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Exposure of mice to secondhand smoke elicits both transient and long-lasting transcriptional changes in cancer-related functional networks

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Secondhand smoke (SHS) has long been linked to lung cancer and other diseases in nonsmokers. Yet, the underlying mechanisms of SHS carcinogenicity in nonsmokers remain to be elucidated. We investigated the immediate and long-lasting effects of SHS exposure on gene expression in mice *in vivo*. We exposed mice whole body to SHS for 5 h/day, 5 days/week for 4 months in exposure chambers of a microprocessor-controlled smoking machine. Subsequently, we performed microarray gene expression profiling, genome-wide, to construct the pulmonary transcriptome of SHS-exposed mice, immediately after discontinuation of exposure (T0) and following 1-month (T1) and 7-month (T2) recoveries in clean air. Sub-chronic exposure of mice to SHS elicited a robust transcriptomic response, including both reversible and irreversible changes in gene expression. There were 674 differentially expressed transcripts immediately after treatment (T0), of which the majority were involved in xenobiotic metabolism, signaling, and innate immune response. Reduced, yet, substantial numbers of differentially expressed transcripts were detectable in mice after cessation of SHS-exposure (254 transcripts at T1 and 30 transcripts at T2). Top biofunctional networks disrupted in SHS-exposed mice, even after termination of exposure, were implicated in cancer, respiratory disease, and inflammatory disease. Our data show that exposure of mice to SHS induces both transient and long-lasting changes in gene expression, which impact cancer-related functional networks. The pattern of transcriptional changes in SHS-exposed mice may provide clues on the underlying mechanisms of lung tumorigenesis in nonsmokers. Our findings underscore the importance of eliminating SHS from environments where nonsmokers are unavoidably exposed to this carcinogen.

Secondhand smoke (SHS), also referred to as environmental tobacco smoke, passive smoke, or involuntary smoke, has long been linked to lung cancer and other diseases in nonsmokers.[1–3] Approximately 600,000 deaths per year are attributable to SHS exposure, worldwide.[3,4] In the United States alone, it is estimated that about 10,000 to 15,000 people who have never smoked die from lung cancer annually.[5] Between 2000 and 2004, nearly 3,400 nonsmoking US adults died yearly from lung cancer associated with exposure to SHS.[6] Notwithstanding the established link between SHS exposure and lung cancer in nonsmokers, the underlying mechanisms of SHS carcinogenicity remains unknown.[7] Understanding the mechanisms of action of SHS in the genesis and progression of lung cancer in nonsmokers is fundamental to identifying potential targets of intervention for prevention, diagnosis, therapy, and prognosis of this disease.[7]

Research in our laboratory[8–11] and others' (reviewed in Refs. [12–15]) has shown that tobacco smoke or its constituents cause genetic and epigenetic alterations that may lead to transcriptional changes in cancer-related genes. Many differentially expressed genes in human cancers are re-adjustable through pharmacologic/genetic approaches, *e.g.*, epigenetic therapy or genetic manipulations.[16,17] Thus, an effective strategy to investigate the underlying mechanisms of tobacco-smoke associated carcinogenesis is to uncover the alterations of transcriptome caused by exposure to tobacco smoke carcinogens *in vivo*. Identifying the reversible and irreversible transcriptional changes that occur due to exposure to SHS may highlight molecular pathways and functional networks that are disrupted during the initiation and progression of lung cancer in nonsmokers. This mechanistic knowledge will be critical to developing biological markers that can best predict the evolution of nonsmokers' lung cancer. The premise of these biomarkers will lie in their utility for prevention, early detection, treatment, and monitoring of the progression of lung cancer in nonsmokers.

Mouse models of human cancer have proven to be invaluable systems to study the underlying mechanisms of carcinogenesis.[18,19] It has been shown that despite the interspecies differences, modulation of gene expression by tobacco smoke is mostly similar between humans and mice.[20] This

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What's new?
While the epidemiological association between second-hand smoking (SHS) and lung cancer is well established, the molecular mechanisms underlying SHS carcinogenesis are not well understood. To gain insights into these mechanisms, the authors globally analyzed pulmonary gene expression profiles in mice sub-chronically exposed to SHS. By identifying the biological networks associated with SHS they uncover new candidate strategies potentially strengthening prevention, diagnosis, and therapy of lung cancer in nonsmokers in the future.

underscores the utility of mouse models for studying tobacco-smoke associated carcinogenesis in humans. In this study, we have investigated the modulation of gene expression consequent to *in vivo* exposure of mice to SHS. We have used a microprocessor-controlled smoking machine and exposure chambers to sub-chronically expose mice whole body to SHS. Subsequently, we have performed microarray gene expression profiling, genome-wide, to construct the pulmonary transcriptome of SHS-exposed mice, immediately after discontinuation of exposure (T0) and following 1-month (T1) and 7-month (T2) recoveries in clean air. For validation purposes, we have also conducted quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis[21] on randomly selected target genes identified by the array analysis in SHS-exposed mice. We demonstrate that sub-chronic exposure of mice to SHS induces both transient and long-lasting transcriptional changes, which mainly affect cancer-related functional networks. The differentially expressed genes in SHS-exposed mice, even after discontinuation of exposure, are implicated in cancer, inflammatory response, and respiratory disease.

Material and Methods

Animals

Thirty male adult mice (6–8 weeks old) on a C57BL/6 genetic background (Stratagene, Santa Clara, CA) were randomly divided into two groups of (*i*) Experimental (SHS exposure; $n = 15$) and (*ii*) Control (sham-treatment: clean air; $n = 15$), each subdivided into three categories (five mice, each), including (T0) 4 months exposure; (T1) 4 months exposure + 1 month recovery; and (T2) 4 months exposure + 7 months recovery. The mice assigned to each experimental or control group were kept in polypropylene cages in groups of two to three animals per cage, and housed in air-conditioned animal rooms with ambient temperature of $21 \pm 1^{\circ}C$ and relative humidity of 55%, with 12-h light/dark cycle. Throughout all experiments, including the exposure phase and recovery periods, the mice had access to food (PicoLab Rodent Diet 20, PMI Nutrition International, LLC., Brentwood, MO) and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee in accordance with the recommendations of the National Institutes of Health provided in the Guide for the Care and Use of Laboratory Animals. We note that although C57BL/6 mice are less sensitive to pulmonary carcinogens as compared to other strains, such as A/J mice, the latter mice, however, develop spontaneously lung tumors, *e.g.*, adenomas, at high frequencies.[22] Work in our laboratory has shown that SHS is a comparatively weak mutagen and carcinogen, whose biological effects can be established in the C57BL/6 mice with high specificity.[8,10,11] We have also verified that male C57BL/6 mice are sufficiently sensitive to the genotoxic and epigenetic effects of chemical carcinogens.[8–11,23] Our genome-wide and epigenome-wide studies in these mice have also shown that five animals per experimental/control groups are sufficient to yield statistically significant results.[8–11,23] In our preliminary studies, we have also confirmed that short-term and long-term effects of SHS can be established in this mouse model 1 month and 7 months, respectively, post-treatment. The 1-month and 7-month recoveries of SHS-exposed mice in clean air correspond to 3.7% and 25.8% of the average lifespan of these animals.

Smoking machine and SHS exposure

We used a custom-made smoking machine (model TE-10, Teague Enterprises, Davis, CA) to generate SHS for experimental exposure of mice, as described in Ref. [11]. The TE-10 smoking machine is a microprocessor-controlled unit, which can continuously smoke up to 10 cigarettes. A multi-component accessory system collects, ages, and dilutes the smoke generated in the smoking chamber, and converts the high levels of smoke to concentrations needed for various applications in the accompanying exposure chambers. The machine can produce mainstream smoke, sidestream smoke or a combination of the two in different proportions. Included in the TE-10 smoking machine are calibration and recording features that document the number of smoked cigarettes at a given flow rate, and measure total suspended particulate (TSP) levels in the exposure chambers. Cigarettes are smoked according to the Federal Trade Commission (FTC) method, which includes 2-s puffs of 35 cc at 1-min intervals.[11,24] We programmed the TE-10 smoking machine to generate a mixture of sidestream smoke (89%) and mainstream smoke (11%), which is conventionally used as SHS for experimental purposes.[8,25] We used the 3R4F Reference Kentucky cigarettes (University of Kentucky, Lexington, KY), which have a declared content of 11.0 mg total particulate matter, 9.4 mg tar, and 0.73 mg nicotine per cigarette. Each cigarette was smoked using the FTC method for nine min at a flow rate of 1.05 L/min. All experimental mice underwent

an acclimatization period during which they were gradually exposed to incremental doses of SHS (see Supporting Information Fig. S1 panels *c* and *d*). Following the acclimatization period, the mice were maintained on a SHS exposure regimen that consisted of whole body exposure to SHS for 5 h per day, 5 days per week, for 4 months. Control mice were handled similarly to SHS-exposed animals, and underwent sham-treatment with filtered high-efficiency particulate-air (HEPA). At the end of all experiments (T0, T1, and T2), mice were euthanized by CO_2 asphyxiation, and lungs were harvested, snap-frozen in liquid nitrogen, and preserved at $-80^{\circ}C$ until further analysis.

Genome-wide gene expression profiling and data analysis

We used the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc., Santa Clara, CA) to establish the gene expression profile in the lungs of mice exposed to SHS, immediately after discontinuation of exposure (T0) and following 1-month (T1) and 7-month (T2) recoveries in clean air, in comparison with age-matched controls. The microarray platform used in the present study enables interrogation of over 39,000 transcripts and variants from more than 34,000 well-characterized mouse genes. Probe intensity data (.CEL) were generated using Affymetrix Command Console v1.1 and analyzed using Partek® Genomics Suite software v.6.6 (Partek Inc., St. Louis, MO, USA), as described in Ref. 26. Briefly, CEL files from all arrays were imported into the Partek software and included in a single analysis. Data were normalized according to Partek predefined criteria, including RMA Background Correction, Quantile Normalization, and Log (base 2) transformation. The significantly over- and under-expressed genes in experimental mice relative to respective controls were detected using One-way ANOVA tool. Relevant genes meeting the criteria of a twofold change and a FDR-corrected p value of <0.05 were subjected to further analysis. Additional relaxed criteria included an unadjusted p value of <0.05 and a fold change of ± 2. To establish gene expression trends within each experimental group as well as across all groups, significant gene lists were examined by Partek® hierarchical clustering. The full series of microarray datasets have been deposited in the Gene Expression Omnibus database at NCBI (htttp://www.ncbi.nlm.nih.gov/geo/) under accession number GSE41421.

Gene ontology and canonical pathways analysis

To examine genes and functional processes affected by *in vivo* exposure of mice to SHS, we have analyzed the relevant gene lists generated by Partek® using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Tool v.6.7.[27] The Functional Clustering Analysis tool in DAVID was used to group together redundant GO categories. Functional identification of gene networks and canonical pathways was further investigated using the Ingenuity Pathway Analysis® (IPA®) v.9 tool (Ingenuity Systems, Redwood, CA; www.ingenuity.com).

Quantitative real-time reverse transcription-PCR

Standard qRT-PCR was used to determine the expression level of individual genes identified by microarray analysis, as previously described.[28] Briefly, total RNA was extracted from the lungs of experimental and control mice using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. DNase-treated RNA (0.5 μg) was reverse transcribed into complementary DNA (cDNA) using SuperScript® VILO™ cDNA Synthesis kit (Invitrogen Corp., Carlsbad, CA). The mRNA expression level of target genes was determined by qRT-PCR using the TaKaRa SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (Clontech Laboratories, Inc., Mountain View, CA) and the CFX96 Touch™ Real-Time PCR detection system (BioRad Laboratories, Hercules, CA). All reactions were performed in triplicate and fold changes were determined using the $2^{-\Delta\Delta Ct}$ method.[28] The primer sets used for qRT-PCR are available upon request.

Results

Mice survival and body weight

All mice from both experimental and control groups well tolerated the SHS exposure/sham-treatment without exhibiting any sign of stress or discomfort. The survival rate of mice in the experimental and control groups alike was 100% at the end of all experiments. Whereas control mice gained steadily body weight during the sham-treatment and recovery periods, the experimental mice showed a nearly flat pattern of body weight during the 4 months SHS exposure period. Nonetheless, the SHS-exposed mice gained body weight progressively during the 1-month and 7-month recovery periods (see, Supporting Information Fig. S1*a* and S1*b*).

External dosimetry of SHS exposure in mice

We gravimetrically measured the concentrations of TSP in the exposure chambers of our smoking machine twice daily. As shown in Supporting Information Figure S1*c*, after the acclimatization period, the average concentration of TSP in the exposure chambers was 233.0 ± 15.4 mg/m^3. This TSP concentration corresponds to SHS generated through continuous smoking of 8.0 ± 0.5 cigarettes (see Supporting Information Fig. S1*d*). The fluctuations of TSP concentration in the exposure chambers of the smoking machine were mostly due to temporal variations in airflow, which were adjusted after each TSP measurement by regulating the number of loaded cigarettes.

Time-course gene expression profiling in SHS-exposed mice

Using an Affymetrix microarray platform (Affymetrix Inc.), we have globally analyzed the gene expression profile of lung tissues from mice exposed to SHS for 4 months, immediately after discontinuation of exposure (T0) and at ensuing recovery times (T1: 1-month recovery and T2: 7-month recovery). Compiled lists of differentially expressed targets identified by

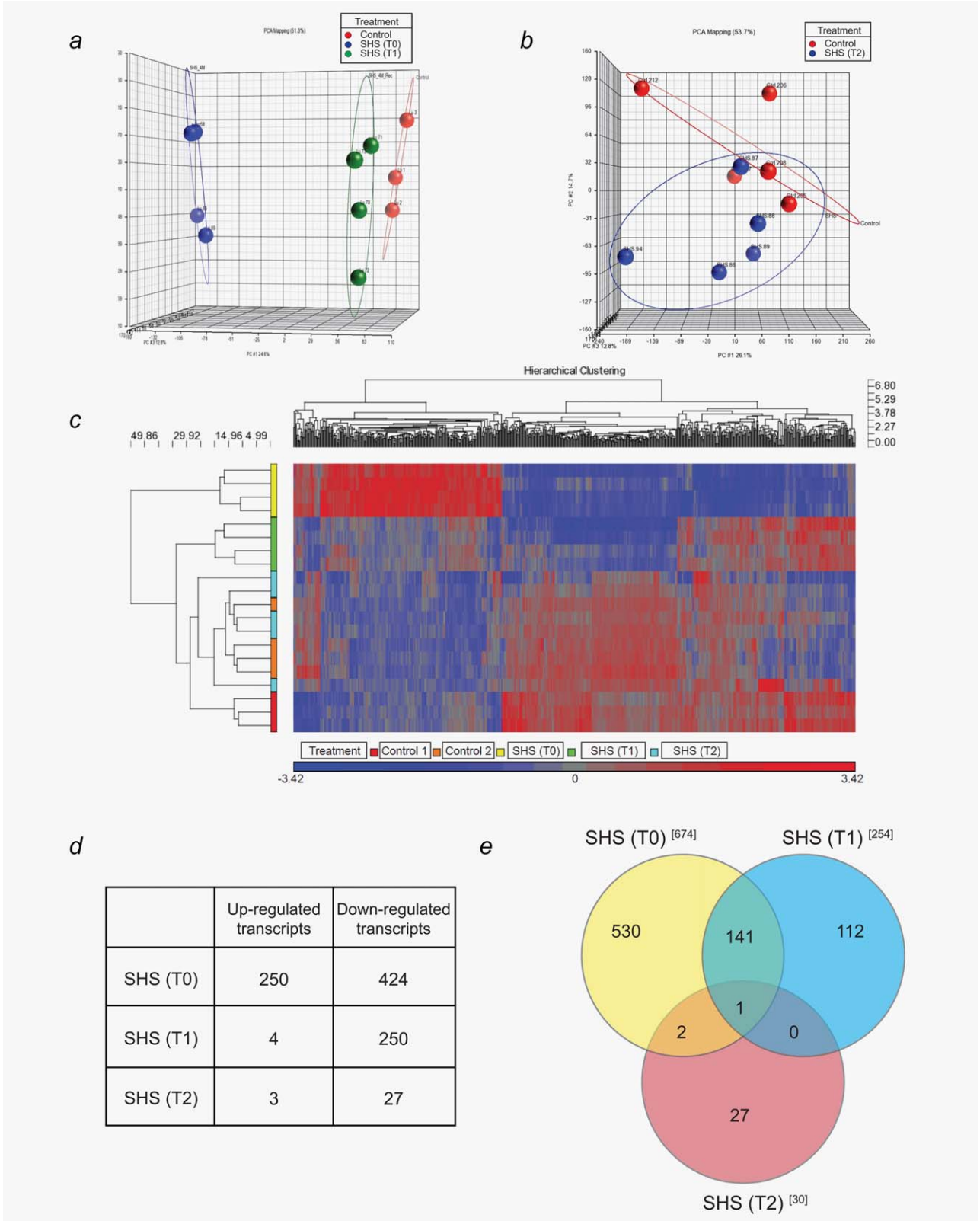
microarray analysis in the three experimental groups relative to corresponding controls are shown in Supporting Information Table S1. Principal Component Analysis (PCA) and Hierarchical Clustering by Partek® confirmed that datasets in the same experimental groups clustered closely together and stayed distinct from those of respective controls (Figs. 1*a*–1*c*). As shown in Figure 1*d*, sub-chronic exposure of mice to SHS at T0 resulted in differential expression of 674 transcripts (with a fold change of ±2 and a FDR-corrected p value of <0.05). Of these, 250 transcripts were up-regulated (with fold changes ranging from +2 to +20.66), and 424 transcripts were down-regulated (with fold changes ranging from −7.96 to −2) (Fig. 1*d* and Supporting Information Table S1). A reduced, yet, substantial number of differentially expressed transcripts (with fold change ranges between +3.34 and −4.82) was detected in SHS-mice that had undergone one-month recovery in clean air (T1) (Fig. 1*d* and Supporting Information Table S1). Of the 254 differentially expressed transcripts in mice at T1, 142 transcripts overlapped with those found in mice at T0, while the remaining 112 being unique to T1 mice (Fig. 1*e*). This observation indicates that although the immediate transcriptomic effects of SHS exposure in mice may become attenuated over time, a subset of these effects remains persistent and a new set of "delayed" effects manifests. Furthermore, we found a smaller number of differentially expressed transcripts ($n = 30$), with fold changes between + 21.75 and −2.50, in SHS-mice following a prolonged recovery of 7 months in clean air (T2) (Fig. 1*d* and Supporting Information Table S1 and Fig. S2). Of significance, 27 of the differentially expressed targets were unique to T2 mice, which reaffirms that *in vivo* long-term and short-term effects of SHS exposure on the transcriptome are different from one another.

Identification of the biological pathways and networks affected in SHS-exposed mice

Using a combination of the GO functional annotation clustering analysis (DAVID v. 6.7) and the Ingenuity Pathway Analysis® (IPA® v. 9.0), we obtained detailed gene ontology information for the differentially expressed genes identified in the three experimental groups. According to DAVID, SHS-exposed mice, upon termination of exposure (T0), had significant enrichment of over-expressed gene targets involved in functional processes, such as signaling, response to oxidative stress, and glutathione and xenobiotic metabolism (Fig. 2*a*, left panel). Down-regulated targets in T0 mice were mostly implicated in innate immune response, GTP-ase activity, transcription regulation, and cell adhesion (Fig. 2*a*, right panel). Furthermore, IPA® analysis revealed that of the 674 differentially expressed targets in T0 mice, approximately 50% were associated with cancer (Fig. 2*b*). Top functional networks included cancer, cellular and hematological system development, drug and amino acid metabolism, and protein synthesis (Fig. 2*c*). Here, the convergence of genes involved in pathways frequently perturbed in cancer (*i.e.*, *Ras*, *Erk* pathways) is noticeable (Fig. 2*c*). DAVID analysis of the differentially expressed genes in T1 mice that had undergone 1-month recovery in clean air identified ten relevant biological categories (Fig. 3*a*). Gene targets involved in intracellular organelle, chromosome organization, transcription regulation, and mRNA processing were highly represented (Fig. 3*a*). The latter observation supports a potential role for SHS in modulating mRNA post-translational modifications and chromatin remodeling. As shown in Figure 3*b*, cancer remained the top listed disease associated with the differentially expressed targets in T1 mice (164 out of 254 identified transcripts: ~65%), as determined by IPA® analysis. Top functional networks generated by IPA® confirmed enrichment of genes involved in gene expression, protein synthesis, cell morphology, RNA post-transcriptional modification, cellular assembly and molecular transport (Fig. 3*c*). Moreover, changes in gene expression were also detected in T2 mice, *albeit* to a smaller extent than those found in T0 and T1 mice (30 differentially expressed targets). DAVID analysis of the deregulated transcripts in T2 mice showed high enrichment of genes involved in functional categories, such as signaling, serine protease inhibitor, and inflammatory response (Fig. 4*a*). According to IPA®, the top canonical pathways disrupted in T2 mice included the caveolar-mediated endocytosis signaling, LXR/RXR activation, atherosclerosis signaling, IL-12 signaling and production of macrophages, and acute phase response signaling pathways. Of significance, these pathways are mostly implicated in inflammatory response and inflammatory disease and remain activated months after discontinuation of SHS exposure (Figs. 4*a*–4*d*). The top functional network showed enrichment of genes involved in hematological system development, hematopoiesis, and humoral immune response, in connection with pathways frequently disrupted in cancer, *i.e.*, Mek/Erk, Akt pathways (Fig. 4*c*). The link between inflammation and cancer is better highlighted in Figure 4*d*. Several differentially expressed genes in T2 have the potential to either promote or inhibit inflammatory response and trigger tumorigenesis (Fig. 4*d*).

Quantitative real-time reverse transcription-PCR validation of microarray results

To validate the gene-expression profiling data, we randomly selected several up- and down-regulated targets identified by Affymetrix microarray analysis in the three experimental groups (T0, T1 and T2), and examined their transcription levels by qRT-PCR. Figure 5 shows the mean normalized expression levels of several transcripts in SHS-mice and age-matched controls. We analyzed several Cytochrome P450 (CYP-450) enzymes that are known to play crucial roles in xenobiotic metabolism, and more specifically, in activating or detoxifying tobacco smoke carcinogens.[29] Robust up-regulation of the CYP-450 family-1 member-a1 (*Cyp1a1*), CYP-450 member-b1 (*Cyp1b1*), and Ahr repressor (*Ahrr*) mRNAs was detectable in SHS-exposed mice at T0, indicating that overexpression of these genes in mice is an



	Up-regulated transcripts	Down-regulated transcripts
SHS (T0)	250	424
SHS (T1)	4	250
SHS (T2)	3	27

Figure 1. Global gene expression profiling in SHS-exposed mice *versus* control. Genome-wide gene expression profiling was performed on total RNA samples extracted from the lungs of SHS-exposed and control mice using the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix Inc.). (*a*) Principal component analysis (PCA) of microarray data obtained from SHS-treated mice at T0 (blue) and T1 (green) and corresponding controls (red) using the Partek® software. (*b*) PCA of microarray data obtained from SHS-treated mice at T2 (blue) and corresponding controls (red) using the Partek® software. (*c*) Heatmap of gene expression profiles in the lungs of mice at T0, T1, and T2, by Hierarchical Clustering Analysis in Partek®. (*d*) Differentially expressed targets in the three experimental groups (T0, T1, and T2) were identified by one-way ANOVA, as described in the text. (*e*) Venn diagrams of aberrantly regulated transcripts identified in SHS-exposed mice at T0 (yellow), T1 (blue), and T2 (pink) *versus* age-matched controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

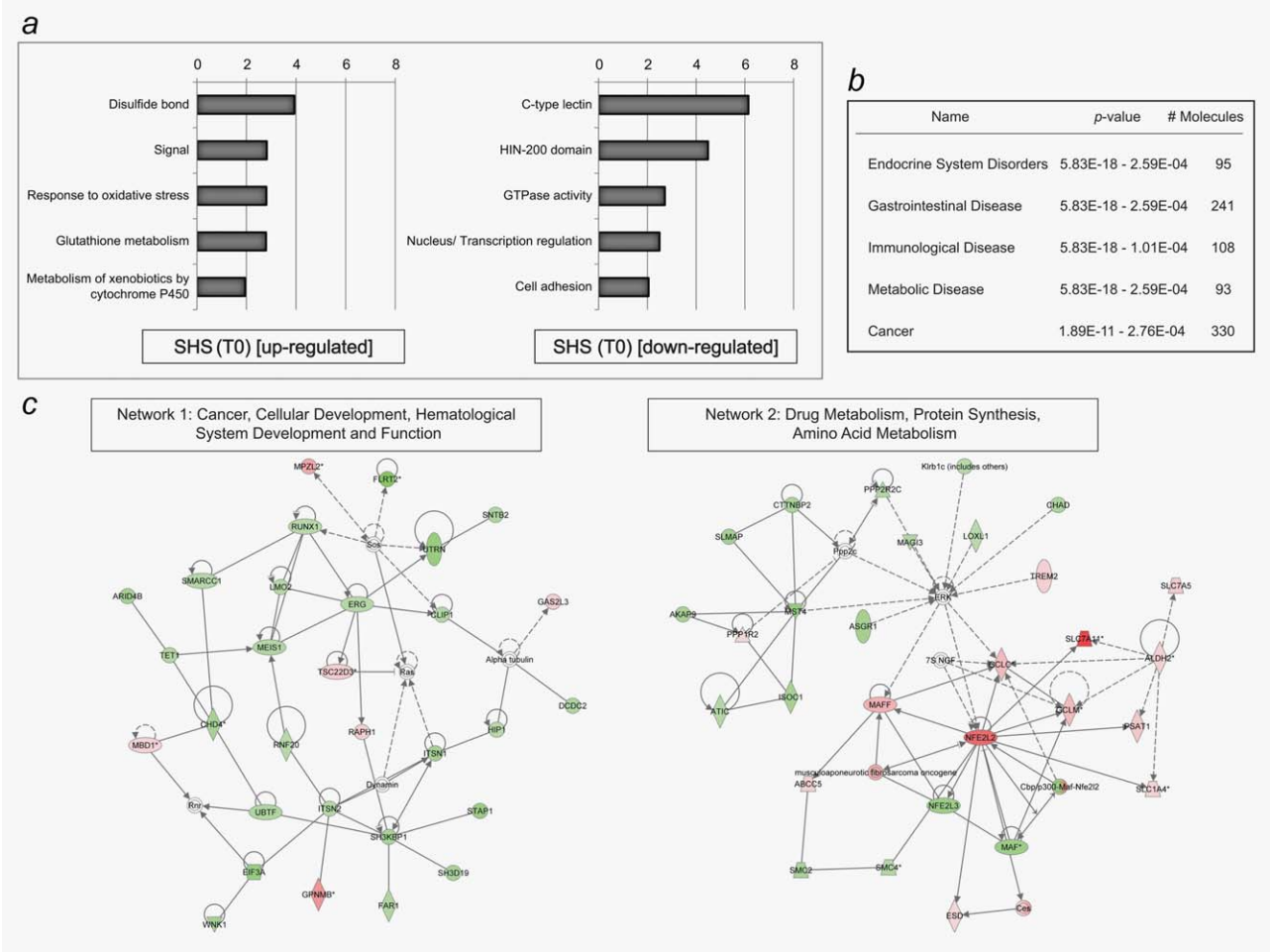


Figure 2. Gene-set enrichment and functional pathway analysis in SHS-exposed mice immediately after termination of exposure (T0). A combination of the GO functional annotation clustering analysis (DAVID v. 6.7) and the Ingenuity Pathway Analysis® (IPA® v. 9.0) was used to obtain detailed gene ontology information for the aberrantly regulated genes identified in SHS-exposed mice at T0. (*a*) The top GO categories with an enrichment score of >2 (*x*-axis) are listed on the *y*-axis. GO analysis of the 250 up-regulated (left) and 424 down-regulated transcripts (right) in T0 mice relative to controls (with FDR-corrected $p < 0.05$ and ± 2-fold change) are shown. (*b*) Top diseases and disorders identified by IPA® in T0 mice relative to controls. (*c*) Top functional networks were generated by IPA® in T0 mice relative to controls. The convergence of genes involved in pathways frequently perturbed in cancer is noticeable. Red and green nodes represent up-regulated and down-regulated genes, respectively. White nodes show molecules that are not included in the datasets but interact with other components of the networks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immediate response to SHS exposure (Fig. 5*a*). In T1 mice, however, transcription of *Cyp1a1*, *Cyp1b1*, and *Ahrr* returned to baseline levels (Fig. 5*a*), which is consistent with the removal of these mice from the SHS-filled environment (during the 1-month recovery in clean air). Likewise, down-regulation of the cancer susceptibility candidate 4 (*Casc4*), the alpha thalassemia/mental retardation syndrome X-linked (*Atrx*), and the cyclin-dependent kinase inhibitor 1B (*Cdkn1b*) genes was confirmed in T0 mice, followed by reversal of their transcription mostly to baseline levels in T1 mice (Fig. 5*b*). Moreover, up-regulation of the zinc finger and BTB domain containing 16 (*Zbtb16*), the family with sequence similarity 107, member A (*Fam107a*), the period circadian clock 2 (*Per2*), and *Ggt1* genes was verified in T0 mice and extended to T1 mice, although to varying degrees (Fig. 5*c*). These findings indicate that a subset of transcriptomic effects caused by SHS exposure in mice is reversible in time; however, other transcriptional changes remain persistent dependent on target genes. Lastly, we confirmed up-regulation of a number of genes, including lactotransferrin (*Ltf*), glycoprotein 2 (*Gp2*), *Ggt1*, and *Retnla*, in T2 mice that had undergone 7 months recovery in clean air (Fig. 5*d*).

Discussion

To gain insights into the underlying mechanisms of SHS carcinogenicity, we have analyzed the pulmonary gene expression profile of mice exposed whole body to SHS for a duration of 4 months, immediately after discontinuation of exposure (T0) and following 1-month (T1) and 7-month (T2) recoveries in clean air. We found that sub-chronic exposure of mice to SHS elicited a robust transcriptomic response, with several hundred transcripts being differentially expressed

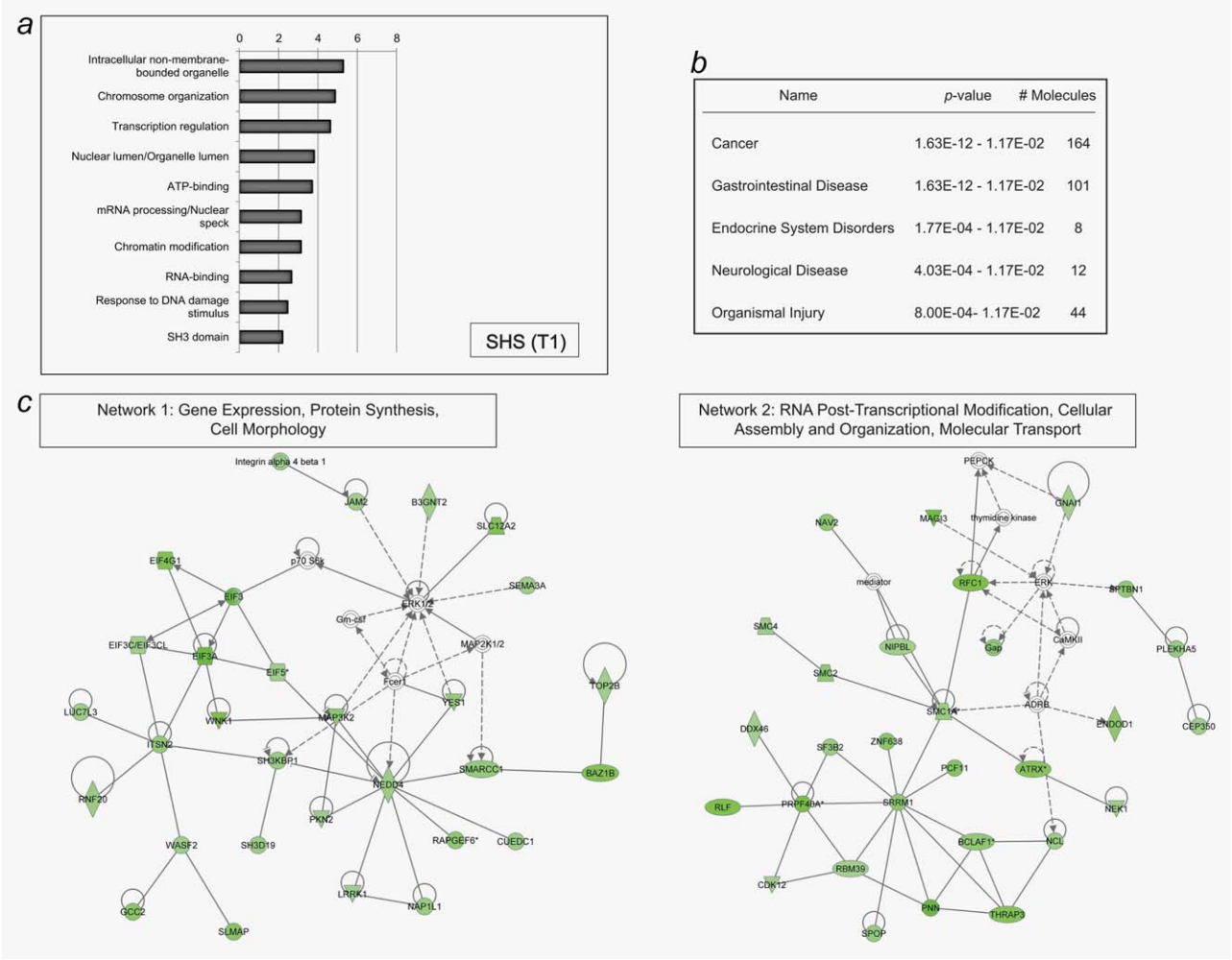


Figure 3. Gene-set enrichment and functional pathway analysis in SHS-exposed mice following one-month recovery in clean air (T1). A combination of the GO functional annotation clustering analysis (DAVID v. 6.7) and the Ingenuity Pathway Analysis® (IPA® v. 9.0) was used to obtain detailed gene ontology information for the aberrantly regulated genes identified in SHS-exposed mice at T1. (*a*) The top GO categories with an enrichment score of ≥ 2 (*x*-axis) are listed on the *y*-axis. GO analysis of the deregulated transcripts in T1 mice relative to controls (with FDR-corrected $p < 0.05$ and ± 2-fold change) is shown. (*b*) Top diseases and disorders identified by IPA® in T1 mice. (*c*) Top functional networks were generated by IPA® in T1 mice relative to controls, and showed enrichment of genes involved in pathways frequently perturbed in cancer. See, also legend to Figure 2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

upon termination of SHS exposure (T0) (Figs. 1*d* and 1*e*; Supporting Information Table S1). The differentially expressed transcripts in T0 mice belong to gene families that play crucial roles in xenobiotic metabolism and signaling, as well as innate immune response (Fig. 2*a*). Top bio-functional networks disrupted in SHS-exposed mice at T0 consisted of cancer, cellular development, hematological system development and function, and drug metabolism (Fig. 2*c*).

Short-term recovery of SHS-exposed mice in clean air (T1) resulted in attenuation of the induced transcriptomic effects (Fig. 1*d*). Over one-half of the differentially expressed transcripts in T1 mice overlapped with those found in T0 mice (*i.e.*, 142 common transcripts), whereas the remainder 112 were unique to T1 mice (Fig. 1*e*). Prolonged recovery of SHS-exposed mice in clean air (T2) led to further dilution of the induced transcriptomic effects (Fig. 1*d*). The overwhelming majority of the differentially expressed transcripts in T2 mice (27 out of 30) were distinct from those found in T0 and/or T1 mice (Fig. 1*e*). The only common target across T0-, T1-, and T2-mice was an unknown transcript (1425470_at) (Fig. 1*e*; Supporting Information Table S1). The resistin like alpha (*Retnla/RELMα/Fizz1*) gene was one of the two targets identified in both T0 and T2 mice. Expression of *RELMα* is usually low in normal mouse lung, but it is greatly increased during hypoxia-induced pulmonary hypertension, allergic airway inflammation, bleomycin-induced lung fibrosis, and asthma.[30] Kolosova *et al.* showed that *RELMa* induces robust proliferation of mesenchymal stem cells (MSC) in mouse lung dependent on *Pi3k/Akt* and *Erk* activation.[30] The gamma-glutamyltransferase 1 (*Ggt1*) gene was another common target in T0 and T2 mice. Gamma-glutamyltransferase (GGT) catalyzes the first step in the degradation of

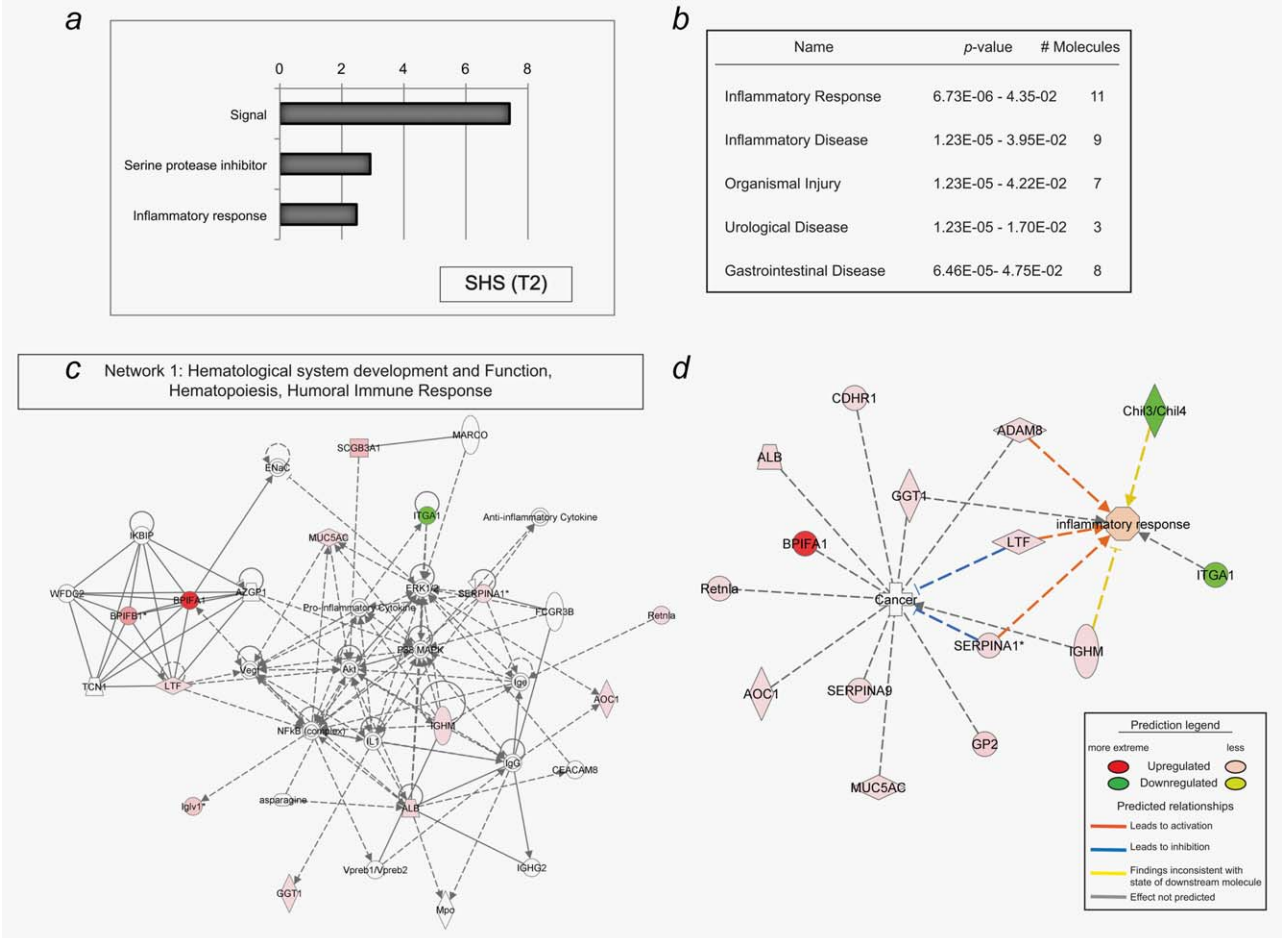


Figure 4. Gene-set enrichment and functional pathway analysis in SHS-exposed mice following 7-month recovery in clean air (T2). A combination of the GO functional annotation clustering analysis (DAVID v. 6.7) and the Ingenuity Pathway Analysis® (IPA® v. 9.0) was used to obtain detailed gene ontology information for the aberrantly regulated genes identified in SHS-exposed mice at T2. (*a*) The top GO categories with an enrichment score of ≥ 2 (*x*-axis) are listed on the *y*-axis. GO analysis of the deregulated transcripts in T2 mice relative to controls (with unadjusted $p < 0.05$ and ± 2-fold change) is shown. (*b*) Top diseases and disorders identified by IPA® in T2 mice. (*c*) Top functional networks were generated by IPA® in T2 mice relative to controls. Genes converged into the *Mek/Erk* pathway, which is known to be frequently disrupted in cancer. (*d*) Several deregulated genes at T2 are involved in inflammatory response (*Adam 8*, *Chil3/Chil 4*, *Ggt1*, *Ighm*, *Itga1*, *Ltf* and *Serpina 1*; $p = 6.73\text{E-}06$). The link between inflammation and cancer is highlighted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

extracellular glutathione (GSH), thus facilitating the recovery of GSH amino acids, in particular cysteine. Differential expression of GGT has been observed in a variety of human malignancies and tumor-derived cell lines, suggesting a role for GGT in tumor progression via oxidative stress pathways.[31] Elevated GGT has been associated with mortality from all causes, including cancer, in the US population.[32] Also, a 65-gene expression signature, including *GGT1*, has been found to be associated with non-small cell lung carcinoma (NSCLC) in nonsmokers with high expression levels of CHRNA6 and CHRNB3 proteins.[33] Our overall findings indicate that the immediate transcriptomic effects of SHS exposure in mice are mitigated over time, however, persistent effects as well as novel "delayed" effects are also manifest dependent on the length of time post-exposure.

We found a reversal of transcriptomic effects that had impacted the CYP-450 gene family in T0 mice in animals that had undergone recovery in clean air (Fig. 5*a* and Supporting Information Table S1). Of note, many members of the superfamily of the CYP-450 enzymes are known to metabolize tobacco smoke carcinogens.[29] The reversibility of the induced transcriptional changes in the CYP-450 gene family in SHS-exposed mice maintained in clean air is consistent with the removal of these animals from the source of exposure. We also observed both persistent and new transcriptional changes in SHS-exposed mice after discontinuation of exposure. The differentially expressed transcripts in T1 mice remained predominantly associated with cancer (~65%), and consisted of genes involved in transcription regulation, chromosome organization, and chromatin

modifications among others (Figs. 3*a*–3*c*). The transcriptional changes in T2 mice impacted three major biological processes, including signaling, serine protease inhibitors, and inflammatory response (Fig. 4*a*). The differentially expressed genes in T2 mice were primarily associated with inflammatory response and inflammatory disease (Fig. 4*b*). The perturbation of gene expression in the inflammatory response and disease pathways in SHS-exposed mice months after discontinuation of exposure is highly significant considering the established link between inflammation and lung cancer in humans.[34–36] Continued exposure to SHS has been shown to cause pulmonary inflammatory disorders, including chronic obstructive pulmonary disease (COPD) in both human and animal models.[37] Of note, chronic inflammation is known to create a pre-malignant cell environment, eventually leading to tumor initiation and progression.[35,36,38]

Of the 30 differentially expressed transcripts in T2 mice, thirteen, all up-regulated, map to genes that are altered during tumorigenesis, including *Bpifa1*, *Gp2*, *Alb*, *Serpina1*, *Ltf*, *Muc5ac*, *Ighm*, *Ggt1*, *Aoc1* (*Abp1*), *Retnla*, *Serpina9*, *Cdhr1*, *Adam8* ($p = 3.29E\text{-}02$ IPA® analysis). Of these, eight are known to be deregulated/mutated in lung cancer and other lung-associated diseases (Table 1).[33,39–45] Of significance, the overexpressed *Retnla* gene in T2 mice (Fig. 5*d*) is known to be up-regulated in rat lung tissues following chronic exposure to cigarette smoke.[44] *RELMα* is expressed by macrophages and bronchial epithelial cells, as well as by pulmonary vascular cells during hypoxia.[30] *RETNLβ*, the human homolog of the murine *Retnla*, is considered a marker of inflammatory response in lung diseases[46] and is located on chromosome 3q13, a region that is recurrently amplified in NSCLC.[46,47] Histopathologically, NSCLC is the best-described lung cancer in nonsmokers.[33,48] Other differentially expressed genes of interest in T2 mice are the up-regulated *Ggt1* and *Ltf* and the down-regulated *Itga1*. In humans, expression of *GGT1* is associated with NSCLC,[33] whereas expression of *LTF* is linked to pulmonary exacerbation[43] (Table 1). In mice, mutation of the *Itga1* gene is shown to reduce the incidence and growth of lung epithelial tumors initiated by oncogenic *Kras* (Table 1).[45]

We have recently demonstrated a genotoxic mode of action for SHS based on the induction of DNA damage and

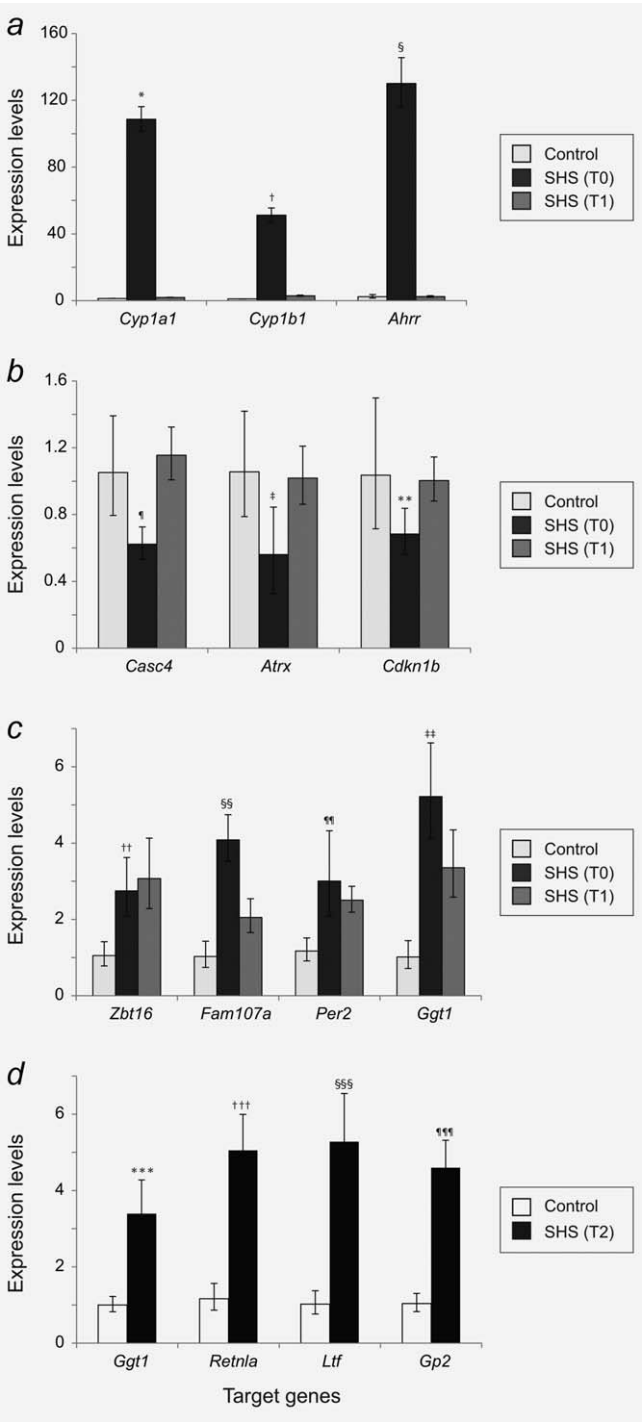


Figure 5. Quantification of gene expression by qRT-PCR in SHS-exposed mice *versus* control. The expression status of individual target genes identified by Affymetrix microarray analysis was examined by quantitative RT-PCR using the $2^{-\Delta\Delta Ct}$ method.[28] Bars represent the mean normalized expression (±SD) of triplicate samples in SHS-exposed mice and age-matched controls. Data were normalized using the endogenous housekeeping gene, *Gapdh*, as reference. Relative transcription levels of *Cyp1a1*, *Cyp1b1* and *Ahrr* (*a*), *Casc4*, *Atrx* and *Cdkn1b* (*b*), *Zbt16*, *Fam107a*, *Per2* and *Ggt1* (*c*), and *Ggt1*, *Retnla*, *Ltf* and *Gp2* (*d*) in SHS-exposed mice *versus* controls. *SHS (T0) *vs*. control: $p = 0.01606$; SHS (T0) *vs*. SHS (T1): $p = 0.01648$. †SHS (T0) *vs*. Control: $p = 0.00453$; SHS (T0) *vs*. SHS (T1): $p = 0.0056$; SHS (T1) *vs*. control: $p = 0.00198$. §SHS (T0) *vs*. control: $p = 0.00968$; SHS (T0) *vs*. SHS (T1): $p = 0.00969$. ¶SHS (T0) *vs*. control: $p = 0.09671$; SHS (T0) *vs*. SHS (T1): $p = 0.01214$.‡SHS (T0) *vs*. control: $p = 0.06063$; SHS (T0) *vs*. SHS (T1): $p = 0.00529$. **SHS (T0) *vs*. control: $p = 0.08387$. ††SHS (T0) *vs*. control: $p = 0.00019$; SHS (T1) *vs*. control: $p = 0.06881$. §§SHS (T0) *vs*. control: $P < 0.000004$; SHS (T0) *vs*. SHS (T1): $p = 0.01658$. ¶¶SHS (T0) *vs*. Control: $p = 0.00493$. ‡‡SHS (T2) *vs*. control: $p = 0.00045$; SHS (T1) *vs*. control: $p = 0.05178$. ***SHS (T2) *vs*. control: $p = 0.00282$. †††SHS (T2) *vs*. control: $p = 0.00202$. §§§SHS (T2) *vs*. control: $p = 0.01229$. ¶¶¶SHS (T2) *vs*. control: $p = 0.08328$.

Table 1. List of differentially expressed genes associated with lung disease in T2 mice

Gene	Fold change[1] (T2 *vs.* control)	Disease	References
Adam8	+2.19	Upregulation of human ADAM8 mRNA in lung carcinoma is associated with lung cancer in human.	Mochizuki and Okada, 2007[39]
Alb	+3.53	Up-regulation of human albumin [ALB] protein in pulmonary edema fluid is associated with acute lung injury in human.	Bowler *et al.*, 2004[40]
Aoc1 (Abp1)	+2.35	Somatic missense mutant human AOC1 gene is associated with adenocarcinoma in human lung.	Seo *et al.*, 2012[41]
Bpifa1	+21.75	Up-regulation of human PLUNC [BPIFA1] mRNA in lung tumor from human is associated with non-small cell lung cancer in human.	Kim *et al.*, 2007[42]
Ggt1	+2.45	Expression of human GGT1 mRNA in non-small cell lung carcinoma expressing human CHRNA6 protein and human CHRNB3 protein is associated with non-small cell lung carcinoma in human.	Lam *et al.*, 2007[33]
Ltf	+3.36	Up-regulation of human LTF mRNA in PBMCs is associated with severe acute respiratory syndrome in human.	Reghunathan *et al.*, 2005[43]
Retnla	+2.3	Expression of rat Relm alpha [Retnla] mRNA occurs in alveolar epithelial cells from lung of rat exhibiting pulmonary fibrosis.	Liu *et al.*, 2004[44]
Serpina1	+2.25	Up-regulation of human SERPINA1 mRNA in PBMCs is associated with severe acute respiratory syndrome in human. Mutated SERPINA1 is associated with chronic obstructive pulmonary disease and pulmonary emphysema.	Reghunathan *et al.*, 2005[43]
Itga1	−2.00	In mouse, homozygous mutant mouse INTEGRIN ALPHA 1 [Itga1] gene (knockout) decreases vascularization of lung tumor in mouse that is increased by transgenic oncogenic mutant mouse Kras protein.	Macias-Perez *et al.*, 2008[45]

[1]A "positive" (+) fold change number indicates up-regulation while a "negative" (−) fold change number indicates down-regulation.

mutagenesis in the same mouse model as used in the present study.[8,11] The mutation signature identified in the lungs of SHS-exposed mice was remarkably similar to that found in the human *TP53* gene in lung tumors of nonsmokers, supporting a link between SHS exposure and lung cancer development in nonsmokers.[8] We have also explored whether SHS can exert epigenetic effects by investigating a prime epigenetic alteration, namely aberrant DNA methylation, in the lungs of SHS-exposed mice.[10] However, whole methylome profiling revealed that there was no significant change in the patterns of DNA methylation between SHS-exposed mice and controls. This finding implies that epigenetic alterations other than aberrant DNA methylation, *e.g.*, histone modifications and variants, chromatin remodeling, nucleosome positioning, and microRNA deregulation[17,49,50] may play a role in SHS carcinogenicity. In confirmation, our T0- and T1-mice showed persistent transcriptional changes in a large number of targets ($n = 142$) that are known to be involved in chromosome organization, chromatin modification, and RNA processing (Fig. 1*e*). Future functional studies are needed to verify whether the transcriptionally altered pathways identified in the present study are causally linked to lung cancer in nonsmokers who have a history of exposure to SHS.

In summary, our time-course gene expression profiling analysis provides strong evidence that *in vivo* SHS exposure induces both transient and long-lasting transcriptional changes in cancer-related functional networks. The enduring transcriptomic effects may trigger inflammation and chronic respiratory diseases, which may ultimately lead to lung carcinogenesis. Our data underscore the need to implement and/or improve smoke-free policies to protect nonsmokers from the deleterious effects of SHS, ideally in all places and at all times.

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