Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium

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SUMMARY

To clarify the mechanisms that regulate neuroendocrine differentiation of fetal lung epithelia, we have studied the expression of the mammalian homologs of achaete-scute complex (Mash1) (Ascl1 – Mouse Genome Informatics); hairy and enhancer of split1 (Hes1); and the expression of Notch/Notch-ligand system in the fetal and adult mouse lungs, and in the lungs of Mash1- or Hes1-deficient mice. Immunohistochemical studies revealed that Mash1-positive cells seemed to belong to pulmonary neuroendocrine cells (PNEC) and their precursors. In mice deficient for Mash1, no PNEC were detected. Hes1-positive cells belong to non-neuroendocrine cells. In the mice deficient in Hes1, in which Mash1 mRNA was upregulated, PNEC appeared precociously, and the number of PNEC was markedly increased. NeuroD (Neurod1 – Mouse Genome Informatics) expression in the lung was detected in the adult, and was enhanced in the fetal lungs of Hes1-null mice. Expression of Notch1, Notch2, Notch3 and Notch4 mRNAs in the mouse lung increased with age, and Notch1 mRNA was expressed in a Hes1-dependent manner. Notch1, Notch2 and Notch3 were immunohistochemically detected in non-neuroendocrine cells. Moreover, analyses of the lungs from the gene-targeted mice suggested that expression of Delta-like 1 (Dll1 – Mouse Genome Informatics) mRNA depends on Mash1.

Thus, the neuroendocrine differentiation depends on basic helix-loop-helix factors, and Notch/Notch-ligand pathways may be involved in determining the cell differentiation fate in fetal airway epithelium.

Key words: Lung, Neuroendocrine cell, Differentiation, Mash1, Hes1, Notch/Notch ligand, Mouse

INTRODUCTION

Mammalian airway epithelium is composed of various cell populations, and pulmonary neuroendocrine cells (PNEC) are a relatively minor cell population distributed throughout airway epithelium from the lobar bronchus to the alveolar duct. They occur both as solitary cells and clusters, and the latter are called neuroepithelial bodies when they are innervated (Lauweryns et al., 1972). PNEC seem to function as oxygen sensors in adult life (Youngson et al., 1993). In the fetal period, PNEC are the first cell type to differentiate in humans and animals (Cutz, 1987; Cutz et al., 1984; McDowell et al., 1985; Sarikas et al., 1985), and far more PNEC are seen in fetal lungs than in adult ones. As PNEC secrete gastrin-releasing peptide or calcitonin-gene related peptide, which may be local mitogenic hormones, they have been considered to control growth and morphogenesis of fetal lung (Hoyt et al., 1991; Sunday et al., 1993). The developmental mechanisms of differentiation of PNEC have not been fully elucidated. A classical explant culture study has revealed that PNEC develop from immature lung epithelia, and that mesenchyme and nervous tissue are not always crucial for neuroendocrine differentiation of the epithelial cells (Ito et al., 1997). A recent study demonstrated the significance of a proneural, basic helix-loop-helix (bHLH) transcriptional factor, Mash1, which is essential for neuroendocrine cell differentiation of lung epithelium as mice deficient for Mash1 do not have PNEC (Borges et al., 1997).

In the past few years, the molecular mechanisms of neurogenesis have become increasingly clear, and it has been shown that fundamental mechanisms are conserved between Drosophila and mammals (Jan and Jan, 1994; Lee, 1997). bHLH factors control cell fate determination during neurogenesis and myogenesis, which are regulated by two distinct groups of genetically defined bHLH activator and repressor genes (Jan and Jan, 1993; Kageyama and Nakanishi, 1997). In Drosophila, one group of bHLH factors encoded by proneural genes such as Mash1 activates neural differentiation in the ectoderm, whereas another group of bHLH factors encoded by Hes1 represses differentiation into neurons. In mammals, bHLH genes such as Mash1, neurogenin (Neurod3 – Mouse Genome Informatics) and mammalian atonal...
Involvement of Notch/Notch-ligand system in the cell-fate decision in the lung epithelial system. We show the localization of Mash1 and Hes1 expression immunohistochemically, and the modulation of their mRNAs in fetal developing lungs to reveal the relationship between neuroendocrine differentiation and bHLH factors. To clarify the significance of these genes in neuroendocrine differentiation in fetal lung epithelium, we analyzed the lungs from Mash1- or Hes1-deficient mice and quantified PNEC levels in them. In addition, we examined the expression of Notch receptors and a ligand, Dll1, in the lung to show that the Notch/Notch-ligand pathway, associated with the bHLH factors, may play a role in the airway epithelial cell differentiation.

MATERIALS AND METHODS

Animals

Mice were mated overnight, and the day of the discovery of the vaginal plug was counted as E0. The lung tissues from fetal (E12, E14, E16 and E18), neonatal (N1) and adult ICR mice, purchased from Japan SLC (Shizuoka, Japan), were used for immunohistochemical, northern blot, and reverse transcription-polymerase chain reaction (RT-PCR) analyses. Mash1 and Hes1 mutant fetuses were obtained by mating heterozygous males and females for Mash1- (Guillemot et al., 1993) and Hes1- (Ishibashi et al., 1995) targeted mutations, respectively. Crown-rump lengths and longitudinal lengths of left lungs were measured in neonatal mice with various Mash1 statuses and in fetal mice (E18) with various Hes1 statuses. The fetuses were genotyped by PCR analysis of DNA obtained from the fetus head tissue, as previously reported (Guillemot et al., 1993; Ishibashi et al., 1995). The lung tissues from fetal (E13, E15 and E17) and neonatal mice deficient for Mash1, and the lung tissues from fetal mice (E13, E15, E17 and E18) deficient for Hes1 were used for the immunohistochemical study. The lung tissues from neonatal mice deficient in Mash1 and the lungs from fetal mice (E18) deficient in Hes1 were used for northern-blot and RT-PCR analyses.

Immunohistochemistry

Lung tissues from ICR mice, Mash1-deficient mice and Hes1-deficient mice were fixed overnight in phosphate-buffered 4% paraformaldehyde and frozen. Frozen sections, 5 μm thick, were incubated with mouse monoclonal antibody against Mash1 (courtesy of Dr David J. Anderson; Sommer et al., 1995), rabbit polyclonal antibody against a C-terminal peptide of Hes1 (SPSSGSSLTSDSMWPRWNR), or goat polyclonal antibodies against Notch1, Notch2, Notch3 and Notch4 (Santa Cruz Biotechnology, USA). For detection of neuroendocrine cells, sections were treated with mouse monoclonal antibody against protein gene product 9.5 (PGP9.5; Ultraclone) or rabbit polyclonal antibody against calcitonin-gene-related peptide (CGRP; Sigma Chemicals, USA) or alpha-subunit of GTP-binding protein Gq (Goq; MBL, Japan). If necessary, serial sections were made, and, besides hematoxylin and eosin (HE) staining, immunostaining for Mash1, Hes1, Notch1, Notch2, Notch3, PGP9.5, CGRP or Goq was carried out to clarify relationship between neuroendocrine cells and expression of Mash1, Hes1, Notch1, Notch2 and Notch3. In addition, to detect the occurrence of Clara cells, a type of non-PNEC cell, the lung sections from Mash1-deficient mice (N1) and Hes1-deficient mice (E18) were treated with a rabbit polyclonal antibody against Clara cell protein (courtesy of Dr G. Singh). The sections immunostained for Mash1 or Hes1 were pretreated with a microwave-antigen-retrieval method (Shi et al., 1995) in a citrate buffer solution (pH 6.0, 97°C, 15 minutes). After washing in the phosphate buffer, sections were incubated with biotinylated anti-
mouse (for Mash1 and PGP9.5 stainings), anti-rabbit (for Hes1, CGRP, Goa and Clara cell protein stainings) or anti-goat (for Notch1, Notch2, Notch3 and Notch4 stainings) IgG (Chemicon, USA), and followed by treatment with fluorescein isothiocyanate-conjugated avidin (Sigma). Background staining with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) or 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI; Sigma) was also added to depict cell contour or cell nuclei, respectively. After staining, sections were observed using an epifluorescence microscope and a confocal laser microscope (Olympus model LSM 200UV). Negative controls were performed by applying nonimmune sera to all pertinent subclasses of immunoglobulin.

The numbers of PNEC (including single and clustered PNEC) and PNEC clusters in the lungs from the mice with various Mash1 statuses (5 animals with each genotype) and from the mice with various Hes1 statuses (5 animals with each genotype) were counted in the sections immunostained for PGP9.5, CGRP or Goa, and the incidence of PNEC in a given lung area was calculated (Ito et al., 1999). The incidence of Clara cells in the mice with various Mash1 or Hes1 statuses was calculated as the number of Clara cell protein-positive cells/the total airway cells observed. The values determined in these experiments were subjected to the unpaired t test for analyzing significance. P values below 0.05 were considered to indicate significant differences.

Northern-blot analysis
Total RNA was isolated from the lungs from fetal (E12, E14, E16 and E18), neonatal, adult ICR mouse, from the neonatal lungs of mice with various Mash1 statuses and from the fetal lungs (E18) of mice with various Hes1 statuses using the Trizol reagent (Life Technologies, USA). Total RNA25 μg/lane) was electrophoresed on 1% agarose, 2.2 M formaldehyde gels, and transferred onto nylon membranes. The probes used for Mash1 and Hes1 were full-length cDNAs (Ishibashi et al., 1995). Notch1, Notch2, Notch3, Notch4 and Dll1 cDNA probes were amplified by RT-PCR from RNA extracted from mouse lung or brain. The primers used were: Notch1, 5'-agaagggctgaacgcggcgctggac-3' and 5'-tgaaagagaagttgagagtcctc-3' (494 bp product); Notch2, 5'-gcaagaggcttgagatgctccc-3' and 5'-ggactgccaatactccacctctc-3' (408 bp product, 786-1193) and Dll1, 5'-tgaaaagggaagttgagagtcctc-3' and 5'-agcgataggagccgatctcattg-3' (467 bp product, 1930-2515). PCR products were separated by agarose gel electrophoresis and eluted from the gel with a QIAEX II gel extraction kit (Qiagen, Germany), subcloned into the TA cloning vector using the pGEM-T easy vector system (Promega, USA) and sequenced. Probes were labeled with [α-32P]deoxycytidine triphosphate using a BcaBEST labeling kit (Takara, Japan). Hybridization was performed as previously described (Ishibashi et al., 1995). Membranes were exposed to X-ray film (Kodak, USA) at ~80°C. Northern-blot analyses were repeated four times.

RT-PCR analysis
Total RNA was isolated from the lungs of fetal (E12, E14, E16 and E18), neonatal and adult ICR mice, and from the neonatal lungs of mice with various Mash1 statuses and from the fetal lungs (E18) of mice with various Hes1 statuses using the ThermoScript RT-PCR system (Life Technologies). The primers used were followings: NeuroD, 5'-aagctggcagcggctggcag-3' and 5'-tgagaggaatggcctttatatg-3' (513 bp product); Hes5, 5'-tcggctctaatgcgctctg-3' and 5'-agcgegegegcaacggat-3' (494 bp product) and β-actin 5'-ctggccgctgcctgcaaca-3' and 5'-ttggtcgtattggtgcggg-3' (243 bp product). Adult ICR mouse lung brain were used as positive controls.

RESULTS

Immunohistochemistry for bHLH factors and Notch in mouse lungs
In the ICR mouse lungs, immunohistochemical analysis revealed that PNEC, which were immunostained for CGRP, PGP9.5 and Goa, occurred in the lobar bronchus by E14, and that most PNEC formed clusters; some were innervated late in the fetal period and in adult lungs. Mash1-positive cells were detected in the lobar bronchus and distributing bronchiole as early as E14. They formed clusters in the proximal lobar bronchus, and solitary Mash1-positive cells were often seen in the peripheral bronchus. In the E14 mouse lung, more Mash1-positive cells were seen in the peripheral bronchus than CGRP, PGP9.5 or Goa-positive PNEC (data not shown), but thereafter in the late fetal period and in the adult, eventually all Mash1-positive nuclei cells had the immunohistochemical properties of PNEC (Fig. 1B,C). Hes1 immunostaining was localized in non-neuroendocrine cells in the lung epithelium (Fig. 1C,D). Immunostainings for Notch1 and Notch3 revealed that positive cells were non-neuroendocrine cells in the late fetal and adult lungs (Fig. 1C,E,F). Immunostaining for Notch2 revealed that some non-neuroendocrine cells were positive in the airway (data not shown). Immunostaining for Notch4 was not detected in the airway epithelium.

Expression of bHLH factors in normal mouse lungs and in the lungs of Mash1- or Hes1-deficient mice
Northern-blot analyses revealed that Mash1 and Hes1 mRNAs were detected as early as E12 and E14, respectively (Fig. 2A). Expression of Mash1 mRNA was enhanced in the lungs of the Hes1-deficient mice (Fig. 2B). Mild elevation of Mash1 mRNA levels was observed in the mouse lungs with heterozygous Hes1 gene (Fig. 2B).

NeuroD mRNA, the product of which is downstream of Mash1 in the regulatory pathway of development of olfactory neuron (Cau et al., 1997), was detected by the RT-PCR method in the neonatal and adult mouse lungs, but not in the fetal lungs (Fig. 3A). However, in the fetal lungs from Hes1-deficient mice (E18), NeuroD mRNA was detected (Fig. 3B). Hes5, another repressive bHLH protein, was not detected by the RT-PCR method in the fetal or adult mouse lungs, regardless of the status of the Mash1 or Hes1 genotypes (Fig. 3A,B).

PNEC and Clara cells in Mash1- or Hes1-deficient mice
Body size and lung length of the Hes1-deficient mice were smaller than those of wild-type and heterozygous mice (Table 1). Although size of the lungs was small in Hes1-deficient mice, histological architecture of the lungs seemed normally developed except for many airway nodular lesions (Fig. 4A,D), which corresponded to PNEC clusters (Fig. 4E,F). A quantitative study of PNEC and PNEC clusters with immunostaining for CGRP, PGP9.5 and Goa revealed that they were not detected in the lungs of Mash1-deficient mice throughout the observation period (Fig. 5A). However, in the Hes1-deficient mice, a few precocious PNEC were seen in E13 mice (Figs 4J and 5B), and thereafter, far more PNEC occurred in the Hes1-deficient mice than in wild-type and heterozygous mice (Figs 4E,F and 5B). The numbers of PNEC and PNEC clusters were intermediate in Mash1- and Hes1-deficient mice.
heterozygous mice (Fig. 5A,B). Notch1 immunostaining decreased in Hes1-deficient mice (Fig. 4G,H).

A quantitative study of the occurrence of Clara cells in the lobar bronchus and terminal bronchiole was performed in the neonatal lungs of mice with various Mash1 statuses and in the fetal lungs of mice with various Hes1 statuses (E18). In Mash1-deficient mice, Clara cells appeared in similar numbers as in the wild-type and heterozygous mice, but in the Hes1-deficient mice, Clara cells were significantly reduced compared with the numbers in wild-type and heterozygous mice (Table 2).

Expression of Notch receptors and Notch ligand, Dll1 in normal mouse lungs and in the lungs of Mash1- or Hes1-deficient mice
Northern-blot analyses detected expression of Notch1, Notch2, Notch3 and Notch4 mRNAs in mouse lung tissues. Expression of Notch1, Notch3 and Notch4 mRNAs increased with fetal age and strong expression in the adult lungs (Fig. 6A). Dll1 mRNA was detected in the fetal mouse lungs by E14; the expression increased with fetal age and was marked in the adult lungs (Fig. 6A). In the lungs from Hes1-deficient mice, northern-blot...
analyses revealed that marked decrease of Notch1 mRNA was detected (Fig. 6B). Moreover, in the lungs of Mash1-deficient mice, Dll1 mRNA disappeared, but was upregulated in the lungs of Hes1-deficient mice (Fig. 6B).

## DISCUSSION

In the present study, PNEC showed positive immunostaining for Mash1 in their nuclei. However, in the lungs of E14 mice, more Mash1-positive cells were seen in the peripheral bronchus than PNEC that were positively immunostained for CGRP, PGP9.5 or Gao. In the early PNEC developing stage, Mash1-positive cells may include precursor cells for PNEC. Although PNEC often form clusters in the fetal period (McDowell et al., 1994; Ito et al., 1999), many solitary Mash1-positive cells were seen in the E14 mouse lungs. The fate of the solitary cells is unclear, but these cells could join to form PNEC clusters as the Mash1-positive cells were usually present as clusters after E15. The significance of Mash1 in the development of PNEC has been shown by observations of the lungs from neonatal Mash1-deficient mice (Borges et al., 1997). We confirmed their finding and further showed that PNEC were not present in the lungs from fetal Mash1-deficient mice. Regulation of the expression of Mash1 has not previously been studied in the lung. Bone morphogenic protein (BMP) 2 and the related BMP4 have been shown to induce expression of Mash1 and to promote autonomic neural differentiation in neural crest cells (Shah et al., 1996; Lo et al., 1997). In the fetal mouse lungs, BMP4 is present at high levels in the epithelium of the terminal bud (Bellusci et al., 1996). Contrary to localization of BMP4, PNEC are not seen in the distal part of the developing airway epithelium and they occur preferentially around the airway bifurcation (Sarikas et al., 1985). It seems interesting to study effect of growth factors such as BMPs on Mash1 expression and PNEC induction with explant culture study.

### Table 1. Length of bodies and lungs of mice (mean±s.d.)

<table>
<thead>
<tr>
<th>Animal</th>
<th>Number of neonates and fetuses</th>
<th>Crown-rump length (mm)</th>
<th>Lung length (mm)</th>
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<tbody>
<tr>
<td>N1</td>
<td>Mash1+/+</td>
<td>26.7±0.9</td>
<td>6.6±0.4</td>
</tr>
<tr>
<td></td>
<td>Mash1+/-</td>
<td>26.6±0.7</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td></td>
<td>Mash1-/-</td>
<td>26.1±0.7</td>
<td>6.4±0.5</td>
</tr>
<tr>
<td>E18</td>
<td>Hes1+/+</td>
<td>24.5±1.7‡</td>
<td>6.5±0.4‡</td>
</tr>
<tr>
<td></td>
<td>Hes1+/-</td>
<td>24.3±1.6‡</td>
<td>6.2±0.4‡</td>
</tr>
<tr>
<td></td>
<td>Hes1-/-</td>
<td>20.9±1.6‡</td>
<td>5.1±0.5‡</td>
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*Longitudinal length of the left lung. The crown-rump length and lung length of Hes1+/+ mice are significantly shorter than those of Mash1+/+ or Hes1+/+ mice (‡P<0.001).

In the early PNEC developing stage, Mash1-positive cells may include precursor cells for PNEC. Although PNEC often form clusters in the fetal period (McDowell et al., 1994; Ito et al., 1999), many solitary Mash1-positive cells were seen in the E14 mouse lungs. The fate of the solitary cells is unclear, but these cells could join to form PNEC clusters as the Mash1-positive cells were usually present as clusters after E15. The significance of Mash1 in the development of PNEC has been shown by observations of the lungs from neonatal Mash1-deficient mice (Borges et al., 1997). We confirmed their finding and further showed that PNEC were not present in the lungs from fetal Mash1-deficient mice. Regulation of the expression of Mash1 has not previously been studied in the lung. Bone morphogenic protein (BMP) 2 and the related BMP4 have been shown to induce expression of Mash1 and to promote autonomic neural differentiation in neural crest cells (Shah et al., 1996; Lo et al., 1997). In the fetal mouse lungs, BMP4 is present at high levels in the epithelium of the terminal bud (Bellusci et al., 1996). Contrary to localization of BMP4, PNEC are not seen in the distal part of the developing airway epithelium and they occur preferentially around the airway bifurcation (Sarikas et al., 1985). It seems interesting to study effect of growth factors such as BMPs on Mash1 expression and PNEC induction with explant culture study.

Contrary to the observations in Mash1-deficient mice and in

### Fig. 4. Histological features of the lungs from Hes1-null mice (D-F,H,J) in comparison with those of heterozygous mice (A-C,G,I) at E18 (A-H) and E13 (I,J). HE-stained sections (A,D) show similar histological pattern, but many nodular lesions are seen in the airway epithelium of a Hes1-null mouse (D). Serial sections of the boxed areas in HE sections (A,D) are shown immunostained for CGRP (B,E), Mash1 (C,F) and Notch1 (G,H). Note that many CGRP-positive and Mash1-positive PNEC are seen in the lungs from a Hes1-null mouse (D,F). In these mice, Notch1 staining in the lung is weak (G,H). At E13 (I,J), the precocious appearance of PGP9.5-positive PNEC clusters (arrowheads, light blue) is seen in the lobar bronchus (J). In (B,C, E-H), the arrow s indicate PNEC clusters. In (J), the arrowheads indicate PNEC clusters at E13. Note that false positive staining is diffusely seen in the background of the sections stained for Mash1 (C,F). Counterstaining is achieved using TRITC-conjugated phalloidin (B,E; red) and nuclear staining with DAPI (I,J; dark blue). Bars, 100 µm (A-H); 20 µm (I,J).
Hes1-deficient mice, Mash1 gene expression was enhanced, and precocious neuroendocrine differentiation and a marked increase of PNEC during the fetal period were demonstrated. The increase in PNEC in Hes1-null mice can be explained by the suppression by Hes1 of the binding of Mash1 to DNA through protein-protein interaction (Sasai et al., 1992), and by the repression of Mash1 transcription by Hes1 (Sasai et al., 1992; Ohsako et al., 1994; Van Doren et al., 1994; Chen et al., 1997). In neuronal differentiation, Mash1 transactivates a cascade of neuronal bHLH genes such as NeuroD, and thereby promotes differentiation (Ma et al., 1996; Cau et al., 1997). In our study, NeuroD mRNA was detected only in the adult mouse lungs, but in the Hes1-deficient mice, expression of NeuroD in the fetal lung was upregulated. Although NeuroD is not suspected to be crucial for neuroendocrine differentiation in the fetal lung epithelium, NeuroD might be necessary to induce a subtype of PNEC, as NeuroD is reported to play roles in the development of some subpopulations of entero-neuroendocrine cells (Naya et al., 1997) and as some subpopulations of PNEC develop after birth (McDowell et al., 1994). Thus, the development of PNEC is regulated by bHLH activator and repressor; Mash1 is a dominant activator for neuroendocrine differentiation, and Hes1 inhibits neuroendocrine differentiation through suppressing Mash1 activity.

Table 2. Incidence of Clara cells in bronchial and bronchiolar epithelium

<table>
<thead>
<tr>
<th>Animal</th>
<th>Incidence of Clara cells (%, mean±s.d.)</th>
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<tr>
<td></td>
<td>Bronchus</td>
</tr>
<tr>
<td>Age</td>
<td>Genotype</td>
</tr>
<tr>
<td>N1</td>
<td>Mash1+/+</td>
</tr>
<tr>
<td></td>
<td>Mash1+/−</td>
</tr>
<tr>
<td></td>
<td>Mash1−/−</td>
</tr>
<tr>
<td>E18</td>
<td>Hes1+/+</td>
</tr>
<tr>
<td></td>
<td>Hes1+/−</td>
</tr>
<tr>
<td></td>
<td>Hes1−/−</td>
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</table>

Clara cells were immunohistochemically defined as Clara cell protein-positive cells.

The number of Clara cells in the lungs from Hes1−/− mice was significantly decreased in the bronchus (compared with Hes1+/+ mice, *P*<0.01) and in the bronchiole (compared with Hes1+/+, *P*<0.001; and with Hes1+/−, *P*<0.05).

In situ hybridization for Hes1 mRNA has disclosed Hes1 expression in the mouse airway epithelium (Sasai et al., 1992). In our immunohistochemical study, Hes1 was detected in the nuclei of non-neuroendocrine airway epithelial cells, but not in PNEC. Although it could be supposed that Mash1 itself or signalling pathways involved in Mash1 expression represses Hes1 directly or indirectly, the mechanisms by which Hes1 expression is suppressed in PNEC are not well understood. Immunohistochemically, Notch1, Notch2 and Notch3 were not detected in PNEC, and the undetectable expression of Hes1 in the PNEC nuclei may result in the inactive Notch/Notch-ligand pathway in these cells. Alternatively, some Notch inhibitors, such as Numb, of which the asymmetrical localization has been shown to influence a Notch-mediated interaction in a central nervous cell lineage (Guo et al., 1996), might be present to regulate Notch activity in PNEC. The significance of Hes1 in the development of PNEC was clearly demonstrated by the findings of Hes1-deficient mice. However, even without Hes1, not all fetal airway epithelial cells differentiated into neuroendocrine cells, and most cells differentiated to non-neuroendocrine cells such as Clara cells, although, owing to the increase of PNEC, the proportion of Clara cells in the airway epithelium was a little lower in the lungs from Hes1-deficient mice than in the lungs of the wild-type and heterozygous mice. This could mean that Hes1 has redundancy in its inhibitory activity of neuroendocrine differentiation in the lungs. One of the candidates that might compensate Hes1 function is Hes5 (Ohtsuka et al., 1999). However, in fetal and adult lungs, expression of Hes5 mRNA was not detected by the RT-PCR method, neither was it found in the lungs of Hes1-deficient mice, although Hes5 is reported to be upregulated in the brains of Hes1-deficient mice (Ishibashi et al., 1995). Other candidates for inhibition of neuroendocrine differentiation from immature epithelium might include members of the Id family, especially Id2 or Id4, whose expression has been detected in airway epithelium (Jen et al., 1996).

Expression of Notch1, Notch2, Notch3 and Notch4 mRNAs in the mouse lungs increased with fetal age, and high levels of expression of Notch1, Notch3 and Notch4 were detected in the adults. In the lungs from Hes1-deficient mice in the late fetal period (E18), marked decrease of Notch1 expression was

Fig. 5. (A) Occurrence of CGRP-positive PNEC in fetal (E13-E17) and neonatal (N1) mice with various Mash1 statuses. Note the absence of PNEC in the Mash1-null mice. (B) Occurrence of CGRP-positive PNEC in fetal (E13-E18.5) mice with various Hes1 statuses. Note the precocious appearance of PNEC at E13 and the increased number of PNEC in the Hes1-null mice. The occurrence of PNEC was evaluated by the number of PNEC per lung area. *P*<0.01; **P*<0.05 versus Mash1 or Hes1 homozygous null mice on each day.
Fig. 6. (A) Northern-blot analysis of Notch3, Notch1, Notch4, Dll1 and GAPDH mRNAs in fetal (E12- E18), neonatal (N1) and adult (Ad) ICR mouse lungs. (B) Expression of Notch1, Notch2, Notch3, Notch4 and Dll1 mRNAs in the neonatal lungs from Mash1-null mice and in the fetal lungs from Hes1-null mice (E18). In Hes1-null mice, a decrease of Notch1 expression is seen. Expression of Dll1 mRNA is downregulated in the lungs from Mash1-null mice, but upregulated in Hes1-null mice. The northern-blot analyses were repeated four times, and the expression patterns were reproducible.

deterred. These observations suggest that Notch1 expression in the lung is strongly dependent on Hes1, but that other types of Notch expression are not dependent on Hes1. It has been reported that activation of Hes1 through Notch1 signalling can also upregulate the expression of both Notch1 and Notch2 in mouse myoblasts, and that the upregulation of Notch expression by Hes1 is likely to be indirect (Weinmaster, 1997). However, transient transfection analyses have demonstrated that the active form of Notch can upregulate expression of Hes1 and Hes5 (Nishimura et al., 1998), and in Notch1-deficient mice, enhanced neurogenesis is observed with upregulation of Mash1 and NeuroD, and with downregulation of Hes5 (de la Pompa et al., 1997). Although the regulatory system of the Notch receptors is affected directly or indirectly by the repressive bHLH, the Hes1-dependent expression of Notch1 seen in the fetal lung could mean that a positive-feedback system works in the non-neuroendocrine cells to reinforce the Notch1/Hes1 pathways. Moreover, the immunohistochemical study revealed that Notch1- or Notch3-positive cells were Hes1-positive non-neuroendocrine cells. These suggest that Notch receptors can play important roles in differentiation toward non-neuroendocrine cells.

Expression of Delta mRNA requires Mash1 in Drosophila (Kunisch et al., 1994; Heitzler et al., 1996). In this study, expression of Dll1 seems to be concomitant with the appearance of PNEC during fetal mouse lung development. Furthermore, Dll1 expression was downregulated in the lungs from Mash1-deficient mice, and upregulated in Hes1-deficient mice. These observations suggest that Dll1 expression is dependent on Mash1 and neuroendocrine differentiation in the mouse lungs, and that Hes1 represses Dll1 expression through inhibition of Mash1 activity. Expression of a Delta-like protein has also been reported in PNEC neoplasms (Laborda et al., 1993). Taken together, these studies suggest that Dll1 is expressed in the PNEC. However, it is not well understood how the positive regulation of Dll1 by Mash1 works in the autocrine or paracrine loops that are involved in the processes of differentiation into neuroendocrine cells, or how Dll1 interacts with Notch-receptor-expressing non-neuroendocrine cells. As in Xenopus retinogenesis, Delta-expressing cells inhibit their neighbors from neuronal differentiation, but can enhance the neuronal differentiation pathway in particular environments (Dorsky et al., 1997). It is possible that Dll1 could maintain or promote neuroendocrine differentiation through a positive-feedback loop.

Lung morphogenesis and differentiation of cells into non-neuroendocrine cells such as Clara cells and type 2 alveolar cells are regulated by other types of transcription factors, such as thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factor 3 (Hackett et al., 1996; Whitsett, 1998). It is

Fig. 7. Schematic diagram of proposed regulatory mechanisms of cell fate determination in fetal mouse lung epithelium. Mash1 and Hes1 are involved in neuroendocrine versus non-neuroendocrine cell fate specification in the lung epithelium: for neuroendocrine differentiation, Mash1 is crucial and Hes1 inhibits neuroendocrine differentiation through inactivation of Mash1. Notch receptors, which can activate Hes1, are expressed in non-neuroendocrine cells and are also regulated by Hes1. Hes1 and Notch expression may function to keep non-neuroendocrine cell fate. In neuroendocrine cells, Mash1 upregulates Dll1. The significance of Dll1 in neuroendocrine differentiation and in interaction with Notch receptor-expressing non-neuroendocrine cells remains to be determined.
interesting that differentiation of thyroid C cells, neuroendocrine cells in the thyroid, is dependent on Mash1 (Lanigan et al., 1998) and TTF1 (Suzuki et al., 1998). In this study, we have shown that Mash1 is a positive regulator for the neuroendocrine differentiation in the lung, and that Hes1 is a negative regulator. Moreover, Notch receptors and their ligand are suspected to take part in the determination of cell differentiation through interaction with active and repressive bHLHs (Fig. 7). Besides the Notch/Notch-ligand pathways, extrinsic factors including BMP4, or other signalling pathways such as Sonic hedgehog (Nakagawa et al., 1996; Belluscii et al., 1997), could play roles in the regulation of airway epithelial cell differentiation, and transcription factors other than bHLH, which are involved in lung morphogenesis and non-neuroendocrine cell differentiation, might be involved directly or indirectly in the process of neuroendocrine differentiation. In future studies, we hope to elucidate the precise interactions between the bHLH network and the Notch/Notch-ligand pathways; to clarify whether other active and repressive bHLH factors participate in the regulatory mechanisms of the bHLHs; and to study the interaction of the neuronal bHLH factors with other factors involved in lung morphogenesis and development.

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