Functional diversity of notch family genes in fetal lung development

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Kong, Yanping, Jonathon Glickman, Meera Subramaniam, Aliakbar Shahsafaei, K.P. Allamneni, Jon C. Aster, Jeffrey Sklar, and Mary E. Sunday. Functional diversity of notch family genes in fetal lung development. Am J Physiol Lung Cell Mol Physiol 286: L1075–L1083, 2004; 10.1152/ajplung.00438.2002.—In Drosophila, developmental signaling via the transmembrane Notch receptor modulates branching morphogenesis and neuronal differentiation. To determine whether the notch gene family can regulate mammalian organogenesis, including neuroendocrine cell differentiation, we evaluated developing murine lung. After demonstrating gene expression for notch-1, notch-2, notch-3, and the Notch ligands jagged-1 and jagged-2 in embryonic mouse lung, we tested whether altering expression of these genes can modulate branching morphogenesis. Branching of embryonic day (E) 11.5 lung buds increased when they were treated with notch-1 antisense oligodeoxynucleotides in culture compared with the corresponding sense controls, whereas notch-2, notch-3, jagged-1, or jagged-2 antisense oligos had no significant effect. To assess cell differentiation, we immunostained developing lung bud cultures for the neural/neuroendocrine marker PGP9.5. Antisense to notch-1 or jagged-1 markedly increased numbers of PGP9.5-positive neuroendocrine cells alone without affecting neural tissue, whereas only neural tissue was promoted by notch-3 antisense in culture. There was no significant effect on cell proliferation or apoptosis in these antisense experiments. Cumulatively, these observations suggest that interactions between distinct Notch family members can have diverse tissue-specific regulatory functions during development, arguing against simple functional redundancy.

branching morphogenesis; cell differentiation; neuroendocrine cells; neurons; antisense oligonucleotides; mouse embryos; immunohistochemistry

NOTCH WAS INITIALLY IDENTIFIED as a transmembrane signaling protein that is located on the cell surface and is involved in Drosophila neurogenesis (4, 17). Whereas “proneural” genes promote neuronal cell commitment, “neurogenic” genes such as notch restrict neurogenesis (4, 40). Complete loss of function of a neurogenic gene such as notch can lead to massive neuronal hyperplasia. Notch, Delta, and Serrate/Jagged are transmembrane molecules with EGF-like repeats (4, 10). Delta and Serrate act as Notch ligands, activating a common intracellular signaling pathway leading to cleavage of the cytoplasmic domain of Notch, which then translocates to the nucleus (3, 4). It is generally believed that all notch genes can interact functionally in similar developmental pathways in vertebrates and invertebrates (17). In vertebrates, multiple notch genes have been identified as well as multiple genes encoding the Notch ligands Delta and Jagged/Serrate (3, 17).

The observation that neuroblasts rapidly decrease Notch expression as they begin to differentiate suggested that Notch functions to inhibit the neuronal phenotype (10). However, notch gene expression occurs in many fetal and adult tissues, especially central nervous system, hematopoietic tissues, and the lung (1, 5, 9, 20, 33, 34, 36, 40). Although different Notch ligands might have distinct functions in hematopoiesis (11, 16), there has been little evidence for divergent functions of the multiple Notch proteins (11), especially with regard to specific developmental processes.

The function of Notch has been recently explored in genetically deficient cells and mice and by antisense oligonucleotide strategies. In the vertebrate retina, Austin et al. (6) used antisense oligonucleotides to demonstrate that the number of ganglion cells produced was inversely related to the level of Notch-1 activity. This is a versatile system for examining the Notch pathway in a specific cell fate decision. It is clear that Notch is effectively an ideal morphogen that can influence cell fate, proliferation, apoptosis, cell-cell adhesion (3, 10), and border formation: that is, everything that goes into building a tissue. Furthermore, the outcome of Notch signaling is not stereotyped but highly dependent on dose, timing, and context (5). Therefore, we investigated how Notch signaling influences branching morphogenesis and neuroendocrine cell differentiation during murine lung development.

MATERIALS AND METHODS

Animals. Timed pregnant Swiss-Webster mice were obtained from Taconic Laboratories (Germantown, NY) at gestational day 7 (E7). The National Research Council Guide for the Care and Use of Laboratory Animals was strictly adhered to throughout all phases of this study. The Animal Care Committee of the Brigham & Women’s Hospital and Children’s Hospital reviewed and approved the protocols used in this study.

Preparation of RNA and cDNA. Lungs were harvested from E12 to E18 fetal mice and from neonatal mice between the day of birth (postnatal day 1 = P1). Total RNA was prepared from snap-frozen lung tissue with TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions as described previously (42). RNA integrity was evaluated by examination of 18S and 28S bands after separation on ethidium bromide-stained 1.5% agarose/formaldehyde gels. cDNA was prepared using SuperScript 2 RNase Reverse Transcriptase (GIBCO-BRL) according to the manufacturer’s instructions.

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The medium culture containing fresh oligodeoxynucleo-
tides (1 μM). The culture medium was refreshed daily. The number of branch points per lung bud
was close to identical within each litter (variability ± 1–2 branch
points at E11.5). The sequences of the notch-1 antisense and sense
oligonucleotides have been previously published (6). The sequences
of the remaining oligodeoxynucleotides are given as follows: Notch-2
(corresponds to bp 1,953–1,971); antisense 5′ CACTACAGAT-
GCTCCCAT, sense 5′ ATGGGAGCCATCGTAGTG; Notch-3 (cor-
responds to bp 2,132–2,150); antisense 5′ GTTCGCCAGTAG-
GCAAAGT, sense 5′ ACTTTGCTACTTGCGGAAC; Jagged-1
(corresponds to bp 726–749); antisense 5′ GTTTATCATGCC-
GAGTGGAAGC, sense 5′ GCTTCTCCTACGAGCATGATA-
AAC; Jagged-2 (corresponds to bp 817–836); antisense 5′ CTGTG-
CACAGAACCTGGCCCT, sense 5′ AGGGCAAGTCTCTGTGACAGG.

To verify the specificity of the antisense-mediated effects, we
immunostained paraffin sections of the cultured lung buds for
Notch-1, Pan-Notch, Jagged-1, and Jagged-2 with the same antibodies
given in Fig. 2.

Neuroendocrine/neural cell differentiation in lung buds cultured
with antisense oligonucleotides: computerized image analysis. E11.5
murine lung buds were cultured for 7 days with antisense or sense
oligonucleotides (sequences given above) before harvesting, fixation,
and routine processing into paraffin blocks as described above. Serial
sections through the whole block for each group of buds were
immunostained for the neural/neuroendocrine cell-specific marker
PGP9.5 (30, 39). Computerized image analysis (density slice deter-
mination) was used to measure the tissue area of the cross section
of each lung bud as detailed below. Images were captured under a Nikon
Eclipse E600 microscope with bright-field illumination at ×20 magnifi-
cation, which was connected to a Nikon digital camera DXM1200.
The camera, in turn, interfaced with a Dell Dimension 8200 computer
via Nikon ACT-I software for capturing digital images. Subsequently,
we optimized contrast using Adobe Photoshop 6.0 before carrying out
measurements using the Scion Image 1.62b program.

PGP9.5-positive neuroendocrine cells were quantified both as the
number of PGP9.5-positive cell clusters per cm² of tissue and as the
total number of PGP9.5-positive cells per cm² of tissue. Neuroendo-
crine cells in developing murine lung are localized to both the
cartilaginous airways and the smaller, more distal airways. Due to this
scattered distribution of PGP9.5-positive neuroendocrine cells, the
results of quantitative morphometry were normalized for the area
of lung tissue in question.

Similarly, we used computerized image analysis to determine the
relative area of PGP9.5-positive mesenchymal tissue by measuring the
area of nonepithelial PGP9.5-positive tissue and normalizing this for
the total area of the lung bud.

Statistical analyses. Numerical data were analyzed by unpaired
Student’s t-test and/or ANOVA. Results are expressed as mean group
values ± SE.

RESULTS AND DISCUSSION

Notch family gene expression in developing murine lung. In
the present study, we investigated the expression of the genes
encoding Notch-1, -2, -3 and Jagged-1 and -2 in developing
murine lung between E10 and P14, corresponding to the period
of morphogenesis and cytodifferentiation in this organ. We
chose to investigate these selected genes encoding Notch and
Notch ligands as a paradigm of Notch signaling, rather than an
exhaustive analysis of all identified genes in the Notch signal-
ning pathway. Results of representative semiquantitative RT-
PCR analyses are given in Fig. 1. Murine notch-1, -2, and -3
genes are expressed from E11 through P14; only notch-1
mRNA is detected on E10. Similarly, notch-1 is the first of
these genes to be expressed in the developing pancreas (23). In
contrast, notch-3 mRNA is only marginally detectable on E14

RNA analysis: RT-PCR. Synthetic oligodeoxynucleotides were
designed to span at least one intron and were purchased from Oligos
Etc. (Wilsonville, OR). The conditions for semiquantitative RT-PCR
were determined such that 40 cycles for notch and jagged RT-PCR
yielded a positive control signal in the midlinear region of the curve
(cycle number vs. integrated band intensity). RT-PCR was carried out
as described previously (42) with the following primer pairs for PCR,
based on murine sequences from GenBank (accession numbers given
below). Notch-1 forward (F), caagttgacattacgaagct; Notch-1 reverse
(R), cacgacacaaacagacag; Notch-1 probe, caatctgtgctgtctctct;
Notch-1 band size, 421 bp; Notch-1 GenBank accession number,
NM_008714. Notch-2 F, gcacatactacatagctagctg; Notch-2 probe,
tcatcaagactgtggtctac; Notch-2 band size, 643 bp; Notch-2 GenBank accession
number, D32210. Notch-3 F,tgtagtagagggagaagtgtc; Notch-3 R, tcagacgacaa-
cagaaag; Notch-3 probe, caagctggtctcagctcct; Notch-3 band size, 617 bp;
Notch-3 GenBank accession number, X74760. Jag-1 F, cagaggtgcagctgctagtggt; Jag-1 R, arectctgacacgacag; Jag-1 probe,
cagtagctgsacaggt; Jag-1 band size, 147 bp; Jag-1 GenBank accession
number, AA021036. Jag-2 F, ctagagtccttacacagcttggt; Jag-2 R, tgcagacgagcaggtac; Jag-2 probe, gcactgagcagctgacag; Jag-2 band size, 557 bp; Jag-2 GenBank accession number,
AF101937.

Band identities were verified by DNA sequencing and by probing
RT-PCR Southern with an internal primer (sequences of which are
given above).

PCR reactions were carried out with Taq polymerase (Boehringer
Mannheim, Indianapolis, IN) as described previously (42). Each
PCR reaction was subjected to 40 cycles, except β-actin was amplified for
22 cycles. Each cycle included denaturation for 1 min at 94°C,
annealing for 1.5 min, and extension for 2 min at 72°C with a thermal
cycler (M. J. Research, Watertown, MA). Negative controls consisted
of an equal volume of diethyl pyrocarbonate-treated water substituted
for the volume of RNA in the above reaction.

PCR products were fractionated on 1.5% agarose gels and blotted
onto nitrocellulose according to standard protocols (42). Internal
oligomeronucleotide probes were end-labeled with T4-polymerase ki-
nase and hybridized according to standard protocols (42). These
conditions were defined to yield semiquantitative results as previously
described (42), with linear detection of positive control bands over a
2-log range of input control RNA. Relative amounts of specific mRNAs could subsequently be normalized with actin mRNA
in the same RT reaction mixture.

Immunoperoxidase analyses. Murine E14 lung tissues were fixed for
12–18 h in 4% paraformaldehyde before being processed into paraffin.
Three-micrometer paraffin sections were prepared on Fisher “Plus”
slides. Immunostaining was carried out via the avidin-biotin complex
immunoperoxidase technique, with dianinobenzidine as substrate, as
described (42). Either hematoxylin or methyl green was used as coun-
terstain. Affinity-purified antibodies to Notch-1, Pan-Notch, Jagged-1,
and Jagged-2 were described previously (5, 12, 27). Brief descriptions are
given as follows. The Notch-1 antibody preparation is an affinity-purified
cytoplasmic domain of human
Notch-1, termed TC, which shows no homology to Notch-2 or Notch-3.
The antipan-Notch antibodies are affinity-purified rabbit antibodies
specific for a cytoplasmic domain of human Notch-1, termed T3, which
is specific for Notch-1, -2, and -3. Similarly, Jagged-1- and Jagged-2-
specific antibodies were raised against unique cytoplasmic domains of the
two molecules that were used to immunize rabbits. The anti-Jagged
antibodies were affinity purified with the appropriate antigen as described
(27). Negative controls run in parallel consisted of normal preimmune
rabbit IgG.

Branching morphogenesis of lung buds cultured with antisense
oligonucleotides. Embryonic lung branching morphogenesis was as-
sayed after 48 h of culture as described previously (21) using E11.5
murine lung buds (7–13 branch points per lung bud at time zero)
treated with antisense or sense phosphorothioate oligodeoxynucleo-

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Pan-Notch immunoreactivities are localized to both cytoplasm and nuclei (Fig. 2A), suggesting that activated Notch-1 may be translocated to the nucleus. More intense Notch-1 immunostaining occurs on mitotic figures in anaphase (Fig. 2A).

By comparison, on E14, Jagged-1 is strongly detectable in distal (undifferentiated) airway epithelium and scattered undifferentiated mesenchymal cells (Fig. 2B). Epithelium of proximal conducting airways has low to undetectable levels of Jagged-1. However, Jagged-2 is strongly detected in all epithelial cells and undifferentiated mesenchyme, but not in airway-associated smooth muscle (Fig. 2B). Jagged-2 immunostaining is visible in both cytoplasm and nuclei of many cells.

Role of notch family members in lung branching morphogenesis. These observations prompted us to analyze potential functional roles for notch and jagged genes in E11.5 lung branching morphogenesis using lung buds treated with genespecific oligonucleotides (sense or antisense). Branching is regulated by epithelial-mesenchymal interactions (29). E10–E12 lung bud explants from embryonic rodents have been studied for decades as a simple, straightforward and yet elegant system for directly observing mammalian branching morphogenesis. The branching that occurs in vitro has been demonstrated to be highly similar to the in vivo process, although clearly lacking neural input and active circulation (8, 14, 29, 38, 41). We observed the most striking effects after 48 h with notch-1 antisense (Fig. 3). Compared with the corresponding sense controls using lung buds derived from the same litter of mice (Fig. 3, top, left), notch-1 antisense (Fig. 3, top, right) led to significantly increased numbers of peripheral branch points (P < 0.004).

The results of quantification of the numbers of peripheral branch points were pooled and are summarized in Fig. 3 (bottom). notch-1 antisense resulted in a ~25% increase in branching compared with the notch-1 sense control (P < 0.002 by ANOVA). Intermediate results were obtained using a 5-bp mismatched “scrambled” oligonucleotide (M5) corresponding to the notch-1 antisense (6): cultures with M5 had a trend toward reduced branching compared with notch-1 antisense (P < 0.10 by ANOVA). Results with M5 were essentially identical to those with the notch-1 sense control (P = 0.42). There was no significant difference between the untreated negative control and N15 or M5. There was no reproducible effect on branching morphogenesis with notch-2, notch-3, jagged-1, or jagged-2 antisense oligonucleotides compared with the corresponding sense controls (data not shown).

Furthermore, there was no difference between any of the sense and antisense groups with regard to cell proliferation [assessed by 5-bromo-2’-deoxyuridine incorporation and proliferating cell nuclear antigen immunostaining (30), nor with regards to numbers of apoptotic cells (as assessed by terminal deoxyxynucleotidyltransferase-mediated dUTP nick end labeling staining in situ and nuclear morphology for apoptotic bodies) (42).

We verified the specificity of these antisense-mediated effects by immunostaining paraffin sections of the cultured lung buds for Notch family proteins (see Fig. 4).

Notch family members regulate cell differentiation in cultured lung buds. To evaluate the potential role of these five genes in neuroendocrine cell differentiation, we maintained E11.5 lung buds in culture for 7 days before harvest and routine processing into paraffin blocks. Serial sections through

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**Fig. 1.** mRNAs encoding Notch-1 (N-1), N-2, N-3 (A) and Jagged-1 (Jag-1) and Jagged-2 (B) in developing murine lung. Each lane represents RNA prepared from a pool of at least 10 murine lungs from 2 or more litters. Lung tissue was harvested daily between embryonic day (E) 10 (the initial lung bud forms ~E9.5) to E18, and postnatal day 1 (P1, defined as the day of birth) through P14, as indicated. This is 1 of 2 independent experiments carried out at separate times and with fresh RNA pools and freshly prepared oligonucleotides. Band identities were verified by DNA sequencing and by probing RT-PCR Southern blots with an internal primer (sequences of which are given above).

The RNA integrity is verified by comparable detection of β-actin transcripts in all lanes. jagged-1 is also expressed from E11 through P14, except for E13. In contrast, jagged-2 is expressed in a more temporally restricted fashion: E11, E12, E16 (peak), E18, and P14, with marginally detected levels on P1 and P7. The expression of all three notch and two jagged genes within the time frame from E10 to E12 suggests that any of these could potentially function as regulators of early branching morphogenesis. Furthermore, the expression of all five notch family genes at some time point between E15 and E18 suggests that any of these could potentially function as regulators of cell differentiation, which takes place from E14 to P1. We focused the remainder of this investigation on distribution and function of Notch family members during fetal lung development.

**Localization of Notch family proteins in E14 murine lung.** To determine the cellular distribution of Notch and Jagged proteins, we carried out immunostaining of E14 fetal mouse lung using four different affinity-purified antisera specific for Notch-1, Pan-Notch (domains shared within the family), Jagged-1, or Jagged-2 (5). Representative photomicrographs are given in Fig. 2. On E14, Notch-1 and Pan-Notch immunoreactivities are clearly visible in both the epithelial and mesenchymal compartments. However, there is relative sparing of airway-associated smooth muscle. Also, on E14, Notch-1 and
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the whole block for each group of buds were immunostained for the neural/neuroendocrine cell-specific marker PGP9.5 (30, 39). Increased numbers of neuroendocrine cells were demonstrated in airway epithelium of the lung buds cultured with antisense to notch-1 or jagged-1 (Fig. 4, A and C, respectively) compared with buds from the same litters that were cultured with the corresponding sense control oligonucleotides (Fig. 4, B and D). Treatment with the notch-1 antisense resulted in a fourfold increase in the numbers of PGP9.5-positive foci (Fig. 4E, P < 0.0003) or cells (Fig. 4F, P < 0.004). Likewise, jagged-1 antisense induced a ~4- to 10-fold increase in numbers of PGP9.5-positive foci or cells (P < 0.012 and 0.008, respectively). Comparable results were obtained for four such experiments (6-9 buds per group).

To verify the specificity of the notch-1 antisense-mediated effects, we immunostained paraffin sections of the cultured lung buds for Notch family proteins. Representative photomicrographs are given in Fig. 4G (top). In lung buds cultured with notch-1 sense or notch-2 antisense, Notch-1 immunopositivity is present in airway epithelial cells, but this immunoreactivity is notably absent from buds treated with notch-1 antisense (Fig. 4G). Similarly, notch-3, jagged-1, or jagged-2 antisense oligonucleotides had no effect on Notch-1 immunostaining (data not shown).

To verify the specificity of the jagged-1 antisense-mediated effect, we immunostained paraffin sections of the cultured lung buds for Jagged-1. Representative photomicrographs are given in Fig. 4G (bottom). Jagged-1 immunopositivity is present in airway epithelial cells of lung buds cultured with jagged-1 sense or jagged-2 antisense but is notably absent from buds treated with jagged-1 antisense. Similarly, antisense to notch-1, -2, or -3 had no effect on Jagged-1 immunostaining (data not shown).

In the course of these experiments, we made the unexpected observation that lung buds treated with notch-3 antisense oligonucleotides developed a marked (approximately fivefold) increase in the relative area of PGP9.5-immunopositive mesenchymal tissue (Fig. 5, A and C). This tissue is composed of two distinct groups of PGP9.5-positive cells. First, there are large clusters of cells that immunostain only weakly to moderately for PGP9.5-positive mesenchymal tissue compared with any of the other sense or antisense groups. One of the largest masses of PGP9.5-positive cells in a sense control is given in Fig. 5B, which is morphologically most consistent with more mature neural tissue, strong PGP9.5-positive immunostaining, and the appearance of ganglia. Most of the sense controls have smaller masses of PGP9.5-positive mesenchymal tissue compared with any of the other sense or antisense groups. One of the largest masses of PGP9.5-positive cells in a sense control is given in Fig. 5B, which is morphologically most consistent with more mature neural tissue, strong PGP9.5-positive immunostaining, and the appearance of ganglia. Most of the sense controls have smaller masses of PGP9.5-positive mesenchymal tissue (Fig. 5D), which consists of delicate nerve fibers and ganglia, as well as pulmonary neuroendocrine cells. However, there was no difference in relative numbers of
neuroendocrine cells between the notch-3 sense and antisense groups (Fig. 5, C vs. D). The pooled quantitative results of three experiments are given in Fig. 5E. The buds cultured with notch-3 antisense oligonucleotides have greater than a fivefold increase in the proportional area of PGP9.5-positive mesenchymal tissue per lung bud (P < 0.007).

Summary and interpretation of functional investigations. In summary, we observe increased branching morphogenesis in lung buds cultured with antisense to notch-1, increased neuroendocrine cell differentiation with notch-1 or jagged-1 antisense, and increased neural tissue with notch-3 antisense (Table 1). Several molecular mechanisms are suggested by these observations. With regard to regulation of branching morphogenesis, there appears to be a unique role for Notch-1 in the regulation of branching of the developing lung. With regard to the appearance of PGP9.5-positive neuroendocrine cells in lung buds, wild-type Notch-1 and Jagged-1 may function as a receptor-ligand pair to inhibit neuroendocrine cell differentiation in the lung, similar to Notch-mediated inhibition of neurogenesis in Drosophila (17). Surprisingly, only Notch-3 regulates the differentiation of PGP9.5-positive neural tissue. Cumulatively, these observations suggest that Notch ligands other than Jagged-1 or -2 may be involved in the Notch-mediated regulation of branching morphogenesis and neural differentiation.

The effects of Notch signaling on branching morphogenesis could be mediated via altered cell adhesion to other cells and/or to extracellular matrix components and/or altered cell motility. This interpretation would be consistent with the observations that Notch signaling via Delta (26) or Jagged (25) can regulate cell adhesion and/or motility in neurons, keratinocytes, and endothelial cells, which may occur through a novel pathway involving the actin cytoskeleton (11, 26, 37). In the present study, the absence of changes in cell proliferation or apoptosis further suggests that altered cell adhesion and/or migration is a likely mech-

Fig. 3. Functional role for Notch-1 in lung branching morphogenesis. Embryonic lung branching morphogenesis was assayed after 48 h of culture as described previously (17) using E11.5 murine lung buds treated with antisense or sense phosphorothioate oligodeoxynucleotides. Top: representative lung buds from the same litter after 48 h of treatment with notch-1 sense (middle) or notch-1 antisense (right). Compared with the sense controls (left), notch-1 antisense-treated buds had a marked increase in peripheral branch points. Bottom: pooled results of quantification of the numbers of peripheral branch points are presented from 5–6 independent experiments, with 3–6 buds per group per experiment. Data are expressed initially as the fold increase in branch points for each bud relative to the same lung bud at time 0 [baseline numbers of branch points ranged from 7 to 11 at time 0, with minimal variability within each litter (± 1–2 branch points)]. Then each individual result was expressed as its percent change compared with the mean of the corresponding sense control. notch-1 antisense (N1AS) resulted in ~30% more branching (P < 0.003) compared with the notch-1 sense control (N1S). The untreated negative controls (Neg) did not differ significantly from the sense controls. M5 is a 5-bp mismatched control for N1AS that yielded results similar to the sense and negative controls. There was no significant difference in branching morphogenesis between sense and antisense oligonucleotides for notch-2, notch-3, jagged-1, or jagged-2.
Fig. 4. Pulmonary neuroendocrine cell differentiation is modulated by notch family genes. E11.5 murine lung buds were cultured for 7 days with antisense or sense oligonucleotides before harvest and routine processing into paraffin blocks. Serial sections through the whole block for each group of buds were immunostained for the neural/neuroendocrine cell-specific marker PGP9.5 (24, 31). L, airway lumen. Magnification: ×200 in A and B, ×400 in C and D. A and B: increased numbers of neuroendocrine cells are demonstrated in airway epithelium of lung buds cultured with notch-1 antisense (A, arrows) compared with buds from the same litters that were cultured with the corresponding sense control oligonucleotides (B). PGP9.5 immunoreactivity is also present in nerve fibers within the lung explants (B, medium arrow, bottom right corner). C and D: increased numbers of neuroendocrine cells are present in lung buds cultured with jagged-1 antisense (C, arrows) compared with control buds that were cultured with jagged-1 sense control oligonucleotides (D). PGP9.5 immunoreactivity is also present in small nerve fibers within the lung explants (D, small arrows). There was no significant effect of any of the other antisense oligonucleotides on PGP9.5-positive neuroendocrine (NE) cells. E and F: computerized image analysis was used to measure the tissue area of the cross section of each lung bud. The PGP9.5-positive cells were quantified both as the number of PGP9.5-positive cell clusters per cm² of tissue (E) and as the total number of PGP9.5-positive cells per cm² of tissue (F). In a representative experiment, treatment with notch-1 antisense resulted in a 4-fold increase in the numbers of PGP9.5-positive foci or cells (P < 0.0003 and 0.004, respectively). Similarly, jagged-1 antisense induced a 4–10-fold increase in numbers of PGP9.5-positive foci or cells (P < 0.012 and 0.008, respectively). Similar results were obtained for 4 such experiments (6–9 buds per group). G: To verify the specificity of the above effects, we immunostained paraffin sections of lung buds cultured for 7 days with notch-1 antisense for Notch-1. Representative photomicrographs are given at top: Notch-1 positivity is present in airway epithelial cells of lung buds cultured with notch-1 sense (left) or notch-2 antisense (right) but is notably absent from buds treated with notch-1 antisense (middle). To verify the specificity of the jagged-1 antisense (J1AS)-mediated effect, we immunostained paraffin sections of the cultured lung buds for Jagged-1. Representative photomicrographs are given at bottom of G: Jagged-1 positivity is present in airway epithelial cells of lung buds cultured with jagged-1 sense (J1S, left) or jagged-2 antisense (J2AS, right) but was notably absent from buds treated with jagged-1 antisense (middle). L, airway lumen, circled for visibility in J1S and J2AS, bottom.
anism underlying altered branching with Notch family antisense oligonucleotides.

With regard to cell differentiation, diminished Notch-1 or Jagged-1 leads to increased pulmonary neuroendocrine cells, analogous to the function of Notch signaling during Drosophila neurogenesis. These data implicate Notch-1 and Jagged-1 as a receptor-ligand pair regulating differentiation of neuroendocrine cells in the developing lung, consistent with our observation of Jagged-1 immunostaining predominantly localized to epithelial cells in the fetal lung during the period of cell differentiation (E14). These data are consistent with the observation of Notch immunostaining only in nonneuroendocrine cells in the lung (15). In other murine systems, Notch signaling has been demonstrated to play a role in differentiation of pancreatic endocrine cells (2) and endodermal endocrine differentiation in the gut (18, 43).

Our unexpected observation of a specific role for Notch-3 in regulating neural (but not neuroendocrine) differentiation suggests two possibilities. Either these two cell types (neuroendocrine vs. neural) are derived from distinct pluripotent precursor cells (epithelial vs. mesenchymal) in the lung and/or a common precursor cell can follow one or the other lineage pathways, depending on whether Notch-1 and Jagged-1 or Notch-3 is downregulated.

Finally, our data suggest that the Notch ligand(s) interacting with Notch-1 and/or Notch-3 to regulate branching and neural differentiation is neither Jagged-1 nor Jagged-2. Delta-1 or -2 are likely candidates, but we cannot rule out the involvement of a novel Notch ligand.

Relevance to other cellular systems. Differential effects of Notch ligands have been observed in other systems. Exogenous

Table 1. Summary of observations in murine lung buds cultured with antisense oligodeoxynucleotides

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<th>#NE Cells</th>
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The results of experiments carried out in 2 different assay systems: 48-h cultures of embryonic day 11.5 (E11.5) lung buds to assess branching morphogenesis, and 7-day lung bud cultures to assess neural/neuroendocrine (NE) cell differentiation using the specific marker PGP9.5.
Delta-1 or Jagged-1 promotes natural killer cell differentiation of hematopoietic progenitors, whereas only Delta-1 is permissive for the emergence of CD4/CD8-positive cells (16). Similar functions have been observed for Notch ligands in other systems (11). Soluble forms of both Delta-1 and Delta-4 regulate mitogenesis of cultured hematopoietic precursors (20). Jagged-1 deficiency causes Agalile syndrome in humans (24), whereas Notch-3 deficiency is associated with a degenerative vascular disorder (19). Mice deficient for Notch-2 or Jagged-1 have a similar phenotype with hypoplastic kidneys and myocardium (28). Evidence suggesting divergent functions for different notch genes comes from studies of transgenic mice overexpressing activated Notch-1 or Notch-3 in thyocytes (7, 35) or in the pancreas (2).

Conclusions. The present study demonstrates a major role for Notch signaling in the regulation of cytodifferentiation in murine embryonic lung, with additional involvement in branching morphogenesis of the developing bronchial tree. Cumulatively, our observations suggest that different Notch and Jagged family members can have diverse tissue-specific regulatory functions during development, with only partial redundancy (as with the effects of Notch-1 and Jagged-1 in neuroendocrine cell differentiation). This functional complexity could be due, in part, to Notch ectodomain-binding cofactors such as Fringe (13, 22, 32) or Scabrous (22, 31), which in turn could modulate ligand selection or signal transduction by Notch. The specification of unique cell fates by such combinatorial signaling is becoming recognized as an important mechanism in mammalian lung embryogenesis.

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