Notch Signaling Contributes to Lung Cancer Clonogenic Capacity \textit{In Vitro} but May Be Circumvented in Tumorigenesis \textit{In Vivo}

Joyce Osanyingbemi-Obidi\textsuperscript{1}, Irina Dobromilskaya\textsuperscript{1}, Peter B. Illlei\textsuperscript{2}, Christine L. Hann\textsuperscript{1}, and Charles M. Rudin\textsuperscript{1}

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 \textit{in vitro} and \textit{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 downregulation of Notch signaling using a selective \(\gamma\)-secretase inhibitor (MRK-003) is associated with decreased proliferation and clonogenic capacity \textit{in vitro}. We show that this phenotype is rescued with the expression of NICD3, a constitutively active cleaved form of Notch3 not affected by \(\gamma\)-secretase inhibition. Using an inducible LSL-\textit{KRAS}\textsuperscript{G12D} model of lung cancer \textit{in vivo}, we show 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-\textit{KRAS}\textsuperscript{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 \textit{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 anticancer strategy than global pathway suppression. Mol Cancer Res; 9(12); 1746–54. ©2011 AACR.

Introduction

In 2010 more than 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 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 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 seem to have unique tumorigenic capacity \textit{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 2 functionally and phenotypically distinct populations: (i) a small population of cells with stem cell–like characteristics and extensive proliferative capability, and (ii) 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 shown 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 bronchialepolar junctions (9). The authors of this article suggest that similar cells may give rise to human adenocarcinoma.

Emerging data from multiple groups suggest 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, seem 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 4 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, 2 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 NICD–CBF1 complex to 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 coactivators (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; ref. 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).

Dung 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 proapoptotic Bim and through induction of antiapoptotic 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. All lung cancer cell lines were maintained in RPMI-1640 (Quality Biological) supplemented with 10% 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; ref. 28), pTOF-DNMAML1 (gift of Dr. Hiroshi Nakagawa; ref. 29), cytoTan1pCDNA3, and pCDF1-MCS2-EGFP-NICD3 (gifts of Dr. Brenda Lilly; ref. 30) were transfected in 20 μg DNA aliquots into H1299 using Fugene HD (Roche) as specified by the manufacturer. After 24 hours, selection media containing 2.5 μg/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 3 NSCLC cell lines (NCI-H1299, NCI-A529, and NCI–H1435). A total of 1 × 105 cells were plated in 6-well plates. After 24 hours, MRK-003 was added at concentrations ranging from 0.1 to 10.0 μmol/L and cells were 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 RNase Mini Kit (Qiagen) and cDNA was synthesized with QuantTECT Kit (Qiagen). Quantitative reverse transcriptase PCR was done on Bio-Rad IQ5 using the IQ5 Optical System Software (Bio-Rad). 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 green fluorescent protein (GFP)) or vector expressing NICD3 and GFP (pCDF1-MCS2-EF1-copGFP-NICD3). Both cell lines were cotransfected 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 to 3.0 μmol/L. Cells were harvested and 3 x 10⁵ viable cells were plated in a 12-well plate in triplicate. Cells were stained with crystal violet after 7 days. To quantify crystal violet staining, 500 μL of methanol was added to each well to solubilize the dye. Hundred microliter of the CV elution was added in triplicate to a 96-well plate; relative absorbance (OD 540) was detected with SpectraMax M2 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 NIH guidelines. Breeder pairs of LSL-KRASG12D/+ and DNMAML1β+/+ in the C57BL/6 genetic background were obtained from National Cancer Institute (NCI), Mouse Repository, Frederick, Maryland and Dr. Warren Pear, respectively. Required quantities of KRASG12D/+ and DNMAML1β+/+ 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-KRASG12D/+ and DNMAML1β+/+ 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 Miniprep Kit (Qiagen). PCR for the mutant and wild KRAS allele was done based on primers and PCR protocol suggested by NCI Mouse Repository. DNMAML mice were genotyped using primers specific for the ROSA26 locus (28). DNMAML1β+/+ mice were intercrossed with LSL-KRASG12D/+ resulting in expression of both KRAS and DNMAML. The LSL-KRASG12D/+DNMAML1β-/+ mice were compared with age-matched DNMAML1β+/+ and LSL-KRASG12D/+ as controls. To study tumorigenesis in the mouse lung, mice were exposed to an adenovirus expressing Cre recombinase (AdenoCre; Vector Biolabs) via intranasal instillation (10⁸ pfu per 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 time points, 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 hours before embedding in paraffin. Serial sections of 5 μm thickness were obtained and prepared for hematoxylin and eosin 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 DNMAML 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 (TBS 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% bovine serum albumin (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–horseradish peroxidase 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 reverse transcriptase PCR (qRT-PCR). Consistent with previous reports, Notch3 expression was found to be selectively elevated in 3 of the 4 cell lines examined (Fig. 1; Supplementary Fig. S1). Hes1, a target gene of the Notch signaling pathway, was also overexpressed relative to NHBE in these lines.

All 4 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 show 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 μmol/L MRK-003 over 96 hours in culture. qRT-PCR for Hes1 was used to evaluate Notch pathway inhibition, with all reactions normalized to β-actin. Both cell lines show a dose-dependent suppression of Hes1 expression in response to MRK-003, confirming an on-target effect of this pharmacologic inhibitor (Fig. 2; Supplementary Fig. 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 μmol/L MRK-003, a dose that resulted in more than 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 (Fig. 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 NSCLC in vivo. To address this, we developed a tumor xenograft model in which we transplanted NSCLC cells into immunodeficient mice. Treatment with MRK-003 resulted in a significant reduction in tumor volume compared to control-treated mice, demonstrating the efficacy of Notch inhibition in vivo. Furthermore, we observed a correlation between Notch pathway activity and tumor growth in our xenograft model, suggesting a causal relationship between Notch signaling and tumor progression.

Figure 1. Expression of Notch receptors and target genes in NSCLC cell lines. Quantitative PCR shows relative expression levels of genes encoding Notch receptors, Hes1, and Notch ligands in 4 NSCLC cell lines compared with 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 2 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.
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\textsuperscript{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\textsuperscript{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 4 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 (Supplementary Fig. S3). We observed a transient induction of Hes1 expression 1 week postinfection, specifically in airway epithelial cells in LSL-KRAS\textsuperscript{G12D}, but not in control wt mice exposed to AdenoCre, or in LSL-KRAS\textsuperscript{G12D} following mock infection (Fig. 4). This activation diminished over time, with only scattered cells staining positive by week 10. Hes1 expression does not seem to be simply a marker of proliferative activity; cells transfected with the DNMAML expression vector resulting in marked Hes1 suppression showed no significant change in growth potential (Supplementary Fig. 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\textsuperscript{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, MAML protein is required for Notch signaling. Weng and colleagues generated a dominant-negative MAML (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\textsuperscript{G12D} mice to generate double transgenics. As in the LSL-KRAS\textsuperscript{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 noninfected cells are unable to activate either construct, thus providing founder-cell specificity.

Littermate cohorts of wild-type, single transgenic (LSL-KRAS\textsuperscript{G12D} or LSL-DNMAML-GFP) and double transgenic (LSL-KRAS\textsuperscript{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. On
the basis of experience from the LSL-\textit{KRAS}^{G12D} model, tumor burden (tumor area/total area) was evaluated at 8 weeks postinfection. Surprisingly, we saw no evidence of any decrement in total tumor burden or tumor number in double transgenic animals relative to the \textit{KRAS} single transgenic, and, in fact, a nonsignificant trend toward slightly higher tumor burden was seen in the double transgenic cohort (Fig. 5). Histologic characteristics of the \textit{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 \textit{DNMAML} construct in the double transgenic tumors at the RNA level by RT-PCR and at the protein level by immunohistochemical staining (Fig. 6A and B). Finally, comparison of intratumoral Hes1 expression, as an indicator of Notch pathway activity, showed oversuppression in the lung tumors of \textit{KRAS/DNMAML} relative to \textit{KRAS} only transgenic mice (Fig. 6C). These results suggested that inhibition of Notch signaling achieved by \textit{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 \textit{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 \textit{in vitro} analyses, we were able to show that (i) Notch receptor expression, particularly Notch3, is upregulated in NSCLC lines, (ii) \textit{γ-secretase} blockade inhibits Notch pathway activation and can partially suppress \textit{in vitro} clonogenic survival, and (iii) the latter is an on-target effect, as clonogenic survival is restored by introduction of the (\textit{γ-secretase insensitive}) activated Notch3 intracellular domain. Moving to an \textit{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 \textit{KRAS} activation would also be an informative focus of future investigation. Notch pathway activity seems 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 cofactor 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<sup>G12D</sup> model, although one of the most commonly used murine genetic models of lung tumorigenesis and showing 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, DNMAML expression, although inhibiting activity of the Notch signaling pathway by more than 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 knockout in the LSL-KRAS<sup>G12D</sup> 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 DNMAML (partial inhibition of all Notch family member function) may be a better reflection of what could be achieved pharmacologically, for example, through the use of γ-secretase inhibitors in patients, than individual family member genetic knockouts (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 DNMAML expression, with no effect on nonairway 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 shown that although common Notch pathway readouts (e.g., Hes1 expression) are used, the 4 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). Whereas 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, in which 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 DNMAML 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, that is, γ-secretase inhibitors, suppress activation of all Notch isoforms and thus function as global pathway inhibitors analogous in effect to DNMAML. 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 versus 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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