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Notch signaling contributes to lung cancer clonogenic capacity *in vitro* but may be circumvented in tumorigenesis *in vivo*

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Abstract

The Notch signaling pathway is a critical embryonic developmental regulatory pathway that has been implicated in oncogenesis. In non-small cell lung cancer (NSCLC), recent evidence suggests that Notch signaling may contribute to maintenance of a cancer stem or progenitor cell compartment required for tumorigenesis. We explored whether intact Notch signaling is required for NSCLC clonogenic and tumorigenic potential *in vitro* and *in vivo* using a series of genetically modified model systems. In keeping with previous observations, we find that *Notch3* in particular is upregulated in human lung cancer lines, and that down regulation of Notch signaling using a selective γ -secretase inhibitor (MRK-003) is associated with decreased proliferation and clonogenic capacity *in vitro*. We demonstrate that this phenotype is rescued with the expression of NICD3, a constitutively active cleaved form of Notch3 not affected by γ -secretase inhibition. Using an inducible LSL-*KRAS*^{G12D} model of lung cancer *in vivo*, we demonstrate a transient upregulation of Notch pathway activity in early tumor precursor lesions. However, a more rigorous test of the requirement for Notch signaling in lung oncogenesis, crossing the LSL-*KRAS*^{G12D} mouse model with a transgenic with a similarly inducible global dominant negative suppressor of Notch activity, LSL-DNMAML (dominant negative mastermind-like), reveals no evidence of Notch pathway requirement for lung tumor initiation or growth *in vivo*. Distinct Notch family members may have different, and potentially opposing, activities in oncogenesis, and targeted inhibition of individual Notch family members may be a more effective anti-cancer strategy than global pathway suppression.

Introduction

In 2010 over 160,000 Americans died of lung cancer, more than from prostate, breast, and colon cancers combined, making this by far the leading cause of cancer deaths (1). Approximately 85% of all lung cancers are of non-small cell histology. Although current therapies have improved survival and quality of life for patients with advanced non-small cell lung cancer (NSCLC), this remains a nearly universally fatal disease. New therapeutic approaches are critically needed.

Progenitor subpopulations of cancer cells with high clonogenic and tumorigenic potential, termed cancer stem cells or tumor progenitor cells, have recently been described within

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many solid tumors including cancers of the breast, prostate, and brain (2). Differentiated tissues of the body are all derived from pluripotent precursors, the most primitive of which are termed stem cells. Stem cells maintain a unique ability for self-renewal, and for production of progeny capable of differentiation along multiple developmental lineages. Similarly, recent data from a large number of laboratories using a variety of approaches suggest that the differentiated and heterogeneous cells that comprise human tumors are derived from relatively small populations of precursors with selective self-renewal and differentiative potential (3). These self-renewing precursors appear to have unique tumorigenic capacity in *in vivo* models. Taken together these several lines of evidence support what has become known as the cancer stem cell hypothesis: that a small but variable percentage of cells in a tumor are capable of extensive self-renewal and tumor propagation, and that tumors are comprised of at least two functionally and phenotypically distinct populations: 1) a small population of cells with stem cell-like characteristics and extensive proliferative capability, and 2) a more differentiated population with limited proliferative (and essentially no long-term tumorigenic) potential.

The isolation and initial phenotypic characterization of stem-like cells within cancers with unique clonogenic/tumorigenic potential was first demonstrated in the context of Acute Myeloid Leukemia and more recently in breast, brain, prostate and other malignancies (4-8). Definitive isolation and characterization of precursor populations for NSCLC has not been reported, although data from a murine model of lung adenocarcinoma suggests that tumors arise from a small compartment of specialized cells in the terminal bronchoalveolar junctions (9). The authors of this report suggest that similar cells may give rise to human adenocarcinoma.

Emerging data from multiple groups suggests that survival, proliferation, and differentiation of cancer stem cells are regulated by differential activity of key embryonic signaling pathways (10). Survival, proliferation and differentiation of normal stem cells and somatic precursors are tightly regulated by key developmental signal pathways. Many of these pathways, including the Notch, Wnt, and Hedgehog pathways, appear to be active, and aberrantly regulated, in cancers and in defined cancer precursor populations (2). Elevated expression of Notch family members, as well as expression of *Hes1* (hairy and enhancer of split 1), a downstream target of the Notch pathway, has been reported in NSCLC, consistent with functional pathway activity (11-13).

The Notch signaling pathway is a highly evolutionarily conserved developmental regulatory pathway (14-16). In mammalian species including mice and humans, there are four Notch receptors: Notch1, Notch2, Notch3 and Notch4 (17). Notch receptors are single-pass transmembrane proteins with a large extracellular portion and relatively small intracellular domain. Notch signaling is initiated upon Notch ligand (Jagged or Delta-like) binding to the extracellular domain. Because Notch ligands are also transmembrane proteins, the Notch cascade is normally triggered by direct cell-to-cell contact. Upon ligand/receptor interaction, two sequential proteolytic cleavage events of the engaged Notch receptor occur, involving α -secretase and γ -secretase, releasing the Notch intracellular domain (NICD). The NICD can then translocate to the nucleus where it converts the complex CSL (comprised of CBF1 and RB) from a transcriptional repressor into a transcriptional activator (18, 19). The NICD-CBF1 complex is bound by Mastermind-like (MAML) proteins, which serve as a scaffold to recruit co-activators (i.e. p300), driving the expression of downstream targets such as *Hes1*, encoding a basic helix loop helix transcriptional repressor, and other genes that promote cell growth and proliferation.

In addition to its multiple roles in controlling cell fate and differentiation decisions in embryogenesis, dysregulation of the Notch pathway has been implicated in many cancers,

first and most definitively T-cell acute lymphoblastic leukemia (T-ALL) (20). Approximately 60% of T-ALL harbor activating mutations in *Notch1*, which functions as a critical driver mutation for this type of cancer. The roles of aberrant Notch signaling in other malignancies are more complex, with both Notch pathway activating mutations, and inactivating mutations being defined (21-23).

Dang and colleagues first linked the dysregulation of Notch3 to human lung cancer when they observed an upstream chromosome translocation in tumor from a nonsmoker and later observed overexpression of *Notch3* in 40% of NSCLC (24, 25). Oncogenic mutations in *Notch1* in lung cancer have also been described (11). Among other suggested effects, it has been reported that Notch activity promotes NSCLC survival through inhibition of pro-apoptotic Bim, and through induction of anti-apoptotic Survivin (26, 27). A putative tumor progenitor cell subset in NSCLC lines, defined by aldehyde dehydrogenase upregulation, was recently found to be specifically dependent on Notch activity for maintenance of clonogenic potential (12). Taken together, these observations suggest that the inhibition of Notch signaling represents a potential therapeutic strategy in NSCLC.

In summary several lines of indirect evidence suggest that Notch signaling may regulate proliferation, survival, and differentiation of a subpopulation of clonogenic precursors or cancer stem cells in NSCLC. We sought to explore the roles of Notch signaling in lung cancer development, and in particular to more definitively test the hypothesis that Notch signaling was critical to initial stages of lung carcinogenesis in early precursor lesions using *in vivo* models of NSCLC tumorigenesis.

Materials and Methods

Cell lines and inhibitors

NSCLC cell lines NCI-H1299, NCI-H1435, NCI-A549 and NCI-H1993 were obtained from American Type Culture Collection (ATCC). All lung cancer cell lines were maintained in RPMI-1640 (Quality Biological) supplemented with 10% fetal bovine serum (FBS; Gemini). Cultures were incubated at 37°C with 95% air / 5% CO₂ in a humidified incubator. The γ -secretase inhibitor MRK-003 (Merck & Co., Inc.; obtained through a Material Transfer Agreement) was dissolved in dimethyl sulfoxide (DMSO) for *in vitro* use (21).

Plasmids and stable transfectants

MSCV-Mam(12-74)-EGFP (gift of Dr. Warren Pear, (28)), pTOF-DNMAML1 (gift of Dr. Hiroshi Nakagawa, (29)), cytoTan1pCDNA3²³ and pCDF1-MCS2-EF1-copGFP-NICD3 (gifts of Dr. Brenda Lilly, (30)) were transfected in 20 μ g DNA aliquots into H1299 using Eugene HD (Roche) as specified by the manufacturer. After 24 hours selection media containing 2.5 μ g/ml puromycin was added and stable lines were created after growing in selection media for 14 days.

Pharmacologic Notch inhibition

Inhibition of Notch signaling using MRK-003 was evaluated in two NSCLC cell lines (NCI-H1299, NCI-A529 and NCI-H1435). 1×10^5 cells were plated in 6-well plates. After 24 hours MRK-003 is added at concentrations ranging from 0.1 μ M to 10.0 μ M and cells are harvested 96 hours later. Cells were either treated with MRK-003 or with vehicle alone (DMSO at a final concentration of 0.03%). mRNA was isolated from cells following treatment.

RNA isolation and quantitative PCR analysis

NSCLC cell lines were treated with DMSO (final concentration 0.3%) or MRK-003 for 96 hours and then harvested. RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA was synthesized with QuantTECT kit (Qiagen). Quantitative reverse transcriptase PCR was performed on Biorad iQ5 using the $\Delta\Delta$ CT protocol using TaqMan inventoried primers/probes (Applied Biosystems) for Hes1 and β -actin (as internal control). Analysis was performed using IQTM5 Optical System Software (Biorad). Integrated DNA Technologies Prime Time qPCR Assays was used to detect expression of *DNMAML*.

Cell viability and regrowth assays

H1299 cells were transfected with either empty vector (expressing GFP) or vector expressing NICD3 and GFP (pCDF1-MCS2-EF1-copGFP-NICD3). Both cell lines were co-transfected with a linear puromycin resistance construct (Clontech) to establish a cell line that can be grown in selection media. After 7 days in selection media clones were picked and expanded. Established cell lines (H1299-GFP and H1299-N3) were treated with MRK-003, a gamma secretase inhibitor, for 8 days, at concentrations between 0.1 - 3.0 μ M. Cells were harvested and 3×10^3 viable cells were plated in a 12 well plate in triplicate. Cells were stained with crystal violet after 7 days. In order to quantify crystal violet staining, 500 μ l of methanol was added to each well to solubilize the dye. 100 μ l of the CV elution was added in triplicate to a 96 well plate; relative absorbance (OD 540) was detected with SpectraMax M2^e spectrophotometer and SoftMax Pro software (Molecular Devices).

Mice

All animal experiments were done in accordance with approved protocols from the Johns Hopkins University, School of Medicine Animal Care and Use Committee and National Institutes of Health guidelines. Breeder pairs of LSL-KRAS^{G12D/+} and *DNMAML*^{f/+} in the C57BL/6 genetic background were obtained from NCI, Mouse Repository, Frederick, Maryland and Dr. Warren Pear, respectively. Required quantities of KRAS^{G12D/+} and *DNMAML*^{f/+} mice were generated as described below. Animals were housed in ventilated cages under standardized conditions (21°C, 60% humidity, 12-h light/12-dark cycle, 20 air changes per hour) in the Johns Hopkins University rodent barrier facility.

Breeding, genotyping analysis, gene activation and expression

LSL-KRAS^{G12D/+} and *DNMAML*^{f/+} mice were maintained in a C57BL/6 heterozygous genetic background. The genetic background of murine pups was confirmed by DNA extraction from tail tips. Briefly, genomic DNA was extracted from snap-frozen tail tissue samples using the mini-prep kit (Qiagen). PCR for the mutant and wild KRAS allele was performed based on primers and PCR protocol suggested by NCI Mouse Repository. *DNMAML* mice were genotyped using primers specific for the ROSA26 locus (28). *DNMAML*^{f/+} mice were intercrossed with LSL-KRAS^{G12D}, resulting in expression of both KRAS and *DNMAML*. The LSL-KRAS^{G12D};*DNMAML*^{f/+} mice were compared with age-matched *DNMAML*^{f/+} and LSL-KRAS^{G12D} mice as controls. To study tumorigenesis in the mouse lung, mice were exposed to an adenovirus expressing Cre recombinase (AdenoCre; Vector Biolabs) via intranasal instillation (10^8 pfu/mouse), resulting in expression of mutant KRAS or both mutant KRAS and *DNMAML* specifically in the lung.

Lung tissue preparation and analysis

At the indicated timepoints, the lungs were inflated to allow for further analysis. The animals were sacrificed and the lungs were inflated with 1.5 ml of 0.6% agarose/PBS via an intratracheal catheter. The lungs were then removed and fixed in 10% buffered formalin for 24 h before embedding in paraffin. Serial sections of 5 μ m thickness was obtained and

prepared for H & E staining and immunohistochemistry. Three sections of each lung were histologically evaluated by a pathologist blinded to the experimental groups. To quantify tumor burden, the lung area and tumor area were analyzed for each animal using ImageScope Viewer Software (Aperio).

Immunohistochemistry

To assess Notch signaling and DNAML expression Hes1 and GFP were evaluated by immunohistochemistry. Briefly, for immunohistochemistry of Hes1, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions, and washed in TBST (Tris Buffered Saline with 0.1% Tween 20). Antigen retrieval was carried out by heating the sections in EDTA/Citrate Buffer (pH 8) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in methanol for 60 minutes. Nonspecific binding sites were blocked using protein block containing 5% milk, 5% BSA, and 20% goat serum in TBST for 60 minutes. Sections were then incubated at room temperature for 60 minutes with 1:50 dilution of polyclonal Rabbit Anti-Hes1 (Chemicon). After several washes with TBST, the slides were incubated with appropriate biotinylated-secondary antibody for 2 hours and then washed and incubated with Streptavidin-HRP complex reagent (ImmunoCruz; Santa Cruz Biotechnology). After rinsing with PBS, the slides were incubated with the chromogen, Vector VIP Peroxidase Substrate Kit (Vector Labs) for 3 minutes, then rinsed and counterstained with hematoxylin. Substituted nonimmune rabbit immunoglobulins for primary antibodies were used as negative controls. Anti-GFP (Abcam) was evaluated using a similar protocol, however, the protein block contained 5% milk, 5% BSA, and 10% donkey serum and detected with Vector NovaRED Peroxidase Substrate Kit. (Vector Labs). The JHMI Tissue Microarray Core scanned all slides using ScanScope (Aperio) and images were analyzed with ImageScope.

Results

Inhibition of Notch signaling can suppress NSCLC clonogenicity *in vitro*

Previous investigators have reported elevated expression of Notch receptors, most commonly Notch3, in human NSCLC (25). To identify cell lines representative of this phenotype, we assessed expression levels of key Notch pathway components in 4 NSCLC cell lines relative to levels in normal human bronchial epithelial cells (NHBE) by quantitative RT-PCR. Consistent with previous reports, *Notch3* expression was found to be selectively elevated in 3 of the 4 cell lines examined (Figure 1; Figure S1). *Hes1*, a target gene of the Notch signaling pathway, was also overexpressed relative to NHBE in these lines.

All four Notch receptors are dependent on γ -secretase cleavage for release of the intracellular domain and downstream signaling via nuclear translocation. To evaluate the effect of targeted Notch pathway inhibition on clonogenic survival of NSCLC *in vitro*, we first sought to demonstrate that the potent and selective γ -secretase inhibitor MRK-003 would in fact suppress pathway activity in our cells. Two NSCLC cell lines (NCI-H1299, and NCI-H1435) were treated with doses up to 10 μ M MRK-003 over 96 hours in culture. Quantitative reverse transcription-PCR (qRT-PCR) for *Hes1* was used to evaluate Notch pathway inhibition, with all reactions normalized to β -actin. Both cell lines demonstrate a dose-dependent suppression of *Hes1* expression in response to MRK-003, confirming an on-target effect of this pharmacologic inhibitor (Figure 2; Figure S2).

Notch pathway activation has been implicated in maintenance of clonogenicity in a number of *in vitro* cancer models. However, γ -secretase can cleave multiple substrates other than the Notch receptors (31). To evaluate the ability of MRK-003 to affect clonogenicity, and to

assess whether the effects of this inhibitor are in fact Notch pathway-dependent, we first transfected H1299 NSCLC cells with either an empty vector control or the same vector expressing NICD3, the constitutively active intracellular domain of *Notch3*. Stable transfectants were selected in puromycin. Cell lines with or without NICD3 were either mock treated (0.03% DMSO), or incubated for 8 days with 3 μ M MRK-003, a dose that resulted in over 50% inhibition of Notch pathway activity. Equal numbers of surviving cells were then replated, and clonogenic capacity evaluated by crystal violet staining. MRK-003 exposure markedly suppressed clonogenic regrowth of control cells, but had essentially no effect on cells transfected with the constitutively active NICD3 (Figure 3). These data confirm that targeted inhibition of Notch signaling by MRK-003 can affect clonogenic survival *in vitro*. More importantly, this experimental design confirmed that this effect is pathway-specific, not attributable to off-target effects of the γ -secretase inhibitor.

Evaluating temporal and spatial activation of Notch signaling in NSCLC tumorigenesis

An effect of Notch inhibition on clonogenic capacity *in vitro* does not necessarily translate into the more biologically relevant endpoint of an effect on tumorigenicity *in vivo*. To begin to examine the latter endpoint, we turned to a well-characterized *in vivo* model of NSCLC tumorigenesis, the LSL-*KRAS*^{G12D} transgenic mouse initially developed in the Jacks laboratory (32). In this model an oncogenic *KRAS* allele is silenced by a transcriptional stop site flanked by loxP sites (lox-stop-lox). Inhalation of an adenoviral vector containing the Cre recombinase promotes rearrangement between lox sites, deletion of the transcriptional stop, and selective high level expression of mutant *KRAS* in cells lining the bronchial and alveolar airways (15). As oncogenesis in this model is tightly temporally and spatially regulated, it serves as an ideal platform for examining activation of the Notch signaling pathway in the earliest stages of tumorigenesis.

We hypothesized that Notch signaling would be activated in the initial stages of tumorigenesis and that early lesions in particular might be highly dependent on Notch signaling. LSL-*KRAS*^{G12D} mice were exposed to an adenovirus expressing Cre recombinase (AdenoCre) via intranasal instillation, resulting in expression of mutant *KRAS* specifically in lung epithelium. We evaluated lesions at serial stages of tumorigenesis. Mice were divided into four groups (n=5 animals per time point) and sacrificed at predetermined intervals at 1, 3, 6 and 10 weeks after Cre-mediated activation. Notch pathway activation was monitored in microscopic precursor lesions by immunohistochemical staining for Hes1 expression at the various stages of tumorigenesis. We confirmed the specificity of the antibody used for immunohistochemistry by evaluation of cell lines transfected with an expression vector encoding DNMAML (Figure S3). We observed a transient induction of Hes1 expression 1 week post infection, specifically in airway epithelial cells in LSL-*KRAS*^{G12D}, but not in control wt mice exposed to AdenoCre, or in LSL-*KRAS*^{G12D} following mock infection (Figure 4). This activation diminished over time, with only scattered cells staining positive by week 10. Hes1 expression does not appear to be simply a marker of proliferative activity; cells transfected with the DNMAML expression vector resulting in marked Hes1 suppression demonstrated no significant change in growth potential (Figure S4). These data suggest that Notch activation may have a selective role in initial steps of tumorigenesis, consistent with a role in a tumor progenitor population.

Genetic disruption of Notch signaling in the LSL-*KRAS*^{G12D} model

Transient activation of Notch signaling in early stages of lung oncogenesis does not necessarily imply that this activation is required for cell transformation or tumor growth. To evaluate whether Notch pathway activation is required in lung carcinogenesis, we sought a mechanism to interrupt this pathway specifically in the same lung epithelial precursor cells in which oncogenic *KRAS* was being activated. As noted above, Mastermind-like (MAML)

protein is required for Notch signaling. Weng and colleagues generated a dominant-negative Mastermind-like (DNMAML) expression vector by fusing the N-terminal domain of MAML to GFP (33). This construct specifically inhibits Notch signaling by all 4 Notch family members. DNMAML was used as the basis for constructing an inducible DNMAML knock-in murine model (28). Rosa 26 *DNMAML-GFP* mice, on a C57BL/6 background, were provided as a gift from Dr. Warren Pear, and were crossed with *LSL-KRAS^{G12D}* mice to generate double transgenics. As in the *LSL-KRAS^{G12D}* mice, *DNMAML-GFP* expression in these mice was under the control of an upstream LOX-stop-LOX cassette, which can be activated with inhaled AdenoCre. Airway epithelia infected with the adenoviral Cre delivery vector rapidly rearrange proximal LOX sites, resulting in concomitant coordinated activation of both oncogenic KRAS and DNMAML-GFP. Adjacent non-infected cells are unable to activate either construct, thus providing founder-cell specificity.

Littermate cohorts of wild-type, single transgenic (*LSL-KRAS^{G12D}* or *LSL-DNMAML-GFP*), and double transgenic (*LSL-KRAS^{G12D}/LSL-DNMAML-GFP*) mice, at least 5 mice per cohort, were treated with AdenoCre via intranasal instillation, resulting in expression of mutant KRAS and/or DNMAML-GFP in airway epithelial cells. Based on experience from the *LSL-KRAS^{G12D}* model, tumor burden (tumor area/total area) was evaluated at 8 weeks post-infection. Surprisingly, we saw no evidence of any decrement in total tumor burden or tumor number in double transgenic animals relative to the *KRAS* single transgenic, and in fact a non-significant trend toward slightly higher tumor burden was seen in the double transgenic cohort (Figure 5). Histologic characteristics of the *KRAS^{G12D}* and double transgenic tumors, evaluated by an experienced clinical pathologist in our lung cancer program, were indistinguishable. We confirmed successful activation of the *DNMAML* construct in the double transgenic tumors at the RNA level by RT-PCR and at the protein level by immunohistochemical staining (Figure 6A and B). Finally, comparison of intratumoral Hes1 expression, as an indicator of Notch pathway activity, demonstrated over suppression in the lung tumors of *KRAS/DNMAML* relative to *KRAS* only transgenic mice (Figure 6C). These results suggest that inhibition of Notch signaling achieved by DNMAML expression *in vivo* is not sufficient to suppress cellular transformation or progressive tumor growth in the context of a strongly transforming oncogene such as mutant KRAS.

Discussion

Several lines of indirect evidence have implicated the Notch pathway in the development of a wide array of solid tumors (15). Through a series of *in vitro* analyses we were able to demonstrate (1) that Notch receptor expression, particularly Notch3, is upregulated in NSCLC lines, (2) that γ -secretase blockade inhibits Notch pathway activation and can partially suppress *in vitro* clonogenic survival, and (3) that the latter is an on-target effect, as clonogenic survival is restored by introduction of the (γ -secretase-insensitive) activated Notch3 intracellular domain. Moving to an *in vivo* model of NSCLC development, we were able to show transient upregulation of Notch pathway activity early in the course of tumorigenesis, with maintenance of high level pathway activity in a small minority of cells in bulk tumors. We did not analyze transient Notch activation in the context of DNMAML, which would be of interest for future studies in this model system. A more detailed time-course of the transient induction of Notch signaling in airway epithelium in the context of KRAS activation would also be an informative focus of future investigation. Notch pathway activity appears to be transiently upregulated in distal airway epithelium after KRAS induction. Upregulation of Notch signaling in response to expression of oncogenic Ras has been previously reported in other systems although the precise molecular signaling pathways linking these events have not been fully defined (34, 35). All of these results are consistent with Notch signaling having a role in maintenance of a tumorigenic progenitor cell in lung

cancer, and are generally consistent with prior reports in the literature implicating Notch signaling in this context.

Strikingly, however, genetic disruption of Notch signaling in the same *in vivo* model through introduction of a dominant negative inhibitor of the critical co-factor MAML did not have any evident suppressive effect on tumor initiation or growth *in vivo*, calling into question whether Notch activity is in fact required for lung tumorigenesis. This result comes with several important caveats. First, the LSL-*KRAS*^{G12D} model, while one of the most commonly used murine genetic models of lung tumorigenesis, and while demonstrating many key features of clinical NSCLC, may not adequately reflect the complexity of the human disease. Differential oncogenic requirements in murine and human tissues have been well documented (36). Formally testing the requirement for intact Notch signaling in multiple distinct models of lung cancer development would be of interest. Second, DN*MAML* expression, while inhibiting activity of the Notch signaling pathway by over 50%, is only partially suppressive, and sufficient Notch activity may remain to allow tumor initiation and growth. This could be more rigorously tested by tissue-specific Notch or MAML gene knock-out in the LSL-*KRAS*^{G12D} mouse, although the potential redundancy among 4 Notch family members and 3 MAML isoforms might preclude a definitive interpretation. The global pathway suppression achieved by expression of DN*MAML* (partial inhibition of all Notch family member function) may be a better reflection of what could be achieved pharmacologically, e.g. through the use of γ -secretase inhibitors in patients, than individual family member genetic knock-outs (complete loss of function for a single Notch family member). Clinical dose escalation of γ -secretase inhibitors as Notch pathway inhibitors has been attenuated by severe and dose-limiting adverse effects, notably large volume secretory diarrhea (37-39). Thus tumor-specific pathway suppression by induction of DN*MAML* expression, with no effect on non-airway tissues, is likely to overestimate the potential efficacy of any systemic Notch inhibitor therapies currently in clinical development. Our observations, if applicable to human disease, would call into question the utility of global Notch inhibitor therapies in the treatment of lung cancer.

Observations from several groups have demonstrated that although common Notch pathway readouts (e.g. *Hes1* expression) are used, the four Notch family members may have very different, and in fact sometimes opposing, activities *in vivo* (40-42). These data are beginning to offer an explanation for what have been conflicting observations that Notch pathway activation can have either oncogenic or tumor suppressor effects (43). While oncogenic activities have gained by far the most attention, tumor suppressor effects associated with Notch signaling have been clearly defined in skin/keratinocyte transformation (44-46), small cell lung cancer (47-49), and myeloid leukemia (21). These opposing context-dependent effects parallel the complex functions of this pathway in embryonic development, where the Notch pathway can either maintain progenitor/stem cell characteristics or drive tissue-specific differentiation (50). It is clear that not all Notch family members contribute equally to lung tumorigenesis. In NSCLC, Notch3 upregulation has been most consistently observed, although gain-of-function Notch1 mutations have also been reported (11). It is possible that individual Notch isoforms have opposing effects on lung oncogenesis, and that DN*MAML* expression, by inhibiting all Notch signaling, is ineffective even though individual Notch isoforms are important therapeutic targets. The Notch pathway inhibitors farthest along in clinical development, i.e. γ -secretase inhibitors, suppress activation of all Notch isoforms, and thus function as global pathway inhibitors analogous in effect to DN*MAML*. More recent development of isoform-specific inhibitors, such as antagonistic humanized monoclonal antibodies specific for individual Notch family members (51), may offer a strategy to more narrowly target relevant oncogenic pathways. It will be of interest to test the effects of these and similar isoform-specific agents in models such as that described here.

A final observation from these data derives from the apparent dichotomy between our *in vitro* and *in vivo* data. *In vitro*, we have observed that Notch pathway inhibition can decrease cancer cell line clonogenic potential, and that this effect can be reversed by expression of a constitutively active form of Notch3. *In vivo*, in contrast, we see no evident effect on tumorigenicity from induced expression of a dominant negative Notch pathway inhibitor. One factor in this dichotomy may be, as noted above, differential effects of individual Notch isoforms vs. global suppression of Notch signaling. A second contributing factor may be the distinction between clonogenic survival in tissue culture and tumorigenic capacity in complex multicellular biological environments. Continued attention to development and testing of relevant *in vivo* models of human disease is essential, particularly with regard to preclinical evaluation of therapies designed to affect tumor progenitor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of Notch receptors and target genes in NSCLC cell lines

Quantitative PCR demonstrates relative expression levels of genes encoding Notch receptors, Hes1, and Notch ligands in four NSCLC cell lines compared to those of normal human bronchial epithelial cells. Relative expression was evaluated using Taqman probes (ABI). All reactions were normalized to β -actin (*Notch4* levels were undetectable in all lung and cell line samples).

Figure 2. Gamma secretase inhibition suppresses notch signaling

Downregulation of Notch signaling using a selective γ -secretase inhibitor (MRK-003; Merck) was observed in two NSCLC cell lines: (A) NCI-H1299 and (B) NCI-H1435. Cells were grown to 50% confluence and treated with MRK-003 at the indicated doses for 96 hours. Relative expression of *Hes1* was evaluated using qRT-PCR normalized to β -actin. NT: not treated.

Figure 3. Role of Notch3 in clonogenic survival

H1299-GFP and H1299-N3 were mock-treated, or treated with 3 μ M MRK-003 for 8 days.

(A) Relative Notch pathway activity, as determined by *Hes1* expression by qRT-PCR (all reactions are normalized to β -actin) (B) 3×10^3 viable cells were plated in a 12-well plates in triplicate, allowed to form colonies for 7 days, and were then stained with crystal violet ($p=0.003$ for the difference between cell lines).

Figure 4. Transient induction of Hes1 in airway epithelial precursors after mutant KRAS induction

LSL-KRAS^{G12D} transgenic mice were given inhaled adenoviral Cre, leading to oncogene activation in the transgenic animals. Lungs were harvested from cohorts of mice pretreatment and at 1, 3, 6 and 10 weeks post-infection. **Top:** hematoxylin/eosin stain. **Bottom:** immunohistochemistry for Hes1. A transient induction of Hes1 is seen in airway epithelial cells at 1 week, with scattered positive cells in subsequent growing tumors. Representative images are shown. No Hes1 induction over baseline staining was observed in concomitantly treated non-transgenic littermate controls (not shown).

Figure 5. KRAS induced tumorigenesis is not suppressed by DNMAML

(A) Lung sections stained with hematoxylin/eosin reveal similar tumor histologies from mutant KRAS and KRAS/DNMAML mice. (B) Total tumor burden, calculated as a fraction of total lung area, is similar in KRAS only and double transgenic KRAS/DNMAML mice. No tumors were observed on control wildtype or DNMAML only littermates. Analysis was performed at 8 weeks after infection.

Figure 6. *DNMAML* expression and modulation of Notch pathway *in vivo*

(A) Strong *DNMAML* gene expression was seen by qRT-PCR in double transgenic (*DNMAML/KRAS*) tumors, but not in tumors from *KRAS* only transgenics. *DNMAML* only transgenic lungs did not form tumors, and lower level expression is seen, reflecting that only a fraction of epithelial cells are infected. (B) Immunohistochemical staining with an anti-GFP antibody of tumors from *KRAS* only and *KRAS/ DNMAML* tumors. The anti-GFP antibody recognizes the GFP component of the *DNMAML* fusion protein. (C) Relative expression of intratumoral *Hes1* from *KRAS* and *KRAS/ DNMAML* mice.