Achaete-Scute Homologue-1 Tapers Neuroendocrine Cell Differentiation in Lungs after Exposure to Naphthalene

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The basic helix-loop-helix transcription factor achaete-scute homologue-1 (ASH1) plays a critical role in regulating the neuroendocrine (NE) phenotype in normal and neoplastic lung. Transgenic (TG) mice that constitutively express human ASH1 (hASH1) under control of the Clara cell 10-kDa protein (CC10) promoter in non-NE airway lining cells display progressive epithelial hyperplasia and bronchiolar metaplasia or bronchiolization of the alveoli (BOA). However, little is known about the involvement of hASH1 in regeneration of the conducting airway. In this study, we investigated the impact of hASH1 on airway cell injury and repair in the TG mice following an intraperitoneal injection of naphthalene, which specifically ablates bronchiolar Clara cells and induces pulmonary NE cell hyperplasia. We discovered an overall attenuation of NE maturation coupled with increased proliferation in TG mice during post-naphthalene repair. In addition, BOA lesions revealed enhanced epithelial cell proliferation while preserving Clara cell markers CC10 and the principal naphthalene-metabolizing enzyme cytochrome P4502F2. These data suggest that ASH1 may play an important role in maintaining a progenitor phenotype that promotes renewal of both NE and epithelial cells. Moreover, ASH1 may propagate a stem cell microenvironment in BOA where epithelium becomes resistant to naphthalene toxicity.

Key Words: achaete-scute homologue-1; bronchiolization of the alveoli; lung repair; neuroendocrine; transgenic mouse.

The pulmonary neuroendocrine (NE) cell (PNEC) system (solitary PNECs and neuroepithelial bodies or NEBs) consists of a small subpopulation of airway cells that act as local modulators of growth and differentiation via their amine and peptide products during lung development (Linnoila, 2006). Although their function in normal adult human lung is less well defined, PNECs proliferate in response to a variety of insults including exposure to environmental pollutants, suggesting a key role during epithelial cell regeneration following lung injury. Achaete-scute homologue-1 (ASH1) is selectively expressed in normal PNECs and many lung cancers with NE

features (Borges *et al.*, 1997). Deletion studies support the role of ASH1 in the development of PNECs by virtue of the fact that ASH1 null mice lack PNECs, whereas mice deficient in the ASH1 repressor hairy and enhancer of split 1 have increased numbers of PNECs (Ito *et al.*, 2000). We have previously generated an ASH1 transgenic (TG) mouse that constitutively expresses human ASH1 (hASH1) throughout conducting airway epithelium under the control of the major Clara cell 10-kDa protein (CC10) promoter. ASH1 TG mice develop progressive hyperplasia of the conducting airways and an outgrowth of cells resembling bronchiolar epithelium that extend into proximal alveolar spaces (Linnoila *et al.*, 2000b). This form of alveolar metaplasia resembles human bronchiolization of the alveoli (BOA), a histologically distinct lesion that occurs in a variety of pathologic conditions such as inflammation, chemical irritation, and exposure to carcinogens (Nettesheim and Szakal, 1972).

One widely used experimental system for studying epithelial cell repair in the adult lung is naphthalene exposure, which specifically ablates bronchiolar Clara cells (Peake *et al.*, 2000; Reynolds *et al.*, 2000b; Stevens *et al.*, 1997). Naphthalene is a ubiquitous environmental pollutant shown to be carcinogenic in rats and thought to contribute to human cancer risk through occupational (incomplete combustion of hydrocarbons and vaporization from diesel and jet fuels) and/or casual (moth repellents and cigarette smoke) exposure (Preuss *et al.*, 2003). The species-selective toxicity of naphthalene in mice is attributed to the naphthalene-metabolizing enzyme cytochrome P-450 isozyme 2F2 (CYP2F2), which generates a cytotoxic response in Clara cells resulting in Clara cell ablation, rapidly followed by Clara cell reconstitution coincident with PNEC hyperplasia and subsequent regeneration of the airway lining (Mahvi *et al.*, 1977; Plopper *et al.*, 1992; Shultz *et al.*, 1999). The degree and distribution of injury in the airway epithelium correlates with the dose of naphthalene and the strain of mice.

Because bronchiolar Clara cells are the principal progenitors for injured airway epithelium, we utilized their

regenerative capacity to study the impact of hASH1 on epithelial cell repair and regeneration in TG mice exposed to naphthalene. The timing and extent of Clara cell regeneration along the airways was unaffected. Contrary to the expected increase in NE differentiation, TG mice showed an overall attenuation of cells that express NE markers during post-naphthalene repair. However, the PNECs had a significantly higher proliferation index after naphthalene treatment, suggesting that ASH1 nevertheless may contribute to their reconstitution. Moreover, BOA lesions retained CC10 and CYP2F2 expression, along with significantly increased 5-bromo-2-deoxyuridine (BrdU) incorporation. Our study suggests that ASH1 may play an important role in maintaining the readiness of PNECs to proliferate during repair. ASH1 TG mice may also harbor a unique microenvironment (BOA) resistant to naphthalene-induced epithelial damage resembling stem cell niche (Kim *et al.*, 2005; Reynolds *et al.*, 2000a). Because many lung disorders are thought to result from altered mechanisms of lung regeneration, the ASH1 TG mice may provide a useful model to study biological and molecular events implicated in the progression of pulmonary disease.

MATERIALS AND METHODS

Cell culture and establishment of stable hASH1 expression in lung epithelial cell lines. DMS53, a small cell lung cancer (SCLC) line, was a gift from Dr Douglas Ball (Johns Hopkins University, Baltimore, MD). It was grown in Waymouth's medium (Life Technologies, Rockville, MD) supplemented with 16% fetal bovine serum (FBS), 100 units of penicillin, and 100 μ g of streptomycin per ml. NCI-H441 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 100 units of penicillin, and 100 μ g of streptomycin per ml at 37°C and 5% CO₂. The human bronchial epithelial cells transformed by SV40 T-antigen (BEAS-2B cells) (American Type Culture Collection) were cultured in bronchial epithelial cell growth medium with "bullet kit" additives (Cambrex Bio-science, Walkersville, MD) composed of growth factors and antibiotics.

For making the stable expression of hASH1 in NCI-H441 and BEAS-2B cell lines, hASH1 gene was inserted into pcDNA 3.1/V5-His-TOPO TA plasmid and transfected into TOP10 *Escherichia coli* cells using the Invitrogen Expression Kit (Invitrogen, Carlsbad, CA). After selection and analysis of the TOP10 *E. coli* cells, purified plasmid was prepared for transfection. Two micrograms of plasmid DNA were transfected into H441 and BEAS-2B cells using Lipofectamine-Plus Reagent Kit (Invitrogen) in 100-mm dish. Following G418 (Invitrogen) treatment, cells derived from a single colony were cultured at least 4 weeks with a selective antibiotic G418 for stable hASH1 expression. The expression of the gene was tested by reverse transcription PCR (RT-PCR) or quantitative real-time PCR (qRT-PCR) and immunostaining.

RT-PCR and qRT-PCR. Total RNA was extracted from cultured cells using RNeasy Minikit (Qiagen, Valencia, CA) followed the manufacturer's protocol. qRT-PCR was performed as previously described (Wang *et al.*, 2007). The PCR reactions for the target gene and β -actin were performed in separate tubes to avoid possible competition and/or interference in a single reaction tube. The sequences of primers for RT-PCR and qRT-PCR are included in the Supplementary table 1.

Wild-type and ASH1 TG mice. Adult FVB wild-type mice were housed under specific pathogen-free conditions under a 12-h light/dark cycle with

access to food and water *ad libitum* according to a protocol approved by NIH Animal Care and Use Committee. ASH1 TG mice were generated as previously described (Linnoila *et al.*, 2000b). In brief, the construct was generated by fusing the entire coding sequence of the hASH1 gene downstream of the CC10 promoter. Phenotypes were confirmed by the identification of BOA lesions and diffuse ASH1 immunostaining throughout the conducting airways of ASH1 TG. ASH1 TG mice were genotyped with DNA obtained from 0.5-cm tail clips using the REDExtract-N-Amp Tissue PCR Kit according to the vendor's instructions (Sigma, St Louis, MO). Two sets of PCR primers were used for genotyping analysis; one recognizing hASH1 and the other against the acetylcholine receptor as an internal PCR control for both wild-type and ASH1 TG mice. Briefly, PCR was carried out in a 40- μ l PCR reaction mixture containing 4 μ l of PCR grade water, 20 μ l of REDExtract PCR Reaction Mix, 2 μ l of a 10 μ M concentration of each primer, and 8 μ l of DNA. Each sample was subjected to 40 cycles, and each cycle consisted of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, and extending at 72°C for 1 min, with a final step at 72°C for 10 min. Ten microliters of each sample was loaded onto a 2% agarose gel and run at 118 volts for 1 h in 1 $\times$ Tris-Borate-EDTA solution at room temperature.

Naphthalene treatment and tissue preparation. A total of 38 mice approximately 6 months in age and representative of both sexes were assigned to four groups: (1) wild-type mice 5 days after exposure to oil ($n = 8$); (2) wild-type mice 5 days after exposure to naphthalene ($n = 11$); (3) ASH1 TG mice 3–5 days after exposure to oil ($n = 9$); and (4) ASH1 TG mice 3–5 days after exposure to naphthalene ($n = 10$). Exposed mice received a single intraperitoneal injection of naphthalene (Sigma Aldrich) dissolved in Mazola corn oil (300 mg/kg body weight), whereas control animals were given a comparable volume of corn oil alone (10 ml/kg body weight). Mice were sacrificed 3 or 5 days following treatment. We administered BrdU (70 mg/g body weight) by intraperitoneal injection 2 h prior to sacrifice to label cell undergoing proliferation. The left lung was infused via intratracheal instillation with fresh 4% paraformaldehyde under 15 cm H₂O pressure and placed in fresh fixative overnight. Lungs were cut longitudinally to expose airways, paraffin embedded, and sectioned at 5 μ m onto poly-L-lysine-coated slides.

Immunohistochemistry. Paraffin-embedded tissue sections were deparaffinized, hydrated, and stained using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) following the vendor's instructions with modifications as described (Linnoila *et al.*, 1988). Microwave antigen retrieval in 0.01M citrate buffer (pH 6.0) was performed for all slides stained with antibodies to calcitonin gene-related peptide (CGRP) (1:3000, Amersham Biosciences, Piscataway, NJ), synaptophysin (SYN) (1:100, Zymed, San Francisco, CA), murine ASH1 (mASH1) (1:50, BD Biosciences Pharmingen, San Diego, CA), and BrdU. Clara cell differentiation was evaluated using a rabbit anti-murine antibody raised against the recombinant murine CC10 protein (CC10) (1:100,000, a kind gift from Dr Franco DeMayo, Baylor School of Medicine, Houston, TX) (Ray *et al.*, 1996). A rat anti-BrdU antibody (1:300, Accurate Chemical, Westbury, NY) was used to detect BrdU incorporation in terminal airway epithelium and BOA lesions undergoing cell proliferation. Signals were visualized using 3,3'-diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with Mayer's hematoxylin or Light Green.

Immunohistochemistry (IHC) for NE markers in terminal bronchioles, small bronchioli (cross sections 50–500 μ m in diameter), and alveoli was quantified by calculating/Airway Ratio (F/A) as previously described (Castro *et al.*, 2000). NE marker expression was computed for each animal by dividing the number (sum) of solitary PNECs and NEBs by the total number of airways analyzed. In TG mice, BOAs were included, and results were expressed as NE foci per structure. All airways and BOAs from every mouse (6–11 per group) were analyzed. Consequently, this mounted on an average of 30 (range 16–48) airways and 6–8 BOAs (TG mice only).

Diffuse CC10 staining expression was assessed using a staining index as previously described (Jensen *et al.*, 1994), which analyses both the intensity of immunoreactivity and number of positive cells.

FIG. 1. Overexpression of hASH1 in pulmonary epithelial cells does not induce NE differentiation. (A) Photomicrographs of hASH1 expression following stable transfection of empty vector (EV; top panel) and hASH1 gene in NCI-H441 cell line. Note nuclear immunoreactivity of hASH1 protein in the lower panel. (Immunoperoxidase stain). (B) RT-PCR of transfected pulmonary epithelial cells. No mRNA for chromogranin A (ChrA), calcitonin, or gastrin-releasing peptide (GRP) in hASH1 overexpressing BEAS-2B and NCI-H441 cell lines was found. An SCLC cell line (DMS53) was used as a positive control. (C). Increased expression of the neural stem cell marker nestin mRNA in hASH1-transfected BEAS-2B and NCI-H441 lung epithelial cells (qRT-PCR).

Quantification of BrdU incorporation was assessed in terminal bronchioles and BOA lesions using MetaMorph imaging software (Universal Imaging, Westchester, PA). In brief, we counted the number of positive epithelial cells within 200 μ m of terminal bronchiolar epithelium starting at the bronchioloalveolar duct junction (BADJ) using a $\times 20$ objective (Nikon Eclipse E400 Microscope). BOA lesions were defined as an outgrowth of terminal bronchiolar epithelium along alveolar walls. Comparable structures were chosen randomly for analysis. In order to calculate the BrdU labeling index, over 1000 cells per animal were observed, derived from 8 to 10 terminal bronchioles and 4 to 6 BOAs in each case. A labeling index was calculated by dividing the number of BrdU-positive nuclei by the total number of nuclei examined per animal. Total mice per group were six to nine.

Nonradioactive in situ hybridization. In order to detect the expression of CYP2F2 messenger RNA (mRNA), linearized plasmids containing the full length (1.4 kb) of the murine CYP2F2 coding region (a kind gift from Dr J. Ritter, Virginia Commonwealth University, Richmond, VA) served as template for the generation of sense and antisense RNA probes in the presence of digoxigenin-labeled uridine triphosphate according to the vendor's instructions (DIG RNA Labeling Kit, Roche Applied Sciences, Indianapolis, IN). Alkaline hydrolysis was performed after labeling to reduce probe length to approximately 200 bp. In brief, 12 μ l of 200mM Na_2CO_3 and 8 μ l of NaHCO_3 were added to 20 μ l of each probe and incubated for 30 min at 60°C. Probes were purified by ethanol precipitation, quantified by UV spectrophotometry (Nanodrop, Wilmington, DE), and resuspended in sterile molecular biology grade water at a concentration of 10–50 μ g/ml. Prior to hybridization, sections were deparaffinized in xylene, rehydrated in a series of graded alcohols, postfixed in fresh 4% para-formaldehyde at 37°C for 10 min, and digested in 10 μ g/ml proteinase K in 2 $\times$ saline-sodium citrate (SSC) buffer at 37°C for 30 min. All solutions used for *in situ* hybridization were prepared with diethylpyrocarbonate-treated water. Hybridization conditions were performed as described in the nonradioactive *in situ* hybridization application manual (Roche Applied Sciences) with the following modifications: after overnight hybridization at 50°C, slides were washed under stringent conditions in 2 $\times$ SSC/0.1% SDS four times for 5 min each at room temperature and then in 0.1 $\times$ SSC/0.1% SDS at 42°C two times for 10 min. Sections were subject to RNase treatment (10 μ g/ml, Sigma) for 15 min at 37°C to reduce binding of nonspecific RNA. Immunological detection of digoxigenin-labeled RNA was performed using the Dig Nucleic Acid Detection Kit (Roche Applied Sciences) according to the vendor's instructions. Digoxigenin staining in bronchioles and BOA lesions was quantified using the staining index as previously described.

Statistical analysis. Given that there were significant interaction effects between genotype and treatment, individual comparisons were performed using the Mann-Whitney rank sum test. Mean values for all parameters were used to

determine statistical significance ($p \leq 0.05$). Fold-change differences in response to treatment (naphthalene vs. oil) between wild type and ASH1 TG were determined by comparing mean values between groups.

RESULTS

Overexpression of hASH1 In Vitro Not Sufficient for NE Differentiation in Epithelial Cells

To test if hASH1 overexpression in well-characterized airway epithelial cell lines induces NE differentiation, we established stable hASH1-expressing cells in two human airway epithelial cells: the immortalized bronchial epithelial BEAS-2B cells and the peripheral airway cell carcinoma NCI-H441 cells (Figs. 1A and B). Using RT-PCR or qRT-PCR, we found intense expression of hASH1 in both transfected cell lines although no mRNA for the three markers of mature NE cells, chromogranin A, gastrin-releasing peptide, and calcitonin, was detected (Fig. 1B). However, the hASH1-expressing clones showed 148- and 18-fold increased in expression of neural stem cell marker nestin in BEAS-2B and NCI-H441 cells, respectively (Fig. 1C). These data indicate that overexpression of hASH1 alone in non-NE airway epithelium may not be enough to drive NE differentiation, but hASH1 may play a role in maintaining a more immature NE phenotype.

ASH1 Expression Patterns in Mice after Exposure to Naphthalene

ASH1 expression was examined by IHC after naphthalene or corn oil (control) treatment using a monoclonal mASH1 antibody, which recognizes both mASH1 and hASH1. Because it has been previously reported that a single dose of naphthalene at 300 mg/kg of body weight results in rapid depletion of Clara cells followed by PNEC hyperplasia in 3–5 days, we used a similar regimen (Poulsen *et al.*, 2008; Reynolds *et al.*, 2000a) (Supplementary figure 1). Naphthalene treatment leads to a statistically significant increase in endogenous mASH1 immunoreactivity in the nuclei of solitary PNECs and cell clusters known as NEBs within bronchioles of wild-type mice ($p < 0.001$) (Fig. 2A). Alternatively, as expected, constitutive nuclear

FIG. 2. ASH1 expression patterns in wild-type and ASH1 TG mice after naphthalene treatment. (A) Top panel, a photomicrograph of endogenous nuclear mASH1 in an NEB from a wild-type mouse 5 days after naphthalene exposure (solid arrow; Lu, lumen; immunoperoxidase staining; bar = 50 μ m). Lower panel, a bar graph of an increased incidence of mASH1-positive NE foci in wild-type mice following naphthalene exposure. (B) A photomicrograph of widely distributed hASH1-positive nuclei in BOA and the epithelium of a terminal bronchiolus (TB) of a TG mouse (Immunoperoxidase staining).

hASH1 expression in ASH1 TG was evident throughout the majority of bronchiolar epithelium regardless of treatment regimen making the detection of endogenous mASH1 staining in solitary PNECs or NEBs problematic (Fig. 2B). Although we were unable to differentiate between endogenous (NE related) mASH1 immunoreactivity and constitutive hASH1 staining in bronchioles of TG mice, no significant change of total ASH1 expression in airways and BOA of ASH1 TG mice was found between oil control and naphthalene treatment group (Fig. 2B).

PNEC Hyperplasia Is Attenuated despite Increased Proliferation in Naphthalene-Treated ASH1 TG Mice

Because constitutive expression of ASH1 in Clara cells of TG mice failed to induce NE phenotype in normal lungs (Linnoila *et al.*, 2000b), we examined the impact of ASH1 on

PNEC hyperplasia during airway cell repair following Clara cell ablation resulting from acute naphthalene toxicity (Peake *et al.*, 2000; Stevens *et al.*, 1997). We investigated PNEC differentiation by IHC using antibodies to the well-established NE markers CGRP and SYN (Fig. 3). As expected, a statistically significant increase in the number of CGRP- and SYN-positive NE foci was observed in bronchioles of wild-type mice 5 days after naphthalene exposure ($p < 0.001$, $p < 0.01$, respectively; Figs. 3B and C). A significant post-naphthalene increase in SYN was also detected in bronchioles of exposed ASH1 TG mice ($p < 0.01$); however, elevated CGRP expression was not statistically significant. Overall, the data on post-naphthalene days 3 and 5 were similar and thus combined (Fig. 3). It was notable that the number of both CGRP- and SYN-containing NE foci remained lower in bronchioles of exposed ASH1 TG compared with wild-type controls ($p < 0.05$, Figs. 3B and C). These results suggest that constitutive hASH1 may attenuate PNEC differentiation by impairing maturation, evidenced by the decrease of CGRP in response to naphthalene toxicity.

The lung phenotype of ASH1 TG includes hyperplasia of bronchiolar airway epithelium and progressive BOA (Linnoila *et al.*, 2000b). BOA lesions consist of ciliated and nonciliated epithelial cells and occasional PNECs (Jensen *et al.*, 1994). Given that acute naphthalene toxicity induces PNEC hyperplasia in bronchioles of wild-type and ASH1 TG mice, we investigated whether a similar response occurs in BOA. Similar to our previous observations in humans (12), we show that NE foci are rare in murine BOA (Figs. 3B and C).

To further examine the impact of ASH1 on NE differentiation during post-naphthalene repair, we analyzed BrdU incorporation within CGRP-containing cells in naphthalene-exposed wild-type and TG mice. We first counted BrdU-positive NEBs. In post-naphthalene lungs, there was a three- to sixfold increase in NEBs that contained one or more BrdU-labeled cells when compared with lungs from untreated mice, but the difference between wild-type and TG mice was not statistically significant (data not shown). Next, we evaluated individual CGRP-positive cells for BrdU staining. Although a trend toward increased proliferation was seen in PNECs within bronchioles of treated wild-type mice, these findings were not statistically significant. Alternatively, a significant increase in CGRP-containing PNECs that were also BrdU positive in ASH1 TG mice was observed. Because there were only few PNECs in BOA, we combined the data on labeled PNECs of airways and BOA lesions of ASH1 TG after naphthalene treatment ($p = 0.03$, Fig. 4). Our results suggest that constitutive expression of hASH1 may augment PNEC proliferation although it decreases their maturation during post-naphthalene repair.

BOA Retains Clara Cell Markers during Post-Naphthalene Injury and Repair

Bronchiolar Clara cells serve as the principal source of progenitors for the reconstitution of injured airway epithelium

FIG. 3. NE differentiation in ASH1 TG mice after naphthalene exposure. (A) Photomicrographs of CGRP-containing NEBs and solitary PNECs in a bronchiole of wild type (WT), ASH1 TG, and BOA of ASH1 TG mice 3–5 days after naphthalene treatment (solid arrows). BL, bronchiole; (Immunoperoxidase staining, 200 $\times$). (B) Quantification of relative incidence of CGRP staining NE foci (solitary PNECs and NEBs) per structure 3–5 days after naphthalene exposure. Structures include BL and BOA. (** $p < 0.001$, * $p < 0.05$, Mann-Whitney rank sum test) (C) Quantification of relative incidence of SYN staining NE foci (solitary PNECs or NEBs) per structure 3–5 days after naphthalene exposure. Structures include BL and BOA. (** $p < 0.01$, * $p < 0.05$, Mann-Whitney rank sum test.).

(Evans *et al.*, 1978). Clara cells are specific targets for acute naphthalene toxicity by virtue of the fact that they contain high levels of CYP2F2, which is needed to metabolize naphthalene to its toxic form responsible for destroying Clara cells (Plopper *et al.*, 1992). In order to study the impact of constitutive hASH1 expression on Clara cell reconstitution, we evaluated expression of the Clara cell markers CC10 and CYP2F2 in bronchioles and BOA by IHC and RNA-RNA *in situ* hybridization, respectively. CC10 protein and CYP2F2 mRNA were strongly expressed in bronchioles of both unexposed wild-type and ASH1 TG mice (Figs. 5A and B and 6A and B). As expected, quantification of CC10-containing cells revealed a significant decrease 3–5 days after naphthalene exposure regardless of genotype (Figs. 5A and B), confirming Clara cell ablation/repair and suggesting that constitutive hASH1 has no effect on Clara cell damage or regeneration following acute naphthalene toxicity. Likewise, a significant reduction in CYP2F2 mRNA was observed in bronchioles of exposed

ASH1 TG mice ($p < 0.05$, Figs. 6A and B), revealing a similar post-naphthalene response as seen in the CC10-containing cells in these mice (Figs. 5A and B).

In an effort to further understand the role of constitutive ASH1 expression in post-naphthalene repair, we also examined expression of the Clara cell markers CC10 and CYP2F2 in BOA. Morphologically BOA is a metaplastic lesion composed of bronchiolar-type epithelium (Nettesheim and Szakal, 1972). However, unlike the bronchiolar compartment of ASH1 TG, which showed a significant decrease after naphthalene treatment, BOA epithelium retained most of its CC10- and CYP2F2-containing cells (Figs. 5C and D and 6C and D). In fact, CC10 expression was significantly higher in BOA compared with bronchioles of the post-naphthalene lungs of ASH1 TG mice at 5 days (staining index 4.74 ± 0.35 vs. 2.43 ± 0.56 , $p = 0.007$). Although there was a decrease in CYP2F2 expression in bronchioles of the TG mice, the relative expression of this marker in BOA remained stable (Figs. 6C

FIG. 4. Increased proliferation of PNECs in ASH1 TG mice after naphthalene exposure. (A) Photomicrograph of a CGRP-containing NEB in bronchiolar epithelium of an ASH1 TG mouse 5 days after naphthalene treatment (arrow). (B) Photomicrograph of BrdU incorporation in the same structure (arrow) in the adjacent section. (A, B, immunoperoxidase stain, bar = 50 µm). (C) The percentage (%) of BrdU-labeled CGRP-containing PNECs is increased in BL and BOA of ASH1 TG 3–5 days after naphthalene treatment ($p = 0.03$). Data represent the mean $\pm$ standard error of measurement. The Mann-Whitney rank sum test was used to compare mean values.

and D). These results suggest that constitutive hASH1 expression may alter the response to post-naphthalene injury or repair in BOA epithelium. In other words, BOA appears to escape the toxic effects of naphthalene observed in the bronchioles.

Post-Naphthalene Epithelial Cell Proliferation Is Efficient in the Distal Airways of ASH1 TG Mice

Previous studies have demonstrated that the region of terminal bronchioles and BADJ comprises a critical regenerating zone in the mouse lung (Giangreco *et al.*, 2002; Kim *et al.*, 2005). Accordingly, we focused on the terminal bronchioles and utilized BrdU incorporation to explore the impact of constitutive ASH1 expression on cellular dynamics in our current model. Mice were injected with BrdU 2 h prior to sacrifice, and IHC was used to quantitate BrdU incorporation in the most distal portion of regenerating terminal airway epithelium (200 µm from the BADJ, Fig. 7A). The percentage

of labeled cells was calculated by dividing the number of BrdU-positive cells by the total number of cells analyzed (~1000 per animal) and multiplying by 100. Consequently, we were able to show a dramatic and statistically significant increase in BrdU incorporation in terminal airways of exposed wild-type and ASH1 TG mice ($p < 0.001$), indicating a similar post-naphthalene proliferation response in terminal bronchioles regardless of genotype (Figs. 7A and C).

Our previous studies on human non-SCLC resection material showed high levels of the cell proliferation marker PCNA in human BOA, indicating an increased potential for cell growth in these lesions (12). In an effort to relate these findings to the current model, we examined the proliferation response of BOA epithelium in ASH1 TG mice by quantifying BrdU incorporation in 20 lesions from each treatment group. Our current findings show that BOA lesions have a significantly higher rate of cell proliferation compared with terminal bronchioles in unexposed ASH1 TG mice ($p < 0.01$) (Figs. 7B and C). Furthermore, a dramatic and statistically significant increase in BrdU labeling was detected in BOA 3–5 days after naphthalene exposure, comparable to the proliferative response observed in terminal bronchioles after naphthalene treatment ($p < 0.01$) (Figs. 7B and C).

DISCUSSION

ASH1 was initially identified as a neural marker and a basic helix-loop-helix transcription factor in the developing central nervous system. Knockout of ASH1 resulted in the absence of PNECs in mice, suggesting that ASH1 plays a critical role in NE differentiation (Borges *et al.*, 1997). However, we have previously reported that constitutive hASH1 expression in Clara cells of mice does not enhance NE differentiation in airways, and there is a lack of NE differentiation in hASH1-induced BOAs. Likewise, our current results showed that using the same lung-specific promoter, overexpression of hASH1 in lung epithelial cells did not induce the expression of NE markers, whereas there was evidence that the neural stem cell marker nestin was induced in the transfectants. Using a lentiviral system, Osada *et al.* (2008) were able to show upregulation of NE-related genes and downregulation of tumor suppressors in a different ASH1-transduced non-SCLC cell line. The results suggest that the *in vitro* data are highly dependable on the cell lines, reagents, and techniques. Nevertheless, it appears that ASH1 alone was not enough to drive NE differentiation in the mouse lung but may need additional factors or a particular microenvironment for NE differentiation.

Following naphthalene injury, PNECs undergo proliferation with concomitant increase in NE marker expression (Stevens *et al.*, 1997). Given that ASH1 TG mice are known to develop PNEC hyperplasia of the conducting airways during simian virus 40 large T antigen-induced carcinogenesis (Linnoila

FIG. 5. Clara cell markers are retained in BOA of ASH1 TG mice after naphthalene treatment. (A) Photomicrographs of strong CC10 expression in the majority of Clara cells lining the bronchioles of control mice, which is decreased to a few scattered positive cells 5 days after naphthalene administration. (Lu, lumen; immunoperoxidase stain; bar = 50 μ m.) (B) Quantification of CC10 immunoreactivity (staining index [0–6] = intensity [0–3] plus distribution [0–3]) in bronchioles 3–5 days after naphthalene administration. CC10 expression was reduced in bronchioles (* p < 0.05) but retained in BOA 3–5 days after naphthalene (panels C, D). (C) Photomicrographs of the BOA epithelium that retained strong CC10 immunoreactivity regardless of treatment regimen. (Lu, bronchiolar lumen; bar = 50 μ m.) (D) Quantification of CC10 staining index (staining index [0–6] = intensity [0–3] plus distribution [0–3]) in BOAs of exposed ASH1 TG. Data represent the mean $\pm$ standard error of measurement. The Mann-Whitney rank sum test was used to compare mean values of staining.

et al., 2000b), we expected to see an increase in PNECs in bronchioles of naphthalene-exposed TG mice. In contrast, a principal finding of the present study was that post-naphthalene NE marker expression in bronchioles of ASH1 TG mice was significantly lower than that observed in wild-type animals, whereas the proliferation rate of PNECs in TG mice increased. As only mature PNECs express CGRP and other neuropeptides, a higher proliferation rate may actually preclude full maturation or differentiation of PNECs. A comparable situation exists in the early development of central and peripheral nervous systems where the expression of ASH1 gene is largely restricted to mitotically active precursor cells (Schuurmans and Guillemot, 2002; Sommer *et al.*, 1995). Once the neural cells terminally differentiate, they no longer express ASH1.

Recently, Jiang *et al.*, (2009) found that hASH1 directly regulates the stem cell marker genes *CD133* and *aldehyde dehydrogenase 1A1* in lung NE carcinoma cells of SCLC. Moreover, hASH1 also modulates the tumor-initiating capacity of SCLC cells. We were able to show that overexpression of hASH1 in a lung epithelial (non-NE) cell line increased the expression of the neural stem cell marker nestin, but not mature

NE makers. Take this together, it suggests that hASH1 may play an important role in maintenance of cell stemness and prevent differentiation into mature NE phenotype.

Although there is a wealth of information on the cellular response to acute naphthalene toxicity in murine distal lung (Peake *et al.*, 2000; Reynolds *et al.*, 2000a; Stevens *et al.*, 1997; Stripp *et al.*, 1995; Van Winkle *et al.*, 1995), this is the first report to document mechanisms of injury and repair in airways constitutively expressing the NE transcription factor ASH1 in Clara cells. CC10, composing 7% of the total protein in normal bronchioloalveolar lavage fluid, is the major protein product of the nonciliated secretory or Clara cells that are precursors for neoplastic and nonneoplastic peripheral airway epithelia (Bernard *et al.*, 1992; Singh *et al.*, 1988). The CC10 promoter was used to direct ASH1 expression into the airways in TG mice in the current study (Linnoila *et al.*, 2000b). On the other hand, it has been previously reported that during development and injury, PNECs may also express CC10, suggesting a close relationship between the cells (Khor *et al.*, 1996; Reynolds *et al.*, 2000a). Specifically, the research by Reynolds *et al.* (2000a) revealed that during post-naphthalene

FIG. 6. CYP2F2 mRNA expression in the mouse lung. (A, B) Abundant CYP2F2 mRNA expression was observed throughout bronchioles in wild-type mice with little change at 5 days after exposure to naphthalene. CYP2F2 mRNA expression is also shown in bronchioles of ASH1 TG with reduced levels 5 days after naphthalene treatment. (C, D) CYP2F2 mRNA expression levels remained high in BOAs (arrows). (RNA-RNA *in situ* hybridization; Lu, bronchiolar lumen; bar = 50 μ m; the Mann-Whitney rank sum test was used to compare mean values of staining, $*p < 0.05$).

repair, most of the regions or foci of CGPR-containing PNECs were located at the sites of nascent CC10-containing Clara cells. Moreover, PNECs were proliferating, and a subset of cells associated with NEBs expressed both CGRP and CC10. In light of this, one interpretation of our current data is that the expression of hASH1 in the CC10-containing (Clara) cells contributes to the increased post-naphthalene proliferation rate in PNECs of TG mice.

As reported by others, we show depletion of bronchiolar Clara cells in airways of naphthalene-treated wild-type mice followed by a significant increase in overall epithelial cell proliferation. Moreover, a similar response to injury was observed in bronchioles of ASH1 TG mice, suggesting that constitutive hASH1 has little or no effect on Clara cell restoration following acute airway injury. The results are in accordance with our recent study showing that the NE phenotype has limited impact on post-naphthalene repair in murine lung (Linnoila *et al.*, 2007).

Another intriguing finding in our current study was the retention of CC10-containing (Clara) cells in BOA of ASH1 TG mice exposed to naphthalene. Functionally, the BOA cells were reminiscent of the previously described minor naphthalene-resistant Clara cell population, attributed to the lack of the major naphthalene-metabolizing enzyme, CYP2F2

(Giangreco *et al.*, 2002). Although we were not able to prove with certainty that these Clara cells were chemoresistant, they were clearly distinct from those lining bronchioles, which were readily ablated by naphthalene treatment. On the other hand, our results showed that BOA epithelium retained CYP2F2 expression after naphthalene treatment, implying an alternative mechanism for naphthalene resistance in BOA of ASH1 TG mice. Possible explanations include that other metabolizing enzymes such as CYP2S1 play a role in naphthalene-associated cytotoxicity in murine BOA or that the epithelium becomes tolerant to naphthalene exposure (Bui and Hankinson, 2009). Moreover, *in vitro* and *in vivo* studies have shown that acute naphthalene toxicity is mediated through a substantial loss of glutathione (GSH), which may be prevented by elevated levels of GSH (Phimister *et al.*, 2005). Repeated exposures to naphthalene may result in tolerance to naphthalene through induction of γ -glutamylcysteine synthetase (γ -GCS) expression (West *et al.*, 2002). Further studies will be needed to determine whether γ -GCS/GSH plays a role in naphthalene resistance of BOA in ASH1 TG mice. Finally, we cannot rule out the possibility that the region-specific location of BOA (alveolar compartment) may alter the cellular response to naphthalene and protect the epithelium from toxic metabolites.

FIG. 7. Post-naphthalene epithelial cell proliferation is enhanced in BOA lesions of ASH1 TG mice. (A) Rare BrdU-positive cells are detected along 200 μ m of terminal bronchiolar epithelium in control mice (arrows). An increase in the number of BrdU-positive cells was observed 5 days after naphthalene treatment (arrows). (B) Whereas only occasional BrdU immunoreactivity was observed in bronchioles or BOA lesions in unexposed ASH1 TG mice (arrows), a dramatic increase in the number of BrdU-containing cells was detected throughout BOAs (arrows) 3 days after naphthalene treatment. (TB, terminal bronchiolus; bar = 50 μ m.) (C) Quantification of BrdU-labeled cells in the most distal part (200 μ m) of TB and BOA is shown for each animal group (mean $\pm$ standard error of measurement). Individual percentages were derived by dividing the number of BrdU-positive cells by the total number of cells analyzed per animal ($\sim$ 1000) multiplied by 100. BOA lesions have a significantly higher rate of cell proliferation compared with terminal bronchioles in unexposed ASH1 TG mice ($p < 0.01$). A significant increase in cell proliferation was observed in bronchioles of wild type (WT) and ASH1 TG (TG) 3–5 days after naphthalene exposure (Mann-Whitney rank sum test, $**p < 0.01$, $***p < 0.001$). Whereas a comparable increase was detected in BOA after naphthalene exposure, the overall labeling index was also higher than in terminal bronchioles.

Naphthalene-induced Clara cell ablation has been widely used to study stem/progenitor cell populations contributing to the reconstitution of injured airway epithelium in murine distal

lung (Kim *et al.*, 2005; Peake *et al.*, 2000; Phimister *et al.*, 2005; Stripp *et al.*, 1995). Furthermore, bronchioalveolar stem cells (BASCs), which are located at the BADJ, are resistant to

bronchiolar and alveolar damage and proliferate during epithelial cell renewal *in vivo* when the mice are exposed to naphthalene (Kim *et al.*, 2005). Based on our observations, BOA formation starts from BADJs, structures of BASCs' niche (Giangreco *et al.*, 2002; Linnoila *et al.*, 2000a; Wang *et al.*, 2007). It is possible that BASCs in TG mice contribute to the formation of BOA epithelium that is composed of cells closely resembling mature bronchiolar Clara cells.

Although lung tissue is normally quiescent with minimal proliferative activity, discrete groups of pulmonary epithelial cells contribute to the mitotic cell population of distal airway epithelium in response to naphthalene injury (Stripp *et al.*, 1995). Although we saw no evidence to suggest that constitutive hASH1 expression alters the epithelial proliferative response in terminal bronchioles, it is notable that BOA lesions in unexposed ASH1 TG mice revealed a higher proliferation index than the adjacent terminal bronchiolar epithelium. Interestingly, these findings are in concert with our previous study demonstrating an increased growth potential in BOA lesions present in human lung tissue (Jensen-Taubman *et al.*, 1998). Supporting evidence for the increased growth potential in ASH1 TG also comes from recent reports showing that inhibition of ASH1 by RNA interference can significantly suppress growth and induce apoptosis of lung cancer cells with ASH1 expression *in vitro* and *in vivo* (Osada *et al.*, 2005; Wang *et al.*, 2007).

In summary, this study is the first to demonstrate two critical new findings. The first one is that ASH1 attenuates NE maturation coupled with increased proliferation in TG mice during post-naphthalene repair. This finding supports the idea that ASH1 may play an important role in maintaining a progenitor phenotype that promotes renewal of both NE and epithelial cells. The second novel observation is that BOAs, potential premalignant lesions of lung cancer, retain CC10 and CYP2F2 expression, along with significantly increased BrdU incorporation. It indicates that constitutive expression of ASH1 may propagate a unique microenvironment reminiscent of stem cell niche. Further studies are needed to determine the mechanism how ASH1 tapers naphthalene-induced pulmonary NE differentiation as well as mediates the resistance to naphthalene toxicity in BOA of hASH1 TG mice.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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