The Transcriptome of \(Nrf2^{-/-}\) Mice Provides Evidence for Impaired Cell Cycle Progression in the Development of Cigarette Smoke–Induced Emphysematous Changes

Stephan Gebel,* Svenja Diehl,* Jan Pype,† Bärbel Friedrichs,* Horst Weiler,* Jutta Schüller,* Haiyan Xu,† Keiko Taguchi,‡ Masayuki Yamamoto,‡ and Thomas Müller*†

*Philip Morris Research Laboratories GmbH, Philip Morris International Research & Development, 51149 Köln, Germany; †Philip Morris Research Laboratories bvba, Philip Morris International Research & Development, 3001 Leuven, Belgium; and ‡Department of Medical Biochemistry, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai 980-8575, Japan

† To whom correspondence should be addressed at Philip Morris Research Laboratories GmbH, Fuggerstr. 3, 51149 Köln, Germany. Fax: +49-2203-303362. E-mail: thomas.mueller@pmintl.com.

Received October 19, 2009; accepted January 29, 2010

Cigarette smoke (CS) imposes a strong oxidative burden on exposed tissues resulting in a severely disturbed oxidant/antioxidant balance, which in the context of chronic exposure is assumed to be a key contributor to CS-related diseases. Because of its emerging central role in orchestrating the general cellular antioxidant response, the pathway leading to the activation of the transcription factor Nrf2 has received mounting attention over the past decade in investigations aimed at elucidating CS-induced pathophysiological mechanisms. To comprehensively characterize the impact of Nrf2 in acute and subchronic smoking scenarios, \(Nrf2^{-/-}\) mice and their wild-type (wt) ICR littermates were exposed to either ambient air (sham exposure) or one of three doses of CS for up to 5 months, with two postexposure endpoints of 1 and 13 days. The lungs of the mice were monitored for transcriptomic changes on a genome-wide level, which confirmed an impaired expression of antioxidant and phase 2–related genes in CS-exposed \(Nrf2^{-/-}\) mice. Importantly, in comparison to wt mice, an attenuated cell cycle/mitotic response and intensified stress gene expression pattern were observed in exposed \(Nrf2^{-/-}\) mice, which was paralleled by clear dose-dependent effects on alveolar destruction and impaired lung function. In contrast, the inflammation-related transcriptional response and scores for various bronchioalveolar inflammation parameters were qualitatively and quantitatively similar in CS-exposed mice of both genotypes. Taken together, these results confirm the protective nature of Nrf2 in oxidative stress scenarios and suggest that the enhanced emphysematous phenotype exhibited by CS-exposed \(Nrf2^{-/-}\) mice is more likely caused by an imbalance in cell loss and regeneration than by increased inflammation.

Key Words: cigarette smoke; Nrf2; gene expression profiling; cell cycle; emphysema.
polymorphisms in the human NRF2 gene have been associated with human respiratory disease, especially emphysema. While no close connection was seen between the risk of COPD and NRF2 promoter–resident single nucleotide promoter polymorphisms (SNPs) in a group of Japanese patients (Yamamoto et al., 2004), it was recently reported that SNPs −686G/A (rs35652124) and CCG trinucleotide repeats are in almost complete linkage disequilibrium with SNP rs2364723 (Siedlinski et al., 2009). This latter SNP was found to be associated with a lower forced expiratory volume in 1 s (FEV1) level, a strong indicator of emphysema. Interestingly, effects induced by SNP rs2364723 are more prominent in ever smokers, while patients harboring the −617 A SNP face a significantly elevated risk for developing acute lung injury (Marzec et al., 2007).

In mechanistic terms, Nrf2 is strictly controlled in the cytosol in the absence of cellular stress by a sophisticated dual negative control mechanism involving a fine-tuned two-site (“hinge-latch”) binding via its ETGE and DLG motifs to the C-terminal DC domains of homodimerized Keap1, the functional negative regulator of Nrf2 (Yamamoto et al., 2008). In this condition, Keap1, while natively complexed to Cullin 3 (Cul3), thus constituting a unique ubiquitin E3 ligase, promotes the polyubiquitination and rapid degradation of Nrf2. However, if, owing to changes in their microenvironment, cells are confronted with an impaired redox homeostasis arising from intruding electrophiles and oxidants, this inhibitory mechanism is abruptly abrogated based on conformational changes in the Keap1 homodimer, resulting in the release of Nrf2. As demonstrated by transgenic mutation analysis, the switch from the functionally repressing conformation to the Nrf2 activating conformation is induced by the oxidation of three essential cysteine residues within the Keap1 protein, i.e., at positions 151, 273, and 288 (Yamamoto et al., 2008). Once activated, Nrf2 demonstrates increased stability and localizes to the nucleus to initiate the transcriptional response in conjunction with an appropriate dimerization partner, by targeting its recognition sequence known as antioxidant response element (ARE) in the promoter region of potential client genes.

Regarding the sulfhydryl (SH)-modifying mechanism fundamental to Nrf2 activation as briefly summarized above, it is important to note that previous investigations in vitro and in vivo demonstrated the biological relevance not only of the electrophilic potential inherent to CS in general but also of specific compounds either delivered directly from CS or formed from CS-dependent precursors in an aqueous milieu. Compounds identified in this context include peroxynitrite, benzoquinone, and CS-related aldehydes, in particular acrolein, all of which are known to biologically act, at least in part, through their strong SH-reactiveness (for review, see Müller and Gebel, 2006). The activation of Nrf2 was finally proven in CS-exposed cells in vitro by its strong stabilization, nuclear localization, and ARE binding as well as by the abrogated transcriptional activation of presumptive Nrf2 response genes, such as NAD(P)H:quinone oxidoreductase (nqo1) and heme oxygenase-1 (hmx1), under the regimen of Nrf2 down-regulation by RNA interference (Knörr-Wittmann et al., 2005).

To our knowledge, the current literature contains two reports describing the effects of CS exposure on the lungs of Nrf2−/− mice in comparison to their wild-type (wt) counterparts (Iizuka et al., 2005; Rangasamy et al., 2004). Both studies report an increased accumulation of inflammatory cells in the lungs of CS-treated Nrf2−/− versus Nrf2+/- mice, thus potentially explaining the susceptibility of Nrf2−/− mice for emphysema as highlighted, e.g., by greater mean values of the mean linear intercept. In addition, the paper by Rangasamy et al. (2004) provided evidence for a profound protective effect of Nrf2 on several other oxidative stress- and inflammation-related parameters. However, while presenting data solely from acutely exposed animals (Rangasamy et al., 2004) or from a small number of known Nrf2 target genes (Iizuka et al., 2005), both papers, in terms of exposure duration and dose, are limited regarding alterations of the gene expression patterns in CS-treated mice of both genotypes. Here, we report on the genome-wide transcriptomic changes observed in the lungs of Nrf2−/− and wt mice exposed to three doses of CS or to ambient air for up to 5 months with and without two postexposure endpoints of 1 and 13 days. Our data demonstrate that in response to CS exposure, wt and Nrf2−/− mice develop distinct gene expression patterns, suggesting that the susceptibility of CS-exposed Nrf2−/− mice for emphysema formation may be more likely caused by the inability to compensate for cell loss rather than by increased inflammation.

MATERIALS AND METHODS

Cigarette and cigarette smoke generation. The Standard Reference Cigarette 2R3F was obtained from the Tobacco and Health Institute at the University of Kentucky. It is a filter cigarette with reported mainstream smoke yields per cigarette of 11.6 mg total particulate matter (TPM), 0.81 mg nicotine, and 12.3 mg carbon monoxide (CO). All cigarettes were conditioned and smoked according to International Organization for Standardization methods as described (Gebel et al., 2004). Mainstream smoke was diluted with filtered conditioned air to a target concentration of 750 μg TPM/L. To monitor the stability and reproducibility of smoke generation, TPM, CO, nicotine, aldehydes (formaldehyde, acetaldehyde, and acrolein), and particle size distribution were determined (Hausmann et al., 1998).

Animals and exposure. The Nrf2 knock out mouse (ICR background) (Reg. No. 00984) was provided by RIKEN BRC, which is participating in the National BioResource Project of the Ministry of Education, Culture, Sports, Science, and Technology, Japan. Female Nrf2−/− mice, bred under specific pathogen-free conditions at Charles River Laboratories Lyon (France), were used. Embryos originated from crossing an ICR female with a heterozygous male. Number of backcross generations was 11 † 1 on ICR (Itoh et al., 1997). Before delivery, the genotype of each individual mouse was confirmed by the breeder, and the mice were grouped by genotype upon arrival. The Nrf2−/− mice and their wt littermates, bred under specified pathogen-free conditions at Charles River Laboratories Lyon (France), were used. Embryos originated from crossing an ICR female with a heterozygous male. Number of backcross generations was 11 + 1 on ICR (Itoh et al., 1997). Before delivery, the genotype of each individual mouse was confirmed by the breeder, and the mice were grouped by genotype upon arrival. The Nrf2−/− mice and their wt littermates were between 12 and 13 weeks old at the start of exposure. Histopathological evaluation and serological screening confirmed the good health status of the mice at the beginning of the study. Care and use of the mice was in conformity with the American Association for Laboratory Animal Science Policy on the Humane Care and Use of Laboratory Animals (http://www.aalas.org/). Animal experiments were approved by the Institutional
Animal Care and Use Committee of Philip Morris Research Laboratories, Belgium. Mice were exposed in whole-body exposure chambers to diluted mainstream CS at a target concentration of 750 µg TPM/l for 2, 3, or 4 h/day (low, medium, and high dose, respectively) or to conditioned fresh air (sham exposure) for 4 h/day, 5 days/week. The CS exposure started with a dose adaptation period, i.e., 125 µg TPM/l on day 1 and was gradually increased to reach the target concentration by day 17. Each exposure group included 34–47 mice per genotype dedicated for four different endpoints (histopathology, bronchoalveolar lavage [BAL] cell analysis, respiratory mechanic measurements, and gene expression analysis). All mice were exposed for 5 months to CS except those used for gene expression analysis, which were subjected to the exposure schedule outlined below and in Table 1. Daily exposure was continued throughout the weekend before the 5-month necropsy.

Lungs from nine mice per group were fixed for histopathological evaluation and morphometrical evaluation of emphysematous changes in the lung parenchyma. BAL fluid was collected from nine mice per group by five consecutive cycles of filling and emptying of the lungs via the trachea with 1 ml MgCl2-free phosphate-buffered saline containing 0.3% bovine serum albumin. The differentiation of free lung cells was implemented by flow cytometry using antibodies against Ly6G (clone 1A8; fluorescein isothiocyanate conjugated; BD Biosciences) and F4/80 (clone CI:A3-1; allophycocyanin conjugated; BD Biosciences). Multianalyte profiling (RodenMAP) was performed by Rules-Based Medicine Inc. (Austin, TX) in supernatants from the first BAL cycle. Nine mice per study group were used for respiratory mechanic measurements (see below). For these endpoints, dissection was performed at 18–24 h after the last exposure.

Gene expression analysis (four mice/genotype/exposure group/time point) was assessed at five different time points: after 1 day (exposure at 125 µg TPM/l), at 2 months, at 5-months CS-exposure, and at 5-months CS-exposure plus 1- or 13-day postexposure. Low- and high-dose CS groups were included for the two 5-month time points: exposure plus 1-day postexposure (Table 1). For gene expression analysis, mice were sacrificed 1–3 h after the last exposure (except for postexposure groups). To monitor smoke uptake and exposure, carboxyhemoglobin (HbCO) concentrations in blood were determined during the exposure period. The HbCO concentrations in blood were determined during the exposure period. The HbCO proportions were comparable for three CS-exposed groups (low, medium, and high dose) of the same genotype were limited. Nevertheless, the high-dose group consistently had the lowest mean body weight gain in each genotype. At the end of the 5-month exposure period, the mean body weight of the high-dose neverreached mice and wt groups was decreased by 41 and 24%, respectively, compared to sham-exposed groups.

**TABLE 1**

**Summary of Study Concept: X, Dissection Time Points Used for Genome-Wide Expression Analysis, and Y, Dissection Time Point Used for Phenotypic Analyses. For Details, See Text and “Materials and Methods” Section**

<table>
<thead>
<tr>
<th>Exposure Postexposure</th>
<th>Acute</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 months</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 months</td>
<td>X/Y</td>
<td>X/Y</td>
<td>X/Y</td>
<td>X/Y</td>
</tr>
<tr>
<td>5 months 1 day</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>5 months 13 days</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**RNA preparation.** For RNA preparation, lungs were deep-frozen in liquid N2 immediately after dissection and stored at −70°C. Left lobes were minced and RNA was extracted using the RNeasy Midi Kit (Qiagen, Hilden, Germany). RNA integrity was determined using the Agilent 2100 Bioanalyzer. All RNA samples showed a RNA integrity number > 8.

**Reverse transcriptase real-time quantitative PCR.** Reverse transcriptase real-time quantitative PCRs (RT-qPCRs) were conducted using the cDNA Archive Kit and the following Assay-on-Demand Kits from Applied Biosystems (Darmstadt, Germany): eukaryotic β-actin (assay ID: Mm00466851_m1), cyp1A1 (Mm00487217_m1), cxxl (Mm00433859_m1), npo1 (Mm00500554_m1), nqo1 (Mm00500821_m1), aerka (Mm01248177_m1), slpi (Mm00441529_g1), ccna2 (Mm00438064_m1), and birc5 (Mm00599750_g1). For first-strand complementary DNA (cDNA) synthesis, 2 µg of total RNA was used in a final reaction volume of 100 µl. The qPCRs were performed with 20 ng first-strand cDNA/reaction in triplicate (10 µl final volume each) in 384-well plates. Of the panel of housekeeping genes tested, β-actin showed the most constant expression levels and was therefore used as an endogenous control for normalization.

Analysis of the RT-qPCRs was performed by Comparative Ct Method for Relative Quantification.

**Microarray procedure.** Transcriptome analysis was performed by Microarray Facility Tübingen (Germany) according to the manufacturer’s recommendation in the Affymetrix GeneChip Expression Analysis Technical Manual (Santa Clara, CA). After synthesis of double-stranded cDNA, biotinylated complementary RNA (cRNA) was generated and fragmented. The degree of fragmentation and the length distribution of this fragmented biotinylated cRNA was checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer. Ten micrograms of biotinylated fragmented cRNA was hybridized to GeneChip Mouse Genome 430 2.0 for 16 h at 60 rpm in a 45°C GeneChip Hybridization Oven 640 (Affymetrix). The arrays were washed and stained on the GeneChip Fluidics Station 450 (Affymetrix), followed by antibody signal amplification, washing, and staining using streptavidin R-phycocerythin. The arrays were scanned using the Agilent GeneArray Scanner 3000 7G. Scanned image files were visually inspected for artifacts and then analyzed. Each image was scaled to the same all probe set intensity for comparison between chips. The GeneChip Operating Software was used to control the fluids station and the scanner, to capture probe array data, and to analyze hybridization intensity data, applying the default parameters provided in the Affymetrix data analysis software package.

**Quality control of microarray data.** The quality of the Affymetrix Microarray data was checked using the Bioconductor package sleaply. No outlier chips were identified using the following quality parameters: percentage of present calls, average background, scaling factor, and GAPDH/β-actin ratios.

**Gene expression data analysis.** Gene expression data were processed using R and Bioconductor packages affy, germa, and genefilter. Raw data were background corrected, normalized, and summarized using the GeneChip-Robust Multi-array Average algorithm (Wu et al. 2004). For comparison of two different groups, the t-test was performed using the default settings from the genefilter package. The fold change was calculated using nonlogarithmic values. Probe sets showing absolute fold changes greater than or equal to 3 and p values less than 0.05 were identified.

**Unsupervised clustering.** Fold changes were set to Unweighted Pair Group Method with Arithmetic mean clustering using correlation as the similarity measure (Spotfire DecisionSite for Functional Genomics). Clustering was limited to 1486 probe sets for the time course data set and to 1399 probe sets for the dosage data set by filtering the absolute Fold Change to 3 and the p value to 0.05.

**Functional analysis.** Probe sets from the data sets belonging to the time course cluster 1.1, 1.2, 1.3, or 2.1 or to the dosage cluster 3 or 4 were analyzed using “Ingenuity Pathways Analysis” (IPA) (Ingenuity Systems, http://www.ingenuity.com). Fischer’s exact test was used to calculate a p value, determining the probability that each biological function assigned to that subcluster is due to chance alone.

**Nucleotide pattern search.** Genomic sequences including the 10-kbp upstream region of a number of genes were isolated from the Mouse Genome.
RESULTS

Study Concept

To build a comprehensive picture of the Nrf2-dependent transcriptional network induced by CS exposure in vivo, the transcriptomes of Nrf2−/− and wt mice were monitored on a genome-wide level as a function of exposure duration, dose, and smoking cessation (postexposure period). Therefore, four mice per group and genotype were whole-body exposed once to 375-μg TPM (acute exposure) or for 2 months to 2250 μg TPM/day (medium dose) or for 5 months to 1500 μg TPM/day (low dose) (Table 1). To explore the impact of smoking cessation on Nrf2-dependent gene regulation, two postexposure endpoints following the 5-month exposure schedule were included: 1 day (all dose groups) or 13 days (medium dose group only) before sacrifice (Table 1). Using the Affymetrix mouse gene chip “Mouse Genome 430 2.0,” whole-genome–based gene expression profiles were established from whole-lung tissues of individual animals and analyzed in silico as described under “Materials and Methods” section. The complete raw data set discussed in this publication has been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus (Edgar et al., 2002) and is accessible through Gene Expression Omnibus Series accession number GSE18344 (http://www.ncbi.nlm.nih.gov/geo). The validity of the microarray studies was further checked for selected genes (see “Materials and Methods” section) by RT-qPCR, which, in quantitative terms, generally confirmed the data obtained by microarray analysis (Table 2 and 3). In order to correlate the transcriptomes emerging from this exposure matrix with potential disease outcomes, groups of mice of both genotypes were subjected to the 5-month exposure regimen. The lungs of these animals were investigated for several disease markers related to emphysema (see “Materials and Methods” section).

TABLE 2
CS Exposure–Dependent Differential Expression of Selected Genes Obtained by RT-qPCR Analysis. Time (exposure period) Dependency

<table>
<thead>
<tr>
<th>Applied biosystems assay ID</th>
<th>Gene symbol</th>
<th>Gene expression (( \text{wt/k0}))</th>
<th>Acute</th>
<th>2 months</th>
<th>5 months</th>
<th>5 m, 1-day pe</th>
<th>5 m, 13-day pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn00487217_m1</td>
<td>Cyp1A1</td>
<td>187.8/197.7</td>
<td>327.7</td>
<td>116.4</td>
<td>258.6</td>
<td>465.1</td>
<td>1.3/1.3</td>
</tr>
<tr>
<td>Mn00500554_m1</td>
<td>Mmp-12</td>
<td>1.1/1.4</td>
<td>41.1</td>
<td>25.7</td>
<td>29.3</td>
<td>15.6</td>
<td>22.0/28.7</td>
</tr>
<tr>
<td>Mn00500821_m1</td>
<td>Nqo1</td>
<td>8.5/2.0</td>
<td>18.0</td>
<td>2.5</td>
<td>25.4</td>
<td>1.4</td>
<td>1.6/0.8</td>
</tr>
<tr>
<td>Mn00433859_m1</td>
<td>Ccl2</td>
<td>1.2/1.8</td>
<td>22.3</td>
<td>20.4</td>
<td>11.2</td>
<td>6.5</td>
<td>17.9/13.0</td>
</tr>
<tr>
<td>Mn01248177_m1</td>
<td>Aurka</td>
<td>−1.5/−1.3</td>
<td>2.1</td>
<td>2.0</td>
<td>2.3</td>
<td>1.9</td>
<td>2.5/1.8</td>
</tr>
<tr>
<td>Mn00599750_g1</td>
<td>Birc5</td>
<td>−1.6/−1.3</td>
<td>3.1</td>
<td>2.5</td>
<td>3.1</td>
<td>2.2</td>
<td>3.0/2.5</td>
</tr>
<tr>
<td>Mn00438064_m1</td>
<td>Cen2</td>
<td>−1.5/−1.3</td>
<td>2.9</td>
<td>2.5</td>
<td>3.1</td>
<td>2.1</td>
<td>3.1/2.5</td>
</tr>
<tr>
<td>Mn00441529_g1</td>
<td>Slpi</td>
<td>−3.0/1.9</td>
<td>1.9</td>
<td>1.4</td>
<td>1.6</td>
<td>1.7</td>
<td>2.4/2.5</td>
</tr>
</tbody>
</table>

Note. RNA samples prepared from lungs of sham-exposed nrf 2 knock out (ko) and wt mice or mice exposed to CS (2250 μg TPM/l per day, 5 days/week) for the exposure and postexposure (pe) periods indicated were analyzed.
TABLE 3
CS Exposure–Dependent Differential Expression of Selected Genes Obtained by RT-qPCR Analysis. Dose Dependency

<table>
<thead>
<tr>
<th>Applied biosystems assay ID</th>
<th>Gene symbol</th>
<th>wt</th>
<th>ko</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm00487217_m1</td>
<td>Cyp1A1</td>
<td>242.7</td>
<td>258.6</td>
</tr>
<tr>
<td>Mm00500554_m1</td>
<td>Mmp-12</td>
<td>36.2</td>
<td>29.3</td>
</tr>
<tr>
<td>Mm00500821_m1</td>
<td>Nqo1</td>
<td>14.0</td>
<td>25.4</td>
</tr>
<tr>
<td>Mm00433859_m1</td>
<td>Cxcl1</td>
<td>8.9</td>
<td>11.2</td>
</tr>
<tr>
<td>Mm01248177_m1</td>
<td>Aurora1</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Mm00599750_g1</td>
<td>Birc5</td>
<td>2.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Mm00438064_m1</td>
<td>Ccna2</td>
<td>2.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Mm00441529_g1</td>
<td>Slpi</td>
<td>1.5</td>
<td>1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Medium High</td>
</tr>
<tr>
<td>246.1  265.1  454.4</td>
</tr>
</tbody>
</table>

Note. RNA samples prepared from lungs of sham-exposed Nrf2 ko and wt mice or mice exposed to CS (1500 µg [low], 2250 µg [medium], or 3000 µg [high]) TPM/l per day, 5 days/week for 5 months were analyzed (for technical details on RT-qPCR, see “Materials and Methods” section).

Subclusters 1.2 and 1.3 identify genes that are major targets of CS-dependent differential expression after either 2 months (cluster 1.2) or 5 months (cluster 1.3) of exposure (Supplementary fig. 2, Table 4). Importantly, the upregulation of clustered genes in CS-exposed Nrf2−/− mice is dependent on the exposure period, whereas no or comparatively less expression is seen in the corresponding wt reference groups. It is of note that several of these genes are related to (1) phase 1 detoxification, such as cyp1a1, cyp1b1, and genes encoding their principal transcriptional regulator proteins, i.e., the aryl hydrocarbon receptor (Ah) and its dimerization partner Arnt; (2) antioxidant defense regulation, such as metallothionein 1(mti) and 2(mti2), which are also known to be involved in metal binding and detoxification (Theocharis et al., 2003); and (3) inflammation, such as mmp8, adam8, il1r2, and il4ra. Based on this profile, it is tempting to speculate that the stronger expression of cell protective genes in smoke-exposed Nrf2−/− mice is an attempt made by the cellular stress sensing and transcription control system to compensate for the lacking Nrf2-regulated line of defense. Since these mice also show an attenuated expression of cell cycle progression and mitosis execution–related genes (see below), the same logic may explain the increased expression levels of genes involved in cell growth regulation, such as elf3 (ets domain transcription factor) and pgf (placental growth factor). Interestingly, the group of stress-responsive genes preferentially expressed by CS-exposed Nrf2−/− mice includes genes known to be partly regulated by Nrf2, as a result of the presence of ARE sequence(s) in their corresponding composite promoters, i.e., gsr (glutathione-S-reductase) and serpina3m (a serine proteinase inhibitor). Another potential candidate of partial Nrf2 regulation observed in this study is ald3h3a1 (subcluster 1.2), a key component of aldehyde detoxification. Exhibiting an (Nrf2-independent) expression profile characteristic for phase 1 genes, which is also supported by a previous promoter analysis (Xie et al., 1996), this gene was found to be constitutively upregulated >-10-fold in lung tissue of unexposed mice exhibiting a Clara cell–specific conditional knock out in the kep1 gene (Jon Maher and Keiko Taguchi, unpublished observation). Thus, we conclude that ald3h3a1 is targeted by both the Ah receptor and the Nrf2 pathway, supporting the concept that drug-processing genes may be activated by an AhR-Nrf2 signaling axis (Yeager et al., 2009).

Genes showing apparent Nrf2 dependency contained in cluster 2 (Fig. 1, Table 5) demonstrate a completely different expression behavior compared to the canonical Nrf2 target genes in cluster 1.1, which, as outlined above, are characterized by immediate activation upon acute CS exposure and prompt inactivation upon exposure cessation. Instead, genes in cluster 2 are generally slightly downregulated during acute exposure but become strongly upregulated upon subchronic exposure and even progressively expressed during postexposure in Nrf2 wt mice, whereas they are only moderately expressed, if at all, in CS-exposed Nrf2−/− mice (Fig. 1, Table 5). In particular, this genotype-specific dichotomy in gene expression is most...
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Acute</th>
<th>2 months</th>
<th>5 months</th>
<th>1-day pe</th>
<th>13-day pe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cl 1.1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nqo1</td>
<td>14.2</td>
<td>2.1</td>
<td>36.2</td>
<td>1.9</td>
<td>47.7</td>
</tr>
<tr>
<td>Scl7a11</td>
<td>6.7</td>
<td>1.1</td>
<td>14.4</td>
<td>1.1</td>
<td>14.9</td>
</tr>
<tr>
<td>Akr1b8</td>
<td>3.9</td>
<td>1.6</td>
<td>8.4</td>
<td>2.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Adh7</td>
<td>2.2</td>
<td>1.8</td>
<td>7.1</td>
<td>1.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Rab27a</td>
<td>2.4</td>
<td>2.3</td>
<td>9.6</td>
<td>1.5</td>
<td>8.9</td>
</tr>
<tr>
<td>Krt85</td>
<td>5.4</td>
<td>−1</td>
<td>7.2</td>
<td>−2</td>
<td>5.3</td>
</tr>
<tr>
<td>Gsta2</td>
<td>2.5</td>
<td>−1.4</td>
<td>3.5</td>
<td>−2.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Mmp8</td>
<td>1.6</td>
<td>1.1</td>
<td>3.2</td>
<td>1</td>
<td>3.4</td>
</tr>
<tr>
<td>Gpx2</td>
<td>2.9</td>
<td>4.2</td>
<td>5.1</td>
<td>2</td>
<td>4.2</td>
</tr>
<tr>
<td>Ces1</td>
<td>2.5</td>
<td>−1.1</td>
<td>4.4</td>
<td>1</td>
<td>4.4</td>
</tr>
<tr>
<td>Ikbkg</td>
<td>1.6</td>
<td>1.7</td>
<td>3</td>
<td>1.6</td>
<td>3.4</td>
</tr>
<tr>
<td>Srcn1</td>
<td>4.5</td>
<td>1.7</td>
<td>3.3</td>
<td>1.3</td>
<td>5.1</td>
</tr>
<tr>
<td>Adam8</td>
<td>4.1</td>
<td>2</td>
<td>3.2</td>
<td>1.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Stk25</td>
<td>5.8</td>
<td>2.3</td>
<td>3.8</td>
<td>1.4</td>
<td>5.6</td>
</tr>
<tr>
<td>Gcm</td>
<td>2.4</td>
<td>1.5</td>
<td>3.3</td>
<td>1.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Mcola3</td>
<td>−1.1</td>
<td>−1.4</td>
<td>1.4</td>
<td>1.7</td>
<td>9.4</td>
</tr>
<tr>
<td>Gsta2</td>
<td>1.5</td>
<td>−1.3</td>
<td>1.5</td>
<td>2.9</td>
<td>12.8</td>
</tr>
<tr>
<td>Cte</td>
<td>−2.3</td>
<td>1.5</td>
<td>−1.9</td>
<td>1.2</td>
<td>15.4</td>
</tr>
<tr>
<td>Akr1c19</td>
<td>1.3</td>
<td>1.2</td>
<td>2.2</td>
<td>2.7</td>
<td>7.2</td>
</tr>
<tr>
<td>Sgcb3a1</td>
<td>2.4</td>
<td>1.4</td>
<td>4</td>
<td>4.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Ut2r</td>
<td>1.2</td>
<td>−1.2</td>
<td>2.3</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Arnt2</td>
<td>1.1</td>
<td>1.3</td>
<td>2.6</td>
<td>5.7</td>
<td>3.6</td>
</tr>
<tr>
<td>Slc13a2</td>
<td>1</td>
<td>1.2</td>
<td>2.5</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Aap4</td>
<td>−1.3</td>
<td>1.9</td>
<td>3</td>
<td>3.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Clec4a</td>
<td>1.2</td>
<td>1.8</td>
<td>2</td>
<td>4.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Clecl</td>
<td>1.1</td>
<td>1</td>
<td>1.5</td>
<td>3.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Aldh3a1</td>
<td>4.9</td>
<td>6.4</td>
<td>15.3</td>
<td>15.2</td>
<td>10.9</td>
</tr>
<tr>
<td>Sirp1</td>
<td>−1.1</td>
<td>1.4</td>
<td>1.1</td>
<td>11</td>
<td>−3</td>
</tr>
<tr>
<td>Dmbt1</td>
<td>1</td>
<td>−1</td>
<td>1.9</td>
<td>12.7</td>
<td>−1.4</td>
</tr>
<tr>
<td>Agr2</td>
<td>1.5</td>
<td>3.7</td>
<td>3.6</td>
<td>13.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Cad177</td>
<td>5.9</td>
<td>1.6</td>
<td>169.1</td>
<td>259.4</td>
<td>39.4</td>
</tr>
<tr>
<td>Mmp8</td>
<td>1.6</td>
<td>1.7</td>
<td>12.8</td>
<td>9.7</td>
<td>9.8</td>
</tr>
<tr>
<td>Adam8</td>
<td>2.2</td>
<td>2.2</td>
<td>6.4</td>
<td>8.6</td>
<td>6.8</td>
</tr>
<tr>
<td>Cdxn1a</td>
<td>1.6</td>
<td>2.5</td>
<td>1.7</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Shh25</td>
<td>1.6</td>
<td>−1</td>
<td>2.3</td>
<td>−1.6</td>
<td>−1.3</td>
</tr>
<tr>
<td>Appl1a</td>
<td>1.1</td>
<td>−1</td>
<td>1.6</td>
<td>−1</td>
<td>1.9</td>
</tr>
<tr>
<td>Hip3</td>
<td>−1.1</td>
<td>1</td>
<td>1.2</td>
<td>−1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Tsmap11</td>
<td>1.8</td>
<td>1.3</td>
<td>1.8</td>
<td>1.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Atp11a</td>
<td>1.3</td>
<td>1.2</td>
<td>1.9</td>
<td>2.6</td>
<td>4.1</td>
</tr>
<tr>
<td>Ramp3</td>
<td>−1.1</td>
<td>−1.8</td>
<td>1.7</td>
<td>−1</td>
<td>2.9</td>
</tr>
<tr>
<td>Rab27a</td>
<td>−1.1</td>
<td>1.1</td>
<td>1.9</td>
<td>1.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Serpina3n</td>
<td>1.1</td>
<td>1.1</td>
<td>2.3</td>
<td>2.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>
In support of this notion, 

in silico

Selection criteria for clustering: fold change /C21 genes involved in "General Mitosis" (analysis (IPA) confirmed a significant overrepresentation of than or equal to threefold expression change (p

regulation, such as involved in cell cycle and mitotic (spindle organization) the core of this cluster is dominated by several key players pronounced after 13 days of postexposure. Most importantly, the core of this cluster is dominated by several key players involved in cell cycle and mitotic (spindle organization) regulation, such as aurka, aurkb, ccna2, and survivin (birc5).

In support of this notion, in silico knowledge-based pathway analysis (IPA) confirmed a significant overrepresentation of genes involved in "General Mitosis" (p < 6 × 10^-13) in cluster 2. The low expression of genes (compared to wt mice) involved in cell cycle progression and mitosis execution suggests an impaired cell cycle progression and proliferation phenotype in CS-exposed lung cells of Nrf2^-/- mice. This conclusion is supported by mechanistic investigations in vitro showing that untreated Nrf2^-/- lung epithelial cells undergo partial cell cycle arrest, especially G2/M checkpoint arrest, based on a compromised glutathione (GSH) metabolism in Nrf2^-/- cells (Reddy et al., 2008). While these data point to a more indirect effect caused by the lack of Nrf2 on cell proliferation, promoter analysis for ARE presence revealed that several of these genes are equipped with presumptive ARE consensus sequences in their 5' upstream region (Table 5), thus leaving the possibility that Nrf2 might directly control the expression of these genes in a context-dependent manner.

In contrast to previous reports (Iizuka et al., 2005; Rangasamy et al., 2004), it is important to note that Nrf2 does not affect the inflammatory response to a major extent according to the current data set. In fact, CS-exposed mice show a strong activation of inflammation-related genes, which is almost the same in both genotypes, as revealed by in silico analysis in general (Supplementary fig. 3) and by expression patterns of key inflammatory mediators, such as ccl1, ccl2, ccl3, ccl6, ccl20, mmp12, etc. in particular (Table 6). The inflammatory response on the transcriptional level, in both qualitative and quantitative terms, correlates with a similar lung inflammatory phenotype in CS-exposed mice of both genotypes (see below).

CS-Induced Nrf2 Genotype-Specific Transcriptome: Effects of Dosage

In order to evaluate the current data set for CS-dependent dose effects, expression data from Nrf2^-/- and wt mice exposed for 5 months to all three doses (low, medium, and high, see above) were analyzed. While again limiting the number of responsive genes to those showing a greater than or equal to threefold expression change in at least one instance, the resulting overall heat map image exhibits extensive areas of dose-dependent gene regulation, partly reflecting distinct genotype specificity referred to as clusters 3 and 4 (Supplementary fig. 4, Supplementary table 2). In particular, the core of cluster 3 (subcluster 3.1) (Table 7, Supplementary fig. 5) comprises genes which generally remain silent in Nrf2^-/- mice but were dose-dependently upregulated in wt mice in the low and medium dose groups, while a ceiling effect for the expression rates of these genes was observed in the high-dose groups. Intriguingly, subcluster 3.1 is composed to a high degree of genes belonging either to the group of antioxidant "canonical" Nrf2 client genes, such as nqo1, trxrd1, and gpx2, or to the group of cell cycle regulatory genes, such as aurka, ccna2, and cep55, also represented by clusters 1.1 and 2, respectively. The apparent close Nrf2-dependent relation in expression patterns between genes covered by clusters 1.1 and 2 is further underlined by the finding that almost all

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene symbol</th>
<th>wt</th>
<th>ko</th>
<th>wt</th>
<th>ko</th>
<th>wt</th>
<th>ko</th>
<th>wt</th>
<th>ko</th>
</tr>
</thead>
<tbody>
<tr>
<td>1428942_at</td>
<td>Mti</td>
<td>1.2</td>
<td>2.9</td>
<td>1.9</td>
<td>3</td>
<td>2.4</td>
<td>6.6</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1422557_s_at</td>
<td>Mti</td>
<td>1.1</td>
<td>1.7</td>
<td>2.4</td>
<td>2.6</td>
<td>2.4</td>
<td>4.2</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>1416612_at</td>
<td>Cyp1b1</td>
<td>18.2</td>
<td>16.7</td>
<td>45.7</td>
<td>100.5</td>
<td>74.3</td>
<td>182.9</td>
<td>7.3</td>
<td>13.3</td>
</tr>
<tr>
<td>1416613_at</td>
<td></td>
<td>11.8</td>
<td>9.1</td>
<td>40.6</td>
<td>55.8</td>
<td>80.1</td>
<td>144.1</td>
<td>9.2</td>
<td>10.2</td>
</tr>
<tr>
<td>1422217_a_at</td>
<td>Cyp1a1</td>
<td>295.6</td>
<td>235.7</td>
<td>642.4</td>
<td>226.3</td>
<td>317.8</td>
<td>714.9</td>
<td>-1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>1418153_at</td>
<td>Lama1</td>
<td>1.6</td>
<td>2.7</td>
<td>5.9</td>
<td>3.0</td>
<td>6.2</td>
<td>7.6</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>1416576_at</td>
<td>Soc3</td>
<td>1.4</td>
<td>1.7</td>
<td>1.3</td>
<td>2.2</td>
<td>1.9</td>
<td>3.4</td>
<td>3.2</td>
<td>2.2</td>
</tr>
<tr>
<td>1421816_at</td>
<td>Gsr</td>
<td>2.0</td>
<td>1.4</td>
<td>2.8</td>
<td>1.4</td>
<td>2.5</td>
<td>3.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>1419532_at</td>
<td>Ilvx2</td>
<td>1.2</td>
<td>3.3</td>
<td>11.0</td>
<td>12.7</td>
<td>9.0</td>
<td>23.0</td>
<td>8.7</td>
<td>10.5</td>
</tr>
<tr>
<td>1416916_at</td>
<td>Elf3</td>
<td>1.5</td>
<td>1.9</td>
<td>1.7</td>
<td>2.0</td>
<td>2.3</td>
<td>3.4</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>1423996_a_at</td>
<td>Htra</td>
<td>1.4</td>
<td>1.6</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
<td>3.1</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>1418471_at</td>
<td>Pgf</td>
<td>1.7</td>
<td>1.6</td>
<td>1.8</td>
<td>1.7</td>
<td>1.5</td>
<td>3.1</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td>1420796_at</td>
<td>Ahr</td>
<td>1.0</td>
<td>1.0</td>
<td>4.0</td>
<td>3.4</td>
<td>8.2</td>
<td>8.5</td>
<td>1.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Note. nrf2 knock out (ko) and wt mice were exposed to CS (750 μg TPM/l × h, 3 h/day, 5 days/week) for the exposure and postexposure (pe) periods indicated. Selection criteria for clustering: fold change ≥ 3 (p < 0.05) in at least one instance. Numbers marked in bold refer to expression values, which meet the greater than or equal to threefold expression change (p < 0.05) criterion applied in this study for gene detection.
genes in these two clusters are generally found in cluster 3 (Supplementary table 2).

Regarding transcriptomic changes in the context of increasing CS dosage and genotype, another relevant cluster ("cluster 4") is disclosed by the overall heat map image shown in Supplementary figure 6 (Supplementary table 2). Genes contained in this broad cluster are generally characterized by less or marginal differential regulation (mainly downregulation after acute exposure) in wt mice upon exposure to increasing doses of CS, whereas these genes become upregulated in Nrf2−/− mice, especially in animals exposed to the high dose (Supplementary fig. 6). Knowledge-based in silico analysis by IPA revealed that genes represented in this cluster are involved mainly in disease pathways, especially "neoplasia" \( (p < 3.7 \times 10^{-12}) \) and "respiratory disease" \( (p < 1.1 \times 10^{-9}) \), indicating that Nrf2−/− mice are considerably more sensitive to CS-dependent cellular insults, especially at higher doses, as also indicated by a considerably increased number of differentially expressed genes (Supplementary table 2).

It is of special note that cluster 4 also includes the gene encoding Nrf2's negative regulator, keap1. While this gene is not subject to any differential regulation in CS-exposed wt mice, it becomes dose-dependently upregulated (to approximately fivefold \( [p < 0.05] \)) in Nrf2−/− mice in the high-dose group. This finding, which to our knowledge is described here for the first time, might suggest that keap1 transcriptional regulation is the target of a complex feedback mechanism, with Nrf2 acting as a negative regulator under cell stress conditions.

**CS-Induced Nrf2-Dependent Lung Phenotype**

In order to relate the CS-dependent genotype-associated transcriptomes described above to lung-specific phenotypic differences, lungs from Nrf2−/− and wt mice (sham and all three dose groups after 5 months of continuous exposure, see Table 1) were investigated for effects on alveolar structures and on lung function (using "forced pulmonary maneuvers") and for the appearance of bronchioalveolar inflammation.
While CS-exposed Nrf2−/− mice showed clear dose-dependent effects on alveolar destruction (as indicated by a more than 20% increase in “mean chord length” in the high-dose group) and lung function (as indicated by increasing scores for “Cp0,” “TLC,” “FVC,” and decreasing scores for the ratio “FEV during the first 20 s of the maneuver/FVC” [FEV20/FVC]), only minor effects for these parameters were observed in wt mice (Table 8, Supplementary figs. 7A–D). In general, these results show that the lack of Nrf2 results in an increased loss of lung elasticity during CS-exposed Nrf2−/− mice have an enhanced susceptibility to develop emphysema (Iizuka et al., 2005; Rangasamy et al., 2004).
In striking contrast to the aforementioned reports, however, no impact of the genetic availability of Nrf2 was observed regarding lung inflammatory parameters. In fact, similarly strong increases in the total number of free lung cells, including neutrophils, lymphocytes, and macrophages in BAL fluid, show that CS exposure provoked severe lung inflammation in a dose-dependent manner in mice of both genotypes (Table 8, Supplementary figs. 7E–G), thus indicating that Nrf2, at least in a dose-dependent manner similar for both genotypes (Fig. 2). In this context, it should be reemphasized that the lack of Nrf2-dependent differences in CS-induced lung inflammation described here, although contrary to previously published data (Iizuka et al., 2005; Rangasamy et al., 2004), is in line with the aforementioned observation that similar inflammation-related gene expression patterns are obtained for both genotypes.

**DISCUSSION**

Since its first description as the principal regulator of the cell’s first line of defense (Ishii et al., 2000), Nrf2 has emerged as prime cellular target of CS-induced stress (for review, see Kensler et al., 2007). In fact, the genetic availability and the control of Nrf2 activity have been linked directly with pathological outcomes of CS exposure, i.e., emphysema and lung cancer (summarized in Hayes and McMahon, 2009). Focusing on the genome-wide transcriptomic changes of acutely and subchronically exposed Nrf2−/− and wt mice, and supported by lung function and morphometry assessments, the results of the current study support the general conclusion highlighted in two recent reports (Iizuka et al., 2005; Rangasamy et al., 2004) that the responsiveness of Nrf2 is...
TABLE 7
Cluster 3.1: nrf-2 Genotype-Dependently Regulated Genes Following Expression Algorithms Underlying Clusters 1.1 and 2 are Similarly Regulated in a CS Dose-Dependent Manner. Expression Fold Changes of Genotype-Dependently Regulated Genes as Identified by One-Dimensional Unsupervised Hierarchical Clustering (Supplementary fig. 5)

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene symbol</th>
<th>Cluster appearance in exposure duration</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wt</td>
<td>low</td>
</tr>
<tr>
<td>1452242_at</td>
<td>Cep55</td>
<td>2</td>
<td>4.8</td>
</tr>
<tr>
<td>1417911_at</td>
<td>Ccn2</td>
<td>2</td>
<td>3.6</td>
</tr>
<tr>
<td>1424511_at</td>
<td>Aukka</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>1423627_at</td>
<td>Nqo1</td>
<td>1.1</td>
<td>25.7</td>
</tr>
<tr>
<td>1450110_at</td>
<td>Adh7</td>
<td>1.1</td>
<td>4.6</td>
</tr>
<tr>
<td>1421058_at</td>
<td></td>
<td>3.6</td>
<td>6.4</td>
</tr>
<tr>
<td>1420413_at</td>
<td>Slc7a11</td>
<td>1.1</td>
<td>7.3</td>
</tr>
<tr>
<td>1455454_at</td>
<td>Akr1c19</td>
<td>1.1</td>
<td>4.4</td>
</tr>
<tr>
<td>1442486_a_at</td>
<td>Tnmd1</td>
<td>1.1</td>
<td>2.9</td>
</tr>
<tr>
<td>1460185_at</td>
<td>Krt85</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>1449279_at</td>
<td>Gpx2</td>
<td>1.1</td>
<td>2.6</td>
</tr>
<tr>
<td>1423714_at</td>
<td>Akr1c19</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>1429022_at</td>
<td>Acoxap1r1</td>
<td>1.9</td>
<td>3.4</td>
</tr>
<tr>
<td>1431320_a_at</td>
<td>Myosu</td>
<td>3.8</td>
<td>5.1</td>
</tr>
<tr>
<td>1452040_a_at</td>
<td>Cdxa3</td>
<td>2</td>
<td>2.9</td>
</tr>
<tr>
<td>1424305_at</td>
<td>Igj</td>
<td>2</td>
<td>2.6</td>
</tr>
<tr>
<td>1417926_at</td>
<td>Luzp5</td>
<td>2.3</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Note. nrf2 knock out (ko) and wt mice were exposed to the "low," "medium," or "high" CS dose (for details, see "Materials and Methods" section) for 5 months. Selection criteria for clustering: fold change ≥ 3 (p < 0.05) in at least one instance. Numbers marked in bold refer to expression values, which meet the greater than or equal to threefold expression change (p < 0.05) criterion applied in this study for gene detection.

TABLE 8
Comparison of Genes as Identified by One-Dimensional Unsupervised Hierarchical Clustering (Supplementary fig. 5). In contrast to these findings, however, no significant genotype-specific differences in lung inflammation parameters were seen in the current study. In fact, CS-exposed mice of both genotypes developed a strong inflammatory response, generally in a dose-dependent manner, which, on the transcriptional level, is reflected by similar expression rates of general and key drivers in inflammation (Table 6, Supplementary fig. 3) and, on the phenotypic level, by high scores for general lung inflammation (data not shown) and inflammatory parameters in BAL fluid (Fig. 2, Table 8, Supplementary fig. 7). In contrast to one (lizuka et al., 2005) of the two previous studies, which discussed slpi encoding a leukoprotease inhibitor described to be effective as α-1 antitrypsin (Smith and Johnson, 1983) as a potential source of CS-induced inflammation because of its supposed regulation by Nrf2, the expression rates for slpi in the current study were similar in CS-exposed Nrf2−/− and wt mice (Tables 2, 3, and 6).

In relation to the two previous reports (lizuka et al., 2005; Rangasamy et al., 2004), our data suggest a different causal relationship for the increased susceptibility of CS-exposed Nrf2−/− mice to develop emphysema, indicating that Nrf2 may have a functional role in cell cycle progression and mitosis. Following unsupervised hierarchical clustering, a distinct area ("cluster 2") of differentially expressed genes specific to genotype was observed. These genes are characterized by a general slight repression after acute exposure, which is followed by a strong upregulation in subchronically exposed wt mice but by relatively low to moderate expression rates in corresponding Nrf2−/− exposure groups. This genotype-specific differential expression behavior becomes most obvious after 13 days of postexposure, when the expression fold changes of the genes were generally two to five times higher in wt mice compared to knockout mice (Fig. 1, Table 5). Intriguingly, this rather small cluster shows an extreme density of genes that are, functionally, closely related in cell cycle progression and mitosis execution (15 out of 20 genes, Table 5), e.g., genes encoding proteins involved in guiding the mitotic process, spindle organization and stability, and chromatin binding (for recent reviews, see Fu et al., 2007; Vader et al., 2006). In support of this observation, Reddy et al. (2008) reported that untreated cultured lung epithelial cells from Nrf2−/− mice are partially cell cycle arrested, especially in the G2 phase of the cell cycle. In mechanistic terms, this G2/M checkpoint arrest was found to be based mainly on Cdk1 inactivation consequent to the inability of Nrf2−/− cells to produce adequate levels of intracellular GSH required for smooth cell cycle progression (Reddy et al., 2008). Clearly, oxidative stress induced by CS exposure would intensify this effect because of its strong potential to deplete intracellular GSH levels (Müller and Gebel, 1998). Because a pronounced apoptotic phenotype, especially in endothelial and type II epithelial lung cells, is also exhibited by CS-exposed Nrf2−/− mice (Rangasamy et al., 2004; Sussan et al., 2008), it is tempting to conclude that the increased emphysematous phenotype exhibited by CS-exposed Nrf2−/− mice relates, at least in part, to the attenuated ability of these mice to compensate for net cell loss based on an imbalance of cell death and regeneration.

a major determinant for CS-induced emphysema, although signs of compensation for the lacking Nrf2-dependent line of defense were detected in the transcriptome of CS-exposed Nrf2−/− mice. In particular, genes identified in subclusters 1.2 and 1.3, which in comparison to wt reference groups are preferentially upregulated in CS-exposed Nrf2−/− mice, are functionally related to (phase 1) detoxification, antioxidant defense, and growth regulation. In this context, it is of special interest that two of these genes, i.e., Mtr1 and Mtr2, are reported to be included in a group of 13 genes whose expression remained perturbed in the lungs of former smokers who stopped smoking for more than 20 years (Spira et al., 2004).

In mechanistic terms, the studies by Rangasamy et al. (2004) and lizuka et al. (2005) point to an increase in general lung inflammation as one major factor for the enhanced emphysematous phenotype seen in CS-exposed Nrf2−/− mice. In striking contrast to these findings, however, no significant genotype-specific differences in lung inflammation parameters were seen in the current study. In fact, CS-exposed mice of both genotypes...
Importantly, because most of the cell cycle progression and mitotic genes represented by cluster 2 were found to be equipped with putative ARE sequences in their 5’ upstream region, Nrf2 might (co)direct the expression of these genes in a context-dependent manner to create an environment for smooth cell cycle progression and mitosis execution. Finally, it is equally important that the conclusions drawn here from the transcriptomic data set are supported by our phenotypic observation of significantly reduced lung elasticity in CS-exposed Nrf2−/− mice (Table 8), which is most conceivably explained by insufficient tissue regeneration.

The discrepancy in the experimental results described in the current study and the two previous reports on CS-exposed Nrf2−/− mice (Iizuka et al., 2005; Rangasamy et al., 2004), as outlined above, needs to be addressed. A preliminary explanation for the different outcomes might be the different types of study design used in the various investigations relating to exposure parameters, such as time, dosage, and type of test cigarette smoked for exposure. For example, a mixture of sidestream smoke (89%) and mainstream smoke (11%) as used by Rangasamy et al. (2004) is supposed to harbor different irritating and therefore also different toxicological properties than pure mainstream smoke. Hence, depending on the exposure conditions (i.e., acute vs. chronic exposure; mainstream smoke vs. mixed mainstream and sidestream smoke, both in the context of dose), different functional activities of Nrf2 may prevail. Moreover, since the current study was based on the analysis of whole-lung tissue, the genetic lack of nrf2 needs to be discriminated for the different cell types involved in lung tissue assembly to potentially further mechanistically correlate CS-dependent transcriptomic effects with a particular disease phenotype. Finally, it is important to note that our finding of an Nrf2 genotype–dependent differential expression of cell cycle and mitosis-related genes in CS-exposed mice remains to be substantiated mechanistically on the functional level in vivo, e.g., by cytometric investigations also in a cell type–specific manner.

In conclusion, the transcriptomic analysis of CS-exposed mice differing in the genetic availability of Nrf2 as presented in this manuscript provides evidence for the involvement of the Keap1-Nrf2 pathway in lung tissue homeostasis and regeneration, which is probably consequent to tissue injury and inflammation. Future mechanistic studies are clearly required to reveal the impact of the increased relevance of Nrf2 in fundamental cellular and tissue processes. For example, increased knowledge of the interfaces of the potential Nrf2 cross talk with cell cycle and inflammation-signaling pathways would help to further understand the role of this transcription factor in physiological and pathological scenarios. In particular, the more abundant Nrf2-dependent expression of genes controlling cell cycle progression and mitosis execution under (sub)chronic cell- and tissue-stressing conditions should be investigated according to the more recent finding that sustained activation of Nrf2 represents a selective advantage for tumor

### TABLE 8

| CS Exposure Induces Dose-Dependent Effects on the Mean Linear Intercept and Lung Function Parameters in nrf 2−/− (ko) Mice but not in wt Mice, Whereas Similar Increases in Inflammatory Lung Cells in BAL Fluid are Observed by CS Exposure in Both Genotypes (see also Supplementary fig. 7; for technical details, see text and “Materials and Methods” section) |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | Sham | Low | Medium | High | p value |
| Mean ± SE                  |      |     |        |      |         |
| Lung morphometry            |      |     |        |      |         |
| (alveolar structure)        |      |     |        |      |         |
| Mean chord length (µm)      | wt   | 29.5 ± 0.91 | 32.56 ± 0.6 | 31.97 ± 1.59 | 33.19 ± 0.93 | 0.07 |
|                            | ko   | 29.9 ± 1.28 | 31.71 ± 1.32 | 34.72 ± 0.73 | 34.69 ± 1.57 | 0.035 |
| Lung function (forced       |      |     |        |      |         |
| pulmonary maneuver)         |      |     |        |      |         |
| Cpt (ml/cm H2O)             | wt   | 0.041 ± 0.0032 | 0.057 ± 0.0050 | 0.066 ± 0.0083 | 0.056 ± 0.0062 | 0.045 |
|                            | ko   | 0.046 ± 0.0049 | 0.059 ± 0.0045 | 0.099 ± 0.036 | 0.120 ± 0.032 | 0.12 |
| TLC (ml)                    | wt   | 1.19 ± 0.079 | 1.38 ± 0.13 | 1.52 ± 0.089 | 1.43 ± 0.127 | 0.18 |
|                            | ko   | 1.07 ± 0.064 | 1.52 ± 0.11 | 1.66 ± 0.082 | 1.87 ± 0.118 | < 0.0001 |
| FVC (ml)                    | wt   | 0.97 ± 0.079 | 1.14 ± 0.094 | 1.23 ± 0.075 | 1.17 ± 0.11 | 0.23 |
|                            | ko   | 0.89 ± 0.056 | 1.24 ± 0.095 | 1.43 ± 0.11 | 1.59 ± 0.15 | 0.0004 |
| FEV2/FVC                    | wt   | 0.39 ± 0.037 | 0.31 ± 0.024 | 0.28 ± 0.022 | 0.31 ± 0.041 | 0.11 |
|                            | ko   | 0.36 ± 0.039 | 0.30 ± 0.020 | 0.27 ± 0.02 | 0.23 ± 0.02 | 0.0088 |
| Free lung cells in BAL fluid (× 10^6) |      |     |        |      |         |
| Neutrophils                 | wt   | 0.06 ± 0.029 | 3.25 ± 0.435 | 7.63 ± 1.037 | 7.55 ± 0.939 | < 0.0001 |
|                            | ko   | 0.11 ± 0.097 | 2.99 ± 0.486 | 6.9 ± 0.684 | 8.24 ± 0.922 | < 0.0001 |
| Lymphocytes                 | wt   | 0.06 ± 0.01 | 1.01 ± 0.196 | 1.57 ± 0.425 | 1.07 ± 0.097 | 0.001 |
|                            | ko   | 0.07 ± 0.019 | 0.94 ± 0.139 | 1.52 ± 0.209 | 1.5 ± 0.227 | < 0.0001 |
| Macrophages                 | wt   | 4.1 ± 0.51 | 4.9 ± 0.66 | 5.1 ± 1.06 | 5.4 ± 0.66 | 0.71 |
|                            | ko   | 3.1 ± 0.47 | 3.6 ± 0.47 | 4.8 ± 0.51 | 4.7 ± 0.53 | 0.042 |
| ∑ free lung cells           | wt   | 4.3 ± 0.49 | 9.1 ± 1.03 | 14.3 ± 2.28 | 14.0 ± 1.5 | < 0.0001 |
|                            | ko   | 3.3 ± 0.46 | 7.5 ± 0.81 | 13.3 ± 1.04 | 14.5 ± 1.33 | < 0.0001 |
FIG. 2. Multianalyte profiling in BAL fluid of sham and CS-exposed nrf2 knock out (ko) and wt mice. nrf2 ko and wt mice were exposed to CS (1500 μg [low], 2250 μg [medium], or 3000 μg [high] TPM per day, 5 days/week) for 5 months (for technical details, see "Materials and Methods" section).
development, especially in lung tumorigenesis (Hayes and McMahon, 2009).

SUPPLEMENTARY DATA

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

FUNDING

This work was supported in part by Philip Morris USA, Inc. prior to the spin-off of Philip Morris International, Inc. by Altria Group, Inc. on March 28, 2008.

ACKNOWLEDGMENTS

We thank Lynda Conroy for her engagement on the advancement of this manuscript and her expert editorial support. Moreover, we acknowledge the expert technical assistance of V. Böhm, U. Neumann, and A. Völkel (all Philip Morris Research Laboratories, Cologne).

REFERENCES


