

# Early Changes in Gene Expression Induced by Tobacco Smoke: Evidence for the Importance of Estrogen within Lung Tissue

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## Abstract

Lung cancer is the leading cause of cancer deaths in the United States, surpassing breast cancer as the primary cause of cancer-related mortality in women. The goal of the present study was to identify early molecular changes in the lung induced by exposure to tobacco smoke and thus identify potential targets for chemoprevention. Female A/J mice were exposed to either tobacco smoke or HEPA-filtered air via a whole-body exposure chamber (6 h/d, 5 d/wk for 3, 8, and 20 weeks). Gene expression profiles of lung tissue from control and smoke-exposed animals were established using a 15K cDNA microarray. Cytochrome P450 1b1, a phase I enzyme involved in both the metabolism of xenobiotics and the 4-hydroxylation of 17 $\beta$ -estradiol (E<sub>2</sub>), was modulated to the greatest extent following smoke exposure. A panel of 10 genes were found to be differentially expressed in control and smoke-exposed lung tissues at 3, 8, and 20 weeks ( $P < 0.001$ ). The interaction network of these differentially expressed genes revealed new pathways modulated by short-term smoke exposure, including estrogen metabolism. In addition, E<sub>2</sub> was detected within murine lung tissue by gas chromatography-coupled mass spectrometry and immunohistochemistry. Identification of the early molecular events that contribute to lung tumor formation is anticipated to lead to the development of promising targeted chemopreventive therapies. In conclusion, the presence of E<sub>2</sub> within lung tissue when combined with the modulation of cytochrome P450 1b1 and other estrogen metabolism genes by tobacco smoke provides novel insight into a possible role for estrogens in lung cancer. *Cancer Prev Res*; 3(6): 707–17. ©2010 AACR.

## Introduction

Lung cancer is the leading cause of cancer death in the United States and has surpassed breast cancer as the primary cause of cancer-related mortality in women (1). Exposure to cigarette smoke is estimated to account for approximately 90% of all lung cancers (2). Epidemiologic and clinical data suggest a gender difference in the biology of lung cancer (3, 4). Women appear to have an increased susceptibility to tobacco carcinogens but have

a better prognosis after lung cancer diagnosis as compared with men (3). It has been suggested that estrogens may affect the susceptibility of women to lung cancer (2, 5–7).

The individual variation in efficiency of cellular processes such as detoxification, metabolic activation, adduct formation, and DNA repair influences individual susceptibility to the tumorigenic effects associated with tobacco smoke exposure. Detoxification enzymes play a critical role in the metabolism of both tobacco-related carcinogens and endogenous compounds (including hormones) within the human lung (8). Activation of the aryl hydrocarbon receptor signaling pathway by components of tobacco smoke such as polycyclic aromatic hydrocarbons leads to transcriptional upregulation of a number of genes including members of the cytochrome P450 (CYP) family, in particular, *CYP1B1* and *CYP1A1*. These phase I enzymes activate procarcinogens such as benzo(a)pyrene (BaP) to reactive electrophilic intermediates, which are in general detoxified to inactive water-soluble conjugates for excretion by phase II enzymes (i.e., glutathione S-transferase, UDP-glucuronosyl-transferase, sulfotransferase, and catechol-O-methyltransferase). Upregulation or downregulation of these enzymes, in turn, can influence

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the fate of reactive intermediates in the body, leading to the accumulation or clearance of metabolites; thus influencing cancer incidence.

It is well established that estrogen can stimulate cell signaling and proliferation in the breast via estrogen receptor (ER)-dependent and ER-independent pathways (9). The role of estrogen as a procarcinogen is also based on the ability of its metabolites (catechol estrogens) to induce genetic damage. The formation of catechol estrogens is catalyzed by CYP1B1, CYP1A1, CYP1A2, and CYP3A4 in several tissues. The most carcinogenic catechol estrogen is 4-hydroxyestradiol (4-E<sub>2</sub>), an estrogen agonist that binds to the ER with greater affinity and for longer periods of time than the parent compound 17 $\beta$ -estradiol (E<sub>2</sub>; ref. 10). 4-E<sub>2</sub> is produced primarily by CYP1B1 and is quickly oxidized to highly reactive quinones that bind to DNA, forming depurinating DNA adducts (11) that induce genetic mutations.

Although smoking cessation decreases the risk of lung cancer, ex-smokers remain at a significantly increased risk for cancer for decades. Development of an efficacious chemopreventive regimen for lung cancer has been hindered by our inability to identify early molecular targets for intervention as well as those individuals who would benefit most from treatment. The majority of the molecular targets that have been identified to date represent late events in tumorigenesis, as indicated by their presence in established tumors. These include biomarkers of tumor cell proliferation (i.e., EGFR, TP53, KRAS, RB, and BCL2) and angiogenesis stimulation factors (i.e., VEGF, FGF, and MMP), among others (12). Whereas these late events aid in selecting treatment options and predicting prognosis, early events (in particular, those occurring during the preneoplastic phase) can be used as targets for chemopreventive intervention, thus blocking tumor formation. Limited attention has been given to the identification of early biomarkers of lung cancer risk.

The A/J mouse and its genetically related A/HeJ strain have been shown to be the strains that are most responsive to cigarette smoke exposure as compared with AKR, BALB/C, C3H/HeJ, C57BL/6, CAST/Ei, DBA, SWR, and 129/Sv mice (13). A/J mice develop lung adenomas/adenocarcinomas spontaneously and, following exposure to tobacco smoke, readily develop additional lung tumors that are similar to human lung adenocarcinomas with respect to pathologic features, genetic alterations, and aberrant signaling pathways (14). By allowing the animals to recover in fresh air for 16 weeks following 20 weeks of smoke exposure, Witschi and colleagues (15) were successful in enhancing lung tumor development in smoke-exposed mice.

The goal of the present study was to identify early changes in gene expression that are induced within the nonneoplastic lung tissue of female A/J mice following exposure to tobacco smoke. Such early alterations in gene expression may yield insight into tumor initiation and promotion and represent both early biomarkers of lung cancer development and molecular targets for chemopreventive intervention. In this study, we compared the gene expression profile of lung tissue from mice exposed to tobacco smoke for 3 to 20 weeks with that of lungs from control animals main-

tained in ambient air for the same period of time. Our enhanced understanding of the early molecular events that contribute to lung tumor development is anticipated to aid in the identification of promising chemopreventive targets that can be examined readily for their efficacy in the A/J model of current and former smoke exposure.

## Materials and Methods

### Tobacco smoke exposure

Female A/J mice, 8 to 9 weeks of age, were purchased from The Jackson Laboratory and maintained with free access to food (AIN-93G, Harlan Teklad Global Diets) and water. Body weights were recorded weekly to monitor growth. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

Mice were randomized into six treatment groups (six per group) and exposed to either tobacco smoke or HEPA-filtered ambient air (control). Exposure of mice to sidestream smoke was carried out in whole-body chambers for 6 hours per day, 5 days per week for 3, 8, and 20 weeks. Sidestream smoke was generated from the University of Kentucky reference cigarette (2R4F), and the suspended smoke particulate concentration in the chamber atmosphere was maintained at approximately 40 to 45 mg/m<sup>3</sup>.

Exposure of mice to smoke was ascertained by measuring levels of urinary cotinine, ethoxyresorufin deethylase activity in lung microsomes, and cytochrome P450 1a1 (Cyp1a1) protein. Significant increases of these markers were observed in smoke-exposed groups over controls at all time points. Cotinine was measured routinely in urine samples (ELISA Kit, OraSure Technologies) collected from three to five mice per group over 24 hours. Cotinine values ranging from 4 to 10  $\mu$ g cotinine/mg creatinine were observed in urine from smoke-exposed mice as compared with picogram levels in the unexposed control groups. Lobes of lungs from three to four animals per treatment group were pooled, and lung microsomes were prepared by differential centrifugation (9,000  $\times$  g and then 100,000  $\times$  g for 60 minutes). The protein content of the resulting fraction was determined using a commercial kit from Bio-Rad. The ethoxyresorufin deethylase activity of lung microsomes was determined using a modified fluorimetric resorufin assay (16). Aliquots of the microsomal protein were separated on 12% SDS-polyacrylamide gels and subjected to Western blot analysis. Blots were probed with a polyclonal rabbit anti-rat Cyp1a1 antibody (diluted 1:800) from XenoTech LLC and secondary antirabbit antibody conjugated with alkaline phosphatase. Typical ethoxyresorufin deethylase activity levels (mean  $\pm$  SEM) were 1.21  $\pm$  0.3 and 9.74  $\pm$  1.4 pmol/min/mg protein for control and smoke-exposed groups, respectively. Immunoblots of lung microsomes probed with anti-Cyp1a1 antibody also showed significantly higher levels of protein in smoke-exposed mice.

At the time of sacrifice, lung tissue was excised from each animal, immediately placed in TRIzol (Invitrogen Corp.), and stored at  $-80^{\circ}$ C for subsequent RNA isolation

and analysis. The remaining lung tissue was snap-frozen in liquid nitrogen for Cyp1a1 analyses.

### RNA extraction and probe preparation

Frozen tissues were homogenized in TRIzol using a Polytron System PT 1200C (Kinematica AG), and total cellular RNA was extracted as recommended by the manufacturer. RNA concentration was determined by absorbance at 260 nm, and quality was assessed by monitoring the integrity of the 28S and 18S rRNAs by agarose gel electrophoresis.

Equal amounts of RNA from three animals were pooled, yielding two control and two smoke-exposed RNA pools per time point (3 and 8 weeks). To circumvent the potential contamination of normal lung tissue with neoplastic cells, samples collected at 20 weeks were evaluated individually, totaling six control and six smoke-exposed samples.

A total of 20 pooled (3 and 8 weeks) and individual (20 weeks) RNA samples (1  $\mu$ g each) were subjected to one round of T7-based linear RNA amplification using the RiboAmp Kit (Molecular Devices). Universal mouse total RNA (Clontech Laboratories, Inc.) was also subjected to one round of amplification and served as a common reference for all hybridizations. cDNA probes were synthesized in duplicate by a standard reverse transcription reaction using 2  $\mu$ g of each amplified RNA and labeled by indirect (amino-allyl) incorporation of Cy3 or Cy5 (CyDye Post-Labeling Reactive Dyes, Amersham Biosciences Corp.; dye-flip replicates). The concentration of the labeled cDNA probe was determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).

### cDNA microarray hybridization

The expression profile of samples was established using a mouse microarray containing 15,552 (15K) cDNA clones obtained from the Institute of Aging, NIH (sequence information is available at <http://lgsun.grc.nia.nih.gov/cDNA/15k.html>) and printed at Fox Chase Cancer Center. Probe hybridizations were performed following standard procedures. Following hybridization, the slides were scanned with a GMS 428 scanner (Affymetrix) at full laser intensity and variable photomultiplier tube voltage settings, capturing the full dynamic range for each slide in each respective channel. Image segmentation and spot quantification were done with the ImaGene software, version 5.6.1 (BioDiscovery), using the original default settings of the software. The mean intensities of signal and local background were extracted for each spot and subjected to analysis.

### Mathematical analyses

Microarray data were processed and analyzed using R (<http://www.r-project.org/>) and the Bioconductor (17) platform. Only spots with GenBank accession entries ( $n = 15,245$ ) were considered for analysis. Background correction was carried out using the *normexp* method (18) implemented in the Bioconductor package *limma* (Linear Models for Microarray Data), with an offset of 50. This method has been found to be preferable to local background subtraction

in most cases. LOWESS (locally weighted regression and smoothing scatter) normalization was used to correct for intensity-dependent dye bias. Dye-swap replicates were considered as replicates for statistical comparisons. To identify genes that were differentially expressed between smoke-exposed and reference samples, an empirical Bayes moderated *t* test, as implemented in *limma* (19), was used. Due to differences in the sample design, the differential expression analysis was carried out separately for samples at 3 and 8 weeks (pooled samples) and 20 weeks (individual samples). Lists of differentially expressed genes for downstream analyses were selected using a *P* value threshold of 0.001.

Ingenuity Pathways Analysis (IPA version 6.3; <http://www.ingenuity.com/>) was used to search for underlying biological pathways and molecular networks. IPA provides a rich functional annotation of genes and proteins and protein-protein interactions as well as the role of genes in various diseases. The genes differentially expressed at all time points (3, 8, and 20 weeks) were uploaded into IPA along with the corresponding fold change values. These genes are searched in the IPA functional annotation database called Ingenuity Pathways Knowledge Database (IPKB). Depending on the input gene list, the Ingenuity software models networks and pathways through a statistical computation using functional relationships such as interaction, activation, and localization between proteins, genes, complexes, cells, tissues, drugs, and diseases. Given a list of genes and their expression values or fold changes, IPA computes a score (*P* value) for network eligible genes. A higher score implies a significant composition of genes in a network.

### Gas chromatography-coupled mass spectrometry

Female A/J mice ( $n = 8$ ) at 8 weeks of age were purchased from The Jackson Laboratory. At the time of sacrifice, the lung was perfused with 30 mL of saline and excised, and four lobes were stored at  $-80^{\circ}\text{C}$  for subsequent analysis by gas chromatography/mass spectrometry (GC/MS). Frozen lung tissues were homogenized in 30 mmol/L potassium phosphate buffer (pH 6.0) containing 0.5 mmol/L ascorbic acid. After adding methanol (60%, v/v), the homogenate was extracted twice with 1 volume of hexane. The aqueous phase was filtered using a 0.7- $\mu$ m glass microfiber filter, extracted with 2 volumes of ethyl acetate, and evaporated to dryness. The samples were derivatized in acetonitrile using *N,O*-bis-(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. Deuterium-labeled  $\text{E}_2$  ( $\text{d}_5\text{-E}_2$ ; C/D/N Isotopes, Inc.) was used as internal standard and was added before splitless injection into a HP6890 GC/MS instrument with capillary column (20 m  $\times$  0.18 mm  $\times$  0.18  $\mu$ m, DB-5ms, Agilent JW Scientific Columns, Agilent Technologies). Selective ion monitoring [ $m/z$  342, 416, and 421 for estrone ( $\text{E}_1$ ),  $\text{E}_2$ , and  $\text{d}_5\text{-E}_2$ , respectively] and retention times relative to  $\text{d}_5\text{-E}_2$  were used to identify each compound.

### Immunohistochemistry

Perfused lungs from three female A/J mice were fixed in 10% formalin for 24 hours and subsequently embedded in

paraffin for immunohistochemical analysis. Sections (4 μm) were dewaxed through incubation in xylene, followed by a graded alcohol series, ending in distilled water. Steam heat-induced epitope recovery was used before incubation with the primary antibody. Rabbit polyclonal antibodies for E<sub>2</sub> (AR038-5R, Biogenex), ERβ (51-7700, Zymed Laboratories), and a mouse monoclonal antibody for ERα (clone ER88, Biogenex) were used. All sections were developed using standard immunohistochemical protocols.

**Quantitative real-time PCR**

Primers specific for each murine gene of interest were purchased from Applied Biosystems, Inc, as follows: *Cyp1b1* (assay ID: Mm00487229\_m1), *Cry1* (assay ID: Mm00514392\_m1), *Cbr3* (carbonyl reductase 3; assay ID: Mm00557339\_m1), *Ces3* (carboxylesterase 3; assay ID: Mm00474816\_m1), *Col3a1* (collagen, type III, α1; assay ID: Mm00802331\_m1), *Hdc* (histidine decarboxylase; assay ID: Mm00456104\_m1), *Tef* (thyrotrophic embryonic factor; assay ID: Mm00457513\_m1), *Ugt1a6a* (UDP-glycosyltransferase 1 family, polypeptide A6; assay ID: Mm01967851\_s1), and *Hprt1* (hypoxanthine guanine phosphoribosyl transferase; assay ID: Mm00446968\_m1). Total RNA (1 μg) was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR reactions were done in quadruplicate in an Applied Biosystems 7900HT Fast Real-Time PCR System using universal conditions. Data for each test gene and the housekeeping gene (*Hprt1*) were obtained in the form of threshold cycle number (C<sub>t</sub>) for each time point (3, 8, and 20 weeks) and treatment condition (control and smoke treated). The C<sub>t</sub> values for each gene (at each time point) were normalized to the housekeeping gene, and ΔC<sub>t</sub> values for samples from smoke-treated and control groups were compared using the Mann-Whitney test. The step-up method of Benjamini and Hochberg (20) was used to account for multiple hypotheses testing, and the false discovery rate (FDR) was computed for each gene. An FDR cutoff of 0.10 was used to declare statistical significance. The fold change in the transcript levels of samples from smoke-treated and control groups was computed at each time point using the comparative C<sub>t</sub> method (ΔΔC<sub>t</sub>; Applied Biosystems Reference Manual, User Bulletin #2).

**Western blot analysis**

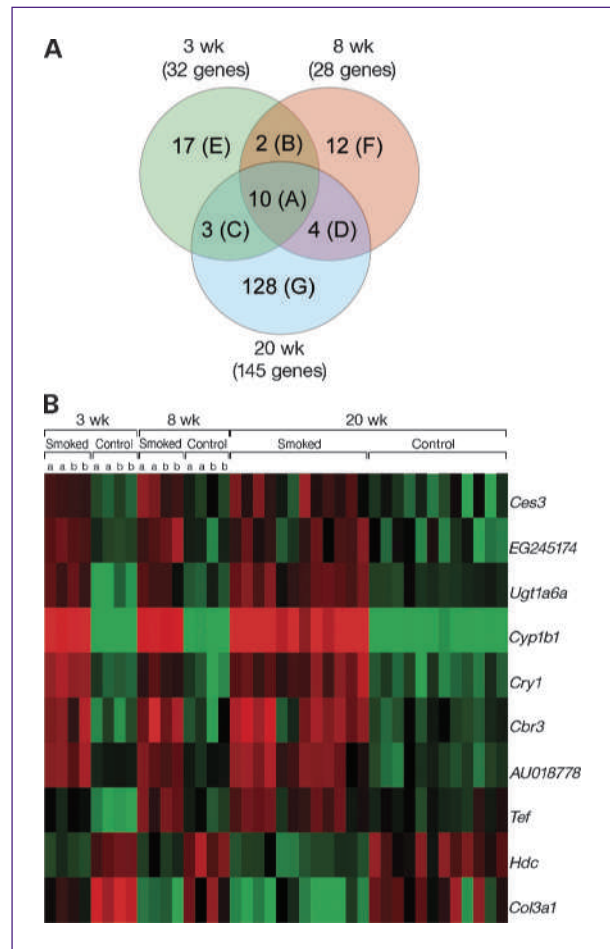
Fifty micrograms of pulmonary microsomal protein isolated from human smokers and nonsmokers (XenoTech LLC) were separated by 10% SDS-PAGE (Bio-Rad) and electroblotted onto a polyvinylidene fluoride membrane. Membranes were blocked for 1 hour at room temperature in TBS with Tween 20 [TBST; 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat milk and incubated overnight at 4 °C with primary antibodies. Primary antibodies against CYP1B1 and HPRT were purchased from Imgenex Corp. and Abcam, Inc., respectively. After washing three times with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad) for

1 hour at room temperature, rinsed with TBST, and visualized using ECL Western Blotting Detection Reagents (GE Healthcare).

**Results**

**Genes modulated by tobacco smoke exposure**

Using a 15K mouse cDNA array, the global gene expression profile of murine lung tissue from female A/J mice exposed to tobacco smoke was compared with that of age-matched control mice maintained in HEPA-filtered ambient air. After normalization, a strong correlation ( $r > 0.8$ ) was observed among all dye-swap replicates as



**Fig. 1.** Genes differentially expressed following 3, 8, and 20 wk of smoke exposure. A, Venn diagram showing the distribution of differentially expressed genes ( $P < 0.001$ ) between control lungs and lungs exposed to smoke for 3, 8, and 20 wk. The 10 genes depicted in Group A are modulated to all three time points. Groups B to D correspond to genes in common between 3 and 8 wk (B), 3 and 20 wk (C), and 8 and 20 wk (D), whereas Groups E to G represent genes identified only at 3 wk (E), 8 wk (F), or 20 wk (G). The genes represented in each group are listed in Table 1. B, a heat map representing the median normalized expression values for the 10 genes altered at all three time points. Data for technical (same letter, i.e., aa) and biological (different letters, i.e., ab) replicates are included.

**Table 1.** Genes differentially expressed following 3, 8, and 20 wk of smoke exposure

Group A				Group G					
Gene symbol	3 wk	8 wk	20 wk	Gene symbol	20 wk	Gene symbol	20 wk	Gene symbol	20 wk
<i>Cyplbl</i>	11.38	8.19	9.81	<i>Scel</i>	2.06	<i>Bcnpl</i>	1.27	<i>Lmbrl</i>	0.71
<i>Cry1</i>	2.66	2.04	2.18	<i>Igh-6</i>	2.01	<i>1600020E01Rik</i>	1.27	<i>Myole</i>	0.71
<i>Cbr3</i>	2.53	2.58	2.02	<i>Acsl1</i>	1.90	<i>Tnk2</i>	1.27	<i>Arf1</i>	0.71
<i>Ugt1a6a</i>	2.52	1.67	1.73	<i>Adprhl2</i>	1.85	<i>Trhde</i>	1.26	<i>Bbx</i>	0.71
<i>AU018778</i>	1.88	1.63	2.02	<i>Nr1d2</i>	1.83	<i>Gtf2a2</i>	1.25	<i>Cbx5</i>	0.71
<i>EG245174</i>	1.85	1.90	1.58	<i>Mylpf</i>	1.81	<i>Trub2</i>	1.25	<i>Tbcl15</i>	0.71
<i>Ces3</i>	1.80	1.74	1.64	<i>Cxcl1</i>	1.80	<i>Bach1</i>	1.25	<i>Sdc4</i>	0.70
<i>Tef</i>	1.79	1.79	1.38	<i>Gclc</i>	1.78	<i>Nfatc4</i>	1.24	<i>Comtd1</i>	0.70
<i>Hdc</i>	0.52	0.52	0.55	<i>Gpx2</i>	1.72	<i>Zfp612</i>	1.24	<i>Xbp1</i>	0.70
<i>Col3a1</i>	0.42	0.41	0.49	<i>LOC100039206</i>	1.66	<i>Krtap16-10</i>	1.24	<i>Lpcat1</i>	0.70
<b>Group B</b>				<i>4922501L14Rik</i>	1.61	<i>Khdc1a</i>	1.23	<i>Igtp</i>	0.69
<b>Gene symbol</b>	<b>3 wk</b>	<b>8 wk</b>	<b>20 wk</b>	<i>Slc40a1</i>	1.58	<i>Ncdn</i>	1.22	<i>Ywhaq</i>	0.69
<i>Ier3</i>	2.00	1.86	—	<i>Fbln2</i>	1.53	<i>Ptp4a1</i>	1.21	<i>9030425E11Rik</i>	0.68
<i>Ttc21a</i>	0.54	0.50	—	<i>Gmnn</i>	1.50	<i>Pygb</i>	0.84	<i>Rnf113a1</i>	0.68
<b>Group C</b>				<i>Fabp3</i>	1.48	<i>Cyhr1</i>	0.84	<i>Hsp90ab1</i>	0.67
<b>Gene symbol</b>	<b>3 wk</b>	<b>8 wk</b>	<b>20 wk</b>	<i>Bclaf1</i>	1.48	<i>Ptpn6</i>	0.83	<i>Tcfcp2l1</i>	0.67
<i>Akr1c14</i>	2.60	—	3.02	<i>Tsix</i>	1.46	<i>Maged2</i>	0.83	<i>Ipo5</i>	0.66
<i>Pdia6</i>	0.64	—	0.60	<i>Cldn12</i>	1.45	<i>Col4a1</i>	0.83	<i>Ahcyl2</i>	0.65
<i>Igfbp3</i>	0.47	—	0.54	<i>Tex12</i>	1.44	<i>Abca3</i>	0.82	<i>Ndst1</i>	0.65
<b>Group D</b>				<i>Cox7a2</i>	1.42	<i>Slc15a4</i>	0.82	<i>LOC100043546</i>	0.65
<b>Gene symbol</b>	<b>3 wk</b>	<b>8 wk</b>	<b>20 wk</b>	<i>Slc39a4</i>	1.41	<i>Ankrd17</i>	0.81	<i>Aspm</i>	0.64
<i>Fkbp5</i>	—	2.36	2.35	<i>Bmp1</i>	1.41	<i>Itgb7</i>	0.81	<i>H2-K1</i>	0.64
<i>Gsta4</i>	—	2.02	1.81	<i>Pigc</i>	1.41	<i>Ubr5</i>	0.80	<i>Hbb-b1</i>	0.64
<i>Hsp90aa1</i>	—	0.58	0.68	<i>Ak3l1</i>	1.41	<i>Wdr79</i>	0.79	<i>Thra</i>	0.63
<i>Ncald</i>	—	0.54	0.71	<i>Gas1</i>	1.40	<i>Srebf1</i>	0.79	<i>2810025M15Rik</i>	0.63
<b>Group E</b>				<i>1700020C11Rik</i>	1.40	<i>Rhbdd3</i>	0.79	<i>Leo1</i>	0.62
<b>Gene symbol</b>	<b>3 wk</b>	<b>8 wk</b>	<b>20 wk</b>	<i>Elof1</i>	1.40	<i>Stmn1</i>	0.79	<i>5830443L24Rik</i>	0.60
<i>Dnajb7</i>	2.32	—	2.53	<i>LOC100043812</i>	1.39	<i>Ubx2</i>	0.79	<i>Rlbp11</i>	0.60
<i>Wipf3</i>	1.98	—	2.28	<i>Ndufc1</i>	1.39	<i>Ptms</i>	0.79	<i>Lrp2</i>	0.58
<i>Ppap2b</i>	1.84	—	1.78	<i>Gsto1</i>	1.38	<i>Pdia5</i>	0.78	<i>Spnb2</i>	0.57
<i>Gstm1</i>	1.64	—	1.76	<i>Cabc1</i>	1.38	<i>Azin1</i>	0.78	<i>Sparc</i>	0.56
<i>Lonrf3</i>	1.64	—	1.57	<i>Prl3d1</i>	1.38	<i>Zdhhc18</i>	0.78	<i>Smc1b</i>	0.54
<i>Tspan15</i>	0.63	—	1.56	<i>Hnmpu</i>	1.37	<i>Gm2a</i>	0.76		
<i>Igf1</i>	0.62	—	0.61	<i>Fancm</i>	1.37	<i>Bst2</i>	0.75		
<i>Col1a2</i>	0.60	—	0.53	<i>Zfp11</i>	1.37	<i>Rac2</i>	0.75		
<i>Cd34</i>	0.58	—	0.52	<i>Apbb2</i>	1.35	<i>Selplg</i>	0.75		
<i>Arhgap28</i>	0.58	—	0.52	<i>Snx14</i>	1.34	<i>Diablo</i>	0.74		
<i>Plekha6</i>	0.58	—	0.50	<i>Akr1c19</i>	1.34	<i>Dhrs3</i>	0.74		
<i>Fbp2</i>	0.56	—	0.38	<i>Tmem57</i>	1.33	<i>Tia1</i>	0.73		
<i>Npr3</i>	0.53	—	0.50	<i>Esd</i>	1.32	<i>Mpv17</i>	0.73		
<i>Igsf10</i>	0.52	—	0.50	<i>Josd3</i>	1.32	<i>Hba-a1</i>	0.73		
<i>D0H4S114</i>	0.47	—	0.50	<i>Sorbs1</i>	1.31	<i>Banp</i>	0.73		
<i>Slc38a5</i>	0.45	—	0.38	<i>Appl2</i>	1.31	<i>Phtf1</i>	0.73		
<i>Npnt</i>	0.40	—	0.38	<i>D19Ert652e</i>	1.30	<i>Gcap14</i>	0.72		
				<i>Xdh</i>	1.30	<i>Cybb</i>	0.72		
				<i>Vdac1</i>	1.30	<i>Stip1</i>	0.72		
				<i>Lias</i>	1.29	<i>Laptm5</i>	0.72		
				<i>RP23-292J1.1</i>	1.27	<i>Reps1</i>	0.72		

NOTE: Differentially expressed genes were identified using the *limma* package with a *P* value threshold of 0.001. Categorization of genes (Groups A-G) corresponds to the same classification in Fig. 1A. Values represent the fold change in linear scale.

**Table 2.** Gene expression analyses by quantitative real-time PCR

Gene	3 wk			8 wk			20 wk		
	P	FDR	Fold change	P	FDR	Fold change	P	FDR	Fold change
<i>Cyp1b1</i>	2.16e-03	4.33e-03	22.58	2.16e-03	2.89e-03	22.58	2.16e-03	3.46e-03	19.05
<i>Cry1</i>	2.16e-03	4.33e-03	4.30	2.16e-03	2.89e-03	2.79	2.16e-03	3.46e-03	2.94
<i>Cbr3</i>	2.16e-03	4.33e-03	3.56	2.16e-03	2.89e-03	2.55	2.16e-03	3.46e-03	2.79
<i>Ces3</i>	6.49e-02	6.49e-02	2.38	8.66e-03	9.89e-03	1.68	4.11e-02	4.70e-02	1.57
<i>Tef</i>	2.16e-03	4.33e-03	2.53	6.49e-02	6.49e-02	1.54	4.33e-03	5.77e-03	1.87
<i>Ugt1a6a</i>	6.49e-02	6.49e-02	1.71	2.16e-03	2.89e-03	2.58	6.49e-02	6.49e-02	1.46
<i>Col3a1</i>	1.52e-02	2.42e-02	0.54	2.16e-03	2.89e-03	0.30	2.16e-03	3.46e-03	0.38
<i>Hdc</i>	6.49e-02	6.49e-02	0.91	2.16e-03	2.89e-03	0.43	2.16e-03	3.46e-03	0.43

NOTE: Total RNA was assayed by reverse transcription-PCR. Samples were analyzed in quadruplicate, and the resulting data were expressed as the average cycle threshold ( $C_t$ ). The housekeeping gene *Hprt1* was used for data normalization ( $\Delta C_t$ ). Comparisons between control and smoke-exposed samples for statistical significance were determined using the Mann-Whitney test ( $P$  value) and the step-up method of Benjamini and Hochberg (FDR). Fold change was calculated using the comparative  $C_t$  method ( $\Delta\Delta C_t$ ). Genes induced or repressed following smoke exposure are indicated by a fold change  $>1$  or  $<1$ , respectively.

well as between two pools of samples ( $n = 3$  mice per pool) from the same treatment group (data not shown).

To identify early molecular changes induced by tobacco smoke, gene expression profiles from control and smoke-exposed lung tissues were determined following 3, 8, and 20 weeks of exposure. The expression of 32, 28, and 145 genes was modulated significantly by smoke following 3, 8, and 20 weeks, respectively ( $P = 0.001$ ; Fig. 1A). Ten genes were identified as differentially expressed at all time points (Group A, Table 1). The heat map represents fold change in the expression of these genes following 3, 8, and 20 weeks of exposure (Fig. 1B). Hierarchical clustering of the median corrected expression values of this subset of genes showed a precise separation of control and smoke-exposed samples (data not shown). Interestingly, the magnitude of the change in expression was similar for all smoke-exposed groups.

The single gene differentially expressed to the greatest extent (9- to 12-fold increase) in all smoke-exposed groups as compared with controls was *Cyp1b1* (Table 1), a phase I detoxification enzyme involved in both the activation of carcinogens such as BaP and the metabolism of  $E_2$ .

A surprising finding is the significant upregulation of *cryptochrome 1* (*Cry1*), one of the key transcriptional regulators of circadian rhythm, in response to smoke exposure. After *Cyp1b1*, *Cry1* was the gene differentially regulated to the greatest extent following 3 weeks of cigarette smoke exposure (fold change, 1.99; Table 1) and one of the 10 genes altered at all three time points after smoke exposure (Fig. 1).

#### Quantitative real-time PCR and Western blot analysis

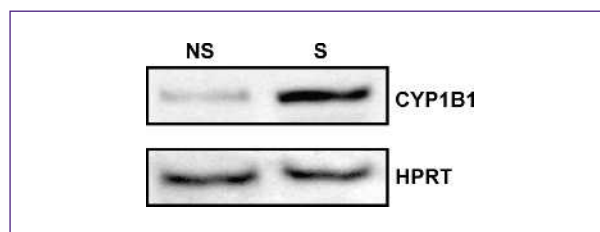
Differential expression of the genes that were modulated at all three time points and have a known function (7 of the 10 genes, Group A, Table 1) was validated by real-time PCR. All genes tested showed at least a 2-fold change in relative quantitation or had a FDR of  $<0.10$ , thereby validating the cDNA microarray results. The results for the 3-, 8-, and

20-week time points are presented in Table 2. A strong correlation was observed between the fold changes in gene expression determined by cDNA microarray and reverse transcription-PCR (Spearman's  $\rho$  was 0.9, 0.79, and 0.9 for samples obtained after 3, 8, and 20 weeks of smoke exposure, respectively).

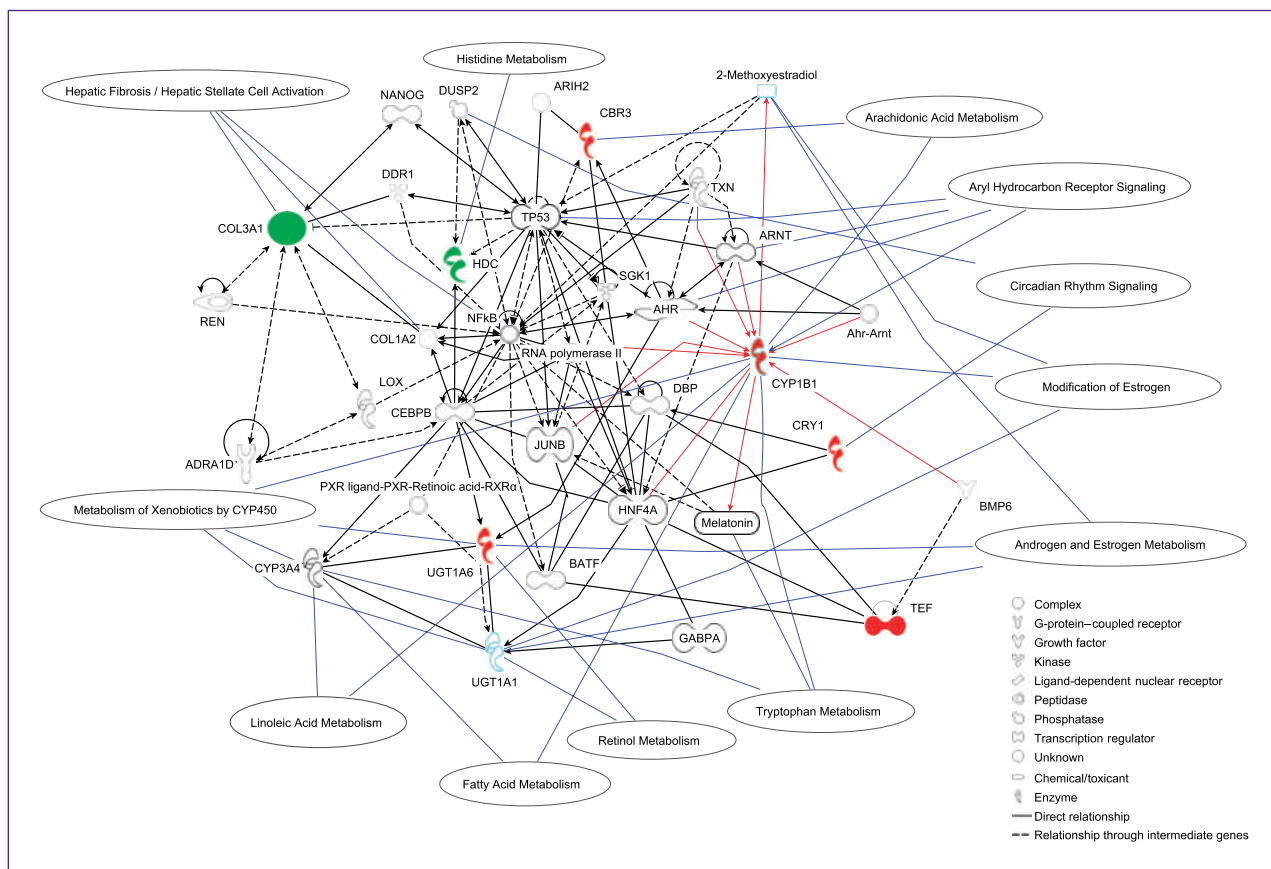
Examination of three commercial antibodies for their specificity for mouse CYP1B1 indicated that none were highly specific for the protein of interest when analyzed by Western blot. For this reason, the ability of smoke to increase CYP1B1 mRNA expression was validated at the protein level using pooled preparations of human lung microsomes. Western blot analyses revealed a significant elevation in CYP1B1 protein in microsomes from smokers as compared with nonsmokers. These data not only confirm that both CYP1B1 mRNA and protein levels are elevated after smoke exposure but also indicate the relevance of the murine finding to humans (Fig. 2).

#### Identification of biological pathways and networks modulated by tobacco smoke

In addition to identifying individual genes differentially expressed (fold change), a complementary strategy was



**Fig. 2.** Western blot analysis of CYP1B1 in human pulmonary microsomes from nonsmokers (NS) and smokers (S). Each sample (50  $\mu$ g) contains a pool of microsomal protein from four individuals of mixed genders. HPRT was used as a loading control.



**Fig. 3.** Network of genes differentially expressed in common following 3, 8, and 20 wk of smoke exposure. Differentially expressed genes ( $n = 7$ ) are depicted as a network with overlaid functions and pathways according to Ingenuity Pathways Analysis Software. The green and red colors represent downregulated and upregulated genes, respectively. The remaining genes are involved in the network through direct or indirect interactions. Red lines connecting *Cyp1b1* to other genes indicate a direct relationship with *Cyp1b1* (protein-protein interaction or protein-DNA). For example, the Ahr-Arnt complex increases transcription of *Cyp1b1* in mammals (47). Solid blue lines with balloons indicate gene function or a pathway in which a gene is involved.

used to identify biological pathways and networks modulated by short-term exposure to tobacco smoke. The fold changes of the 10 genes differentially expressed at all time points (3, 8, and 20 weeks of smoke exposure) were mapped through a statistical computation method (Ingenuity Pathways Analysis Software). Figure 3 depicts a high-scoring network based on 7 of 10 genes that were eligible for network construction. In addition, canonical pathways and significant functions were mapped onto this network.

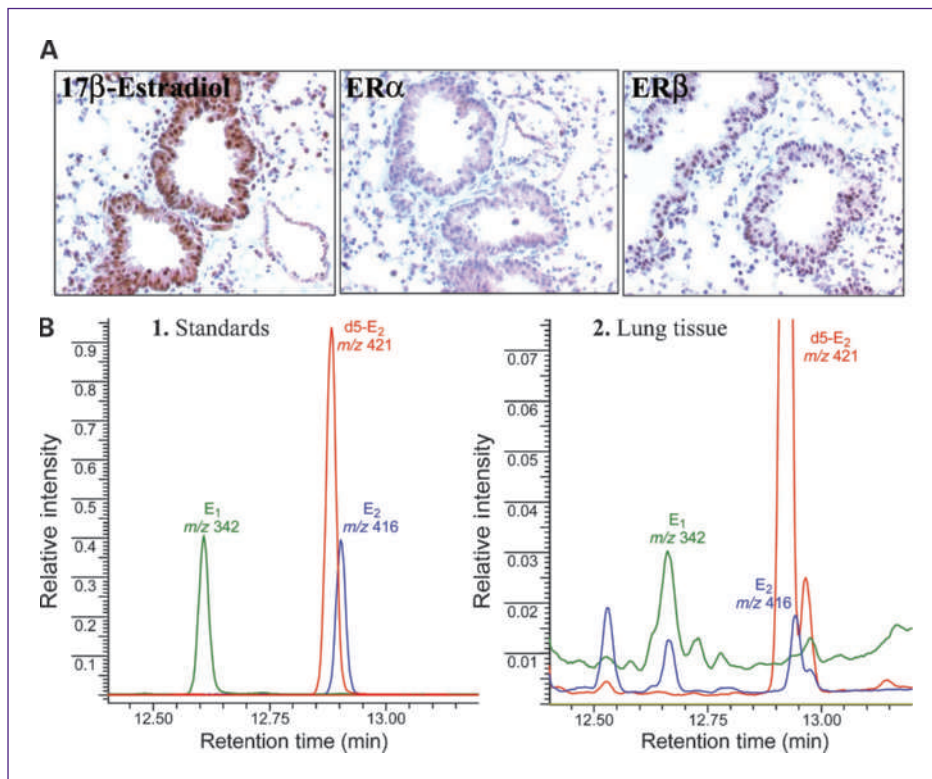
Consistent with induction of *Cyp1b1* and its role in estrogen metabolism, oxidation of estrogen was identified as part of the network significantly modulated by tobacco smoke exposure, as highlighted in Fig. 3. *Cyp1b1* was also present in several other pathways identified as being altered by smoke exposure, including metabolism of xenobiotics by cytochrome P450, linoleic acid metabolism, fatty acid metabolism, and tryptophan metabolism.

Additional pathways modulated by smoke exposure are indicated in Fig. 3 and include hepatic fibrosis, histidine metabolism, arachidonic acid metabolism, aryl hydrocarbon receptor signaling, and circadian rhythm signaling. Further studies are required to determine if either activa-

tion or repression of these biological pathways contributes to smoke-induced lung tumorigenesis.

### Detection and localization of estrogens within murine lung tissue

Although metabolism of estrogen is an important activity of *Cyp1b1*, this hormone had not been detected in murine lung tissue previously. We established a sensitive GC/MS method for the detection of  $E_2$  and  $E_1$ , which can be converted to  $E_2$ . Solvent extraction and GC/MS protocols were developed using standard solutions of the compounds  $E_1$  and  $E_2$ . Lung tissue extracts were mixed with known amounts of  $E_1$  and  $E_2$  before extraction to assess recovery. Representative chromatograms of standards and extracts of lung tissue, illustrating the ions monitored, are presented in Fig. 4. Analysis of lung tissue from eight untreated female mice clearly showed the presence of both  $E_1$  and  $E_2$  in extracts. The limit of detection per injection for  $E_1$  and  $E_2$  standards was 0.03 pmol, and the recoveries were 93% and 91%, respectively. However, the recovery of each compound from tissue was lower ( $E_1$ , 29%;  $E_2$ , 28%). Therefore, the actual concentration of estrogens in lung



**Fig. 4.** Detection of estrogens within murine lung tissue. Lung tissue from female A/J mice was subjected to immunohistochemical and GC/MS analyses. A, detection of E<sub>2</sub>, ERα, and ERβ in lung epithelial cells by immunostaining. The bronchioalveolar epithelium (BAE) stained positive for all antigens evaluated. Subcellular staining was observed as follows: E<sub>2</sub>, strong nuclear and cytoplasmic staining in the BAE and some pneumocytes; ERα, cytoplasmic staining of the BAE; and ERβ, nuclear staining in the BAE and some pneumocytes. B, selective ion monitoring of trimethylsilyl derivatives of E<sub>1</sub> and E<sub>2</sub> (1.1 pmol each) and d5-E<sub>2</sub> (2.6 pmol) as standards (B1) and in the murine lung tissue (B2). Each trace represents different ions monitored. Deuterium-labeled E<sub>2</sub> represents the internal standard. Unmarked peaks in B2 denote unknowns; the upper part of the chromatogram was cropped to enhance the visualization of small peaks.

tissue could not be quantified due to low recovery. Efforts are under way to optimize the recovery and expand the methods to measure a full panel of estrogen metabolites.

The cellular localization of E<sub>2</sub> within female murine lung tissue was determined by immunohistochemistry. Estrogen receptors (ERα and ERβ) were also examined to determine if ER-mediated estrogen signaling could occur within lung tissue. Staining for all antigens was localized primarily to the bronchial and bronchioalveolar epithelium. Strong nuclear and cytoplasmic staining of E<sub>2</sub> was observed, whereas staining of ERα and ERβ was localized primarily to the cytoplasm and nucleus, respectively (Fig. 4). No positive staining was detected in sections incubated with nonimmune IgG (negative control; data not shown). Because this observation was purely qualitative, additional quantitative analyses of immunostained sections of untreated and smoke-exposed lung tissues are required to validate the observed subcellular localization of ER expression. Nevertheless, intracellular localization of E<sub>2</sub> when combined with its detection by GC/MS in perfused lungs, as done in this study, ensures that estrogens are present within murine lung tissue (as opposed to only in the circulation).

## Discussion

Witschi and colleagues (15) have shown that A/J mice that have been exposed to tobacco smoke for 20 weeks develop an increased multiplicity of lung tumors when allowed to recover in ambient air for 16 weeks before

sacrifice, as compared with those exposed to tobacco smoke continuously for 36 weeks. This model suggests that early changes in gene expression within a smoke-exposed lung lead to irreversible cellular damage that is sufficient to initiate lung carcinogenesis. Thus, genes whose expression is altered very early in the carcinogenesis process represent cellular alterations that may serve as early targets for intervention in smoke-induced lung carcinogenesis. The expression of genes that were altered consistently after 3, 8, and 20 weeks of active smoke exposure [*Cyp1b1*, *Cry1*, *Cbr3*, *Ugt1a6a*, *Ces3*, *Tef*, *Hdc*, and *Col3a1* (Fig. 1, Group A; Tables 1 and 2)], as determined by cDNA microarray, was validated by quantitative real-time PCR. It should be noted that not all of the genes with an established role in smoke-induced carcinogenesis were represented on the microarray, including *Cyp1a1*.

*Cyp1b1*, a phase I enzyme, was the gene induced to the greatest extent within the lungs following continuous exposure to tobacco smoke. *CYP1b1* transcript levels were elevated as early as 3 weeks and remained upregulated for the duration of smoke exposure (20 weeks; Fig. 1; Tables 1 and 2). Upregulation of *Cyp1b1* by tobacco smoke is of utmost interest because of its role in the metabolism of not only the polycyclic aromatic hydrocarbons found in tobacco smoke (e.g., BaP) but also E<sub>2</sub>, resulting in the generation of highly reactive catechols and quinone metabolites. These derivatives are known to form DNA adducts and cause genotoxicity (21, 22). Administration of 4-E<sub>2</sub>, the major product of *CYP1B1* metabolism of estrogen, to Syrian hamsters and CD-1 mice has led to the



development of renal cancer (23) and uterine adenocarcinomas (24), respectively. In humans, higher levels of 4-E<sub>2</sub> are present in endometrial and breast cancers as compared with normal tissue (25).

Transcriptional induction of pulmonary *Cyp1b1* by tobacco smoke has not been reported previously for A/J mice (14, 26). A 2-fold increase in *Cyp1b1* mRNA levels was observed within the lungs of Sprague-Dawley rats exposed to mainstream tobacco smoke for 3 hours, but, surprisingly, not after 3 weeks of exposure (27). These preclinical data are consistent with reports of CYP1B1 induction in human smokers (28, 29). The dual role of *Cyp1b1* in the metabolism of both constituents of tobacco smoke and estrogens, the pulmonary induction of *Cyp1b1* by short-term tobacco smoke exposure, and validation of the response of CYP1B1 to tobacco smoke in human microsomes, as observed in the present study, provide strong support for the A/J mouse as a highly relevant model system in which to investigate the role of hormones in smoke-induced lung carcinogenesis.

Although results from previous studies suggest that ER-mediated signaling can occur within the lung, the present study is the first to report the detection of E<sub>1</sub> and E<sub>2</sub> within murine lung tissue. It is likely that tobacco smoke modulates estrogen levels within the lung by altering the expression of estrogen metabolism genes, including *Cyp1b1*. The GC/MS protocol established in this study focuses on the detection of parent estrogens. Based on the important role of estrogen signaling and estrogen metabolites in breast carcinogenesis, a detailed analysis of the biological significance of this hormone in lung tissue is warranted. Analysis of estrogen metabolites in human breast tissue by high-performance liquid chromatography revealed that the levels of 4-E<sub>2</sub> and 4-E<sub>1</sub> as well as the quinone conjugates are significantly elevated in breast tissue from women with cancer as compared with women with benign disease (30). Although data on the detection of estrogens within tissue have been reported primarily for the breast, the ability of human bronchial epithelial BEAS-2B cells to metabolize estrogen has been demonstrated *in vitro*. Accumulation of 2-E<sub>2</sub> and 4-E<sub>2</sub> was observed in lung cells treated with BaP in the presence of E<sub>2</sub> (31). In summary, detection of hydroxylated metabolites and estrogen conjugates (methoxy and others) within the lungs, as well as estrogen-associated DNA adducts, is a promising tool to elucidate the mechanism by which estrogen induces lung carcinogenesis.

E<sub>2</sub> as well as ER $\alpha$  and ER $\beta$  was detected immunohistochemically in murine bronchioloalveolar cells (Fig. 4). Atypical cytoplasmic localization of ER $\alpha$  was observed in the present study. Although this finding is in agreement with the reported cytoplasmic localization of ER $\alpha$  in human lung tissue (normal and tumor; ref. 32) and normal human bronchial epithelial cells (13), the biological significance of extranuclear localization of ER $\alpha$  remains unclear. In breast cancer cells, the ER can be present in the cytoplasm or in the cell membrane where it binds to growth factor receptors, such as the EGF receptor, and exerts its signaling through downstream kinases (9).

Although the functionality of ERs within the murine lung was not investigated in this study, numerous studies suggest that estrogen signaling within the lung can promote cell proliferation (13, 32, 33).

Data generated in the present study with the A/J mouse model complement the findings from several epidemiologic and preclinical analyses that suggest the involvement of estrogen in lung carcinogenesis, either alone or in combination with smoking (7, 34, 35). Similar to humans, the effect of hormones on lung carcinogenesis in animals has been suggested (36, 37). The multiplicity of lung tumors in mice exposed to mainstream cigarette smoke early in life was significantly higher in females than in males (36). Moreover, exposure of mice to diethylstilbestrol, a synthetic estrogen, increases the incidence and multiplicity of lung tumors induced by urethane administration (37).

Following *CYP1B1*, *Cry1* was the gene most differentially expressed in the present study (Figs. 1 and 3; Table 1). *Cry1* is one of the regulators of circadian rhythm, controlling physiologic, biochemical, and behavioral functions with a periodicity of approximately 24 hours. A prior study in rats exposed to tobacco smoke describes a distinct cyclic pattern of expression of other circadian rhythm genes such as *Arntl*, *Dbp*, and *Nr1d2*, but not *Cry1* (38). "Clock genes" are emerging as central players in cell cycle control and proliferation, and the altered expression of these genes has been observed in both endometrial (39) and breast (40) cancers. Interestingly, levels of melatonin, an important neuroendocrine output of circadian rhythm, are also affected by smoke exposure (41), and CYP1B1 is capable of metabolizing melatonin to 6-hydroxy melatonin (42). Our data depict the direct interaction between enzyme (*Cyp1b1*) and substrate (melatonin) and the overexpression of *Cry1* (Fig. 3). These data, when combined, provide support for further evaluation of the role of the circadian rhythm genes in tobacco smoke-induced lung carcinogenesis.

Following *Cyp1b1* and *Cry1*, the other genes differentially regulated at all time points following smoke exposure (Group A) are involved in metabolism of both endogenous and exogenous compounds (*Cbr3*, *Ugt1a6a*, *Ces3*, and *Hdc*), signal transduction (*Tef*), and the extracellular matrix (*Col3a*), and their expression has been associated with other cancer types. *Cbr3* (carbonyl reductase 3) catalyzes the reduction of many endogenous and xenobiotic carbonyl compounds, including steroids and prostaglandins, to their corresponding alcohols. Its expression is reduced in oral squamous cell carcinoma cells when compared with premalignant dysplasias and hyperplasias and has been associated with reduced cell growth and motility (43). Histidine decarboxylase (*Hdc*) is a member of the histidine metabolism pathway and is responsible for the biosynthesis of histamine. *Hdc* has been suggested as a new marker for neuroendocrine differentiation, inflammatory pathologies, and several leukemias and highly malignant forms of cancer, including small-cell lung carcinoma (44). The involvement of histamine in the growth of mouse and rat tumors has been suggested (45). Thyrotrophic embryonic

factor (Tef) is a transcription factor that controls the expression of many enzymes and regulators involved in detoxification and drug metabolism, such as cytochrome P450 enzymes, carboxylesterases, and constitutive androstane receptor (46).

In summary, this study identifies gene expression changes that are induced by smoke exposure (3, 8, and 20 weeks). It is the first to report the successful detection of estrogen within murine lung tissue and a network of CYP1B1-associated genes that are modulated by smoke exposure. The ability of tobacco smoke to induce alterations in the expression of genes related to estrogen metabolism within the lung provides new insight into the molecular basis of smoke-induced lung cancer, in particular, female lung cancer. Alteration of circadian rhythm and other pathways is also reported, which may represent novel targets for lung cancer prevention.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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