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Review

# Significance of proneural basic helix-loop-helix transcription factors in neuroendocrine differentiation of fetal lung epithelial cells and lung carcinoma cells

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**Summary.** In this brief review article, we describe how cell fate determination by which the airway epithelial cells become neuroendocrine or non-neuroendocrine is regulated by a network of basic helix-loop-helix transcription (bHLH) factors in a similar manner to neuronal differentiation, and how this system could work to determine cell differentiation of human lung carcinomas. Immunohistochemical studies reveal that mammalina achaete-scute complex homologue (Mash)1 is expressed in pulmonary neuroendocrine cells (PNEC), while hairy and Enhancer of split (Hes)1 is expressed in pulmonary non-neuroendocrine cells (non-PNEC). Studies using gene-deficient mice for the bHLH factors revealed that in *Mash1* homozygous null mice no PNEC are detected, while PNEC increase markedly in *Hes1* homozygous null mice. These observations suggest that Mash1 is an essential positive factor for neuroendocrine differentiation of lung epithelium, and that Hes1 is one of the repressive factors for neuroendocrine differentiation. Moreover, immunohistochemical studies revealed that Notch receptors are detected in non-PNEC, and thus the Notch signalling pathway could play a role in the determination of airway epithelial cell differentiation.

In human lung carcinomas, a similar bHLH network should operate to determine cell differentiation phenotypes. Generally, expression of the human homologue of Mash1 (HASH1) is detected in small cell carcinoma and carcinoids, while Hes1 seems to be expressed mainly in non-small cell carcinoma.

Thus, proneuronal bHLH factors may play roles in cell fate determination of the airway epithelial system, and may regulate human airway epithelial cells in diseased conditions.

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## Introduction

The lung epithelium is derived from the foregut endoderm, and contains various epithelial cell types which differentiate from primitive undifferentiated cells. Cytodifferentiation of the fetal lung epithelium seems morphologically to follow a centrifugal pattern and to occur in a specific order (Cutz, 1987). The first cell type which manifests a differentiated phenotype and can be morphologically defined is the neuroendocrine cell in humans and animals (Cutz, 1987; Ito, 1999). Afterwards pulmonary neuroendocrine cells (PNEC), ciliated cells, secretory cells such as Clara cells, and basal cells appear in developing fetal airway epithelium. Type 2 alveolar cells occur with development of the alveolus. Regulatory mechanisms of differentiation of lung epithelial cells have been extensively studied in recent years, and it has been shown that the establishment of a cell phenotype requires the presence of transcription factors (Table 1) that activate or repress expression of specific genes (Hackett et al., 1996; Whitsett, 1998). Hepatocyte nuclear factor-3 family members, homeodomain proteins such as thyroid transcription factor-1 (TTF-1) and zinc finger proteins such as GATA-6 play important roles in the development and differentiation of the tissues and cells from the foregut endoderm. HNF-3ß, TTF-1 and GATA-6 are reported to be expressed in undifferentiated airway epithelial cells and to be involved in differentiation of Clara cells or type 2 alveolar cells through regulation of the gene expression of Clara cell secretory protein or surfactant apoproteins (Bingle and Gitlin, 1993; Bohinski et al., 1994; Ikeda et al., 1996; Kimura et al., 1996; Zhou et al., 1997; Bruno et al., 2000). On the other hand, HNF-3/forkhead homologue 4 (HFH-4) is associated with differentiation of ciliated cells with expression of ß-tubulin (Blatt et al., 1999;

Tichelaar et al., 1999).

In contrast to the extensive studies of transcriptional regulation of cell differentiation of pulmonary non-neuroendocrine cells (non-PNEC) such as Clara cells and alveolar type 2 cells, the mechanisms of transcriptional regulation involved in PNEC development have not been clarified until recently (Borges et al., 1997; Ito et al., 2000). PNEC are a relatively minor cell population distributed throughout the airway epithelium from the lobar bronchus to the alveolar duct. They occur both as solitary cells and clusters, which are called neuroepithelial bodies when they are innervated (Lauweryns and Peuskens, 1972). PNEC seem to function as oxygen sensors in adults (Youngson et al., 1993). The embryonic origin of PNEC has not been determined, but the endodermal origin of these cells is generally accepted as PNEC occur in immature fetal epithelium in vitro (Emura et al., 1994; Ito et al., 1997) and as neuroendocrine carcinoma seems to be derived from normal epithelium (Sidhu, 1979). PNEC are considered to belong to the diffuse neuroendocrine system, as this cell type contains endocrine features and neuron-specific phenotypes (Ito, 1999). The term "diffuse neuroendocrine system" has been used on the basis of the common characteristics of the endocrine-like cells and neurons (Pearse and Takor Takor, 1979). The cells in the diffuse neuroendocrine system, which include pancreatic islet, gastrointestinal and respiratory neuroendocrine cells, thyroid C cells, adrenal medulla cells and pituitary cells, share common phenotypes with neuronal cells. The cells comprising the diffuse neuroendocrine system seem to require similar transcription factors to express common neuroendocrine proteins during the differentiation process, although a number of different transcription factors have been reported to be involved in differentiation of the cells (Table 2).

**Table 1.** Expression of transcription factors for differentiation of various cell types of the lung.

| LUNG CELL TYPE | TRANCRIPTION FACTOR |
|---|---|
| Clara cell | TTF-1, HNF-3, GATA-6 |
| Ciliated cell | HFH-4 |
| Neuroendocrine cell | Mash1 |
| Type 2 alveolar cell | TTF-1, HNF-3, GATA-6 |

TTF-1: thyroid transcription factor 1; HNF-3: hepatocyte nuclear factor 3; HFH-4: HNF-3/forkhead homologue; Mash1: mammalian achaete-scute complex homologue 1

Basic helix-loop-helix (bHLH) genes control cell fate determination in various tissues, and are categorized into two distinct groups of genetically defined bHLH activator and repressor genes (Jan and Jan, 1994; Kageyama and Nakanishi, 1997). In mammals, bHLH genes such as mammalian achaete-scute complex homologue-1 (Mash1), neurogenin and mammalian atonal homologue (Math)-1 are expressed in neural precursor cells, and they upregulate late-expressing bHLH genes such as NeuroD and Math2 to direct terminal differentiation (Ma et al., 1996; Cau et al., 1997). On the other hand, Hes1, one of the hairy and Enhancer of split homologues, represses neuronal differentiation by suppression of proneural bHLH factors. The suppressive mechanisms of Hes1 on proneural bHLH factors are suggested to involve two pathways: suppressing the formation of Mash1/E2A complex through protein-protein interaction and by repressing Mash1 transcription (Sasai et al., 1992; Chen et al., 1997a,b). The balance between these two antagonistic bHLH factors is important for the timing of differentiation and for generation of the correct number of neurons (Kageyama and Nakanishi, 1997). Repressive bHLH factors such as Hes1 are regulated by the Notch pathway (Jarriault et al., 1995; Hsieh et al., 1997; Schroeter et al., 1998). Notch transduces intercellular signals controlling the cell differentiation fate (Kimble and Simpson, 1997; Greenwald, 1998; Weinmaster, 1998; Artavanis-Tsakonas et al., 1999). In Drosophila, when Notch is activated by its ligand, Delta or Serrate,

**Table 2.** Transcriptional regulation of differentiation of diffuse neuroendocrine system.

| TRANSCRIPTION FACTOR | MOTIF/DOMAIN | ORGAN | SPECIFIC CELL TYPE | AUTHORS (YEAR) |
|---|---|---|---|---|
| Pit1/GHF-1 | POU | pituitary | lactotrope,somatotrope, thyrotrope | Bodner et al., 1988; Ingraham et al., 1988 |
| Lhx3/P-Lim | homeodomain | pituitary | lactotrope,somatotrope, thyrotrope, gonadotrope | Sheng et al., 1996 |
| Ptx1/P-OTX | homeodomain | pituitary | corticotrope | Lamonerie et al., 1996; Szeto et al., 1996 |
| Mash1 | bHLH | lung<br>thyroid<br>adrenal | PNEC<br>calcitonin cell<br>medulla, sympathetic cells | Borges et al., 1997; Ito et al., 2000<br>Lanigan et al., 1998<br>Ernsberger et al., 1995 |
| NeuroD/BETA2 | bHLH | pancreas<br>intestine | insulin cell<br>secretin cell and cholecystokinin cell | Naya et al., 1997 |
| GATA-6 | zinc finger | stomach | neuroendocrine cell | Dimaline et al., 1997 |
| Nkx6.1 | homeodomain | pancreas<br>stomach | insulin cell<br>gastrin cell and serotonin cell | Rudnick et al., 1994<br>Øster et al., 1998 |
| Pax6 | homeodomain | pancreas | glucagon cell | St.-Onge et al., 1997 |

Suppressor of Hairless (Su[H]) translocates to the nucleus and activates Enhancer and split gene expression, which antagonizes achaete scute complex and inhibits neural differentiation.

In this review article, we describe how cell differentiation to become neuroendocrine or non-neuroendocrine is regulated by bHLH factors, as revealed by experiments using gene-deficient mice for a positive proneural bHLH factor, Mash1 and a repressive bHLH factor, Hes1. Moreover, in human lung neoplasms, these factors are expressed, and could play roles in cell differentiation of the cancer cells, although the mechanisms underlying tumor cell differentiation seem more complicated than those of normal fetal mouse lung.

## Immunohistochemical study of expression of proneural bHLH factors in fetal mouse lung

PNEC share similar patterns of protein expression with neural cells and the neuroendocrine cells in other organs. These common expression patterns are seen in association with cell surface specialization, signal transduction systems, cytoplasmic components, secretory pathways, cytoskeleton, and transcription factors (Ito, 1999). Although similar expression phenotype and ultrastructural features are seen in PNEC from various animal species, the secretory products are different depending on the species (Ito, 1999). In mammalian lungs, PNEC occur as solitary cells or clustered cell nests. In human lungs, they are often present as solitary cells, but in rodents, clustered PNEC are more often encountered. In normal fetal mouse lungs, PNEC appear in the lobar bronchus by gestational day 14.5. They proliferate in a centrifugal pattern, and some are innervated late in the fetal period and in adult lungs (Ito et al., 1999a,b). The expression pattern seen in vivo is similarly observed in explant cultures of fetal lungs (Ito et al., 1997).

In the developing fetal lungs of mice, Mash1-positive cells are detected in the lobar bronchus and distributing bronchiole as early as gestational day 14. They form clusters in the proximal lobar bronchus, and solitary Mash1-positive cells are often seen in the peripheral bronchus. In the mouse lung of gestational day 14, more Mash1-positive cells are seen in the peripheral bronchus than calcitonin gene-related peptide (CGRP)-, PGP9.5- or α subunit of GTP-binding protein Go (Goα)-positive PNEC, but thereafter in the late fetal period and in the adult, eventually all cells with Mash1-


**Fig. 1.** Serial sections of normal fetal mouse lung (gestational day 18) with fluorescent immunostaining for Mash1 (left), Goα (center) and Hes1 (right). A Goα-positive neuroendocrine cell cluster (arrow) possesses Mash1-positive nuclei, and cells surrounding the cluster show positive nuclear staining for Hes1. x 700


**Fig. 2.** Serial sections of normal fetal mouse lung (gestational day 18) with fluorescent immunostaining for Notch1 (left), Mash1 (center) and Notch3 (right). Mash1-negative non-neuroendocrine cells surrounding a Mash1-positive neuroendocrine cell cluster (asterisk) show positive staining for Notch1 and Notch3. x 800

positive nuclei are found to have the immunohistochemical properties of PNEC (Fig. 1). This observation suggests that precursor cells for PNEC, which do not possess a general neuroendocrine phenotype, might show positive staining for Mash1 (Ito et al., 2000). On the other hand, early in lung development, Hes1 immunostaining seems to be localized in the airway epithelial cytoplasm, and shows discrete nuclear staining in the late gestational period and in adults (Fig. 1). As Northern blotting analysis reveals that Hes1 mRNA is expressed early in fetal lung development (Ito et al., 2000), the cytoplasmic staining of Hes1 seen in the fetal lungs of the early developmental stage may suggest that Hes1 protein has been produced but is functionally immature. An immunohistochemical study revealed that cells positively stained for Notch1 and Notch3 immunostainings are non-PNEC in late fetal and adult lungs (Fig. 2). Notch4 is detected in the lungs and is reported to be localized mainly in alveolar endothelial cells (Uyttendaele et al., 1996).

## PNEC in the lungs from gene-deficient mice for Mash1 gene

In *Mash1* homozygous null mice, differentiation of autonomic neurons, olfactory and retinal epithelia is defective, and neonates without the gene die soon after birth (Guillemot et al., 1993; Sommer et al., 1995; Tomita et al., 1996a). Grossly, the lungs from the gene-targeted mice appear normally developed. As first reported by Borges et al. (1997), in *Mash1* homozygous null mice, the histological lung architecture seems normal except for the absence of PNEC, and that intrapulmonary nerve tissues are formed in the animals (Fig. 3). The study by Borges et al. (1997) indicated that Mash1 is essential for development of PNEC in the fetal mouse lung. It will next be interesting to know how Mash1 gene expression is regulated by extrinsic or intrinsic factors during fetal lung development, as lung development is strongly influenced by growth factors and the extracellular matrix (Keyzer and Post, 1999). We have examined the effects of various growth factors on PNEC formation in fetal lung explants of mice, but no remarkable increase of PNEC has been induced by the growth factors examined so far (Ito, 1999). As 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), it will be interesting to study the possible roles of BMPs in PNEC development. Between the Mash1 gene and the genes for neuroendocrine phenotypes, a cascade of bHLH factors are suspected to function in the process of neurogenesis (Kageyama and Nakanishi, 1997). There has not yet been any study to clarify the signalling pathway downstream of Mash1 gene in PNEC development, but histochemical studies of candidate molecules, molecular analysis of the lungs of *Mash1* homozygous null mice or gene-targeting studies could answer these questions in the near future.

Regarding the functional aspects of PNEC in the fetal lungs, the *Mash1* mutant mice have afforded interesting findings. As a relatively large number of PNEC are present in the late fetal period in comparison with the number in adult life, PNEC have been considered important in stimulating the growth and development of fetal lungs through their secretion of growth factors (Sunday et al., 1990; Hoyt et al., 1991). It has been reported that exogenous bombesin induces a modest increase of branching in fetal mouse lung explants, and PNEC may play a role in branching morphogenesis (Aguayo et al., 1994). However, even though no PNEC are detected in the fetal lungs in the mice lacking *Mash1*, no morphological abnormalities of lung size or of morphogenesis of airways or alveoli are found (Borges et al., 1997; Ito et al., 2000). These observations suggest that PNEC might not be critical in mouse lung development. However, the mice lacking *Mash1* die with cyanosis and fasting in one day (Guillemot et al., 1993). The pathogenesis of respiratory insufficiency seen in the *Mash1* deficient neonates has not been studied, but considering that PNEC may work as $O_2$-sensing receptors (Youngson et al., 1993), it can

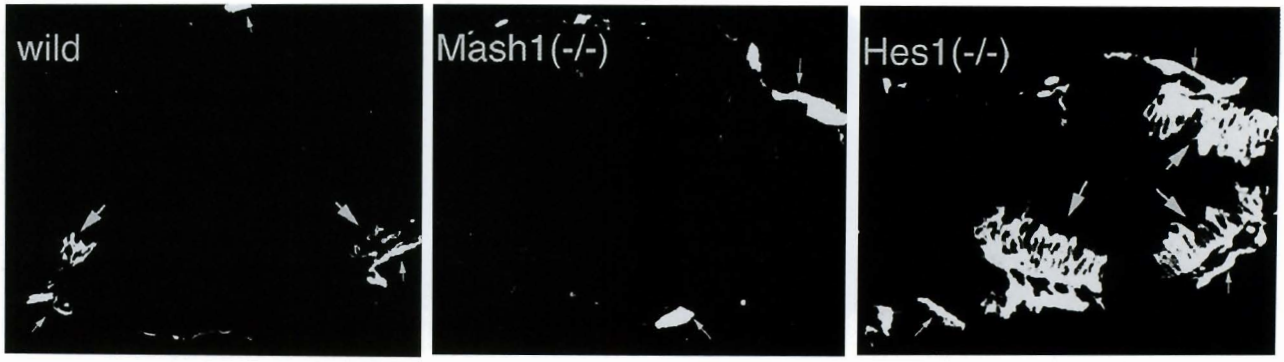


**Fig. 3.** Goα fluorescent immunostaining of fetal lungs from wild-type (left), *Mash1* deficient (center) and *Hes1* deficient (right) mice. In the lung from a *Mash1* deficient mouse, no PNEC are detected, although nerve fibers are formed (center). In contrast, in the lung from a *Hes1* deficient mouse, a marked increase of PNEC is seen (right). PNEC clusters (large arrows), nerve fibers (small arrows). x 200

be speculated that the absence of PNEC in the gene-deficient animals might induce respiratory failure due to the defective $O_2$-sensors, or that some undetectable immaturity of the lung owing to the absence of PNEC might cause respiratory failure.

### PNEC in the lungs from gene-deficient mice for Hes1 gene

In *Hes1* homozygous null mice, differentiation of neuronal precursors is accelerated, resulting in neural tube defects and eye anomalies (Ishibashi et al., 1995; Tomita et al., 1996b). In a recent study of the gene-deficient mice, the pancreas showed severe hypoplasia caused by depletion of pancreatic epithelial cell precursors due to accelerated differentiation of post-mitotic endocrine cells, and precocious and excessive differentiation of multiple endocrine cell types in the gastrointestinal tract was also observed (Jensen et al., 2000). Most mice lacking *Hes1* die before birth, and neonates cannot survive long. Although the size of the body and lungs of the *Hes1* homozygous null fetal mice are much smaller than those of wild-type or heterozygous mice, histologically the lung architecture appears normal except for many airway nodular lesions, which consist of PNEC. In the *Hes1* deficient mice, a few PNEC are precociously seen in the mice of gestational day 13, and thereafter, far more PNEC, with accompanying enhanced Mash1 expression, occur in the *Hes1* homozygous null mice than in wild-type or heterozygous mice (Fig. 3). Other epithelial cell types such as Clara cells seem to develop normally, but the incidence of non-PNEC in the airway in the gene-deficient mice is lower in proportion to the increased number of PNEC (Ito et al., 2000). It is suspected that the increase of PNEC in the *Hes1* homozygous null mice is due to the loss of Mash1 inactivation by Hes1. These observations using mice lacking the genes of active and repressive bHLH factors indicate that determination of cell differentiation in the fetal airway epithelium is regulated by a network of bHLH factors (Fig. 4) as seen in neurogenesis and myogenesis. As not all airway epithelial cells differentiate into PNEC, repressive bHLH factors other than Hes1 are suggested to cooperate to induce the undifferentiated cells to differentiate into non-PNEC. As Hes5 has been reported to operate with Hes1 in neurogeneis (Ohtsuka et al., 1999), we examined expression of Hes5 mRNA in the mouse lung, but Hes5 expression was not detected (Ito et al., 2000). Other Hes or Id family members may regulate the inhibition of neuroendocrine differentiation of the fetal airway epithelium.

In vertebrates, the intracellular domain of Notch interacts with RBP-Jκ (Su[H] homologue), and activates the Hes1 promoter (Jarriault et al., 1995; Honjo, 1996). As Notch1 is immunohistochemically detected in non-PNEC and its expression is downregulated in the lungs from *Hes1* deficient mice (Ito et al., 2000), an autoregulatory pathway to maintain the cellular differentiation of non-PNEC could work through the Notch1-Hes1 signalling system. In mice lacking RBP-Jκ or Delta-like-1, reduced Notch signalling leads to increased proneural genes and to promotion of pancreatic endocrine cells (Apelqvist et al., 1999). Thus, a defect in Notch signalling can induce accelerated neuroendocrine differentiation in the pancreas, as seen in *Hes1*-gene-deficient mice (Jensen et al., 2000). Lung abnormalities of mice lacking the genes involved in Notch signalling such as Notch1, RBP-Jκ or Delta-like-1 have not been reported as the mice die during the intrauterine period before development of the lung (de la Pompa et al., 1997; Hrabe de Angelis et al., 1997), but the mechanism of the Notch pathway involvement in lung epithelial cell differentiation should be studied in the near future.

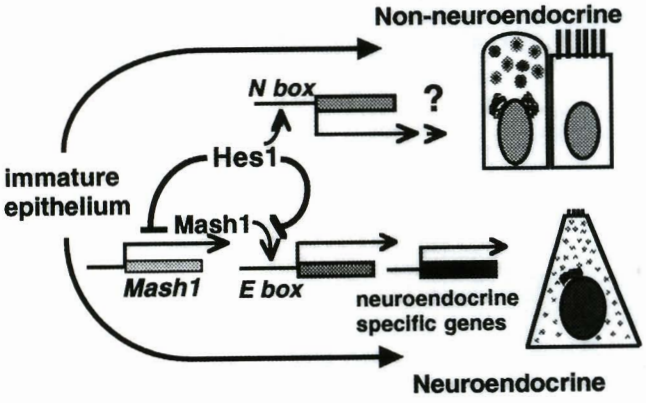


**Fig. 4.** Schematic diagram of cell fate determination of fetal airway epithelium regulated by active and repressive bHLH factors. Neuroendocrine phenotypes are dependent on Mash1. In non-neuroendocrine cells, Hes1 represses Mash1 activation and maintains the cells as non-neuroendocrine. Moreover, Notch1 activates Hes1, and Hes1 probably indirectly regulates Notch1 expression.

### Human lung carcinomas and proneuronal bHLH

The human homologue of Mash1, HASH1, has been reported to be expressed in some human neuroendocrine neoplasmas, including small cell carcinoma of the lung, neuroblastoma, pheochromocytoma, thyroid medullary carcinoma and brain primitive neuroectodermal tumors (Ball et al., 1993; Chen et al., 1996, 1997a,b; Borges et al., 1997; Rostomily et al., 1997; Gestblom et al., 1999;

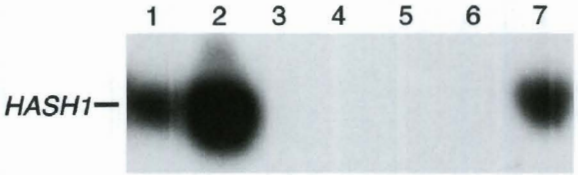


**Fig. 5.** Northern blotting analysis of HASH1 expression in human cell lines: lanes 1 and 2 (small cell carcinoma of the lung), lanes 3-5 (non-small cell carcinoma of the lung), lane 6 (human bronchial epithelial cell) and lane 7 (neuroblastoma).

Soderholm et al., 1999). In cell lines from human lung carcinomas, HASH1 mRNA is exclusively expressed in small cell carcinoma cell lines, but not in non-small cell carcinoma cell lines (Ball et al., 1993; Borges et al., 1997; Soderholm et al., 1999) (Fig. 5). Tissue-in situ hybridization for HASH1 mRNA reveals positive staining for small cell carcinoma and carcinoid of the lung (Fig. 6). In neuroblastoma cell lines, HASH1 expression is not correlated with induction of differentiation, suggesting that HASH1 expression does not indicate the degree of neuroendocrine differentiation (Soderholm et al., 1999). However, in small cell carcinoma cell lines, an antisense oligonucleotide against HASH1 represses neuroendocrine differentiation, and in human lung carcinomas, HASH1 is crucial for neuroendocrine differentiation (Borges et al., 1997). The repressive bHLH factor Hes1 has not been extensively studied in human neoplasms. According to a Western blotting analysis by Chen et al. (1997b), HASH1-positive cell lines of human lung carcinoma, most of which are small cell carcinoma, possess only trace amounts of Hes1 protein. On the other hand, HASH1-negative cell lines, which are derived from non-small cell carcinomas, express Hes1 protein (Chen et al., 1997b). Furthermore, they showed that HASH1 expression is reduced by transfection of Hes1 in small cell carcinoma cell lines. These studies suggest that cell differentiation in human lung carcinomas is determined by a network of bHLH factors as seen in fetal lung epithelial cells, although it has not been completely clarified whether an intact network is preserved in lung carcinomas. As mentioned above, Hes family members are regulated by Notch signalling, and as a result, Notch signalling can affect neuroendocrine cell differentiation via Hes1 activation. Although in human T cell lymphoma/leukemia, the NOTCH1 gene is known to be altered (Ellisen et al., 1991), changes in expression or mutation of Notch receptors and ligands have not been extensively studied in human lung carcinomas.

## Conclusion

We suppose that studies of fetal lung development will afford many fundamental findings to advance our understanding of cellular and molecular aspects of human lung carcinoma, as fetal lung cells and lung carcinoma cells share common biological characteristics including active cell proliferation and immaturity in differentiation (Ito et al., 1999a). We have described here the significance of a network of bHLH factors in cell fate determination in fetal lung epithelium, and a similar mechanism seems to operate in the determination of the

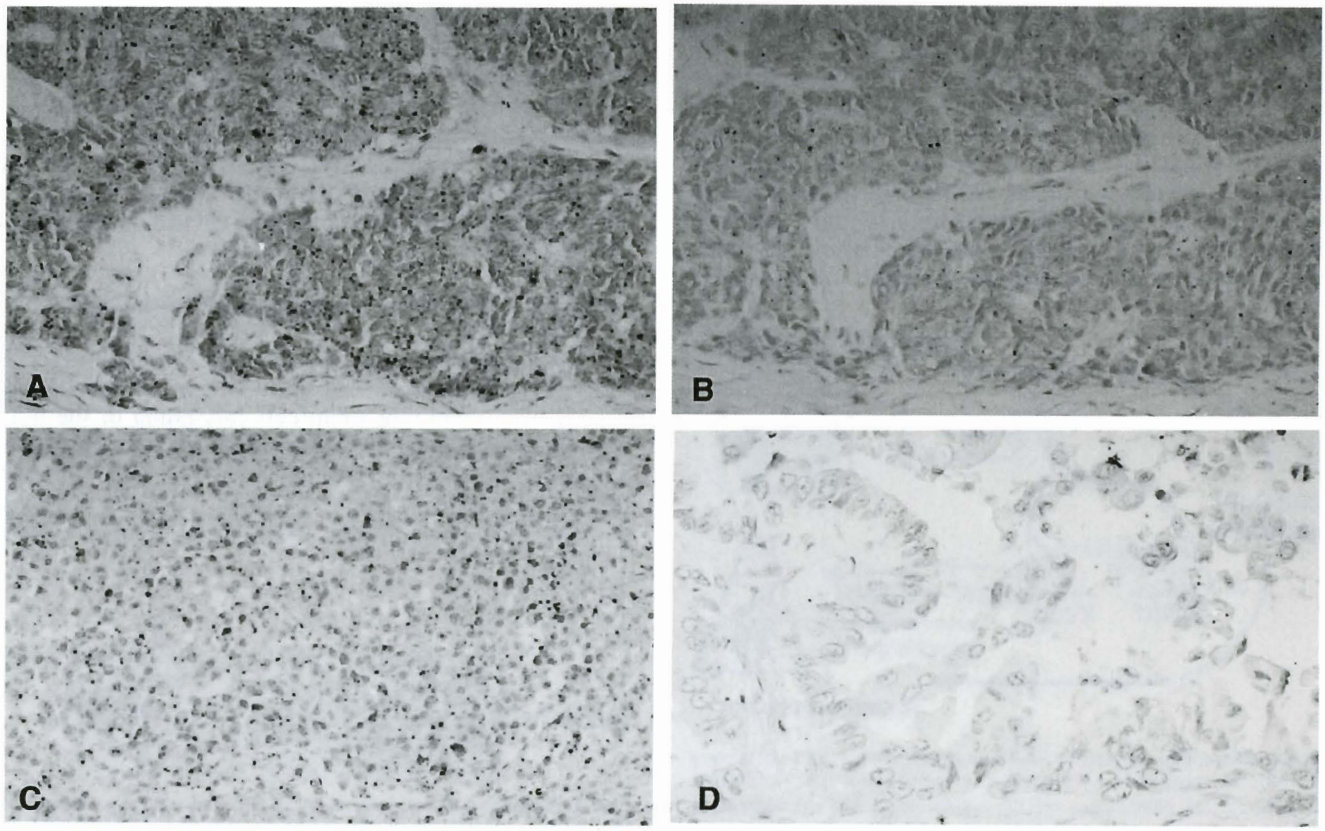


**Fig. 6.** In situ hybridization for HASH1 in small cell carcinoma **(A)**, carcinoid **(C)** and adenocarcinoma **(D)** of the lung. **B.** Control section of small cell carcinoma with sense probe. Counterstained with hematoxylin. x 250

cellular differentiation of human lung carcinomas. In spite of relatively simple mechanisms of neuroendocrine differentiation in the fetal mouse lung epithelial system, the mechanisms underlying the determination of human lung cancer cell differentiation seem more complicated, as lung carcinomas have numerous gene mutations, and as the histological features vary widely depending on the cases (Gazdar and Carbone, 1994). It is expected that further studies of the molecular mechanisms of mouse lung epithelial differentiation will give insights into the mechanisms of determination of differentiation of human lung carcinomas, and vice versa, studies of human lung carcinomas will provide valuable information about the mechanisms of normal differentiation of lung epithelial cells.

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