inhibitors of α4 or α7 nAChRs independently, did not inhibit nicotine-induced proliferation; inhibition of proliferation required that receptors be targeted concomitantly with a broad-spectrum inhibitor. Similar observations were made

when the receptors were silenced separately with shRNA; however cells showed increased proliferation at baseline when silenced for nAChRs. In LLC cells silenced for α4 nAChRs we observed fewer colonies on soft agar, decreased migration, and decreased apoptosis in response to cisplatin, when compared to untransfected cancer cells and cells transfected with control shRNA. Cells silenced for α7 nAChRs did not differ from untransfected cancer cells or cells transfected with control shRNA, with respect to colony formation, migration, and apoptosis. In a lung cancer xenograft model, silencing of α4 and α7 nAChRs in cancer cells resulted in no significant differences in tumor size, and did not alter overall survival. While exploring the role of host cell receptors, no differences were observed in tumor number or size in a spontaneous tumor formation model in animals carrying *KRAS* and α7 nAChR mutations. In contrast, larger tumors were observed in α7 nAChR knockout mice injected with wildtype LLCs. These studies suggest differential roles for α4 and α7 nAChRs in murine lung carcinoma cells, with α4 nAChRs having a predominant role *in vitro*. However, studies performed in animals suggest that targeting these receptors independently in tumor cells may not affect tumor progression *in vivo*, while targeting host receptors may.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Lung cancer is the leading cause of cancer death in men and women in the United States[1]. In 2015 alone, lung cancer is estimated to take the lives of over 158,000 individuals, which represents 27% of all cancer deaths[2]. Lung cancer has a dismal 17% five-year survival rate that has not changed substantially over the past 35 years, despite the development of new surgical procedures, and the use of new radio- and chemotherapeutic protocols[3]. This underscores a desperate need for new strategies in prevention, early detection, and treatment of this deadly disease. Tobacco use represents the major risk factor and is responsible for 71% of global lung cancer deaths[4]. Tobacco smoke is extremely complex, consisting of thousands of compounds, and over 60 carcinogens[5]. Several carcinogens, in particular, thought to be responsible for lung cancer development and progression include the polycyclic aromatic hydrocarbons and the nicotine-derived nitrosamines, N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), among others. The metabolites of these compounds cause mutations in vital genes such as *Rb*, *p53*, and *KRAS*[6]. However, it was recently shown that expression of oncogenic KRAS and knockdown of the tumor suppressor protein p53 were insufficient to confer a full malignant phenotype in bronchial epithelial cells, suggesting that the role these genes and their products play in lung cancer development may have been

overemphasized[7]. This, along with emerging data suggesting an important role of the microenvironment in the etiology of many cancers, leads us to believe that other factors are involved.

We have focused our attention on nicotine, a major plant alkaloid in tobacco. Historically, nicotine was thought to only mediate smoking dependence and addiction by working through reward centers in the brain to cause a release of dopamine and a general sense of euphoria[8]. More recent data, however, reveal that, even though nicotine may be unable to initiate oncogenic transformation, it promotes cancer cell proliferation *in vitro* and may stimulate tumor progression[9–11]. Nicotine mediates its effects through nicotinic acetylcholine receptors (nAChRs) by mimicking acetylcholine, the endogenous ligand for these receptors[12]. NAChRs comprise a family of multimeric cation channel proteins that act as central regulators of a vital network of excitatory and inhibitory neurotransmitters that governs the function of all organs and cells in the mammalian organism. They are located in the plasma membrane and can exist as homo- or heteropentamers. Previously thought to exist only in the nervous system and neuromuscular junctions, they are now recognized as being universally expressed in mammalian cells, and in many cancers[13]. Upon ligand binding in the alpha subunit, the receptors undergo conformational changes that open the channel, allowing cations to rush down their concentration gradient, resulting in depolarization of the plasma membrane and activation of intracellular signal transduction pathways[14].

The link between nAChRs and lung cancer was recently strengthened by studies identifying a common variant in the nAChR gene cluster on chromosome 15q25 with a predisposition to lung cancer[15]. This locus was found to account for 14% (attributable risk) of lung cancer cases. In tumor cells, activation of nAChR signaling stimulates cell proliferation, tumor progression, and metastasis through induction of the synthesis and release of growth, angiogenic, apoptotic, and metastatic factors[11]. However, both tumor cells and host cells of the tumor microenvironment express several of these receptors and the true contribution of distinct nAChRs in lung cancer remains unclear. Furthermore, studies evaluating these receptors have relied on chemical inhibitors, which are notorious for their off-target effects. Thus, the true role of distinct nAChRs in lung cancer remains incompletely understood.

Here, we evaluate the relative contribution of α7 and α4 nAChRs in lung cancer using Lewis Lung Carcinoma cells silenced for these receptors using shRNA technology. Our data suggest that although these receptors play distinct roles in several biological processes in tumor cells tested *in vitro*, with a predominant role for α4 nAChRs, targeting them individually did not lead to significant beneficial effects for the host in a rodent xenograft model. However, in experiments performed in genetically engineered animals to test the role of host cell α7 nAChRs, we observed larger tumor growth compared to wildtype animals. These studies underscore the need to carefully define the relative contributions of these receptors, in both host and tumor cells, prior to the development of interventions for treatment in humans.

MATERIALS AND METHODS

Reagents and Cell Culture

Lewis Lung Carcinoma (LLC) cells were purchased from ATCC (CRL-1642; ATCC, Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceburg, GA), 50 IU/ml penicillin/ streptomycin, and 1 mg amphotericin (Corning Cellgro) at 37 ºC in a humidified 5% CO2 incubator. The mouse nAChR α4 or α7 subunit or control nontarget shRNA plasmid DNA constructs were purchased from Sigma (St. Louis, MO). Polyclonal antibodies specific for α4 nAChR (SAB2100424) and α7 nAChR (AV13018) were purchased from Sigma Aldrich (St. Louis, MO). Chemical antagonists were purchased from Tocris Bioscience (Ellisville, MO). The Cell Titer-Glo® Luminescent Cell Viability Assay and Caspase-Glo® 3/7 Assay were obtained from Promega (Madison, WI). Cell transformation detection assay (colony formation) kit was obtained from Millipore (Temecula, CA).

Silencing of α4 and α7 nAChRs with shRNA

LLC cells ($2.3x10^7$ cells/mL) were permanently transfected with 160 µg of control, α4 or α7 shRNA plasmid DNA as previously described[16]. Briefly, cells were harvested by trypsinization, washed, and resuspended in buffer containing

20 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 6 mM dextrose at pH 7.05. Afterwards, the cells were added to a 0.4-cm-gap cuvette containing the shRNA plasmid DNA, and transfected with a Gene Pulser II electroporation apparatus set at 390v and 500 mF (Bio-Rad, Hercules, CA). Cells were then plated onto 75-mm^2 tissue culture flasks. ShRNA-expressing cells were selected by the addition of 5 µg/ml puromycin antibiotic for a minimum of 2 weeks. To obtain individual clones, cells were serially diluted into 96-well tissue culture plates. Single colonies were then tested for nAChR levels by Western Blot analysis.

Western Blot

Western blot analysis was performed on wildtype (WT) LLC cells and cells transfected with control shRNA or shRNAs to α4 and α7 nAChRs as previously described[17]. Protein (40-50 µg) was heated at 90 ºC for 5 minutes and briefly centrifuged. Proteins were transferred onto nitrocellulose membranes using a BioRad Trans-Blot® SD Semi-Dry Transfer Cell machine for 2 hours at 25V. Blots were incubated overnight in 15mL conicals in 5mL 5% non-fat dry milk in TBST at 4ºC with anti-α4 and/or anti-α7 nAChR (1:500) antibody, washed 3 times for 10 minutes in TBST, and incubated with a secondary anti-rabbit IgG HRP (1:20,000) for 1 hour at RT. Blots were again washed (3 x 10 minutes), transferred to Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) for 1 minute and exposed to Genemate Blue

Basic Autorad film (Bioexpress, Kaysville, UT) for up to 1 hour. Protein densitometry was completed using GS-800 Calibrated Densitometer (Bio-rad).

Proliferation Assay

WT LLC cells and cells transfected with control shRNA or shRNAs targeting α4 or α7 nAChRs (500 cells/well) were plated in white-walled, clear-bottom 96-well plates and cultured with or without nicotine for 5 days in media containing DMEM with 2% FBS and 25mM HEPES buffer. Media was replaced every 2 days. Cell proliferation was evaluated using the Promega CellTiter-Glo® Luminescent Cell Viability Assay (Madison, WI), in a Luminoskan Ascent Luminometer (Beckman Coulter), according to the manufacturer's instructions. The CellTiter-Glo® Luminescent Cell Viability Assay is determines the number of viable cells in culture based on the quantitation of the ATP present, which signals the presence of metabolically active cells.

Migration Assay

Cells were grown to ~70% confluence in six-well plates in culture media containing DMEM with 10% FBS. A sterile 1000-µl pipette tip was used to create a scratch in 3 separate locations within each well. Media was aspirated and pictures taken at 0 hours to measure the width of the unveiled area. Afterwards, the media (5 mL) was replaced and cells were allowed to recover for 48 hours, after which pictures were taken again to measure the covered area.

Colony Formation Assay

A 0.8% base agar layer was prepared and added to an equal amount of culture media (250 µL/12-/well). Plates were placed at 4ºC for at least 30 minutes to allow base agar layer to gel. Top agar (0.4%) layer was prepared by mixing 0.8% solution with equal amount of culture media (DMEM). Plates were incubated at 37ºC for 5 minutes prior to addition of cells (500/well) followed by incubation for 21 days. Fresh media (500 µl/well) was added every 3-4 days, after aspiration of old media. Afterwards, colonies were photographed and quantified using a Beckman plate reader and cell quantification solution (OD 490 nM).

Apoptosis Assay

WT LLC cells and control and α4 and α7 nAChR shRNA-transfected LLC cells ($3x10^3$) were plated in white-walled, clear-bottom 96-well plates and cultured in DMEM with 10% FBS with or without 20 µM cisplatin for 24 hours. Apoptosis was then evaluated using the Promega Caspase-Glo® 3/7 Assay (Madison, WI), in a Luminoskan Ascent Luminometer (Beckman Coulter), according to the manufacturer's instructions. This assay works by providing a luminogenic substrate (Z-DEVD- aminoluciferin) for caspases 3 and 7, that upon cleavage, releases aminoluciferin, which then becomes a substrate for luciferase.

Animal Studies

All mice used in this study were obtained from Jackson Laboratories. The institutional animal care and use committee of the University of Louisville approved all experiments. LLC cells were plated out 24 hours prior to injections and were harvested at ~50% confluence to insure cells were in adequate growth phase. LLC cells (5×10^5 /100 µl sterile PBS) stably transfected with control non-target shRNA, α4 shRNA, or α7 shRNA were injected subcutaneously into the hind flank of wildtype C57BL/6 mice. Afterwards, tumors were monitored and measured weekly. A tumor size >15mm in any direction was considered the endpoint, according to IACUC regulations. Animals were sacrificed and tissues were harvested for analysis. For experiments using α7 nAChR KO mice, 5×10^5 WT LLC cells/100 µl sterile PBS were injected subcutaneously into the hind flank and followed as described above. Animals lacking α7 nAChR and expressing heterozygous mutations for *KRAS* were developed by breeding mice heterozygous for α7 nAChR with mice heterozygous for *KRAS*. The double heterozygous progeny were then bred for four generations to develop animals lacking α7 nAChR and expressing heterozygous mutations for *KRAS*; mutations were confirmed via PCR. These animals developed spontaneous tumors and were sacrificed at 90 days at which time the lungs were harvested for analysis.

Histological Analysis

Animals were euthanized by exposure to carbon dioxide in a closed chamber. Lungs were isolated and inflated at standard pressure, formalin-fixed,

paraffin-embedded, and sectioned (6 μm) using a JUNG RM2055 microtome (Leica, Buffalo Groce, IL). They were then transferred onto glass microslides for histological analysis. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E) to evaluate lung tumors.

Analysis of Data

Means plus standard deviations of the mean were calculated for all experimental values. Significance was assessed by using the Student's *t* test. All experiments were repeated a minimum of 3 times with each sample group containing a minimum number of 3. For survival distributions, the Log-rank test and/or Wilcoxon test were used to determine significance.

RESULTS

Nicotine stimulates LLC cell proliferation, but its mitogenic effects are not affected by separate chemical inhibition of α7 or α4 nAChRs

Nicotine has been shown to stimulate lung carcinoma cell proliferation *in vitro*[18]. To begin to identify key receptors mediating these effects, we exposed WT LLC cells to nicotine and tested the effects of nAChR inhibitors. As presented in **Figure 1A-C,** nicotine (50μg/mL) stimulated LLC cell proliferation. The mitogenic effects of nicotine were decreased by a broad, non-specific nAChR inhibitor, mecamylamine (**Figure 1A**). However, dihydro-β-erythroidine (DhβE), a more specific inhibitor of α4 nAChRs had little effect (**Figure 1B**). MG624, a more specific inhibitor of α7 nAChRs, also had no affect (**Figure 1C**). Interestingly, we observed mild increases in proliferation in cells treated with MG624 alone, suggesting a role for α7 nAChR in control of proliferation at baseline in unstimulated cells.

LLC cells silenced for α4 nAChR show decreased migration, colony formation, and apoptosis, while cells silenced for α7 nAChR do not

We transfected WT LLC cells with control shRNA (LLC/Con^{sh}) or shRNA against α4 nAChR (clones LLC/α4^{CLA} and LLC/α4^{CLB}) or α7 nAChR (clones

LLC/$\alpha7^{CLC}$ and LLC/$\alpha7^{CLD}$). These clones were found to have less than ~75% protein expression for $\alpha4$ and $\alpha7$ nAChRs, and these were used for further studies (**Figure 2A**-**2C**, respectively). To ensure that the transfection procedure by itself did not affect responsiveness to nicotine, LLC/Con^{sh} cells were exposed to nicotine and treated with DhβE and MG624. Again, nicotine stimulated the proliferation of cells transfected with control shRNA cells and, as before, DhβE and MG624 did not affect the response dramatically (not shown).

We then examined the effect of $\alpha4$ and $\alpha7$ nAChR silencing on nicotine-induced LLC proliferation. As presented in **Figure 3**, proliferation was again significantly increased in nicotine-treated LLC/UT (untransfected) and LLC/Con^{sh} cells. Consistent with the findings related to the chemical inhibitors, silencing of $\alpha4$ or $\alpha7$ nAChRs did not prevent-nicotine-induced proliferation. However, lack of $\alpha4$ and $\alpha7$ nAChRs resulted in mild increases in proliferation of unstimulated cells.

We next examined the role of $\alpha4$ and $\alpha7$ nAChRs in cellular migration. Cells were grown to ~80% confluence, after which time, a wound was created using a sterile 1 mL pipette tip. After 48 hours, only ~30% of the initial wound area remained in LLC/UT and LLC/Con^{sh} cells (**Figure 4A** and **4B**). In contrast, after 48 hours, ~85% and ~75% of the initial wound area remained in the LLC/$\alpha4^{CLA}$ and LLC/$\alpha4^{CLB}$ cells, respectively; LLC/$\alpha7^{CLC}$ and LLC/$\alpha7^{CLD}$ cells showed no difference compared to controls.

Figure 1

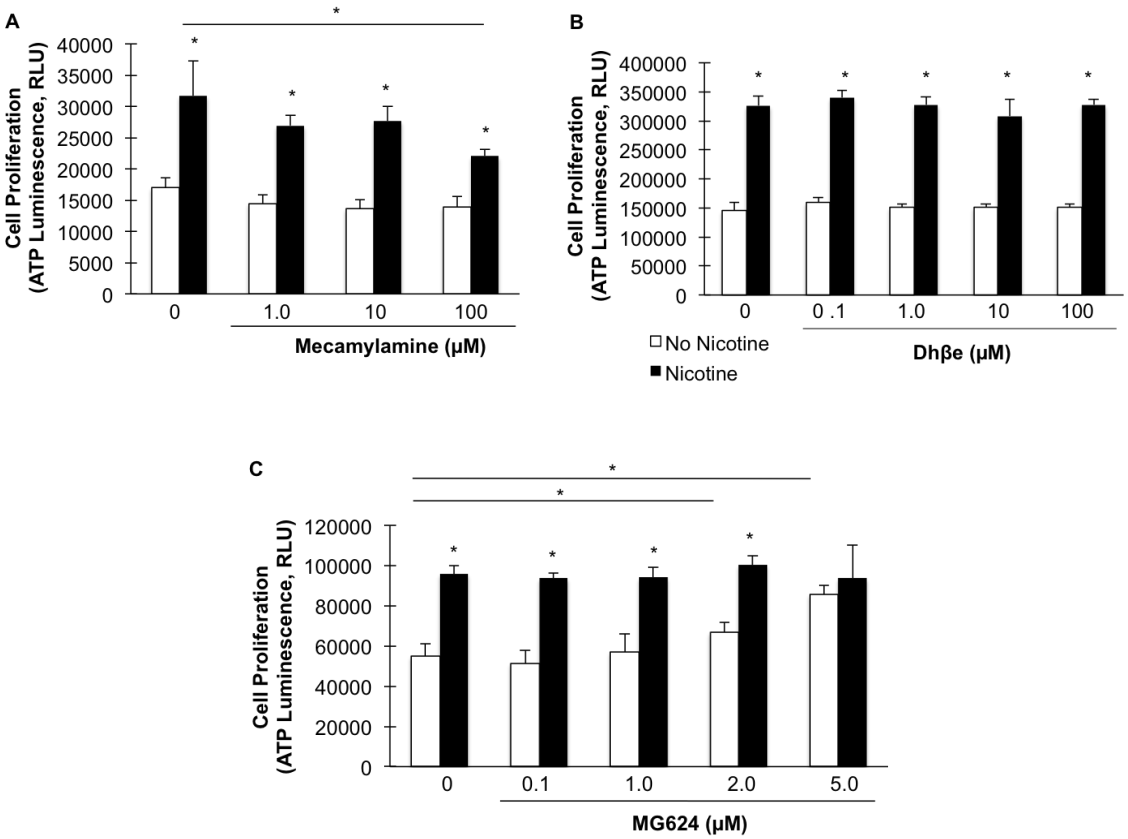

Figure 1. Role of nAChRs in nicotine-induced lung cancer cell proliferation
(A) WT LLC cells were cultured with increasing concentrations of mecamylamine in the presence or absence of nicotine (50 μg/mL) for 5 days. Fresh media containing nicotine was added every 2 days. The number of viable cells was detected using Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega) Mecamylamine significantly decreased nicotine-induced proliferation at 100 μM. **(B)** WT LLC cells were cultured with increasing concentrations of dhβe in the presence or absence of nicotine (50 μg/mL) for 5 days and treated and processed as described above. Note that dhβe had no effect on nicotine-induced proliferation. **(C)** WT LLC cells were cultured with increasing concentrations of MG624 in the presence or absence of nicotine (50 μg/mL) for 5 days and treated and processed as described above. MG624 mildly increased baseline proliferation in a dose-dependent manner in untreated cells, and had no effect on nicotine-induced proliferation. All data are depicted as means +/- SD. Asterisks indicate a significant difference from untreated control.

Figure 2

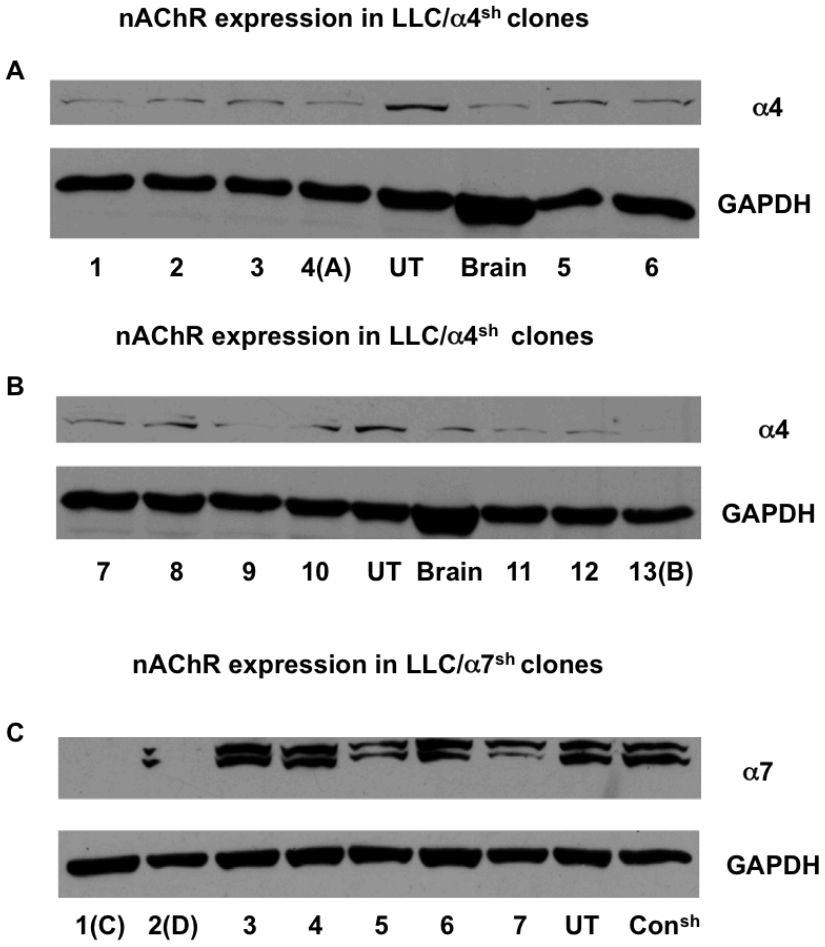

Figure 2. Characterization of LLC cells silenced for nAChRs
After transfection with shRNA targeting nAChRs, LLC cells were grown to ~70-80% confluency and were harvested via cell scraping in PBS. No trypsin was used. Total protein (40 µg) was isolated from LLC/UT, LLC/Con^{sh}, LLC/α4^{CLA}, LLC/α4^{CLB}, LLC/α7^{CLC}, or LLC/α7^{CLD} cells and Western Blot analysis for α4 **(A,B)** and α7 **(C)** was performed. Anti-GAPDH antibody was used to control for gel loading. UT = untransfected cells. Brain = positive control. Clones labeled A and B were chosen for α4 knockdown, whiles clones labeled C and D were chosen for α7 knockdown.

Figure 3

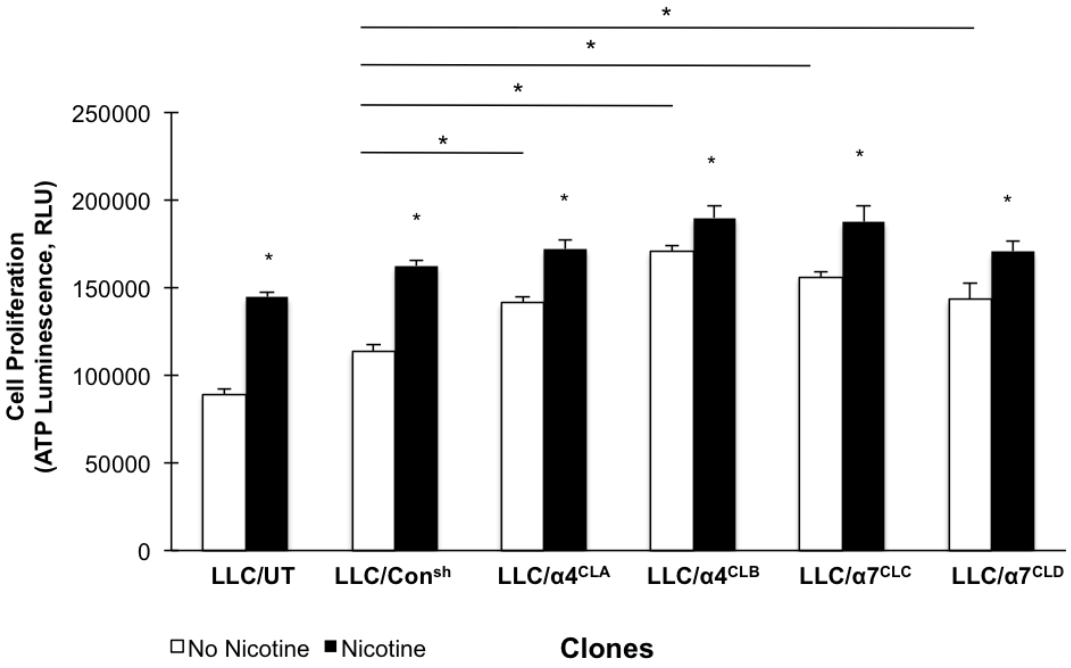

Figure 3. Silencing of α4 and α7 nAChRs does not inhibit nicotine-induced proliferation

Cells were cultured with or without nicotine (50 μg/mL) for 5 days. Fresh media containing nicotine was added every 2 days. The number of viable cells was detected using Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega). Nicotine significantly increased cellular proliferation in LLC/UT, LLC/Con^{sh}, LLC/α4^{CLA}, LLC/α4^{CLB}, LLC/α7^{CLC}, and LLC/α7^{CLD} cells. At baseline, cells silenced for α4 and α7 showed increased cell proliferation, but nicotine-induced proliferation was unchanged.

Figure 4

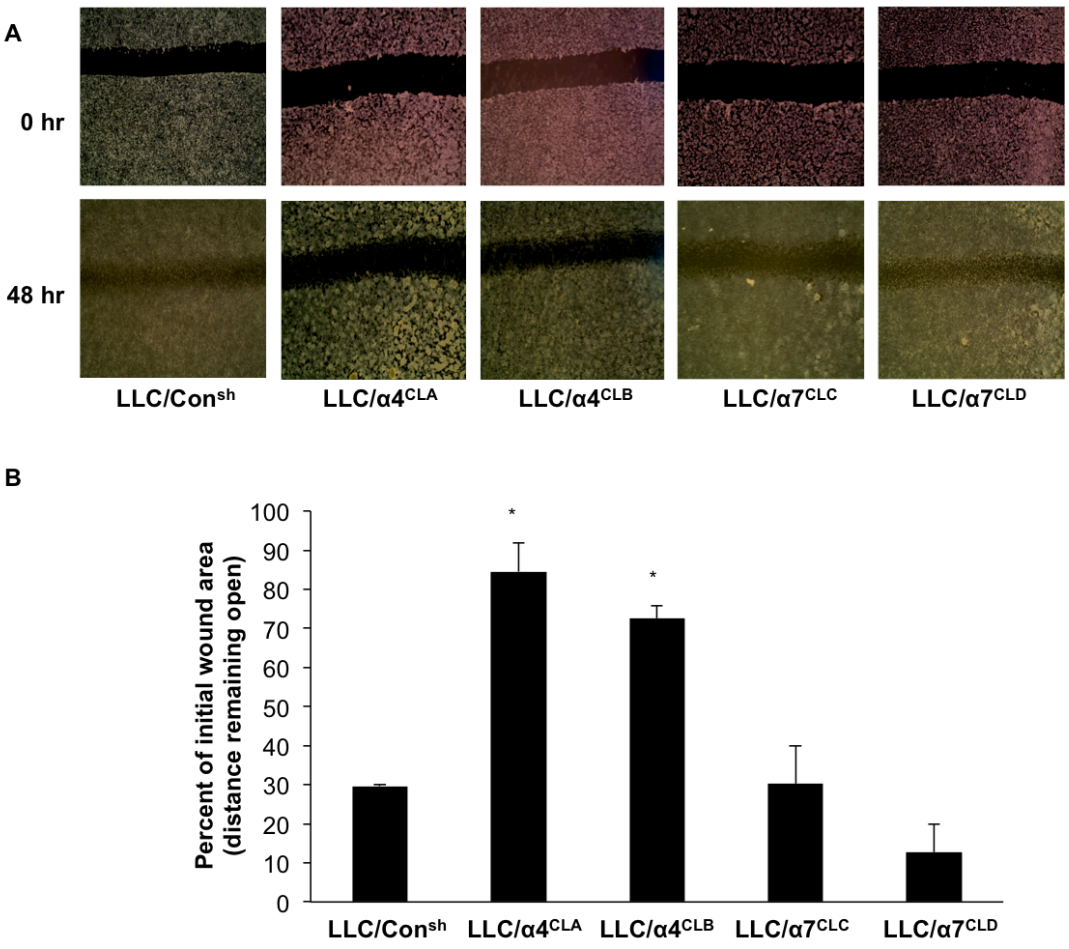

Figure 4. Migration is decreased in LLC cells silenced for α4 nAChR, but not in cells silenced for α7 nAChR

(A) Control and nAChR-silenced LLC cells were grown to ~80% confluency in six-well plates. Afterwards, a sterile 1000 µL pipette tip was used to create a scratch in three separate locations. Media was then aspirated to remove floating cells. DMEM (8mL) containing 10% FBS was then added and the cells were allowed to recover for 48 hours. Wound sites were photographed at 0 hours and 48 hours. **(B)** Quantification of wound length in photographs was performed by measuring the distance of the initial wound area in 3 separate locations at 48 hours, which was subtracted from the initial distance at time 0 hours.

Next, we tested the effects of α4 nAChRs on colony formation on soft agar. As depicted in **Figure 5A**, nicotine significantly increased the number of colonies in LLC/UT and transfected cells. At baseline, overall colony formation was reduced in cells silenced for the α4 nAChR, but the cells remained responsive to nicotine. In contrast, cells silenced for α7 showed no difference in colony number when compared to controls (**Figure 5B**).

Lastly, we examined the role of α4 and α7 nAChRs in drug-induced apoptosis. As depicted in **Figure 6**, silencing of α4 nAChR significantly reduced the amount of cisplatin-induced apoptosis when compared to LLC/UT cells, as determined by caspase 3/7 activity (**Figure 6A**) and ATP (**Figure 6B**) levels, with a greater effect noticed in LLC/$\alpha4^{CLA}$ cells. Note that α4 nAChR silencing did not affect the number of viable cells. Silencing of α7 nAChRs had no effect.

Mice injected subcutaneously with cancer cells silenced for α4 or α7 nAChR do not display changes in survival compared to controls

Having examined the properties of the α4 and α7 nAChR-silenced clones *in vitro,* we proceeded to test their role *in vivo*. To this end, C57BL/6 mice were injected subcutaneously with LLC/Con^{sh} cells, LLC/ $\alpha4^{sh}$ (clones A and B) cells, or LLC/ $\alpha7^{sh}$ (clones C and D) cells. Tumor formation and size were followed for up to 12 weeks. 73% of mice injected with LLC/Con^{sh} cells developed tumors over the course of the experiment. In comparison, only 33.3% and 66.7% of the mice injected with LLC/ $\alpha4^{sh}$ cells (clones A and B, respectively) developed tumors, but this difference was not statistically significant. There was also no

Figure 5

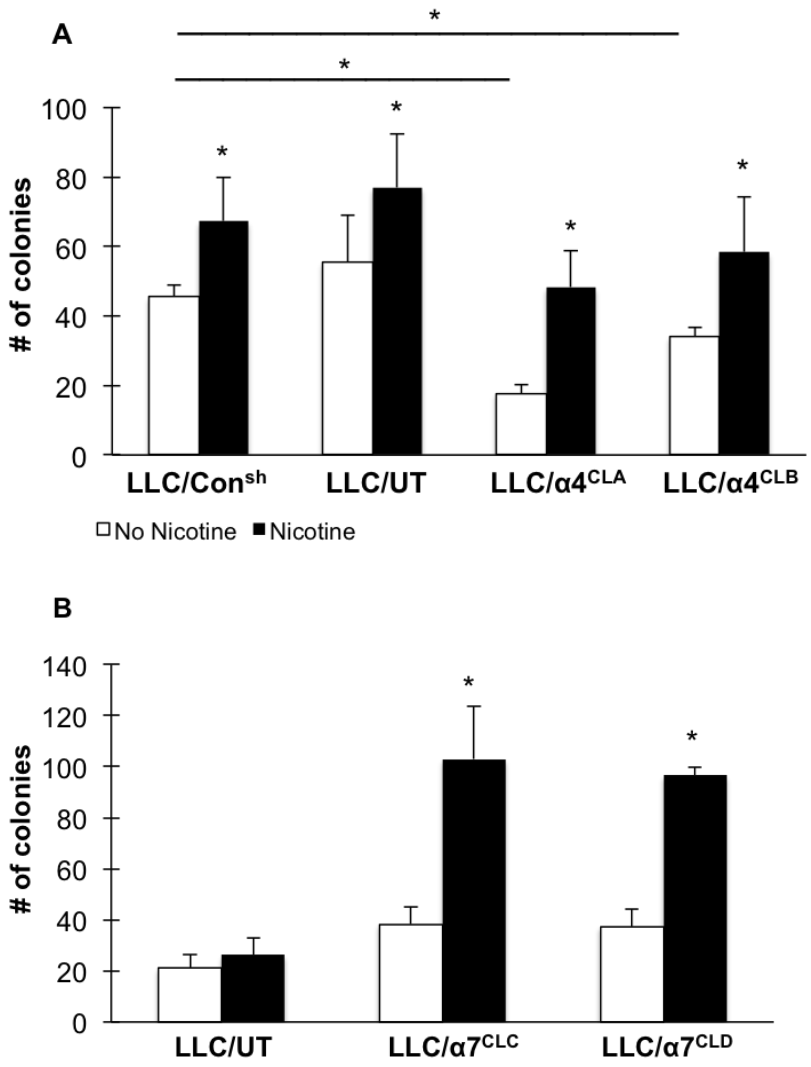

Figure 5. Colony formation is decreased in LLC cells silenced for α4 nAChR, but not in cells silenced for α7 nAChR

Control and nAChR-silenced LLC cells (500) were suspended in 0.4% top agar layer and plated on a 0.8% base agar layer, which was prepared according to Millipore's instructions. After 12 hours, 500 µL DMEM was added. Media was replaced every 3-4 days. Colonies were followed for 21 days and then counted. **(A)** Colony formation was decreased in LLC/$\alpha4^{CLA}$ and LLC/$\alpha4^{CLB}$ cells compared to LLC/Con^{sh} and LLC/UT cells, but nicotine stimulation was observed. **(B)** There was no difference in colony number in LLC/$\alpha7^{CLC}$ and LLC/$\alpha7^{CLD}$ cells when compared to controls at baseline, but nicotine stimulation was observed.

Figure 6

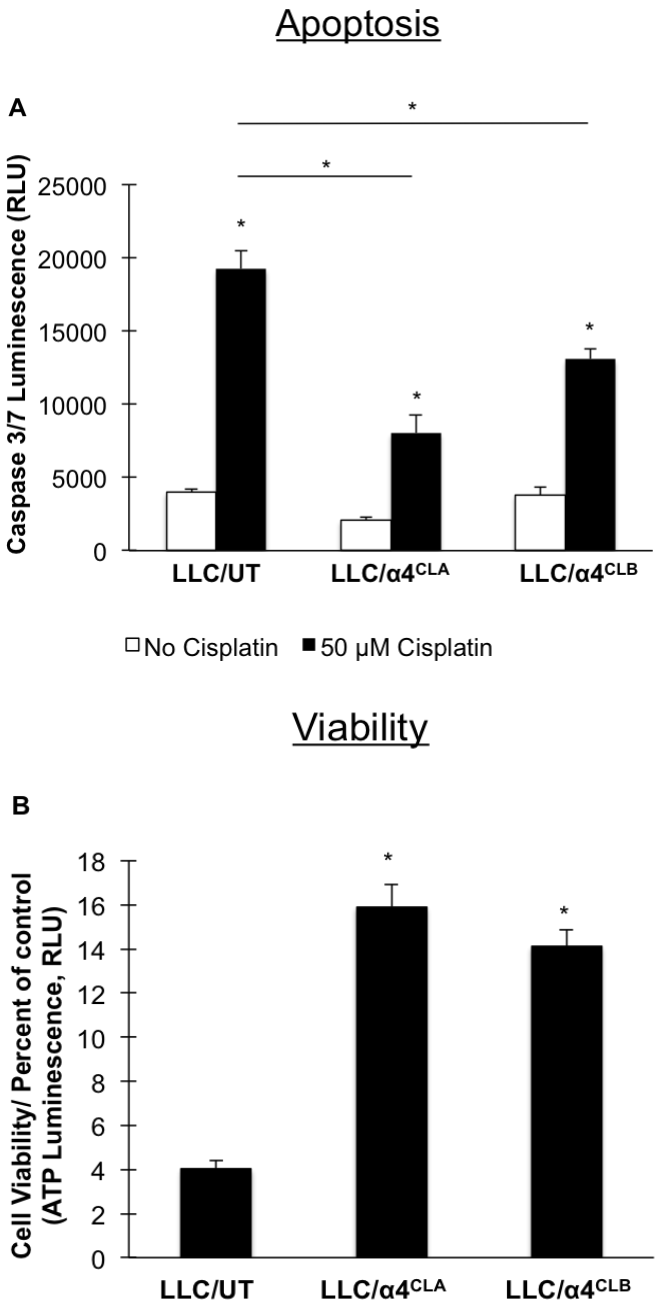

Figure 6. Cisplatin-Induced death is decreased in LLC Cells silenced for α4 nAChR

Cells were cultured in complete DMEM containing 50 μM Cisplatin for 24 hours. Caspase 3/7 activity was detected using the Caspase-Glo 3/7 Assay Kit (Promega). Caspase 3/7 activity was decreased in LLC/$\alpha4^{CLA}$ and LLC/$\alpha4^{CLB}$ cells compared to LLC/Con^{sh} cells **(A)**. The number of viable cells was detected using Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega) **(B)**. Cell viability was increased ~4 fold in LLC/$\alpha4^{CLA}$ and LLC/$\alpha4^{CLB}$ cells compared to LLC/UT cells in cisplatin-treated cells.

difference in tumor size or survival amongst the experimental groups **(Figure 7A,B).** Animals injected with α7 nAChR-silenced clones, also showed no difference in tumor number or size, and did not display changes in survival when compared to those injected with control clones **(Figure 7C,D)**.

Role of host cell α7 nAChRs in lung cancer

To begin to investigate the role of host cell nAChRs, we injected WT LLC cells into WT C57BL/6 and α7 nAChR knockout mice. As depicted in **Figure 8A,** animals deficient in α7 nAChRs grew tumors faster compared to wildtype animals, and thus displayed decreased survival between 20 and 30 days (**8B)**.

By their very nature, xenograft models of lung cancer allow for the study of tumor growth and metastasis, but not tumor development. To test this, we chose to study animals with *KRAS* mutations, which spontaneously develop tumors. *KRAS* mutations are relatively frequent in non-small cell lung cancer in humans[19]. Animals with both *KRAS* mutations and α7 nAChR mutations were sacrificed at 90 days to examine spontaneous tumor development. As presented in **Figure 9**, no differences were noted between *KRAS* and *KRAS*/α7 nAChR knockout animals with respect to tumor number or size in the lungs.

Figure 7

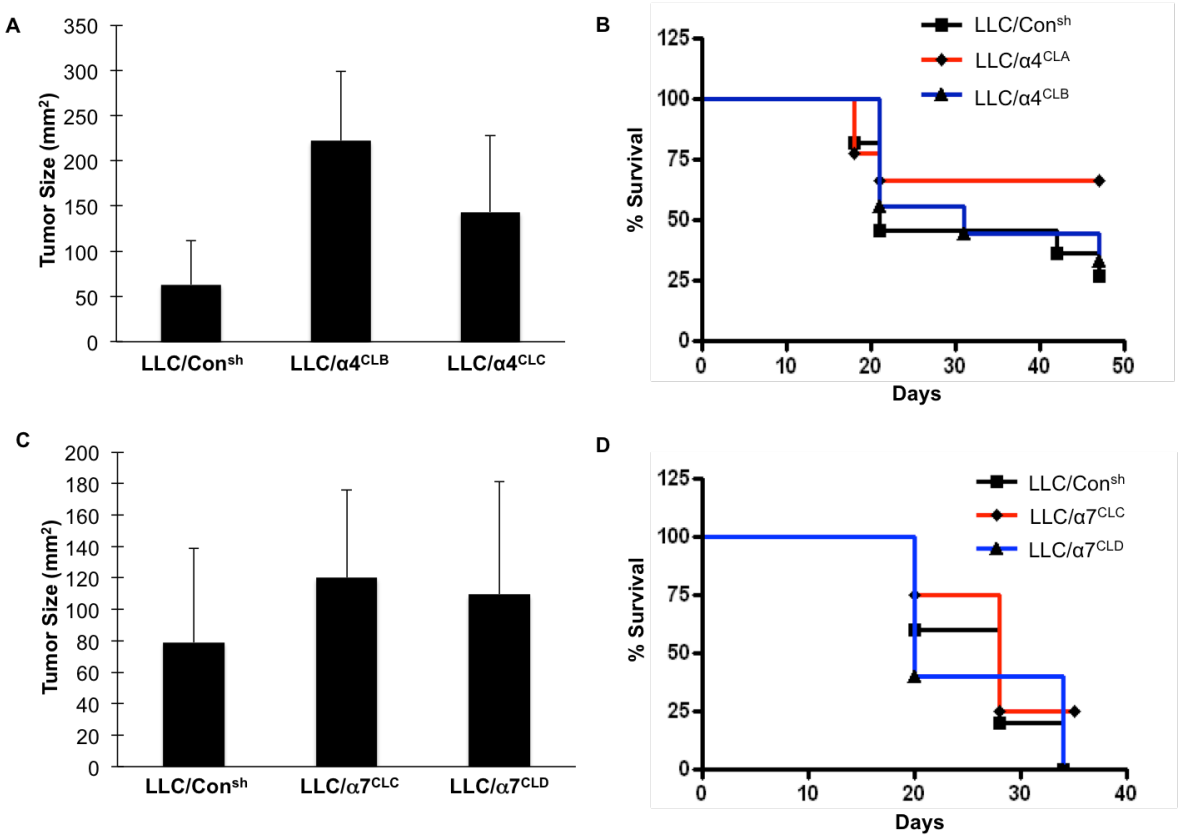

Figure 7. Mice injected with LLC cells silenced for α4 nAChR demonstrate no differences in tumor size compared to control, and do not show differences in survival

(A,B) LLC/Con^{sh} (n=11), LLC/α4^{CLA} (n=9), LLC/α4^{CLB} (n=9) cells (5x10^5) were injected into the hind flank of WT C57BL/6 mice. Tumor formation and size were followed up to 12 weeks. A tumor size of ≥15mm in length or width was established as the endpoint according to IACUC regulations. Mice were then sacrificed and lungs harvested and processed for examination of metastases. No differences were noted amongst groups when evaluating tumor size **(A)** or survival **(B)**. **(C,D)** LLC/Con^{sh} (n=5), LLC/α7^{CLC} (n=5) and LLC/α7^{CLD} (n=4) (5x10^5) cells were injected into the hind flank of WT C57BL/6 mice Tumor formation and size were followed up to 12 weeks. A tumor size of ≥15mm in length or width was established as the endpoint according to IACUC regulations. Mice were then sacrificed and lungs harvested and processed for examination of metastases. No differences were noted amongst groups when evaluating tumor size **(C)** and survival **(D)**.

Figure 8

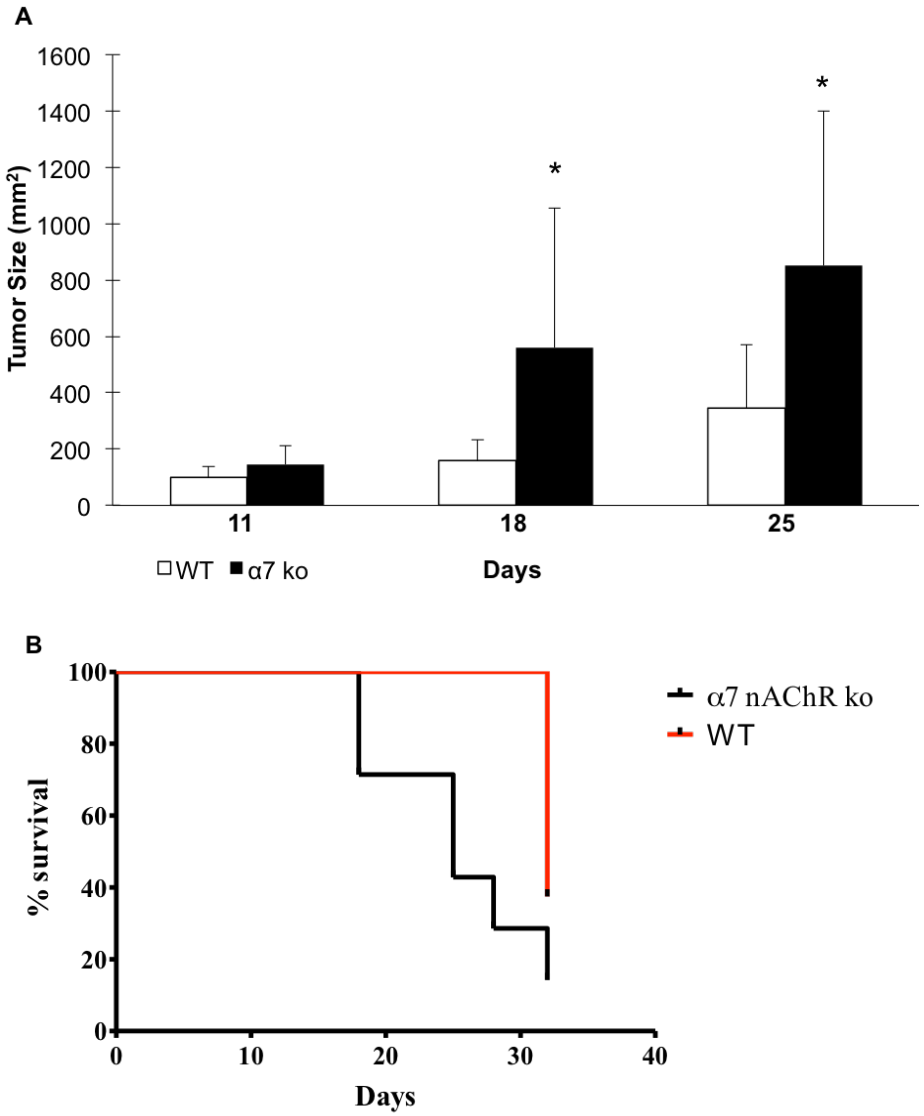

Figure 8. Tumor growth is increased α7 nAChR deficient animals
LLC/WT cells ($1x10^6$) were injected into the hind flank of WT C57BL/6 mice (n=8) or α7 nAChR knockout mice (n=7). Tumor formation and size were followed up to 6 weeks. A tumor size of ≥15mm in length or width was established as the endpoint according to IACUC regulations. Mice were then sacrificed and lungs harvested and processed for examination of metastases. Larger tumors were observed in α7 nAChR knockout mice **(A)**, which led to decreased survival **(B).**

Figure 9

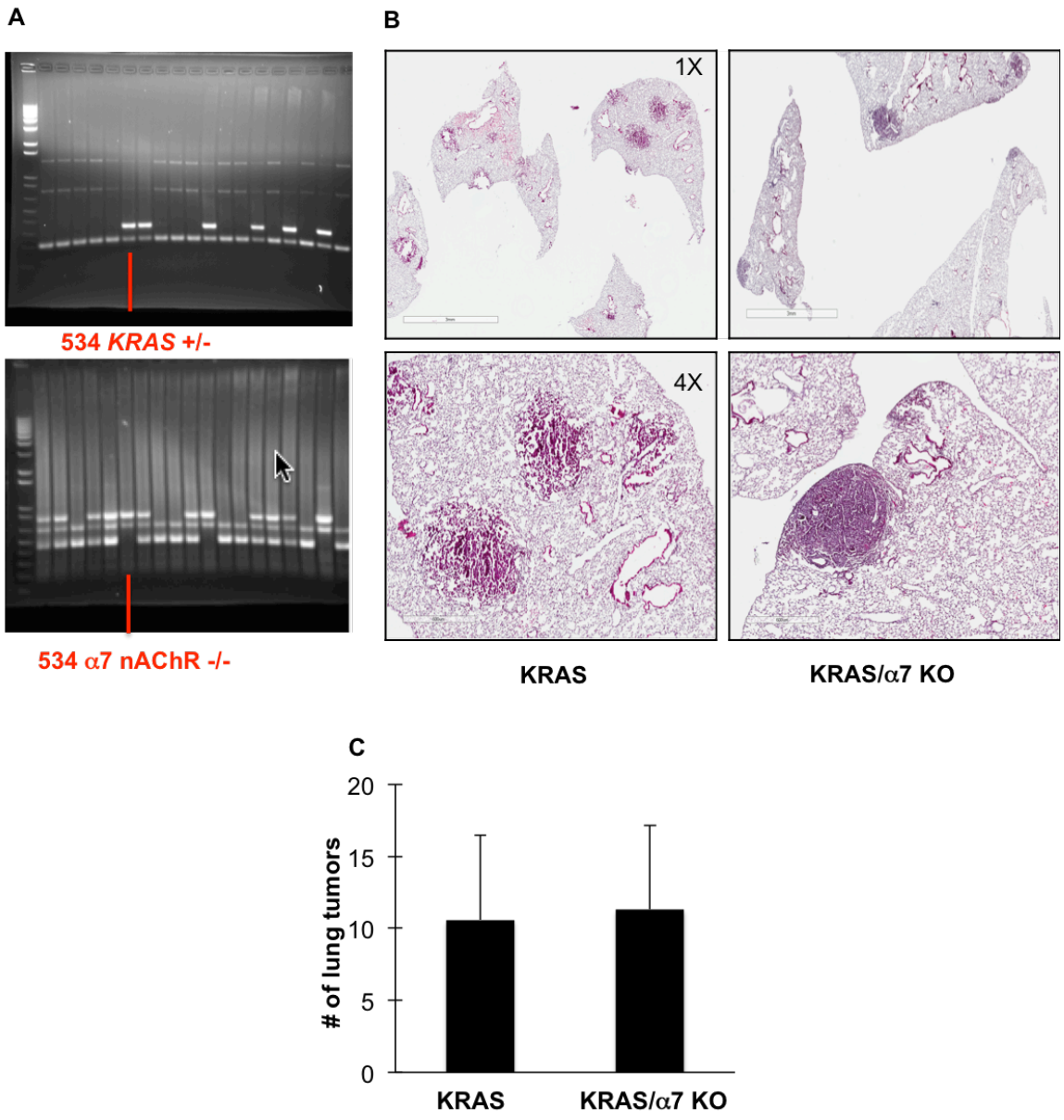

Figure 9. Spontaneous tumor development is unchanged in KRAS/α7 nAChR knockout mice when compared to KRAS mice

KRAS (n=5) and KRAS/α7 nAChR knockout mice (n=6) were sacrificed at 90 days and lungs harvested and processed for examination of tumors. Representative PCR gel images shown for genotyping **(A)**. Representative lung tumor images shown at 1x and 4x **(B).** No differences in lung tumor number or size were observed **(C)**.

DISCUSSION

It is well established that tobacco use is the number one risk factor for development of lung cancer, which will take the lives of millions of people worldwide this year[4]. Despite major increases in survival rates of many cancers, little progress has been made in the treatment of lung cancer. Data, however, are emerging showing important roles for nAChRs in cancer biology, which places emphasis on the role of nicotine, an important component of tobacco and an exogenous ligand for these receptors[20]. The mitogenic effects of nicotine are well known and a number of investigators have shown that these effects can be abolished by using chemical nAChR antagonists, such as α-bungarotoxin[21–23]. However, results obtained from experiments using such antagonists must be viewed with caution due to off-target effects. An important example is varenicline, a prescription drug used for smoking cessation. The mechanism of action of varenicline is reported to be through partial agonism of $\alpha4\beta2$ nAChRs receptors[24], but recent data suggest that this agent also binds to $\alpha3$- and $\alpha6$-containing nAChRs and displays full agonism on $\alpha7$ nAChRs[25]. These off-target effects make it difficult to examine the role of individual nAChRs when using such chemicals. We thus used shRNA technology to knockdown a single, specific nAChR subunit at a time, allowing for careful delineation of the roles of the different receptors. We tested LLCs because of the extensive literature available

using these cells to study lung cancer in models that allow their use in normal, immunocompetant C57BL/6 mice.

First, we confirmed that nicotine exerted mitogenic effects on WT LLC cells. Note that, as previously reported[18], this effect was relatively small (~30-40%), which is likely due to the fact that the cells were not starved prior to stimulation as others have reported[11,26]. At least one group has shown that the ability of nicotine to increase expression of Akt, Erk, and mTOR activation *in vitro*, was largely dependent on the absence of serum which causes lower basal levels of pathway activation[27]. Instead, the cells were cultured in 2% fetal bovine serum, which we believe is more physiologically relevant. Although the most robust effect was noted at the highest dose tested (276% increase over control), we chose to use doses more physiologically relevant for further experiments. As expected, based on the literature, we found that a non-specific nAChR chemical inhibitor (e.g., mecamylamine) diminished nicotine-induced LLC cell proliferation, although the effect was relatively small. However, more specific inhibitors targeting α4 or α7 nAChRs independently did not inhibit nicotine-induced cell proliferation, while the α7 nAChR antagonist had a small mitogenic effect at higher doses. This lack of a significant inhibitory activity was confirmed in cells silenced specifically for either the α4 or the α7 nAChR subunits. This may suggest that nAChRs containing the α4 and α7 subunits play little role in nicotine-induced proliferation, and that effects seen in other studies performed using chemical antagonists were indeed due to off-target effects. On the other hand, we believe it is more likely that nicotine-induced proliferation in LLC cells is

not mediated by a single nAChR, but several, and that blocking one or the other independently is not sufficient to completely inhibit proliferation. In other words, both α4 and α7 nAChRs, and perhaps others, need to be targeted to inhibit nicotine-induced cell proliferation. One interesting observation that remains unexplained relates to the mild induction of proliferation observed in unstimulated α7 nAChR-silenced cells. We have observed a similar effect in lung fibroblasts (unpublished data); this seemingly paradoxical effect has been reported in the literature in airway epithelial basal cells[28]. Our data suggest that α7 nAChRs might restrain proliferation in unstimulated cells, while this effect is overcome in cells lacking this receptor. This raises the possibility of multiple receptors mediating these effects, but this requires further investigation.

In contrast to our observations regarding cell proliferation, we found that cells silenced for α4 nAChRs, but not α7 nAChRs, showed reduced migration and ability to form colonies on soft agar when compared to controls. Deficiency of α4 nAChRs also resulted in resistance to cisplatin-induced apoptosis.

Having characterized the clones *in vitro*, we turned our attention to testing the roles of these receptors in an *in vivo* model of lung cancer. For this, cells silenced for α4 or α7 nAChR subunits were injected subcutaneously into C57BL/6 mice. Mice injected with cells silenced for α4 nAChRs developed fewer tumors, but this was not statistically significant. No differences were observed in animals injected with control or α7 nAChR deficient cells. Tumor size was also not different between groups. Importantly, silencing α4 or α7 nAChRs independently had no significant effect on the survival of animals. Note that

tumor growth and progression (and animal survival) were tested in untreated animals suggesting that these nAChRs play insignificant roles in tumor progression in the absence of exogenous stimulation. Whether these receptors play roles in tumor progression in the setting of nicotine exposure awaits further exploration.

Finally, we turned our attention to host cell nAChRs. We focused on α7 nAChRs because of the availability of C57BL/6 mice with α7 nAChR knockout mutations[29]. In the xenograft model, we found increased tumor progression in α7 nAChR knockout animals, which is consistent with our *in vitro* proliferation data for α7 nAChR silencing in unstimulated cells. However, when we created animals with double mutations in *KRAS* and α7 nAChRs to test spontaneous tumor development, the number and size of lung tumors was similar to that in *KRAS* mice with wildtype expression of α7 nAChRs. Together, these studies suggest that host cell α7 nAChRs may not play critical roles in tumor development driven by mutations, but may play a vital role in cancers driven by oncogenic microenvironments.

Another explanation is that other nAChRs overcompensate when α7 nAChRs are absent. As stated before, in cultured tumor cells (this report) and in lung fibroblasts (unpublished observations), absence of α7 nAChRs leads to increased cellular proliferation.

In summary, our studies shed light into the differential roles that α4 and α7 nAChRs play in LLC cells when tested *in vitro*. Specifically, α4 nAChRs were found to promote lung carcinoma cell migration and colony formation on soft

agar. α4 nAChRs were also found to play a role in cisplatin-induced apoptosis. α7 nAChRs appear to have little to no effect on these processes, but both receptors seem to play roles in cellular proliferation at baseline in unstimulated cells. However, studies in animals suggest that neither receptor in the tumor cell is essential for tumor development, growth, and progression *in vivo* in the absence of exogenous nicotine stimulation. Rather, our *in vivo* studies point to an important role of these receptors, specifically the α7 nAChRs, in the host. Together, our studies suggest that targeting one receptor alone might be insufficient to inhibit tumor progression *in vivo,* while targeting multiple nAChRs might prove more successful. Further exploration of the differential roles of nAChRs in both cancer cells and host cells, especially in humans, will undoubtedly enhance our understanding of how they influence cancer and other biological processes. This work is expected to better direct our efforts towards the development of effective strategies for intervention in the setting of lung cancer and other tumors.

SUMMARY AND CONCLUSIONS

CAVEATS AND WEAKNESSES

Our main goal in this study was to determine if nAChRs played an important role in the development and progression of lung cancer. Previous studies implicating these receptors in the etiology of lung cancer utilized chemical antagonists in their methodology, which are notorious for their off-target effects. While we believe our approach using shRNA technology is more specific and targeted to individual receptors, it is important to note that knocking down the expression of one receptor could lead to a compensatory increase or decrease in the expression of other nAChRs and/or other relevant molecules.

While our data suggest the α4 and α7 nAChRs may play little to no role, individually, in lung cancer progression, our data utilizing knockout animals suggest an important role of the α7 nAChR in the host. We are now in the process of developing an α4 nAChR knockout animal to further study the role of these receptors in the host. However, this study did not offer any insight into what cells and mechanisms might be mediating these effects. A vast number of cell types in the body express nAChRs, and so knockout animals could have a large number of processes affected compared to wildtype animals. Additional studies are needed to examine exactly what is causing these effects in the α7 nAChR deficient animals.

FUTURE WORK

The future directions described here will seek to address the questions that remain unanswered, as well as new questions that have arisen, in our attempts to better understand the role that nAChRs play in lung cancer development and progression. Our data presented here suggest an important role for the host in lung cancer progression. For our future studies, we thus chose to examine three aspects of the host that we believe important in helping us better understand lung cancer development and progression.

Role of host nAChRs

Amidst the perceived importance of host nAChRs in the etiology of lung cancer, we hypothesize that activation of nAChRs in lung fibroblasts promotes an oncogenic microenvironment that renders the host susceptible to lung cancer development and progression. We will examine this by exposing animals chronically with nicotine in their drinking water and then injecting them with LLC cells and examining tumor development and metastases. Presumably, any differences we observe will be through affects on the host. However, signaling through nAChRs has been shown to affect a number of different host responses, such as inflammation, alterations in the immune system, and angiogenesis. We will also examine these processes and investigate what cells and mechanisms are mediating these effects.

Role of tumor cell-stromal interactions

Recent studies have implicated the tumor microenvironment as a new chemotherapeutic target, by demonstrating the importance of tumor cell-stromal interactions in tumor progression. However, the exact mechanisms of how tumor cell-stromal interactions drive lung cancer progression remain undefined. We suspect host fibroblasts represent an important component of the tumor microenvironment that may help drive tumor progression. Consistent with the latter, we found that human NSCLC cells show alterations in cell morphology, increased proliferation, and increased colony formation on soft agar when exposed to conditioned media harvested from IMR-90 lung fibroblasts. Interestingly, the fibroblast-derived conditioned media also promoted tumor cell resistance to cisplatin-induced apoptosis. We plan on investigating these interactions further, performing *in vivo studies*, and determining exactly what soluble factors are mediating these responses.

Role of aging in the etiology lung cancer

Cancer incidence is known to rise exponentially with age. This is thought to be due to the accumulation of oncogenic mutations, as well as changes in the tissue microenvironment. Consistent with this, we found that aged animals showed greater metastases to the lung in our LLC model when compared to young animals (**Figures 10-12**). We began to inquire into the mechanisms for this observation. Dean Jones at Emory University, after testing hundreds of healthy and diseased individuals, has recently shown that the physiological

cysteine/cystine redox potential found in the plasma of healthy subjects is around -80 mV. In subjects with disease, this redox potential may be oxidized to values between -62 to -20 mV. Relevant to our proposal is the fact that aging is associated with alterations in this mechanism. We have shown that this oxidative stress can activate nAChRs, and lead to increased expression of extracellular matrix proteins. We have also shown that aged lungs show alterations in the expression of extracellular matrices. We thus hypothesize that aging, by activation of nAChRs in the lung via oxidation of the cysteine/cystine redox potential, promotes an oncogenic microenvironment that renders the host susceptible to lung cancer development and progression. If this is true, we deduce that we can prevent lung metastasis in old animals through dietary interventions. Cysteine and cystine are derived from dietary sulfur amino acids and Dean Jones has also shown that oxidation of the cysteine/cystine redox potential could be mimicked in rodents exposed to a diet with low sulfur content, while a diet with high sulfur content could reverse the effect[30]. Thus, we predict that a diet with supplementation of sulfur can reverse the oncogenic microenvironment observed in aged animals.

Figure 10

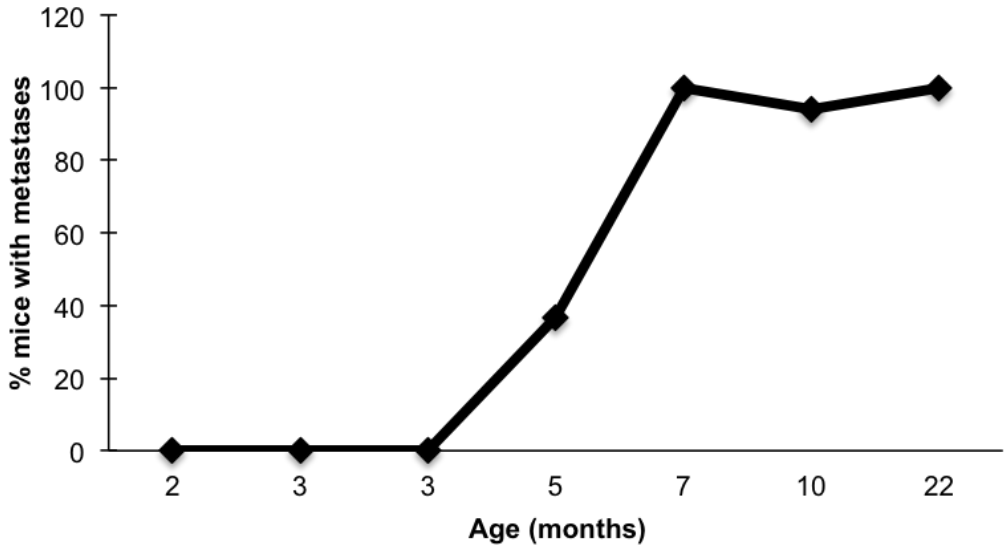

Figure 10. Metastases in young versus old mice
Retrospective analysis of 6 *in* vivo experiments was performed. Each experiment included WT C57BL/6 animals injected with WT LLC cells. Mice 3 months of age and younger failed to develop metastases, while metastasis in mice after 7 months of age was almost consistently 100%.

Figure 11

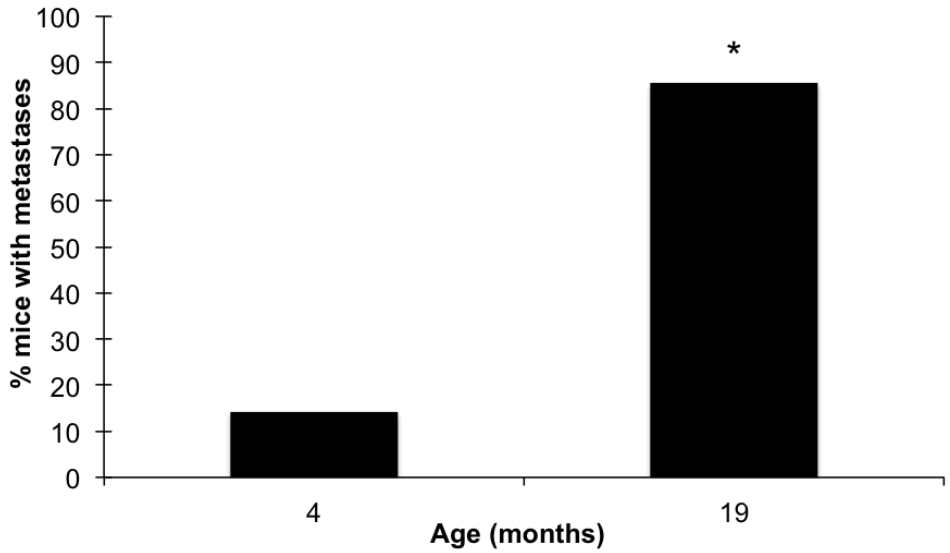

Figure 11. Old animals are more susceptible to lung cancer metastasis
Young (4 months, n=7) and old (19 months, n=7) WT C57BL/6 animals were injected with $1x10^6$ WT LLC cells into the hind flank. Tumor formation and size were followed up to 2 weeks. A tumor size of ≥15mm in length or width was established as the endpoint according to IACUC regulations. Mice were sacrificed at 2 weeks and lungs harvested and processed for examination of metastases. All animals developed tumors at the site of injection, with no differences in size observed. 1/7 animals in the young group developed metastases, while 6/7 animals in the old group developed metastases.

Figure 12

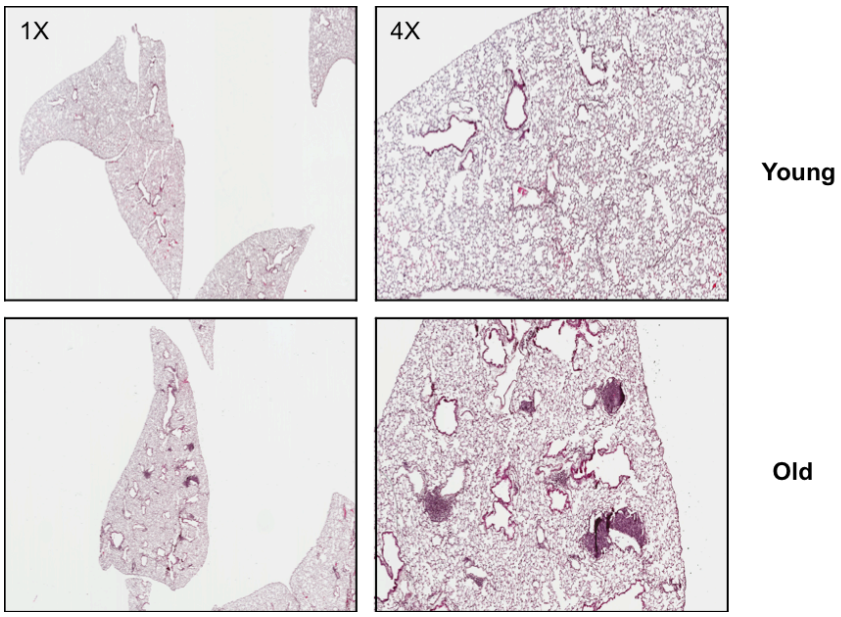

Figure 12. Lung images of young versus old mice injected with LLC cells
Young (4 months, n=7) and old (19 months, n=7) WT C57BL/6 animals were injected with $1x10^6$ WT LLC cells into the hind flank. Tumor formation and size were followed up to 2 weeks. A tumor size of ≥15mm in length or width was established as the endpoint according to IACUC regulations. Mice were sacrificed at 2 weeks and lungs harvested and processed for examination of metastases.

REFERENCES

1. Jemal, A. *et al.* Cancer Statistics , 2009. *J CLIN* **59,** 225–249 (2009).

2. American Cancer Society. Cancer Facts & Figures 2015. (2015).

3. Gilligan, D. *et al.* Preoperative chemotherapy in patients with resectable non-small cell lung cancer : results of the MRC LU22 / NVALT 2 / EORTC 08012 multicentre randomised trial and update of systematic review. *Lancet* **369,** 1929–1937 (2007).

4. WHO REPORT on the global TOBACCO epidemic, 2011 Warning about the dangers of tobacco. (2011).

5. Talhout, R. *et al.* Hazardous compounds in tobacco smoke. *Int. J. Environ. Res. Public Health* **8,** 613–628 (2011).

6. Sekido, Y., Fong, K. M. & Minna, J. D. Molecular genetics of lung cancer. *Annu Rev Med* **54,** 73–87 (2003).

7. Sato, M. *et al.* Multiple oncogenic changes (K-RAS(V12), p53 knockdown, mutant EGFRs, p16 bypass, telomerase) are not sufficient to confer a full malignant phenotype on human bronchial epithelial cells. *Cancer Res.* **66,** 2116–28 (2006).

8. D'Souza, M. S. & Markou, A. Neuronal mechanisms underlying development of nicotine dependence: implications for novel smoking-cessation treatments. *Addict. Sci. Clin. Pract.* **6,** 4–16 (2011).

9. Schuller, H. M. Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochem. Pharmacol.* **38,** 3439–42 (1989).

10. Shi, D. *et al.* Nicotine Promotes Proliferation of Human Nasopharyngeal Carcinoma Cells by Regulating α7AChR, ERK, HIF-1α and VEGF/PEDF Signaling. *PLoS One* **7,** 1–11 (2012).

11. Dasgupta, P. *et al.* Nicotine induces cell proliferation by β -arrestin – mediated activation of Src and Rb – Raf-1 pathways. *J. Clin. Invest.* **116,** 2208–2217 (2006).

12. Carlisle, D. L. *et al.* Nicotine signals through muscle-type and neuronal nicotinic acetylcholine receptors in both human bronchial epithelial cells and airway fibroblasts. *Respir. Res.* **16,** 1–16 (2004).

13. Wessler, I. & Kirkpatrick, C. J. Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans. *Br. J. Pharmacol.* **154,** 1558–71 (2008).

14. Kunzelmann, K. Ion Channels and Cancer. *J. Membr. Biol* **205,** 159–173 (2005).

15. Hung, R. J. *et al.* A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature* **452,** 633–637 (2008).

16. Roman, J., Ritzenthaler, J. D., Roser-Page, S., Sun, X. & Han, S. Alpha5Beta1-Integrin Expression Is Essential for Tumor Progression in Experimental Lung Cancer. *Am. J. Respir. Cell Mol. Biol.* **43,** 684–91 (2010).

17. Roman, J., Ritzenthaler, J. D., Bechara, R., Brown, L. A. & Guidot, D. Ethanol stimulates the expression of fibronectin in lung fibroblasts via kinase-dependent signals that activate CREB. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288,** L975–87 (2005).

18. Zheng, Y., Ritzenthaler, J. D., Roman, J. & Han, S. Nicotine stimulates human lung cancer cell growth by inducing fibronectin expression. *Am. J. Respir. Cell Mol. Biol.* **37,** 681–90 (2007).

19. Bos, J. L. ras Oncogenes in Human Cancer : A Review ras Oncogenes in Human Cancer : A Review. *Cancer Res.* 4682–4689 (1989).

20. Lindstrom J, Anand R, Gerzanich V, Peng X, Wang F, W. G. Structure and Function of Neuronal Nicotininc Acetylcholine Receptors. *Prog Brain Res* **109,** 125–137 (1996).

21. Paleari, L. *et al.* Role of alpha7-nicotinic acetylcholine receptor in human non-small cell lung cancer proliferation. *Cell Prolif.* **41,** 936–59 (2008).

22. Davis, R. *et al.* Nicotine promotes tumor growth and metastasis in mouse models of lung cancer. *PLoS One* **4,** e7524 (2009).

23. Codignola, a *et al.* Serotonin release and cell proliferation are under the control of alpha-bungarotoxin-sensitive nicotinic receptors in small-cell lung carcinoma cell lines. *FEBS Lett.* **342,** 286–290 (1994).

24. Fagerström, K. & Hughes, J. Varenicline in the treatment of tobacco dependence. *Neuropsychiatr. Dis. Treat.* **4,** 353–63 (2008).

25. Mihalak, K. B., Carroll, F. I. & Luetje, C. W. Varenicline Is a Partial Agonist at α4β2 and a Full Agonist at α7 Neuronal Nicotinic Receptors. *Mol. Pharmacol.* **70,** 801–805 (2006).

26. Dasgupta, P. *et al.* Nicotine induces cell proliferation, invasion and epithelial- mesenchymal transition in a variety of human cancer cell lines. *Int J Cancer* **124,** 36–45 (2009).

27. Maier, C. R. *et al.* Nicotine does not enhance tumorigenesis in mutant K-Ras-driven mouse models of lung cancer. *Cancer Prev. Res.* **4,** 1743–1751 (2011).

28. Maouche, K. *et al.* {Alpha}7 Nicotinic Acetylcholine Receptor Regulates Airway Epithelium Differentiation By Controlling Basal Cell Proliferation. *Am. J. Pathol.* **175,** 1868–82 (2009).

29. Orr-Urtreger, a *et al.* Mice deficient in the alpha7 neuronal nicotinic acetylcholine receptor lack alpha-bungarotoxin binding sites and hippocampal fast nicotinic currents. *J. Neurosci.* **17,** 9165–71 (1997).

30. Nkabyo, Y. S., Gu, L. H., Jones, D. P. & Ziegler, T. R. Thiol/disulfide redox status is oxidized in plasma and small intestinal and colonic mucosa of rats with inadequate sulfur amino acid intake. *J. Nutr.* **136,** 1242–1248 (2006).