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# Bromodeoxyuridine (BrdU)-label-retaining cells in mouse terminal bronchioles

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**Summary.** Adult male mice were continuously treated with bromodeoxyuridine (BrdU) for 1, 2, or 4 weeks by an osmotic pump. To detect BrdU-label-retaining cells (LRCs), putative progenitor/stem cells, other animals were continuously treated with BrdU for 2 weeks, and were then kept without any treatments for 2, 6, or 18 months. The lungs were fixed with 4% paraformaldehyde, and were paraffin-embedded. We observed terminal bronchioles with BrdU immunostaining alone or with BrdU immunostaining accompanying immunostaining for Clara cell secretory protein (CCSP), forkhead box protein J1 (FoxJ1), or calcitonin gene-related peptide (CGRP).

The average incidences of BrdU-incorporated cells in the terminal bronchioles after 1, 2, and 4 weeks of continuous BrdU infusion were 6.2%, 11.9%, and 23.1%, respectively. Most BrdU-incorporated cells in these periods were CCSP-immunoreactive (91.7%, 91.3%, and 88.2%, respectively), which means progenitor function of Clara cells. FoxJ1-immunoreactive BrdU-incorporated cells were fewer (5.4%, 3.0%, 2.7%, respectively). The average incidences of BrdU-LRCs in the terminal bronchioles after 2, 6, and 18 months were 7.2%, 4.3, and 2.7%, respectively. Most BrdU-LRCs were CCSP-immunoreactive (91.0%, 92.7%, and 89.6%, respectively), and FoxJ1-immunoreactive BrdU-LRCs were fewer (6.0%, 5.7%, and 2.1%, respectively). CGRP-positive BrdU-incorporated cells were occasional. CGRP-positive BrdU-LRCs were detected in 17.6% of neuroepithelial bodies (NEBs) at 2 months, but disappeared at 6 months. BrdU-positive stem cell candidates, which locate at the brochiolo-alveolar duct junction or cover NEB, were few throughout this study.

In conclusion, in the lungs treated only with BrdU, CCSP-immunoreactive cells are important to maintain homeostasis in the terminal bronchiolar epithelium.

**Key words:** BrdU-label-retaining cell, Lung, Clara cell secretory protein, FoxJ1, Calcitonin-gene related peptide

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## Introduction

In the adult mouse lung, the airway (bronchial and bronchiolar) and alveolar epithelia contain various cells, and Clara cells are the most frequent cell in the bronchiolar epithelium, followed by ciliated cells, while neuroendocrine cells are rare. The turnover of the mouse airway epithelium has been shown to be slow, although the turnover time has varied largely depending on the inflammatory or injury status, strains, and ages of the animals examined. In the bronchioles, Clara cells were shown to serve as a progenitor cell in injured bronchial epithelia using various methods, including autoradiographical studies with transmission electron microscopy (Evans et al., 1976, 1978). However, under normal conditions, the proliferative activity of bronchiolar epithelial cells such as Clara cells is low, with the labeling index of tritiated thymidine varying by less than 1% (Divertie et al., 1968; Kauffman, 1971). The BrdU labeling index was shown to be similar to that of tritiated thymidine (Ito et al., 1994; Okudela et al., 1999).

Regarding stem cells and stem cell niches in the mouse lung, basal cells can function as classical stem cells in the large airways (trachea and main bronchi), and self-renew and produce ciliated and secretory cells (Rawlins and Hogan, 2006). In the more distal airways,

in which there are no basal cells, previous studies have suggested that subpopulations of Clara cells near neuroepithelial bodies (variant Clara cells) in specific micro-environments can self-renew and give rise to different cell types after injury (Stripp et al., 1995; Hong et al., 2001). The transitional region between the terminal bronchiole and the alveolus is known as the bronchiolo-alveolar duct junction (BADJ), and Kim et al. (2005) demonstrated the significance of bronchioloalveolar stem cells (BASCs), which have dual differentiation markers for Clara cells and alveolar type 2 cells at the BADJ. BASCs propagate after naphthalene injury, and have been demonstrated to differentiate to alveolar cells and bronchiolar cells *in vitro*. However, the role of lung epithelial stem/progenitor cells in the maintenance and repair of the adult lung has still not been fully clarified. Giangreco et al. (2009) recently showed using green-fluorescent protein chimeric mice that randomly distributed progenitor cells maintained normal epithelial homeostasis and, in contrast, stem cells such as those at the BADJ and variant Clara cells adjacent to neuroepithelial bodies (NEBs) appear to restore injured airway epithelia.

The tritiated thymidine or BrdU-label-retaining cell (LRC) assay is an approach used to identify the location of epithelial stem cells or proliferation locations both *in situ* and *in vivo* (Liu et al., 2006). A few studies have used this assay to study lung tissues. In the larger airways, LRCs appear to cluster in the intercartilage regions, and these non-epithelial cells are supposed to be components of a special niche (Borthwick et al., 2001). A naphthalene injury study revealed that a label-retaining subpopulation of Clara cells is located adjacent to NEBs in the bronchi/bronchioles or at the BADJs (Stripp et al., 1995; Hong et al., 2001; Giangreco et al., 2002). The latter may be the same as putative BASCs (Kim et al., 2005).

A single injection of BrdU can only label a few cells in normal adult lungs (Ito et al., 1994; Okudela et al., 1999); therefore, it is difficult to chase BrdU-LRCs after a long observation period. In the present study, at first, we treated mice continuously with BrdU up to 4 weeks to discover the profile of the BrdU-labeled terminal bronchiolar cells with immunohsitochemistry for bronchiolar epithelial cell specific markers, and seek the proper time period of continuous labeling of BrdU for the following BrdU-LRC study. Next, the animals; pretreated with continuous BrdU labeling for 2 weeks, were kept for 2,6 and 18 months without any treatments, and BrdU-LRCs in the terminal bronchiole were detected. We clarified modulations in the number of BrdU-LRCs, their localization, and cell types with double immunostainings for BrdU/CCSP, BrdU/FoxJ1 and BrdU/CGRP to find out candidate stem cell population in the adult mouse terminal bronchiole and its localization.

## Materials and methods

### *Animal and BrdU labeling*

Male ICR mice (CRl:CD1; 10 week old) were purchased from Japan SLC (Shizuoka, Japan). They were kept with free access to water and food, and were maintained on a 12-h daylight/dark cycle under pathogen-free conditions. This study was approved by the Animal Care Committee of Kumamoto University.

To label S-phase cells and their descendants (Fig. 1), an osmotic pump (ALZET micro-osmotic pump Model 1002, Durect Corporation, Cupertino, CA, USA) was planted in the subcutaneous tissue of the mouse back under pentobarbital anesthesia. The pump was filled with sterile 60 mg/ml 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) dissolved in 50% DMSO, and according to the manufacturer's instructions, an average of 0.21 $\mu$l/hr BrdU solution was delivered. To obtain mouse lungs that were continuously labeled with BrdU for 1 or 2 weeks, animals were sacrificed by an intraperitoneal injection of an overdose of pentobarbital after 1 or 2 weeks of the infusion. The

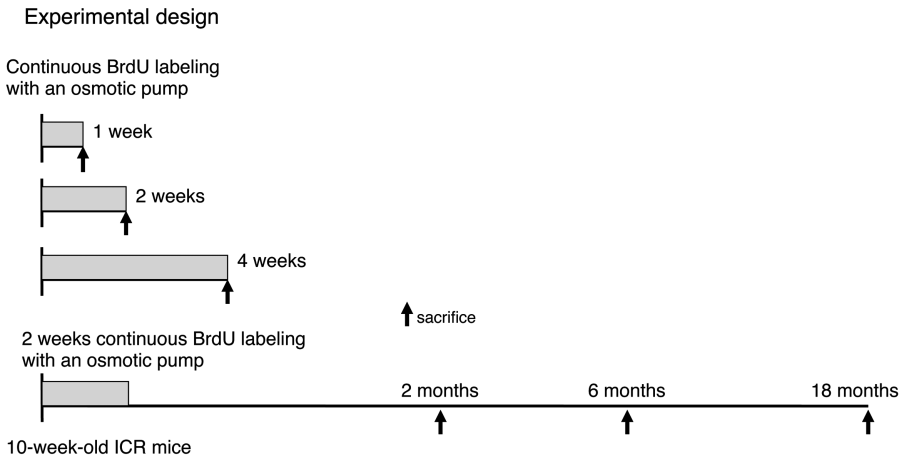



**Fig. 1.** Experimental design. ICR mice (4-5 animals in each experimental group) were treated with a continuous infusion of bromodeoxyuridine (BrdU) for 1, 2, or 4 weeks with an osmotic pump, and were then sacrificed. To detect BrdU-label-retaining cells (BrdU-LRCs), the mice were treated with a continuous infusion of BrdU for 2 weeks, and were sacrificed 2, 6, or 18 months after the removal of osmotic pumps(4-5 animals in each experimental group).

lungs were fixed with the inflation of phosphate-buffered 4% paraformaldehyde for one day, and were then embedded in paraffin. To obtain mouse lungs that were continuously labeled with BrdU for 4 weeks, the osmotic pump was replaced by another pump filled with the BrdU solution after 2 weeks, and the lungs were fixed with phosphate-buffered 4% paraformaldehyde after 4 weeks, followed by paraffin embedment.

To study BrdU-LRC, the osmotic pump was removed from mice after the BrdU infusion for 2 weeks under pentobarbital anesthesia, and animals were sacrificed 2, 6, or 18 months after the removal of the pump. The lungs were fixed by phosphate-buffered 4% paraformaldehyde, followed by paraffin embedment.

Mini-osmotic pump implantation and surgical procedures did not induce any pathological changes in the lungs.

### *Immunostaining for BrdU*

Paraffin-embedded sections were rehydrated with xylene and graded ethanol solutions. After being treated with 0.3% hydrogen peroxide, the sections were heated at 95°C for 40 min in antigen retrieval solution (pH8, Nichirei, Tokyo, Japan). After being treated with 2.5% normal goat serum (Vector Laboratories, Burlingame, CA) for 20 min, the sections were treated with rat anti-BrdU monoclonal antibody (BU1/75, Abcam, Cambridge, England) for 1 hr at room temperature. After washing, the sections were treated with anti-rat IgG conjugated with the HRP polymer (ImmPress Reagent, Vector) for 30 min at room temperature. The sections were further treated with diaminobenzidine-hydrogen peroxide solution, and counterstained with hematoxylin.

### *Double immunostaining for BrdU and either CCSP, FoxJ1, or CGRP*

We determined cell type of the BrdU-potitive cells using antibodies against CCSP for Clara cells, FoxJ-1 for ciliated cells, and CGRP for neuroendocrine cells (Rawlins and Hogan, 2006). Paraffin-embedded sections were rehydrated with xylene and graded ethanol solutions. After being treated with 0.3% hydrogen peroxide, the sections were heated at 95°C for 40 min in the antigen retrieval solution for CCSP immunostaining (DAKO REAL Target Retrieval Solution, pH 6, DAKO, Carpinteria, CA) or in the solution (pH 8, Nichirei) for FoxJ1 or CGRP immunostaining. After being treated with 4% skimmed milk in PBS for 20 min at room temperature, the sections were treated with goat anti-CCSP polyclonal antibody (Santa Cruz Biotechonolgy, Santa Cruz, CA), mouse ant-FoxJ1 monoclonal antibody (Santa Cruz), or goat anti-CGRP polyclonal antibody (Abcam) for 1hr at room temperature. After washing, the sections were treated with the secondary antibodies conjugated with the HRP polymer for the corresponding animal IgGs (for goat, MAXPO(G), Nichirei; for mouse, ImmPress Reagent, Vector) for 30 min at room temperature. A Vulcan Fast™ Red Choromogen Kit (Biocare Medical, Concord, CA) was used for CCSP and CGRP immunostainings, and a Vina Green™ Chromogen Kit (Biocare Medical) was used for FoxJ1 immunostaining. After washing in PBS three times, the sections were again heated at 95°C for 40 min in antigen retrieval solution (pH 8, Nichirei). After being treated with 2.5% normal goat serum (Vector) for 20 min, the sections were treated with rat anti-BrdU monoclonal antibody for 1hr at room temperature. After washing, the sections were treated with anti-rat IgG conjugated with the HRP-polymer (ImmPress Reagent, Vector) for 30min at room temperature. The sections were treated with diaminobenzidine-hydrogen peroxide solution, and counterstained with hematoxylin.

BrdU-incorporated cells were counted in the terminal bronchioles of the left lungs of each experimental group, and 5-10 bronchioles were analyzed from 4-5 animals in each experimental group. The labeling index was calculated as the total number of BrdU-positive terminal bronchiolar epithelial cells per the total number of terminal bronchiolar epithelial cells counted in each animal. The number of terminal bronchiolar cells counted is listed in Table 1. The incidence of BrdU-incorporated epithelial cells was calculated as positive cells/total cells counted in a terminal bronchiole, and the average and standard deviation were calculated from the summation of each incidence. We focused on the epithelial cell types of BrdU-incorporated cells in the terminal bronchioles with double immunostaining. We measured the incidence of CCSP-immunoreactive cells and FoxJ1- immunoreactive cells in all BrdU-incorporated bronchiolar cells.

Neuroepithelial bodies (NEBs), which are neuroendocrine cell clusters, were stained positively with CGRP immunostaining. We counted BrdU-labelled cells in an NEB, and calculated the incidence of BrdU-labeled cells in an NEB to clarify how often BrdU-labeled neuroendocrine cells were present and to find out their fate. Moreover, we focused on BrdU-positive variant Clara cells that were negative for CGRP and were located adjacent to an NEB, and calculated the incidence of BrdU-incorporated variant Clara cells in an NEB.

In addition, we focused on the localization of BrdU-positive cells in the terminal bronchiolar epithelium, especially localization of cells related to the BADJ. We selected 12-25 terminal bronchioles, including BrdU-positive cells, in each mouse, and counted BrdU-positive bronchiolar epithelial cells in the five cells adjacent to the BADJ, those in the next 5 proximal cells, and those in 5 even more proximal cells (Fig. 9).

The mean ± SD for each experimental group (for each recovery; n=4-5) is expressed in bar graphs. Differences between means were considered significant when $P<0.05$, as determined by the unpaired Student's t test. The Spearman's correlation coefficient was used for continuous BrdU labeling to determine correlations between the labeling index and BrdU infusion period.

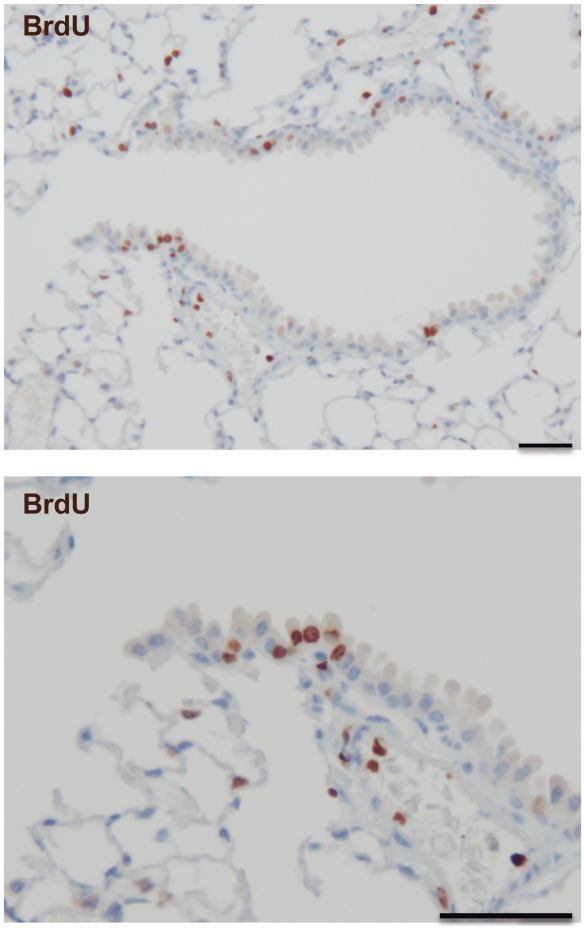
## Results

### *BrdU-positive cells in the terminal bronchiolar epithelium after 1, 2, and 4 weeks of continuous BrdU labeling*

The average incidences of BrdU-incorporated cells in the terminal bronchioles after 1, 2, and 4 weeks of continuous BrdU labeling were 6.2%, 11.9%, and 23.1%, respectively (Fig. 2), and a significant correlation was observed between the labeling incidence and the observation periods (Y=5.626X+0.5635, R2=0.86068). Most BrdU-incorporated cells were CCSP-immunoreactive; 91.7%, 91.3%, and 88.2% in each observation period from 1 week to 4 weeks (Fig. 3), and there was a small proportion of FoxJ1-immunoreactive BrdU-incorporated cells; 5.4%, 3.0%, 2.7% in each observation period from 1 week to 4 weeks (Fig. 4).

### *BrdU-LRC in the terminal bronchiolar epithelium 2,6,and 18 months after 2 weeks of continuous BrdU labeling*

The average incidences of BrdU-LRCs in the mouse bronchiolar epithelium after 2, 6, and 18 months were 7.24%, 4.34, and 2.72%, respectively (Fig. 5). These



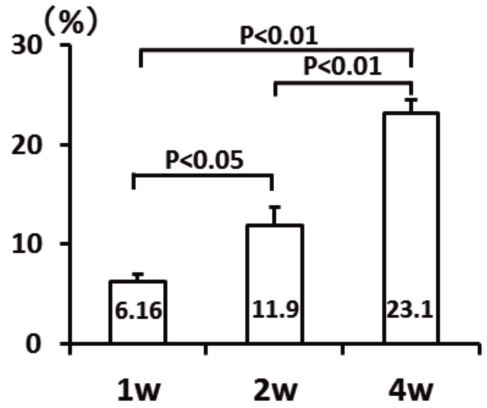




**Fig. 2.** BrdU immunostaining in the mouse terminal bronchiolar epithelium after 1,2, or 4 weeks of continuous BrdU labeling. **Left panel.** In the terminal bronchiole, BrdU-positive epithelial cells are occasionally seen after 2 weeks of continuous BrdU. Note that BrdU-labeled cells do not concentrate at the bronchiolo-alveolar duct junction (BADJ). Counterstained with hematoxylin. Bar: 50 $\mu$m. **Right panel.** Labeling indices of BrdU-positive cells in the terminal bronchiolar epithelium after 1, 2, or 4 weeks of continuous BrdU. Note that BrdU-positive epithelial cells increase significantly with the observation periods.

**Table 1.** List of the number of BrdU-positive cells and terminal bronchiolar epithelial cells counted in each mouse.

| Experimental Group | Number of animals | No. of BrdU-positive cells/No. of terminal bronchiolar epithelial cells counted in each mouse |
|---|---|---|
| 1 week BrdU labeling | 4 | (414/6493), (232/5134), (222/4162), (471/5604) |
| 2 weeks BrdU labeling | 5 | (239/1889), (293/2304), (80/1604), (327/2162), (376/2728) |
| 4 weeks BrdU labeling | 4 | (600/2386), (654/2598), (560/2455), (455/2385) |
| 2 months after 2 weeks BrdU | 5 | (136/1863), (196/2800), (73/1520), (76/853), (189/2304) |
| 6 months after 2 weeks BrdU | 4 | (31/1536), (222,2677), (141/2606), (24/1470) |
| 18 months after 2 weeks BrdU | 4 | (71/1770), (32/1429), (55/1367), (12/1921) |

incidences decreased with the observation periods, but were not significantly different after 6 months. Most BrdU-LRCs were CCSP-immunoreactive; 91.0%, 92.7%, and 89.6%, respectively (Fig. 6), and there was a small portion of FoxJ1-immunoreactive BrdU-LRCs; 6.0%, 5.7%, and 2.1%, respectively (Fig. 7).

*BrdU-incorporated cells in neuroepithelial bodies*

In this study, CGRP-positive BrdU-incorporated cells were occasionally seen in the NEBs, CGRP-positive cell clusters (Fig. 8). The incidences of BrdU-incorporated cells in NEBs were 20%, 31.8%, and 18.2%, respectively, in each observation period with the continuous labeling of BrdU from 1 week to 4 weeks (Table 2). CGRP-positive BrdU-LRCs were detected in 17.6% of NEBs at 2 months (Fig. 8), while no CGRP-positive LRCs were detected at 6 or 18 months (Table 2). Rare cells, which were defined as variant Clara cells as they covered NEBs and were negative for CGRP, were positive for BrdU (Table 2).

*Localization of BrdU-positive cells near the brochiolo-alveolar duct junction (BADJ)*

We focused on BrdU-incorporated cells in the BADJ, and examined whether the localization of BrdU-incorporated bronchiolar cells accumulated in the BADJ. We counted BrdU-positive bronchiolar epithelial cells in

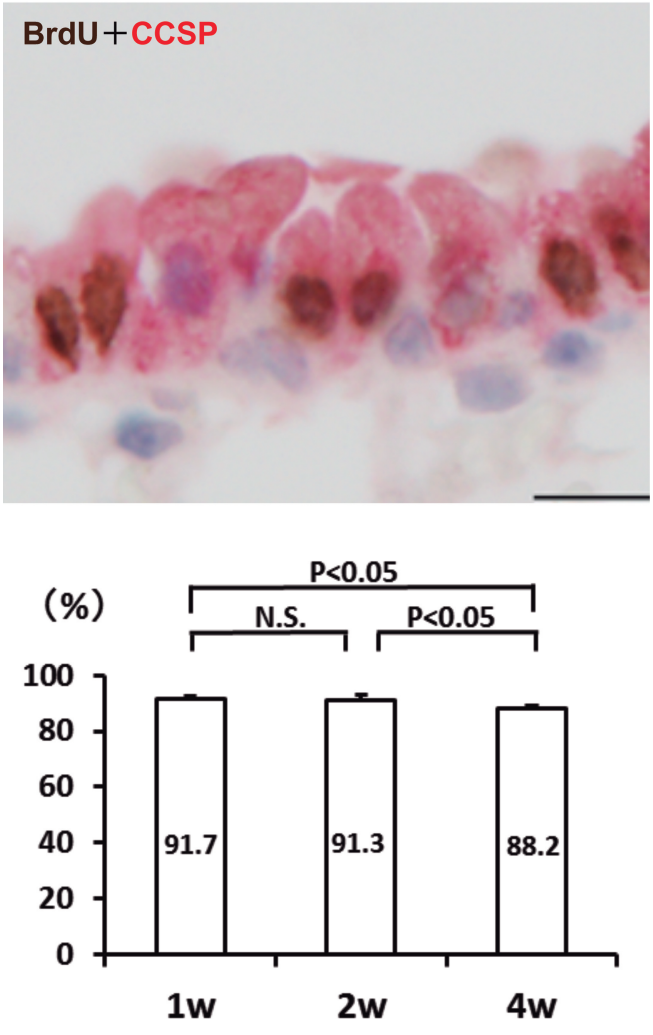



**Fig. 3.** BrdU-positive Clara cells in the mouse terminal bronchiolar epithelium after 1, 2, or 4 weeks of continuous BrdU labeling. Double immunostaining for BrdU (brown) and Clara cell secretory protein (CCSP, red) reveals many double positive cells (upper panel). Counterstained with hematoxylin. Bar: 10 $\mu$m. CCSP-positive cells are dominant in the BrdU-incorporated cells throughout the observation periods (lower panel).

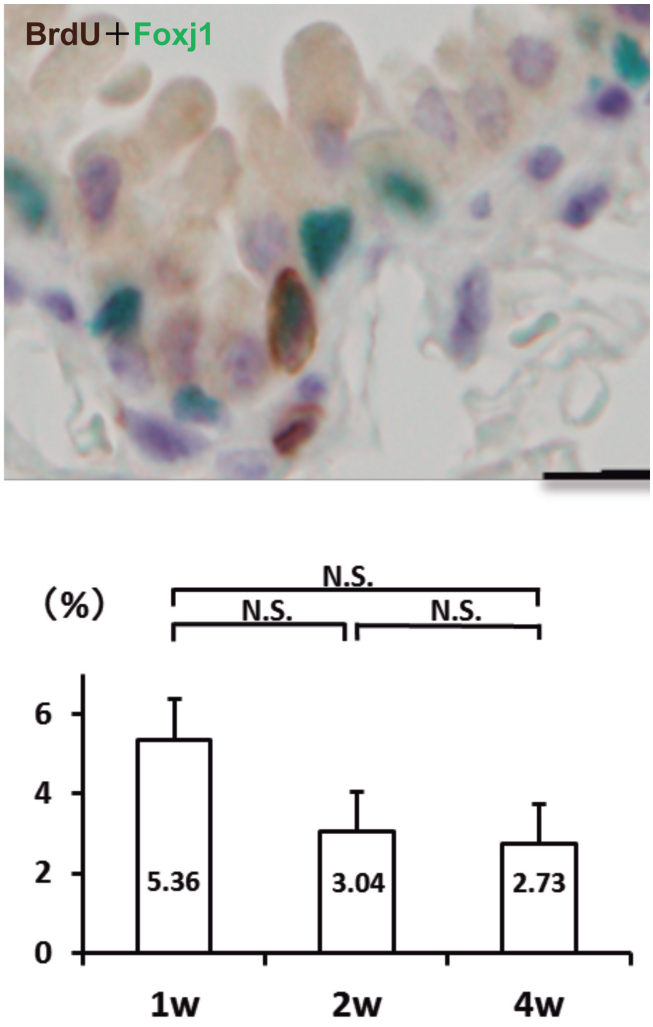



**Fig. 4.** BrdU-positive ciliated cells in the mouse terminal bronchiolar epithelium after 1, 2, or 4 weeks of continuous BrdU labeling. Double immunostaining for BrdU (brown) and forkhead box protein J1 (FoxJ1, green) reveals a few double positive cells (upper panel). Counterstained with hematoxylin. Bar: 10 $\mu$m. The incidence of double positive cells in all BrdU-positive cells was constantly low (lower panel).

five cells adjacent to the BADJ (zone A), those in the next 5 proximal cells (zone B), and those in 5 even more proximal 5 cells (zone C) (Fig. 9A). After 2 weeks of continuous BrdU labeling, the labeling indices were 22.9% (zone A), 15.6% (zone B), and 18.8% (zone C) (Fig. 9B), and no significant differences were found. In the BrdU-LRC study, a significant difference was detected only in BrdU labeling index of the distal terminal bronchioles at 6 months (Fig. 9C).

**Table 2.** BrdU-positive cells in neuroepithelial bodies (NEBs).

| Experimental group | Total number of CGRP- and BrdU-double positive cells in NEBs | Total number of variant Clara cells* in NEBs |
|---|---|---|
| 1 week BrdU labeling | 8/40 | 6/40 |
| 2 weeks BrdU labeling | 14/44 | 4/44 |
| 4 weeks BrdU labeling | 6/33 | 2/33 |
| 2 months after 2 weeks BrdU | 6/34 | 2/34 |
| 6 months after 2 weeks BrdU | 0/37 | 0/37 |
| 18 months after 2 weeks BrdU | 0/30 | 0/30 |

*: Variant Clara cells are defined as cells covering CGRP-positive NEBs and negative for CGRP.

## Discussion

In the present study, many bronchiolar epithelial cells were labeled with BrdU, which was continuously infused with osmotic pumps. The incidence of BrdU-incorporated bronchiolar epithelial cells increased depending on the infusion periods from 1 week to 4 weeks, and the labeling index at 4 weeks was almost twice that at 2 weeks, and four times that at 1 week. This suggests that most BrdU-incorporated cells in the bronchioles did not re-enter into the S-phase of the cell cycle during these periods, which could reflect a very slow turnover of the lung epithelial population (Plopper and Dungworth, 1987). Time needed for all terminal bronchiolar epithelial cells labeled with BrdU is calculated as 17.7 weeks in the present study. The time seems reasonable, as doubling time is 8 weeks in rat tracheal epithelium of 7-week old rat and the time

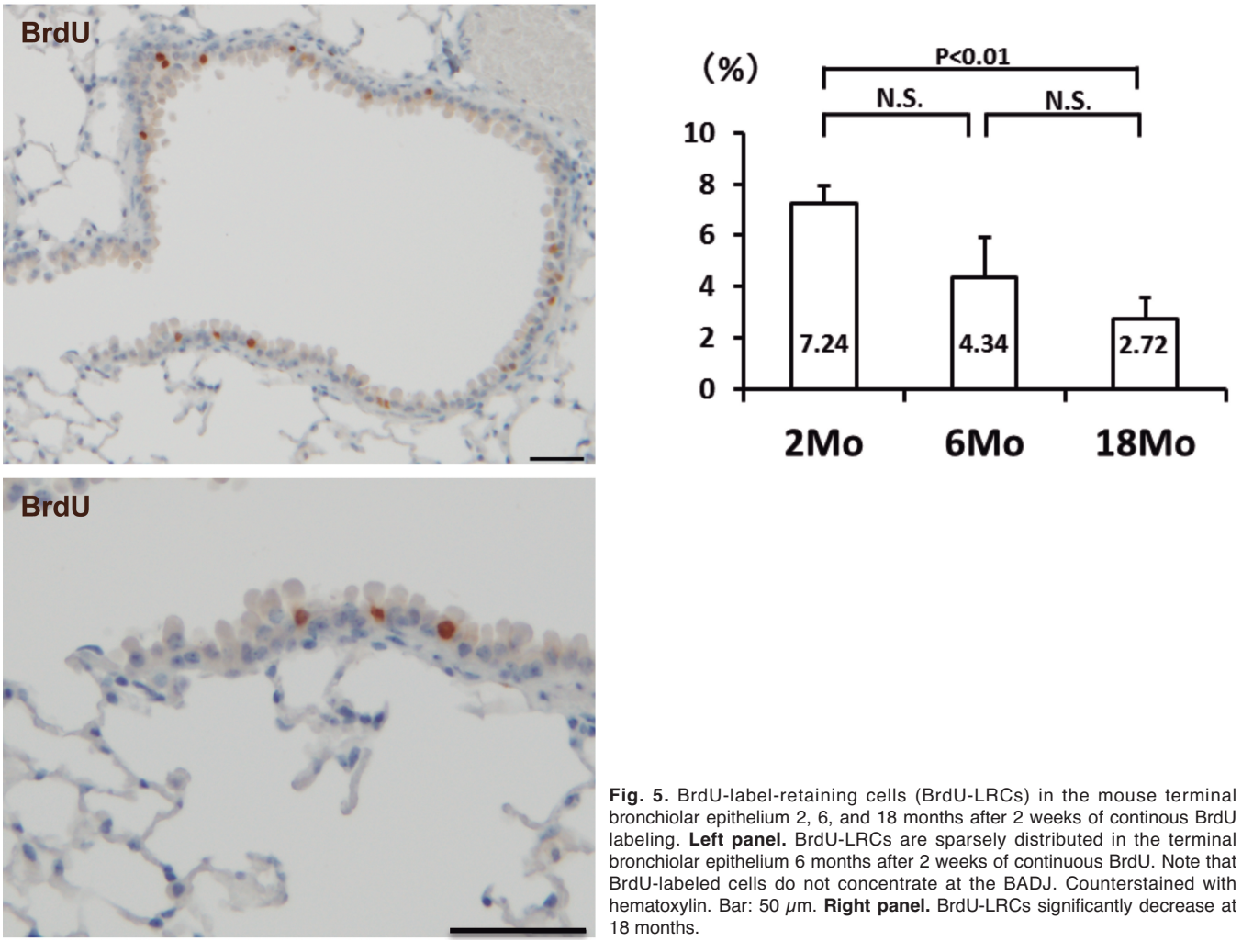



**Fig. 5.** BrdU-label-retaining cells (BrdU-LRCs) in the mouse terminal bronchiolar epithelium 2, 6, and 18 months after 2 weeks of continous BrdU labeling. **Left panel.** BrdU-LRCs are sparsely distributed in the terminal bronchiolar epithelium 6 months after 2 weeks of continuous BrdU. Note that BrdU-labeled cells do not concentrate at the BADJ. Counterstained with hematoxylin. Bar: 50 $\mu$m. **Right panel.** BrdU-LRCs significantly decrease at 18 months.

increases with age (Kauffman, 1980). The principal progenitor cell in the bronchiole following epithelial injury was previously shown to be Clara cells (Evans et al., 1976), and, in the present study, regardless of the observation periods, most BrdU-labeled bronchiolar epithelial cells were Clara cells that were positive for CCSP. A small proportion of BrdU-labeled cells were ciliated cells, which were positive for FoxJ1 (Rawlins et al., 2007). FoxJ-1-positive cells occupied about 3-5% of all BrdU-labeled bronchiolar epithelial cells during the 1-4-week continuous labeling periods. This observation suggests that most BrdU-incorporated Clara cells should become Clara cell predominantly after mitosis, and that 3-5% of the BrdU-incorporated Clara cells could enter into ciliated cell differentiation after mitosis. Since the occurrence of ciliated cells in the normal mouse bronchiolar epithelium is about one third (Plopper, 1983), the low level of BrdU labeling observed in the ciliated cells suggests that the turnover time of this cell population must be markedly longer than that of Clara cells under normal conditions. According to Evans' single pulse chase study (Evans et al., 1978), about 30% of tritiated thymidine-labeled cells in the bronchioles 1 week after injury were shown to be ciliated cells. The high tritiated thymidine-labeling of ciliated cells may suggest that the experimental conditions applied had strong injurious effects on ciliated cells.

Studies using the BrdU-LRC assay with longer time observation periods are necessary in cells or tissues with a long turnover time, such as the liver and lung. Long observation times for tissues with a rapid turnover time has been inadequate, and, for example, in the present study, no BrdU-LRC was detected in the small intestine.

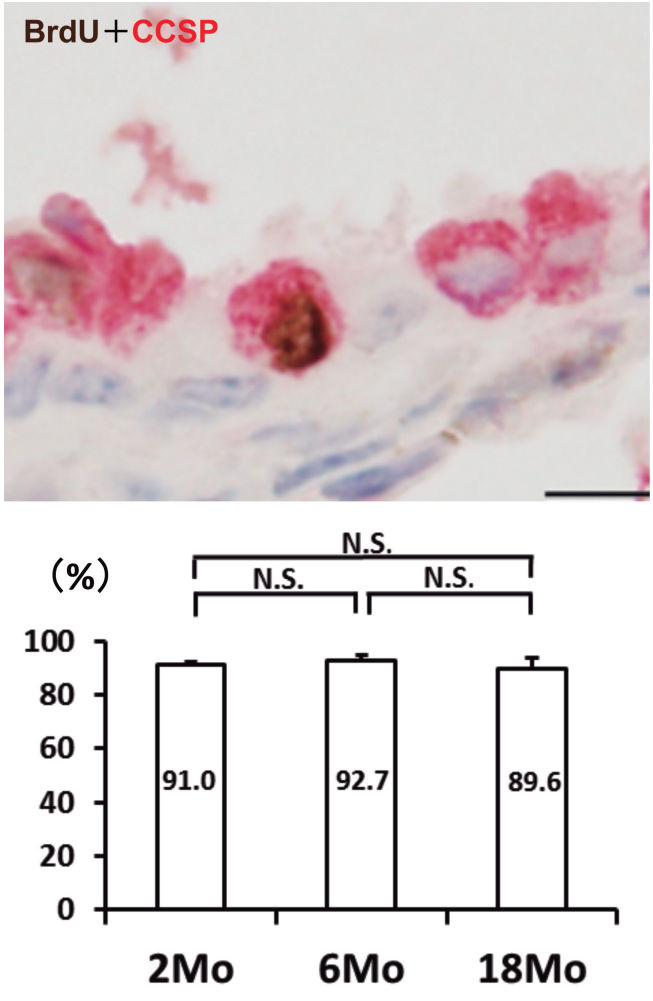



**Fig. 6.** BrdU-label-retaining Clara cells in the mouse terminal bronchiolar epithelium 2, 6, and 18 months after 2 weeks of continous BrdU labeling. Double immunostaining for BrdU (brown) and CCSP (red) reveals occasional double positive cells at 18 months (upper panel). Counterstained with hematoxylin. Bar: 10 $\mu$m. CCSP-positive cells are dominant in the BrdU-incorporated cells throughout the observation periods (lower panel).

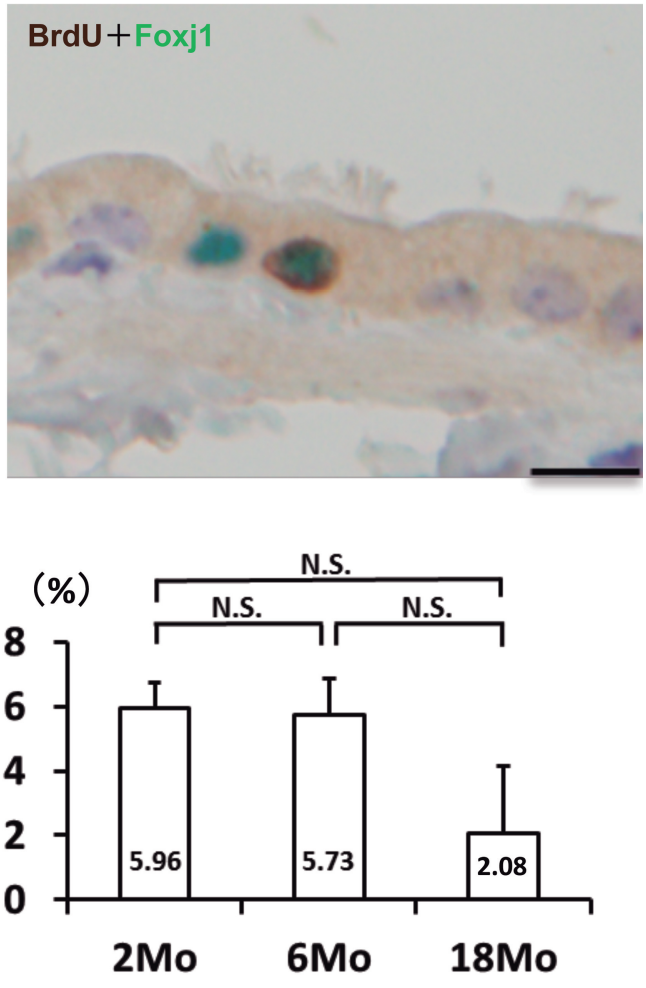



**Fig. 7.** BrdU-label-retaining ciliated cells in the mouse terminal bronchiolar epithelium 2, 6, and 18 months after 2 weeks of continous BrdU labeling. Double immunostaining for BrdU (brown) and FoxJ1 (green) revealed a few double positive cells at 6 months (upper panel). Counterstained with hematoxylin. Bar: 10 $\mu$m. The incidence of FoxJ1-positive BrdU-LRCs in all BrdU-LRCs was constantly low (lower panel).

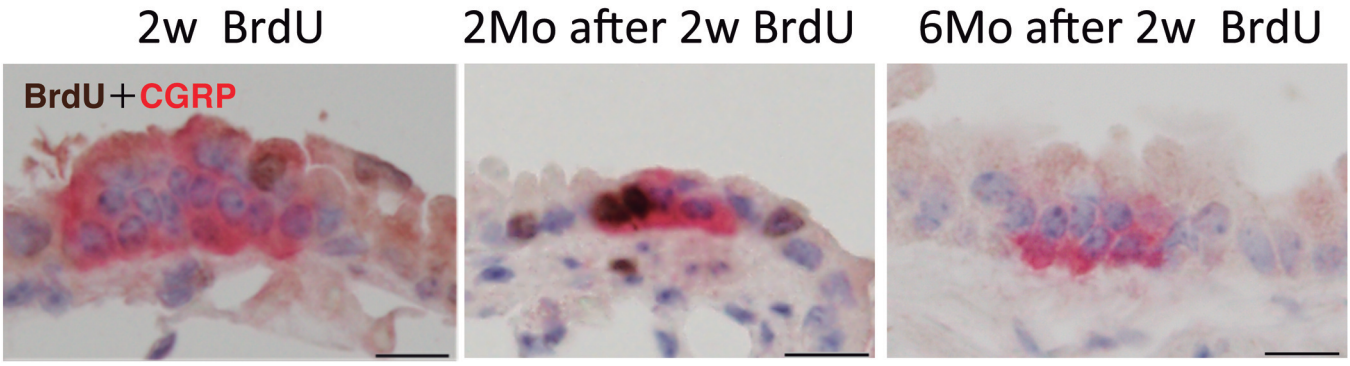



**Fig. 8.** Double immunostaining for BrdU and calcitonin gene-related peptide (CGRP) in the bronchiolar neuroepithelial bodies (NEBs). **Left.** Double positive cells for CGRP and BrdU were seen in an NEB of the mouse lung treated with BrdU for 2 weeks. **Middle.** CGRP-positive BrdU-LRCs were only detected in the mouse lung at 2 months, and note the two neighboring cells that were positive in an NEB. **Right.** No CGRP-positive BrdU-LRCs were detected after 6 months. Bar=10ìm.

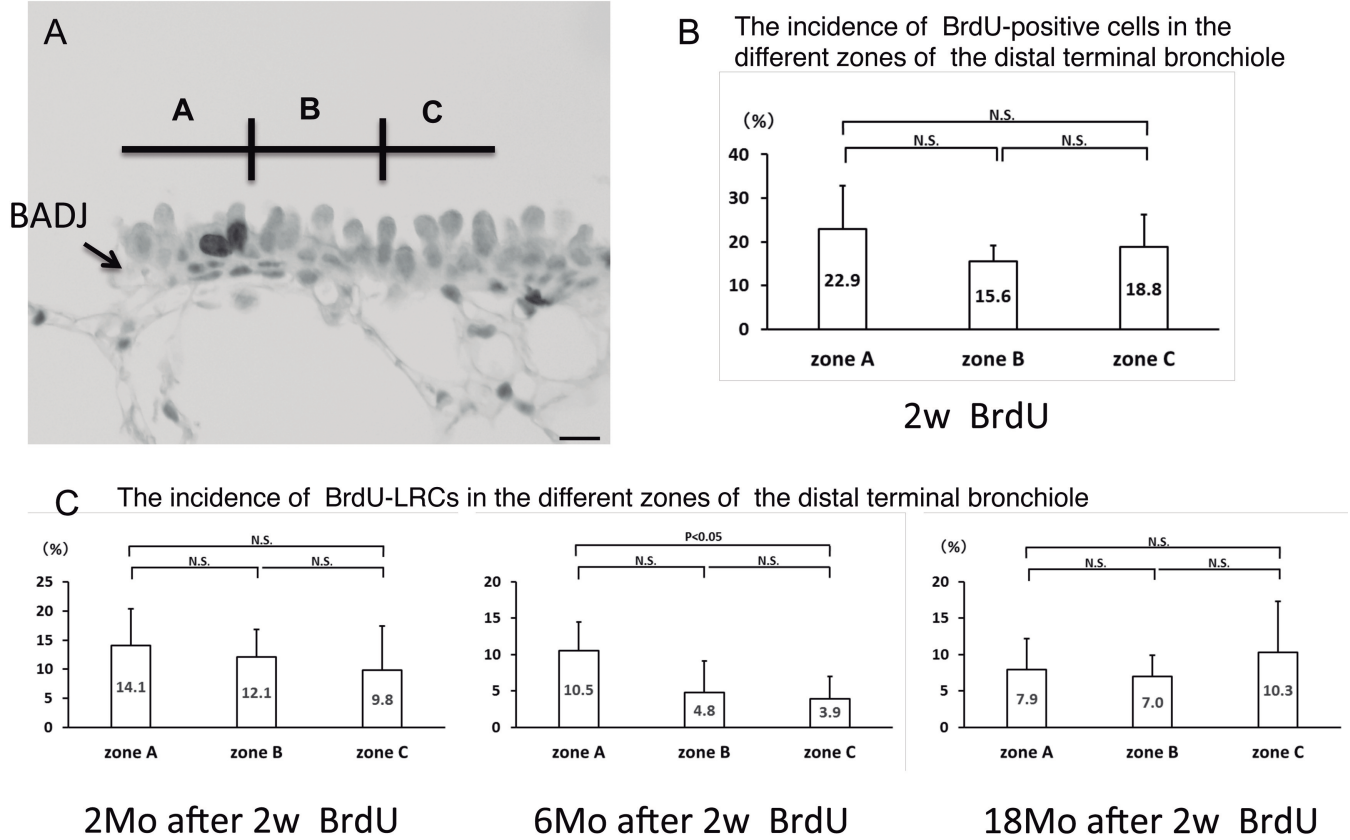



**Fig. 9.** BrdU-positive epithelial cells in the distal terminal bronchioles. A comparison of incidences among the different zones proximal to the BADJ. **A.** The distal terminal bronchiolar epithelium was subdivided into three zones: (zone A) 5 epithelial cells just adjacent to the BADJ, (zone B) 5 epithelial cells proximal to the A region, (zone C) 5 epithelial cells proximal to the B region, and the incidence of BrdU-positive cells were calculated in these regions. **B.** After 2 weeks of continuous BrdU labeling, no significant difference of the incidence of BrdU-positive cells was detected among the three zones. **C.** In the BrdU-LRC study, significant difference was detected only in BrdU labeling index of the distal terminal bronchioles at 6 months, but was not evident at 2 and 18 months.

However, in a long-term observation study, BrdU-LRC assays have revealed significant findings in various organs, such as the liver (Li et al., 2010), prostate (Tsujimura et al., 2002), and bladder (Kurzrock et al, 2008). These studies succeeded in showing the localization of BrdU-LRCs and their phenotypic characteristics. The number of BrdU-LRCs in the terminal bronchioles decreased depending on the observation period after 2 weeks of continuous BrdU labeling. The labeling index had not decreased significantly after 6 months, and this observation suggests that BrdU-LRCs are very quiescent cells in the cell cycle after 6 months, and do not undergo mitosis for one more year. Similar observations were demonstrated in the liver, in which the incidence of BrdU-LRC was not altered after 2 months. Most BrdU-LRCs were Clara cells, and only a small population was ciliated cells. In the present study, BrdU-LRCs in the bronchiolar epithelium were not concentrated at the BADJ nor around the NEBs, which suggests that cell turnover and replacement should proceed slowly and at random under normal physiological conditions. In the present study, differences in the functional and immunohistochemical properties of BrdU-label-retaining Clara cells and other Clara cells were not examined; however, Ling et al. (2006) proposed that an Oct4-positive Clara cell population may function as stem/progenitor cells in a lung injury model. In the present study, only a few BrdU-label-retaining ciliated cells were present throughout the observation period. In this context, some of the BrdU-label-retaining ciliated cells were visible after 2 months -from the 2 weeks BrdU infusion-, and based on the facts that cells remaining up to 18 months as ciliated cells are considered to be terminally differentiated cells (Rawlins et al., 2007) and that new production of ciliated cells from BrdU-label-retaining Clara cells in these 16 months may be small, thus the life span of BrdU-labeled ciliated cells appears to be long.

Although the proliferative response of pulmonary neuroendocrine cells (PNECs) has been reported under various experimental conditions (Ito, 1999), the cell kinetics of PNECs in normal adult lungs have yet to be studied. In the fetal developing lung, PNECs appear to arise mainly from undifferentiated cells (Ito et al., 2000); a few PNECs have been shown to undergo cell division (Sorokin et al., 1982; Cutz et al., 1985; McDowell et al., 1985). According to Reynolds et al. (2000), PNECs in NEBs can proliferate and produce PNECs in Clara cell ablation conditions. In the present study, double immunostainig for BrdU and CGRP revealed that BrdU-incoporated PNECs were present under normal conditions, although the number of CGRP-positive PNECs was small. In some NEBs, two neighboring PNECs were positively stained for BrdU. These observations suggest that PNECs can self-renew and undergo cell division in normal lungs and do not always come from variant Clara cells surrounding the PNECs. This is supported by Rawlins et al. (2009), who showed that PNECs did not belong to CCSP-positive Clara cell lineage in a lineage tracing study using *CCSP-CreER*™ -knock-in mice. Sutherland et al. (2011) demonstrated in a recent study on the origin of small cell carcinoma of the lung, a high grade malignant lung carcinoma subtype with neuroendocrine differentiation, that neoplasms could come from PNECs. Taken together, it is suggested that PNECs could be a cell type with cell renewal potential under normal and pathological conditions. Also, CGRP-positive BrdU-LRCs were only detected at 2 months, but not at 6, 18 months, which suggests that the number of labeled cells were much fewer than Clara cells and ciliated cells and that PNECs might have shorter turnover time than Clara cells and ciliated cells.

As the present study aimed to clarify cell kinetic study in the normal terminal bronchiole of the lung, two fundamental factors, such as postnatal growth and ageing of the lung epithelial system and cell injury by BrdU must be considered to carry forward this study. Though we started this study using 10-week old adult mice, lung airway epithelial was able to grow continuously after then, and showed ageing after a long period (Kauffman, 1980). In the present study, the proportion of CCSP-positive cells and FoxJ1-positive cells in the terminal bronchiole was similar regardless of mouse age (data not shown), which suggests that, although postnatal growth and ageing must induce various changes, their effect on BrdU labeling in each cell type couold be small. Moreover, we must consider the injurious effects of BrdU (Ross et al., 2008) on terminal bronchiolar epithelial cell kinetics. According to Zhou et al. (2011), BrdU induces cell senescence in Clara cells when the mice are treated with naphthalene, a Clara cell-specific toxin, while BrdU treatment alone does not alter the number of Clara cells in the vehicle control mice. As it is not clear how BrdU incorporation could change BrdU labeling indices in the terminal bronchiole in comparison with those of true normal mouse without any treatment, the injurious effects of BrdU on the cell kinetics of the mouse lung was not considered in the present study, as no pathological changes such as epithelial cell death and inflammation were seen.

The present study clearly demonstrated that the BrdU-LRC assay is very useful for identifying progenitor/stem cells under normal conditions after continuous BrdU labeling in tissues such as the lung, in which the cell proliferative rate is very low. Furhtermore, double immunostaining for BrdU and cell specific markers was also valuable for identifying the cell types of BrdU-LRC and their kinetics. In conclusion, this study revelaed that in the almost normal condition, Clara cell serves as a progenitor/stem cell in the terminal bronchiole, and BASCs or variant Clara cells do not always work as a progenitor/stem cell.

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