

# NeuroD1 mediates nicotine-induced migration and invasion via regulation of the nicotinic acetylcholine receptor subunits in a subset of neural and neuroendocrine carcinomas

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**ABSTRACT** Cigarette smoking is a major risk factor for acquisition of small cell lung cancer (SCLC). A role has been demonstrated for the basic helix-loop-helix transcription factor NeuroD1 in the pathogenesis of neural and neuroendocrine lung cancer, including SCLC. In the present study we investigate the possible function of NeuroD1 in established tumors, as well as actions early on in pathogenesis, in response to nicotine. We demonstrate that nicotine up-regulates NeuroD1 in immortalized normal bronchial epithelial cells and a subset of undifferentiated carcinomas. Increased expression of NeuroD1 subsequently leads to regulation of expression and function of the nicotinic acetylcholine receptor subunit cluster of  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 4$ . In addition, we find that coordinated expression of these subunits by NeuroD1 leads to enhanced nicotine-induced migration and invasion, likely through changes in intracellular calcium. These findings suggest that aspects of the pathogenesis of neural and neuroendocrine lung cancers may be affected by a nicotine- and NeuroD1-induced positive feedback loop.

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## INTRODUCTION

Signature characteristics of tumor pathogenesis include the acquisition of qualities that enable unrestrained growth and metastasis. Many of the genes expressed during tumorigenesis regulate developmental gene programs initiated during embryogenesis and organogenesis (Wong *et al.*, 2008; Ben-David and Benvenisty, 2011; Ben-David *et al.*, 2011). Small cell lung cancers (SCLCs) 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;

Ball *et al.*, 1993; Rostomily *et al.*, 1997; Hu *et al.*, 2004; Syder *et al.*, 2004; Frattucci *et al.*, 2007; Jiang *et al.*, 2009; Osborne *et al.*, 2013b).

SCLC accounts for 15–20% of all lung cancers. It is a highly aggressive form of lung cancer, is strongly associated with cigarette smoking, and is characterized by neuroendocrine morphological features, distinguished by large nuclei, little cytoplasm, and expression of classical neuroendocrine markers such as synaptophysin and chromogranin A, fast growth, early dissemination, and a high frequency of metastasis, especially to brain (Quan *et al.*, 2004; Jackman and Johnson, 2005; van Meerbeeck *et al.*, 2011). Tumors of patients who develop lung cancer and SCLC in particular as a result of smoking have a significantly higher number of mutations per megabase of DNA in specific genes (such as TP53) than do nonsmokers with the same subtypes of lung cancer (Pleasant *et al.*, 2010; Govindan *et al.*, 2012).

Nicotine induces factors that participate in multiple signal transduction pathways throughout the body, including brain and 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 nicotinic acetylcholine receptors (nAChRs; Lam *et al.*, 2007; Improgio *et al.*, 2010a).

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Abbreviations used: ERK2, extracellular signal-regulated kinase 2; MAPK, mitogen-activated protein kinase; nAChR, nicotinic acetylcholine receptors; NeuroD1, neuronal differentiation 1; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

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Cell line	Cell type	p53 status	KRAS mutation	Additional mutation
H69	SCLC-V	Mutant	No	PIK3CA/RB1
H82	SCLC-V	Mutant	No	RB1
H358	NSCLC	Null	Yes (G12V)	<sup>a</sup>
H460	NSCLC	Wild type	Yes (Q61H)	PIK3CA/CDKN2A/STK11
H524	SCLC-V	Mutant	No	RB1
H526	SCLC-V	Mutant	No	RB1
H889	SCLC-V/C	Mutant	No	–
H1155	NSCLC-NE	Mutant	Yes (Q61H)	PTEN/MSH6
H1184	SCLC-C	Mutant	No	<sup>a</sup>
H2171	SCLC-V	Mutant	No	<sup>a</sup>
H2227	SCLC-C	Mutant	No	RB1
SHSY5Y	Neuroblastoma	Wild type	No	ALK
NTERA2	Germ cell tumor	Wild type	No	SF3B1
HCC4017	NSCLC	Unknown	Yes (G12C)	–
HBEC3KTRL53-Clone 5	NSCLC-NE	Constitutive knockdown	Overexpress (G12V)	–
HBEC3KT-53	Immortalized bronchial epithelial	Constitutive knockdown	No	–
HBEC3KT	Immortalized bronchial epithelial	Wild type	No	–
HBEC4KT	Immortalized bronchial epithelial	Wild type	No	–
HBEC30KT	Immortalized bronchial epithelial	Wild type	No	–

C, classic; K, CDK4; T, hTERT; V, variant.

<sup>a</sup>Many more in COSMIC database (<http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>).

TABLE 1: Mutational status of cell lines.

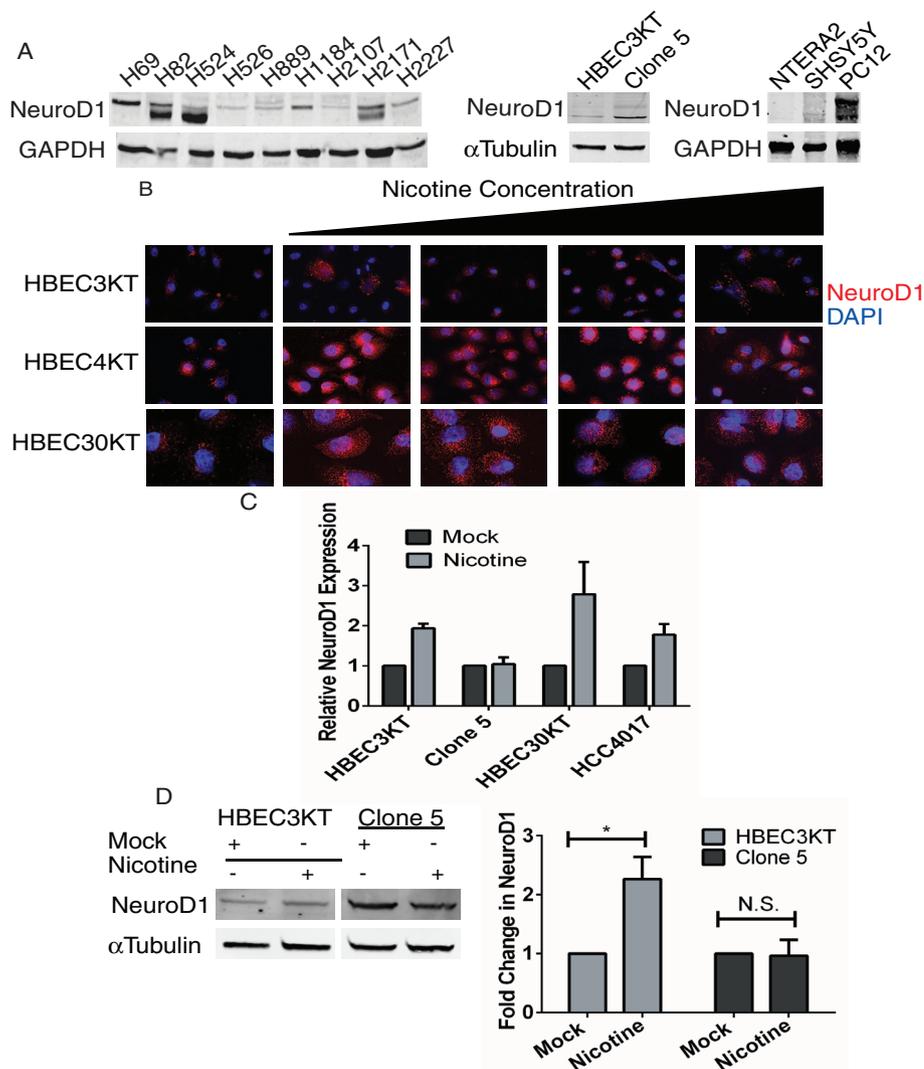
Originally studied as neuronal and muscle ion channels, these receptors, when activated by their ligand, acetylcholine or nicotine, are selectively permeable to calcium. Formed from a combination of nine  $\alpha$  (2–10) and three  $\beta$  (2–4) subunits of specific homomeric and heteromeric combinations, these pentameric nAChRs are also expressed in many nonneuronal and nonmuscle cells, such as macrophages, keratinocytes, and normal lung epithelia (Tournier *et al.*, 2006; Kalamida *et al.*, 2007; Lam *et al.*, 2007; Improgo *et al.*, 2010a).

It has been suggested that nicotine induces ASCL1 expression, and in turn ASCL1 induces expression of the nAChR gene cluster of CHRNA5/A3/B4, specifically  $\alpha$ 3 and  $\beta$ 4 (Linnoila, 2006; Improgo *et al.*, 2010a). During development, expression of the transcription factors ASCL1 and NeuroD1 is temporally separated, with the expression of ASCL1 preceding that of NeuroD1, specifically in hypothalamic neuroendocrine cells, olfactory neuronal progenitors, and neuroendocrine cells located in pulmonary epithelia (Cau *et al.*, 1997; Ito *et al.*, 2000; McNay *et al.*, 2006; Neptune *et al.*, 2008). We demonstrated that NeuroD1 is essential for survival and metastasis of a subset of lung cancers with neuroendocrine features, acting primarily through its downstream targets, the receptor tyrosine kinase TrkB and the neural cell adhesion molecule (Osborne *et al.*, 2013b). In this study in lung epithelial cells and several tumors, we found that nicotine induces NeuroD1 and TrkB in a cell type-specific manner, leading to up-regulation of the nAChR gene cluster of CHRNA5/A3/B4. Our findings support the conclusion that NeuroD1 is induced by nicotine. One consequence is increased expression of the nAChR gene cluster of CHRNA5/A3/B4, ultimately contributing to nicotine-induced migration.

## RESULTS

### Nicotine induces expression of NeuroD1 in immortalized bronchial epithelial cells and some lung cancers

To gain perspective on the mechanism of NeuroD1 action, we examined its expression in several neural/neuroendocrine cancer cell lines, focusing predominantly on lung cancer and neuroblastoma models (Table 1). In addition to patient-derived SCLC cell lines, we used several human bronchial epithelial cell (HBEC) lines. The cells were immortalized by expressing cyclin-dependent kinase 4 and human telomerase and were from different donors (indicated by numbers, HBEC3KT, HBEC4KT, and HBEC30KT). We also used an isogenic manipulated tumor-forming clone (HBEC3KTRL53-Clone 5, referred to hereafter as Clone 5) derived from HBEC3KT by stable depletion of p53 and expression of oncogenic KRAS, because it displayed an increase in NeuroD1 relative to HBEC3KT parent cells (Sato *et al.*, 2006, 2013; Osborne *et al.*, 2013b; Figure 1A). Previously, we characterized actions of NeuroD1 in Clone 5 and found that loss of the protein decreased migration and formation of colonies in soft agar (Osborne *et al.*, 2013b). In addition, we used several non-small cell lung cancer (NSCLC) lines, including HCC4017, which was taken from the contralateral lung of the HBEC30KT donor. 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 (referred to hereafter as NTERA2), which we confirmed does not (Figure 1A). NTERA2 is reported to have stem cell characteristics and can be differentiated along a neuroectodermal lineage with retinoic acid (RA), which causes expression of NeuroD1 (Andrews, 1984; Mavilio, 1993; Przyborski *et al.*, 2000; Huang *et al.*, 2011). NeuroD1 expression was also detected in several



**FIGURE 1:** Effect of nicotine on expression of NeuroD1 in patient-derived lung cancer, bronchial epithelial, and neuroendocrine cell lines. (A) Patient-derived SCLC, HBEC3KT, Clone 5, SHSY5Y, NTERA2, and rodent PC12 cell lines were lysed, and 25  $\mu$ g of total protein was resolved on gels and immunoblotted for NeuroD1; GAPDH was the loading control. (B) Immortalized bronchial epithelial cells HBEC3KT, HBEC4KT, and HBEC30KT were treated with 0.25, 0.5, 1, and 2  $\mu$ M nicotine for 24–48 h. Cells were fixed and immunostained for NeuroD1 and 4',6-diamidino-2-phenylindole (DAPI). (C) qRT-PCR analysis of NeuroD1 expression in HBEC3KT, Clone5, HBEC30KT, and NSCLC HCC4017 with or without exposure to 0.25  $\mu$ M nicotine for 48 h, plotted as relative expression. The control was 18s RNA. One of two independent experiments in duplicate. (D) HBEC3KT and Clone 5 cells were treated with 1  $\mu$ M nicotine for 48 h, and 25  $\mu$ g of lysate protein was immunoblotted as in A with tubulin as control. Relative expression quantified using LI-COR infrared imaging in three independent experiments; \* $p$  < 0.05.

of the patient-derived neuroendocrine lung cancer cell lines, as well as in the rodent pheochromocytoma PC12 cell; its expression was undetectable in the undifferentiated germ cell tumor NTERA2 and low in the immortalized normal HBEC lines (Figure 1, A and B).

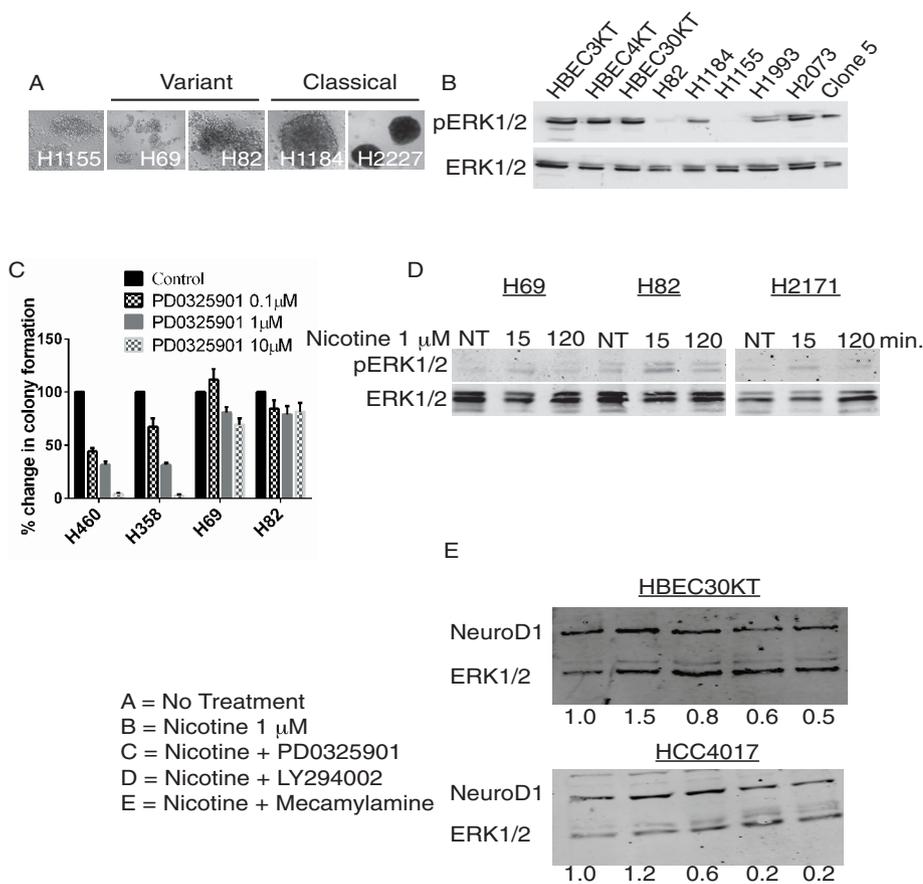
Cigarettes contain various carcinogens, including the addictive component nicotine, which has been linked to the increased onset of lung cancer (Hecht, 1999). Of all the subtypes of lung cancer, SCLC has the highest occurrence in patients with a history of tobacco use (Jackman and Johnson, 2005). Nicotine has a half-life in the circulation of 6–8 h and a measured average blood concentration (depending on consumption, type, etc.) ranging from 25 to 1000 nM (Russell et al., 1980; Hukkanen et al., 2005; Pillai et al.,

2011). To investigate potential effects of nicotine action on NeuroD1 early in tumor pathogenesis, we treated normal immortalized bronchial epithelial cell lines HBEC3KT, HBEC4KT, and HBEC30KT with increasing concentrations of nicotine for 24–48 h. An increase in NeuroD1 expression was observed after exposure of cells to concentrations of nicotine achieved in smokers (Figure 1B). Increased NeuroD1 mRNA and protein expression was detected at the lowest concentration of nicotine in both HBEC3 and 30KT lines and HCC4017 cells; however, further increase was not observed in Clone 5 (Figure 1, C and D).

### Nicotine-induced NeuroD1 expression is ERK1/2 pathway independent in SCLC, NSCLC, clone 5, HBECs, and SHSY5Y neuroblastoma cells

Phosphorylation of serine residues in NeuroD1 by ERK1/2 is required for proper trans-activation activity on the insulin promoter in pancreatic  $\beta$ -cells; however, the importance of these phosphorylation events for protein accumulation and activity seems to be cell type specific (Khoo et al., 2003; Dufton et al., 2005). Activating mutations of the MAPK-ERK1/2 pathway in SCLC are rarely observed; in addition, activation of this pathway via overexpression of Raf-1 in several SCLC cell lines results in growth arrest (Mitsudomi et al., 1991; Alessi et al., 1993; Ravi et al., 1998; Sun et al., 2007). The term “variant” refers to a subclass of SCLC cells with morphological features similar to those of NSCLC large cells, which in culture are floating, loosely bound, multicell aggregates that contrast with classic SCLC cells, which form tightly packed spheres (Carney et al., 1985a,b; Gazdar et al., 1985; Figure 2A). We observed that variant SCLC (e.g., H69 and H82) had higher NeuroD1 expression than classic SCLC (Figures 1A and 2A). The variant SCLC cell line H82 had lower basal phosphorylated ERK1/2 than the classic SCLC cell line H1184 (Figure 2B). Treatment of the SCLC cell lines H69 and H82 with the MEK1/2 inhibitor PD0325901 did not decrease anchorage-independent growth; however, growth inhibition was observed in the NSCLC lines H358 and H460 (Figure 2C). In addition, we found that nicotine caused an increase in phosphorylated ERK1/2 within minutes in several of the SCLC cell lines (Figure 2D), consistent with previous work. (Dajas-Bailador et al., 2002b; Jin et al., 2004; Xu and Deng, 2004; Chen et al., 2008).

Nicotine acts through nicotinic acetylcholine receptors (nAChRs). These ion channels have many possible subunit compositions and have been shown to be expressed in normal lung and lung cancer cells, specifically SCLC (Lam et al., 2007; Improgo et al., 2010a). Thus we tested the effects of mecamylamine, a nonselective and noncompetitive antagonist of several nAChRs. Mecamylamine caused >50% reduction in nicotine-induced NeuroD1 expression in HBEC30KT



**FIGURE 2:** Effect of nicotine and common signaling inhibitors on NeuroD1 expression and colony formation in lung cancer and immortalized bronchial epithelial cells. (A) Differential interference contrast images at 10× magnification of SCLC and NSCLC-neuroendocrine (NE) lines. (B) ERK1/2 and pERK1/2 were immunoblotted in 25 μg of lysate protein from HBEC, SCLC, and NSCLC cells as in Figure 1. (C) Formation of colonies in soft agar by SCLC (H69, H82) and NSCLC (H358, H460) cells after 4-wk exposure to 0, 0.1, 1, and 10 μM PD0325901. Colony number plotted as percentage of control. (D) SCLC H82, H69, and H2171 lines were starved for 18 h in 0.1% FBS and then untreated (NT) or treated with 1 μM nicotine for 15 and 120 min. ERK1/2 and pERK1/2 were immunoblotted as in B. (E) HBEC30KT and HCC4017 cells in normal medium were treated for 2 h as follows: A, control; B–E, 1 μM nicotine; C, +0.5 μM PD0325901; D, +10 μM LY294002; E, +10 μM mecamylamine. Lysates were immunoblotted for NeuroD1 and ERK1/2 as before. One of three independent experiments.

and HCC4017 cells (Figure 2E), confirming that nAChRs can mediate actions of nicotine on the transcription factor. We also asked whether inhibition of ERK1/2 or phosphatidylinositol 3-kinase (PI3K) affected nicotine-induced NeuroD1 expression. Effects of inhibitors of the MEK1/2 or PI3K pathway on nicotine-induced NeuroD1 expression varied (Supplemental Figures S1 and S2), suggesting that neither pathway is essential for NeuroD1 expression, but instead multiple pathways can affect nicotine-induced NeuroD1 expression.

### Nicotine can up-regulate NeuroD1 without causing differentiation of SHSY5Y and NTERA2 cells

Because NeuroD1 was originally identified as a gene required for neuronal differentiation, we investigated whether 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 after treatment with RA. The resulting cells

have biochemical and morphological similarities to neurons (Andrews, 1984; Lopez-Carballo et al., 2002). Treatment of these cell lines with RA induced expression of NeuroD1 and one of its key targets, TrkB, as well as of a marker of mature neurons, NeuN. In contrast, treatment with nicotine caused an increase in NeuroD1 and TrkB but no significant increase in NeuN (Figure 3, A and B), suggesting that it was not able to induce a differentiated state. We examined expression of transcription factors ASCL1 and NGN3, which lie upstream of NeuroD1. NGN3, like ASCL1, is a bHLH transcription factor found in brain and pancreas during development (Cau et al., 1997; Huang et al., 2000; Ito et al., 2000; McNay et al., 2006; Neptune et al., 2008). RA caused decrease or no change in expression of ASCL1 or NGN3 in either cell line (Figure 3B), whereas nicotine caused increased ASCL1 and NGN3 in the NTERA2 cell line, supporting the idea that nicotine is not promoting a more differentiated state.

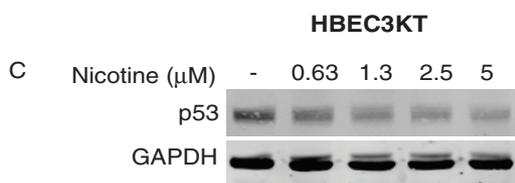
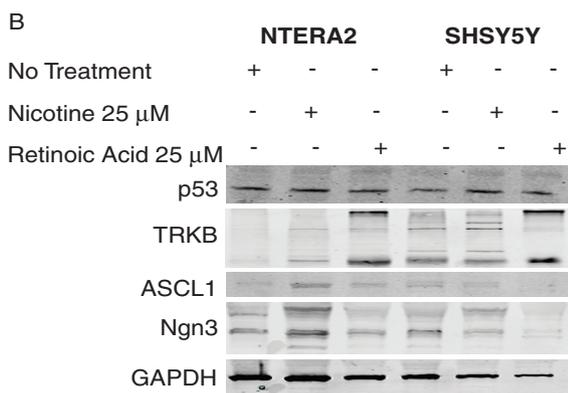
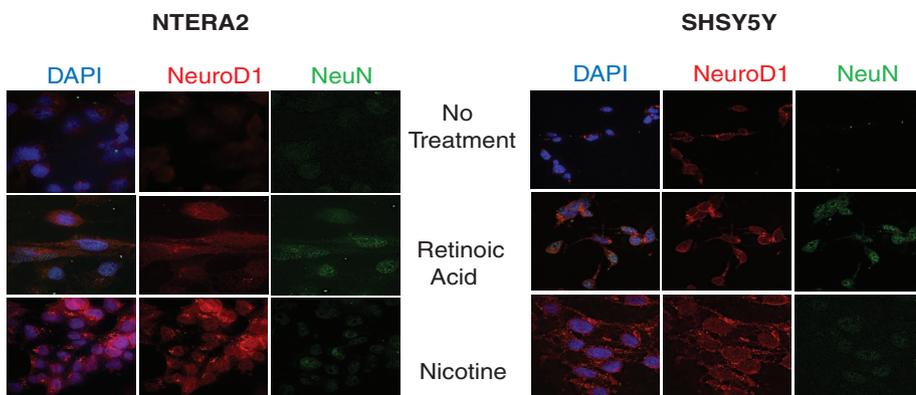
### Nicotine decreases p53 expression in a cell type-specific manner

We demonstrated that loss of p53 led to increased NeuroD1 expression in a cell type-specific manner (Osborne et al., 2013a). Nicotine induces proliferation and leads to down-regulation of p53 (Pfeifer et al., 2002; Dasgupta and Chellappan, 2006; Dasgupta et al., 2006; Sato et al., 2008; Puliyappadamba et al., 2010). Treatment of SHSY5Y and the NTERA2 cells with nicotine did not suppress p53 expression (Figure 3B). However, p53 expression was reduced by nicotine in HBEC3KT (Figure 3C). This leads to the idea that suppression of p53 in nonepithelial tissues may not be required to increase NeuroD1 expression.

### NeuroD1 regulates the expression of nAChR α3 and α5 but not β4 subunits in SHSY5Y neuroblastoma, SCLC, and HBEC3KT

Several genome-wide studies mapped a specific region on chromosome 15q25 as a locus for lung cancer susceptibility, and this locus contains the genes that encode the nAChR subunit cluster of α3, α5, and β4 (Amos et al., 2008; Hung et al., 2008). ASCL1 can regulate the α3 and β4 subunits (Improgo et al., 2010a). We detected higher expression of the nAChR subunits and NeuroD1 in the cancer lines than in the HBEC lines (Figure 4A). Several of the subunits are heavily glycosylated and acetylated to enable proper localization to the plasma membrane, which may explain the extra bands visualized (Albuquerque et al., 2009). Using low concentrations of nicotine, we found that NeuroD1 and all three AChR subunit mRNAs increased in SHSY5Y cells (Figure 4B). An increase in each of the subunits was also detected in the HBEC3KT cells with exposure to nicotine (Supplemental Figure S3A). Increase in neither NeuroD1

A



**FIGURE 3:** Nicotine can increase NeuroD1 expression without causing neuroendocrine differentiation. (A, B) NTERA2 and SHSY5Y cells were treated with 25  $\mu$ M nicotine or 25  $\mu$ M retinoic acid for 14 d to induce differentiation. (A) Cells were fixed and immunostained for NeuroD1 and NeuN (mature neuronal marker) and DAPI. Representative of three independent experiments. (B) Cell lysates were immunoblotted for NeuroD1, TrkB, ASCL1, NGN3, and p53. GAPDH was the loading control. (C) HBEC3KT cells were treated with 0.63, 1.25, 2.5, and 5  $\mu$ M nicotine over 24–48 h. Cell lysates were immunoblotted for p53; GAPDH was the loading control.

(Supplemental Figure S3C) nor subunit expression was observed in the HBEC3KT53, an HBEC cell line with constitutive knockdown of p53 (Supplemental Figure S3A). The inability of nicotine to cause a significant increase in NeuroD1 and AChR subunit expression was also observed in the NSCLC cell lines Clone 5 and H358 (both of which have oncogenic KRAS and have lost p53; Supplemental Figure S3B; see Table 1 for cell-line oncogenotype).

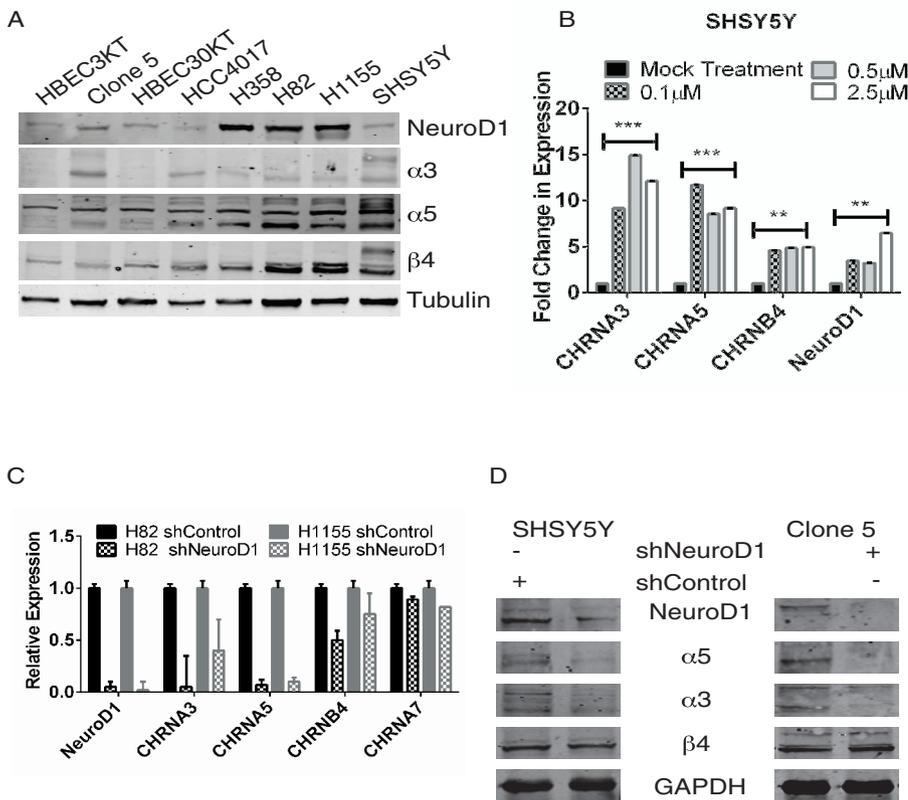
Next we knocked down NeuroD1 in the cancer cells and examined expression of the nAChR subunits. In the neuroendocrine lung cancer cell lines, knockdown of NeuroD1 led to a significant decrease in both  $\alpha$ 3 and  $\alpha$ 5, but less so in  $\beta$ 4 or  $\alpha$ 7, nAChR receptor subunits (Figure 4C). Similarly, knockdown of NeuroD1 in SHSY5Y and Clone 5 cells led to a significant decrease in both  $\alpha$ 3 and  $\alpha$ 5 but not  $\beta$ 4 subunits (Figure 4D). This suggests that increased expression of the  $\alpha$ 3 and  $\alpha$ 5 in response to nicotine may be mediated by NeuroD1.

### NeuroD1 regulates nicotine-induced invasion by SHSY5Y neuroblastoma and Clone 5 via regulation of the $\alpha$ 3 and $\alpha$ 5 subunits

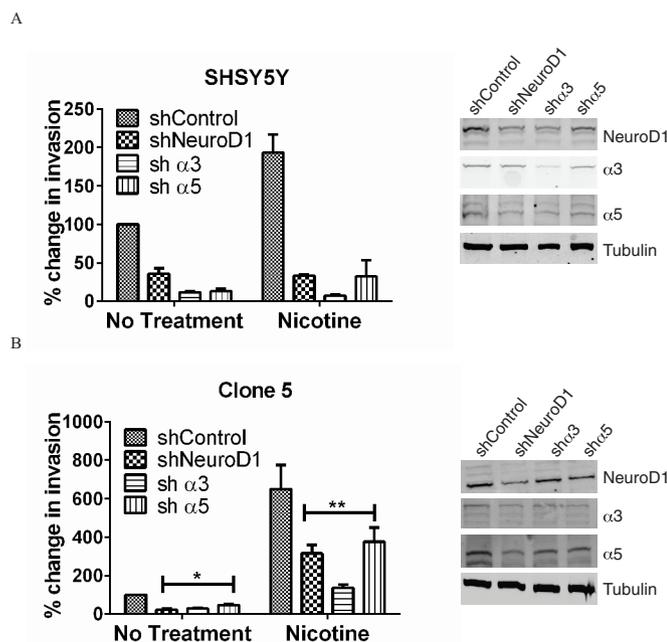
NeuroD1 regulates survival and the metastatic capabilities of several neural/neuroendocrine carcinomas (Huang *et al.*, 2011; Osborne *et al.*, 2013b). Nicotine acts through various combinations of nAChR subunits to promote epithelial-to-mesenchymal transition and metastasis (Xu and Deng, 2004; Tournier *et al.*, 2006; Guo *et al.*, 2008; Dasgupta *et al.*, 2009; Pillai *et al.*, 2011). Thus we sought to investigate the possibility that NeuroD1 could modulate nicotine-induced invasion in our cell lines. Nicotine exposure was able to increase invasion of Clone 5 and SHSY5Y lines through Matrigel, whereas knockdown of NeuroD1 led to >50% reduction in nicotine-induced migration in both cell lines (Figure 5, A and B). Loss of either  $\alpha$ 3 or  $\alpha$ 5 expression reduced invasion with or without nicotine treatment. The  $\alpha$ 5 results were not anticipated, because this receptor subunit is thought of as an accessory or an orphan subunit that is unable to yield functional receptors when expressed alone and speculated to increase receptor sensitivity to ligand (Ramirez-Latorre *et al.*, 1996; Gerzanich *et al.*, 1998; Jackson *et al.*, 2010). Taken together, these data suggest that nicotine acts through nAChR subunits to mediate cell invasion and migration by a NeuroD1-dependent mechanism.

### The NeuroD1 target TrkB regulates expression of several nAChR subunits in H69 and H82 SCLC

In addition to increased expression of NeuroD1, we observed that nicotine increased expression of TrkB in NTERA2 cells (Figure 3B). Nicotine via nAChR receptor subunit  $\alpha$ 7 was reported to stimulate proliferation in the SHSY5Y cell line via release of brain-derived neurotrophic factor (BDNF), the ligand for TrkB (Serres and Carney, 2006). On the basis of this idea, we asked whether phosphorylated TrkB bound the nAChR subunit complex. Immunoprecipitation experiments failed to detect interaction between TrkB and any of the complexes containing  $\alpha$ 5 (Supplemental Figure S4). We then examined whether there was a relationship between TrkB expression and expression of any other nAChR receptor subunits. Loss of TrkB led to decrease in expression of not only the cluster subunits of  $\alpha$ 3,  $\alpha$ 5, and  $\beta$ 4, but also of  $\alpha$ 7, in three of the neuroendocrine lung cancer cell lines examined—H69, H82, and the neuroendocrine NSCLC H1155 (Figure 6A). Next we investigated whether TrkB activity was required for changes in AChR expression. The pan-Trk inhibitor lestaurtinib decreased expression of the nAChR cluster receptor subunits, with the most pronounced effects on  $\alpha$ 3,  $\alpha$ 5, and  $\beta$ 2 subunits (Figure 6B). Nicotine combined with BDNF increased expression of the  $\alpha$ 3 and to some extent the  $\alpha$ 5 subunit (Figure 6C and Supplemental Figure S4A, lanes 1 and 4),



**FIGURE 4:** NeuroD1 regulates the expression of  $\alpha 3$  and  $\alpha 5$ , but not  $\beta 4$ , nAChR subunits in SHSY5Y, Clone 5, SCLC, and NSCLC-NE lines. (A) NeuroD1 and  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 4$  subunits were immunoblotted in 25  $\mu$ g of lysate protein from SCLC, NSCLC, HBEC3KT, Clone 5, and SHSY5Y cells as in Figure 1A. (B) SHSY5Y was treated with 0.1, 0.5, or 2.5  $\mu$ M nicotine for 48 h. qRT-PCR of NeuroD1 and CHRNA3, A5, and B4 subunits; 18s RNA control. One of three independent experiments. \*\*\*  $p < 0.001$ , \*\*  $p < 0.005$ . (C, D) Knockdown of NeuroD1 in H82, H1155, and Clone 5 lung and SHSY5Y neuroblastoma lines. H82 and H1155 cells were stably infected with shNeuroD1 or shControl vectors. Cells were selected by puromycin resistance and sorted for expression of green fluorescent protein. Then (C) NeuroD1 and CHRNA3, A5, A7, and B4 expression was analyzed by qRT-PCR and plotted relative to 18s RNA, or (D) lysate proteins were immunoblotted for NeuroD1 and  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 4$  with GAPDH as control.



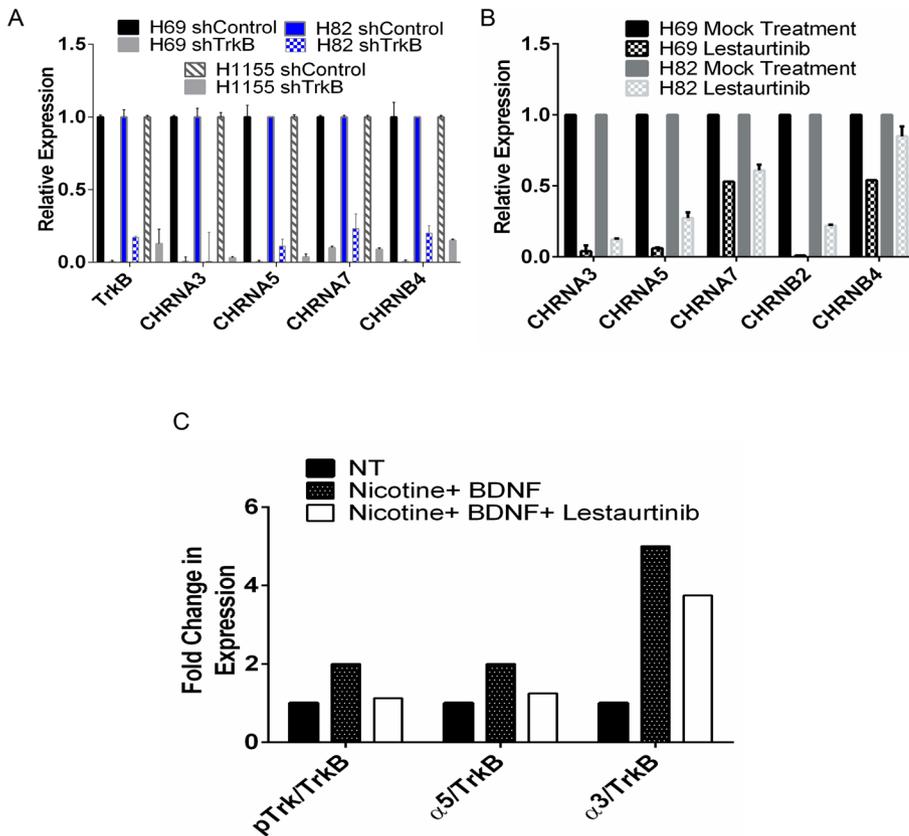
**FIGURE 5:** Acetylcholine receptor subunits  $\alpha 3$  and  $\alpha 5$  contribute to invasion induced by nicotine in SHSY5Y and clone 5 cells. (A) SHSY5Y and (B) clone 5 cells were infected with shNeuroD1, sh $\alpha 3$ , sh $\alpha 5$ , or shControl vectors to deplete the targeted proteins. Cells were dissociated, and 25,000 cells were placed in 0.2 ml of growth factor-reduced Matrigel. Invasion was scored as described in *Materials and Methods*. SHSY5Y,  $N = 2$ ; Clone 5,  $N = 3$ . \*  $p < 0.05$ , \*\*  $p < 0.005$ . Right, proteins remaining 48 h after treatment with shRNAs, with tubulin as the loading control.

consistent with decreased expression of both subunits with lestaurtinib (Figure 6C and Supplemental Figure S4A, lanes 1, 4, and 7). Taken together, these data suggest that nicotine may regulate expression of nAChR subunits by both NeuroD1-dependent and TrkB-dependent mechanisms.

### NeuroD1 regulates changes in intracellular calcium in SHSY5Y neuroblastoma cells

The function of nAChR receptors upon ligand binding is to elevate cytoplasmic free calcium. Nicotine is believed to promote the influx of calcium predominantly through the  $\alpha 7$  subunit, based on effects of a selective antagonist (Dajas-Bailador *et al.*, 2002a). Calcium influx into cells has a plethora of roles exerting effects on many proteins, including those that enhance cell motility. We determined which of the cell lines displayed changes in intracellular calcium in response to nicotine. Nicotine induced acute changes in intracellular calcium only in SHSY5Y cells (Figure 7A). Segregation of the precise source of nicotine-induced changes in intracellular calcium has been complex. Prior work examining the contributions of  $\alpha 7$ ,  $\alpha 3$ , and  $\beta 2$  subunits revealed that in addition to influx through the nAChR, voltage-gated calcium channels and intracellular stores mediate a large percentage of the nicotine-induced response (Dajas-Bailador *et al.*, 2002b). To ascertain whether the changes we observed were dependent on an extracellular source of calcium, we treated cells with both the general nAChR antagonist mecamylamine and nifedipine, a blocker of L-type voltage-gated calcium channels. Both compounds prevented nicotine-induced changes in intracellular calcium to a similar extent, consistent with the idea that the majority of the response we observed was due to calcium influx (Figure 7B).

We next examined whether knockdown of NeuroD1, in addition to  $\alpha 3$  or  $\alpha 5$  subunits, would have an effect on the function of the receptors. Knockdown of  $\alpha 3$  or  $\alpha 5$  subunits prevented changes in intracellular calcium observed upon short-term exposure of cells to nicotine (Figure 7C). Knockdown of NeuroD1 also led to reduction in nicotine-induced intracellular calcium (Figure 7C). These data support the idea that NeuroD1 can regulate the function of the nAChR receptor subunits via regulation of their expression. These



**FIGURE 6:** TrkB, a downstream target of NeuroD1, regulates expression of several nAChR subunits in H69 and H82 SCLC and H1155 NSCLC-NE lines. (A) H69, H82, and H1155 cells were stably infected with shTrkB or shControl vectors. Cells were selected by sorting as in Figure 5. qRT-PCR analysis of TrkB and CHRNA3, A5, A7, and B4, plotted as expression relative to the 18s RNA control. (B) H69 and H82 cells were treated with 10  $\mu$ M lestauritinib for 8 h, followed by qRT-PCR analysis of TrkB and CHRNA3, A5, A7, B4, and B2 plotted as described. (C) H82 cells were starved for 8 h in 0.1% FBS. Cells were treated with 1  $\mu$ M nicotine and 50 ng/ml BDNF for 1 h with or without lestauritinib. Left, cell lysates immunoblotted with indicated antibodies. Right, blots quantified using LI-COR infrared imaging.

findings suggest the presence of feedback mechanisms that may occur early or throughout neuroendocrine tumorigenesis.

## DISCUSSION

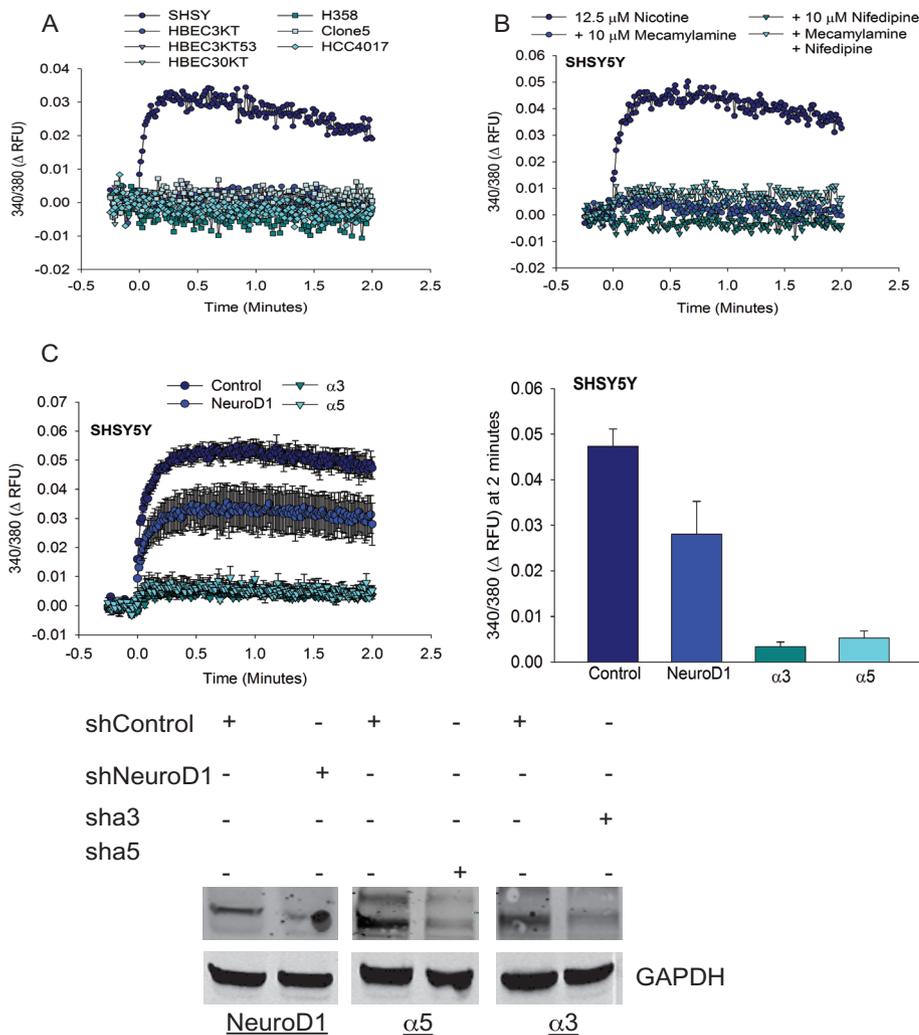
Nicotine induces cell survival and metastasis and also has been shown to inhibit differentiation (Sato *et al.*, 2008). We find that up-regulation of NeuroD1 by nicotine occurs in some but not all cell types. In normal immortalized HBEC, exposure to nicotine causes an increase in NeuroD1; however, this was not the case in several nonneuroendocrine cells that lack p53 alone or in combination with KRAS mutations. This is consistent with previous accounts demonstrating that nicotine does not enhance tumorigenesis in KRAS-driven mouse models of lung cancer (Maier *et al.*, 2011). Our findings also suggest that long-term exposure to nicotine does not induce differentiation but does induce NeuroD1 in neural/neuroendocrine models. Consistent with their roles in maintenance of progenitor states during differentiation of stem cells, we found that transcription factors such as ASCL1 and NGN3 decrease with exposure to retinoic acid but increase with nicotine (Cau *et al.*, 1997; Gu *et al.*, 2002; McNay *et al.*, 2006; Xu *et al.*, 2008; Magenheimer *et al.*, 2011). It is possible that loss of p53 as result of nicotine exposure could lead to increase in NeuroD1 expression during the development of lung cancer. Similarly, overexpression of p53 in embryonic

stem cells has also been shown to promote differentiation and limit proliferation potential, consistent with suppression of NeuroD1 (Li *et al.*, 2012; Menendez *et al.*, 2012).

The ERK1/2-MAPK pathway regulates both transactivation activity and accumulation of NeuroD1 protein in a context-dependent manner. Nicotine activates both AKT and ERK1/2, most likely by calcium-dependent mechanisms (Dajas-Bailador *et al.*, 2002b; West *et al.*, 2003; Tsurutani *et al.*, 2005; Chen *et al.*, 2008; Pillai *et al.*, 2011); however, we find that the increase in NeuroD1 expression as a consequence of nicotine exposure can be independent of both ERK1/2 and PI3K. Calcium influx stimulates other pathways that may control NeuroD1 expression; for example, in neural stem cells this is believed to occur indirectly through calcium, calmodulin-dependent protein kinase II (Schneider *et al.*, 2008). Therefore other pathways need to be investigated that may ultimately be activated by nicotine-induced calcium influx leading directly or indirectly to NeuroD1 expression.

The use of multiple cell lines in this study attempts to direct our understanding of the mechanisms by which nicotine induces NeuroD1 during neuroendocrine carcinogenesis of the lung, as well as in other neuroendocrine tumor types. Although we recently published results indicating that NeuroD1 may enhance metastasis of neuroendocrine carcinomas via regulation of the proto-oncogene TrkB, the link connecting NeuroD1 to active TrkB was not conserved in all cell lines tested (Osborne *et al.*, 2013a). We found that conservation of the regulation of metastatic capability by TrkB depended on the likely cell of origin (i.e., whether the cell expressed neuroendocrine characteristics). This relationship may also be relevant to the actions of nicotine on NeuroD1 in various tissues. Further studies on regulation of NeuroD1 and TrkB by nicotine in other tissues will be valuable to a more thorough examination of this possibility.

We demonstrate that NeuroD1, like ASCL1, regulates the nAChR subunit cluster, specifically  $\alpha$ 3 and  $\alpha$ 5, whereas ASCL1 has been suggested to regulate  $\alpha$ 3 and  $\beta$ 4 (Improgo *et al.*, 2010b). Preliminary data suggest direct binding of NeuroD1 to the promoter regions of the nAChR subunit cluster (unpublished data); further characterization of the exact mode of regulation is necessary. Based on assays of intracellular free calcium, regulation of  $\alpha$ 3 and  $\alpha$ 5 affects the function of the receptors as ion channels. Thus we hypothesize that both NeuroD1 and ASCL1 regulate expression of the nAChR subunit cluster of  $\alpha$ 3,  $\alpha$ 5, and  $\beta$ 4, with different relative action on specific subunits. Considering our present and previous findings, we suggest that NeuroD1 lies in a positive feedback loop through which nicotine enhances the expression of its own receptors (Figure 8). Increased expression of NeuroD1 by nicotine presumably leads to increased expression of the receptor subunits; effects may be both dependent on and independent of TrkB activity. This combinatorial regulation may ultimately lead to changes in the levels of calcium and increased migration and invasion. The exact composition of the



**FIGURE 7:** Depletion of NeuroD1 decreases nicotine-induced increases in intracellular free calcium in SHSY5Y cells. (A) The indicated cell lines were loaded with fura-2AM and then stimulated with 12.5 μM nicotine. Fluorescence was monitored every 0.74 s after stimulation. (B) SHSY5Y cells loaded with fura-2AM were stimulated with 12.5 μM nicotine plus mecamylamine, nifedipine, or both at 10 μM, and fluorescence was monitored as described. *N* = 2. (C) SHSY5Y cells were infected with shNeuroD1, shα3, shα5, or shControl vectors for 48 h and then stimulated with 12.5 μM nicotine. Fluorescence was monitored as described. Representative of (A) four, (B) two, and (C) three independent experiments. Bottom, immunoblots showing effectiveness of depletion.

receptors and the ultimate consequence of regulation of each subunit remain to be defined.

## MATERIALS AND METHODS

### Cell culture

NTERA2 clone D1 cells (American Type Culture Collection [ATCC, Manassa, VA]) were grown in DMEM with 10% fetal bovine serum (FBS). SCLC and NSCLC 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 from ATCC were grown in DMEM/Ham's F12 (1:1) with 10% FBS. Immortalized HBECs (except HBEC3KTRL53-Clone 5; Sato *et al.*, 2006) were cultured in Keratinocyte-SFM (Invitrogen, Grand Island, NY) 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, Madison, WI) and confirmed

to be the same as the DNA fingerprint library maintained by either ATCC or the Hamon Cancer Center. The lines were mycoplasma free, as confirmed by the e-Myco kit (Boca Scientific, Boca Raton, FL).

### Antibodies and reagents

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 and immunoprecipitation: goat NeuroD1 (N-19), rabbit pan-phospho-Trk (E-6), p53 (DO-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; FL-335; Santa Cruz Biotechnology, Santa Cruz, CA); mouse ASCL1 (BD Biosciences, San Jose, CA); rabbit TrkB (Chemicon, Temecula, CA); mouse NGN3 (R&D Systems, Minneapolis, MN); rabbit nAChR subunits (Millipore, Billerica, MA); and mouse pERK1/2, mouse AKT, and rabbit pAKT-S473 (Cell Signaling [Danvers, MA], Sigma-Aldrich [St. Louis, MO], respectively). α-Tubulin hybridoma was purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Lestaurtinib was purchased from LC labs, Woburn, MA; BDNF from R&D Systems; nicotine, PD0325901, and LY294002 was from Cell Signaling. Band intensities were quantified using a LI-COR Odyssey Infrared Imaging System.

### Invasion assays

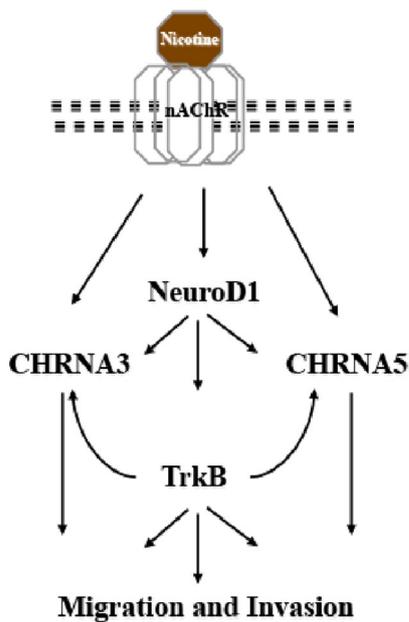
For invasion assays,  $2.5 \times 10^4$  cells were embedded in growth factor-reduced Matrigel in the presence or absence of 1 μM nicotine 48 h after knockdown. Transwell migration was assayed in Transwell permeable supports (3422; Corning, Corning, NY). Cells were seeded in the top chamber in either RPMI with 1% FBS or DMEM/Ham's F12 with 1% FBS and allowed to migrate along a concentration gradient through a polycarbonate membrane with 8-μm pores toward the bottom chambers, which contained medium with 25% FBS. Cells were fixed, stained with methylene blue, and counted.

### Transfection of short hairpin RNAs and plasmids

Virus was generated for infection of human pGIPZ lentiviral short hairpin RNA (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 the Open Biosystems website <http://dharmacon.gelifsciences.com/rnai-and-custom-rna-synthesis/shrna/gipz-lentiviral-shrna/libraries/>). NeuroD1 sh RNA, V2LHS\_152218 (shRNA-1); TrkB shRNA, V2LHS\_63731.

### Calcium assays

Cells were washed twice with phosphate-buffered saline (PBS) and incubated with the fluorescent dye Fura-2AM diluted in



**FIGURE 8:** Model. Nicotine induces activation of NeuroD1 and the nAChR subunits. NeuroD1 then regulates its downstream targets, TrkB and the nAChR subunits, which control invasion and migration in cancer cells.

keratinocyte serum-free medium with 2.5 mM calcium for 1 h. Cells were washed twice and equilibrated for 30 min. Nicotine (25  $\mu$ M) was added using a Synergy microplate reader. Changes in intracellular calcium were assessed by dual excitation of Fura-2 at 340/11 and 380/20 nm and emission at 508/20 nm using Gen5 software.

#### Immunofluorescence and nicotine/differentiation protocol

Cells were treated with nicotine (Sigma-Aldrich; 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 with PBS. Cells were permeabilized with 0.1% Triton X-100 and washed. After incubation with 10% donkey serum/bovine serum albumin at room temperature for 1 h, 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 1 h, washed with PBS, and imaged. Fluorescent Z-stacks (0.2 mm) were acquired and deconvolved using a DeltaVision RT deconvolution microscope.

#### Colony formation assay

Soft agar colony assays were as described previously (Sato *et al.*, 2006). As indicated, PD0325901 was added once per day after seeding and solidification of agar.

#### Quantitative real-time PCR

Total RNA from cell lines was isolated with TRI Reagent. cDNA was synthesized using iSCRIPT cDNA Synthesis Kit (Bio-Rad). RNA were quantified by real-time PCR (RT-PCR) with iTaq (Bio-Rad) master mix using TaqMan probes for 18s rRNA, NeuroD1, and TrkB (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.

Quantitative RT-PCR (qRT-PCR) primers were as follows:  $\alpha$ 3, reverse, TTGCAGAAACAATCCTGCTG, and forward, ATGCTGTGCTGTCCCTCTCT;  $\alpha$ 5, forward, CCAAAGTCTTTGCATGAGA, and reverse, TCCACAGAAACATCCGATCA;  $\alpha$ 7, forward, CCCAAGTG-GACCAGAGTCAT, and reverse, GCCACACTACCCAGAGT;  $\beta$ 4, forward TCCCTGGTCCTTTTCTTCT, and reverse, TGCAGCT-TGATGGAGATGAG; and  $\beta$ 2, forward, GGCATGTACGAGGTGTC-CTT, and reverse, CACCTCACTCTTCAGCACCA.

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