Differential expression of forkhead box transcription factors following butylated hydroxytoluene lung injury.

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BUTYLATED HYDROXYTOLUENE (BHT) is a phenolic antioxidant that was used as a food preservative, and a single dose of 400 mg/kg body wt to mice primarily causes acute lung injury (24). The cytochrome P-4502B enzyme is responsible for converting BHT to its toxic hydroxylated metabolite, resulting in global lung injury (1, 11, 33). BHT-damaged pulmonary cells are replaced through extensive cellular proliferation, which is completed within 9 days post-lung injury. At 2–4 days after BHT lung injury, extensive damage to the bronchiolar and alveolar epithelial cells is observed, with substantial influx of inflammatory cells (1). Concomitantly, these pulmonary epithelial cells undergo proliferation, which is followed by differentiation of alveolar type II cells into type I cells. Subsequently, distal pulmonary endothelial and interstitial cells exhibit BHT-mediated damage and repair between 4 and 7 days after BHT exposure (1). Moreover, biological removal of the BHT metabolites is accomplished through increased expression of phase II detoxifying genes, which are activated by the “cap ’n’ collar” Nrf1 transcription factor, as evidenced by increased susceptibility of Nrf1-deficient mice to BHT-induced mortality (6).

The hepatocyte nuclear factor (HNF)-3α, -3β, and -3γ proteins, which share homology in the winged helix/forkhead DNA binding domain (8, 25), were originally identified as mediating transcription of hepatocyte-specific genes (10, 21, 22). They are a growing family of transcription factors that play important roles in cellular proliferation and differentiation as well as in organ morphogenesis (17). Recently, the nomenclature of the winged helix/forkhead family has been revised to Forkhead box (Fox) genes (16). Subsequent expression and transfection studies demonstrated that HNF-3β (Foxa2) also regulates transcription of genes required for bronchiolar and type II epithelial cell function (2–4, 14, 28, 39). Furthermore, HNF-3β regulates promoter expression of nkn homeodomain transcription thyroid factor-1 (15), which is critical for branching morphogenesis of the lung (18) and regulates expression of the surfactant protein genes (4, 5, 12, 34). Transgenic mouse studies demonstrated that increased expression of HNF-3β in the distal respiratory epithelium blocks lung morphogenesis and vasculogenesis through inhibition of E-cadherin and vascular endothelial growth factor gene expression (38). The regulation of HNF-3β expression by proliferative signals following global lung injury, however, has yet to be determined.

The human winged helix family member HNF-3/forkhead homolog (HFH)-11B, also known as Trident and FOXM1b, is a potent transcriptional activator that is expressed in proliferating cells of mouse embryos (embryonic day 16), including liver, intestine, lung,
and renal pelvis (19, 36). In adult organs, HFH-11 expression is extinguished in the postmitotic, differentiated cells of the liver, lung, and kidney, but its expression continues in proliferating cells of adult tissue, primarily in thymus, testis, small intestine, and colon (19, 36). Consistent with a role in mediating cell cycle progression, hfh11/Trident-deficient embryos display an abnormal polyploid phenotype in embryonic hepatocytes and cardiomyocytes, suggesting that HFH-11 expression is required to link DNA replication with mitosis (20). Reactivation of hepatic HFH-11B levels during partial hepatectomy-induced liver regeneration occurs at the G1/S transition of the cell cycle and continues throughout the period of proliferation, suggesting that HFH-11 expression is a marker for cellular propagation (36). Liver regeneration studies with transgenic mice displaying premature HFH-11B expression revealed that the mice exhibited an 8-h acceleration in the onset of hepatocyte DNA replication and mitosis resulting from earlier expression of cell cycle regulatory genes (35). These results suggest that HFH-11B expression is limiting in proliferating cells and that changing its kinetics of expression will accelerate hepatocyte entry into S phase. Whether HFH-11B expression is also induced in response to lung injury remains to be determined.

Previous in situ hybridization studies have demonstrated that HFH-8 (also known as FREAC-1 and
Foxf1) expression initiates during gastrulation in a subset of mesodermal cells, arising from the primitive streak region that contributes to the extraembryonic mesoderm and lateral mesoderm (26). During organogenesis, HFH-8 expression is restricted to the splanchnic mesoderm contacting the embryonic gut, suggesting that it may participate in the mesenchymal-epithelial induction of lung and gut morphogenesis (23, 26). Consistent with these embryonic expression studies, adult HFH-8 expression is restricted to the mesenchymal cells of the alveolar sac and the lamina propria and smooth muscle of the intestine. The regulation of HFH-8 expression in response to lung injury and repair, however, has not yet been determined.

In this study, we used BHT-mediated lung injury to induce cellular proliferation and examined the expression pattern of three Fox transcription factors during the lung repair process. Although BHT lung injury did not alter epithelial expression of HNF-3β, we show that HFH-11 expression is markedly induced within 2 days following BHT treatment and that its protein levels were sustained throughout the period of cellular proliferation. We also observed a transient 65% reduction in HFH-8 mRNA levels between 4 and 6 days following BHT injury, suggesting that HFH-8 expression decreases during the period of mesenchymal cell proliferation. To determine the cellular expression pattern of the HFH-8 gene, we used heterozygous Hfh-8(+/−) mouse lungs in which the β-galactosidase gene was knocked into the coding region of the mouse HFH-8 gene locus. HFH-8-expressing cells, as detected by nuclear β-galactosidase enzyme staining, were colocalized with platelet endothelial cell adhesion molecule (PECAM)-1-positive alveolar endothelial cells and with α-smooth muscle (α-SM) actin-positive peribronchiolar smooth muscle cells.

MATERIALS AND METHODS

BHT treatment of mice. BHT (3,5-di-tert-butyl-4-hydroxytoluene; Sigma, St Louis, MO) was dissolved in corn oil (Mazola) at 40 mg/ml concentration, and a single intraperitoneal injection of BHT (400 mg/kg body wt) was given to BALB/c males (4–6 wk of age). To determine statistical significance of any observed differences, we used three mice per time point following BHT administration, which included 16 h and 1, 2, 4, 6, 8, and 10 days. The mice were killed by CO2 asphyxiation, and lung tissue was used to prepare total RNA or lungs were inflated with 4% paraformaldehyde and were then paraffin embedded as described previously (35, 39).

Antibodies and immunohistochemical and β-galactosidase enzyme staining. A microtome was used to prepare 5-μm sections of lung tissue, which were deposited onto Superfrost Plus microscope slides (Fisher) and either stained with hematoxylin and eosin or Giemsa for morphological examination or used for immunohistochemical staining with various antibodies. Mouse monoclonal anti-proliferation cell nuclear antigen (PCNA) antibody (clone PC10) was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and used at a dilution of 1:1,000; affinity-purified rabbit polyclonal anti-

Fig. 2. Induction of proliferation cell nuclear antigen (PCNA) expression following BHT mouse lung injury. Microtome sections of paraffin-embedded lungs were prepared at various times after BHT lung injury and used for immunohistochemistry with anti-PCNA monoclonal antibodies. The PCNA protein-antibody complex was visualized using alkaline phosphatase-conjugated secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitro blue tetrazolium as the substrate (stains blue) and nuclei were counterstained with methyl green (stains nuclei green). A: PCNA-negative nuclei (methyl green) counterstained with methyl green (C–F). PCNA expression was detectable throughout the lung parenchyma between days 4 and 8 following BHT lung injury. Magnification for A–C, E, and F is ×50 and for D, it is ×158.
mouse HFH-11 antibody was generated and used at a dilution of 1:100 as described previously (35, 36); rat monoclonal anti-PECAM-1 antibody (clone MEC 13.3) was purchased from PharMingen (San Diego, CA) and used at a dilution of 1:500; mouse monoclonal HNF-3β antibody (clone 4C7) was purchased from Sigma (Clone 1A4) and used at a dilution of 1:400. Briefly, paraffin wax was removed from lung sections with xylene and rehydrated with decreasing graded ethanol washes. Citrate buffer (0.02 M, pH 6.0) was used for microwave retrieval to enhance the antigenic activity as described previously (39). Sections were then blocked with 2.5% normal horse serum for 1 h and incubated at 4°C overnight with primary antibody. Staining for PCNA was performed using horse anti-mouse antibody conjugated with alkaline phosphatase (Vector Laboratories, Burlingame, CA). Staining for HFH-11 was developed using horse anti-rabbit antibody conjugated with biotin followed by avidin-alkaline phosphatase conjugate (all from Vector). A 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt/nitro blue tetrazolium kit from Vector Laboratories was used as a substrate for alkaline phosphatase. Immunohistochemical staining for PECAM-1 was performed after trypsin retrieval (37) using biotinylated goat anti-rat antibody (PharMingen, San Diego, CA), and developed with streptavidin-horseradish peroxidase conjugate and 3,3′-diaminobenzidine substrate kit (Vector). For colocalization studies, sections were stained with rabbit anti-HFH-11 and mouse anti-mouse secondary antibody conjugated with FITC (Vector). All immunohistochemical reactions were carried out in parallel with reactions lacking primary antibodies to ensure the specificity of the observed staining. Slides were counterstained with methyl green (Vector). Student’s t-test was used to determine statistically significant differences in percentages of PECAM-1-positive cells in the lung. Differences of \( P < 0.05 \) were considered significant. Values are given as means ± SD.

An HFH-8 gene disruption targeting vector was generated in which the winged helix DNA binding domain was replaced by a nuclear localizing β-galactosidase gene and phosphoglycerol kinase promoter-driven neomycin gene (see Fig. 7A). The HFH-8 gene targeting vector replaced NH₂-terminal sequences between the NcoI and NotI sites with the nuclear localizing β-galactosidase, which was cloned in frame with the mouse HFH-8 coding region (GenBank accession number L355949). The Transgenic Mouse Facility at the University of Cincinnati used embryonic stem (ES) cell technology to select ES cells with the HFH-8 β-galactosidase gene-targeted locus using procedures described by Clark et al. (7). These targeted ES cells were subsequently used to create \( Hfh8^{+/−} \) mice in which expression of the nuclear localizing β-galactosidase gene was controlled by the HFH-8 DNA regulatory region. To determine HFH-8-expressing cells, \( Hfh8^{+/−} \) lung tissue was stained for β-galactosidase enzyme with 1 mg/ml X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and paraffin embedded, and a microtome was used to deposit sections on a slide as described previously (9). Paraffin wax was removed from lung sections with xylene and rehydrated with decreasing graded ethanol washes followed by immunohistochemical staining (brown) with either PECAM-1 or α-SM actin antibodies as previously described.

**RESULTS AND DISCUSSION**

**Morphological changes and cellular proliferation in the lung after BHT lung injury.** To investigate whether expression of winged helix genes was influenced by cellular proliferation during lung injury, we adminis-

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**Fig. 3. Increased expression of hepatocyte nuclear factor (HNF)-3/b-forkhead homolog (HFH)-11 mRNA after BHT lung injury.** Total mouse lung RNA was prepared by an acid guanidium-thiocyanate-phenol-chloroform extraction method using RNA-STAT-60 (Tel-Test “B,” Friendswood, TX). RNase protection assay was performed with [³²P]UTP-labeled antisense RNA synthesized from plasmid templates with the appropriate RNA polymerase as previously described (10). Approximately \( 2 \times 10^6 \) cpm of each probe was hybridized at 45°C to 20 μg of total RNA in a solution containing 20 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA and 80% formamide overnight. After hybridization, samples were digested 1 h at 37°C by using 10 U/sample of RNase ONE enzyme according to the manufacturer’s protocol (Promega, Madison, WI). The RNase One protected fragments were electrophoresed on an 8% polyacrylamide-8 M urea gel followed by autoradiography. Quantitation of expression levels was determined with scanned X-ray films by using the BioMax 1D program (Kodak). The cyclophilin hybridization signal was used for normalization control between different lung RNA samples. Synthesis of antisense human HFH-11B, rat HNF-3β, and rat surfactant protein (SP) C and mouse cyclophilin RNA probes was described previously (26, 36). Antisense mouse HFH-8 RNA probe was generated from mouse HFH-8 cDNA (nucleotides 437–816), which was cloned in pBl plasmid.
tered a single, nonlethal dose of BHT to wild-type male BALB/c mice (4–6 wk of age). At each of the various time points after BHT injury, three mice were killed and lung tissue was isolated and used for paraffin embedding or preparation of total RNA as described in MATERIALS AND METHODS. Although there was no mortality from the BHT-induced lung injury, the mice developed shallow, rapid breathing between 2 and 6 days after treatment. Bright-field microscopy examination of paraffin sections revealed extensive lung damage by 4 days following BHT treatment, with increased alveolar wall thickness and influx of leukocytes into the lung parenchyma (compare Fig. 1, A and B with C–E). The morphological evidence of lung damage persisted until 6 days after BHT exposure (Fig. 1, F and G) and gradually improved toward the later time points (Fig. 1H). To confirm that we have reproduced global lung proliferation as reported by previous BHT injury studies (1, 11, 33), a commercially available PCNA antibody was used for immunohistochemical staining. Consistent with these previous BHT studies, injured mouse lungs exhibited elevated PCNA staining in bronchiolar and alveolar epithelial cells by 2 days after BHT injury, demonstrating that these cells were undergoing extensive proliferation (Fig. 2B). An increase in PCNA-positive cells was also detected in the alveolar region of the lung between 4 and 8 days after BHT lung injury (Fig. 2, C–F), correlating with proliferation of the pulmonary endothelial cells and connective fibroblasts as previously reported (1).

**BHT lung injury induces expression of HFH-11 (FoxM1B) during cellular proliferation.** We previously demonstrated that HFH-11 expression is induced during liver regeneration following partial hepatectomy, following H2O2 treatment of human microvessel endothelial cells, and in response to tracheal administration of keratinocyte growth factor, the latter of which causes proliferation of type II cells (36). To investigate
whether proliferative signals following lung injury also induced expression of the HFH-11, we performed RNase protection assays with HFH-11 antisense RNA probes and mouse lung RNA isolated at various times after BHT lung injury (Fig. 3A). HFH-11 mRNA levels from three distinct mouse lungs were normalized to the cyclophilin levels and used to determine the means ± SD (Fig. 3B). Consistent with HFH-11 involvement in cellular proliferation, injured mouse lungs displayed a pronounced increase in HFH-11 mRNA levels by 2 days following BHT treatment, and those remained elevated until the 8-day time point (Fig. 3, A and B).

To determine the cellular expression pattern of the HFH-11 protein following BHT lung injury, we used an affinity-purified HFH-11 antibody for immunohistochemical staining of paraffin-embedded lung sections. Consistent with previous studies, HFH-11 protein was not detected in normal adult mouse lungs (Fig. 4A), but as early as 2 days following BHT lung injury, abundant HFH-11 protein staining was evident in both the alveolar (Fig. 4, B and C) and bronchial epithelial cells (Fig. 4D). Localization of the HFH-11 protein was mainly nuclear and most of the HFH-11 staining colocalized with PCNA-positive nuclei (Fig. 5, A and B). At 2 days following BHT injury, HFH-11 protein expression colocalizes with HNF-3β staining (Fig. 5, C–H), which is a marker for both bronchiolar epithelial cells (including Clara cells) and type 2 alveolar epithelial cells (39). Elevated levels of the HFH-11 protein were also sustained in the alveolar region until day 8 following BHT lung injury (Fig. 4, E and H) and were detected in the mesenchymal cells of the arteriole walls (Fig. 4, F and G). Taken together, these results suggest an active involvement of the HFH-11 transcription factor during lung injury repair in response to BHT cellular damage. This conclusion is supported by liver regeneration studies with HFH-11B transgenic mice, demonstrating that premature hepatic expression of the transcription factor leads to liver fibrosis, thus highlighting the role of HFH-11 in cellular proliferation and regeneration.

Fig. 5. Colocalization of HFH-11 with PCNA and HNF-3β proteins in BHT lung injury. Paraffin sections of the lung were prepared 2 days after BHT injection and used for immunohistochemistry with both affinity-purified anti-HFH-11 antibody and monoclonal antibody against either PCNA or HNF-3β protein and visualized using differential immunofluorescence. Positive staining nuclei for the proliferation-specific HFH-11 transcription factor (tetramethylrhodamine isothiocyanate, red) displayed colocalized nuclear staining with either PCNA (FITC, green) in alveolar cells (A and B) or HNF-3β (FITC, green) in bronchiolar epithelial cells (C–F) and alveolar epithelial cells (G–H). Magnification for C and D is ×25 and for A, B, and E–H, it is ×100.
factor HFH-11B caused accelerated hepatocyte entry into the S phase resulting from earlier expression of cell cycle regulatory genes (35). Furthermore, hfh11/Trident-deficient embryos display an abnormal polyploid phenotype in embryonic hepatocytes and cardiomyocytes, suggesting that HFH-11 expression is required for progression of DNA replication into mitosis (20). Likewise, increased pulmonary expression of HFH-11 protein following lung injury may play an important role in the induction of DNA replication and mitosis. Other models have determined the induction of Nrf1, CCAAT enhancer binding protein (C/EBP)-β and -δ, c-jun/c-fos, and nuclear factor (NF)-κB transcription factors following lung injury (6, 13, 14, 29, 31, 32). This study has identified the HFH-11 protein as an additional transcription factor that is stimulated following BHT lung injury and that participates in cellular proliferation during lung injury repair.

**BHT lung injury does not alter HNF-3β levels in proliferating bronchiolar and alveolar epithelial cells.** Previous liver regeneration studies demonstrated that expression of the HNF-3β is sustained in proliferating hepatocytes (27). To determine whether HNF-3β expression is also maintained in proliferating lung cells, total RNA from BHT-injured mouse lungs was analyzed for HNF-3β mRNA expression by RNase protection assay. Although BHT-induced extensive proliferation of bronchiolar and alveolar type II cells (Fig. 2), we observed no changes in either HNF-3β or SP-C mRNA levels (Fig. 6, A and B). To confirm that HNF-3β protein levels were maintained during lung epithelial cell proliferation, we used immunofluorescence to demonstrate that HNF-3β staining was maintained in both bronchiolar (Fig. 5, D and F) and alveolar epithelial cells (Fig. 5H). Furthermore, HNF-3β protein expression colocalizes with the proliferation-specific HFH-11 protein (Fig. 5, C, E, and G), suggesting that pulmonary expression of HNF-3β was not influenced by proliferative signals induced following BHT lung injury. Moreover, previous studies demonstrated that HNF-3β protein is not altered during hepatocyte replication in regenerating liver or following lipopolysaccharide-induced acute-phase response (27). Taken together, these data suggest that HNF-3β maintains transcription of differentiated epithelial cell genes without interfering with progression of cellular replication.

**Colocalization of HFH-8 expressing cells with alveolar endothelial and peribronchiolar smooth muscle cells.** To determine the cellular expression pattern of the HFH-8 gene, we used heterozygous Hfh8+/− mouse lungs in which the normal Hfh8 gene locus was replaced by a nuclear localizing β-galactosidase gene cloned in frame with the mouse HFH-8 coding region (Fig. 7A). Expression of the nuclear localizing β-galactosidase gene was under the control of the HFH-8 DNA-regulatory sequences, and thus staining for β-galactosidase enzyme activity allows identification of HFH-8-expressing cells (a more detailed characterization of the Hfh8+/− mice will be described elsewhere). Heterozygous Hfh8+/− lung tissue was stained for β-galactosidase enzyme activity with X-gal substrate (blue), paraffin embedded, sectioned, and then prepared for immunohistochemical staining (brown) with either PECA1 or α-SM actin antibodies. In the absence of primary antibody, control immunohistochemical staining of Hfh8+/− lung tissue displayed only blue staining for β-galactosidase enzyme activity in the alveolar region and in peribronchiolar smooth muscle cells (Fig. 7, B and C). By contrast, immunohistochem-

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**Fig. 6. Differential expression of HFH-8 and HNF-3β follows BHT lung injury.** Total RNA was prepared from mouse lungs at various times following BHT lung injury, and RNase protection assay was used to analyze for HFH-8, HNF-3β, surfactant protein (SP) C and cyclophilin expression. Photographs of the result (A) and scanning densitometric data (B) are shown. Relative HFH-8 and HNF-3β mRNA expression are presented as the means ± SD. C: decrease of HFH-8 mRNA expression was not due to reduced number of endothelial cells. Paraffin sections of lungs were prepared at various times after BHT injection and used for immunohistochemistry with anti-platelet endothelial cell adhesion molecule (PECAM)-1 antibody, a marker for endothelial cells. Total number of endothelial cells in high-power microscopic fields was counted using 3 randomly picked sections, and the means ± SD for each time point were calculated from 3 different mice.
ical staining of Hfh8(+/-) lung tissue with the PECAM-1 antibody demonstrated that β-galactosidase enzyme activity is colocalized with PECAM-1 staining in the alveolar region but not in the peribronchiolar smooth muscle cells (Fig. 7, D–F). These results demonstrate that alveolar expression of the HFH-8 gene resides in the PECAM-1-positive endothelial cells (30). Pulmonary blood vessels lacked detectable HFH-8 staining, suggesting that HFH-8 expression is restricted to alveolar endothelial cells (data not shown). Furthermore, α-SM actin immunohistochemical staining in Hfh8(+/-) lung tissue colocalizes with β-galactosidase-staining cells surrounding the bronchiolar region, demonstrating that HFH-8 expression also resides in the peribronchiolar smooth muscle cells (Fig. 7G). However, our data cannot rule out the possibility that HFH-8 is also expressed in alveolar smooth muscle cells.

BHT lung injury causes diminished expression of HFH-8 (Foxf1) during proliferation of mesenchymal cells. In contrast to the Fox transcription factors HFH-11 and HNF-3β, HFH-8 expression is restricted to the mesenchymal tissue of the adult alveolar lung and intestine. To examine whether HFH-8 expression changes following BHT lung injury, RNase protection assay was used to examine HFH-8 mRNA levels at different time points following lung damage. This analysis revealed a transient 65% reduction in HFH-8 mRNA between 4 and 6 days following BHT treatment (Fig. 6, A and B), a time period when extensive proliferation of the mesenchymal cells is observed (1). Interestingly, reduction in cellular proliferation correlates
with the restoration of HFH-8 expression levels by 8 days following BHT injury. The decline in HFH-8 levels during mesenchymal cell proliferation is in contrast to sustained expression of the winged helix HNF-3β protein in proliferating epithelial cells (Figs. 5 and 6).

Because HFH-8 is expressed in alveolar endothelial cells of the lung, we wanted to determine whether the decrease in HFH-8 levels was due to selective death of endothelial cells following BHT lung injury. Lung sections for each time point were stained with anti-PECAM-1 antibodies (data not shown), and the number of PECAM-1-positive endothelial cells (30) was counted in high-power microscope fields and plotted in Fig. 6C. This analysis revealed that BHT lung injury did not diminish the number of PECAM-1-positive endothelial cells but rather caused a dramatic reduction in HFH-8 mRNA levels in response to proliferative signals. These results suggest that reduction in HFH-8 levels coincides with the period of mesenchymal cell proliferation.

In summary, we show that BHT lung injury induces expression of the winged helix transcription factor HFH-11 during proliferative stages of the repair process. Although HNF-3β expression is sustained during pulmonary epithelial cell proliferation, BHT lung injury causes significant reduction in HFH-8 expression, coinciding with proliferation of the pulmonary mesenchymal cells. We also used heterozygous Hfh8+/− mouse lungs in which the β-galactosidase gene was knocked into the coding region of the mouse HFH-8 gene locus to determine the HFH-8 cellular expression pattern. HFH-8-expressing cells in the adult mouse lung as detected by nuclear β-galactosidase enzyme staining were cococalized with markers specific to alveolar endothelial cells and peribronchial smooth muscle cells.

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