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Functional facets of the pulmonary neuroendocrine system

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Pulmonary neuroendocrine cells (PNECs) have been around for 60 years in the scientific literature, although phylogenetically they are ancient. Their traditionally ascribed functions include chemoreception and regulation of lung maturation and growth. There is recent evidence that neuroendocrine (NE) differentiation in the lung is regulated by genes and pathways that are conserved in the development of the nervous system from *Drosophila* to humans (such as *achaete-scute homolog-1*), or implicated in the carcinogenesis of the nervous or NE system (such as the retinoblastoma tumor suppressor gene). In addition, complex neural networks are in place to regulate chemosensory and other functions. Even solitary PNECs appear to be innervated. For the first time ever, we have mouse models for lung NE carcinomas, including the most common and virulent small cell lung carcinoma. Moreover, PNECs may be important for inflammatory responses, and pivotal for lung stem cell niches. These discoveries signify an exciting new era for PNECs and are likely to have therapeutic and diagnostic applications.
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Pulmonary neuroendocrine cells (PNECs) are part of the diffuse neuroendocrine system (DNES) distributed throughout the body.[1] They first appeared in the scientific literature in the 1940s, when Feyrter and Fröhlich[2–4] discovered *clear cells* (helle Zelle) in the bronchial epithelium due to their lucid cytoplasm in hematoxylin–eosin-stained sections. The PNEC system (solitary PNECs and neuroepithelial bodies or NEBs) consists of a distinct population of airway epithelial cells displaying endocrine and paracrine secretory mechanisms and associated with nerve fibers. Over the years, many names and functions have been assigned to them.[5,6] By 1993 a consensus,[7] a possible dual role, for these cells had emerged: (1) during early stages of lung organogenesis, PNECs acting via their amine and peptide products could function as local modulators of lung growth and differentiation; and (2) later in fetal life and postnatally, PNECs and particularly innervated NEBs could play a role as airway chemoreceptors. Consequently, many articles have focused on their role during embryonal and neonatal periods.[6,8–14] Their contribution to adult lung is less clear. It is likely that PNECs are pluripotent and the biological or physiologic significance may be context- and time-dependent.

The purpose of this article is to briefly review recent advances in PNEC research, and the functions of these cells in processes ranging from lung development and respiratory physiology to repair, disease and carcinogenesis. Not surprisingly, many of the latest discoveries reflect methodological and technological advances in the biomedical field.[15–17]

The role of PNECs in lung development

Early phases of lung development are dominated by the growth and differentiation of the conducting airways, where PNECs are the first specialized epithelial cell type to appear. The lung primordium starts as an outgrowth of endoderm in the ventral part of the embryonal pharynx. The lung bud epithelium grows into adjacent mesenchyme and starts branching to form the future bronchial tree. The various stages are divided into the embryonic, pseudoglandular, canalicular, saccular and alveolar/microvascular maturation periods.[18] Contrary to the conducting airways, the alveolar compartment develops late in fetal life and continues to further mature postnatally.[18] The early appearance of

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PNECs is a key to their proposed role in lung development.

In humans, ultrastructurally distinct primitive PNECs (pre-NE cells), which contain serotonin and neuron-specific enolase (NSE), can be detected in the beginning of the pseudoglandular period at 8 weeks of gestation. Solitary and clustered PNECs containing bombesin, the major neuropeptide in human lungs, appear at about 10 weeks of gestation.[5] In animals with shorter gestation periods, developmental events are more closely arranged: pre-NE clear cells can first be seen in rabbits (gestation 32 days) on prenatal day 18, in rats (gestation 22 days) on prenatal day 15, and in hamsters (gestation 16 days) in the trachea on prenatal day 12 and in large bronchi on day 13.[19] The maturation and differentiation of the airway epithelium, including the PNECs, follows a centrifugal pattern, which starts in the trachea and proximal airways and progresses distally.

As the distal segments of developing airways (future terminal and respiratory bronchioles) elongate, they are referred as canaliculi, hence the term canalicular period, which takes place at 17–24 weeks of gestation in humans. The order of appeareance of differentiated epithelial cells in this compartment is similar to that observed in proximal airways: PNECs differentiate first, followed by ciliated and secretory (Clara) cells. In parallel with the increasing number of peripheral airways, the number of PNECs also increases. In developing bronchioles, small NEBs composed of 3–5 bombesin and serotonin-immunoreactive cells appear at airway branching points. Rare nerve endings have been demonstrated in contact with NEBs already in human fetal lung.[20]

Recent studies using transgenic mice have demonstrated that there is an early spatial restriction of progenitor cells forming the proximal conducting airways, compared with the peripheral lung. More specifically, PNECs were shown to be derived from a different group of precursors than those forming the peripheral subset of respiratory epithelial cells.[21] This evidence came from experiments where subsets of lung precursor cells were permanently labeled at defined times during development by using Cre recombinase to activate two floxed markers under control of doxycycline-dependent surfactant protein C (SP-C) promoter. Distinct cell lineages that contributed to the peripheral lung, conducting airways, NEBs and tracheal-bronchial glands became established before formation of the definitive lung bud at embryonic day (E) 9.0–9.5. NEBs and tracheal-bronchial glands were never labeled, distinguishing their lineage from the peripheral subset of lung epithelial cells. While this report did not specifically comment on solitary NE cells (or how many NEBs were included), the findings are consistent with the previous studies identifying PNECs early, in the beginning of the pseudoglandular period. Unfortunately, the authors failed to disclose whether they used other NE markers than CGRP to confirm their observations, which makes it difficult to evaluate how comprehensive this study is.

Another piece of evidence that PNECs are a vital part of the proximal airway cell epithelium comes from studies on the role of *Sonic hedgehog* (SHH) in lung development. SHH is a mammalian ortholog of the *Drosophila* gene *Hedgehog*. Cellular events mediated by SHH regulate the tissue patterning and organization that are critical for organogenesis.[22–24] Examination of SHH deficient mice revealed a general maintenance of proximal airway cell patterning, including the presence of PNECs, despite severe branching abnormalities and peripheral lung hypoplasia.[25] The study was prompted by observations that such human congenital pulmonary malformations as lung hypoplasia, tracheoesophageal fistula, and esophageal atresia may be attributed to defective SHH signaling.[26–29] Taken together, these studies illustrate how molecular technology may be used to identify the timing of PNEC fate restriction and lineage relationships during morphogenesis.[17]

In the immature, developing lung markers of NE differentiation are co-expressed with the epithelial markers before they are restricted to specific PNECs, suggesting a common cellular origin for airway cells. Wuenschell *et al*[30] showed that during mouse embryonic days 13–15 of the pseudoglandular period, the NE marker CGRP, Clara cell 10 kDa protein (CC10), and surfactant-associated protein A (SP-A) immunostaining was colocalized in all epithelial cells of the distal airways. Only the later phase, composed of embryonic days 16–18 during the canalicular period, revealed the emergence of the differentiated cell types and distinct lineages. Moreover, the expression patterns were recapitulated in serumless organ culture of developing lungs, demonstrating that the information necessary to generate both phases of gene expression was present in the lung tissue by embryonic day 11. A comparable pattern of diffuse expression of the early NE marker PGP9.5 in airways was seen in human fetal lungs in the second trimester during the pseudoglandular period.[31]

Incidentally, while PGP9.5 has been commonly used as a marker for PNECs and nerves in normal lungs for years, there is emerging evidence that PGP9.5 is also highly expressed in many non-NE lung cancer cell lines and primary tumors.[32,33] In these studies, the increased expression of PGP9.5 was specifically associated with lung cancer development, suggesting it might serve as a helpful marker for the detection of lung cancer. It is not known whether this could represent partial NE differentiation of non-small-cell lung carcinomas (NSCLCs). The postulated mechanism is that PGP9.5, a ubiquitin C-terminal hydrolase, may contribute to $p27^{Kip1}$ degradation by way of a Jun

binding protein, which may contribute to tumor growth.[34]

In addition to appearing early in the developing mammalian lung, PNECs are present in lower vertebrates, suggesting that they are phylogenetically an integral early part of the pulmonary development[35–38] (Figure 1). During the history of vertebrates, the PNEC system in the air-breathing organs has developed in several evolutionary lineages, which are phylogenetically independent from each other. As illustrated in Figures 1 and 2, the structure of NEBs in red-eared turtles closely resembles that of rabbits, rats, hamsters, and mice, including the close relationship with Clara-like cells.

Proposed roles for PNECs in fetal and newborn lung development include regulation of branching morphogenesis[39] as well as cellular growth and maturation. Evidence for this comes from multiple studies showing that NEBs emerge as foci of growth in the non-endocrine epithelium first in perihilar airways, and as the NEBs mature in more peripheral branches, they assume the same function at this location as well.[19] When fetal hamsters were exposed continuously to ^{3}H-thymidine during the formation of the bronchial tree, the cumulative labeling in autoradiographs revealed patterns of cell division centering around NEBs. The mechanism of action involves paracrine secretion of bioactive neuropeptides and growth factors synthesized in PNECs.

The effectors of mitogenic stimulation include CGRP, and gastrin-releasing peptide (GRP), the latter being the major bombesin-like peptide (BLP). CGRP is the major neuropeptide produced by PNECs and NEBs in rodents, whereas GRP is the major neuropeptide produced by PNECs and NEBs in humans and non-human primates.[40] Accordingly, CGRP has been shown to stimulate growth of cultured guinea-pig tracheal epithelial cells[41] and

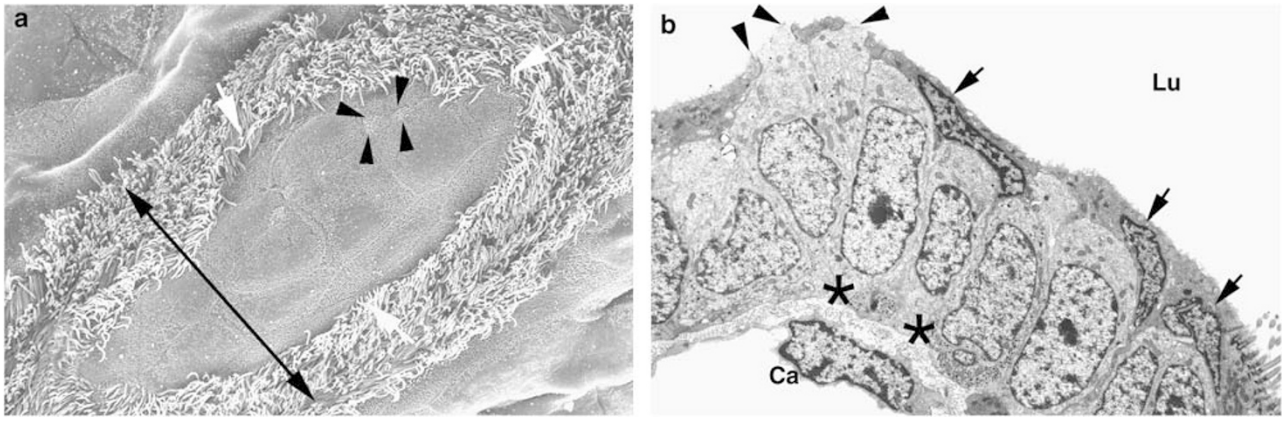

Figure 1 Phylogenetic preservation of NEBs. An NEB of red-eared turtle covered by Clara-like cells illustrated by two ultrastuctural techniques. (**a**) A scanning electron micrograph view from inside the lumen of an airway. An NEB (white arrows) is surrounded by ciliated cells. Contact with airway lumen is limited to few openings like the one indicated by arrowheads. Black double-headed arrow marks the plane of panel b. (**b**) Cross-section of the same NEB using transmission electron microscopy. Groups of dense-core granules appear basally (asterixes), close to an underlying capillary. Arrowheads indicate contact with lumen, arrows mark Clara-like non-NE cells. Lu = airway lumen; Ca = capillary. Panels reproduced and modified from Adriaensen and Scheuerman[207] with permission.

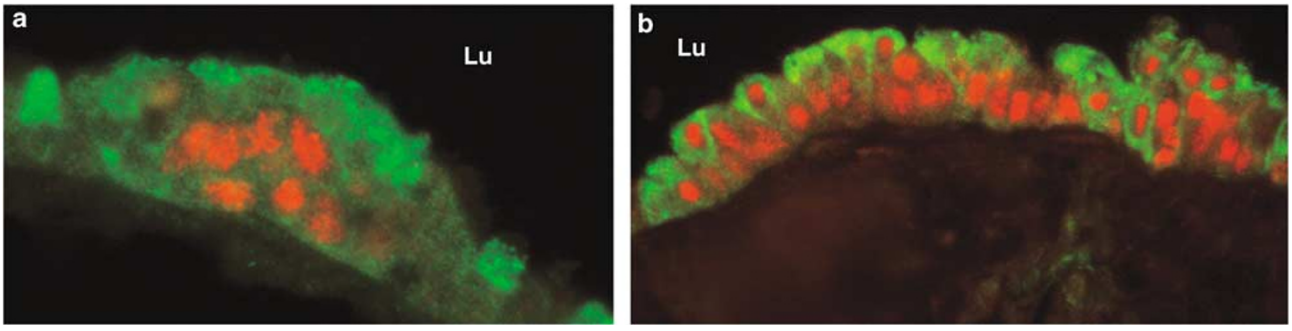

Figure 2 Close relationship of PNECs with airway epithelial cells demonstrated by double labeling using immunofluorescence. Red nuclear staining indicates ASH1 immunoreactivity, green cytoplasmic staining CC10, a major Clara cell protein. (**a**) An NEB in normal hamster lung is frequently covered by a 'cap' of Clara cells. (**b**) A linear PNEC hyperplasia following exposure of hamsters to tobacco-specific nitrosamine NNK for 6 weeks reveals co-localization ASH1 (red nuclei) and CC10 (green cytoplasm) in a number of cells suggesting a close relationship between these two cell types.

fetal rat lung.[42] Addition of bombesin has been shown to result in increased growth and maturation of lungs in mice *in utero*, and in human and murine organ cultures. Moreover, blocking by antibombesin monoclonal antibody MAb 2A11[43] inhibited over 50% of lung automaturation in serum-free organ cultures.[44,45] It is also intriguing that BLPs mediate lung injury in a hyperoxic baboon model, although in normal fetal baboon lung they, accompanied by PNECs, contribute to lung growth and maturation potentially by incorporating related novel receptors and peptides.[39,46] Despite all of these studies, it is not entirely clear whether cellular proliferation and maturation are the result of the specific action of the differentiated PNECs on the surrounding lung tissues, or whether they are part of the general response to the local cues that are also responsible for the differentiation of PNECs at these sites.

The ontogeny of PNECs

PNECs are part of the diffuse endocrine system (DES), characterized by NE and endocrine cells distributed throughout multiple organs in the body.[1] Other components of DES include cell populations in the hypothalamus and adenohypophysis, pineal gland, paraganglia, cell populations in the thymus, pancreatic islets, thyroid C cells, parathyroid glands, endocrine cells of the breast, gastrointestinal and genitourinary tracts, melanocytes and Merkel cells of the skin. It is currently thought that PNECs like their counterparts in the gastrointestinal tract are derived from multipotent epithelial progenitors as alluded before, and that all epithelial cells arise from a single stem cell.[47,48]

In order to define the putative progenitors for the PNEC lineage during lung development Pan *et al*[49] recently turned to the neuronal developmental marker FORSE-1, the forebrain-surface-embryonic monoclonal antibody. The FORSE-1 epitope serves as a marker of progenitor cells in the earliest forebrain region of the mammalian central nervous system. The epitope is related to the Lewis-X and stage-specific embryonic markers (SSEA), which have been extensively studied during embryogenesis, differentiation and neoplasia. In developing rabbit fetal lungs and corresponding cell cultures, it first labeled the primitive airway epithelium during the early stage at E16, then became restricted to PNECs/NEBs, and then decreased with increasing serotonin (5HT) labeling. The presence of few FORSE-1/5HT-positive cells in postnatal lung suggested the retention of progenitors. Co-labeling with antibodies to other SSEA antigens supports the embryonic phenotype of NE-programmed cells that ultimately lose SSEA antigens as NE maturation progresses towards term. The FORSE-1 epitope in the developing lung was associated with a large carrier protein potentially associate with cell adhesion and PNEC/NEB differentiation. The observation of FORSE-1 epitope expression in primitive undifferentiated epithelium before expression of 5HT supports the idea that the primitive undifferentiated pulmonary epithelium expresses a pluripotent phenotype before further commitment and differentiation into respective specialized cell types.[14]

Regulation of pulmonary NE differentiation

Regulation of NE differentiation in the lung is poorly understood. There is recent evidence that the same genes, which are involved in neuronal differentiation in *Drosophila* may regulate PNEC differentiation in mammals.[50,51] An important group of candidates is the family of helix–loop–helix proteins, most of which have *Drosophila* representatives including achaete-scute homologs (*Ascl1, Mash1*), E proteins, atonal, NeuroD, neurogenin, Id proteins, hairy and enhancer of split1 (*Hes1*), and Hes-related proteins. These are transcription factors that utilize the basic helix–loop–helix (bHLH) motif, combining DNA binding with dimerization functions. bHLH genes control cell differentiation in various tissues and are assigned into two distinct groups of genetically defined bHLH activator and repressor genes.[52]

'Proneural' genes such as Achaete-scute-homolog-1 (termed Mash1 in rodents, hASH1 in humans) promote neuronal cell commitment. ASH1 is important in early development of neural and NE progenitor cells in central and peripheral nervous systems. ASH1 is also selectively expressed in PNECs (Figure 2) as well as in a range of lung cancers with NE features.[53,54] Strikingly, newborn mice bearing a disruption of the ASH1 gene have no detectable PNECs[53] (Figure 3a). Otherwise, the lungs appear normal including the presence of CC10-expressing airway epithelial cells and SP-C expressing type II cells.[55] Several observations further support the specific role of ASH1 in PNEC differentiation. First, synaptophysin and CGRP-reactive autonomic neurons persisted in the lungs of mutant mice. Second, enteric neurons and NE cells in the gut and pancreatic islets from mutant mice were positive for multiple NE markers, including CGRP. However, mutant mice died in 12 h after birth with hypoventilation and severe olfactory and central nervous system abnormalities.[56] These results suggest that other cell types in ASH1 deficient mouse lungs were able to compensate for the lack of PNECs during lung development. However, it is conceivable that dysfunctional chemoreception in the lungs due to the lack of proper cells may contribute to the perinatal death of the animals.

To explore the potential of ASH1 to promote NE differentiation in non-NE cells, hASH1 was constitutively expressed in the airway epithelium under

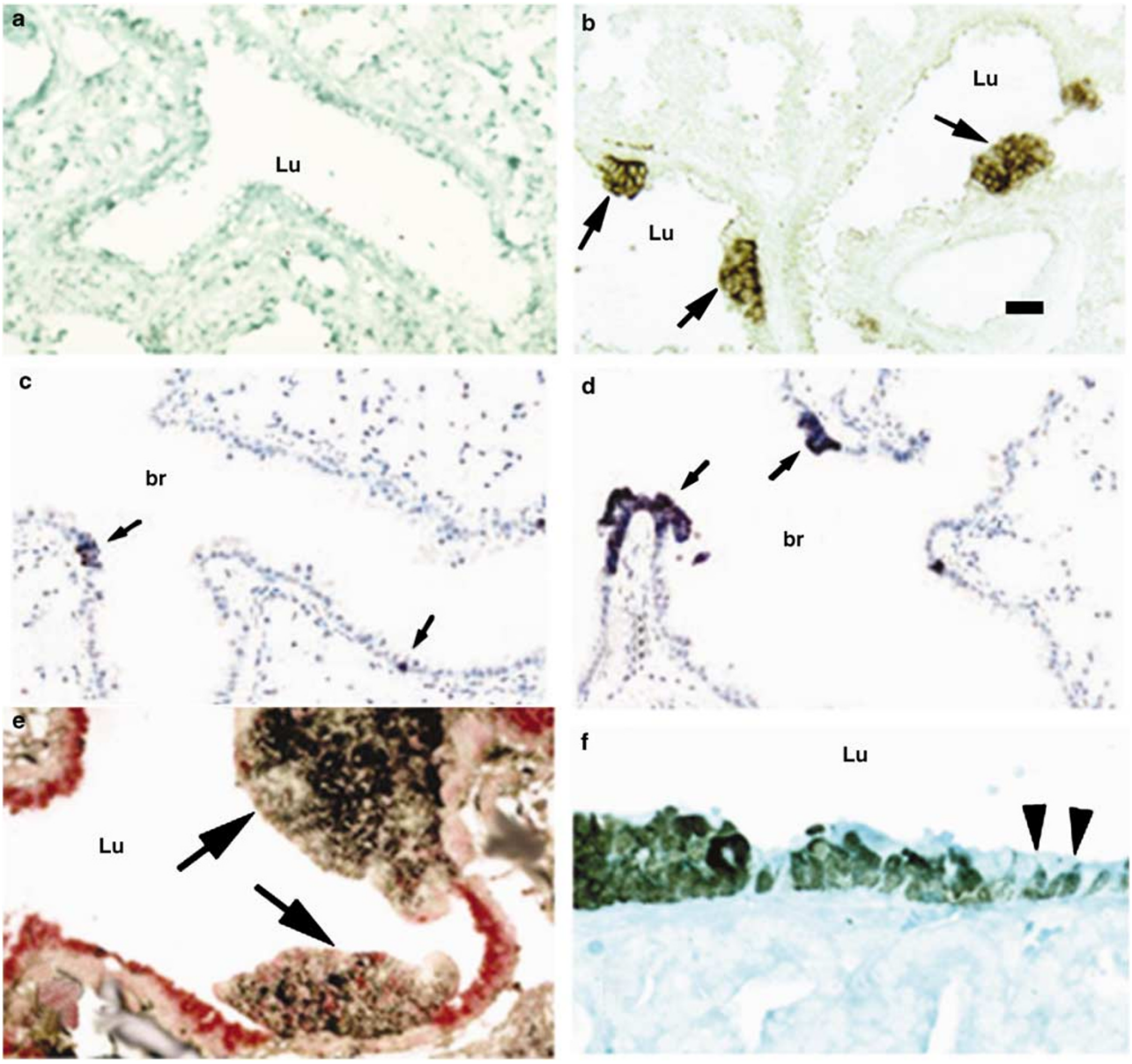

Figure 3 Molecular mechanisms of PNEC differentiation. (**a**) Deficiency of the proneural transcription factor ASH1 leads to the lack of neuroendocrine cells specifically in the lung. Immunohistochemical staining for CGRP reveals no positive cells in the lung of 1-day-old ASH1 knockout mouse. (**b**) Knocking out HES1, which is a repressor of ASH1, results in marked nodular hyperplasia of NEBs (arrows) in 1-day-old mice. Compare with normal lung in panel c. Immunohistochemical staining for CGRP, bar = 25 μm. (**c**) CGRP-containing NEBs in a wild-type control mouse (arrows). (**d**) Ablation of tumor suppressor gene Rb-1 is associated with papillary hyperplasia of NEB (arrows). (**e**) Conditional ablation of both Rb-1 and p53 in the lung yields even larger clusters of PNECs protruding to the airway lumen (arrows). Double staining for ASH1 (brown, nuclear immunoprecipitate in PNECs) and Clara cell-specific protein CC10 (red cytoplasmic immunoreactivity) in adjacent epithelial cells. (**f**) PNEC hyperplasia/dysplasia in the airways of a bi-transgenic SV40-hASH1 mouse is linear and more diffuse, involving also solitary cells (arrowheads). Immunohistochemical staining for synaptophysin. Lu = airway lumen; br = bronchiolus. Original mouse tissue in panel b as courtesy of Dr Takaaki Ito, Yokohama, Japan. Panels c and d reproduced from Wikenheiser-Brokamp[196] with permission.

the CC10 promoter, but failed to induce overt NE differentiation in the heterologous airway epithelium.[57] Taken together, these findings imply that ASH1 is necessary but not sufficient for PNEC differentiation. One interpretation of the results is that the role of PNECs is critically important for the homeostasis of lung, and their proper existence is guarded by multiple redundant mechanisms.

Typically, achaete-scute complex transcription factors induce neuronal fate commitment in undifferentiated ectodermal cells in *Drosophila* and, at the same time, lead to repression of neuronal fate in the adjoining cells via lateral inhibition through the Notch pathway. Notch belongs to a conserved family of transmembrane receptors that transduce intercellular signals controlling cell differentiation fate.

It is one of the 'neurogenic' genes required for proper segregation of neural and epidermal lineages. Complete loss of function of any of the 'neurogenic' genes such as *Notch, Delta, Serrate, Enhancer of split complex* and *Suppressor of Hairless* can lead to massive neuronal hyperplasia.[58,59] Activation of Notch leads to expression of *Hes1*, a major neuronal repressor gene, which antagonizes the ASH1 complex and inhibits neural differentiation. Consequently, in normal mammalian lung, ASH1 expression is associated mainly with PNEC differentiation, while Hes1 positive cells belong to non-NE cells, which express Notch1 and Notch3.[55]

In the lungs of HES1-deficient mice, the expression of Notch1, but not other types of Notch, is reduced and the expression of ASH1 increased. Fetal lungs also reveal enhanced expression of NeuroD, a late-expressing bHLH gene associated with terminal differentiation. Like hASH1-deficient animals, transgenic HES1 knockout mice suffer from severe neural abnormalities and die during gestation or just after birth.[60] Both body and lung sizes of the HES1 deficient mice are smaller than the wild-type mice. Morphologically, lungs reveal precocious PNECs on E13 and hyperplastic clusters of PNECs on E18 (Figure 3b). However, even without HES1, not all fetal airway epithelial cells differentiate into PNECs, and CC10-expressing cells remain abundant. This suggests that Hes1 has redundancy in its inhibitory activity of PNEC differentiation.

In *Drosophila*, achaete-scute complex proteins regulate Notch activation through expression of its ligands Delta or Serrate. Expression of the mammalian Notch ligand Delta-like 1 (Dll 1) in the developing lung shares the pattern of PNECs[61] and the transgenic knockout of ASH1 is associated with total loss of Dll 1.[55] This suggests that activation of the Dll 1 appears to be under the control of ASH1 also in mammals, while the significance of Dll 1 in PNEC differentiation and its interactions with Notch receptor-expressing non-NE cells remains to be determined. Moreover, it is not clear at the present time, what are the local cues that are responsible for turning ASH1 on in some epithelial cells and not others in the mammalian lung.

To further explore the role of Notch signaling, the expression of several related genes has been mapped in the developing mouse lung. Most genes are expressed in specific populations that may contribute to cell diversification.[61] It is notable that specifically altering the expression of *Notch-1* with antisense oligonucleotides in culture results in increased branching of embryonic lung buds. In addition, antisense to *Notch-1* or *Jagged-1* markedly increases numbers of PNECs, while only neural tissue was promoted by *Notch-3* in culture. Together, these studies demonstrate that players of 'neural lateral inhibition' pathway exist and are potentially functional in the lung.[62] Whether this is relevant for PNEC pathobiology still needs to be determined.[63]

Mediators of NE differentiation are dependent on cellular context. In addition to lung, ASH1 is also important for the development and maturation of the chromaffin cells in the adrenal medulla and parafollicular calcitonin-producing C-cells in the thyroid,[64,65] which were found to be affected in ASH1-deficient mice. In contrast, different bHLH transcription factors may be important for other cells of the DES, such as MATH1 in Merkel cells, the NE cells of the skin and their tumors[66,67] or in enteroendocrine cells of the small intestine[68] and for the sensory hair cells in inner ear.[69]

The complexities of the differentiation of DES is further illustrated by the protein tyrosine phosphatase (PTP)-σ-deficient mice.[70] PTP-σ is a transmembrane receptor PTP and a member of the LAR family. PTP-σ expression is tightly controlled and developmentally regulated within epithelial, neuronal, and neuroendocrine (NE) tissues and PTP-σ-deficient mice reveal NE dysplasias and defects in pituitary, pancreas and gut.[58,71] Remarkably, lungs in these animals appear normal, and, unlike in the ASH1 knockout mouse, the distribution of PNECs is unaffected.[70] This is consistent with the idea that NE cells in various organs are under different developmental regulation.

PNECs in stem cell niche

All pulmonary epithelial cells including PNECs and non-NE airway epithelial cells are likely to be derived from a single stem cell.[72] Until recently, stem cell research in the lung has progressed rather slowly due to the anatomical and functional complexities associated with numerous distinct cell types and slowly renewing epithelium. Stem cells can be defined as undifferentiated cells that maintain the capacity to (1) proliferate, (2) self-maintain the progenitor population, (3) produce a large number of differentiated daughter cells, (4) regenerate the tissue after injury, and (5) maintain flexibility in the use of these options or implementation of the spectrum of stemness.[73,74] Two levels of epithelial progenitors are involved in lung morphogenesis: multipotent undifferentiated cells (lung primordial cells) and pluripotent, region-specific (bronchial, bronchiolar and alveolar) cells.[75]

Stem cells are mostly slow-cycling and give rise to transient amplifying cells (TAC), which impact the majority of tissue renewal in the setting of injury. In the peripheral lung epithelium TAC consist of Clara cells and type II cells.[76] Both intrinsic characteristics and the microenvironment influence stem cell commitment to special lineages at their place of residence. Epithelial stem cells in many organs, including the dermis, intestine, and hair follicle are often confined to discretely localized niches that are protected from environmental insults.[77] These niches are important because they provide essential extrinsic clues, which control stem cell proliferation

and commitment into more differentiated progenitor cells and TAC. In the lung, PNECs are associated with stem cell niches in both the proximal and distal airways.[78]

Mouse trachea reveals two stem cell niches: gland ducts in the proximal compartment and select foci near the cartilage–intercartilage junction in the distal trachea.[79] They were identified by localization of label-retaining cells (LRCs) after injury. Prolonged metabolic labeling with BrdU will mark epithelial stem cells, which by virtue of their slow cycle time will retain the label for extended period. Because the basal rate of mitosis in healthy airways is exceedingly low, it has been necessary to injure the epithelium to cause cell proliferation. The experiments revealed a close relationship with PNECs in the distal trachea. However, in neither location were the LCRs PNECs.

One of the intrapulmonary stem cell niches includes NEBs located at airway bifurcations.[80–82] In an adult mouse model of airway injury and repair using naphthalene-ablation of Clara cells, the epithelial regeneration occurs preferentially at airway branch points and is accompanied by NEB hyperplasia.[83,84] Two populations of LRCs and candidate stem cells were identified inside the NEBs: CGRP-expressing cells and a pollutant resistant subpopulation of CC10-expressing cells or variant CC10-containing cells (vCEs). To further investigate contributions made by CC10 and CGRP-expressing cells to epithelial renewal, CC10-expressing cells were ablated through acute administration of ganciclovir to transgenic mice expressing herpes simplex virus thymidine kinase under the control of CC10 promoter. CGRP-containing PNECs proliferated after depletion of CC10-expressing cells, yet were unable to repopulate the entire airway.[82] These data suggest that the NEB microenvironment is critical for the maintenance of a reservoir for pollutant-resistant stem and progenitor cells that respond to depletion of an abundant airway progenitor cell such as Clara cell. Resistance to destruction by environmental agents is an internal characteristic of stem cells in many organs. In this case, it manifests as the lack of detectable cytochrome *P*450 2F2 isozyme (CYP2F2) protein in the subpopulation of Clara cells, as CYP2F2 is required for the metabolism of naphthalene to its cytotoxic effector. Another stem cell niche is at the bronchioloalveolar junction, but PNECs may play a diminished role at this location.[85,86] The significance of these studies is two-fold. They have provided morphological evidence for the existence of airway stem cells and identified a novel role for NEBs as a stem cell niche. It remains to be seen whether NEBs actually act as producers or receivers of the stem cells. In addition, stem cell research on NEB niches may provide important clues for NE carcinogenesis and specifically, for the development of small cell lung carcinoma (SCLC), which is potentially a cancer of stem cells.

External clues in NE and neuronal stem cell niches include Hedgehog (Hh) signaling, an embryonic pathway also implicated in lung development, as discussed earlier.[87,88] For instance, in cerebellar development, a SHH gradient established by Purkinje cells regulates expansion and proliferation of granule cell precursors.[89,90] Hh target genes include its receptor Patched (Ptch) and the transcriptional activator Gli1. In fetal lung, Ptch is colocalized in CGRP-containing NEBs and SHH is positive in adjacent cells suggesting that during normal development, PNEC precursors respond to SHH signal elaborated by adjacent airway epithelial cells.[23,91] In the adult mouse naphthalene model of epithelial cell injury and repair, the expression of both SHH ligand and Gli1 was markedly increased in the epithelial compartment 72 h after napththalene injury. By day 4, Gli1 was not observed in nascent airway NE cells expressing CGRP. These data show that acute airway cell regeneration results in widespread activation of airway intraepithelial Hh signaling, and is consistent with the role of Hh signaling in determining stem cells fates.[92]

Innervation and chemoreception function of PNECs

Since the 1940s, it has been evident that PNECs occur as solitary cells or clusters,[2,4] which Fröhlich referred to as corpuscles (Körperchen). He also suggested that they were innervated and functioned as chemoreceptors. In 1972, Lauweryns and Peuskens introduced the term neuroepithelial body.[93,94] By definition, NEBs are corpuscular, organoid structures composed of PNECs, which are heavily innervated and reach from the basement membrane to the airway lumen. Subsequent studies using electron microscopy, denervation and tracing suggested that NEBs were predominantly innervated by afferent sensory fibers of vagal origin with cell bodies residing in the nodose ganglion.[95–97] Other fibers were also present, which appeared to be efferent, less frequent and had a potential modulatory role. Current PNEC/NEB research focusing on innervation and chemoreception has been greatly empowered by clever combinations of increasingly sophisticated methods, including electrophysiology, ultrasensitive immunohistochemistry, confocal microscopy and transgenic animals.

Brouns and co-workers[98–100] have recently shown that rat NEBs are under dual sensory innervation, which may be subject to the modulating effect of a third system of nerve fibers, intrinsic in the lung. A vagal sensory component with origin in the nodose ganglion revealed calbinding D28K and $P2X_3$ purinoreceptor immunoreactive nerve fibers that were myelinated until they lost their myelin sheaths just before branching and protruding among the cells in NEBs. The branching intraepithelial $P2X_3$-containing fibres were exclusively associated with

quinacrine-stained NEBs, suggesting that ATP might act as a neurotransmitter/modulator through a receptor-mediated pathway.[101] This vagal sensory component was not affected by capsaicin nor expressed capsaicin receptors (vanilloid receptor subtype 1). A second sensory nerve fiber system consisted of thin unmyelinated, nonvagal substanceP/CGRP-immunoreactive nerves that had their origin in dorsal root ganglia and were depleted by systemic capsaicin treatment. Moreover, Brouins *et al*[100] were also able to show that part of the pulmonary NEBs selectively receive extensive nitrergic nerve terminals that originate from intrinsic neurons. All the NEBs receiving neuronal nitric oxide synthase (nNOS)-positive fibers, received CGRP-containing nerves, and half of them were contacted by purinergic fibers as well. The authors estimated that 40–50% of rat NEBs receive purinergic innervation, 56% CPRP-containing nerves and 9–10% nNOS-positive fibers. Based on these results, it is notable that six different combinations of nerves may innervate NEBs. This will allow for a great flexibility in neurally mediated communication and adaptation which, in turn, may prepare NEBS for a variety of potential challenges and functions associated with their physiology.

Not all NEBs seem to be innervated in adult rat, suggesting that they may serve yet another function.[99] There is a decreasing nerve density from proximal to more distal conducting airways, while there remains a frequent interchange of single nerve fibers across epithelial and subepithelial compartments without termination. On the other hand, the studies of Pan *et al*[102] on developing rabbit lung indicate that NEBs and solitary PNECs may be actually interconnected via fine submucosal nerve fibers. The authors were able to show that the density and complexity of NEB innervation increased with advancing gestation, reaching a plateau postnatally. The significance of the results is that the 'connection in parallel' may allow functional signal amplification from dispersed, smaller NEBs (Figure 4). Moreover, the study challenges a prevalent paradigm that solitary PNECs may not be

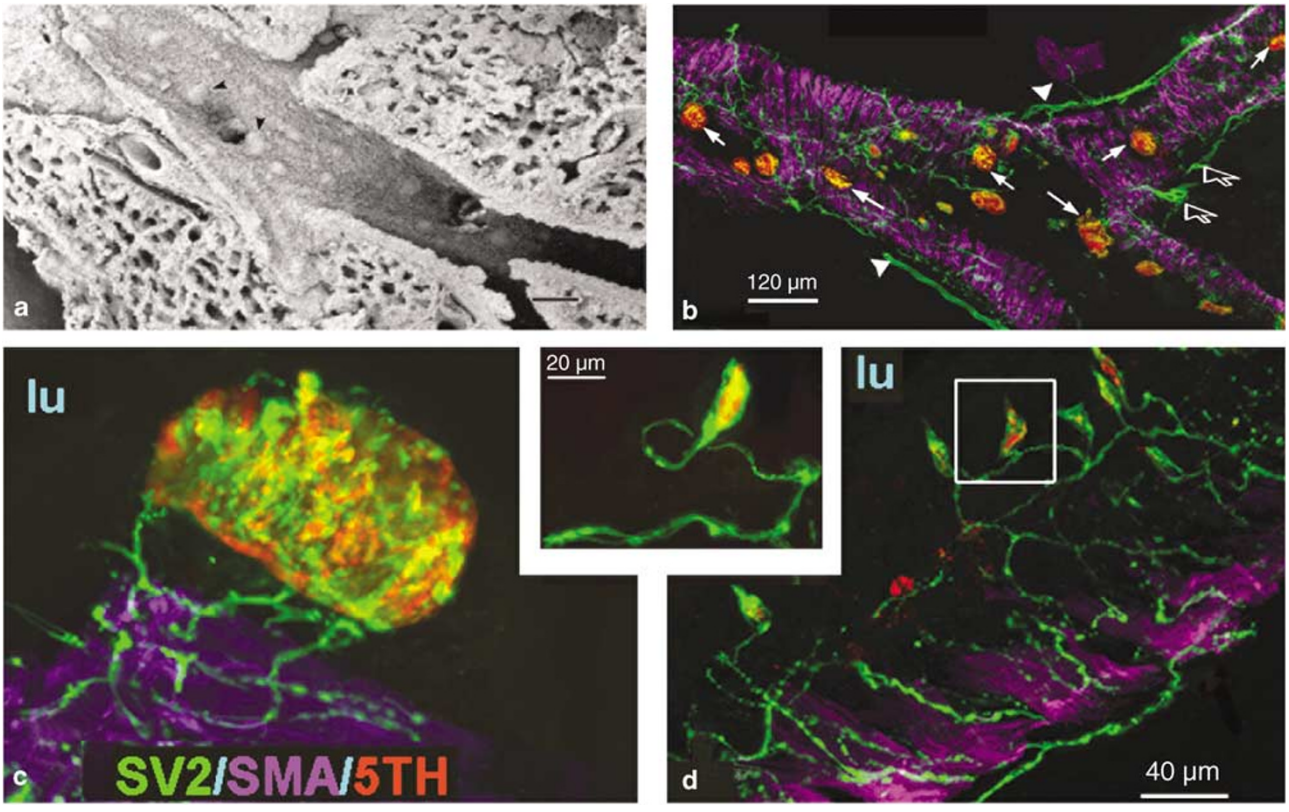

Figure 4 Distribution and innervation of NEBs and solitary PNECs. (**a**) Scanning electron micrograph of the central airway of a hamster fetal lung (15-day gestation) with numerous NEBs (arrow heads) protruding into the airway lumen. Bar = 100 μm. (**b**) Comparable low-magnification view using confocal microscope of a large airway with partially open luminal aspect in fetal rabbit lung at embryonal day 21. Immunostaining for synaptic vesicular protein 2 (SV2) in green, smooth muscle actin (SMA) in purple and serotonin (5 TH) in red. Several compact NEBs (arrows) are evident in airway mucosa. Thick nerve trunks (solid arrowhead) and small ganglia (open arrows) are present in the adventitia of airway wall. (**c**) Close-up of an NEB in the airway of 2-day-old rabbit lung with several nerve fibers arising from submucosal plexus and entering at the base. Nerve density is greatly increased compared to (**b**). (**d**) A portion of airway wall showing a complex neural plexus around smooth muscle. Several flask-shaped solitary PNECs with thin apical processes facing the lumen and nerve fiber contact at cell base are present. *Inset:* Close-up of boxed area revealing a solitary, innervated PNEC is shown in the inset in the middle. Illustrations reproduced and modified from Cutz *et al*[10] (panel a) and Pan *et al*[102] (panels c–d and inset) with permission.

innervated[103] (Figure 4, inset). As an interpretative caution, it should be noted that there are well-known species variations in lung innervation.[104] Likewise, any numerical estimations are subject to methodological variations from one study to the next, including the sensitivity of immunohistological techniques.

Based primarily on physiologic studies using single fiber action potentials, it has been postulated that there may be at least four different categories of receptors in the airways. They include well-characterized slowly adapting stretch receptors (SARs), rapidly adapting stretch receptors (RARs), pulmonary C-fiber receptors, and finally, the more hypothetical sensory receptors involving NEBs.[105] There is increasing evidence that NEBs may be sensors of hypoxia. The possible sensory role for pulmonary neuroepithelial receptors, including a chemoreceptor function to monitor concentration of airway gases, stems from early neuroanatomical studies.[106] In the 1970s, Lauweryns *et al* compared NEBs as hypoxia sensitive airway chemoreceptors with the arterial chemoreceptors, carotid bodies using newborn rabbits.[93,107–109] They showed that NEBs react to airway hypoxia by increasing exocytosis of their dense core vesicles (DCVs) and decrease in cytoplasmic amine content.

The steady improvement of relevant *in vitro* models has been pivotal for characterizing the membrane properties and receptors of PNECs and NEBs that are responsible for the oxygen-sensing function.[5,15,110,111] Using the whole-cell patch clamp technique on cultured NEBs from fetal rabbits, Youngson *et al*[112] showed that NEB cells exhibit features of excitable cells since they express voltage-activated potassium, calcium and sodium currents. Upon exposure to hypoxia (pO^2 25–30 mmHg), there was a reversible reduction (20–30%) in outward potassium current while inward sodium and calcium currents were unaffected by hypoxia. In current clamp mode, the closure of potassium channels by hypoxia produced an increase in spontaneous firing frequency and slope of the depolarization potential in NEB cells. Incidentally, it was recently proposed that voltage-gated ion channels and the membrane 'exitability' seen in SCLCs might also accelerate metastatic progression.[113,114]

The studies by Youngson *et al*[112,115] revealed that NEB cells possess an oxygen-binding protein cytochrome *b*, a NAPDH oxidase located in cellular membranes. Proof that NAPDH oxidase acts as the O_2 receptor came from oxidase-deficient mice.[116] In wild-type animals, hypoxia and diphenylene iodonium, an inhibitor of the oxidase, caused inhibiton of potassium-dependent currents, while there was no effect in the oxidase-deficient mice. Whole-cell currents in NEB cells were recorded from fresh lung slices where the PNECs were identified using vital dye neutral red. Moreover, the oxidase gene was localized in NEBs by *in situ* hybridization. Additional evidence that NAPDH oxidase acts as a functional O_2 sensor was provided by faster and shallower breathing in young oxidase-deficient mice both during normoxia and hypoxia.[117] In contrast, studies have indicated that NADPH may not be the primary O_2 sensor in adrenomedullary chromaffin cells or pulmonary artery smooth muscle cells, which underlies the complexity of O_2-sensing mechanisms.[118,119] Conversely, NADPH oxidase may not be the only oxygen sensor compound in PNECs given the critical function that this sensory mechanism represents in the lung.[120]

A natural stimulus such as O_2 concentration transduced via an oxygen sensor on PNECs could modulate various pulmonary homeostatic processes, including airway tone,[121] pulmonary circulation,[122,123] control of breathing,[117] as well as lung growth and differentiation[14] (Figure 5). The candidate neurotransmitters involved in chemotransduction of hypoxia stimulus in NEBs are similar to those proposed for the carotid body glomus cells,[124] including serotonin (5-HT), acetylcholine (ACh) and ATP.[12] The action may happen through neuronal, paracrine or autocrine mechanisms, and may involve several feedback loops. Classic NEBs respond to hypoxia by the release of serotonin (5-HT), which may result in bronchoconstriction, vasomotor toning and/or growth-factor-like properties[125,126] as well as positive feedback activation through serotonin type 3 autoreceptors residing on PNECs.[127,128]

Direct evidence on hypoxia-induced 5-HT release comes from a study that showed a dose-dependent response in a range of pO^2 95–18 mmHg obtained from NEBs in lung slices by carbon fiber amperometry, which is able to measure in real time the release of single molecules of 5-HT.[127] This was modulated by L-type calcium channels. A recent study by Fu *et al* also revealed that NEBs possess functional purinergic receptors, which may provide additional means for modulation of hypoxia chemotransmission. For example, ATP will act on $P2X_{2/3}$ autoreceptors as a feedback mechanism further augmenting 5-HT release.[129] These data are consistent with the role of ATP as a sensory mediator in gastrointestinal chemosensory function.[130] Moreover, NEBs harbour functional ACh receptors and cholinergic mechanisms may play a role under normal as well as pathological conditions especially those linked to smoking.[131,132] They may also respond to hypercapnia.[133]

The oxygen-sensing properties of NEBs are likely to be very important during the neonatal period. The study of Van Lommel and Lauweryns[134] reported that the relative number of NEBs tended to be higher in animal species with shorter gestation such as rat and hamster, hence requiring airway chemoreceptors to protect against hypoxia due to less mature lungs at birth. Detailed stereological studies revealed that the total volume on NEBs in newborn hamsters was twice the size of carotid bodies, the principal chemoreceptor organ in adults. By 1

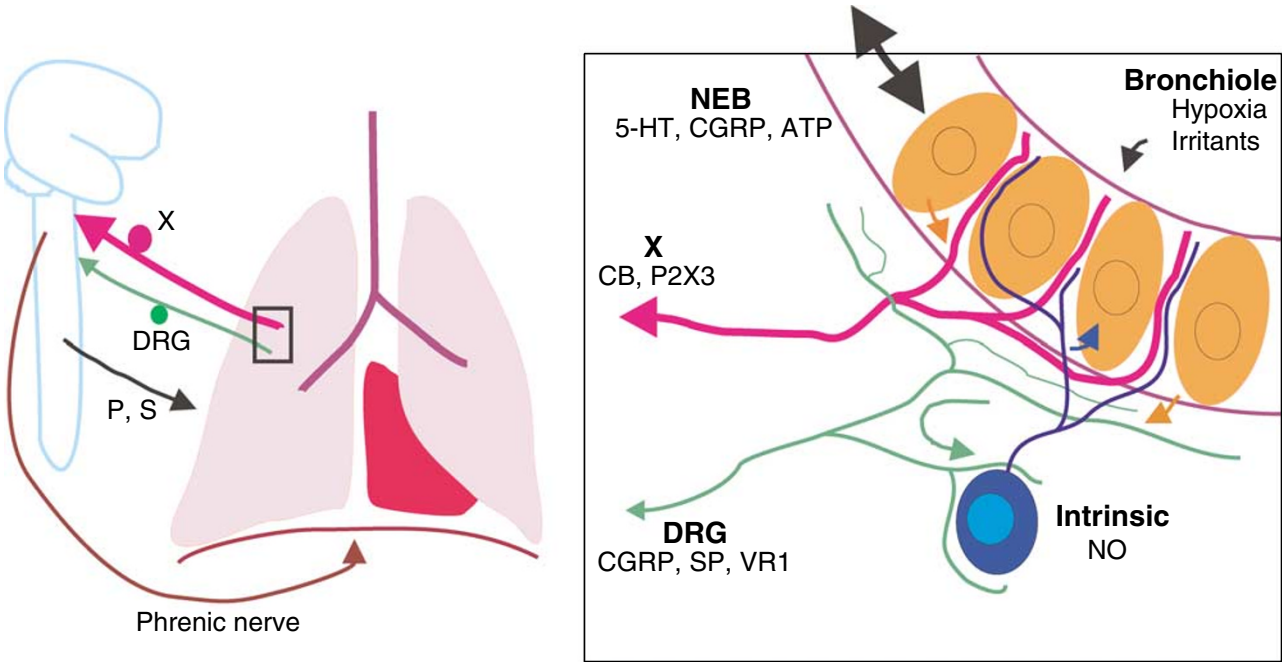

Figure 5 Complex functions require complex structures. Schematic representation of dual afferent innervation of the lung and a proposed local NE modulatory arrangement. *Left panel*: Dual afferent innervation of the lung. Vagal (*X*, red) and dorsal root ganglionic (*DRG*, green) primary afferents innervate intrapulmonary airways and project to brainstem and spinal cord (blue). Various reflex responses are relayed through parasympathetic (*P*), sympathetic (*S*) and phrenic nerve pathways regulating respiration and cardiopulmonary homeostasis. *Right panel*: Close-up view of an NEB. Thick caliber vagal afferents (*red*) distribute terminal branches between cells, while DRG afferent fibers (green) form a subepithelial plexus. Axons originating from nitrergic intrinsic neurons (dark blue) also ramify between NEB cells. Stimuli from the bronchiolar lumen may trigger release of, for example, ATP from NEB cells, thus exciting vagal afferents through $P2X_3$ receptors. Likewise, subepithelial DRG afferents may also be stimulated and could modulate activity of intrinsic neurons via an axon reflex (curved green arrow). Intrinsic neurons, in turn, may regulate sensitivity of NEB cells to luminal stimuli by release of NO from their intraepithelial terminals. Black double-headed arrow indicates mechanical stimulation of NEB during distension of the bronchiole, which also may trigger mediator release from NEB cells. Reproduced from Neuhuber[208] with permission.

Table 1 Human conditions and experimental studies with alterations in PNECs

Neonatal/pediatric	*Adult*	*Animal models*
Hyaline membrane disease	Pneumonia	Acute or chronic hypoxia
Bronchopulmonary dysplasia	Chronic bronchitis, emphysema	Hypercapnia
Pulmonary dysmaturity	Bronchiectasis	Almitrine bismesylate
Asphyxia with brainstem injury	Cigarette smoking	Nitrosamines, nicotine or cigarette smoke
Pulmonary hypertension	Asthma	
Pulmonary hypoplasia	Eosinophilic granuloma	Naphthalene
Cystic adenomatoid malformation	Mechanical ventilation with O_2	Asbestos
Cystic fibrosis	Plexogenic pulmonary arteriopathy	Active sensitization
Central hypoventilation syndrome	Diffuse idiopathic PNEC hyperplasia	Diaphragmatic hernia
Sudden infant death syndrome	Neoplasms (surrounding lung)	Genetically altered mice: transgenic or knockout
Idiopathic NE cell hyperplasia of infancy		

month of age, this was reversed due to rapid involution in NEBs.[134,135] The NEB volume was comparable to that of parathyroid glands, consistent with a significant physiologic function. Other substantial age-related changes include postnatal maturation of innervation. Rabbit carotid body is relatively inactive during fetal and perinatal period. A study that compared the innervation of carotid body with that of NEBs in rabbit showed that carotid bodies reach their maturity for afferent innervation, which is necessary for reflexogenic receptor function later.[136] Consequently, NEBs may complement carotid bodies during early phase of postnatal development. NEBs will function as auxillary chemoreceptors important during neonatal adaptation at the time when the function of carotid bodies is still immature. Even NEBs will acquire more afferent nerve endings postnatally.[134] preparing them for their potential role in adult lungs involving more complex respiratory reflexes (Figure 5).

The PNEC system in pulmonary pathology, carcinogenesis and animal models

Over the years countless articles and two books have been devoted to the theme of how the number, distribution and peptide content of PNECs might be associated with diseases and pathological conditions (Table 1).[5,8,137–139] However, the functional connections have for the most part remained elusive. According to Boers *et al*, normal adult lung has approximately 12.5 PNECs per cm of basement membrane (about 0.41% of all cells) distributed through the airways, which is a three- to four-fold decrease compared to the PNEC numbers in fetal lungs.[140–142] NEBs are rare.[143,144] In Figure 6, Weichselbaum *et al*[143] provides a dramatic 'bird's eye' view of solitary PNECs, whose density ranges from 65 to 250 cells per mm^2 within an individual whole mount of bronchial epithelium. The authors also noted a nonhomogenous distribution, that is, a high PNEC density appeared to be juxtaposed to areas of lower numbers. On the other hand, minimal variation occurs from childhood to old age in the PNEC system of unremarkable human lungs.[145] All numerical estimations are subject to variations of methodologies and markers. Two-thirds of the cells contain BLPs including GRP, and nearly all of the rest contain calcitonin.[140,146] Both BLPs and calcitonin have important functions in human lung pathophysiology.[13,147,148]

Recent studies using baboons and genetically engineered mice have revealed a potential mechanistic link between BLPs derived from PNECs and chronic inflammatory lung disease.[13] BLPs are known to promote normal fetal lung development.[14] Interestingly, BLPs are elevated shortly after birth in premature babies who later develop bronchopulmonary dysplasia (BPD).[149] BPD, which is associated with PNEC hyperplasia, is the most common chronic lung disease in infants in the United States.[9] Remarkably, in baboon models, anti-BLP blocking antibodies abrogate BPD[150] and reduce both the PNEC and mast cell hyperplasia observed in these lungs.[151] Moreover, intratracheally administered bombesin in mice results in an increase of lung mast cells. Cellular mechanisms may include a cascade leading from PNECs to mast cells via BLP family receptors BRS-3 and NMB on mast cells. The significance of these observations is that they strongly implicate PNECs as proinflammatory cells, functionally linked to a myriad of pathological conditions, including asthma, cystic fibrosis, COPD and cancer.[152–155] In addition, they define yet another paracrine role of bioactive peptides in lung.

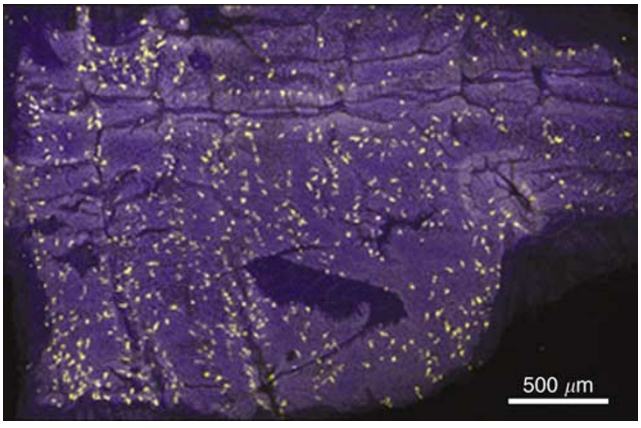

Figure 6 Abundant solitary PNECs in adult human bronchial mucosa. A whole mount of a large piece (approximately 1.3×0.9 cm) of human bronchial mucosa was stained for GRP immunoreactivity and viewed from the luminal surface by confocal microscope. PNECs appear as small yellow spots. Bar = 500 μm. Illustration courtesy of Dr Darryl Knight.[143]

Based on the experimental evidence on NEBs as a chemosensory organ, hypoxia has frequently been proposed as a cause for the numerical increase in PNECs seen in many diseases. There is very little proof that this is actually the case in human lung.[156] Hypoxia may contribute to the increase in PNECs seen in humans and animals living at high altitudes, infants succumbing to sudden infant death syndrome (SIDS) or congenital central hypoventilation syndrome (CCHS or Ondine's curse).[141,157–163] However, common lung pathologies associated with PNEC hyperplasia are more likely to be related to repeated injury, repair, tissue remodeling, fibrosis and inflammation than hypoxia thus implicating a myriad of pathways.

In normal adult human lung, the vast majority of PNECs are solitary and evenly distributed along the epithelium, while in infants and neonates proportionally more of them are in the form of NEBs.[164] The increase of PNECs in most pathological conditions that involve inflammation and fibrosis displays two characteristic patterns: interrupted rows of cells along the basement membrane, and larger disorderly nodular aggregates.[103,164] In the literature, a distinction between responses of solitary PNECs and NEBs has not been made consistently, which may obscure structure–function correlations in diseased lungs. In 1992, Aguayo *et al* described six patients with a rare condition, which is now known as diffuse idiopathic PNEC hyperplasia or DIPNECH.[165,166] By definition, it is a generalized proliferation of solitary, clustered or linear PNECs that may be confined to the airway epithelium, contain foci of an extraluminal proliferation called tumorlets, or be associated with carcinoid tumors as observed by a number of authors over the years.[167–170] Similar entity has been also recognized in children.[171,172] The study of Deterding *et al*[172] described 15 pediatric cases with idiopathic NE cell hyperplasia (NEHI). A distinguishing feature of DIPNECH is that other pathology, which might induce a reactive proliferation of PNECs is absent, while fibrous bronchiolitis with radiographic abnormalities is part of the clinical manifestations of DIPNECH.[173,174] At the moment, the potential significance of this very rare lesion is that it is conceivably a premalignant

lesion similar to atypical adenomatoid hyperplasia (AAH) and squamous cell carcinoma *in situ*.[166,175] Although DIPNECH is already included in the WHO classification of pulmonary neoplasms, its association with carcinogenesis still needs to be established.

It is paradoxical that a large number (one-third) of all human lung cancers display features of NE differentiation, while PNECs are only a minor component of lung epithelium. Clinicopathologically the most important NE carcinoma is SCLC, which accounts for 15–20% of all lung cancers; others include NSCLC with neuroendocrine features (NSCLC-NE), carcinoid and large cell NE carcinoma (LCNEC).[166] While SCLC is the well-established prototype, and the most common and virulent of all human NE neoplasms, its histogenesis and precursor lesions are poorly understood. In 1968, it was noted that SCLC contains DCVs, the ultrastructural hallmark of NE differentiation, similar to those found in PNECs and bronchial carcinoids.[176–178] Curiously, PNEC hyperplasias and dysplasias are common in tumor bearing lungs, but there is little evidence that they are causally related to carcinogenesis rather than being a component of general pathological processes such as inflammation and emphysema frequently found in the same lungs.[179,180] Moreover, exposure of laboratory animals to chemical carcinogens such as tobacco-specific nitrosamines has often lead to marked, albeit reversible PNEC hyperplasias, but distinctly non-NE adenomas and NSCLCs[181–184] (Figure 2). In some instances, the lack of a detailed morphological and biochemical characterization has hindered wider applications of proposed NE lung tumor models.[185–187] This has prompted a search for more relevant models of lung NE carcinogenesis.

In an effort to generate a transgenic model for primary PNEC hyperplasia and neoplasia, Sunday *et al*[188] used v-Ha-ras driven by the neural/NE-specific calcitonin promoter (rascal). In the embryonic lungs, rascal mRNA was seen in undifferentiated epithelium, consistent with expression in a common pluripotent precursor cell.[30,31] However, adult transgenic mice displayed non-NE and PNEC hyperplasias together but mostly non-NE pulmonary carcinomas. One potential explanation for the lack of SCLCs in this model is that human SCLCs do not have mutations in RAS oncogene, which on the other hand is commonly altered in NSCLCs.[189]

The fact that nearly all human SCLCs harbor mutations or alterations in p53 and Rb-1 tumor suppressor genes prompted investigators to knock-out these genes in mouse lung.[189,190] It has been shown before that heterozygous Rb-$1^{-/+}$ mice demonstrate moderate PNEC hyperplasia (author's unpublished observations),[191] but homozygous $-/-$ mice are embryonic lethal. Meuwissen *et al* were able to generate mice that carried conditional alleles for both Rb-1 and p53 (Rb-$1^{-/-}$/$p53^{-/-}$) in lung epithelial cells and developed aggressive lung tumors with striking similarities to human SCLCs including morphology, high expression of ASH1, NE markers and extrapulmonary metastases (Figure 7a and b). Tumors originated from bronchiolar epithelium and revealed a range of premalignant changes (Figure 3e). It appears that Rb function is critical for regulating NE differentiation in normal and neoplastic lung, as the deletion of both alleles of Rb-1 was required for the SCLC phenotype in the tumor model. The significance of this model is that it is the first animal model for SCLC that recapitulates typical features of this NE carcinoma both at the molecular level such as involvement of Rb-1 and p53 tumor suppressor genes, and *in vivo* demonstrating characteristic morphology, NE features and metastatic pattern.[192–194] It provides an important system for investigating fundamental aspects of NE tumor initiation and progression, including the identification of the cell of origin of SCLC and its relationship to PNECs or stem cells in the lung. It also offers means for preclinical testing of early detection, chemoprevention and therapeutic interventions.

Rb is a member of a protein family that also includes p107 and p103, commonly refererred as 'pocket proteins'.[195] In an elegant study, Wikenheiser–Brokamp provided a functional link for these proteins during lung development.[196] Ablation of total Rb family function resulted in opposing effects in distinct cell lineages: pocket proteins were shown to inhibit PNEC fate, but were required for differentiation of other cell types. Lungs with only Rb-1 deficiency reveal PNEC-specific abnormalities. In both cases, the phenotype in mice included prominent PNEC hyperplasias at various stages (Figure 3c and d). It is possible that this occurs through transdifferentiation of airway epithelial cells towards NE phenotype, leaving the role of actual PNECs as SCLC progenitors still elusive. The process of transdifferentiation is in accordance with the idea of common origin for PNECs and epithelial cells.[197] Nevertheless, these data suggest that Rb has PNEC specific functions that play a role in development as well as tumor suppression.

Finally, the concept of NSCLC-NEs is interesting from clinical and histogenetic view.[166] Approximately 10–15% of all NSCLCs may show differentiation towards NE phenotype by immunohistochemistry.[198] Ultrastructural evidence for dual or tripartite (NE, glandular and squamous) differentiation has been reported in the same cell suggesting a common cellular origin.[199] In addition, the original NE phenotype is retained in cultured NSCLC-NE cell lines.[200] Some of these tumors may be responsive to similar cytotoxic treatments as SCLCs, although more studies are needed.[201] Interestingly, mice with constitutive expression of the proneural transcription factor hASH-1, and simian virus large T antigen (TAg), both under the Clara cell 10 kDa secretory protein (CC10) promoter, resulted in aggressive adenocarcinomas with focal NE differ-

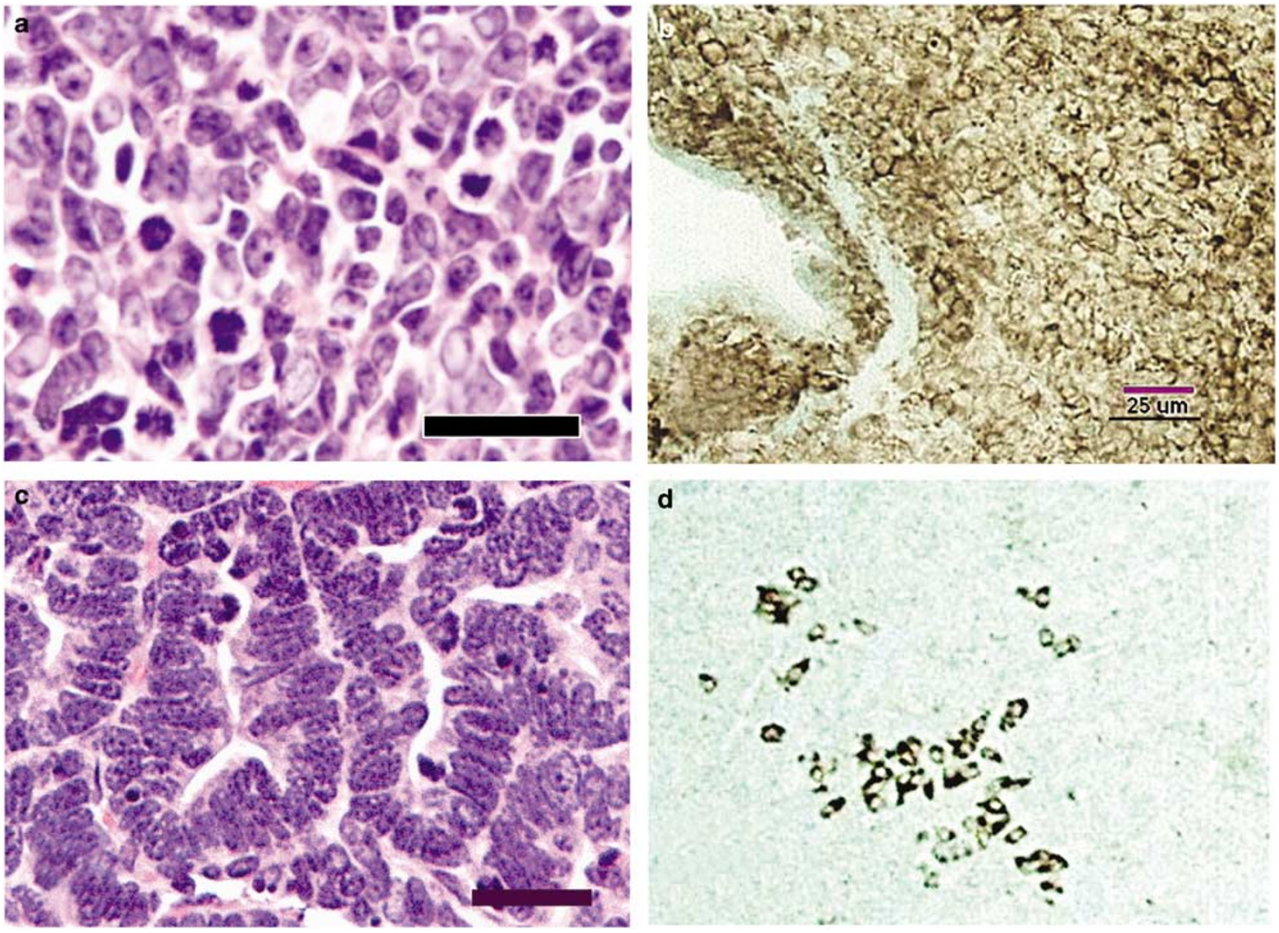

Figure 7 Murine models of lung NE tumors. (**a**) Sheet-like growth pattern of small poorly differentiated cells and numerous mitotic figures in mouse SCLC (MSCLC) tumors (hematoxylin and eosin stain) in conditional Rb-1$^{-/-}$p53$^{-/-}$ mouse. (**b**) MSCLC with intense, diffuse synaptophysin expression (Immunoperoxidase stain). (**c**) A model for human non-SCLC with NE features in bi-transgenic SV40-hASH1 mouse reveals an aggressive adenocarcinoma (hematoxylin–eosin stain) with (**d**) focal positivity for CGRP (Immunoperoxidase stain). Bar = 25 μm.

entiation mimicking human NSCLC-NEs[57] (Figure 7c and d). This was accompanied by profuse bronchiolar PNEC hyperplasia/dysplasia (Figure 3f). The bitransgenic mice died in less than 3 months with massive lung tumors, but unlike the mouse SCLC model there were no metastases.[202] It appears that ASH-1 enhances the tumorigenic effect of TAg in airway epithelium, primarily in Clara cells. At the functional level, this implies that ASH1 may cooperate with defects in p53, pRb or related pathways altered by SV40 TAg in promoting NE lung carcinogenesis. This is in concert with previous studies that have shown that ASH-1 plays a critical role in regulating the NE phenotype in normal lung[53,55] and is part of the molecular signature of an NE subset of human adenocarcinomas.[203]

Summary and conclusions

Research on the pulmonary NE system over the past few years has been very productive. With ever-advancing technologies old venues of investigations have been further defined, and new areas of interest are emerging.

It has been well established that PNECs appear very early during the embryonal development, and are the first specialized cells to differentiate along the airway epithelium. Recent research has revealed that distinct cell lineages contributing to conducting airways with NEBs and glands *vs* peripheral lung become established even before the formation of the definitive lung bud.[21,25] In addition, the development of proximal and distal lung compartments appear to be under different molecular control. This places PNECs in a critical position potentially regulating lung development and physiology.

Phenotypically, PNECs share many features with cells in the nervous system and NE carcinomas (SCLC), such as dense core granules that store bioactive amines and neuropeptides and membrane potentials. It is remarkable that the similarities may extend to molecular mechanisms that control the development of these cells, along with genes that are highly conserved through evolution. Such genes

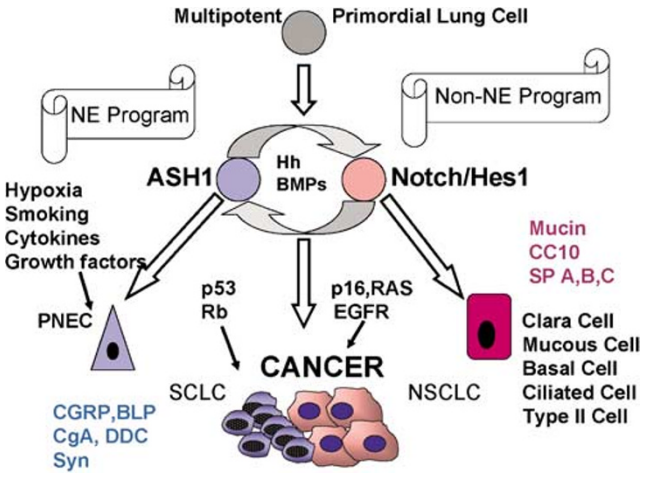

Figure 8 Proposed model for NE differentiation during the development and carcinogenesis of the airway epithelium. A primordial lung cell (stem cell) (gray) gives rise to multipotent progenitor cells that expand during lung development, injury, repair and carcinogenesis. Notch signaling segregates cells towards NE and non-NE programs. Pluripotent epithelial progenitors may express Notch together with low levels of markers from multiple cell lineages such as CGRP and CC10. When Notch is on (pink) cells develop to Clara, mucous, basal ciliated and type II cells, which express epithelial markers such as mucin, CC10, surfactant associated proteins (SP) (A, B, C). When ASH1 is on, the cells (blue) acquire NE properties and become PNECs which express NE markers including CGRP, BLP, Chromogranin A (CgA), L-dopa decarboxylase (DDC) and synaptophysin (Syn). Factors such as Hh signaling and bone morphogenic proteins (BMPs) may maintain or expand the bi-potential pool of cells during development, repair or injury.[91,92] Hypoxia, smoking, cytokines and many growth factors may stimulate PNECs to expand. During carcinogenesis alterations in tumor suppressor genes Rb and p53 result in SCLC, which expresses NE markers. NSCLCs characteristically reveal loss of p16 and activation of RAS and mutations in epithelial growth factor receptors.[189,194] Cancers are frequently heterogenous and can express features of both NE and non-NE differentiation. ASH1 and Notch may also impact survival and proliferation of cancer cells.[204,209]

include ASH1, highly expressed in developing brain and SCLCs, as well as the Notch pathway (Figure 8). Deletion of ASH1 specifically prevents the development of PNECs,[53] and, at the same time, causes severe abnormalities in the nervous system. Inhibition of ASH1 expression in lung cancer cells with ASH1 suppresses growth, potentially rendering this gene as a therapeutic target.[204] SCLCs are characterized by abnormalities of Rb-1 and p53 tumor suppressor genes. Conditional ablation of Rb family proteins leads to PNEC hyperplasia in mice.[196] Moreover, conditional deletion of both suppressor genes Rb-1 and p53 resulted in aggressive lung cancers resembling SCLCs, a first ever mouse model of this disease.[190] These observations provide tools for diagnostic and therapeutic applications.

A novel role for PNECs or NEBs is emerging as guardians of lung stem cell niches.[82] The data suggest that PNECs foster a microenvironment resistant to destruction of environmental agents and promoting stem cell renewal. This has potential implications for carcinogenesis and injury repair, which may extend to chronic lung diseases. Another novel function of PNECs with clinical impact is the regulation of pulmonary inflammation involving mast cells in BPD.[151] PNECs now join other cells such as Clara cells as a source of proteins that can govern the biodefense of airways.[205,206]

It is well established that NEBs are innervated and function as oxygen-sensing chemoreceptors, which may be critical in the transition from intrauterine environment to breathing air. There is recent evidence that even the solitary PNECs are innervated.[102,143] The structural basis for the inherent complexity of neural networks and transmitters that may govern the regulation and modulation of breathing by NEBs continues to unravel.

What is the challenge for future research? As we separate the function from fiction, it is well established that PNECs and NEBs are present in great numbers before birth and during the neonatal period with two disticnt functions: to contribute to lung maturation, and function as oxygen sensors complementing the maturing carotid body. They are less conspicuous in adult lungs, prompting the question what is their role later in life. What is the difference between PNECs and NEBs? Further studies on the role of lung stem cells and PNECs in tissue repair or the role of PNECs in lung carcinogenesis are needed since it is conceivable that these venues for PNECs are more prominent in adult lung. Recent studies have enhanced our knowledge, research means and provide great inspiration, but much remains to be learned.

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References

1 Nylen ES, Becker KL. The diffuse neuroendocrine system. In: Becker KL (ed). Principles and Practice of Endocrinology and Metabolism, 3rd edn. Lippincott Williams and Wilkins: Philadelphia, 2001.
2 Feyrter F. Uber die these von den peripheren endokrinen drusen. Wien Z Innere Med Grenzgeb 1946;10:9–36.
3 Feyrter F. Uber die argyrophilie des hellezellensystems im bronchialbaum des menschen. Z Mikrosk Anat Forsc 1954;761:73–81.
4 Fröhlich F. Die hellezelle der bronchialschleimhaut und ihre beziehungen zum problem der chemoreceptoren. Frankfurter Z Pathol 1949;60:517–559.
5 Linnoila RI. Pulmonary endocrine cells *in vivo* and *in vitro*. In: Kaliner MA, Barnes PJ, Kunz HW, Baraniuk JN (eds). Neuropeptides in Respiratory Medicine. Marcel Dekker: New York, 1994.

6 Van Lommel A, Bolle T, Fannes W, *et al.* The pulmonary neuroendocrine system: the past decade. Arch Histol Cytol 1999;62:1–16.
7 Sorokin SP, Hoyt Jr RF (eds). Proceedings of workshop on pulmonary neuroendocrine cells in health and disease. Anat Rec 1993;236:1–256.
8 Cutz E, Gillan JE, Perrin DG. Pulmonary neuroendocrine cell system: an overview of cell biology and pathology with emphasis on pediatric lung disease. In: Askin FB, Langston C, Rosenberg HS, Bernstein J (eds). Pulmonary Disease. Karger: Basel, 1995.
9 Cutz E. Cellular and Molecular Biology of Airway Chemoreceptors. Landes Bioscience: Austin, 1997.
10 Cutz E, Jackson A. Neuroepithelial bodies as airway oxygen sensors. Respir Physiol 1999;115:201–214.
11 Van Lommel A. Pulmonary neuroendocrine cells (PNEC) and neuroepithelial bodies (NEB): chemoreceptors and regulators of lung development. Paediatr Respir Rev 2001;2:171–176.
12 Cutz E, Fu XW, Nurse CA. Ionotropic receptors in pulmonary neuroepithelial bodies (NEB) and their possible role in modulation of hypoxia signalling. Adv Exp Med Biol 2003;536:155–161.
13 Sunday ME, Shan L, Subramaniam M. Immunomodulatory functions of the diffuse neuroendocrine system: implications for bronchopulmonary dysplasia. Endocr Pathol 2004;15:91–106.
14 Sunday ME, Cutz E. Role of neuroendocrine cells in fetal and postnatal lung. In: Mendelson CR (ed). Endocrinology of the Lung. Development and Surfactant Synthesis. Humana Press Inc.: Totowa, NJ, 2000.
15 Cutz E, Fu XW, Yeger H. Methods to study neuroepithelial bodies as airway oxygen sensors. Methods Enzymol 2004;381:26–40.
16 Whitsett JA, Glasser SW, Tichelaar JW, *et al.* Transgenic models for study of lung morphogenesis and repair: Parker B. Francis lecture. Chest 2001;120:27S–30S.
17 Perl AK, Tichelaar JW, Whitsett JA. Conditional gene expression in the respiratory epithelium of the mouse. Transgenic Res 2002;11:21–29.
18 Cutz E. Cytomorphology and differentiation of airway epithelium in developing human lung. In: McDowell EM (ed). Lung Carcinomas, 1st edn. Churchill Livingstone: Edinburgh, 1987.
19 Sorokin SP, Hoyt Jr RF, Shaffer MJ. Ontogeny of neuroepithelial bodies: correlations with mitogenesis and innervation. Microsc Res Tech 1997;37:43–61.
20 Stahlman MT, Gray ME. Ontogeny of neuroendocrine cells in human fetal lung. I. An electron microscopic study. Lab Invest 1984;51:449–463.
21 Perl AK, Wert SE, Nagy A, *et al.* Early restriction of peripheral and proximal cell lineages during formation of the lung. Proc Natl Acad Sci USA 2002; 99:10482–10487.
22 McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 2003;53:1–114.
23 Miller LA, Wert SE, Whitsett JA. Immunolocalization of sonic hedgehog (Shh) in developing mouse lung. J Histochem Cytochem 2001;49:1593–1604.
24 Unger S, Copland I, Tibboel D, *et al.* Down-regulation of sonic hedgehog expression in pulmonary hypoplasia is associated with congenital diaphragmatic hernia. Am J Pathol 2003;162:547–555.
25 Miller LA, Wert SE, Clark JC, *et al.* Role of Sonic hedgehog in patterning of tracheal–bronchial cartilage and the peripheral lung. Dev Dyn 2004; 231:57–71.
26 Ioannides AS, Henderson DJ, Spitz L, *et al.* Role of Sonic hedgehog in the development of the trachea and oesophagus. J Pediatr Surg 2003;38:29–36.
27 Crisera CA, Maldonado TS, Longaker MT, *et al.* Defective fibroblast growth factor signaling allows for nonbranching growth of the respiratory-derived fistula tract in esophageal atresia with tracheoesophageal fistula. J Pediatr Surg 2000;35:1421–1425.
28 Crisera CA, Connelly PR, Marmureanu AR, *et al.* TTF-1 and HNF-3beta in the developing tracheoesophageal fistula: further evidence for the respiratory origin of the distal esophagus'. J Pediatr Surg 1999;34:1322–1326.
29 Crisera CA, Connelly PR, Marmureanu AR, *et al.* Esophageal atresia with tracheoesophageal fistula: suggested mechanism in faulty organogenesis. J Pediatr Surg 1999;34:204–208.
30 Wuenschell CW, Sunday M, Singh G, *et al.* Embryonic mouse lung epithelial progenitor cells co-express immunohistochemical markers of diverse mature cell lineages. J Histochem Cytochem 1996;44:113–123.
31 Haley KJ, Drazen JM, Osathanondh R, *et al.* Comparison of the ontogeny of protein gene product 9.5, chromogranin A and proliferating cell nuclear antigen in developing human lung. Microsc Res Tech 1997;37:62–68.
32 Hibi K, Liu Q, Beaudry GA, *et al.* Serial analysis of gene expression in non-small cell lung cancer. Cancer Res 1998;58:5690–5694.
33 Hibi K, Westra WH, Borges M, *et al.* PGP9.5 as a candidate tumor marker for non-small-cell lung cancer. Am J Pathol 1999;155:711–715.
34 Caballero OL, Resto V, Patturajan M, *et al.* Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1). Oncogene 2002;21:3003–3010.
35 Zaccone G, Fasulo S, Ainis L, *et al.* Paraneurons in the gills and airways of fishes. Microsc Res Tech 1997;37:4–12.
36 Goniakowska-Witalinska L. Neuroepithelial bodies and solitary neuroendocrine cells in the lungs of amphibia. Microsc Res Tech 1997;37:13–30.
37 Podkowa D, Goniakowska-Witalinska L. Morphology of the air-breathing stomach of the catfish Hypostomus plecostomus. J Morphol 2003;257: 147–163.
38 Podkowa D, Goniakowska-Witalinska L. Adaptations to the air breathing in the posterior intestine of the catfish (Corydoras aeneus, Callichthyidae). A histological and ultrastructural study. Folia Biol (Krakow) 2002;50:69–82.
39 King KA, Torday JS, Sunday ME. Bombesin and [Leu^{8}] phyllolitorin promote fetal mouse lung branching morphogenesis via a receptor-mediated mechanism. Proc Natl Acad Sci USA 1995;92:4357–4361.
40 Li K, Nagalla SR, Spindel ER. A rhesus monkey model to characterize the role of gastrin-releasing peptide (GRP) in lung development. Evidence for stimulation of airway growth. J Clin Invest 1994;94: 1605–1615.
41 White SR, Hershenson MB, Sigrist KS, *et al.* Proliferation of guinea pig tracheal epithelial cells induced by calcitonin gene-related peptide. Am J Respir Cell Mol Biol 1993;8:592–596.
42 Hoyt Jr RF, McNelly NA, Sorokin SP. Calcitonin gene-related peptide (CGRP) as regional mitogen for

tracheobronchial epithelium of organ cultured fetal rat lungs. Am Rev Respir Dis 1993;147:A498.
43 Cuttitta F, Carney DN, Mulshine J, *et al.* Autocrine growth factors in human small cell lung cancer. Cancer Surv 1985;4:707–727.
44 Sunday ME, Hua J, Dai HB, *et al.* Bombesin increases fetal lung growth and maturation in utero and in organ culture. Am J Respir Cell Mol Biol 1990;3: 199–205.
45 Sunday ME, Hua J, Reyes B, *et al.* Anti-bombesin monoclonal antibodies modulate fetal mouse lung growth and maturation *in utero* and in organ cultures. Anat Rec 1993;236:25–32.
46 Emanuel RL, Torday JS, Mu Q, *et al.* Bombesin-like peptides and receptors in normal fetal baboon lung: roles in lung growth and maturation. Am J Physiol 1999;277:L1003–L1017.
47 Baylin SB. 'APUD' cells: fact and fiction. Trends Endocrinol Metab 1990;1:198–204.
48 Cutz E. Neuroendocrine cells of the lung. An overview of morphologic characteristics and development. Exp Lung Res 1982;3:185–208.
49 Pan J, Yeger H, Cutz E. Neuronal developmental marker FORSE-1 identifies a putative progenitor of the pulmonary neuroendocrine cell lineage during lung development. J Histochem Cytochem 2002;50: 1567–1578.
50 Ball DW. Achaete-scute homolog-1 and Notch in lung neuroendocrine development and cancer. Cancer Lett 2004;204:159–169.
51 Ito T, Udaka N, Okudela K, *et al.* Mechanisms of neuroendocrine differentiation in pulmonary neuroendocrine cells and small cell carcinoma. Endocr Pathol 2003;14:133–139.
52 Kageyama R, Nakanishi S. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. Curr Opin Genet Dev 1997;7:659–665.
53 Borges M, Linnoila RI, van de Velde HJK, *et al.* An achaete-scute homologue essential for neuroendocrine differentiation in the lung. Nature 1997;386: 852–855.
54 Westerman BA, Neijenhuis S, Poutsma A, *et al.* Quantitative reverse transcription-polymerase chain reaction measurement of HASH1 (ASCL1), a marker for small cell lung carcinomas with neuroendocrine features. Clin Cancer Res 2002;8:1082–1086.
55 Ito T, Udaka N, Yazawa T, *et al.* Basic helix-loop-helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium. Development 2000;127:3913–3921.
56 Guillemot F, Lo LC, Johnson JE, *et al.* Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 1993;75:463–476.
57 Linnoila RI, Zhao B, DeMayo JL, *et al.* Constitutive achaete-scute homolog-1 promotes airway dysplasia and lung neuroendocrine tumors in transgenic mice. Cancer Res 2000;60:4005–4009.
58 Batt J, Asa S, Fladd C, *et al.* Pituitary, pancreatic and gut neuroendocrine defects in protein tyrosine phosphatase-sigma-deficient mice. Mol Endocrinol 2002; 16:155–169.
59 Artavanis-Tsakonas S, Simpson P. Choosing a cell fate: a view from the Notch locus. Trends Genet 1991;7:403–408.
60 Ishibashi M, Ang SL, Shiota K, *et al.* Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix–loop–helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev 1995;9: 3136–3148.
61 Post LC, Ternet M, Hogan BL. Notch/Delta expression in the developing mouse lung. Mech Dev 2000;98: 95–98.
62 Kong Y, Glickman J, Subramaniam M, *et al.* Functional diversity of notch family genes in fetal lung development 2. Am J Physiol Lung Cell Mol Physiol 2004;286:L1075–L1083.
63 van Tuyl M, Groenman F, Kuliszewski M, *et al.* Overexpression of lunatic fringe does not affect epithelial cell differentiation in the developing mouse lung. Am J Physiol Lung Cell Mol Physiol 2005;288:L672–L682.
64 Huber K, Bruhl B, Guillemot F, *et al.* Development of chromaffin cells depends on MASH1 function. Development 2002;129:4729–4738.
65 Lanigan TM, DeRaad SK, Russo AF. Requirement of the MASH-1 transcription factor for neuroendocrine differentiation of thyroid C cells. J Neurobiol 1998;34:126–134.
66 Leonard JH, Cook AL, Van Gele M, *et al.* Proneural and proneuroendocrine transcription factor expression in cutaneous mechanoreceptor (Merkel) cells and Merkel cell carcinoma. Int J Cancer 2002; 101:103–110.
67 Van Gele M, Boyle GM, Cook AL, *et al.* Gene-expression profiling reveals distinct expression patterns for classic *vs* variant Merkel cell phenotypes and new classifier genes to distinguish Merkel cell from small-cell lung carcinoma. oncogene 2004;23: 2732–2742.
68 Yang Q, Bermingham NA, Finegold MJ, *et al.* Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 2001; 294:2155–2158.
69 Bermingham NA, Hassan BA, Price SD, *et al.* Math1: an essential gene for the generation of inner ear hair cells. Science 1999;284:1837–1841.
70 Batt J, Cutz E, Fladd C, *et al.* Apparent normal lung architecture in protein tyrosine phosphatase-sigma-deficient mice. Am J Physiol Lung Cell Mol Physiol 2003;284:L214–L223.
71 Elchebly M, Wagner J, Kennedy TE, *et al.* Neuroendocrine dysplasia in mice lacking protein tyrosine phosphatase sigma. Nat Genet 1999;21:330–333.
72 Coraux C, Nawrocki-Raby B, Hinnrasky J, *et al.* Embryonic stem cells generate airway epithelial tissue. Am J Respir Cell Mol Biol 2005;32:87–92.
73 Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development 1990;110:1001–1020.
74 Thorgeirsson SS. Hepatic stem cells in liver regeneration. FASEB J 1996;10:1249–1256.
75 Ten Have-Opbroek AAW, Randell SH, Stripp BR. Stem cells in lung morphogenesis, regeneration, and carcinogenesis. In: Sell S (ed). Stem Cells Handbook. Humana Press Inc.: Totowa, 2003.
76 Evans MJ, Cabral-Anderson LJ, Freeman G. Role of the Clara cell in renewal of the bronchiolar epithelium. Lab Invest 1978;38:648–653.
77 Fuchs E, Segre JA. Stem cells: a new lease on life. Cell 2000;100:143–155.
78 Engelhardt JF. Stem cell niches in the mouse airway. Am J Respir Cell Mol Biol 2001;24:649–652.

79 Borthwick DW, Shahbazian M, Krantz QT, *et al.* Evidence for stem-cell niches in the tracheal epithelium. Am J Respir Cell Mol Biol 2001;24:662–670.
80 Reynolds SD, Hong KU, Giangreco A, *et al.* Conditional Clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. Am J Physiol Lung Cell Mol Physiol 2000;278:L1256–L1263.
81 Reynolds SD, Giangreco A, Power JH, *et al.* Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. Am J Pathol 2000;156:269–278.
82 Hong KU, Reynolds SD, Giangreco A, *et al.* Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor depletion. Am J Respir Cell Mol Biol 2001;24:671–681.
83 Stripp BR, Maxson K, Mera R, *et al.* Plasticity of airway cell proliferation and gene expression after acute naphthalene injury. Am J Physiol 1995;269: 791–799.
84 Stevens TP, McBride JT, Peake JL, *et al.* Cell proliferation contributes to PNEC hyperplasia after acute airway injury. Am J Physiol 1997;272: L486–L493.
85 Giangreco A, Reynolds SD, Stripp BR. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. Am J Pathol 2002;161:173–182.
86 Kim CF, Jackson EL, Woolfenden AE, *et al.* Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell 2005;121:823–835.
87 Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001;15:3059–3087.
88 Taipale J, Beachy PA. The Hedgehog and Wnt signalling pathways in cancer. Nature 2001;411: 349–354.
89 Kenney AM, Rowitch DH. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. Mol Cell Biol 2000;20:9055–9067.
90 Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron 1999;22:103–114.
91 Watkins DN, Berman DM, Burkholder SG, *et al.* Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature 2003; 422:313–317.
92 Watkins DN, Berman DM, Baylin SB. Hedgehog signaling: progenitor phenotype in small-cell lung cancer. Cell Cycle 2003;2:196–198.
93 Lauweryns JM, Cokelaere M, Theunynck P. Neuroepithelial bodies in the respiratory mucosa of various mammals. Z Zellforsch 1972;135:569–592.
94 Lauweryns JM, Peuskens JC. Neuro-epithelial bodies (neuroreceptor or secretory organs? In human infant bronchial and bronchiolar epithelium. Anat Rec 1972;172:471–481.
95 Lauweryns JM, Van Lommel AT, Dom RJ. Innervation of rabbit intrapulmonary neuroepithelial bodies. Quantitative and qualitative ultrastructural study after vagotomy. J Neurol Sci 1985;67:81–92.
96 Adriaensen D, Timmermans JP, Brouns I, *et al.* Pulmonary intraepithelial vagal nodose afferent nerve terminals are confined to neuroepithelial bodies: an anterograde tracing and confocal microscopy study in adult rats. Cell Tissue Res 1998;293:395–405.
97 Van Lommel A, Lauweryns JM, Berthoud HR. Pulmonary neuroepithelial bodies are innervated by vagal afferent nerves: an investigation with *in vivo* anterograde DiI tracing and confocal microscopy. Anat Embryol (Berl) 1998;197:325–330.
98 Brouns I, Van Genechten J, Hayashi H, *et al.* Dual sensory innervation of pulmonary neuroepithelial bodies. Am J Respir Cell Mol Biol 2003; 28:275–285.
99 Larson SD, Schelegle ES, Hyde DM, *et al.* The three-dimensional distribution of nerves along the entire intrapulmonary airway tree of the adult rat and the anatomical relationship between nerves and neuroepithelial bodies. Am J Respir Cell Mol Biol 2003; 28:592–599.
100 Brouns I, Van Genechten J, Scheuermann DW, *et al.* Neuroepithelial bodies: a morphologic substrate for the link between neuronal nitric oxide and sensitivity to airway hypoxia? J Comp Neurol 2002;449:343–354.
101 Brouns I, Adriaensen D, Burnstock G, *et al.* Intraepithelial vagal sensory nerve terminals in rat pulmonary neuroepithelial bodies express P2X(3) receptors. Am J Respir Cell Mol Biol 2000;23:52–61.
102 Pan J, Yeger H, Cutz E. Innervation of pulmonary neuroendocrine cells and neuroepithelial bodies in developing rabbit lung. J Histochem Cytochem 2004; 52:379–389.
103 Gosney JR. Pulmonary Endocrine Pathology. Butterworth-Heinemann Ltd: Oxford, 1992.
104 Dey RD, Hung KS. Development of innervation in the lung. In: McDonald JA (ed). Growth and Development of the Lung. Marcel Dekker Inc.: New York, 1997, pp 243–267.
105 Widdicombe J. Airway receptors. Respir Physiol 2001;125:3–15.
106 Sorokin SP, Hoyt RJ. Neuroepithelial bodies and solitary small-granule cells. In: Massaro D (ed). Lung Cell Biology. Marcel Dekker: New York, 1989, pp 191–344.
107 Keith IM, Wiley LA, Will JA. Pulmonary neuroendocrine cells: decreased serotonin fluorescence and stable argyrophil-cell numbers in acute hypoxia. Cell Tissue Res 1981;214:201–205.
108 Lauweryns JM, Cokelaere M. Hypoxia-sensitive neuroepithelial bodies. Intrapulmonary secretory neuroreceptors, modulated by the CNS. Z Zellforsch Mikrosk Anat 1973;145:521–540.
109 Lauweryns JM, Cokelaere M, Lerut T, *et al.* Cross-circulation studies on the influence of hypoxia and hypoxaemia on neuro-epithelial bodies in young rabbits. Cell Tissue Res 1978;193:373–386.
110 Speirs V, Wang Y, Yeger H, *et al.* Isolation and culture of neuroendocrine cell from fetal rabbit lung using immunomagnetic techniques. Am J Respir Cell Mol Biol 1992;6:63–67.
111 Yeger H, Youngson C, Cutz E. *In vitro* models for isolation, culture and characterization of pulmonary neuroendocrine cells and neuroepithelial bodies. In: Cutz E (ed). Cellular and Molecular Biology of Airway Chemoreceptors. Landes Bioscience: Austin, 1997, pp 47–70.
112 Youngson C, Nurse C, Yeger H, *et al.* Oxygen sensing in airway chemoreceptors. Nature 1993;365:153–155.
113 Onganer PU, Djamgoz MB. Small-cell lung cancer (human): potentiation of endocytic membrane activity

by voltage-gated Na(+) channel expression *in vitro*. J Membr Biol 2005;204:67–75.
114 Onganer PU, Seckl MJ, Djamgoz MB. Neuronal characteristics of small-cell lung cancer. Br J Cancer 2005;93:1197–1201.
115 Youngson C, Nurse C, Yeger H, *et al.* Immunocytochemical localization on O2-sensing protein (NADPH oxidase) in chemoreceptor cells. Microsc Res Tech 1997;37:101–106.
116 Fu XW, Wang D, Nurse CA, *et al.* NADPH oxidase is an O2 sensor in airway chemoreceptors: evidence from K+ current modulation in wild-type and oxidase-deficient mice. Proc Natl Acad Sci USA 2000;97:4374–4379.
117 Kazemian P, Stephenson R, Yeger H, *et al.* Respiratory control in neonatal mice with NADPH oxidase deficiency. Respir Physiol 2001;126:89–101.
118 Thompson RJ, Farragher SM, Cutz E, *et al* Developmental regulation of O(2) sensing in neonatal adrenal chromaffin cells from wild-type and NADPH-oxidase-deficient mice. Pflugers Arch 2002;444: 539–548.
119 Archer SL, Reeve HL, Michelakis E, *et al.* O2 sensing is preserved in mice lacking the gp91 phox subunit of NADPH oxidase. Proc Natl Acad Sci USA 1999;96: 7944–7949.
120 Peers C. Oxygen-sensitive ion channels. Trends Pharmacol Sci 1997;18:405–408.
121 Skogvall S, Korsgren M, Grampp W. Evidence that neuroepithelial endocrine cells control the spontaneous tone in guinea pig tracheal preparations. J Appl Physiol 1999;86:789–798.
122 Kemp PJ, Lewis A, Hartness ME, *et al.* Airway chemotransduction: from oxygen sensor to cellular effector. Am J Respir Crit Care Med 2002; 166:17S–24S.
123 Ward JPT, Aaronson PI. Mechanisms of hypoxic pulmonary vasoconstriction: can anyone be right? Respir Physiol 1999;115:261–271.
124 Gonzalez C, Almaraz L, Obeso A, *et al.* Carotid body chemoreceptors: from natural stimuli to sensory discharges. Physiol Rev 1994;74:829–898.
125 Seuwen K, Pouyssegur J. Serotonin as a growth factor. Biochem Pharmacol 1990;39:985–990.
126 Vicaut E, Laemmel E, Stucker O. Impact of serotonin on tumour growth. Ann Med 2000;32:187–194.
127 Fu XW, Nurse CA, Wong V, *et al.* Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. J Physiol 2002;539:503–510.
128 Fu XW, Wang D, Pan J, *et al.* Neuroepithelial bodies in mammalian lung express functional serotonin type 3 receptor. Am J Physiol Lung Cell Mol Physiol 2001;281:L931–L940.
129 Fu XW, Nurse CA, Cutz E. Expression of functional purinergic receptors in pulmonary neuroepithelial bodies and their role in hypoxia chemotransmission. Biol Chem 2004;385:275–284.
130 Bertrand PP, Bornstein JC. ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. J Neurosci 2002;22:4767–4775.
131 Fu XW, Nurse CA, Farragher SM, *et al.* Expression of functional nicotinic acetylcholine receptors in neuroepithelial bodies of neonatal hamster lung. Am J Physiol Lung Cell Mol Physiol 2003;285: L1203–L1212.
132 Schuller HM, Plummer III HK, Jull BA. Receptor-mediated effects of nicotine and its nitrosated derivative NNK on pulmonary neuroendocrine cells. Anat Rec A Discov Mol Cell Evol Biol 2003;270: 51–58.
133 Ebina M, Hoyt Jr RF, McNelly NA, *et al.* Effects of hydrogen and bicarbonate ions on endocrine cells in fetal rat lung organ cultures. Am J Physiol 1997;272: L178–L186.
134 Van LA, Lauweryns JM. Postnatal development of the pulmonary neuroepithelial bodies in various animal species. J Auton Nerv Syst 1997;65:17–24.
135 Bolle T, Van LA, Lauweryns JM. Stereological estimation of number and volume of pulmonary neuroepithelial bodies (NEBs) in neonatal hamster lungs. Microsc Res Tech 1999;44:190–194.
136 Bolle T, Lauweryns JM, Lommel AV. Postnatal maturation of neuroepithelial bodies and carotid body innervation: a quantitative investigation in the rabbit. J Neurocytol 2000;29:241–248.
137 Becker KL, Gazdar AF. The Endocrine Lung in Health and Disease. WB Saunders Company: Philadelphia, 1984.
138 Gosney JR. Pulmonary Endocrine Pathology: Endocrine Cells and Endocrine Tumours of the Lung. Butterworth-Heinemann Ltd.: Oxford, 1992.
139 Mabry M, Nelkin BD, Falco JP, *et al.* Transitions between lung cancer phenotypes—implications for tumor progression. Cancer cells 1991;3:53–58.
140 Boers JE, den Brok JL, Koudstaal J, *et al.* Number and proliferation of neuroendocrine cells in normal human airway epithelium. Am J Respir Crit Care Med 1996;154:758–763.
141 Aita K, Doi M, Tanno K, *et al.* Quantitative analysis of pulmonary neuroendocrine cell distribution of the fetal small airways using double-labeled immunohistochemistry. Forensic Sci Int 2000;113: 183–187.
142 Ito T, Nakatani Y, Nagahara N, *et al.* Quantitative study of pulmonary endocrine cells in anencephaly. Lung 1987;165:297–304.
143 Weichselbaum M, Sparrow MP, Hamilton EJ, *et al.* A confocal microscopic study of solitary pulmonary neuroendocrine cells in human airway epithelium. Respir Res 2005;6:115.
144 Gosney JR, Sissons MC, O'Malley JA. Quantitative study of endocrine cells immunoreactive for calcitonin in the normal adult human lung. Thorax 1985;40:866–869.
145 Gosney JR. Neuroendocrine cell populations in postnatal human lungs: minimal variation from childhood to old age. Anat Rec 1993;236:177–180.
146 Gosney JR, Sissons MC, Allibone RO. Neuroendocrine cell populations in normal human lungs: a quantitative study. Thorax 1988;43:878–882.
147 Nylen ES, Snider Jr RH, Thompson KA, *et al.* Pneumonitis-associated hyperprocalcitoninemia. Am J Med Sci 1996;312:12–18.
148 Nylen ES, O'Neill W, Jordan MH, *et al.* Serum procalcitonin as an index of inhalation injury in burns. Horm Metab Res 1992;24:439–443.
149 Cullen A, Van Marter LJ, Allred EN, *et al.* Urine bombesin-like peptide elevation precedes clinical evidence of bronchopulmonary dysplasia. Am J Respir Crit Care Med 2002;165:1093–1097.
150 Sunday ME, Yoder BA, Cuttitta F, *et al.* Bombesin-like peptide mediates lung injury in a baboon model of

bronchopulmonary dysplasia. J Clin Invest 1998; 102:584–594.
151 Subramaniam M, Sugiyama K, Coy DH, *et al.* Bombesin-like peptides and mast cell responses: relevance to bronchopulmonary dysplasia? Am J Respir Crit Care Med 2003;168:601–611.
152 Adriaensen D, Timmermans JP. Purinergic signalling in the lung: important in asthma and COPD? Curr Opin Pharmacol 2004;4:207–214.
153 Tsukiji J, Sango K, Udaka N, *et al.* Long-term induction of beta-CGRP mRNA in rat lungs by allergic inflammation. Life Sci 2004;76:163–177.
154 Stanislawski EC, Hernandez-Garcia J, de la Mora-Torres MC, *et al.* Lung neuroendocrine structures. Topography, morphology, composition and relation with intrinsic asthma (non-immune). Arch Invest Med (Mex) 1981;12:559–577.
155 Bousbaa H, Fleury-Feith J. Effects of a long-standing challenge on pulmonary neuroendocrine cells of actively sensitized guinea pigs. Am Rev Respir Dis 1991;144:714–717.
156 Gosney JR. The endocrine lung and its response to hypoxia. Thorax 1994;49:S25–S26.
157 Aita K, Doi M, Tanno K, *et al.* Pulmonary neuroendocrine cell distribution in sudden infant death syndrome. Leg Med (Tokyo) 2000;2:134–142.
158 Cutz E, Perrin DG, Hackman R, *et al.* Maternal smoking and pulmonary neuroendocrine cells in sudden infant death syndrome. Pediatrics 1996; 98:668–672.
159 Perrin DG, McDonald TJ, Cutz E. Hyperplasia of bombesin-immunoreactive pulmonary neuroepithelial bodies in sudden infant death syndrome. Pediatr Pathol 1991;11:431–447.
160 Gillan JE, Curran C, O'Reilly E, *et al.* Abnormal patterns of pulmonary neuroendocrine cells in victims of sudden infant death syndrome. Pediatrics 1989;84:828–834.
161 Cutz E, Ma TK, Perrin DG, *et al.* Peripheral chemoreceptors in congenital central hypoventilation syndrome. Am J Respir Crit Care Med 1997;155:358–363.
162 Gosney JR. Pulmonary endocrine cells in native Peruvian guinea-pigs at low and high altitude. J Comp Pathol 1990;102:7–12.
163 Taylor W. Pulmonary argyrophil cells at high altitude. J Pathol 1977;122:137–144.
164 Gosney JR. Pulmonary neuroendocrine cell system in pediatric and adult lung disease. Microsc Res Tech 1997;37:107–113.
165 Aguayo SM, Miller YE, Waldron Jr JA, *et al.* Brief report: idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells and airways disease [see comments]. N Engl J Med 1992;327:1285–1288.
166 Travis WD, Brambilla E, Müller-Hermelink HK, *et al.* Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart. IARC Press: Lyon, 2004.
167 Felton WL, Liebow AA, Lindskog GE. Peripheral and multiple bronchial adenomas. Cancer 1953;6: 555–567.
168 Miller RR, Muller NL. Neuroendocrine cell hyperplasia and obliterative bronchiolitis in patients with peripheral carcinoid tumors. Am J Surg Pathol 1995;19:653–658.
169 Fessler MB, Cool CD, Miller YE, *et al.* Idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells in a patient with acromegaly. Respirology 2004;9:274–277.
170 Cohen AJ, King Jr TE, Gilman LB, *et al.* High expression of neutral endopeptidase in idiopathic diffuse hyperplasia of pulmonary neuroendocrine cells. Am J Respir Crit Care Med 1998;158: 1593–1599.
171 Alshehri M, Cutz E, Banzhoff A, *et al.* Hyperplasia of pulmonary neuroendocrine cells in a case of childhood pulmonary emphysema. Chest 1997;112: 553–556.
172 Deterding RR, Pye C, Fan LL, *et al.* Persistent tachypnea of infancy is associated with neuroendocrine cell hyperplasia. Pediatr Pulmonol 2005;40: 157–165.
173 Gosney JR. Diffuse idiopathic pulmonary neuroendocrine cell hyperplasia as a precursor to pulmonary neuroendocrine tumors. Chest 2004;125:108S.
174 Poletti V, Zompatori M, Cancellieri A. Clinical spectrum of adult chronic bronchiolitis. Sarcoidosis Vasc Diffuse Lung Dis 1999;16:183–196.
175 Ruffini E, Bongiovanni M, Cavallo A, *et al.* The significance of associated pre-invasive lesions in patients resected for primary lung neoplasms. Eur J Cardiothorac Surg 2004;26:165–172.
176 Gmelich JT, Bensch KG, Liebow AA. Cells of Kultchizky type in bronchioles and their relation to the origin of peripheral carcinoid tumor. Lab Invest 1967;17:88.
177 Bensch KG, Corrin B, Pariente R, *et al.* Oat-cell carcinoma of the lung: its origin and relationship to bronchial carcinoid. Cancer 1968;22:1163–1172.
178 Hattori S, Matsuda M, Tateishi R, *et al.* Oat-cell carcinoma of the lung containing serotonin granules. Jpn J Cancer Res 1968;59:123–129.
179 Sheard JD, Gosney JR. Endocrine cells in tumour-bearing lungs. Thorax 1996;51:721–726.
180 Gould VE, Linnoila RI, Memoli VA, *et al.* Neuroendocrine components of the bronchopulmonary tract: hyperplasias, dysplasias, and neoplasms. Lab Invest 1983;49:519–537.
181 Sunday ME, Willett CG, Graham SA, *et al.* Histochemical characterization of non-neuroendocrine tumors and neuroendocrine cell hyperplasia induced in hamster lung by 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone with or without hyperoxia. Am J Pathol 1995;147:740–752.
182 Witschi H, Breider MA, Schuller HM. Failure of ozone and nitrogen dioxide to enhance lung tumor development in hamsters. Cambridge, MA, Health Effects Institute. Res Rep 1993;60:1–38.
183 Linnoila RI, Nettesheim P, DiAugustine RP. Lung endocrine-like cells in hamsters treated with diethylnitrosamine: Alterations *in vivo* and in cell culture. Proc Natl Acad Sci USA 1981;78:5170–5174.
184 Linnoila RI. Effects of diethylnitrosamine on lung neuroendocrine cells. Exp Lung Res 1982;3:225–236.
185 Schuller HM, Becker KL, Witschi HP. An animal model for neuroendocrine lung cancer. Carcinogenesis 1988;9:293–296.
186 Miller MS, Baxter JL, Moore JW, *et al.* Molecular characterization of neuroendocrine lung tumors induced in hamsters by treatment with nitrosamines and hyperoxia. Int J Oncol 1994;4:5–12.
187 Schuller HM, Witschi HP, Nylen E, *et al.* Pathobiology of lung tumors induced in hamsters by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and the modulating effect of hyperoxia. Cancer Res 1990;50:1960–1965.

188 Sunday ME, Haley KJ, Sikorski K, *et al.* Calcitonin driven v-Ha-ras induces multilineage pulmonary epithelial hyperplasias and neoplasms. Oncogene 1999;18:4336–4347.
189 Minna JD, Fong K, Zochbauer-Muller S, *et al.* Molecular pathogenesis of lung cancer and potential translational applications. Cancer J 2002;8:S41–S46.
190 Meuwissen R, Linn SC, Linnoila RI, *et al.* Induction of small cell lung cancer by somatic inactivation of both Trp53 and Rb1 in a conditional mouse model. Cancer Cell 2004;4:181–189.
191 Williams BO, Remington L, Albert DM, *et al.* Cooperative tumorigenic effects of germline mutations in Rb and p53. Nat Genet 1994;7:480–484.
192 Linnoila RI, Aisner SC. Pathology of lung cancer: an exercise in classification. In: Johnson B, Johnson D (eds). Lung Cancer. Wiley-Liss: New York, 1995, pp 73–95.
193 Meuwissen R, Berns A. Mouse models for human lung cancer. Genes Dev 2005;19:643–664.
194 Kaye FJ. Molecular biology of lung cancer. Lung Cancer 2001;34:S35–S41.
195 Classon M, Dyson N. p107 and p130: versatile proteins with interesting pockets. Exp Cell Res 2001;264:135–147.
196 Wikenheiser-Brokamp KA. Rb family proteins differentially regulate distinct cell lineages during epithelial development. Development 2004;131:4299–4310.
197 Anbazhagan R, Tihan T, Bornman DM, *et al.* Classification of small cell lung cancer and pulmonary carcinoid by gene expression profiles. Cancer Res 1999;59:5119–5122.
198 Linnoila RI, Mulshine J, Steinberg S, *et al.* Neuroendocrine differentiation in endocrine and non-endocrine lung carcinomas. Amer J Clin Pathol 1988;90:1–12.
199 McDowell EM, Trump BF. Pulmonary small cell carcinoma showing tripartite differentiation in individual cells. Hum Pathol 1981;12:286.
200 Linnoila RI. Spectrum of neuroendocrine differentiation in lung cancer cell lines featured by cytomorphology, markers, and their corresponding tumors. J Cell Biochem Suppl 1996;24:92–106.
201 Linnoila RI, Piantadosi S, Ruckdeschel JC. Impact of neuroendocrine differentiation in non-small cell lung cancer. The LCSG experience. Chest 1994;106:367S–371S.
202 Linnoila RI, Naizhen X, Meuwissen R, *et al.* Mouse lung neuroendocrine carcinomas: distinct morphologies, same transcription factors. Exp Lung Res 2005;31:37–55.
203 Bhattacharjee A, Richards WG, Staunton J, *et al.* Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci USA 2001;98:13790–13795.
204 Osada H, Tatematsu Y, Yatabe Y, *et al.* ASH1 gene is a specific therapeutic target for lung cancers with neuroendocrine features. Cancer Res 2005;65:10680–10685.
205 Mukherjee AB, Kundu GC, Mantile-Selvaggi G, *et al.* Uteroglobin: a novel cytokine? Cell Mol Life Sci 1999;55:771–787.
206 Chiba Y, Kurotani R, Kusakabe T, *et al.* Uteroglobin-related protein 1 expression suppresses allergic airway inflammation in mice. Am J Respir Crit Care Med, in press. Available from: http://ajrccm.atsjournals.org/cgi/reprint/200503-456OCv1.
207 Adriaensen D, Scheuermann DW. Neuroendocrine cells and nerves of the lung. Anat Rec 1993;236:70–85.
208 Neuhuber WL. Lung sensors: complex functions require complex structures. Am J Respir Cell Mol Biol 2003;28:265–266.
209 Miele L, Osborne B. Arbiter of differentiation and death: Notch signaling meets apoptosis. J Cell Physiol 1999;181:393–409.