THE DEVELOPMENTAL TRANSCRIPTION FACTOR NEUROGENIC DIFFERENTIATION 1 IN MIGRATION AND SURVIVAL OF NEUROENDOCRINE CARCINOMAS

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Dedications

Dedicated with love to my parents, Soleitor and Clarence Osborne, for all the sacrifices they made…It was not in vain.

To the siblings who have stood with me through everything: good, bad and chupid.

To my spouse, all my family, friends and even enemies that have never let me down, given me encouragement and shoulders to cry on, and driven me to always do better.

THE DEVELOPMENTAL TRANSCRIPTION FACTOR NEUROGENIC DIFFERENTIATION 1 IN MIGRATION AND SURVIVAL OF NEUROENDOCRINE CARCINOMAS

By

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DISSERTATION

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Abstract

Differentiation and determination of cell fate during embryogenesis is decided by a collection of transcription factors, including the large family of basic-helix-loop-helix (bHLH) transcription factors. Neurogenic differentiation 1 (NeuroD1) is a bHLH transcription factor responsible for neuronal and neuroendocrine islet differentiation during development of the central and peripheral nervous systems and the pancreas

respectively. NeuroD1 has also been shown to be anomalously expressed in a subset of aggressive neuroendocrine tumors. Initial examination of microarray data revealed that subsets of aggressive small cell lung cancers (SCLC) and certain neuroendocrine non-small cell lung cancers (NSCLC-NE) have high expression of NeuroD1 as compared to human bronchial epithelial cells (HBEC) and other non-small cell lung cancers (NSCLC).

In several neuroendocrine carcinomas, including subsets of neuroendocrine lung cancers, melanoma and some undifferentiated prostate cell lines, NeuroD1 directly induces the expression of signaling pathways that support survival and migration. Loss-of-function/gain-of-function studies in cell lines from each of these cancer types reveled that NeuroD1 regulates both survival and the migration potential of neuroendocrine carcinomas that have lost or mutated p53. Subsequently, loss of p53 has been shown to up-regulate NeuroD1 expression in non-transformed HBECs and cancer cells with neuroendocrine features. The actions of NeuroD1 are carried out by downstream targets which include the signaling molecules, the tyrosine kinase, tropomyosin-related kinase B (TrkB), and the adhesion molecule, neural cell adhesion molecule (NCAM), and the ion channels, the nicotinic acetylcholine receptor subunit cluster of α3, α5, and β4 (nAChR), to name a few. Impaired expression of each of these downstream targets mirrors the various phenotypes associated with loss of NeuroD1. These findings ultimately have implications for the potential of NeuroD1 acting as a lineage-dependent oncogene in neuroendocrine carcinomas.

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List of Abbreviations

AhR-aryl hydrocarbon receptor

Akt- AK strain thymoma

ASCL1- achaete-scute homologue

BDNF- brain derived neurotrophic factor

bHLH- basic helix-loop-helix

cAMP- cyclic adenosine monophosphate

CDK4- cyclin-dependent kinase 4

ChIP-chromatin immnoprecipitation assays

COSMIC- catalogue of somatic mutations in cancer

EMT- epithelial-to mesenchymal transition

EMSA- electrophoretic mobility shift assay

ERK- extracellular-regulated kinase

FGFR- fibroblast growth factor receptor

GAPDH- glyceraldehyde 3-phosphate dehydrogenase

HBEC- human bronchial epithelial cells

HBEC3KT-HBEC immortalized with CDK4 and hTERT

HBEC3KT53 - HBEC3KT with constitutive knockdown of p53

HBEC3KTR$_L$53- HBC3KT with constitutive knockdown of p53 and over-expression of oncogenic KRAS

HIF1α- hypoxia inducible factor 1

hTERT- human telomerase reverse transcriptase

IA-1-Insulinoma 1 (aka INSM1)

MAPK- mitogen activated protein kinase

mTOR- mammalian target of rapamycin

nAChR- nicotinic acetylcholine recptors

NeuroD1- neurogenic differentiation factor 1

NCAM- neural cell adhesion molecule

NGN- neurogenin

NSCLC- non-small cell lung cancer

NSCLC-NE- neuroendocrine non-small cell lung cancer

PAS- Per-arnt-sim

PI3K- phosphatidylinositol 3-kinase

PTEN- phosphatase and tensin homolog

qRT-PCR- quantitative real time- PCR

RA- retinoic acid

ROBO- roundabout receptor

SCLC-small cell lung cancer

shRNA- short hairpin RNA

TrkB- tropomyosin-related kinase B

Chapter One

INTRODUCTION

Biology of bHLH transcription factors

Neurogenic basic helix-loop-helix (bHLH) transcription factors regulate neuronal cell fate and determination during development (Farah et al., 2000); (Lee et al., 1995); (Guillemot and Joyner, 1993; Guillemot et al., 1993). Group A and Class II tissue-specific bHLH transcription factors function as obligatory heterodimers with other bHLH proteins typically the ubiquitous E2A gene products, the E12 and E47 proteins. Biochemical and X-ray crystallographic studies indicate that their basic regions enable binding to canonical DNA recognition consensus sequences on the DNA ,CANNTG, also known as E boxes whereas HLH domains are utilized for dimerization (Murre et al., 1989); (Longo et al., 2008).

There are over 124 bHLH transcription factors that have been characterized in mouse, classified into four groups from A-D, based on distinct binding of consensus sequences(Cherrington et al., 2008). Group A proteins, include several tissue-specific proteins such as neurogenic differentiation 1 (NeuroD1), achaete-scute homologue 1 (ASH1, also known as ASCL1, mASH-1-Mouse; hASH1-human), and MyoD as well as ubiquitously expressed proteins such as E12/E47. These proteins preferentially bind to E-boxes with internal nucleotide bases of GC as in the sequence CAGCTG, In contrast, group B bHLH factors prefer internal bases of CG as in CACGTG (Cherrington et al., 2008; Ledent and Vervoort, 2001). In addition to HLH domains, many group B proteins, such as Myc and SREBP, contain leucine zipper motifs also used for dimerization. Group C proteins, along with HLH domains contain PAS domains, which enable

binding to small molecules. Group D proteins such as Id, contain only an HLH domain and lack basic regions to bind DNA (Ledent and Vervoort, 2001).

HLH proteins are more generally divided into seven classes that also take into account the presence of DNA- binding domains and other motifs or domain structures (Figure 1)(Massari and Murre, 2000). In this classification ubiquitously expressed proteins such as E12/E47 are grouped into Class I, whereas the tissue- specific transcriptional regulators are placed in Class II, (which include MyoD, and NeuroD) and form heterodimers with Class I proteins. Class II proteins generally regulate cell fate and differentiation, which for the two mentioned include neurogenesis and myogenesis (Table 1) (Ishii et al., 2012; Lee et al., 1995; Weintraub et al., 1991). Class III is similar to the Group B classification containing proteins with the additional leucine zipper motifs such the Myc family members and SREBP, whereas Class IV was further sub-divided from Group B to contain Myc-binding proteins such as Mad and Max. Class V proteins comprise the inhibitory or dominant-negative family of Id proteins (Pesce and Benezra, 1993; Tournay and Benezra, 1996). Class VI proteins are segregated for the presence of proline in their basic regions. Pas domains distinguish Class VII proteins and include the Clock family of proteins as well as Hif1α, and AhR.

Normal function of NeuroD1 as a neuronal/neuroendocrine protein

NeuroD1 (also known as BETA2) is a bHLH transcription factor responsible for mediating efficient gene expression of insulin in pancreatic beta cells. Additionally, NeuroD1 is associated with late neuronal differentiation of the peripheral and central nervous system during embryogenesis. NeuroD1 was first recognized for having the capability to convert embryonic epidermal cells into fully differentiated neurons in

Xenopus embryos (Lee et al., 1995). The hamster ortholog, BETA2 (eventually recognized as NeuroD1), was cloned simultaneously from a yeast one-hybrid screen, demonstrating BETA2, along with E47, binds to the bHLH consensus E box on the insulin gene promoter(Naya et al., 1995b).

From embryogenesis to adulthood, NeuroD1 has been shown to have a pattern of tissue-specific expression in cells of neural and/or neuroendocrine origin, usually of the nervous and digestive systems (including the pancreas), in addition to lung epithelial progenitors and adult lung. Both ASCL1 and NeuroD1 are required for formation of pulmonary neuroendocrine cells, whereas NeuroD1 is additionally required for distal lung formation (Ito et al., 2000; Neptune et al., 2008b). NeuroD1 expression is conserved in metazoans including mammals, chick, *Xenopus*, zebrafish, and *C. elegans* (Chae et al., 2004) . During embryogenesis, NeuroD1 is required for development of the endocrine pancreas, as null mice die within five days due to hyperglycemia and failure of endocrine cells to form in the pancreas (Naya et al., 1997).

In the adult mammal, NeuroD1 is expressed in a tissue-specific manner, along with the transcription factors, MafA and PDX-1, to enhance the expression of the insulin gene in pancreatic beta cells (Barrow et al., 2006; Docherty et al., 2005; Zhao et al., 2005). The mitogen-activated protein kinase (MAPK) -extracellular signal-regulated kinase protein 1 and 2 (ERK1/2) also phosphorylate NeuroD1 on four or more serine residues and the specific mutation of serine 274 to alanine causes loss of NeuroD1 transactivation ability on the insulin gene promoter (Khoo et al., 2003). Function of this phosphorylation, however, seemed to be context-dependent because mutagenesis

experiments demonstrated that the same mutations increased NeuroD's ability to induce formation of ectopic neurons (Dufton et al., 2005).

Several mutations of the *NeuroD1* gene have been noted in both Type 1 and Type 2 diabetes (Figure 2) (Chae et al., 2004). *NeuroD1* is located on chromosome 2q32 in a region that has been linked to Type1 diabetes. One mutation, threonine 45 to alanine, seen in Danish, Czech and Japanese populations has been linked to Type 1 diabetes (Chae et al., 2004). Maturity-onset diabetes of the young (MODY), is an autosomal dominant form of type 2 diabetes connected to mutations in several genes required for pancreatic function. One such disease, MODY6, arises from two separate mutations in *NeuroD1.* One of the mutations occurs in the DNA-binding domain which replaces arginine 111 with leucine, ultimately abolishing the E-box binding activity (Malecki et al., 1999a). The other mutation creates a truncated protein. This occurs at the carboxy-terminus, where there is an insertion of a cytosine in a poly-C tract at codon 206 resulting in a frameshift which creates a nonsense peptide from 205 to 242 and a premature stop codon.

NeuroD1 belongs to the *atonal*-related sub-family of bHLH transcription factors, which comprises NeuroD2, NeuroD4, and NeuroD6 (Gasa et al., 2008). During neuronal differentiation the roles of this family have been suggested to be partially redundant, as many areas of the brain show overlapping expression (Schwab et al., 1998). However, additional roles for the paralogs NeuroD2 and NeuroD4 have been shown during pancreatic development similar to that of NeuroD1 (Gasa et al., 2008). As mentioned NeuroD1 plays a role in development of both the pancreas and various neurons throughout the brain. Distinguishing the neuronal from the pancreatic phenotype was

achieved by conditional knockout of NeuroD1, which demonstrated that in addition to neurons of the cerebellum and hippocampus, development of the retina, the vestibular and the auditory systems also showed defects as a result of loss of the protein(Liu et al., 2000; Miyata et al., 1999a; Pennesi et al., 2003).

Other neuronal transcription factors related to NeuroD1

Pancreatic development initiates with the onset of expression of a series of transcription factors, one of the first being the pancreatic duodenal homeobox (PDX-1) homeodomain transcription factor (Lyttle et al., 2008). Commitment of pancreatic progenitor cells to the establishment of the endocrine program during organogenesis begins with the induction of the bHLH transcription factor, neurogenin3 (NGN3) (Table 2) (Xu et al., 2008; Zhou et al., 2008). The three members of the neurogenin (NGN) family 1, 2, and 3 were identified based on sequence similarity; and epistatic analysis indicated that they act upstream of NeuroD1 (Chae et al., 2004). All three neurogenin family members are expressed in the central and peripheral nervous systems during development. However, NGN3 is the only member expressed during pancreatic islet cell formation and was again found to be upstream of NeuroD1 in that context (Gasa et al., 2008; Huang et al., 2000; Lyttle et al., 2008).

In addition to guiding neuronal cell commitment, achaete-scute homologue (ASCL1) is required for development of neuroendocrine lung. ASCL1, a bHLH transcription factor that functions upstream of NeuroD1 in hypothalamic neuroendocrine cells and in neuroendocrine cells located in pulmonary epithelia(Table 2) (Ito et al., 2000; McNay et al., 2006), has also been extensively studied in small cell lung cancer (SCLC). Overexpression of ASCL1 has been shown to increase proliferation and

metaplasia in normal lung airway epithelium (Wang et al., 2007a). Several SCLC cell lines display increased expression of ASCL1 and its knockdown via RNAi has been shown to increase apoptosis in several human lung cancer cell lines (Wang et al., 2007a). ASCL1 has additionally been linked to E-cadherin down-regulation and remodeling of preneoplastic lung epithelia via up-regulation of pAKT/mTOR and matrix metalloproteases 7 (Wang et al., 2007a).

Insulinoma1 (IA-1, INSM1) is a zinc finger transcription factor that is expressed in the developing fetal brain and pancreas. It is reactivated in tumors of neuroendocrine origin, where it was first discovered (Breslin et al., 2003; Liu et al., 2006). Both NeuroD1 and IA-1 have overlapping regions of expression during fetal development as well as in many tumors of neuroendocrine origin. IA-1 and NeuroD1 have been reported to regulate each other. NeuroD1 has been shown via electrophoretic mobility shift assay (EMSA) to be on the IA-1 promoter and up-regulates IA-1 expression in the developing brain, whereas chromatin immunoprecipitation (ChIP) data revealed that IA-1 can bind the NeuroD1 promoter and repress its activity (Breslin et al., 2003; Liu et al., 2006). NGN3, a direct regulator of NeuroD1, has also been shown to act in conjunction with IA-1 during pancreatic development. Recently it is has been shown that NeuroD1 and NGN3 bind the same E-box element on IA-1 promoter (Breslin et al., 2007). However, though NeuroD1 and NGN3 bind to the same E box element, Breslin et. al. demonstrated that NGN3 was a more potent activator.

NeuroD1 downstream targets in cancer

Downstream targets of NeuroD1 have been identified. Among these a significant number were simultaneously shown to also be targets of NGN1 and NGN3

(Gasa et al., 2004; Gasa et al., 2008; Seo et al., 2007). Nevertheless, many of these targets were in fact subsequently confirmed to be direct targets of NeuroD1. Several of these NeuroD1 targets, have been observed to be overexpressed or mutated in a variety of cancers (Cavallaro et al., 2001; Chiaramello et al., 2007; Daniloff et al., 1986; Ditlevsen et al., 2008; Douma et al., 2004; Tournier et al., 2006). Additionally, several of these targets have been linked to an increase in migration potential/ metastasis.

Metastasis of solid tumors accounts for over 90% of cancer related deaths(Gupta and Massague, 2006). Metastasis is the process that enables a cancer cell to escape from the primary site of tumor, migrate through the circulation and invade and become established in distant organs. Among proteins that have been implicated in facilitating the invasiveness of tumors cells, two prominent ones, Slit2 and TrkB, are known downstream targets of NeuroD1 in neuroblastoma.

Liu et al. have demonstrated using ChIP in the neuroblastoma cell line, SH-SY5Y, that NeuroD1 along with E2A proteins bind the *TrkB* promoter (Liu et al., 2004). TrkB is a receptor tyrosine kinase that is activated upon binding of its neurotrophin ligand, brain-derived neurotrophic factor (BDNF), to signal downstream to both phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways(Chiaramello et al., 2007). BDNF/ TrkB signaling has been linked to many cellular responses including, growth, cell survival, differentiation, and synaptic potentiation (Luikart et al., 2008a). Targeted mutations in the catalytic domain of TrkB, lead to several neuronal deficiencies in mice and ultimately neonatal death (Klein et al., 1993) Neuronal movement is essential to proper synapse formation between dendrites and axons of pre-and post-synaptic neurons. TrkB activation stimulates migration of

neurons in the subventricular zone of the lateral ventricle along the rostral migratory stream to the olfactory bulb in rodents and enhance motility in dendritic filapodia (Chiaramello et al., 2007; Ernfors et al., 1994; Luikart et al., 2008a). While TrkB has been found to signal via many molecular pathways, Luikart et al performed experiments demonstrating that TrkB-mediated filopodial motility was specifically mediated via the PI3K pathway. (Luikart et al., 2008b). PI3K signaling is linked to cytoskeletal rearrangements by means of accrual of PIP3, which results in activation of members of the Rho family of small GTP-binding proteins Rac, Cdc42, and Rho, actin polymerization, and subsequent movement of the cell(Kallergi et al., 2007; Kolsch et al., 2008; Qian et al., 2004).

Apoptosis that results from the loss of cell-matrix contacts, also known as anoikis is a theorized obstacle to tumor invasion. Cells that become resistant to anoikis facilitate their survival during intravasation eventually assisting in their metastasis. Overexpression of TrkB in epithelial cells was shown to suppress anoikis resulting in loss of cell-cell contacts, proliferation of spheroids in suspension and metastasis of primary lung tumors in nude mice (Douma et al., 2004). TrkB has also recently been implicated in invasion and metastasis of pancreatic, prostate, and colorectal cancers (Douma et al., 2004; Kupferman et al., 2010; Smit et al., 2009; Thiele et al., 2009).

Slit1 and 2 act as a molecular guidance factor for neuronal migration via its interaction with the roundabout homologue receptors (Robo1, 2, and 3). These receptor /ligand interactions play critical roles in axonal navigation at the ventral midline of the neural tube during development (Stein and Tessier-Lavigne, 2001).

Neuroendocrine tumors and cancer pathology

Immunohistological analysis revealed differential expression of NeuroD1 and ASCL1 in a variety neuroendocrine and non-neuroendocrine tumors (Hiroshima et al., 2006b). Neuroendocrine tumors can initiate from almost all of the organ systems. They are described based on organ of origin, but this feature is not limiting. Many of these tumors share similar pathological characteristics, for example, expression of neuroendocrine markers, such as synaptophysin, chromogranin A and neural cell adhesion molecule (NCAM, also known as CD56). (Abrahamsson, 1999; Cantile et al., 2005; Cindolo et al., 2007b; Eyden et al., 2005a; Eyden et al., 2005b; Pilozzi et al., 2011; Sutherland et al., 2011; Syder et al., 2004a). Neuroendocrine differentiation of tumors has become a topic of interest as differentiation of these tumors from epithelial cells has been hypothesized to be involved in acquisition of invasive/metastatic phenotypes (Cindolo et al., 2007b; Gupta et al., 2008b; Syder et al., 2004a).

Diagnosis and staging of lung cancer comprises radiographic and clinical assessment as well as tissue assessments from biopsies. Based on cellular morphology visualized under a light microscope, the pathology of lung cancer can be categorized into two major classes for treatment, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)(Abrahamsson, 1999; Jackman and Johnson, 2005; Sato et al., 2007). NSCLC accounts for about 80%-85% of all lung cancers where SCLC represents about 15%-20% of all lung cancers. SCLC is a highly aggressive form of lung cancer that is characterized by neuroendocrine morphological features, (distinguished by large nuclei, along with a scant cytoplasm and expression of classical neuroendocrine markers such as synaptophysin and chromogranin A), fast growth, early dissemination, and high frequencies of metastasis especially to brain

(Jackman and Johnson, 2005; Kraus et al., 2002; Quan et al., 2004). More than 95% of patients with SCLC is associated with exposure to cigarette smoking (Jackman and Johnson, 2005). It has been hypothesized that the disease may originate in the minor population of pulmonary neuroendocrine cells that function as oxygen sensors in the adult lung (Ito et al., 2000).

Current treatments for SCLC include surgery for a minority of cases due to high rates of metastasis, platinum-based doublet chemotherapy, commonly cisplatin or carboplatin and etoposide and radiation (Kraus et al., 2002). While SCLC initially responds well to chemotherapy and radiation, there is a development of both chemo- and radio- resistance within three to twelve months and a correspondingly poor prognosis and survival (Jackman and Johnson, 2005; Kraus et al., 2002; Quan et al., 2004).

While targeted drug therapies are making advances in various other devastating cancers, no molecular targets have been developed that have advanced through clinical trials for SCLC (Tiseo and Ardizzoni, 2007). Despite seemingly slow progression, several studies have shed light on the possible series of molecular events responsible for the mutations observed during SCLC tumorigenesis. Amplification of MYC oncogenes have been identified in SCLC for over 30 years (Zhao et al., 2005). Examination of collected from human tumor specimens and development of mouse model system revealed the overwhelming prevalence for the loss of the tumor suppressors *TP53* and *RB1* as causative agents of SCLC (Meuwissen et al., 2003; Sutherland et al., 2011). Recent studies have identified novel mutations established from compilations of data sets from the catalogue of somatic mutations in cancer

(COSMIC) and novel global cancer genome research consortia. In addition to the known hotspots in pathways, e.g., PI3K, including *PIK3CA* and *PTEN*, NOTCH and Hedgehog families,; and *FGFR1,* several new pathways/proteins that may impact treatment of SCLC have come to light. These newly identified mutations arise in, members of the SOX family of transcription factors (*SOX2, SOX3, SOX5, SOX9* and many others), Ras family regulators (*RAB37*, *RASGRF1 and 2*), chromatin modifiers (*MLL2, MED12L*), the glutamate receptors (*GRID1*) and G protein-coupled receptors such as (*GPR55 and GPR113*), to name a few (Table 3) (Peifer et al., 2012; Rudin et al., 2012).

Actions of nicotine in SCLC

As mentioned previously, there is a high correlation with exposure to cigarette smoke, either first or second hand, with the onset of SCLC. Nicotine, the addictive component in cigarettes is well documented to have activity in pleasure centers of the brain, affecting the mood of smokers during as well as after exposure (Paterson and Nordberg, 2000; Paterson et al., 2000; Zhang et al., 2000). Patients that develop lung cancer as a result of smoking have a significantly higher number of mutations per mega-base of DNA in specific genes (such as TP53) when compared to non-smokers with the same subtypes of lung cancer (Govindan et al., 2012). Nicotine induces factors that participate in multiple signal transduction pathways in multiple organs including the brain and the lung (Hukkanen et al., 2005). Nicotine exposure through cigarette smoking has been linked to lung cancer pathogenesis via mechanisms mediated by the pentameric ligand-gated ion channels, the nicotinic acetylcholine receptors (nAChR)

(Improgo et al., 2010a; Lam et al., 2007a). Originally studied as neuronal and muscle ion channels, these receptors when activated, by their most well-known ligands nicotine and acetylcholine, are selectively permeable to calcium. The pentamers are made from various combination of the nine α (α2-10) and three β(β2-4) subunits that form specific homo- and heteromeric combinations. The nAChRs have also been shown to be expressed in many non-neuronal and non-muscle cells such as macrophages, keratinocytes and normal lung epithelia (Improgo et al., 2010a; Kalamida et al., 2007; Lam et al., 2007a; Tournier et al., 2006).

Upon binding to its receptors, nicotine has been reported to induce phosphorylation of both p38-MAPK and Akt (Figure 3) (Carlisle et al., 2004; Carlisle et al., 2007; West et al., 2003). In addition to activating factors responsible for promoting tumorigenesis, nicotine also has been shown to inactivate pro-apoptotic factors including p53 and Bax (Figure 3) (Pfeifer et al., 2002; Puliyappadamba et al., 2010; Xin and Deng, 2005). Recently, nicotine has additionally been shown to increase the expression of ASCL1 in normal lung epithelia (Linnoila, 2006).

This dissertation is focused on elucidating the role of the developmental transcription factor NeuroD1 in aggressive neuroendocrine cancers particularly SCLC. I have demonstrated that inappropriately increased expression of NeuroD1, potentiated by loss of p53, can lead to the increased capability of certain aggressive neuroendocrine cancers to survive during the processes leading up to and throughout metastasis, possibly identifying NeuroD1 as a lineage-restricted oncogene. Interestingly, nicotine leads to an increase in NeuroD1 expression possibly through down-regulation of p53 in HBEC cells. The oncogenic activity of NeuroD1 may be

transmitted through regulation of several signal transduction molecules e.g. TrkB, NCAM, and the nicotinic acetylcholine receptors subunit cluster (α3,α5, and β4), all of which were previously known or suspected to play a role in epithelial-to mesenchymal transition (EMT), increasing metastatic/migration potential, cell survival/proliferation and calcium signaling.

Figure 1-1

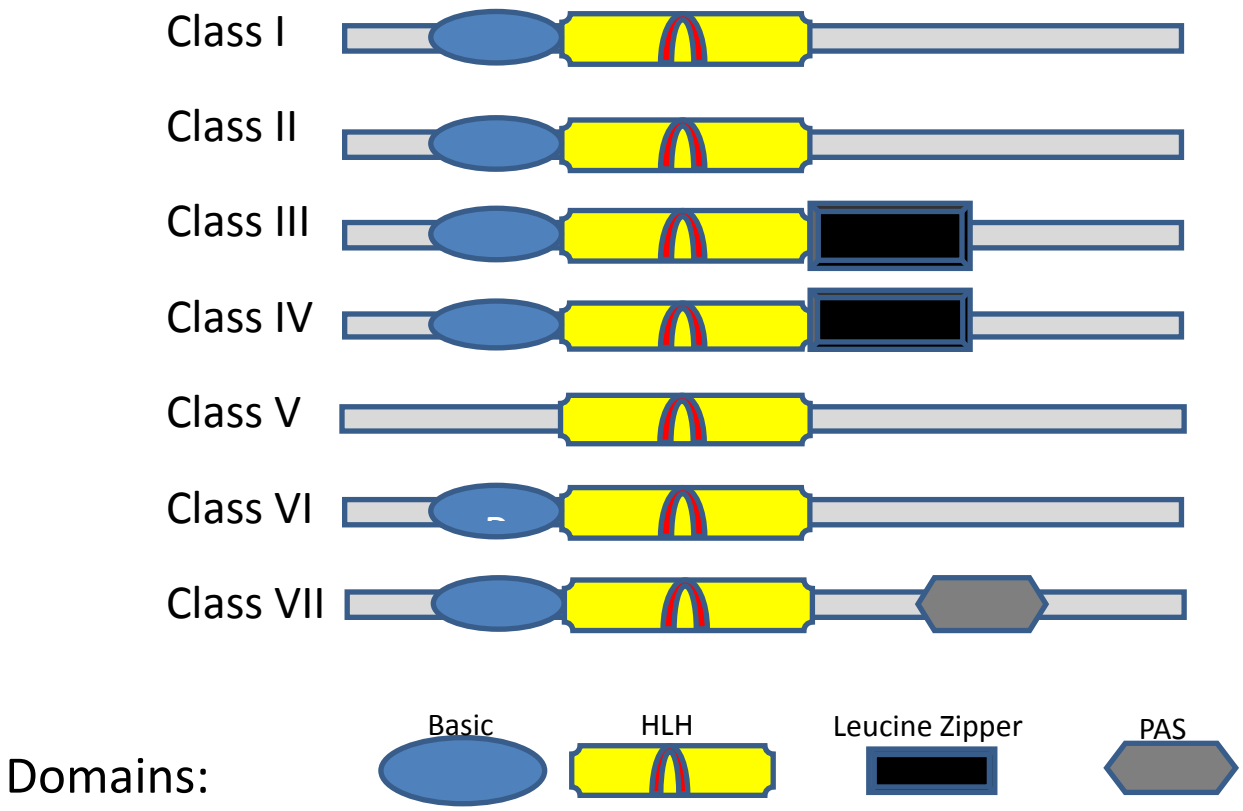


Figure 1. Domains of HLH proteins

HLH proteins are grouped into seven classes, based on presence or absence of DNA binding domains. NeuroD1 belongs to the Class II group of bHLH transcription factors. Also see table 1.

Table 1-1

Classes of HLH proteins and the characterizations

Classification
1. Ubiquitously expressed –example, E2A
2. Tissue Specific- example, NeuroD1
3. Leucine Zipper – examples, Myc family and SREBP
4. Myc binders- examples, Mad and Max
5. No basic domain- example, Id family
6. Proline Rich- example, Hes, Hairy
7. PAS domain- example, Clock

Table 1-2

Tissue-specific Regulation

NeuroD1	ASCL1	NGN
Differentiation Brain-Neurons	Neural Fate-Brain Neurons (autonomic neurons), Oligodendrocytes	Neural Fate-Neurons (sensory lineage)
Neuroendocrine Differentiation-Lung	Neuroendocrine Progenitors- Lung	-
Neuroendocrine Differentiation-Pancreas	-	Neuroendocrine Progenitors-Pancreas (specifically NGN3)

Table1-3

Mutations in SCLC

Previously Known Mutations	Novel Mutations
TP53	SOX Family
RB1	Chromatin Remodelers
PIK3CA	Ras regulators
PTEN	GPCR
FGFR1	Glutamate Receptors

Figure 1-2.

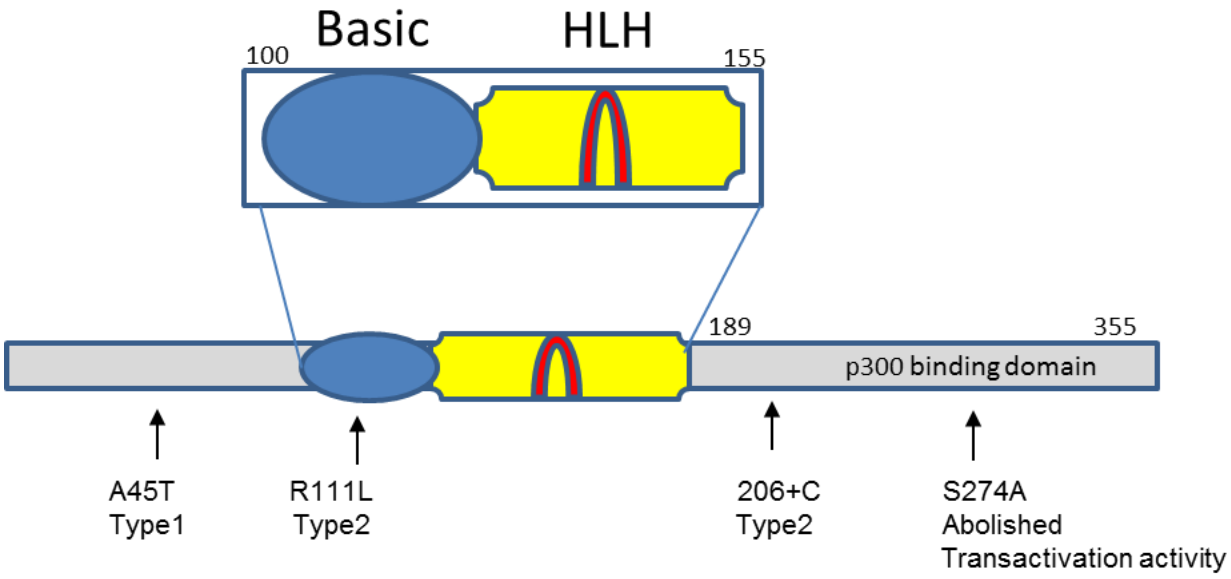


Figure 2. Mutations in NeuroD1.

Several mutations in NeuroD1 are found in different populations around the world. We found that mutations in S274 abolished NeuroD1 transactivation on the insulin promoter (Khoo et al., 2003)(Malecki et al., 1999b).

Figure 1-3

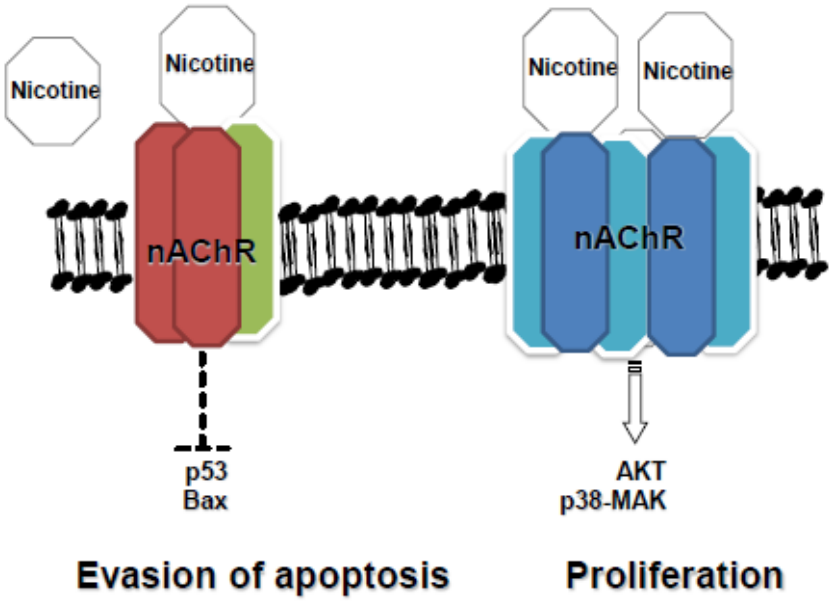


Figure 3. Nicotine induces many signaling pathways

Nicotine induces many signaling pathways including Akt and the p38-MAPK. Nicotine also inhibits several pro-apoptotic factors including Bax and p53. Colors may represent different combination of nAChR receptors

CHAPTER TWO

NeuroD1 Regulates Survival and Migration of Neuroendocrine Lung Carcinomas via Signaling Molecules TrkB and NCAM

Adapted from Osborne J.K., Larsen J.E., Shields M.D., KulKarni, A., Gonzales, J.X., Girard, L., Shames,D.S., Sato, M., Minna J.D., Cobb M.H. (2013). Manuscript submitted to Proceedings of the National Academy of Sciences USA.

Abstract

Small cell lung cancer (SCLC) and other aggressive neuroendocrine cancers are often associated with early dissemination and frequent metastases. We demonstrate that NeuroD1 is a regulatory hub securing cross-talk among survival and migratory-inducing signaling pathways in neuroendocrine lung carcinomas. We find that NeuroD1 promotes tumor cell survival and metastasis in aggressive neuroendocrine lung tumors through regulation of the receptor tyrosine kinase TrkB. Like TrkB, the pro-metastatic signaling molecule NCAM is a downstream target of NeuroD1, whose impaired expression mirrors loss of NeuroD1. TrkB may be a therapeutic target for aggressive neuroendocrine cancers that express NeuroD1.

Introduction

Based on histology, 15-20% of lung carcinomas are categorized as small cell lung cancer (SCLC) and 80-85% as non-small cell lung cancer (NSCLC) (Fischer and Arcaro, 2008; Jackman and Johnson, 2005; Sato et al., 2007; Sun et al., 2007). Lung cancers with neuroendocrine features represent nearly 25% of all lung cancer cases including all SCLC, a subset of NSCLC, and typical and atypical carcinoids. SCLC is the deadliest histological subtype because it is associated with high rates of metastatic disease at time of diagnosis (Fischer and Arcaro, 2008; Jackman and Johnson, 2005). Mixed histological variants containing both SCLC and NSCLC components have previously been linked to a lower overall survival than SCLC alone (Radice et al., 1982). With neuroendocrine differentiation thought to occur spontaneously in 10% to 30% of all NSCLC (Ionescu et al., 2007), a subset of cells within a tumor may promote poorer prognoses and responses than predicted by original pathology.

The neuronal transcription factor neurogenic differentiation 1 (NeuroD1) is overexpressed in a variety of aggressive neural/neuroendocrine carcinomas. NeuroD1 is important for the development and function of several neural/neuroendocrine tissues, the fate of specific neurons in the central and peripheral nervous system and for insulin gene transcription in adult pancreatic β cells (Miyata et al., 1999b; Naya et al., 1995a). NeuroD1 mutations can lead to maturity-onset diabetes of the young 6 (MODY6) (OMIM 606394), the only setting in which NeuroD1 is thus far known to be critical to disease (Malecki et al., 1999b).

To investigate the role of NeuroD1 in tumorigenesis we focused on neuroendocrine lung cancer cells lines and isogenic normal and tumorigenic

immortalized human bronchial epithelial cells (HBECs). We observed that down-regulation of NeuroD1 prevents survival, invasion, and metastasis of several neuroendocrine lung cancer cell lines. TrkB and NCAM are pro-metastatic signaling molecules downstream of NeuroD1 and are responsible at least in part for the phenotypic consequences of NeuroD1 expression.

RESULTS

NeuroD1 is highly expressed in aggressive neuroendocrine lung cancers

NeuroD1 is overexpressed in several aggressive neural/neuroendocrine cancers including SCLC, medulloblastoma, gastric and prostate cancers, and pituitary adenomas (Abrahamsson, 1999; Cantile et al., 2005; Cindolo et al., 2007b; Fratticci et al., 2007; Gupta et al., 2008b; Rostomily et al., 1997; Syder et al., 2004a). To characterize the mechanisms of NeuroD1 action in lung tumor pathogenesis we analyzed a panel of lung cell lines. HBEC cell lines, assigned a number to distinguish lines from different individuals, are immortalized by overexpression of CDK4, and hTERT, (e.g., HBEC3KT) (Ramirez et al., 2004). The immortalized HBEC3KT cell line was sequentially transformed by knockdown of the tumor suppressor p53 and expression of K-RasV12 (HBEC3KTR_L53) (Supplemental Table 1) ((Sato et al., 2006b); and manuscript in preparation). Microarray analysis of lung cell lines revealed that 11 of the 20 SCLC and 3 of the 5 neuroendocrine NSCLC (NSCLC-NE) had significantly higher expression of *NEUROD1* compared to HBEC and NSCLC (Fig. 1A and S1A). We confirmed the neuroendocrine cell lines generally expressed high levels of NeuroD1 compared to HBEC and other lung cancer cell lines (Fig. 1B and 1C). We conducted

further mechanistic studies in three SCLC cell lines (H69, H82 and H2171) and one NSCLC-NE (H1155),

To investigate the indication that various NSCLC tumors spontaneously undergo neuroendocrine differentiation, microarray analysis was performed on an additional 275 NSCLC primary resected patient samples not previously annotated as neuroendocrine (Fig. S1B). Validation of a subset of the NSCLC tumor samples revealed several had relatively high *NEUROD1* expression compared to H69, while the majority of tumor samples expressed more *NEUROD1* than HBEC (Fig. 1D). As we suspected, several tumors annotated as NSCLC (specifically adenocarcinomas and squamous cell carcinomas) may have undergone neuroendocrine differentiation.

NeuroD1 regulates survival and metastasis of neuroendocrine lung cancers

To test if NeuroD1 was essential for tumorigenesis, we established SCLC and NSCLC-NE lines that stably expressed shRNAs against NeuroD1 (Fig. S1C). Depletion of NeuroD1 prevented survival of the neuroendocrine lung cancer cells as measured by reduction of colonies in soft agar (Fig. 1E and S1D)**.** The residual soft agar colonies were less than 25% of the control colony size, suggesting defects in sustained growth (Fig. S1E). This phenotype could be partially rescued by a shRNA-resistant mouse NeuroD1 plasmid (Fig. S1F and S1G). As many patients present with metastases at the time of diagnosis, we investigated the ability of NeuroD1 to regulate migration. Overexpression of NeuroD1 in HBEC3KT resulted in a significant increase in the motility of the cells (Fig. 1F and 1G and S1H), while reduction of NeuroD1 in Clone 5 resulted in a significant decrease in motility, as well as decreased soft agar colony formation (Fig.1I and S2A). To test the effect of NeuroD1 on motility in a non-neuroendocrine NSCLC cell

line, we overexpressed NeuroD1 in the adenocarcinoma cell line H358 and found a three-fold increase in motility (Fig. 1H), suggesting overexpression of NeuroD1 regulates cell migratory potential.

To investigate the tumorigenic role of NeuroD1, neuroendocrine lung cancer cells expressing shNeuroD1 or shControl (pGIPZ) were injected subcutaneously or intravenously into immune-compromised mice. In the subcutaneous xenograft model, knockdown of NeuroD1 in the neuroendocrine lung cancer cells resulted in a substantially reduced rate of tumor growth and weight (Fig. 2A and S2B). We next examined dissemination using H69 stably expressing luciferase (H69-luc). H69-luc expressing shControl colonized to multiple sites, brain, lung, kidney, and lymph nodes, following tail vein injection, whereas knockdown of NeuroD1 prevented colonization and metastasis (Fig. 2B and S2C). These results provide evidence that NeuroD1 is necessary and sufficient for not only anchorage-independent growth and motility but also tumorigenic and metastatic potential.

NeuroD1 downstream targets, TrkB and NCAM, regulate survival and migration in neuroendocrine lung cancer

Expression of both NCAM and TrkB correlates with and is potentially regulated by NeuroD1 during neuronal/neuroendocrine differentiation (Kaplan et al., 1993; Liu et al., 2011; Liu et al., 2004; Seki, 2002; Seo et al., 2007). TrkB was identified in an unbiased screen for genes that overcame anoikis and is implicated in metastases of several cancers (Au et al., 2009; Douma et al., 2004; Kupferman et al., 2010). NCAM, like TrkB, regulates neuronal differentiation, cell survival, neurite outgrowth, migration and also has been implicated in metastasis (Cavallaro et al., 2001; Daniloff et al., 1986;

Hoffman et al., 1986; Vutskits et al., 2001). NCAM and TrkB both exhibited higher expression in neuroendocrine cancer cells than in HBEC or NSCLC (Fig. S3A and S3B). We also observed that active Trk was more highly expressed in lung cancer cells than in HBEC3KT or Clone 8 (a non-tumorigenic clone of HBEC3KTR_L53); however a small amount of phosphorylated Trk was detected in Clone 5 (Fig. S3A). Analysis of the NSCLC lung cancer patient samples confirmed a significant correlation between expression of *NEUROD1* and both *NCAM* and *NTRK2* (TrkB) (Fig. 3A). In neuroendocrine lung cancer cells, expression of NCAM and TrkB was reduced following knockdown of NeuroD1 (Fig. 3B, S3C and S3D).

We confirmed that endogenous NeuroD1 bound to the expected E box of the TrkB promoter in the neuroendocrine cancer cell lines (Fig. 3C). Since the correlation between NCAM and NeuroD1 expression has been made via overexpression data and promoter binding predicted *in silico*, we investigated whether endogenous NeuroD1 directly bound NCAM promoter elements (Kashiwagi et al., 2012; Lee et al., 1995; Neptune et al., 2008b; Seo et al., 2007). We found ten consensus E-box CANNTG binding sites upstream of the transcriptional start site in the NCAM promoter. Two of the E boxes (located at -50 and -2350) were NeuroD1-preferred binding sites (Seo et al., 2007). Enhanced NeuroD1 binding was observed on both sites in the neuroendocrine lung cancer cell lines, whereas no binding was observed in HBEC3KT (Fig.3D).

Next we inquired whether loss of NCAM or TrkB would phenocopy loss of NeuroD1. Reduction in expression of either TrkB or NCAM also led to a decreased ability of neuroendocrine cell lines to form colonies in soft agar (Fig. 3E, 3F and S3E, S3F). Additionally, as with NeuroD1, overexpression of TrkB in HBEC3KT and

HBEC30KT led to two-fold and five-fold increases in motility, respectively as compared to controls (Fig. 3G and S3G). Loss of TrkB also led to a decrease in the sustained rate of tumor growth (Fig. 3H). To further explore the relationship between NeuroD1 and TrkB, we knocked down either NeuroD1 alone or NeuroD1 in conjunction with TrkB. Loss of NeuroD1 alone or together with TrkB decreased the ability of the cells to invade through matrigel (Fig. 3I and S3H). The loss of invasion caused solely by NeuroD1 depletion was substantially restored by overexpression of TrkB. On the other hand, complementation with mouse Neurod1 did not restore invasion caused by depletion of both proteins (Fig. 3I and S3H), confirming that NeuroD1 acts mostly through TrkB.

Lestaurtinib, an inhibitor of the FLT3, JAK2 and Trk tyrosine kinases, has progressed to phase II clinical trials for the treatment of acute myelogenous leukemia (Knapper et al., 2006; Thiele et al., 2009). Sub-nanomolar concentrations of lestaurtinib reduced the ability of NeuroD1-expressing cell lines to form colonies in soft agar (Fig.4A and S4A). In comparison, 1000-fold higher drug concentrations were required to perturb colony formation by HBEC3KT or NSCLC with little NeuroD1 (Fig. 4A, 4B, and S4B), suggesting the potential for a therapeutic window to treat properly selected neuroendocrine cancers in a clinical setting. Treatment with lestaurtinib resulted in a significant reduction in the rate of tumor growth in xenografts, consistent with a reduction in phosphorylated TrkB (Fig. 4C and S4C). We also found that lestaurtinib significantly decreased the ability of the cells to invade matrigel, indicating the importance of TrkB activity in neuroendocrine lung cancer invasion (Fig. 4D).

NCAM signaling was initially thought to occur via tyrosine phosphorylation by the fibroblast growth factor receptor (Abrahamsson, 1999; Kiselyov et al., 2003; Soroka et

al., 2003) and recently by TrkB (Cassens et al., 2010). We investigated whether changing TrkB signaling would alter NCAM modification in SCLC. Treatment with the TrkB ligand, brain-derived neurotrophic factor (BDNF), increased tyrosine phosphorylation while treatment with lestaurtinib decreased its tyrosine phosphorylation, consistent with the evidence that TrkB can phosphorylate NCAM in neuroendocrine lung cancers (Fig 4E). Taken together these data suggest this interaction may rely on the initiation or maintenance of NCAM and TrkB expression by NeuroD1.

Discussion

NeuroD1 and other factors such as the lineage-restricted oncogene, ASCL1 have been shown to be anomalously expressed in a several aggressive neuroendocrine tumors (Rostomily et al., 1997). Initial examination of microarray data revealed that subsets of aggressive small cell lung cancers (SCLC) and certain neuroendocrine NSCLC have high expression of NeuroD1 as compared to HBEC and other NSCLC. Mechanism of action of NeuroD1 is carried out by downstream targets which include the signaling molecules: the tyrosine kinase TrkB, and NCAM. Neuroendocrine differentiation of tumors has become a topic of interest as differentiation of these tumors from epithelial cells has been hypothesized to be involved in acquisition of invasive/metastatic phenotypes (Cindolo et al., 2007a; Gupta et al., 2008a; Syder et al., 2004b). NeuroD1 expression has recently been speculated to contribute to the transformation of epithelial cells to neuronal-like cells (Cindolo et al., 2007a); this transformation may be the onset or termination neuroendocrine differentiation of prostate and other neuroendocrine cancers.

TrkB induces neuronal migration and, similar to NeuroD1, neuronal differentiation (Kaplan et al., 1993; Liu et al., 1997; Luikart et al., 2008a). TrkB has also recently been implicated in invasion and metastasis of pancreatic, prostate, and colorectal cancers (Douma et al., 2004; Sclabas et al., 2005). TrkB is mutated or overexpressed in certain NSCLC, ovarian, prostate, pancreatic, and gastric cancers and can suppress cell death caused by loss of cell-substratum contacts. NCAM, like TrkB, also regulates survival, differentiation and migration of neurons (Abrahamsson, 1999; Cassens et al., 2010; Ditlevsen et al., 2008; Vutskits et al., 2001). NCAM is highly expressed in neuroendocrine lung tumors and immunotherapy using an antibody targeting NCAM linked to the microtubule-depolymerizing agent BB10901 has entered phase II clinical trials for SCLC (Blackhall and Shepherd, 2007). We hypothesize that overexpression of NeuroD1 may contribute to the development and metastasis of extremely aggressive SCLC, via regulation of each of these downstream factors involved in differentiation, cell survival and invasiveness.

Experimental Procedures

Plasmids, Primers, and Luciferase Assays

Stable shNeuroD1 and shTrkB cells lines were generated via infection of human pGIPZ lentiviral shRNA plasmids created by the RNAi Consortium. These were purchased by UT Southwestern as a library (TRC-Hs1.0 (Human)) from Open Biosystems. NeuroD1 short hairpins V2LHS_152218 (shRNA-1), V2LHS_152220 (shRNA-2), TrkB short hair

pins, V2LHS_63731, and NCAM short hair pins V2LHS_111710 and were used (sequences are available online at Open Biosystem website). SCLC cell lines were selected in puromycin (<2 mg/ml) for 6 days. Plasmids were transfected into 293T cells for viral production using FUGENE 6. Oligonucleotides used were: NeuroD1-1 sense CGAAUUUGGUGUGGCUGUA, antisense UACAGCCACACCAAAUUCG-QIAGEN; NeuroD1-AB sense GGAUCAAUCUUCUCAGGCA, antisense UGCCUGAGAAGAUUGAUCC- AMBION; NTRK2-5 sense GACGAGUUUGUCUAGGAAA , antisense UUUCCUAGACAAACUCGUC-QIAGEN. For p53 experiments, cells were transfected with pCMV5, SV40- internal control, pcDNA.1-p53, or pGL3-NeuroD1 constructs using Fugene HD. Luciferase assays used the Promega dual luciferase kit according to manufacturer's protocol

Quantitative Real Time PCR

Total RNA from xenograft tumors and cell lines was isolated with TRI Reagent. RNA from tumor samples was from MD Anderson Cancer Center. cDNA was synthesized using iSCRIPT cDNA Synthesis Kit (BIO-RAD). RNAs for mouse and human NeuroD1, TrkB, NCAM, 18s ribosomal RNA were quantified by RT-PCR with iTaq (Bio-Rad) master mix using TaqMan probes (Applied Biosystems) on an ABI 7500 thermocycler. Relative transcript levels were normalized to 18s rRNA. Transcript amounts in knockdown cells were plotted as fold change relative to control. Data were analyzed using ABI 7500 system software.

Statistical Analyses

Student's *t* test, one-way analysis of variance (ANOVA), Pearson's test, and linear regression were used to determine statistical significance. Statistical significance for all tests, assessed by calculating the P values and was defined as <0.05.

Chromatin Immunoprecipitation

ChIP was performed as previously described (Lawrence et al., 2005). TrkB primers were as described (Liu et al., 2004). NCAM E-box primers -2350 Forward GGGGAGAGAGGTCCAGTGA -2350 Reverse TTCTAGAATGCTGCCCCAGT -50 Forward ATCAAAATATGCAAACTGCTGATTA -50 Reverse CGAACATCAAGGAGGTAAGAGA

Colony Formation and In Vivo Assays

Soft agar and liquid colony assays were as described previously (Sato et al., 2006)(Sato et al., 2006). As indicated, lestaurtinib was added once for 24 hr one day after seeding. In vivo experiments were performed in 6-8 week old NOD/SCID female mice. Viable cells from subconfluent cultures in normal growth medium were counted using Trypan Blue (Invitrogen, Carlsbad, CA). Subcutaneous tumors were elicited by injecting $1.0x10^6$ cells in 0.2 ml PBS into the flank of mice and monitored every 2-3 days. Tumor size was assessed with digital calipers; tumor volume was taken to be equal to the width x length^2 x π/6. The effect of lestaurtinib (LC Laboratories, Woburn, MA) was determined on tumors grown as above. Lestaurtinib or vehicle treatment commenced once tumor volume reached ~200 mm^3. Lestaurtinib was dissolved in 40% polyethylene glycol, 10% polyvinylpyrrolidone, and 2% benzyl alcohol (all from Sigma-Aldrich, St. Louis, MO) in distilled water and administered subcutaneously at 20 mg/kg once daily, five days a week. Tail vein injections of 1.0×10^6 cells in 0.1 ml PBS were used to

compare the metastatic capacity of H69 cells expressing luciferase driven by the CMV promoter (H69-luc). Tumor growth was monitored every week with Bioluminescence Imaging (BLI) following subcutaneous injection of 450 mg/kg D-luciferin oftline (Biosynth, Switzerland) in PBS into anesthetized mice (Paroo et al., 2004). Images were captured ten min after D-luciferin injection with a 60 sec exposure using a CCD camera (charge-couple device, Caliper Xenogen). Animal care was in accord with institutional guidelines and approved IACUC protocols.

Reagents, Antibodies, Immunoblotting

Immunoblot analyses were as previously described using equal amounts of protein from each sample (Lawrence et al., 2005). The following antibodies were used for blotting, immunoprecipitation and ChIP: goat NeuroD1 (N-19), rabbit pan-phospho-Trk (E-6), synaptophysin (H-8), mouse pTyr (PY20), p53 (DO-1), GAPDH (FL-335) (Santa Cruz) ; mouse ASCL1, mouse N-cadherin, mouse E-cadherin (BD biosciences); rabbit TrkB (Chemicon), mouse CD56/NCAM rabbit β-catenin (Cell Signaling), α-tubulin hybridoma was purchased from The Hybridoma Bank Studies at University of Iowa. Lestaurtinib was purchased from LC labs, MA, BDNF from R&D Systems. Band intensities were quantified using LICOR Odyssey Infrared Imaging System.

Immunoprecipitation of NCAM

SCLC cells were lysed in 0.1% SDS, 1% NP40, and 1% sodium deoxycholate with phosphatase inhibitor cocktail (Sigma). NCAM was immunoprecipitated from lysates with anti-NCAM overnight.

Cell Culture

Min6 cells were grown in Dulbecco Modified Eagles medium with 10% fetal bovine serum (FBS). SCLC and NSCLC lines were from the Hamon Cancer Center Collection (UT Southwestern). SCLC, NSCLC-NE, HBEC3KTRL53-Clone 5 (Sato, et al., submitted) and prostate cell lines were cultured in RPMI 1640 with 10% FBS. Melanoma cell lines were cultured in DMEM with 10% FBS. Immortalized HBECs (except HBEC3KTRL53-Clone 5) (Sato et al., 2006b) were cultured in KSFM (Invitrogen) with 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract. The lung cancer cell lines were DNA fingerprinted using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained either by ATCC or the Hamon Cancer Center. The lines were also tested to be free of mycoplasma by e-Myco kit (Boca Scientific).

Migration Assays

For migration assays, cells were seeded 48 hr following either transient expression of mouse NeuroD1 (pCMV-Neurod1) or human TrkB-YFP in HBEC3KT and HBEC30KT or transient knockdown of NeuroD1 (shNeuroD1) in Clone 5. Transwell migration was assayed in a 10-well Boyden chamber (Neuro Probe, Inc.) or Transwell permeable supports (Corning #3422). Clone 5, H358 and HBEC3KT cells were seeded in the top chamber in either RPMI with 1% FBS or KSFM without FBS, respectively, and allowed to migrate along a concentration gradient through a polycarbonate membrane with 8 µm pores to the bottom chamber containing medium with 10% FBS. For TrkB studies, HBECs transfected with TrkB or empty vector were treated with 100 ng/mL BDNF. After 24 hr cells were fixed, stained (with hematoxylin and eosin stain), and counted. For invasion assays 1.5 x10^5 cells were imbedded in Growth Factor Reduced Matrigel in the

presence or absence of 100 nM lestaurtinib in transwell permeable supports. Cells were allowed to migrate for 48 hr across membranes with a gradient of 10% serum in the bottom chamber. In the wound healing assays, 2 x 10^5 HBEC3KT cells were seeded in 6-well dishes in keratinocyte serum-free medium (KSFM) and grown to confluence for 24-48 hours. A wound was created in confluent monolayers using a sterile pipette tip; cell migration was quantified using ImageJ software after 8 hours.

Microarray Analysis

RNA from tumor samples was from MD Anderson Cancer Center. RNA was prepared using the RNeasy Midi kit (Qiagen, Valencia, CA) and analyzed for quality on RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA) with Agilent Bioanalyzer software. Five micrograms of total RNA was labeled and hybridized to Affymetrix GeneChips HG-U133A and B according to the manufacturer's protocol (http://www.affymetrix.com) while 0.5 micrograms of total RNA was used for Illumina BeadChip HumanWG-6 V3 (http://www.illumina.com).These data are available in GEO (accession # GSE4824 and GSE32036). Array data were pre-processed with MAS5 (Affymetrix algorithm for probe summarization) or MBCB (Illumina algorithm for background subtraction [Ding et al, NAR 36:e58, 2008]), quantile-normalized and log-transformed.

.

Figure 2-1

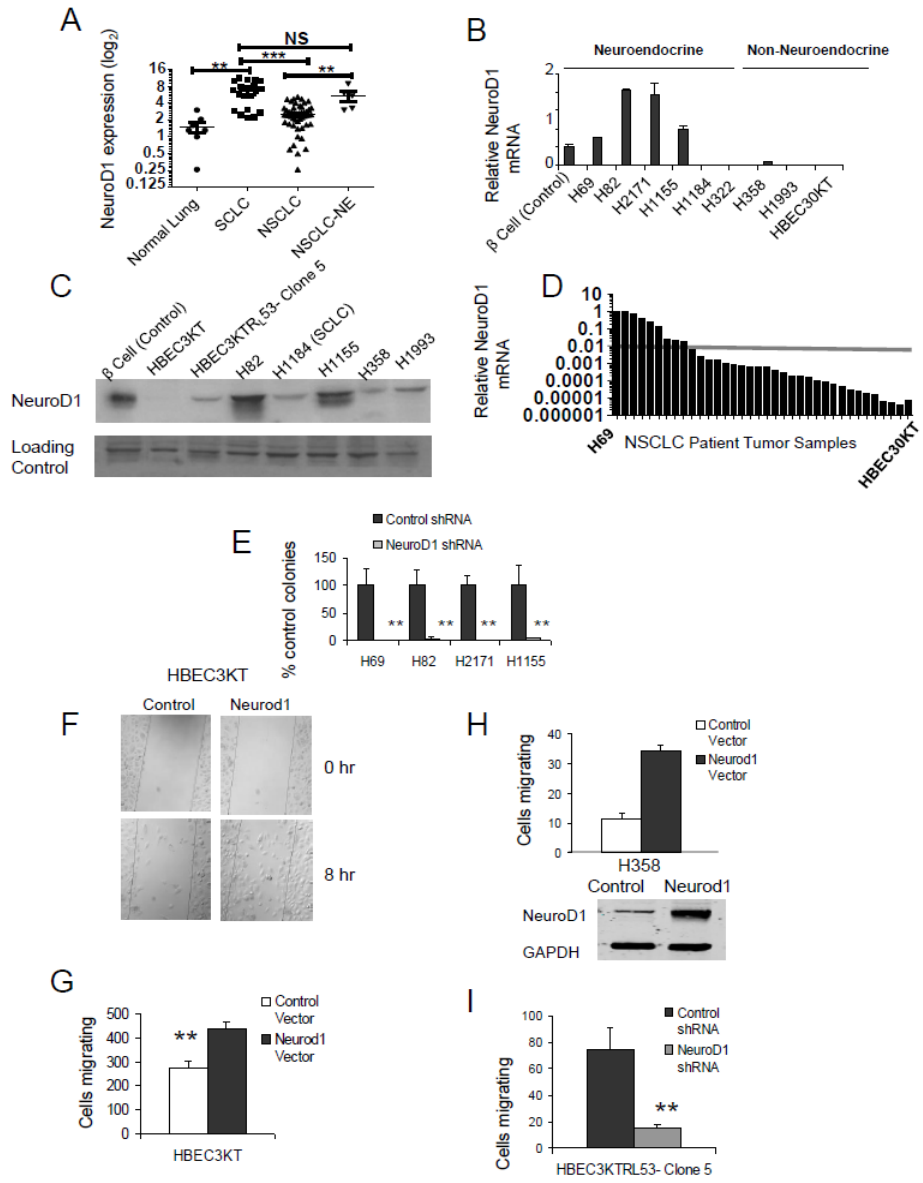


Figure 1. NeuroD1 regulates survival and metastasis of neuroendocrine lung cancers

A) mRNA expression in 86 cell lines, 8 HBEC, 56 NSCLC, and 22 SCLC, was assessed usingAffymetrix HG-U133A & B GeneChips. Cell lines were categorized histopathologically and by *NEUROD1* expression (**p<0.001, ***p<0.0001; two-tailed t-test). B and C) NeuroD1 expression was validated via qRT-PCR and immunoblotting in

cell lines from each type noted above; 50 μg of protein was loaded per lane. Pancreatic β-cell lines are positive controls. D) Thirty-five adenocarcinoma and squamous patient samples analyzed via qRT-PCR to confirm *NEUROD1* expression. Values were normalized to H69 values. The arbitrary line compares expression of *NEUROD1* in the normal bronchial epithelial cell line HBEC30KT. E) Soft agar assays of H69, H82, and H2171 and H1155 infected with shControl or shNeuroD1. Plotted are average number of colonies after two weeks. Error bars, +/-SD of four independent experiments in triplicate (** $p<0.001$; one-way ANOVA). F) G) and H) HBEC3KT and H358 cells were transfected with pCMV-Neurod1, and then subjected to transwell or wound-healing assay. Graph represents mean +/-SD of three independent experiments in duplicate for HBEC3KT (**$p<0.001$; one-way ANOVA). I) Clone 5 cells infected with shNeuroD1 or shControl were subjected to transwell assay. Graph represents mean+/-SD of three independent experiments in triplicate (**$p<0.001$; one-way ANOVA).

Figure 2-2

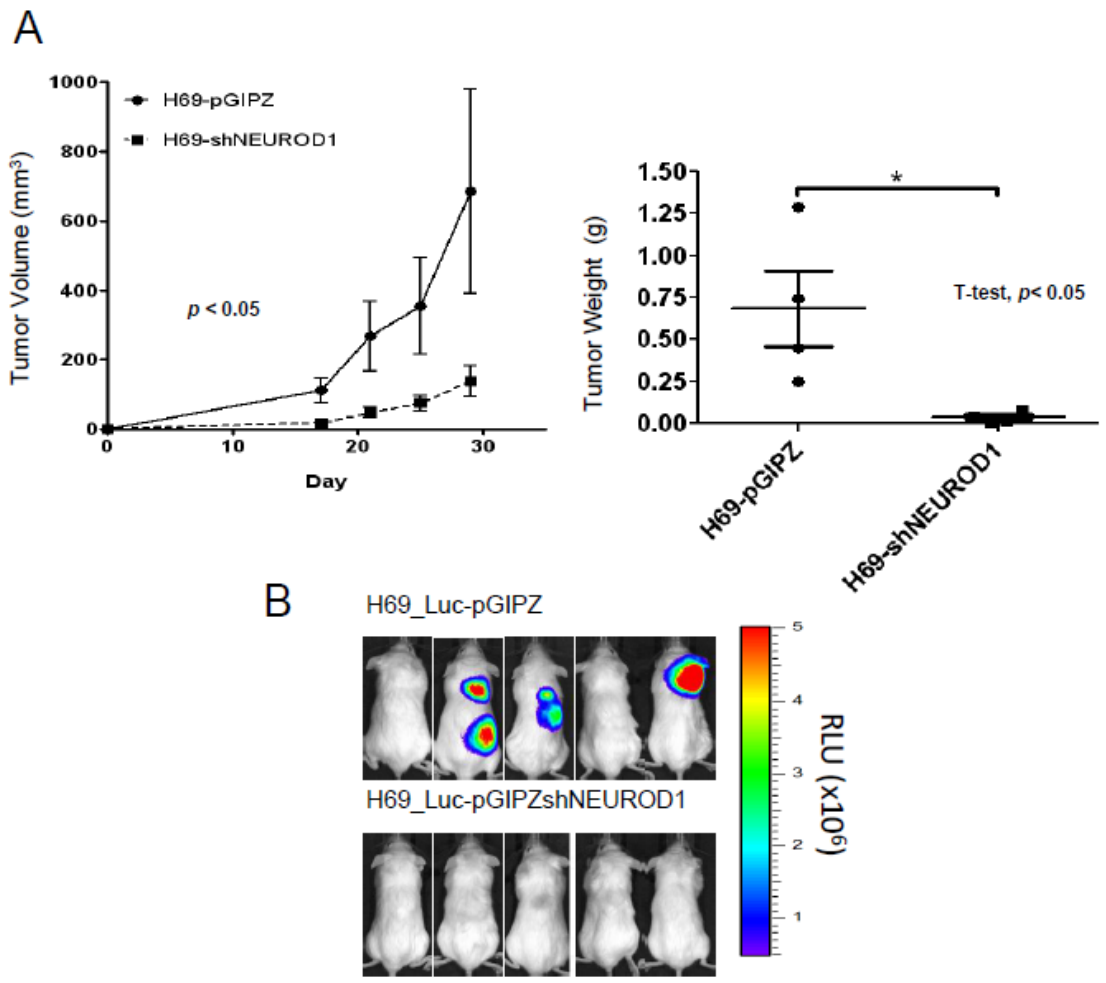


Figure 2-2. NeuroD1 is required for tumorigenesis and metastasis in neuroendocrine lung A) NOD-SCID female mice were injected with 10^6 H69 cells infected with shNeuroD1 or shControl. Tumors were measured every 3-5 days until maximum tumor burden was reached. (N=10, 5 mice per group). *P* values were computed by linear regression (of slopes) for volume measurements and Students' t Test for weights. Means are +/- S.E.M. B) The H69-luc cell line was infected with shcontrol or shNeuroD1. 10^6 cells were injected into the tail vein of mice and monitored for metastases via bioluminescence imaging.

Figure 2-3

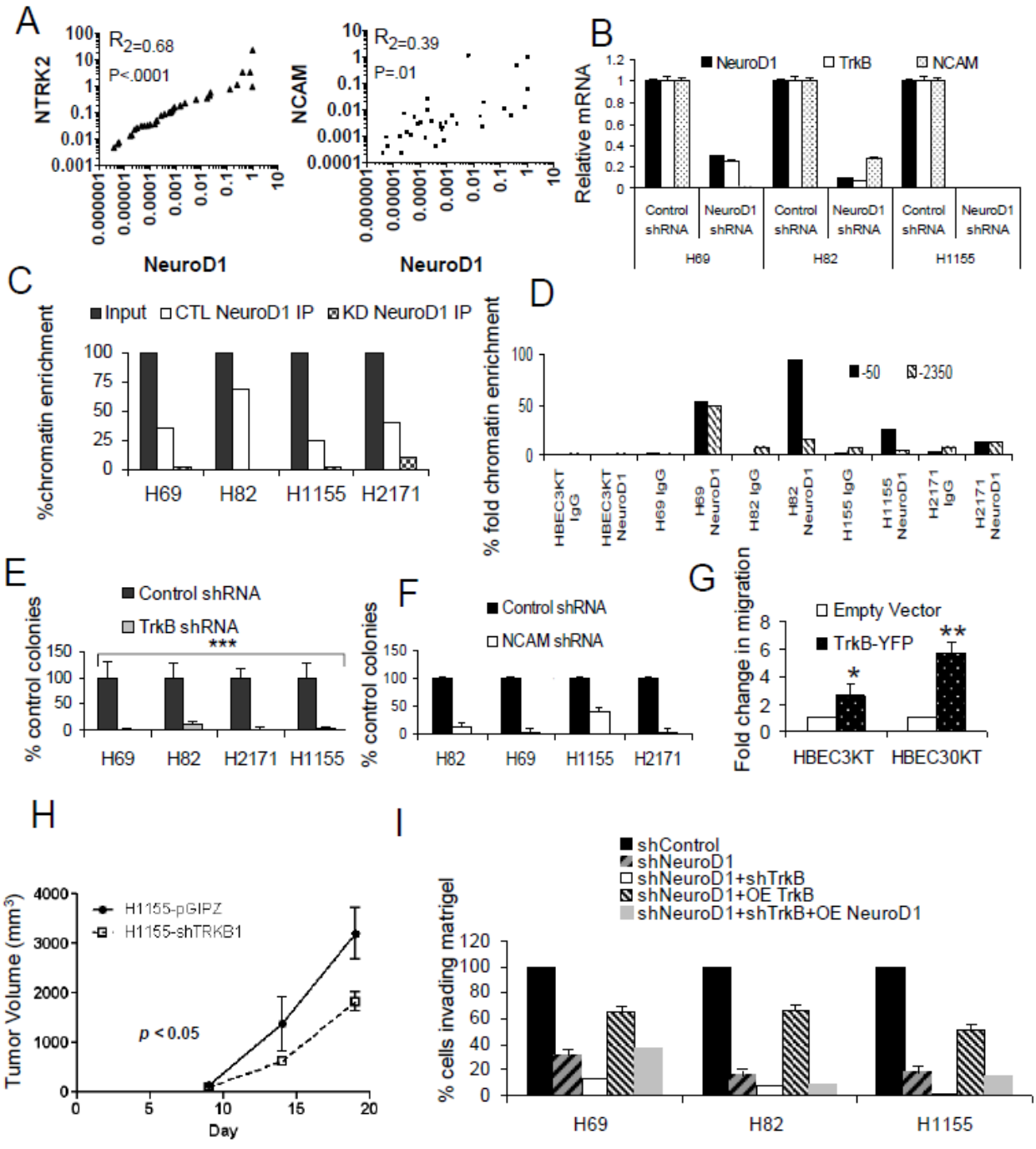


Figure 3. TrkB and NCAM are downstream targets of NeuroD1 that phenocopy loss of NeuroD1 A) XY scatter plots of 35 adenocarcinoma and squamous patient

samples examining correlation between *NEUROD1*, *NRTK2* (TrkB) and *NCAM*. P-values and R^2 values were obtained by Pearson's test. B) qRT-PCR analysis of *NRTK2, NCAM* and *NEUROD1* in lung cancer cells with stable knockdown of NeuroD1. C) ChIP of NeuroD1 on *NTRK2* promoter in cell lines expressing shNeuroD1 or shcontrol (GIPZ). One of four independent experiments in duplicate. D) ChIP of NeuroD1 on two E boxes in the NCAM promoter with NeuroD1 consensus binding sites in HBEC3KT, three SCLC, and a NSCLC-NE. NeuroD1 IP values were compared to input, then plotted as % chromatin fold enrichment normalized to HBEC-3KT. E) Soft agar assay of SCLC and NSCLC-NE lines infected with shControl or shTrkB. The average numbers of colonies after two weeks are shown. Error bars indicate +/-SD from the mean of four independent experiments in triplicate (*** $p<0.001$; one-way ANOVA). F) Cell lines were infected with shControl or shNCAM and subjected to soft agar assay. The average numbers of colonies after two weeks are shown. Error bars indicate +/-SD from the mean of two independent experiments in triplicate. G) HBEC3KT and HBEC30KT cells were transfected with a plasmid encoding human TrkB, then subjected to transwell assay. Graph represents fold mean +/-SD of four and three independent experiments respectively (**$p<0.005$, *$p<0.05$; one-way ANOVA). H) Mice were injected with 10^6 H1155 cells infected with shNeuroD1 or shControl. Tumors were measured until maximum tumor burden was reached. (N=10, 5 mice per group). *P* values were computed by linear regression (of slopes) for volume measurements. Means are +/-S.E.M. I) H69, H82 and H1155 were cell lines were subjected to knockdown of NeuroD1 and/or NeuroD1/TrkB. Knockdown cells were then subjected to overexpression of either

NeuroD1 or TrkB. Cells were then embedded in Growth Factor Reduced Matrigel, for transwell migration assays as described in Methods.

Figure 2-4

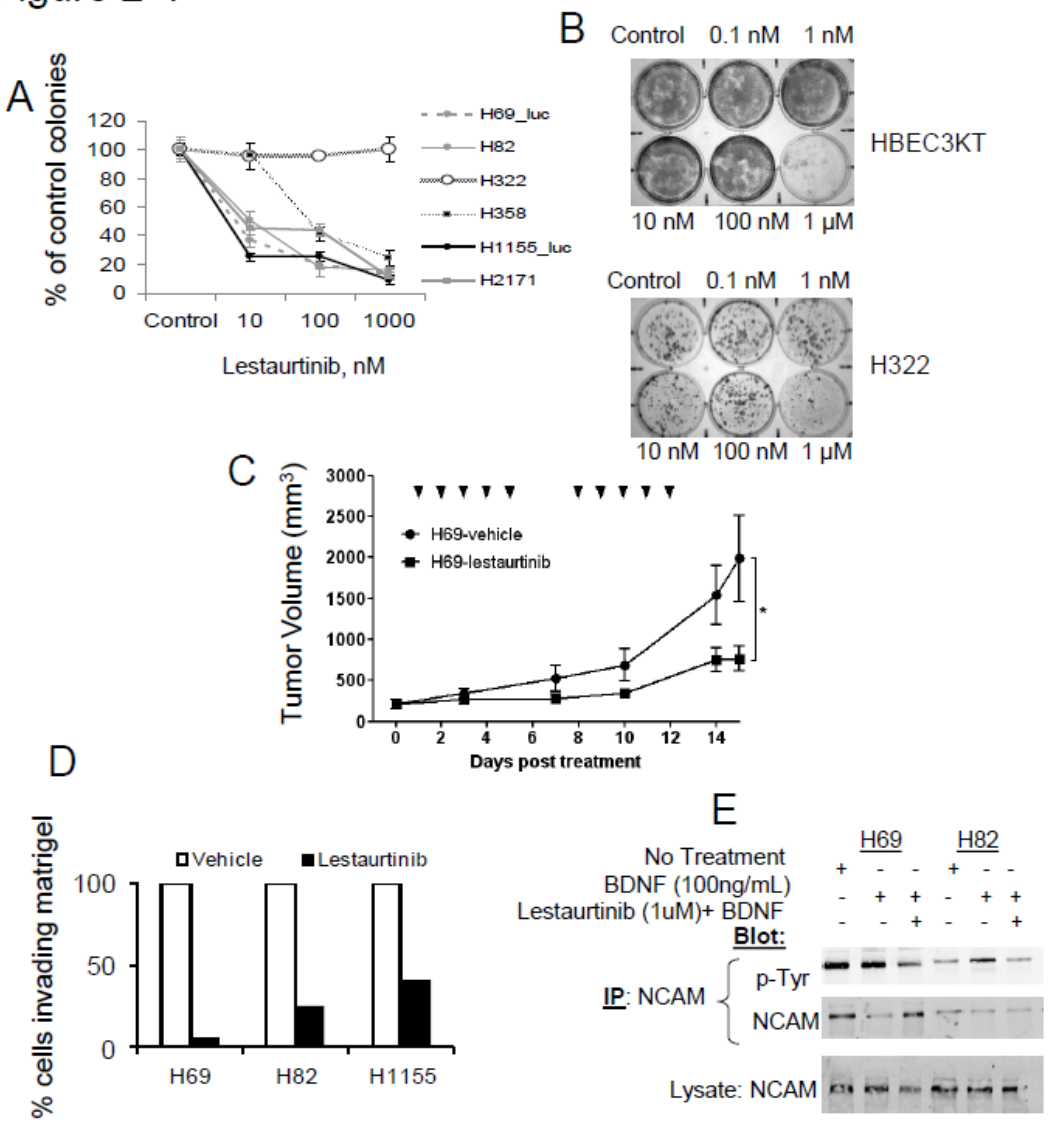


Figure 4. Lestaurtinib regulates survival and invasion

A) Soft agar assays of SCLC and NSCLC-NE cells treated with 10, 100, 1000 nM lestaurtinib. B) Liquid colony assays of HBEC3KT and the NSCLC cell line H322 exposed to increasing concentrations of lestaurtinib. C) 10^6 H69-Luc cells were subcutaneously injected into the flank of mice and monitored every 2-3 days. Lestaurtinib or vehicle treatment commenced once tumor volume reached ~200 mm^3. N=10 for each group. Tick marks represent treatment days. (*p=<0.05; student-T test).

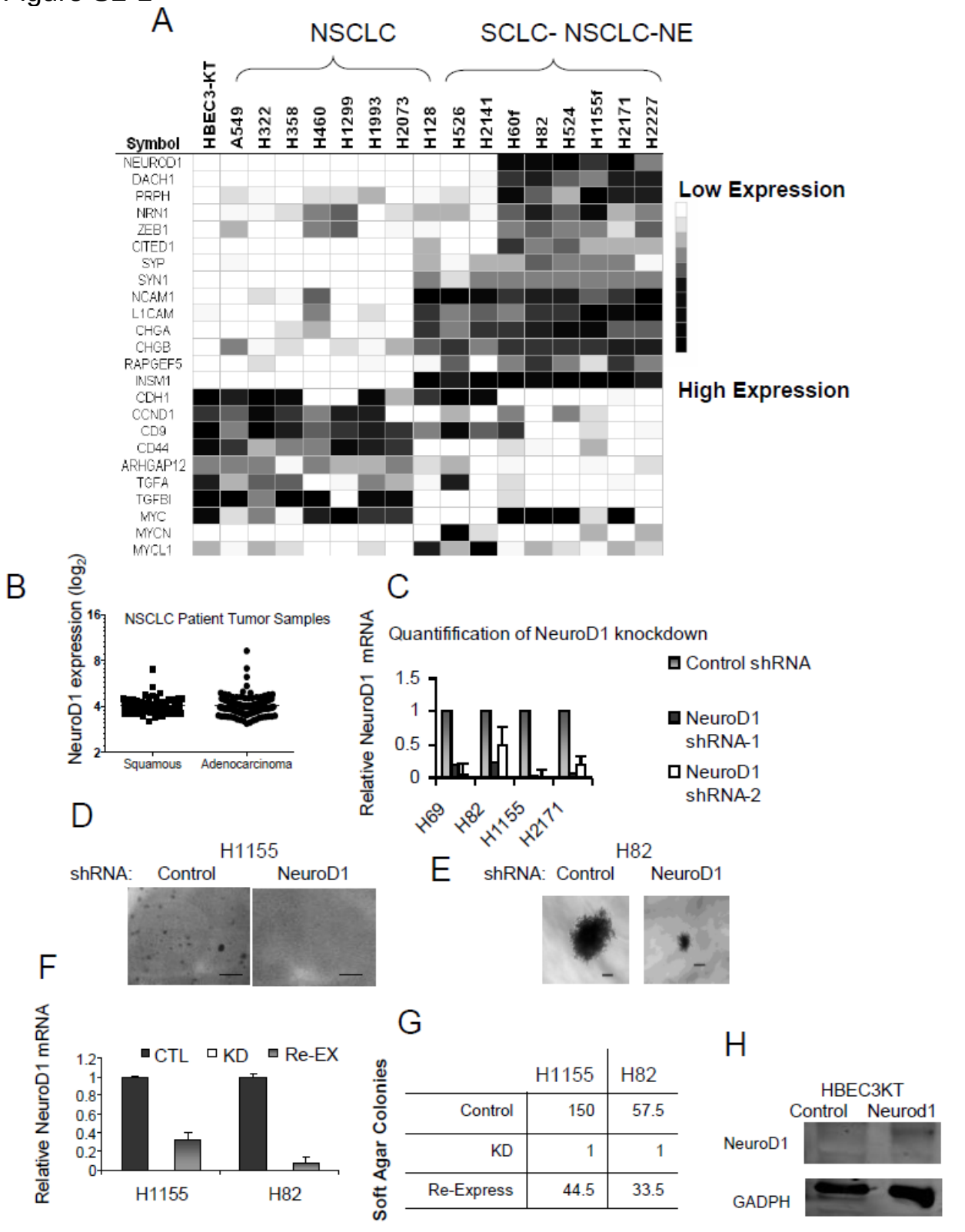
D) Transwell assays of H69, H82 and H1155 embedded in a 1 mm thick layer of Growth Factor Reduced Matrigel with or without 100 μM lestaurtinib. E) SCLC were serum starved for 8 hours then treated with 100 ng/ml BDNF without or with 1 μM lestaurtinib 30 min. NCAM was immunoprecipitated and blotted with anti-phosphotyrosine. Representative of four independent experiments.

Table 2-1

Human Bronchial Epithelial Cell (HBEC) Manipulations

HBEC3-KT HBEC30-KT	CDK4=K, T= hTERT - immortalized, not tumorigenic
HBEC3-KTRL53	CDK4=K, T= hTERT , RL =oncogenic K-Ras 53= knockdown of p53, tumorigenic
HBEC3-KTRL53 Clone 5 Clone 8	Clone 5: CDK4=K, T= hTERT , RL =oncogenic K-Ras 53= knockdown of p53, a tumorigenic clone Clone 8 :CDK4=K, T= hTERT , RL =oncogenic K-Ras 53= knockdown of p53, a non- tumorigenic clone

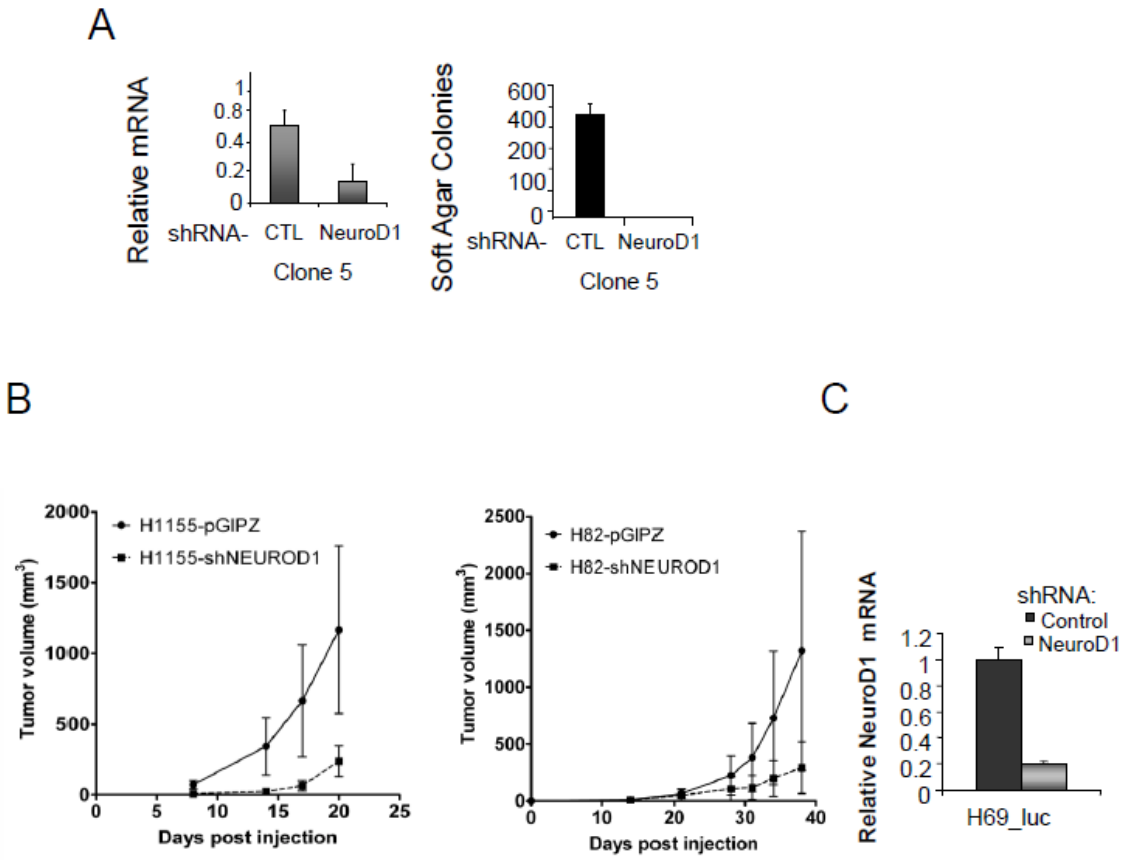
Figure S2-1


A
NSCLC
SCLC- NSCLC-NE
HBEC3-KT
A549
H322
H358
H460
H1299
H1993
H2073
H128
H526
H2141
H60f
H82
H524
H1155f
H2171
H2227
Symbol
NEUROD1
DACH1
PRPH
NRN1
ZEB1
CITED1
SYP
SYN1
NCAM1
L1CAM
CHGA
CHGB
RAPGEF5
INSM1
CDH1
CCND1
CD9
CD44
ARHGAP12
TGFA
TGFBI
MYC
MYCN
MYCL1
Low Expression
High Expression
B
NeuroD1 expression (log2)
NSCLC Patient Tumor Samples
Squamous
Adenocarcinoma
C
Quantifiification of NeuroD1 knockdown
Relative NeuroD1 mRNA
Control shRNA
NeuroD1 shRNA-1
NeuroD1 shRNA-2
H69
H82
H1155
H2171
D
H1155
shRNA: Control NeuroD1
E
H82
shRNA: Control NeuroD1
F
Relative NeuroD1 mRNA
CTL
KD
Re-EX
H1155
H82
G
Soft Agar Colonies
H1155 H82
Control 150 57.5
KD 1 1
Re-Express 44.5 33.5
H
HBEC3KT
Control Neurod1
NeuroD1
GADPH

Supplemenl Figure1. NeuroD1 microarray and functional assay (Associated with figure 1)

A) mRNA expression was analyzed in HBEC, NSCLC and SCLC using Affymetrix HG-U133A & B GeneChips. B) A) mRNA expression in 275 NSCLC lung cancer patient samples assessed using Illumina BeadChip HumanWG-6 V3. C) The stable cell lines created and tested in this supplemental figure were used throughout the paper in experiments employing NeuroD1 knockdown. RNA was extracted from cells, reverse transcribed to cDNA and used for qRT-PCR to quantitate knockdown efficiency. Subsequent studies used shRNA-2 unless otherwise stated. D and E) Formation of colonies in soft agar by H1155 and H82 cells in which NeuroD1 was stably knocked down was measured. Pictures are at 4x (H1155) and 10x (H82) magnification. F) Cells as in (D and E) were transiently transfected with a plasmid encoding mouse Neurod1. Expression was confirmed by mRNA analysis (left).

Figure S2-2

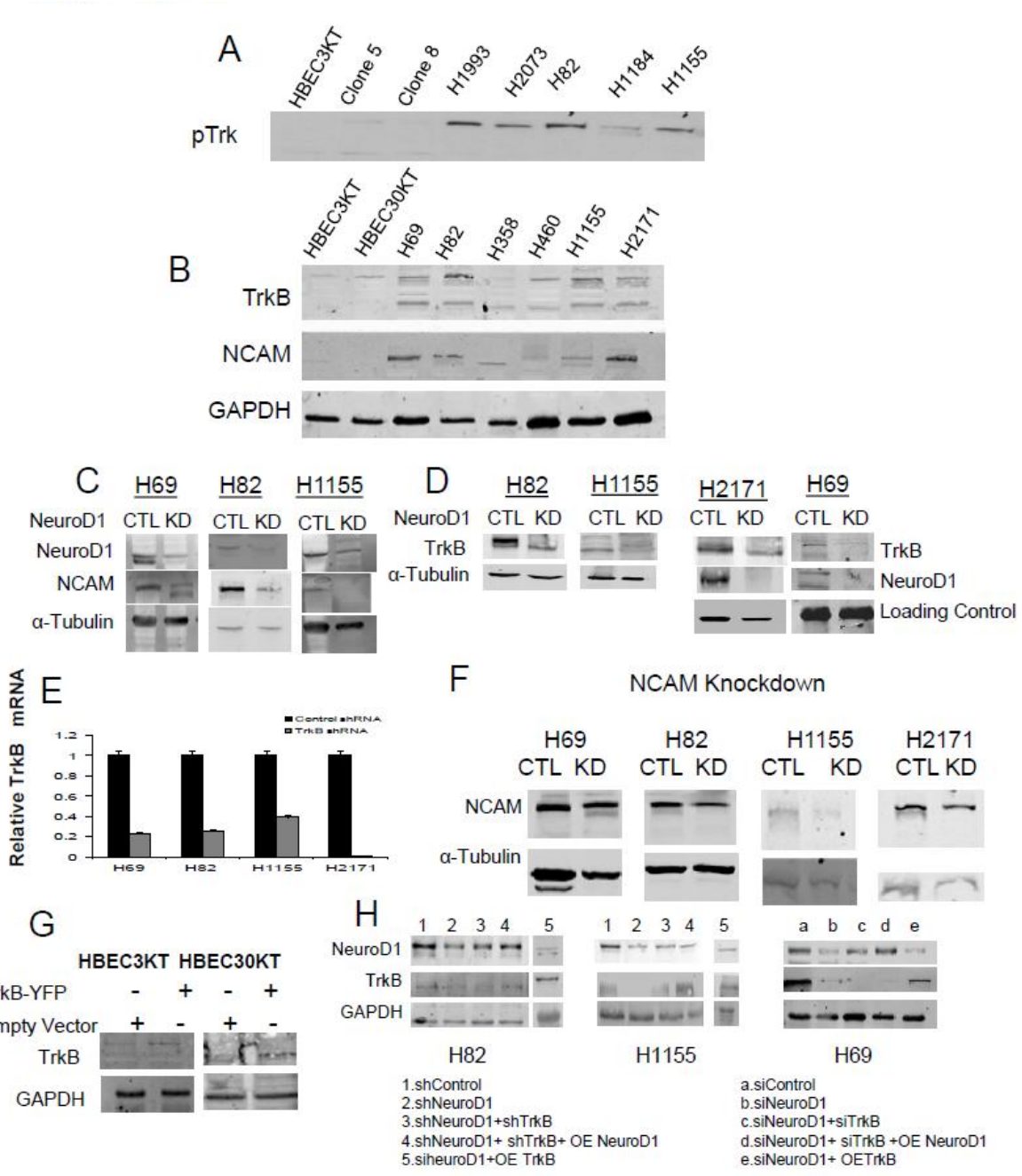


Supplemental Figure 2. Analysis of aspects of NeuroD1 function (Associated with figure 2)

A) HBEC3KTRL53- Clone 5 cells were infected with viruses expressing NeuroD1-1 or control shRNAs. NeuroD1 mRNA was quantitated by qRT-PCR (left). Formation of colonies in soft agar by Clone 5 cells was measured (right). B) NOD-SCID female mice were injected with 10^6 H1155 or H82 cells infected with either control shRNA or NeuroD1 shRNA. Tumors were measured every 3-5 days until maximum tumor burden was reached, N=5 mice per group. *P* values were computed by linear regression (of slopes). Data means are +/- S.E.M. C) The H69-luc cell line was infected with control or

NeuroD1 shRNA and knockdown was quantified via qRT-PCR for Figure 2A and 2B. H) Overexpression of NeuroD1 in HBEC3KT.

Figure S2-3

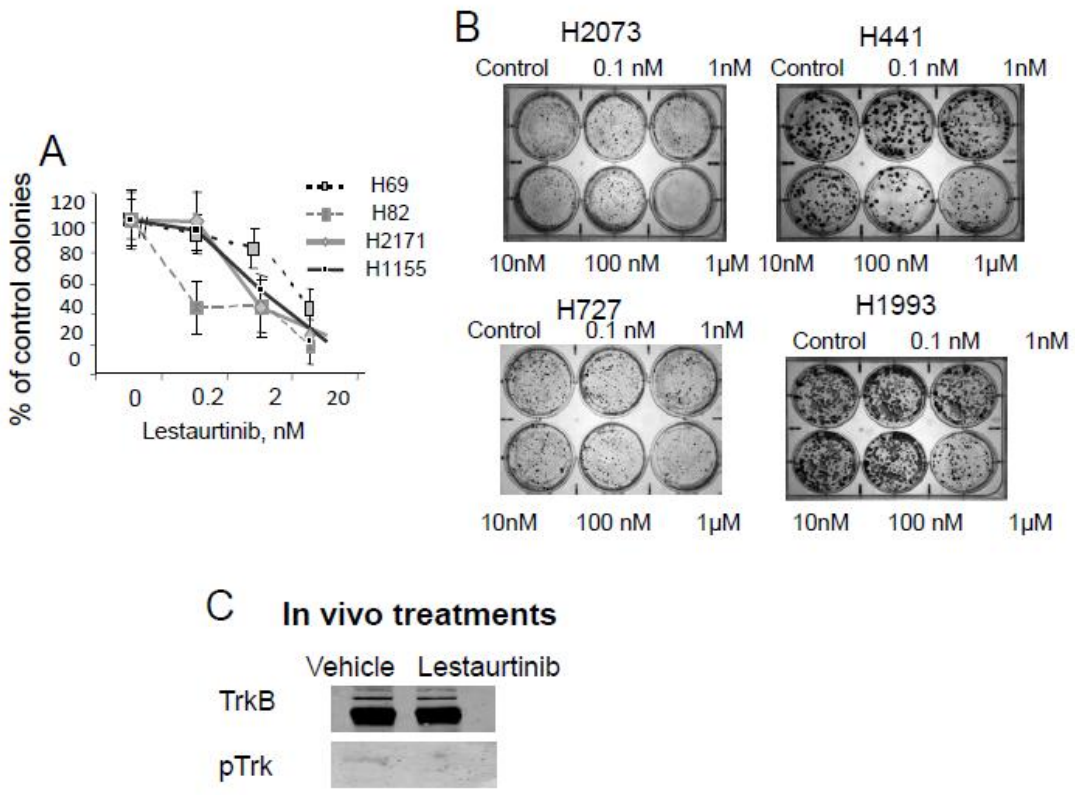


Supplemental Figure 3. Studies of NCAM and TrkB (Associated with figure 3)

A) Lysates of HBEC3KT, Clones 5 and 8, and SCLC, NSCLC and NSCLC-NE were immunoblotted for Active Trk. B) Lysates of HBEC cell lines, SCLC and NSCLC

immunoblotted for NCAM and TrkB. C and D) Lysates of cells in which NeuroD1 was stably knocked down were blotted for NCAM (C) and TrkB (D). (The H82 and H1155 blots accompany NeuroD1 knockdown in Figure 3C) Loading control for H69 is GAPDH, loading control for H2171 isα- tubulin. E) Knockdown of TrkB was quantified by qRT-PCR. F) Knockdown of NCAM was quantified by immunoblotting. G) HBEC3KT and HBEC30KT were transfected with a plasmid encoding TrkB or control vector. Cell lysates were immunoblotted for TrkB and for GAPDH as loading control. H) H69, H82 and H1155 cell lines were transfected with siRNA oligonucleotides or shRNA vectors against NeuroD1 and NeuroD1/TrkB. Knockdown cells were subjected to overexpression of either NeuroD1 or TrkB mammalian expression vectors. Cells were subjected to migration experiments and lysed and immunoblotted for NeuroD1, TrkB and GAPDH as loading control.

Figure S2-4



Supplemental Figure 4. Colony formation with lestaurtinib (Associated with figure 4)

A) Formation of colonies in soft agar was assayed in three SCLC and one NSCLC-NE cell lines then exposed to 0.2, 2, and 20 nM lestaurtinib or control. B) Liquid colony assays was examined in four NSCLC cell lines(H441, H2073, H1993- adenocarcinoma and H727- a neuroendocrine carcinoid) treated once with increasing concentrations of lestaurtinib. Colonies were stained after two weeks. C) Xenograft tumors were homogenized and TrkB was immunoprecipitated and immunoblotted with pTrk antibody

CHAPTER THREE

NeuroD1-expressing neuroendocrine carcinomas are differentially sensitive to TrkB inhibition

Adapted from Osborne J.K., Larsen J.E., Gonzales, J.X., Girard, L., Shames, D.S., Sato, M., Minna J.D., Cobb M.H (2013). Manuscript Submitted to ONCOGENE

Abstract

The developmental transcription factor NeuroD1 is anomalously expressed in a subset of aggressive neuroendocrine tumors. Previously we demonstrated that TrkB and NCAM are downstream targets of NeuroD1 that contribute to its actions in neuroendocrine lung cancer. In this study we examined the importance of NeuroD1 expression in malignant melanoma and prostate cell lines. As in neuroendocrine lung cancers, high levels of NeuroD1 were accompanied by expression of TrkB. Inhibiting TrkB activity decreased invasion in a subset of neuroendocrine pigmented melanomas but not in prostate cell lines, suggesting that sensitivity may be dependent on cell of origin. We also found that loss of the tumor suppressor p53 increased NeuroD1 expression in normal human bronchial epithelial cells and cancer cells with neuroendocrine features. These findings suggest NeuroD1 is a lineage-dependent oncogene acting through its downstream target, TrkB, in multiple cancer types. These results provide insight into the pathogenesis of neuroendocrine cancers.

Introduction

Aberrant expression of basic helix loop helix (bHLH) transcription factors such as neurogenic differentiation 1 (NeuroD1) and achaete-scute homolog1 (ASCL1) has been observed in aggressive neural and neuroendocrine carcinomas (Borges et al., 1997; Cindolo et al., 2007b; Hiroshima et al., 2006a; Rostomily et al., 1997). While the developmental roles of these bHLH proteins are well established, their possible causative roles in the pathogenesis of neuroendocrine carcinomas are less understood. Neuroendocrine tumors can initiate from almost any organ system. While described based on organ of origin, this feature is not limiting, as many of these tumors share pathological characteristics, such as expression of the neuroendocrine markers synaptophysin and chromogranin A, and the neural cell adhesion molecule (NCAM, also known as CD56) (Kaufmann et al., 1997; Klimstra et al., 2010a; Klimstra et al., 2010b). Neuroendocrine tumors are thought to originate from neuroendocrine cells, or undergo an epithelial to neuroendocrine differentiation that leads to more aggressive carcinomas, as observed in melanoma and cancers of the gastrointestinal tract and prostate (Abrahamsson, 1999; Cantile et al., 2005; Cindolo et al., 2007b; Eyden et al., 2005a; Eyden et al., 2005b; Pilozzi et al., 2011; Sutherland et al., 2011; Syder et al., 2004a). To investigate the role of NeuroD1 in tumorigenesis, we examined several tissue types. We report that several pigmented melanoma cell lines express high amounts of NeuroD1 and confirmed previous findings in prostate cell lines (Cindolo et al., 2007b). We find that regulation of TrkB is conserved across multiple tissue types. Additionally, down-regulation of both NeuroD1 and TrkB reduced viability and migration of several carcinomas; however inhibition of TrkB activity only had an effect in the cell

lines expressing neuroendocrine markers. We also determined that loss of p53 is permissive for increased expression of NeuroD1, possibly in a lineage-dependent manner.

Results

NeuroD1 is highly expressed in aggressive neuroendocrine cancers

To investigate the clinical significance, expression of NeuroD1 was examined in a data set including more than 5400 patient samples taken from tumor and normal tissues. Elevated NeuroD1 expression was observed in several malignant tumors including those from neuroendocrine tissues, pancreas, brain, and lung, all of which were SCLC (Figure 1a). Previously, we have demonstrated that NeuroD1 promotes tumor cell survival and metastasis in aggressive neuroendocrine lung tumors through regulation of the receptor tyrosine kinase, TrkB (manuscript submitted). To complement studies in the lung, we examined the role of NeuroD1 in cell lines from non-neural or non-neuroendocrine tissues that undergo neuroendocrine differentiation(Banerjee and Eyden, 2008; Cindolo et al., 2007b). The consequences of NeuroD1 expression have not been investigated in malignant melanoma, even though this factor may induce neuroendocrine differentiation in conjunction with oncogenic B-RAF^{V600E} under certain circumstances (Bhat et al., 2006; Maddodi et al., 2010). NeuroD1 was observed in prostate cancer; however, its expression in several of the commonly used prostate cancer cell lines was only noted upon in vitro differentiation with cAMP (Cindolo et al., 2007b). We observed that NeuroD1, TrkB, and NCAM expression was greater in melanoma cell lines reported to have higher pigmentation and metastatic potential (Cuomo et al., 1991; Kluger et al., 2007) (Figure 1b and Supplemental Figure 1a). The

tendency of increasing expression of the three factors with increasing pigmentation appeared to be independent of the mutational status of B-RAF, as all cell lines with the exception of WM3211 have V600 mutations (Halaban et al., 2009; Woodman et al., 2009; Zheng et al., 2009). As in SCLC, increased expression of the neuroendocrine marker synaptophysin was also detected in melanomas with high NeuroD1 (Figures 1b and 1c). NeuroD1 was also expressed in the undifferentiated malignant prostate cell lines; however, neither of the neuroendocrine markers, synaptophysin or NCAM, were detected (Figure 1d).

Loss of p53 increased NeuroD1 expression

We next sought to investigate specific onco-genotypes possibly responsible for expression of NeuroD1. To do this we utilized HBEC cell lines that were assigned a number to distinguish lines from different individuals and immortalized by overexpression of CDK4, and hTERT, (e.g., HBEC3KT) (Ramirez et al., 2004). The immortalized HBEC3KT cell line was sequentially transformed by knockdown of the tumor suppressor p53 and expression of K-RasV12 (HBEC3KTR$_L$53) (Sato et al., 2006b); (and manuscript in preparation). NeuroD1 expression was increased in HBEC3KT53 cells, a non-tumorigenic derivative with stable knockdown of p53 (Figure 2a). Additionally, an isogenic derivative of HBEC3KT that was transformed from normal to tumorigenic cells followed by clonal selection (HBEC3KTR$_L$53-Clone 5, hereafter called Clone 5) exhibited spontaneous expression of NeuroD1 (Figure 2a). Sustained inactivation of p53 is suggested to enhance tumorigenesis at multiple stages, including initiation and progression (Feldser et al., 2010; Hanahan and Weinberg, 2011; Kemp et al., 1993; Ramirez et al., 2004; Sutherland et al., 2011; Yang et al., 2005). To test a

possible relationship between p53 and NeuroD1, we re-expressed p53 in the tumorigenic cell line Clone 5 and found a substantial decrease in NeuroD1 mRNA (Figure 2b). Next, we utilized a luciferase construct driven by the mouse *Neurod1* proximal promoter to determine if p53 expression affected promoter activity. A 100-fold increase in *Neurod1* promoter activity was observed in immortalized HBEC3KT53 compared to the parental HBEC3KT (Supplemental Figure 1A). Furthermore, re-expression of p53 in HBEC3KT53 and Clone 5 led to a dramatic reduction in *Neurod1* promoter reporter activity (Figure 2c). From this HBEC model we concluded that loss of p53 induced NeuroD1 expression, suggesting that p53 may regulate NeuroD1 early in the pathogenesis of neuroendocrine lung cancer.

To evaluate p53 as a determinant of NeuroD1 expression in neuroendocrine cancers, we analyzed its expression in lung, prostate and melanoma cells with loss (H358, PC3, and YUMAC) or mutant (H1155, M14, SK-MEL-2 and SK-MEL28) p53 (Forbes et al., 2011; Haapajarvi et al., 1999) (Figure 2d). Overexpression of p53 only suppressed NeuroD1 in cells that also had neuroendocrine features, not in the three cell lines with little or no expression of neuroendocrine markers (Figure 2e). Together these results suggest loss p53 is permissive for NeuroD1 expression, not only in neuroendocrine lung cancers but also, as recently suggested, is important for melanoma pathogenesis(Hodis et al., 2012).

NeuroD1 regulates epithelial to mesenchymal transition (EMT)

Loss of cell-cell contacts contributes to oncogenesis and ultimately metastases. Differential expression of NeuroD1 in SCLC has been detected by ourselves and others (manuscript submitted,(Kalari et al., 2012)). We observed that floating, loosely bound

multi-cell aggregates (referred to as variant SCLC) had higher NeuroD1 expression than the floating, but tightly packed spheres (referred to as classical SCLC) (Figure 3a and Table1). The terminology 'variant' refers to a sub-class of SCLC with morphological features similar to NSCLC large cells that in culture are floating, loosely bound multi-cell aggregates. NSCLC-NE and variant SCLC lines that expressed NeuroD1 had little of the lineage-dependent oncogene ASCL1 (Figure 3b). The distinction in morphology among these cell lines has been linked to an increase in both radio-and chemo-resistance as well as an increase in metastatic potential (Doyle et al., 1989). Frequently, loss of E-cadherin is a characteristic of transformed epithelial cells; thus we compared its expression in HBEC and lung cancer cells. Previous microarray data demonstrated an inverse relationship between NeuroD1 and E-cadherin as well as a positive correlation with the EMT transcription factor ZEB1 in NSCLC-NE and SCLC with variant but not classical morphology (manuscript submitted). We confirmed E-cadherin expression was significantly decreased in neuroendocrine lung cancer lines with high NeuroD1 expression, while N-cadherin was unchanged (Figure 3c). Suppressing NeuroD1 resulted in an increase in E-cadherin and ß-catenin, which anchors E-cadherin to the actin cytoskeleton (Figure 3d).

We next investigated if NeuroD1 regulated EMT and/or migration of melanoma and prostate cells. The melanoma lines were more similar to SCLC with respect to E-cadherin expression, as suppression of NeuroD1 increased expression of E-Cadherin; E-cadherin expression was not changed in prostate (Figure 3e). This suggests that NeuroD1 also contributes to metastasis via an increase in EMT.

NeuroD1 and TrkB regulates viability and migration of prostate and melanoma cell lines

Previously, we have demonstrated that knockdown of NeuroD1 and its downstream target TrkB each led to a decrease in survival and migration of neuroendocrine lung cancers. Loss of NeuroD1 reduced TrkB in all lines tested, indicating a conserved connection between NeuroD1 and TrkB across multiple cancer types (Figure 4a). Loss of NeuroD1 in prostate and melanoma lines significantly reduced viability and migration (Figures 4b and 4c). Depletion of TrkB significantly decreased the viability and migration of all the melanoma cell lines (Figures 4d and 4e and Supplemental Figure 2), but prostate cancer cell lines differed, as loss of TrkB decreased migration but not viability (Figures 4d and 4e and Supplemental Figure 2).

TrkB regulates cell survival and migration in melanoma but not prostate cancer cell lines

We next investigated if chemical inhibition of TrkB with lestaurtinib would phenocopy loss of NeuroD1 in melanoma and prostate cancer cells. The melanoma cell lines exhibited a phenotype similar to the neuroendocrine lung cancer cell lines in that treatment with nanomolar concentrations of lestaurtinib reduced cell viability (Figure 5a). The prostate cell lines, however, were resistant to the inhibitor below micromolar concentrations, suggesting a divergence in the actions of TrkB in non-neuroendocrine cells (Figure 5b). The capacity of melanoma cells to invade through matrigel was inhibited by lestaurtinib (Figure 5c), whereas the prostate cell lines were again resistant (Figure 5d). Treatment with lestaurtinib decreased active Trk in the melanoma cell line much as it did on the SCLC cell line, H82 (Figure 5e). Lestaurtinib had no effect on

phosphorylated Trk in the prostate cells lines, offering an explanation of the lack of an effect on migration or viability (Figure 5e). Lestaurtinib did decrease viability and migration of LnCAP; however, these effects were not through a decrease in phosphorylated Trk and may be due to another target of the drug (Figure 5b and 5d). We hypothesized that even if NeuroD1 was highly expressed, its mechanisms of action through TrkB may be dependent on the cell of origin. Neuroendocrine differentiation defined by the presence of synaptophysin, was observed in the melanoma and neuroendocrine lung cancer cell lines, but not in prostate (Figures 1b and 1d). We suggest that NeuroD1 and TrkB regulate migration in neuroendocrine and non-neuroendocrine cancer cells, but the efficacy of targeting TrkB may depend on the cell of origin.

Discussion

Neurogenic bHLH transcription factors, including NeuroD1, are found to have increased expression in neural and neuroendocrine tumors. Whether their expression was causative or solely a consequence of disease had not been determined(Borges et al., 1997; Cantile et al., 2005; Gupta et al., 2008b; Rostomily et al., 1997; Syder et al., 2004a). Recently, NeuroD1 was implicated in the tumorigenesis of neuroblastoma (Huang et al., 2011b). Our data reveal a novel function for NeuroD1 in the induction and coordination of signal transduction pathways that regulate survival and migration of non-neural/neuroendocrine cancers. We now demonstrate that NeuroD1 promotes survival and migration in neuroendocrine lung and other carcinomas at least in part through TrkB.

HBEC models provided a useful system to explore NeuroD1 function. Our studies suggest that p53 negatively regulates NeuroD1 expression not only in HBEC but in carcinomas with neuroendocrine features. Loss of p53 did not unilaterally result in an increase in NeuroD1 expression, as observed in the non-neuroendocrine lung and prostate cell lines. Unlike cells of the prostate, melanocytes derive from neural crest cells migrating from the dorsal neural tube to the dermis making them neuroectoderm in origin (Adameyko et al., 2009; Pavan and Raible, 2012). Furthermore, p53 is not only a potent tumor suppressor but also suppresses self-renewal of adult neural stem cells (Meletis et al., 2006). NeuroD1 has also been shown to enhance proliferation of committed neuronal progenitor cells (Gao et al., 2009). Perhaps p53 mediated inhibition of NeuroD1 in neuroendocrine cells present in nonneural tissues may in some respects parallel its effect on determination of neuronal cell fate.

Surprisingly, inhibition of TrkB kinase activity is apparently not equally significant in the three cancer types examined, lung, melanoma, and prostate. TrkB activity is important for neither migration nor viability in prostate cancer cell lines. We speculate this may be because: 1) prostate cancer cells do not express the neuroendocrine marker synaptophysin; in contrast to melanoma and SCLC, which possibly links them in a neuroendocrine pathology; 2) prostate, unlike melanoma or SCLC, expresses smaller forms of TrkB, possibly TrkB splice variants thought to be inhibitory due to lack of the kinase domain (Ninkina et al., 1997); 3) the effect of NeuroD1 on prostate viability and migration may be mediated by pathways independent of TrkB. Ultimately, additional characteristics must be identified to distinguish TrkB inhibitor-sensitive and insensitive tumor types.

The findings here suggest that NeuroD1 acts as a neuroendocrine lineage-specific regulator of survival and migration. Its expression may be potentiated by loss of p53 and its actions are transmitted mainly through TrkB (Figure 6). The discovery of downstream targets of NeuroD1 in non-neuroendocrine/non-neural tumors is ongoing. The development of drugs that act as inhibitors of transcription factors has proven extremely difficult. Cell surface proteins offer greater opportunities for therapeutic intervention. In particular, TrkB, a receptor and enzyme, has gained attention as a potential target of drug development for neuronal and non-neuronal metastatic carcinomas. NeuroD1-expressing neuroendocrine carcinomas should now also be considered for sensitivity to TrkB inhibitors.

Materials and Methods

Reagents, Antibodies, Immunoblotting

Immunoblot analyses were as previously described using equal amounts of protein from each sample (Lawrence et al., 2005). The following antibodies were used for blotting, immunoprecipitation and ChIP: goat NeuroD1 (N-19), rabbit pan-phospho-Trk (E-6), synaptophysin (H-8), p53 (DO-1), GAPDH (FL-335) (Santa Cruz) ; mouse ASCL1, mouse N-cadherin, mouse E-cadherin (BD biosciences); rabbit TrkB (Chemicon), mouse CD56/NCAM rabbit β-catenin (Cell Signaling), α-tubulin hybridoma was purchased from The Hybridoma Bank Studies at University of Iowa. Lestaurtinib was purchased from LC labs, MA, BDNF from R&D Systems. Band intensities were quantified using LICOR Odyssey Infrared Imaging System.

Quantitative Real Time PCR

Total RNA from xenograft tumors and cell lines was isolated with TRI Reagent. RNA from tumor samples was from MD Anderson Cancer Center. cDNA was synthesized using iSCRIPT cDNA Synthesis Kit (BIO-RAD). RNAs for mouse and human NeuroD1, TrkB, NCAM, 18s ribosomal RNA were quantified by RT-PCR with iTaq (Bio-Rad) master mix using TaqMan probes (Applied Biosystems) on an ABI 7500 thermocycler. Relative transcript levels were normalized to 18s rRNA. Transcript amounts in knockdown cells were plotted as fold change relative to control. Data were analyzed using ABI 7500 system software.

Cell Viability Assay

Cells were plated at a density of 10^5/well and reverse transfected with siRNA for 3 days. Viability after knockdown or drug was assayed using CellTiter- Blue Reagent according to the manufacturer's protocol measuring fluorescence as readout.

Cell Culture

SCLC and NSCLC lines were from the Hamon Cancer Center Collection (UT Southwestern). SCLC, NSCLC-NE, HBEC3KTRL53-Clone 5 (Sato, et al., submitted) and prostate cell lines were cultured in RPMI 1640 with 10% FBS. Melanoma cell lines were cultured in DMEM with 10% FBS. Immortalized HBECs and RWPE(normal immortalized prostate cells) (except HBEC3KTRL53-Clone 5) (Sato et al., 2006b) were cultured in KSFM (Invitrogen) with 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract. The lung cancer cell lines were DNA fingerprinted using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained either by ATCC or the Hamon Cancer Center. The lines were also tested to be free of mycoplasma by e-Myco kit (Boca Scientific).

Migration Assays

For migration assays, cells were seeded 48 hr following knockdown of NeuroD1 or TrkB. Transwell migration was assayed in Transwell permeable supports (Corning #3422). Cells were seeded in the top chamber in either RPMI with 1% FBS and allowed to migrate along a concentration gradient through a polycarbonate membrane with 8 μm pores to the bottom chamber containing medium with 10% FBS. After 24 hr cells were fixed, stained (with hematoxylin and eosin stain), and counted. For invasion assays 1.5 $x10^5$ cells were imbedded in Growth Factor Reduced Matrigel in the presence or absence of 100 nM lestaurtinib in transwell permeable supports. Cells were allowed to migrate for 48 hr across membranes with a gradient of 10% serum in the bottom chamber.

Microarray Analysis

Five micrograms of total RNA was labeled and hybridized to Affymetrix GeneChips HG-U133A and B according to the manufacturer's protocol (http://www.affymetrix.com) while 0.5 micrograms of total RNA was used for Illumina BeadChip HumanWG-6 V3 (http://www.illumina.com).These data are available in GEO (accession # GSE4824 and GSE32036). Array data were pre-processed with MAS5 (Affymetrix algorithm for probe summarization) or MBCB (Illumina algorithm for background subtraction [Ding et al, NAR 36:e58, 2008]), quantile-normalized and log-transformed

Microarray expression data of NeuroD1 mRNA was also compared across diverse benign (N=3879; black dots) and malignant tissues (N=1605; red dots) using the Affymetrix HGU133 Plus v2 GeneChip. These data were obtained from Gene Logic,

Inc. (Gaithersburg, MD). The analysis shown is for probe set ID 206282_at. The microarray data were normalized using the RMA method.

Plasmids, Primers, and Luciferase Assays

Oligonucleotides used were: NeuroD1-1 sense CGAAUUUGGUGUGGCUGUA, antisense UACAGCCACACCAAAUUCG-QIAGEN;
NeuroD1-AB sense GGAUCAAUCUUCUCAGGCA, antisense UGCCUGAGAAGAUUGAUCC- AMBION; NTRK2-5 sense GACGAGUUUGUCUAGGAAA , antisense UUUCCUAGACAAACUCGUC-QIAGEN. For p53 experiments, cells were transfected with pCMV5, SV40- internal control, pcDNA.1-p53, or pGL3-NeuroD1 constructs using Fugene HD. Luciferase assays used the Promega dual luciferase kit according to manufacturer's protocol

Statistical Analyses

Student's *t* test, one-way analysis of variance (ANOVA), Pearson's test, and linear regression were used to determine statistical significance. Statistical significance for all tests, assessed by calculating the P values and was defined as <0.05.

Table 3-1

Cell line	Type of Lung Cancer	Morphology	*NeuroD1* expression	*ASCL1* expression	Features
H69	SCLC	Variant/ spheroid	++++	-	Suspension
H82	SCLC	Variant	++++	+	Suspension
H524	SCLC	Variant	++++	+	Suspension
H526	SCLC	Variant /sheroid	-	-	Suspension
H889	SCLC	Variant	++	+++	Suspension
H1155	NSCLC-NE	Variant	++++	++	Suspension with neuroendocrine properties
H1770	NSCLC-NE	Variant	++++	+	Suspension with neuroendocrine properties
H2171	SCLC	Variant	+++	+	Suspension
H2107	SCLC	Classical Spheroid	-	+++	Suspension
H2227	SCLC	Classical Spheroid	+	-	Suspension and adherent
H1184	SCLC	Classical Spheroid	+	+	Suspension
H322	NSCLC	Adherent	-		
H358	NSCLC	Adherent	+		
H460	NSCLC	Adherent	+		
H1993	NSLC	Adherent	+		
H2073	NSCLC	Adherent	+		

Table 1. Features of the morphologically different SCLC and NSCLC-NE

Characterization of the morphologies of neuroendocrine lung cancers with NeuroD1 and ASCL1 expression

Figure 3-1

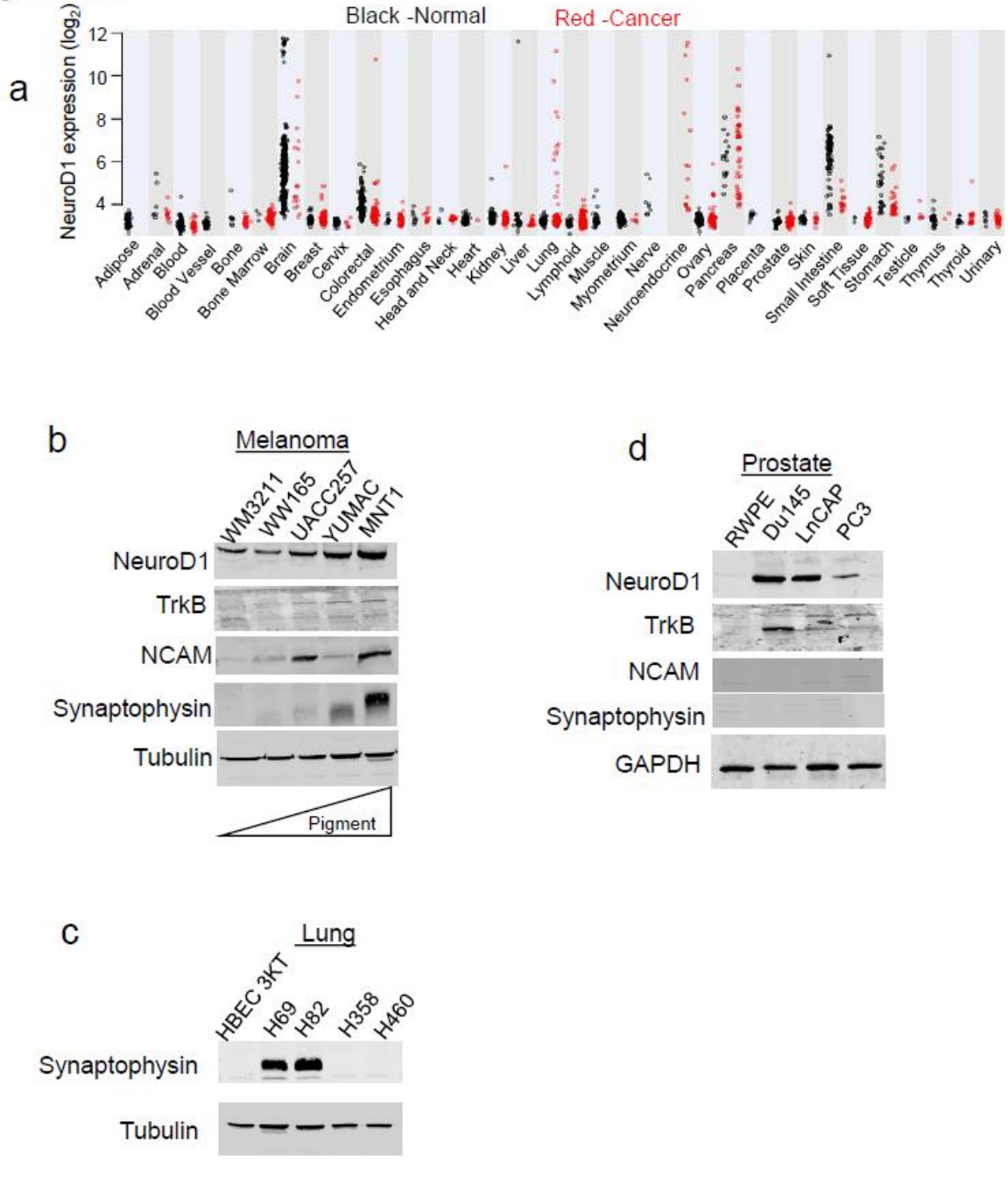


Figure 1. NeuroD1 is expressed in aggressive neuroendocrine cancers

a) Expression of NeuroD1 mRNA compared across diverse normal (N=3879; black dots) and malignant tissues (N=1605; red dots) using the Affymetrix HGU133 Plus v2 GeneChip. b) and d) Melanoma and prostate cancer cell lines were lysed and 25 µg

total protein was loaded then immunoblotted for NeuroD1, TrkB, NCAM, Synaptophysin and Tubulin or GAPDH (as loading controls). Melanoma cell lines were loaded by increasing pigmentation. c) Lung cancer cell lines were lysed and 25 µg of total protein was immunoblotted for synaptophysin and tubulin (as loading controls).

Figure 3-2

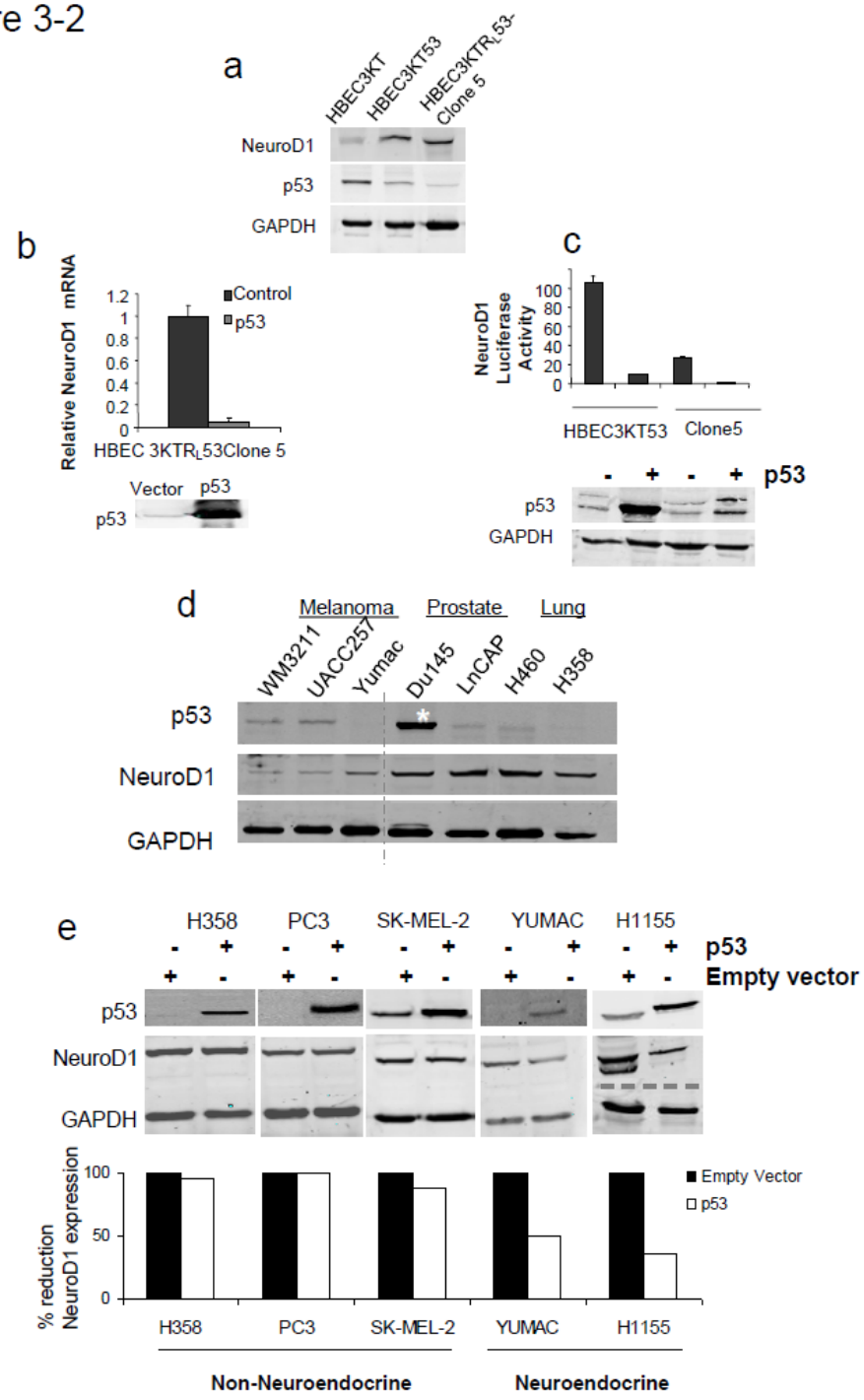


Figure 2. Loss of p53 is permissive for expression of NeuroD1

a) NeuroD1, p53 and GAPDH (loading control) were immunoblotted in lysates of HBEC3KT, HBEC3KT53 and Clone 5. b) qRT-PCR analysis of NeuroD1 in Clone 5 cells

transfected as indicated. Representative p53 immunoblot is shown,one of three independent experiments. c) HBEC3KT53, and Clone 5 were transfected with pGL3-NeuroD1-luciferase with and without p53. p53 was immunoblotted and luciferase activity was measured; one of six experiments shown. d) Melanoma, prostate and lung cancer cell lines were lysed, 50 µg total protein was immunoblotted for p53, NeuroD1, and GAPDH (as loading control). Dashed line indicates discontinuity in gel. Asterisks represent a loss of function mutation in p53 (Gurova et al., 2003).

e) Cell lines with loss of or mutation in p53 were transfected with control vector or vector encoding p53. Cells were lysed and immunoblotted for p53. Overexpression was quantified using Odyssey software.

Figure 3-3

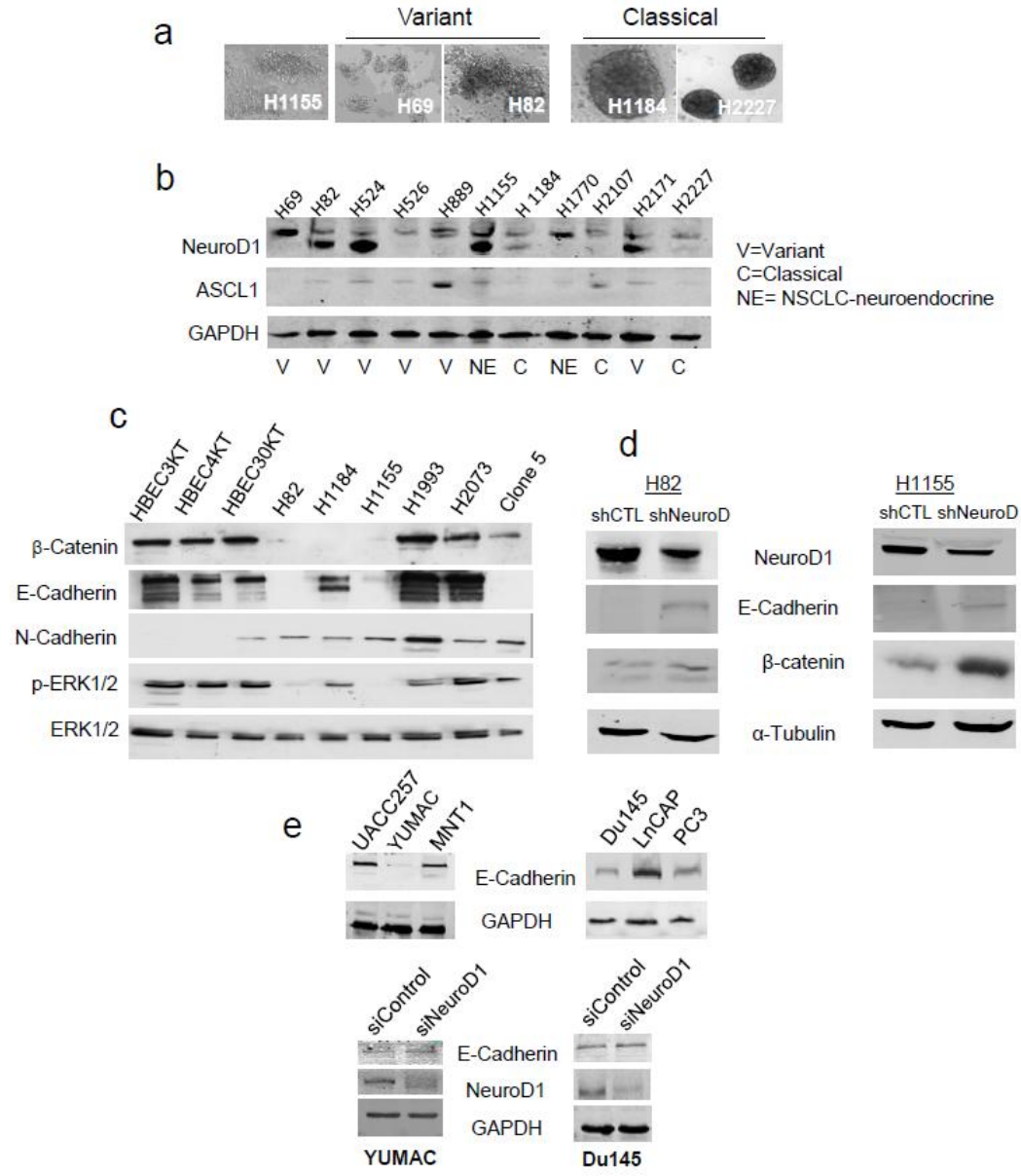


Figure 3. NeuroD1 regulates epithelial to mesenchymal transition

a) DIC images at 10X magnification of various SCLC and NSCLC-NE. b)25 µg of lysate protein was immunoblotted for NeuroD1, ASCL1, and GAPDH (loading control). c) Immunoblots for markers of EMT in HBEC, SCLC and NSCLC lines. d) Expression of NeuroD1, E-cadherin, and β- catenin in H82 and H1155 lines expressing shNeuroD1 or

shcontrol. One of three experiments shown. e) Prostate and melanoma cells were immunoblotted for the E-cadherin and GAPDH (as loading control). Du145 and YUMAC with transient knockdown of NeuroD1 were immunoblotted for E-Cadherin and GAPDH (as loading control).

Figure 3-4

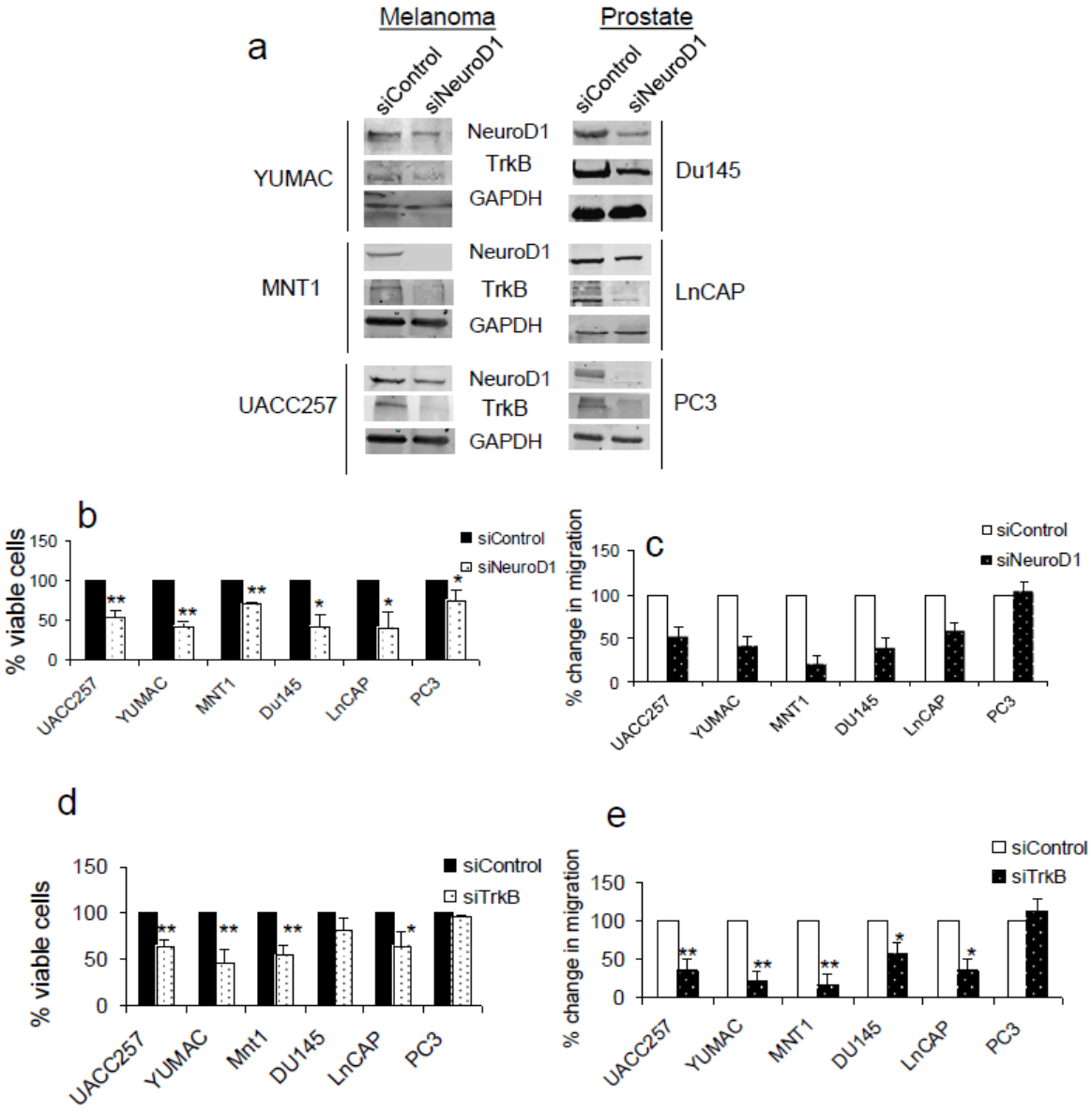


Figure 4. NeuroD1 and TrkB regulates viability and migration of prostate and melanoma

a) NeuroD1 was transiently knocked down in prostate and melanoma cell lines. Lysates were immunoblotted for NeuroD1, TrkB and GAPDH (as loading control).b) NeuroD1 was transiently knocked down in melanoma or prostate in 96 well formats. Cells were

assayed for viability using Cell-Titer Blue. Graph represents fold mean +/-SD of three independent experiments in triplicate (**p<0.005, *p<0.05; one-way ANOVA). c) Knockdown cells in b) were subjected to transwell migration assay. d) TrkB was transiently knocked down in melanoma or prostate in 96 well formats. Cells were assayed for viability using Cell-Titer Blue. Graph represents fold mean +/-SD of three independent experiments in duplicate (**p<0.005, *p<0.05; one-way ANOVA). e) TrkB was transiently knocked down in melanoma or prostate and subjected to transwell migration assay. Graph represents fold mean +/-SD of three independent experiments in triplicate (**p<0.005, *p<0.05; one-way ANOVA)

Figure 3-5

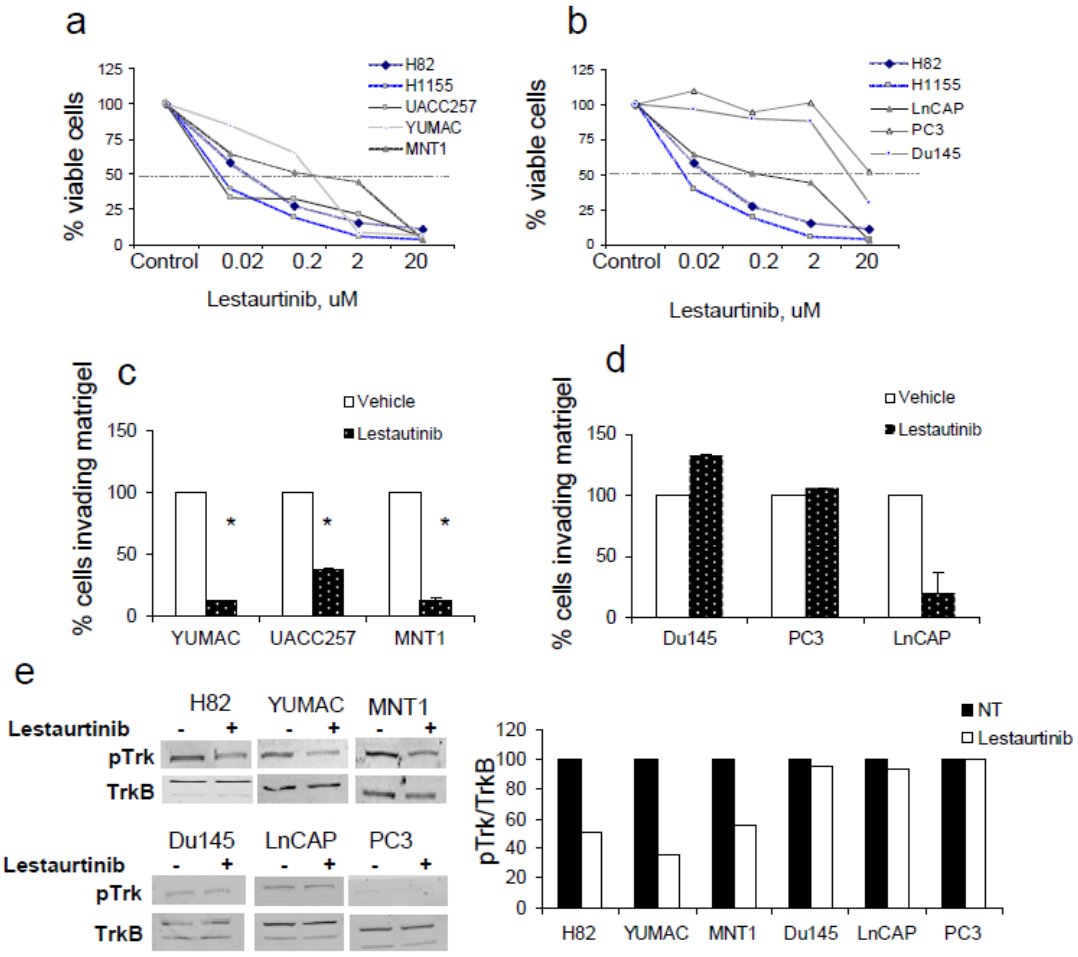


Figure 5. TrkB activity regulates cell survival and migration in melanoma but not prostate cancer cell lines

a) and b) Viability of neuroendocrine lung, prostate and melanoma cells were measured after a treatment with 0.02, 0.2, 2, 20 μM lestaurtinib for 96 hrs, using Cell-Titer Blue. c) and d) Invasion capabilities were measured after being embedded in matrigel along treatment with 2 μM lestaurtinib for 48 hrs. e) SCLC, melanoma and prostate cells were

treated with 10 μM lestaurtinib for 8 hrs. Cells were lysed and immunoblotted with TrkB antibodies. Blots were quantified using Odyssey software.

Figure 3-6

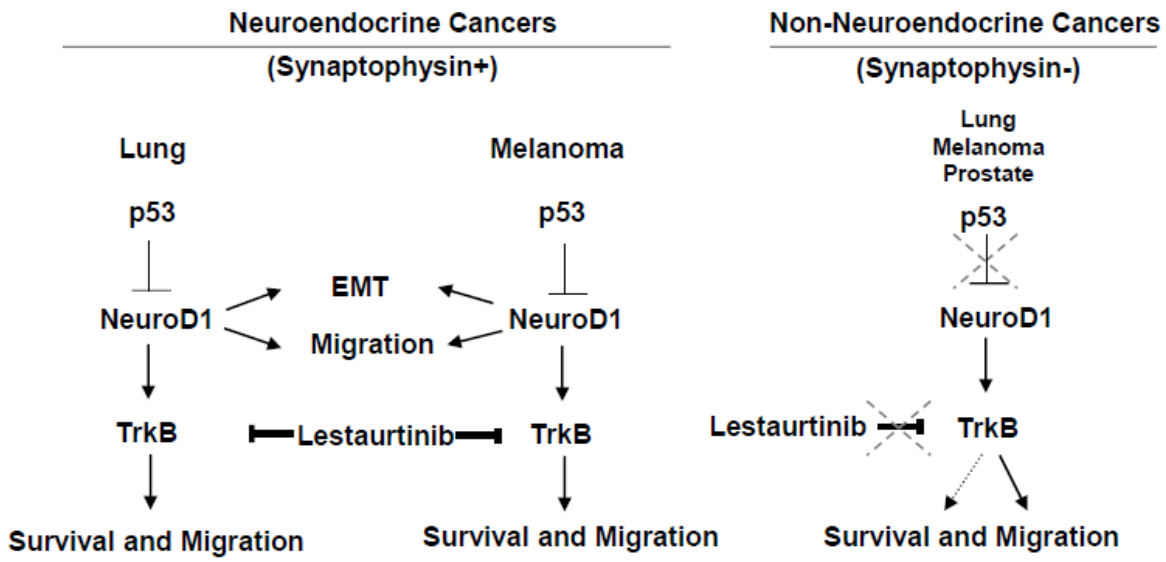


Figure 6. Working model

Figure S3-1

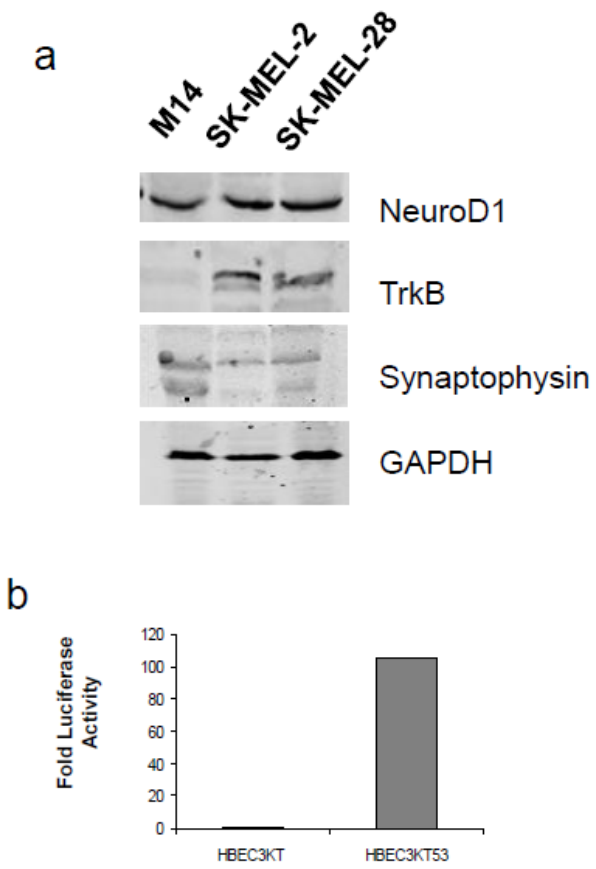


Figure 1. Expression in melanoma with mutant p53 and luciferase assays. a) Melanoma cells were lysed and immunoblotted for TrkB, synaptophysin, and NeuroD1. GAPDH was loading the control. b) HBEC3-KT and HBEC3-KT53 were transfected with a pGL3-NeuroD1-luciferase construct and luciferase activity was measured.

Figure S3-2

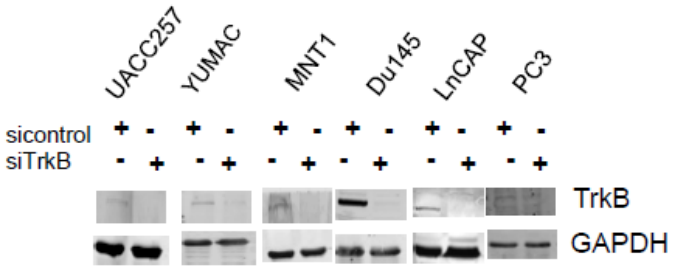


Figure 2. Knockdown of TrkB. TrkB was transiently knocked down in prostate and melanoma cell lines. The lysates were immunoblotted for TrkB and GAPDH was the loading control.

CHAPTER FOUR

Differential Regulation of Nicotinic Acetylcholine Receptors by NeuroD1 and ASCL1

Adapted from Osborne J.K**.,** Borromeo M, McMillan E, Gonzales, J.X., Guerra ML, Johnson, J.E., Cobb, M.H. Manuscript in preparation

Abstract

Cigarette smoking is a major risk factor for acquisition of SCLC. Previously we and others have demonstrated a role for the bHLH transcription factors, NeuroD1 and ASCL1, in the pathogenesis of neuroendocrine lung cancer. In this current study we investigate the similarities and differences in the function of each of these transcription factors in already established tumors, and also the possible actions early on in pathogenesis in response to nicotine. We demonstrate that both NeuroD1 and ASCL1 are up-regulated in normal bronchial epithelial cells (HBECs) and undifferentiated carcinomas in response to nicotine. The increase in expression of each of these proteins subsequently leads to regulation of expression and function of the nicotinic acetylcholine receptor (nAChR) subunit cluster of α3, α5, and β4. Differential regulation of each of these nAChR subunits by NeuroD1 and ASCL1 could be influenced by temporal differences to nicotine exposure. This study suggests aspects of the pathogenesis of neuroendocrine cancers that may occur as a result of nicotine exposure.

Introduction

Signature characteristics of tumor pathogenesis include the acquisition of qualities that enable unrestrained growth and metastasis. Many of the genes that are expressed during tumorigenesis are in fact those that regulate developmental gene programs initiated during embryogenesis and organogenesis (Ben-David and Benvenisty, 2011; Ben-David et al., 2011; Wong et al., 2008). Formerly, we and others demonstrated that small cell lung cancers (SCLC) and other neuroendocrine carcinomas display increased expression of the neuronal basic helix-loop-helix transcription factors, neurogenic differentiation 1 (NeuroD1) and achaete-scute homologue (ASCL1). During development, expression of these transcription factors is temporally separated, with the expression of ASCL1 preceding that of NeuroD1, specifically in hypothalamic neuroendocrine cells, olfactory neuronal progenitors and in neuroendocrine cells located in pulmonary epithelia (Cau et al., 1997; Ito et al., 2000; McNay et al., 2006; Neptune et al., 2008a).

As mentioned, both of these lineage-restricted oncogenes have been shown to be highly expressed in many neuroendocrine lung cancers including SCLC, which accounts for approximately 15-20% of all lung cancers. SCLC is a highly aggressive form of lung cancer that is strongly associated with cigarette smoking and is characterized by neuroendocrine morphological features, i.e. distinguished by large nuclei, along with a scant cytoplasm and expression of classical neuroendocrine markers such as synaptophysin and chromogranin A, fast growth, early dissemination, and high frequencies of metastasis especially to brain (Jackman and Johnson, 2005;

Quan et al., 2004). Patients that develop lung cancer as a result of smoking have a significantly higher number of mutations per mega-base of DNA in specific genes (such as TP53) when compared to non-smokers with the same subtypes of lung cancer (Govindan et al., 2012). Nicotine induces factors that participate in multiple signal transduction pathways in multiple organs including the brain and the lung (Hukkanen et al., 2005). Nicotine exposure through cigarette smoking has been linked to lung cancer pathogenesis via mechanisms mediated by the pentameric ligand-gated ion channels, the nicotinic acetylcholine receptors (nAChR) (Improgo et al., 2010a; Lam et al., 2007a). Originally studied as neuronal and muscle ion channels, these receptors when activated by their most well-known ligands nicotine and acetylcholine, are selectively permeable to calcium. Formed from a combination of nine α (α2-10) and three β(β2-4) subunits of specific homo- and heteromeric combinations, these pentameric nAChRs have also been shown to be expressed in many non-neuronal and non-muscle cells such as macrophages, keratinocytes and normal lung epithelia (Improgo et al., 2010a; Kalamida et al., 2007; Lam et al., 2007a; Tournier et al., 2006).

Recently, it has been suggested that nicotine induces ASCL1 expression and that ASCL1 induces expression of the nAChR gene cluster of CHRNA5/A3/B4, specifically α3 and β4 (Improgo et al., 2010a; Linnoila, 2006). Because NeuroD1 and ASCL1 are expressed in very similar tissues and cancer subtypes our goals were to compare the actions of these transcription factors in disease pathogenesis. We examined the following: 1) if there was overlap of expression that was due to regulation of NeuroD1 by ASCL1; 2) if there was a similar mechanism of activation of their expression; and 3) if there was a convergence on regulation of the same downstream

targets, focusing specifically on the nAChR gene cluster of CHRNA5/A3/B4. To investigate these questions we utilized several neural and lung cancer cell lines. Our findings support the conclusion that, NeuroD1 and ASCL1 are induced by nicotine exposure through unlinked event and share a regulation of the nAChR gene cluster of CHRNA5/A3/B4 with possibly temporal and mechanistic differences.

Results

NeuroD1 is not regulated by ASCL1 in neuroblastoma or lung cancer.

To gain perspective on the mechanism of NeuroD1 action in comparison to ASCL1, we examined NeuroD1 and ASCL1 expression in several neural/neuroendocrine cancer cell lines focusing predominantly on lung cancer and neuroblastoma as our models. Further studies were conducted primarily in four cell lines. For the lung cancer model, we employed the isogenic immortalized normal human bronchial epithelial cell line (HBEC) HBEC3KT and the tumor-forming clone,Clone 5 that displayed NeuroD1 expression (Supplementary Figure S1A and (Sato et al., 2006a); Osborne et.al., manuscript in review). For the neural cancer model, we used the human neuroblastoma cell line, SHSY5Y, previously noted to express NeuroD1, and the human embryonal carcinoma cell line, NTERA2 clone D1 which does not (Supplementary Figure S1A) (referred to hereafter as NTERA2). NTERA2 is reported to have stem cell characteristics and can be differentiated along a neuroectodermal lineage with the

addition of retinoic acid (RA) to cause expression of NeuroD1 (Andrews, 1984; Huang et al., 2011a; Mavilio, 1993; Przyborski et al., 2000).

As previously noted, we observed that both transcription factors were expressed in the patient-derived SCLC cell lines, however rarely to a high extent in the same cell line (Supplementary Figure S1B). We tested the hypothesis that ASCL1 could up-regulate NeuroD1 expression by examining if ASCL1 could rescue NeuroD1 expression. This experiment also allowed us to determine if these neurogenic bHLH transcription factors had redundant functions. Knockdown of NeuroD1 led to a reduction in soft agar colony formation in both the SHSY5Y and the Clone 5 cell lines (Supplementary Figure S1C). Complementation of expression and function was observed with the positive control, the known upstream regulator of NeuroD1, neurogenin 3 (NGN3) (Figure 1A). Functional complementation was also observed of expression and function utilizing a mouse Neurod1 construct. In contrast, ASCL1 did not complement the function of NeuroD1 in either cell line. Nor was it a sufficiently strong inducer of NeuroD1 in the presence of the short hairpin. Furthermore while these proteins may be expressed in similar cell types, their mechanisms of action as lineage-restricted oncogenes appear to be different.

Nicotine leads to increased expression of both NeuroD1 and ASCL1

Cigarettes contain various carcinogens including the addictive component nicotine, which has been linked to the increased onset of lung cancer (Hecht, 1999). Of all the subtypes of lung cancer, SCLC has the highest occurrence in patients with a history of tobacco use(Jackman and Johnson, 2005). To investigate the potential effects of nicotine action on NeuroD1 in tumorigenesis, we treated normal HBEC cell lines (with

low to no NeuroD1 expression, Figure 1A) with increasing concentrations of nicotine for 24-48 hrs. Nicotine has a half-life in the circulation of 6-8 hrs and a measured average blood concentration (depending on consumption, type, etc.) ranging from 25-600 nM (Hukkanen et al., 2005; Russell et al., 1980). An increase in NeuroD1 expression was observed with application of comparable concentrations of nicotine (Figure 2A and Supplementary Figure S2A). NeuroD1 nuclear localization was also detected. To determine if this finding was representative of nicotine action, we examine two other immortalized HBEC cell lines (HBEC30KT and HBEC34KT) and found that NeuroD1 expression was also increased by exposure of these cells to nicotine (Supplementary Figure S2B).

We next examined a time course of a high but non-toxic nicotine concentration. Treatment with 2.5 μM nicotine caused an increase in ASCL1 expression and enrichment of its nuclear localization in as little as 2 hrs, whereas NeuroD1 expression and nuclear localization were more confidently observed at 18 hrs and peaked at 24 hrs (Figure 2B). This suggests that nicotine consumption within the ranges induced by cigarette smoking has the ability to regulate both NeuroD1 and ASCL1.

Nicotine has the ability to up-regulate NeuroD1 without causing differentiation

Because NeuroD1 was originally cloned as a gene required for neuronal differentiation, we investigated if the increase in NeuroD1 expression was also accompanied by neuronal differentiation (Lee et al., 1995). To examine this possibility we used the embryonal carcinoma cell line NTERA2 and the neuroblastoma cell line SHSY5Y. These cell lines were previously shown to differentiate along neuronal lineages, upon long term treatment with retinoic acid (RA). The resulting cells have biochemical and

morphological similarities to neurons (Andrews, 1984; Lopez-Carballo et al., 2002). Treatment of both cell lines with RA induced expression of NeuroD1 as well as the marker of mature neurons, NeuN. Treatment with nicotine caused a dramatic increase in NeuroD1 but no significant increase in NeuN (Figure 3A). Next, we examined expression of ASCL1, the NeuroD1 downstream target TrkB, and the upstream regulator NGN3 in response to both treatments. As was the case with NeuroD1, TrkB expression was induced upon treatment with RA or nicotine in both cell lines (Figure 3A). In contrast, we observed that RA caused a decrease or no change in expression of ASCL1 or NGN3 in either cell line (Figure 3B). Interestingly, in the NTERA2 cell line, nicotine caused an increased in ASCL1 and NGN3. These results lead us to conclude that differentiation leads to the decreased expression of factors responsible for maintenance of progenitor states, such as ASCL1 and NGN3. However, while nicotine leads to an up-regulation of all these factors including NeuroD1, we did not observe increased differentiation.

Nicotine decreases p53 expression in a cell-type specific manner

Formerly, we demonstrated that loss of p53 led to increased NeuroD1 expression in a cell-type specific manner (manuscript submitted). Nicotine has been shown to induce proliferation and lead to a down-regulation of p53 (Dasgupta and Chellappan, 2006; Dasgupta et al., 2006; Pfeifer et al., 2002; Puliyappadamba et al., 2010; Sato et al., 2008). We found down-regulation of p53 expression by nicotine may be cell-type specific. Treatment of the SHSY5Y and the NTERA2 cell lines with nicotine did not lead to a change p53 expression (Figure 3B). However, the loss of p53 expression was observed in HBEC3KT (Figure 3C). This indicates that loss of p53 as result of nicotine

exposure could lead to an increase in NeuroD1 expression during the development of lung cancer. Furthermore, this leads to the conclusion that the suppression of p53 in non-epithelial tissues may not be required to increase in NeuroD1 expression.

Increased nAChR expression in cancer cell lines

Nicotine acts through the nicotinic acetylcholine receptors (nAChR). These pentameric ion channels have many possible compositions, and have been shown to be expressed in normal lung and cancer cells, specifically SCLC (Improgo et al., 2010a; Lam et al., 2007a). A series of genome wide studies have mapped a specific region on chromosome 15q25 as a possible locus for lung cancer susceptibility. This locus contains the genes that encode the nAChR subunit cluster of α3, α5, and β4 (Amos et al., 2008; Hung et al., 2008). Recently these subunits have received more attention, as it was shown that ASCL1 regulates the α3 and β4 subunits (Improgo et al., 2010a). To ascertain if nicotine also causes an increase in expression of the receptor subunits we tested its effects on HBEC3KT cells. Within 2 hrs. we observed an increase in each of the subunits examined (Supplemental Figure 4A). Next we compared expression of the subunit cluster of α3, α5, and β4 in the normal and the cancer cell lines. The HBEC3KT cell line expressed the subunit cluster; however, we observed that the expression of α3 and α5 was increased in the cancer cell lines (Figure 4B and Figure 4C). We also found that the SCLC cell lines seem to have more α5, while β4 remained the same in all cell lines examined (Figure 4C). These results confirmed that cancer cell lines, particularly those with increased expression of NeuroD1, express more of the α3 and α5 subunits than normal cells or NSCLC.

NeuroD1 binds to the nAChR subunits cluster locus.

As mentioned previously, ASCL1 has been shown to regulate the α3 and β4 subunits; however ASCL1 is not expressed in all SCLC cell lines. To investigate whether NeuroD1 could also bind this locus, we performed chromatin immunoprecipitation-sequencing (ChIP-seq) studies in four SCLC cell lines, two with high expression of NeuroD1 and two with high expression of ASCL1. We found that indeed, NeuroD1 did bind to DNA regions within the cluster. Results with ASCL1 showed it bound to different regions (Figure 5A). To further explore this issue we chose a region of DNA that was occupied to some extent by both factors. We chose the sequence labeled peak B, because we observed a peak substantially above background. We confirmed NeuroD1 bound Peak A and B, using HBEC3KT as our control (Supplementary Figure 3A). We performed preliminary ChIP to determine if both ASCL1 and NeuroD1 were bound at various times of nicotine exposure. Interestingly, we found that ASCL1 bound more to peak B in the HBEC3KT cell line for a longer time than NeuroD1 (Figure 5B). However, in the Clone 5 and SHSY5Y cell lines, we found that there was a time-dependent increase in the amount of NeuroD1 that was bound to both regions. Little ASCL1 was observed bound at either sequence in the SHSY5Y cell line (Figure 5C and Figure 5D).

Because we observed an increase in NeuroD1 expression following exposure to either nicotine or RA, we investigated if either drug could enhance binding of NeuroD1 to the nAChR receptor gene locus. We detected that in NTERA2 cells upon differentiation with RA, NeuroD1 bound both peaks A and B (Figure 5E). Furthermore, treatment with nicotine for the same amount of time led to an increase of NeuroD1 bound to peak B and not A; and no binding was detected by ASCL1 in the cells under these conditions (Figure 5E). Taken together these results suggest, both NeuroD1 and

ASCL1 bind to the chromatin in the regions of the nAChR receptor gene cluster following exposure to nicotine.

NeuroD1 regulates the α3 and α5, but not β4 subunits

Because ASCL1 was previously shown to regulate the α3 and β4 subunits, we knocked down NeuroD1 in the cancer cells and examined expression of the subunits. We observed that knockdown of NeuroD1, led to a significant decrease in both the α3 and α5, but not β4 nor the α7 nAChR receptor subunits in the cancer cell lines examined.

The known function of the nAChR receptors upon ligand binding is to promote elevations in intracellular calcium. Previously, it was shown that nicotine promotes influx of calcium predominantly through the α7 subunit using a selective antagonist (Dajas-Bailador et al., 2002). We examined whether loss of NeuroD1, in addition to the α3, α5, and β4 subunits, would have an effect on the function of the receptors. Knockdown of the α3 and α5 subunits led to an inhibition of the increase in intracellular calcium observed upon short term exposure to nicotine. Knockdown of NeuroD1 and the β4 subunit also led to a reduction of intracellular calcium observed upon short term exposure to nicotine. These data support the conclusion that NeuroD1 may regulate the function of the nAChR receptor subunits via regulation of expression.

Discussion

For many years the neurogenic bHLH protein, ASCL1 was thought to be the predominant lineage-restricted oncogene in SCLC. Because ASCL1 was shown to be upstream of NeuroD1 during both brain and lung development, we wanted to investigate if ASCL1 was an upstream regulator of NeuroD1 in cancer. We demonstrate here that while NeuroD1 and ASCL1 may be expressed in very similar cell types, their expression

does not often seem to overlap, leading us to conclude that ASCL1 is not a direct upstream regulator of NeuroD1. In our current study, we find that both NeuroD1 and ASCL1 are up-regulated by nicotine in normal bronchial epithelial cells, however, with apparently different kinetics. Increased expression of each of these factors may act as a potential mechanism for SCLC lung cancer pathogenesis, due to the high association of the disease with cigarette smoking. SCLC is a disease of rapid growth, early dissemination and early metastasis, properties that each of these factors regulate.

Additionally, we demonstrate that long term exposure to nicotine does not induce differentiation, however, but does induce NeuroD1 expression. Nicotine induces proliferation and also has been shown to inhibit differentiation (Sato et al., 2008). Similarly, overexpression of p53 in embryonic stem cells has also been shown to promote differentiation and limit proliferation potential, which in some aspects may mimics it effects on NeuroD1(Li et al., 2012; Menendez et al., 2012).

Finally, we demonstrate that NeuroD1, like ASCL1, binds to the nAChR gene subunit cluster, specifically α3 and α5, suggesting that these subunits may be regulated by NeuroD1, whereas ASCL1 regulates α3 and β4. This regulation of α3 and α5 affects function of the receptors as ion channels, which we demonstrated using calcium influx as measured by the fluorescent dye, Fura2. Taken together these results may lead to the conclusion that both NeuroD1 and ASCL1 regulate expression of the nAChR subunit cluster of α3, α5, and β4. The exact composition of the receptor/s and the ultimate consequence of regulation by each factor still remain to be evaluated.

Materials and Methods

Cell Culture

NTERA2 clone D1 cells (ATCC) were grown in DMEM with 10% fetal bovine serum (FBS). SCLC lines were from the Hamon Cancer Center Collection (UT Southwestern). SCLC and HBEC3KTRL53-Clone 5 and cell lines were cultured in RPMI 1640 with 10% FBS. SHSY5Y were grown in DMEM/F12 with 10%FBS. Immortalized HBECs (except HBEC3KTRL53-Clone 5) (Sato et al., 2006b) were cultured in KSFM (Invitrogen) with 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract. The lung cancer cell lines were DNA fingerprinted using the PowerPlex 1.2 kit (Promega) and confirmed to be the same as the DNA fingerprint library maintained either by ATCC or the Hamon Cancer Center. The lines were also tested to be free of mycoplasma by e-Myco kit (Boca Scientific).

Transfection of short-hairpins and plasmids

Virus was generated for infection of human pGIPZ lentiviral shRNA plasmids against (NeuroD1, CHRNA3/A5/B4) created by the RNAi Consortium. These were purchased by UT Southwestern as a library (TRC-Hs1.0 (Human)) from Open Biosystems (sequences are available online at Open Biosystem website). Plasmids (mouse NeuroD, rat ASCL1, and human NGN3) were transfected using FUGENE 6.

Calcium Assays

Cells were washed twice with PBS and incubated with the fluorescent dye Fura-2AM diluted in KSFM with 2.5 mM calcium for 1 hr. Cells were washed twice and equilibrated for 30 min. Nicotine (25 µM) was added using Synergy microplate reader. Changes in intracellular calcium levels are assessed by dual excitation of Fura-2 at 340/11 and 380/20 and emission at 508/20 using Gen5 software.

Immunofluorescence and Nicotine/Differentiation Treatments

Cells were treated with nicotine (Sigma) (either dose response, time course or long term) or retinoic acid (Invitrogen) (long term) washed with PBS fixed with 4%paraformaldehyde (vol/vol) in PBS for 10 min; and washed PBS. Cells were permeabilized with 0.1% Triton X-100 and washed. After incubation with 10% donkey serum/BSA at room temperature for one hr, cells were incubated with the indicated antibodies at 4 °C overnight. Cells were washed with PBS, incubated with Alexa fluor-conjugated secondary antibody at room temperature for one hr, washed with PBS, and imaged. Fluorescent Z-stacks (0.2 mm) were acquired and deconvolved using the Deltavision RT deconvolution microscope.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as previously described (Lawrence et al., 2005) Primers-ChIP:

Upstream A Forward ACAGGACTCCCTGAGACGAG

Upstream A Reverse ATGAGCTGCCAGACGGTATT

Upstream B Forward TGGCCCCCTTCCTAATTACT

Upstream B Reverse GCAGGTCTGCATAATTTCCTG.

ChIP-seq

Quantitative Real Time PCR

Total RNA from cell lines were isolated with TRI Reagent. cDNA was synthesized using iSCRIPT cDNA Synthesis Kit (BIO-RAD). RNA were quantified by RT-PCR with iTaq (Bio-Rad) master mix using TaqMan probes for 18s, NeuroD1, NGN3 and ASCL1 (Applied Biosystems) on an ABI 7500 thermocycler. Relative transcript levels were normalized to 18s rRNA. Transcript amounts in knockdown cells were plotted as fold change relative to control. Data were analyzed using ABI 7500 system software.

Q-RT-PCR primers: α3 Reverse TTGCAGAAACAATCCTGCTG, α3 Forward ATGCTGTGCTGTCCCTCTCT, α5 Forward CCAAACTGCTTTGCATGAGA, α5 Reverse TCCACAGAAACATCCGATCA, α7 Forward CCCAAGTGGACCAGAGTCAT, α7 Reverse GCCACACACTACCCCAGAGT, β4 Forward TCCCTGGTCCTTTTCTTCCT, β4 Reverse TGCAGCTTGATGGAGATGAG

Colony Formation

Soft agar colony formation assays were as described previously (Sato et al., 2006).

Figure 4-1

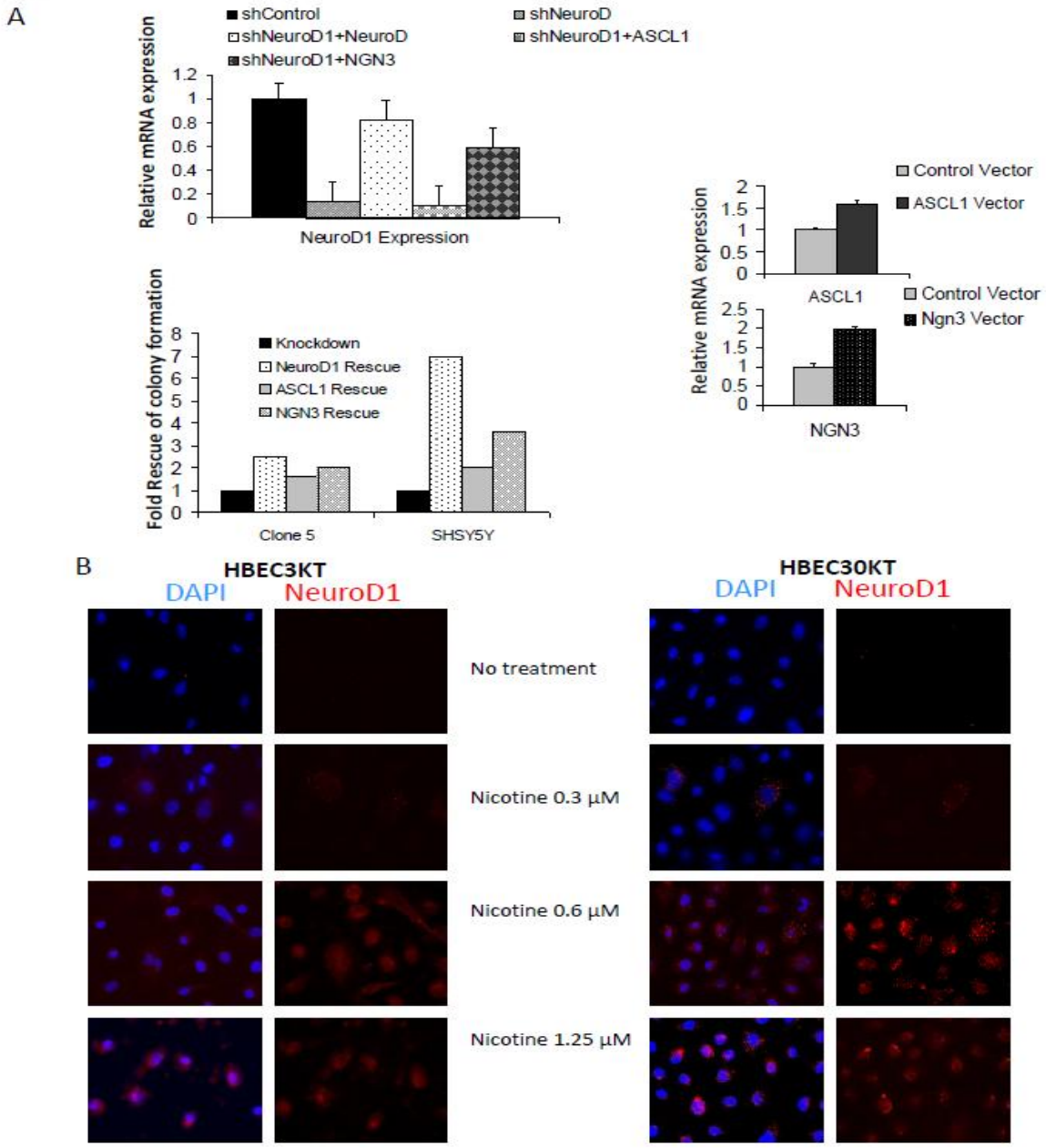


Figure 1. NeuroD1 is not regulated by ASCL1 in neuroblastoma or lung cancer but is regulated by nicotine

A) SHSY5Y and Clone 5 cells were infected with shNeuroD1. Following infection, cells were allowed to recover, then transfected with NeuroD1, ASCL1, or NGN3. Cells were

collected and RNA extracted for q RT -PCR analysis or assayed for soft agar colony formation.

B) HBEC3KT and HBEC30KT were treated with increasing concentrations of nicotine,0.3, 0.6,1.25 μM. Cells were fixed and immunostained for NeuroD1 and DAPI.

Figure 4-2

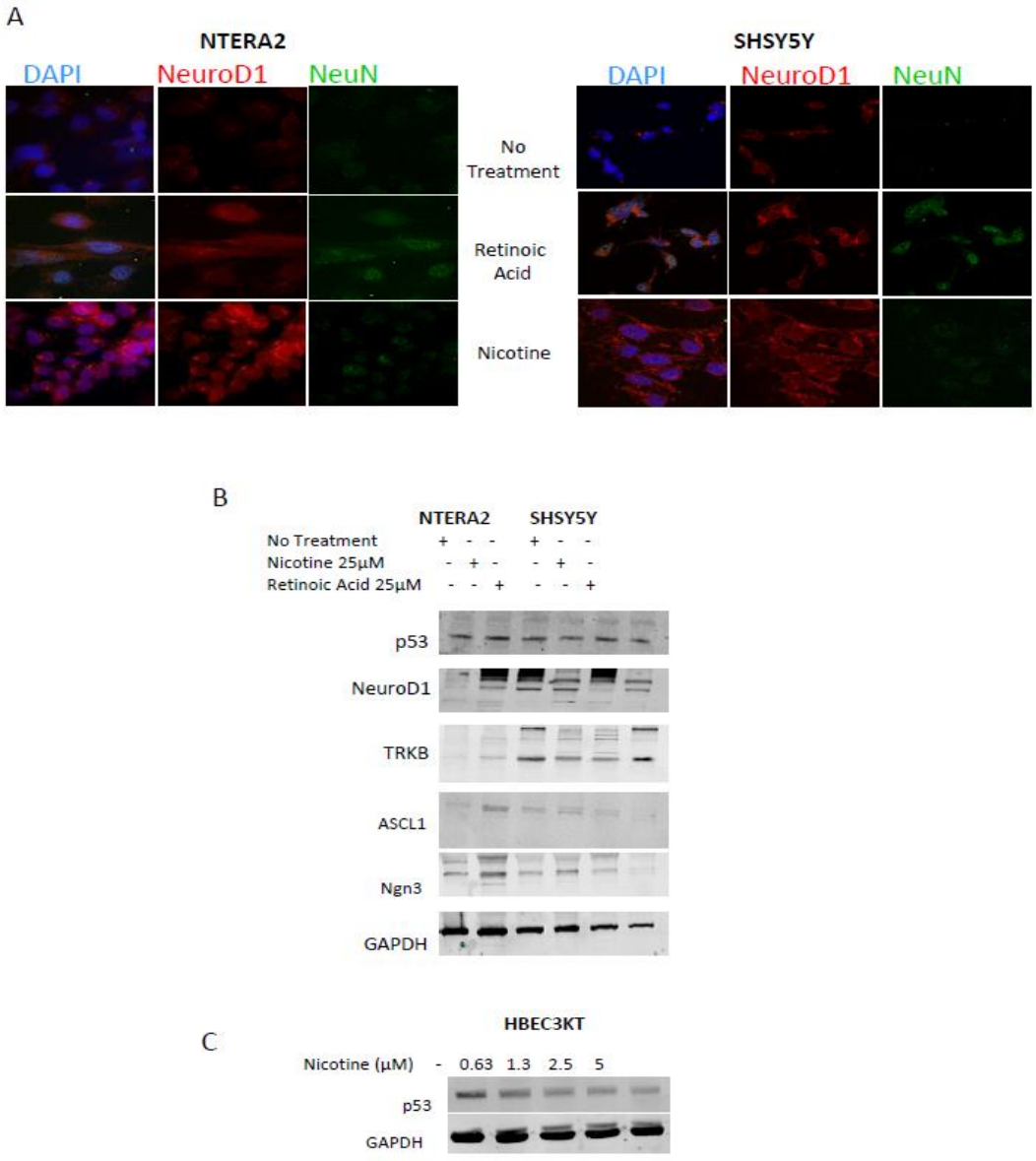


Figure 2. Nicotine has the ability to up-regulate NeuroD1 without causing differentiation

A) NTERA2 and SHSY5Y cells were treated with 25 µM nicotine or retinoic acid for 14 days to induce differentiation. Cells were fixed and immunostained for NeuroD1 and NeuN (mature neuronal marker) and DAPI.

B) Same cells as in (A) were treated with 25 µM nicotine and retinoic acid for 14 days to induce differentiation. Cells were lysed and immunoblotted with NeuroD1, TrkB, ASCL1, NGN3, p53 and GAPDH as loading control.

C) HBEC3KT was treated with 0.63, 1.25, 2.5 and 5 µM nicotine over 24-48 hrs. Cells were lysed and immunoblotted for p53 and GAPDH as loading control

A Figure 4-3

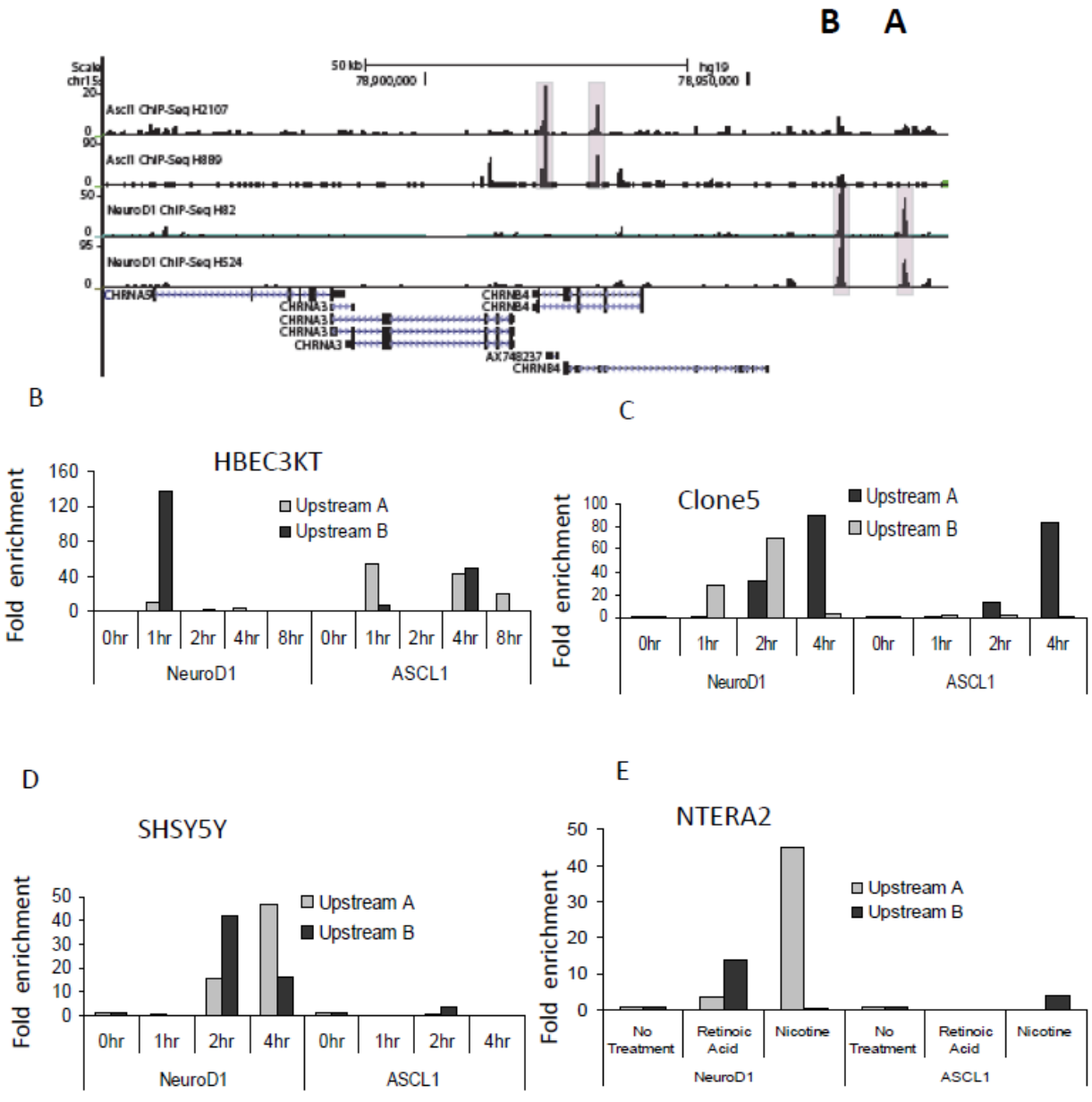


Figure 3. NeuroD1 binds to the promoter region of the nAChR subunits.

A) ChIP-sequencing was performed on four SCLC cell lines, two with high expression of NeuroD1(H82 and H524) and two with high expression of ASCL1 (H889 and H2107).

B) C) D) HBEC3KT, Clone 5, and SHSY5Y were treated with 5 μM nicotine for the indicated times. Cells were fixed and immunoprecipitated with either ASCL1 or NeuroD1. Immunoprecipitates were processed for RT-PCR with 10 ng of starting DNA. All treatments are normalized to time zero.

E) NTERA2 cells as described in Figure 2, were fixed and either ASCL1 or NeuroD1 were immunoprecipitated. Immunoprecipitates were processed for RT-PCR with 10 ng of starting DNA. All treatments are normalized to no treatment.

Figure 4

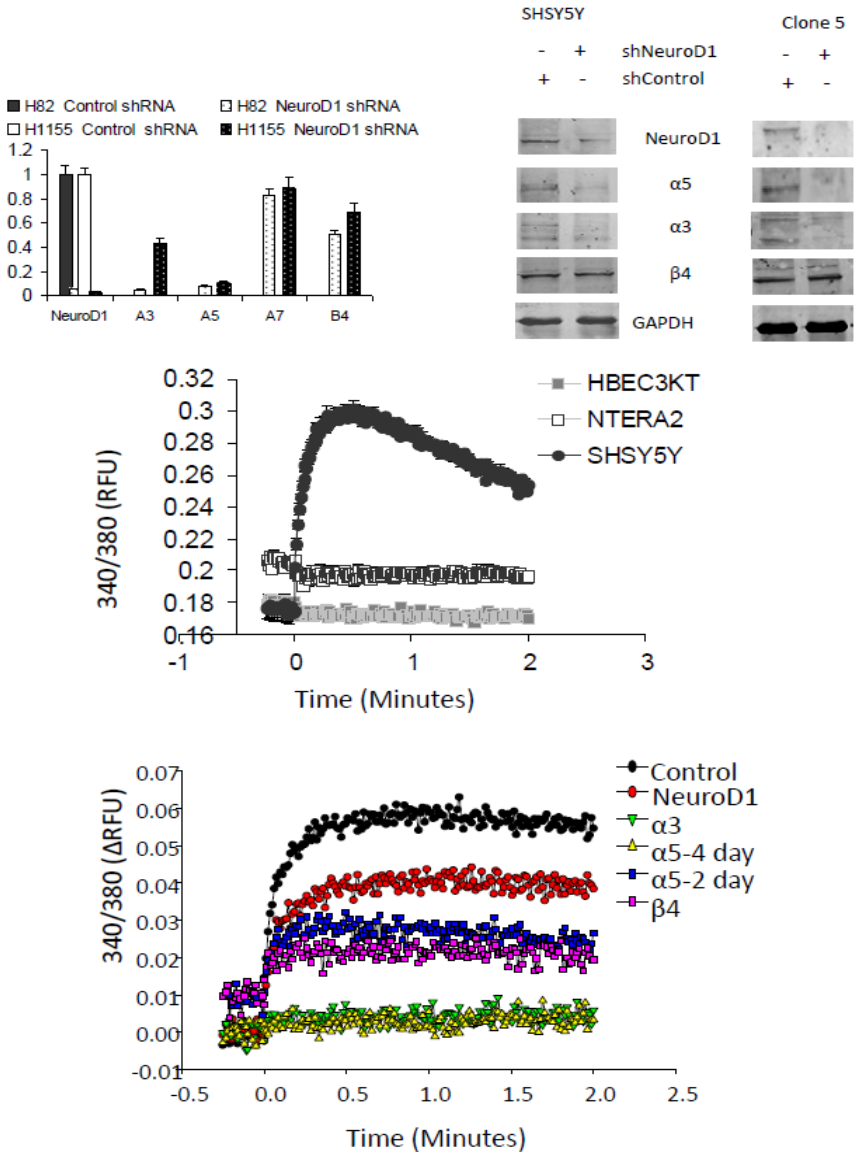


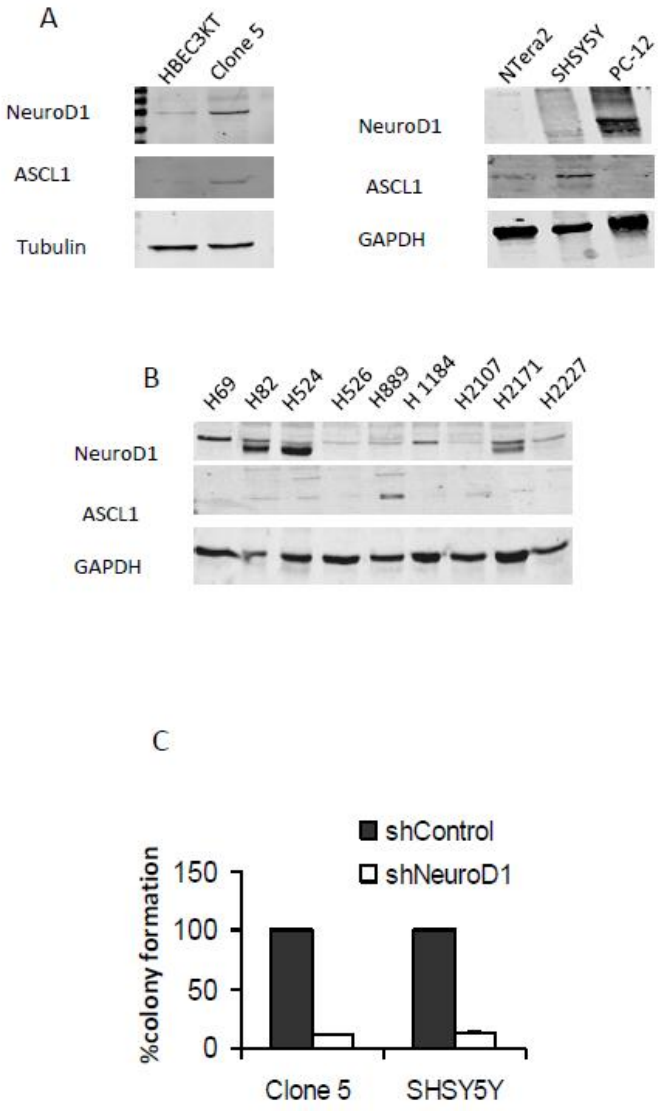
Figure 4. Regulation of nAChR subunit expression and function

A) B) Knockdown of NeuroD1 in lung (H82, H1155, and Clone 5) and neuroblastoma (SHSY5Y) cancer cell lines. Cells were infected with shNeuroD1 or shControl vectors, then either lysed for immunoblotting with antibodies against NeuroD1, α3,α5, β4 or

GAPDH or RNA was extracted for cDNA synthesis for q-RT-PCR analysis of CHRNA3, A5, A7 and NeuroD1.

C) and D) Assays for intracellular free calcium following exposure to nicotine.

Supplementary Figure 4-1



Supplemental Figure Legends

Figure 1. NeuroD1 is not regulated by ASCL1 in neuroblastoma or lung cancer but is regulated by nicotine

A) and B) Patient-derived SCLC cell lines, HBEC3KT, Clone 5, SHSY5Y, and NTERA2, cell lines were lysed. 25µg total protein was immunoblotted for NeuroD1, ASCL1 and GAPDH, as loading control.

C) Clone 5 and SHSY5Y cells infected with shNeuroD1 or shControl vectors were assayed for soft agar colony formation in triplicate. Colonies were counted after 2 weeks.

Supplemental Figure 4-2

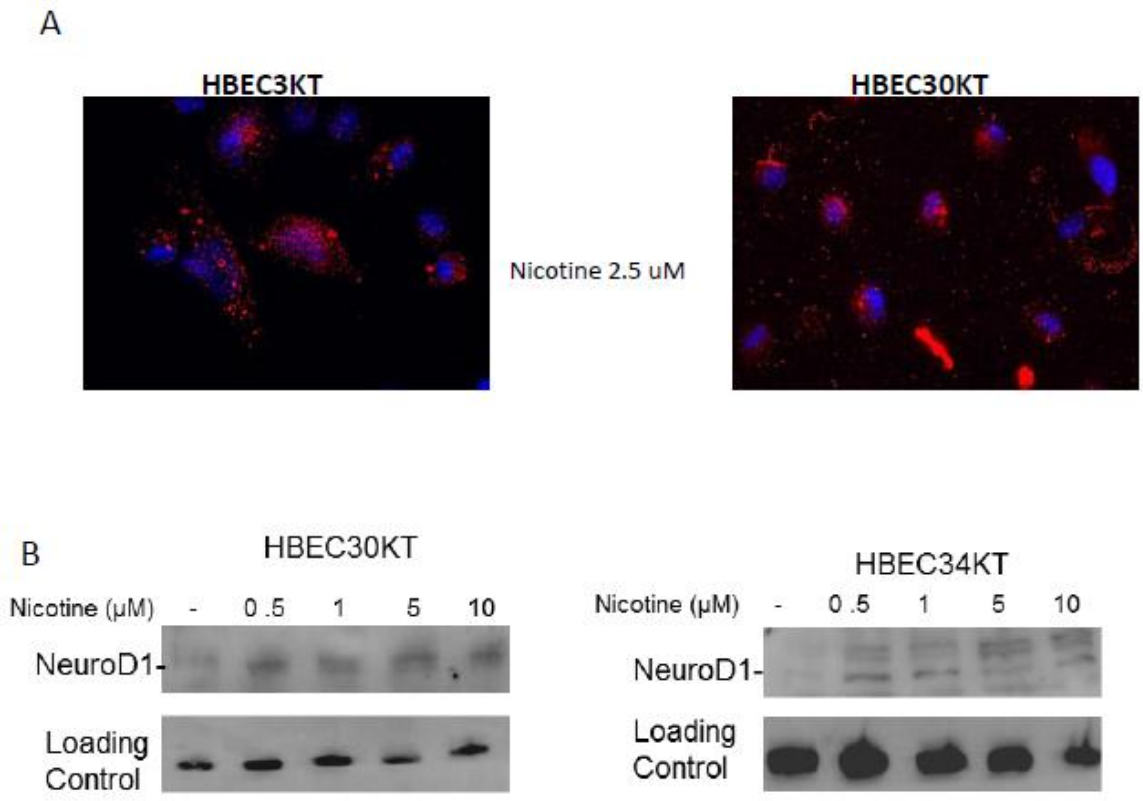


Figure 2. Nicotine leads to increased expression of NeuroD1

A) HBEC3KT and HBEC30KT were treated with 2.5 μM nicotine. Cells were fixed and immunostained for NeuroD1 and DAPI.

B) HBEC30KT and HBEC34KT were treated with 0.63, 1.25, 2.5 and 5 μM nicotine over 24-48 hrs. Cells were lysed and immunoblotted for NeuroD1 and Kif2A as loading control.

Supplemental Figure 4-3

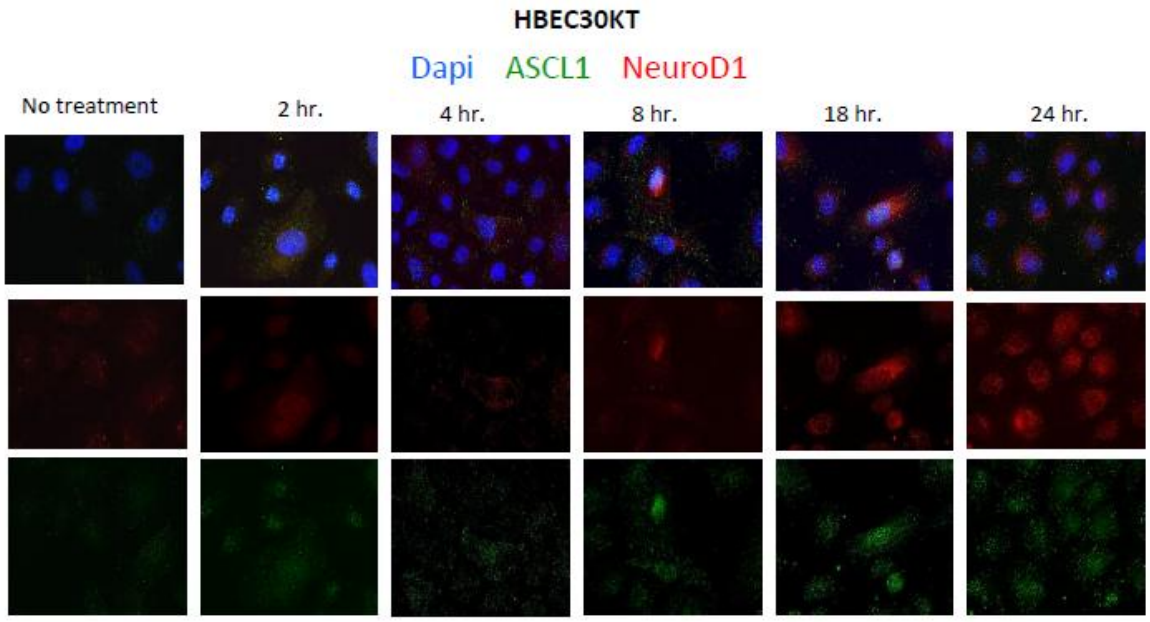


Figure 3. Nicotine leads to increased expression of NeuroD1 and ASCL1

HBEC30KT were treated with 2.5 µM nicotine over a time course of 2, 4, 8, 18, and 24 hrs. Cells were immunostained for NeuroD1 (red), ASCL1 (green) and DAPI.

Supplemental Figure 4-4

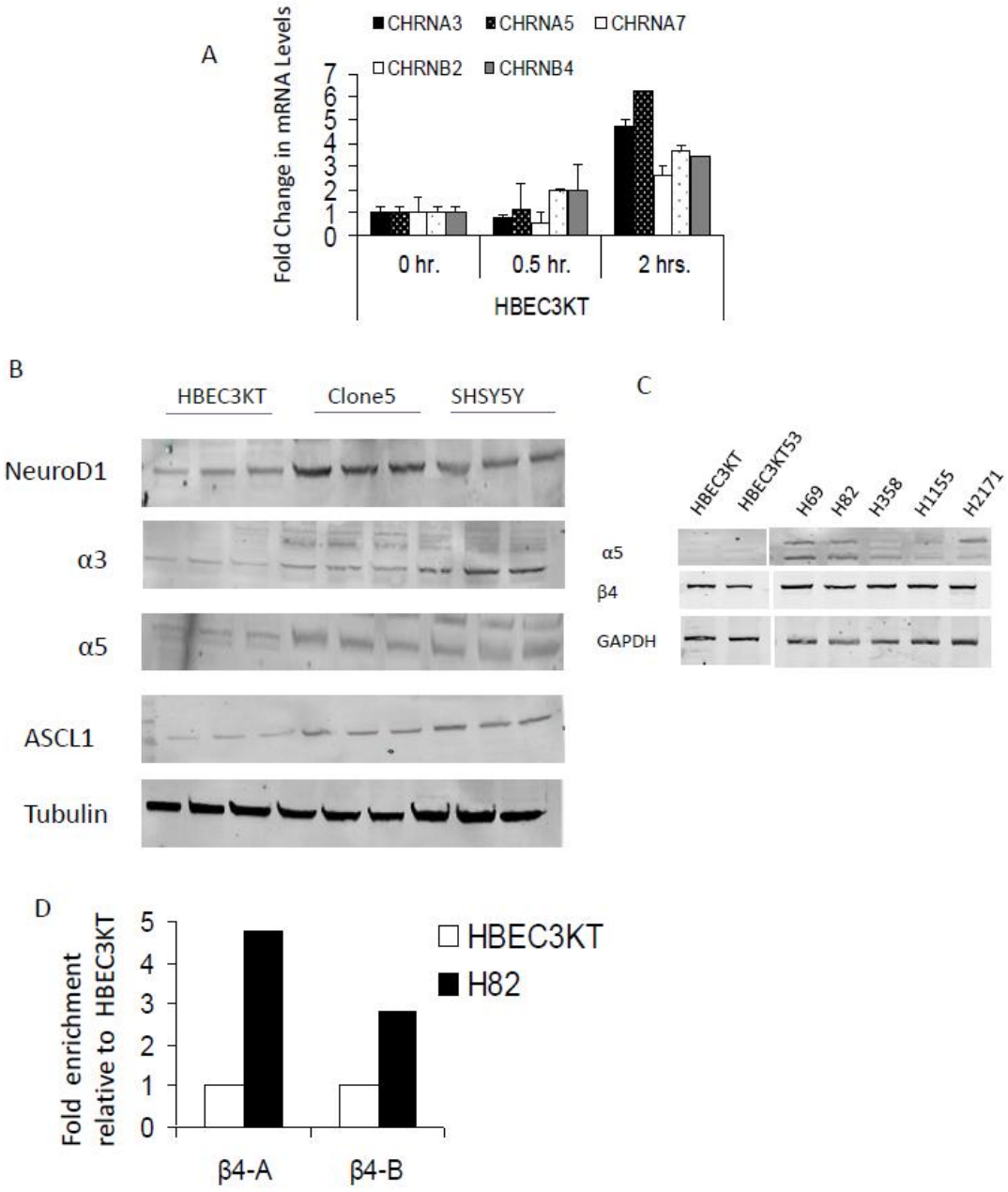


Figure 4. Expression of nAChR subunits in various cell lines

A) HBEC3KT cells were treated with 5 µM nicotine for indicated times. RNA was extracted and cDNA synthesized for q-RT-PCR analysis of CHRNA3, A5, A7, B2, and B4 subunits.

B) HBEC3KT, Clone5 and SHSY5Y cells were lysed and immunoblotted with NeuroD1, ASCL1, α3, α5 and tubulin as loading control. Lanes loaded in triplicate.

C) Various lung cancer cell lines (immortalized- HBEC3KT, HBEC3KT53; SCLC- H69, H82, and H2171; NSCLC- H1155 and H358) were lysed and immunoblotted for α5 and β4 nAChR subunits. D) Confirmation of peaks from ChIP-seq. Cells were and fixed and immunoprecipitated with NeuroD1. Immunoprecipitates processed for RT-PCR with 10ng of starting DNA. PCR was normalized to HBEC3KT.

Chapter Five

Conclusions

During development, the neurogenic bHLH transcription factor NeuroD1 regulates the differentiation of various organs including, brain, lung, the pancreas and gastrointestinal system (Gasa et al., 2004; Ito et al., 2000; Lee et al., 1995; Liu et al., 2011; Miyata et al., 1999b; Syder et al., 2004a). NeuroD1 and the related bHLH factor ASCL1 have been identified repeatedly in neural and neuroendocrine tumors (Borges et al., 1997; Fratticci et al., 2007; Hu et al., 2004; Osada et al., 2008; Rostomily et al., 1997; Wang et al., 2007b). Whether their expression was causative or solely a consequence of disease was an outstanding question.

ASCL1 was previously thought to be the predominant lineage-restricted oncogene in SCLC. ASCL1 is expressed earlier than NeuroD1 during both brain and lung development; thus it seemed likely that increased expression of NeuroD1 in neural and neuroendocrine carcinomas would be a consequence of the aberrant expression of ASCL1. Against this conclusion was the observation that ASCL1 and NeuroD1 were not frequently found together in many tumors; rather, one or the other was more easily detected. Additionally, the morphology and phenotype of the cells that expressed ASCL1 were different from those with NeuroD1.

To begin our investigation of why NeuroD1 was expressed and to identify its mechanisms of action, we were focused on SCLC and NSCLC-NE; both of these types of lung cancer have neuroendocrine features. Until recently, the cells from which these lung cancers originated were unknown. Loss of the tumor suppressors, p53 and RB1, specifically in the Clara cells and neuroendocrine cells of the lung, resulted in large cell neuroendocrine and SCLC, respectively (Meuwissen et al., 2003; Sutherland et al.,

2011). This information was important to further our understanding of how NeuroD1 could be expressed in many cancers, not just neuroendocrine lung. First, we found that NeuroD1 expression was high in the NSCLC-NE cell lines, particularly H1155 and H1770, not just in the SCLC cell lines (Figures 2-1a, and 3-3b). Additionally, there were several reports that detailed uncharacterized driver mutations in melanoma, (particularly Hodis et al., 2012) demonstrating that many patient-derived melanomas had lost p53 as well as RB1 (Halaban et al., 2009; Hodis et al., 2012; Yang et al., 2005). For more than fifteen years, loss of p53 in melanoma was thought to be redundant, and rarely seen, because mutations in the tumor suppressor p16INK4 were thought to predominate (Gruis et al., 1995a; Gruis et al., 1995b; Gruis et al., 1995c; Hussussian et al., 1994; Liu et al., 1995; Ranade et al., 1995). We observed that loss of p53 in our isogenic HBEC model system led to an increase in NeuroD1. Furthermore, we observed that loss or mutation of p53, in the pigmented melanoma cell line YUMAC and in H1155 could be responsible for up-regulation of NeuroD1, as overexpression of p53 in both these cell lines led to a dramatic decrease in NeuroD1 protein (Figure 3-3).

From these findings more questions arose. A causative agent of SCLC was exposure to cigarette smoke. Decades of research on the normal function of pulmonary neuroendocrine cells during development and throughout adulthood, indicated that these cells regulated airway oxygen sensing, pulmonary blood flow, and maintained a stem cell niche in the lung (Ito et al., 2000; Linnoila, 2006; Song et al., 2012; Van Lommel, 2001). SCLC arises from these neuroendocrine cells and nicotine has been shown to specifically target pulmonary neuroendocrine cells (Carlisle et al., 2004;

Carlisle et al., 2007; Improgo et al., 2010b; Improgo et al., 2010c; Improgo et al., 2011; Lam et al., 2007b; Van Lommel, 2001; West et al., 2003).

Additionally, expression of the nAChR subunit gene cluster of α3, α5, and β4 located on the long arm of chromosome 15 is increased in SCLC compared to NSCLC and normal bronchial epithelial cells (Amos et al., 2008; Hecht, 1999; Martinez-Garcia et al., 2010; Pfeifer et al., 2002). A potential connection was suggested because ASCL1 was found to regulate this gene cluster (Improgo et al., 2010a). Interestingly, nicotine was previously shown to decrease expression of p53 (Pfeifer et al., 2002; Sato et al., 2008). Complementing our previous results, we observed that nicotine led to an increase in NeuroD1 expression and a decrease in p53 expression in several different HBEC cell lines. We then developed a hypothesis that nicotine, through mechanisms unknown, causes the down-regulation of p53 in lung epithelial cells, and that this allowed for the increased expression of NeuroD1.

We and others have found that NeuroD1 regulates several proteins that control migration/migration potential in lung cancer, melanoma and prostate cancers. NeuroD1 was recently shown to regulate the neuronal guidance factor Slit2, as well as the receptor tyrosine kinase TrkB, both of which have been linked to regulation of migration and metastasis in neuroblastoma. We confirmed that regulation of TrkB was conserved in neuroendocrine lung cancer cells as well as the prostate and melanoma cells that also expressed NeuroD1 (Figure 3-4). Additionally, we observed that an important group of the actions of NeuroD1 were through TrkB, as TrkB could, to a large extent, rescue the loss of NeuroD1 in neuroendocrine lung cancer cells. TrkB was also a

remarkable target because several inhibitors and antagonists for TrkB have been developed and have undergone clinical trials.

In addition to TrkB, we identified several previously unknown downstream targets of NeuroD1. NCAM was predicted to be a target *in silico,* because its expression during neuronal development has always correlated with NeuroD1. There is a clinical trial of an NCAM inhibitor and TrkB phosphorylates the protein to enhance its signaling. Similarly, loss of NCAM phenocopied loss of NeuroD1 and NeuroD1 directly bound E boxes located in the NCAM promoter.

Most recently, I found that NeuroD1 regulates expression of the nicotinic acetylcholine receptor subunit cluster of α3 and α5. This regulation of α3 and α5 affects function of the receptors as ion channels as measured by changes in intracellular calcium. Through the studies described here, this dissertation project has revealed a novel function for NeuroD1 in the induction and coordination of signal transduction pathways that regulate survival and migration of neuroendocrine and non-neuroendocrine cancers. Furthermore, my work suggests that TrkB is a druggable target that could be useful in treating SCLC.

Future directions for this project should address several unanswered questions. I briefly discuss three here. First, one outstanding issue is exactly how NeuroD1 is induced in these cancers. Loss of p53 can result in enhanced NeuroD1 expression. p53 acts as a repressor of NeuroD1. Does this occur directly through p53 binding to the NeuroD1 promoter or is the mechanism less straightforward? Previously, I searched for p53-consensus binding sites in the NeuroD1 proximal promoter, but found only hexamers, not the canonical dodecamer that p53 is said to bind. Additionally, there are

other non-canonical consensus sequences that have been implicated and could be explored in the NeuroD1 promoter. Investigation into whether there are direct binding sites or intermediate proteins that may be responsible for NeuroD1 repression would be informative. A related issue is why does loss of p53 increase NeuroD1 expression only in certain contexts? Are there cell type-specific repressors of NeuroD1 that cooperate with p53 or activators that are required but antagonized by p53 or a combination of both?

Second, what is the mechanism that leads to down-regulation or inactivation of p53 by nicotine? Because SCLC is linked to cigarette consumption, and SCLC can be caused by loss of p53 and RB1, an exploration of these mechanisms could lead to a better understanding of how these events lead to development of SCLC. And with respect to NeuroD1, the role of RB1 has not been explored.

Some potential answers to these questions can be extracted from the literature. Molecular inhibitors of NeuroD1 have been previously examined during development that may lead to insights into its activation during tumorigenesis. Two NeuroD1-suppressing proteins are the transcription factors SOX2 and Myc, both of which, along with Oct4 and KLF4, have been shown to synergistically induce reprogramming of fibroblasts to stem cells (Masaki et al., 2007; Nakagawa et al., 2008; Takahashi et al., 2007a; Takahashi et al., 2007b). This is interesting also from the observation that many HBEC cell lines have stem-like properties. SOX2 inhibits NeuroD1 expression during neurogenesis, whereas Myc inhibits NeuroD1 function on the insulin promoter (Gao et al., 2009; Kaneto et al., 2002). These observations suggest additional avenues for investigation particularly because p53 and Myc have been shown to reciprocally

regulate each other under context-specific conditions. The mouse model observed in the study by Sutherland et al. was used to show that Myc amplifications were facilitated by the loss of p53 and RB1. Mutations in Myc have been observed for more than thirty years. If Myc has the potential to inhibit NeuroD1 in pancreatic beta cells, are there other neuroendocrine-specific genes also impacted in this manner? It would also be interesting given the large number of pancreatic patient samples that were observed to have increased NeuroD1 expression (see figure 3-1a).

Third, the further examination of downstream targets of NeuroD1 and ASCL1 should yield very interesting results. Observations made thus far lead me to hypothesize that downstream targets of NeuroD1 generate a more metastatic phenotype in cancer cells, whereas downstream targets of ASCL1 lead to a more proliferative phenotype. A more in-depth analysis of the each of their targets and how they differ across various neuroendocrine carcinomas should provide the basis for exploring this hypothesis and could lead to a better understanding of how to treat these cancers.

For many years the treatments of SCLC has included surgery for a minority of cases due to high rates of metastasis, platinum-based doublet chemotherapy, commonly cisplatin or carboplatin and etoposide and radiation. The investigation of the novel mutations that were identified by two independent studies could potentially yield more therapeutic targets (see table 1-3). Cell surface proteins and enzymes are often druggable targets and several of these identified mutations are receptors.

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