

Pulmonary Prostacyclin Synthase Overexpression Chemoprevents Tobacco Smoke Lung Carcinogenesis in Mice

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ABSTRACT

Increased pulmonary production of prostaglandin I₂ (prostacyclin) by lung-specific overexpression of prostacyclin synthase decreases lung tumor incidence and multiplicity in chemically induced murine lung cancer models. We hypothesized that pulmonary prostacyclin synthase overexpression would prevent lung carcinogenesis in tobacco-smoke exposed mice. Murine exposure to tobacco smoke is an established model of inducing pulmonary adenocarcinomas and allows for the testing of potential chemopreventive strategies. Transgenic FVB/N mice with lung-specific prostacyclin synthase overexpression were exposed to mainstream cigarette smoke for 22 weeks and then held unexposed for an additional 20 weeks. All of the exposed animals developed bronchiolitis analogous to the respiratory bronchiolitis seen in human smokers. The transgenic mice, when compared with smoke-exposed transgene negative littermates, had significant decreases in tumor incidence and multiplicity. Significantly fewer transgenics (6 of 15; 40%) developed tumors compared with the tumor incidence in wild-type littermates (16 of 19; 84%; Fisher's exact test, $P = 0.012$). Tumor multiplicity was also significantly decreased in the transgenic animals ($tg^+ = 0.4 \pm 0.5$ versus wild-type = 1.2 ± 0.86 tumors/mouse; $P < 0.001$). Targeted prostaglandin levels at the time of sacrifice revealed significantly elevated prostaglandin I₂ levels in the transgenic animals, coupled with significantly decreased prostaglandin E₂ levels. Gene expression analysis of isolated type II pneumocytes suggests potential explanations for the observed chemoprevention, with Western blot analysis confirming decreased expression of cytochrome p450 2e1. These studies extend our previous studies and demonstrate that manipulation of prostaglandin production distal to cyclooxygenase significantly reduces lung carcinogenesis in a tobacco smoke exposure model, and gene expression studies show critical alterations in antioxidation, immune response, and cytokine pathways.

INTRODUCTION

Lung cancer remains the leading cause of cancer death in both men and women in the United States (1). Worldwide, cigarette consumption has resulted in a lung cancer epidemic with projections that tens of millions of cases will develop in the coming years (2). Tobacco smoke is responsible for the overwhelming majority of lung cancers, and, other than smoking cessation, there are currently no chemopreventive agents that have shown efficacy in preventing lung cancer in humans. The large number of current and former smokers, coupled with the current poor 5-year survival rates for lung cancer (1), additionally emphasizes the need for effective chemopreventive agents.

Animal modeling of lung cancer is imperative to evaluate chemopreventive agents before conducting human trials. Many of the commonly used models involve treatment with chemical carcinogens (the large majority of which are contained in tobacco smoke) that induce

lung tumors. These chemicals induce murine adenocarcinomas that contain many of the same histological and genetic alterations found in human adenocarcinomas (3). To directly address concerns that single or multiple carcinogen injection may not best the model lung tumor development in humans, animal models of tobacco smoke exposure have been developed and tested. Tobacco smoke is a mouse lung carcinogen and can reproducibly induce pulmonary adenocarcinomas (4). The model developed by Witschi *et al.* (5) includes exposure to smoke (a mixture of 89% sidestream and 11% mainstream smoke), followed by a "recovery" period in ambient air. This model of tobacco smoke-induced lung carcinogenesis has been evaluated in inbred murine strains, and the model has shown the ability to induce lung tumors in susceptible strains (4–6). Our results represent the first published report on smoke exposure in FVB/N mice. These experiments will allow for the study of potential chemopreventive agents in a model that better recreates the human condition (either current or former smokers).

Considerable research has focused on products of the arachidonic acid pathway (particularly those generated through cyclooxygenase; COX) as important regulators of lung tumorigenesis. COX inhibition decreases levels of prostaglandins and thromboxanes, and many large epidemiological studies have shown that frequent aspirin users developed fewer lung cancers (7). Human trials evaluating COX inhibition and lung cancer chemoprevention remain to be completed. However, data from murine studies evaluating the role of nonspecific COX or selective COX-2 inhibition have failed to yield overwhelmingly positive results. In fact, COX-2 inhibition by celecoxib leads to no change in tumor multiplicity and an increase in tumor size in an initiator-promoter model of lung tumorigenesis (8). Our research has focused on manipulating prostaglandin production distal to the COX enzymes. Prostacyclin, a prostaglandin H₂ metabolite, is an eicosanoid that has been shown to suppress inflammation (9), inhibit platelets (9), prevent metastases (10), and inhibit the growth of micrometastases (11). We have shown previously that selective pulmonary overexpression of prostacyclin synthase increases pulmonary prostacyclin and chemoprevents murine lung cancer in both a strict carcinogen (ethyl carbamate) and an initiator/promoter model (MCA/BHT) of murine lung cancer (12). The observed significant reduction in lung tumor incidence and multiplicity occurred in a dose-dependent fashion (*i.e.*, animals with a higher degree of prostacyclin synthase expression had a lower incidence and fewer tumors).

To additionally evaluate the chemopreventive properties of prostaglandin I₂, we subjected our prostacyclin synthase overexpressing transgenic animals to a tobacco-smoke lung carcinogenesis model (22 weeks of tobacco smoke followed by a 20-week recovery period in air). All of the animals exposed to smoke developed inflammation at the bronchiolar-alveolar duct junction, a pathological lesion analogous to the respiratory bronchiolitis commonly seen in current and former smokers (13). Exposure to smoke did not alter the pulmonary prostaglandin production when compared with levels measured in non-smoke-exposed animals [*i.e.*, significantly higher prostaglandin I₂ levels and significantly decreased prostaglandin E₂ (PGE₂) levels in

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the transgenics at the time of sacrifice]. We extend our previous findings by using a model of smoke inhalation. Furthermore, extensive microarray analysis strongly suggests potential mechanisms of chemoprevention, including key alterations in antioxidation, immune response, and cytokine pathways. Prostacyclin synthase overexpression significantly decreased both the lung tumor incidence and multiplicity in this model, providing additional evidence that manipulation of prostaglandin production distal to COX may be an attractive lung cancer chemopreventive strategy.

MATERIALS AND METHODS

Development of Transgenic Prostacyclin Synthase Overexpressors. As described previously, transgenic mice were developed using a construct consisting of the human SP-C promoter and full-length rat prostacyclin synthase cDNA (14). The SP-C promoter allows targeted expression to alveolar and airway epithelial cells (15). Transgenic mice were genotyped by performing PCR on genomic DNA isolated from tails as described previously (14). Each line was propagated as heterozygotes. Transgenic mice (Tg⁺) were always bred with wild-type FVB/N (Jackson Labs, Bar Harbor, ME) mice to produce the experimental Tg⁺ mice and transgenic negative littermates (Tg⁻), which were used as controls in all of the experiments. For all of the experiments, mice from our highest expressing line (>250% increase in lung 6-keto prostaglandin F_{1 α} compared with Tg⁻ littermates) were used (14).

Tobacco Smoke-Exposure. Tg⁺ and wild-type littermates from the Denver VA Medical Center aged 12 weeks were transported to Lovelace Respiratory Institute in Albuquerque, NM. After an acclimatization period, mice were placed in wire-bottomed cages in the smoke exposure chamber. The mice were exposed to diluted and slightly aged mainstream cigarette smoke generated from burning IR3 reference cigarettes (Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, KY) as described previously (5, 16). Mice were exposed to smoke concentrations of 100 mg total particulate matter/m³ for the first week, and then the concentration was increased to 250 mg total particulate matter/m³ for the remainder of the exposure. Animal cages were rotated periodically in the chamber, and the duration of exposure was 22 weeks. Study animals were weighed monthly during smoke exposure and at the time of sacrifice. After exposure, mice were transferred to conventional caging with a controlled environment (12 h light-dark cycle, food and water *ad libitum*) for an additional 20 weeks.

Tumor Enumeration. Animals were euthanized via pentobarbital overdose. Tumors were enumerated under a dissection microscope ($\times 5$ magnification) after inflation with formalin at a pressure of 15 cm H₂O. All of the tumors were dissected from the surrounding lung parenchyma. To confirm the presence of pulmonary adenomas and to evaluate the lung parenchyma, the tumors and surrounding uninvolved lung were paraffin-embedded and sectioned, followed by staining with H&E. All of the slides were reviewed with the study pathologist to confirm findings.

Type II Cell Isolation. Murine type II alveolar cells were isolated to determine prostaglandin production and gene expression in a population of cells expressing the transgene. To examine both whole lung and type II cell levels of prostaglandins and prostacyclin synthase expression, the right lower lobe was isolated, both bronchus and vasculature ligated, and lobe placed in radioimmune precipitation buffer for protein analysis and Earle's balanced salt solution (Sigma, St. Louis, MO) for whole enzyme analysis. The remaining lobes were then perfused with 0.9% saline via the pulmonary artery until thorough blanching had occurred. The type II cells were isolated as reported previously (17). The resultant cell suspension was added to antibody plates (CD 45 and CD 32) that had been prewashed twice with DMEM/HEPES and were incubated for 2 h at 37°C in 5% CO₂. After incubation, medium was gently removed from plates and centrifuged (8 min, 130 \times g). Medium was isolated for prostaglandin analysis and cells placed in RNA later for mRNA isolation (Ambion, Austin, TX).

6-Keto Prostaglandin F_{1 α} and PGE₂ Assays to Determine Prostacyclin Synthase and PGE₂ Synthase Activity. Type II cell preparations were obtained as above, and the cells were incubated in medium for 2 h at 37°C with 5% CO₂ followed by removal of the supernatant. Determination of pulmonary 6-keto prostaglandin F_{1 α} and PGE₂ levels by ELISA was performed as described previously (14). The assays were performed in a blinded fashion using

coded sample tubes. Protein concentrations were also performed on an aliquot of the supernatant, and prostaglandins were normalized for protein concentration.

Murine Microarray Analysis. Total RNA was extracted from the type II cell preparations on both Tg⁺ and Tg⁻ mice (two of each) using the RNeasy Total RNA Isolation kit (Qiagen Inc. Valencia, CA). Approximately five μ g of total RNA was collected from each sample and evaluated individually. Fifteen micrograms of the labeled cRNA mixture was applied to the GeneChip microarray analysis (Affymetrix Inc., Santa Clara, CA) as described previously (18). Mouse samples were hybridized to the Affymetrix Murine Genome MOE 430A array. Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described (19).

Statistical Analysis. All of the values were expressed as means \pm SE. For tumor multiplicity and cell counts, the data were normally distributed, and unpaired *t* tests were performed using GraphPad Prism 3.02 for Windows (GraphPad Software for Science Inc., San Diego, CA). For tumor incidence, GraphPad was used to perform Fisher's exact test. Data were considered significant at the *P* < 0.05 level.

Analysis of gene expression was conducted by Affymetrix software. All of the values were expressed as means \pm SE. Means of both groups were compared by two methods, unpaired *t* test and a matrix analysis of all possible combinations of wild-type to transgenic mice. In the matrix analysis, all four of four possible comparisons (a 2 \times 2 table created for both transgenic and wild-type mice) must show concordance in the "increase" or "decrease" call (generated by Affymetrix Microarray Suite 5.0).

Before analysis by any of the statistical tests, variability filters were applied to the dataset to minimize multiple testing errors as described previously (19). The first filter uses the Affymetrix mRNA detection "call" (MAS version 5.0) to exclude all of the genes with an absolute call of "absent" in all of the experiments. The second filter identifies genes with at least moderate variance over the entire experiment; genes that do not vary at all cannot possibly be related to the presence of the transgene. We calculated the variances for each gene. The null hypothesis is that these variances represent random and normally distributed noise. We then compute the statistic $W = (N-1) s_2/\text{median}(s_2)$ where *N* is the number of observations of the gene, which is approximately χ^2 distributed with *N*-1 degree of freedom (20). A *P* is calculated for rejecting the null hypothesis that the gene did not vary, and the false discovery rate multiple testing correction was performed, setting the false discovery rate to be 10%. This results in a list of genes with significantly greater variation than the median variation gene, with at most 10% of that list including genes having true variation less than or equal to median variation. These preprocessing steps screen out genes with low variance and low mRNA levels expression measurements. The remaining gene list is then fed to the Kruskal-Wallis and false discovery rate correction (10% in this case), producing a final gene list of which the expression levels are significantly different between the two genotypes of mice.

Western Blot Analysis. Isolated type II cells were lysed in a cell lysing buffer (5% HEPES, 0.1% dithiothreitol, 0.1% triton, and 10% glycerol) and phosphate buffer saline (Mediatech Inc., Herndon, VA) in a 1:1 mix. The samples were incubated on ice for 45' and were then sonicated for 30 s on ice. Lysates were centrifuged for 10' at 13.4 rpm (16.1 rcf) and supernatants collected. Protein concentration was determined for normalization of protein levels using Bio-Rad Protein Reagent kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated on 4–12% Bis-Tris gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). To verify equal transfer of samples, membranes were stained with Ponceau's solution (0.1% Ponceau in 5% acetic acid; Sigma). Membranes were then blocked for 1 h with 5% TTBS milk and probed overnight with either prostacyclin synthase (1:1000 dilution) or CYP2E1 (1:400 dilution; Chemicon International, Temecula, CA) antibodies. Donkey-antirabbit IgG-horseradish peroxidase (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody. Bound protein was detected with chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA).

Quantitative PCR. The murine type II pneumocytes harvested from both transgenic and wild-type mice (three from each group) were lysed in 1 ml of TRIzol reagent (Invitrogen). Cells were passed through a heavy phase lock gel (Eppendorf, Westbury, NY), and RNA was isolated using an RNA MinElute kit (Qiagen). Reverse transcription-PCR was conducted with 1 μ g of RNA

using a high-capacity cDNA kit (Applied Biosystems, Foster City, CA). Predesigned quantitative PCR probes for β -actin, transforming growth factor- β , peroxisome proliferator-activated receptor (PPAR) γ , and carboxylesterase were obtained from Applied Biosystems. We conducted quantitative PCR in triplicate using TaqMan Universal PCR Master Mix (Applied Biosystems), and results are expressed as message relative to β -actin. A $P < 0.05$ by nonparametric t test was considered statistically significant.

RESULTS

Smoke Exposed Mice Gain Weight Normally and Develop Terminal Bronchiolitis. Animals placed in the smoke exposure chamber were weighed on a monthly basis during exposure and at the time of sacrifice, and the animals continued to consistently gain weight during the smoke exposure. Observed differences in weights were only those expected on the basis of sex. Unlike the marked reduction in weight gain seen in BALB/c mice during smoke exposure (5), FVB/N mice gain weight in a normal fashion. There were no excessive or unexpected deaths among the Tg^+ or Tg^- smoke-exposed animals (data not shown).

One finding consistently present upon histological examination of the lungs at sacrifice was a terminal bronchiolitis. Unlike human lung, murine lungs do not contain respiratory bronchioles, but they do contain terminal bronchioles. Fig. 1 shows representative bronchiolitis in a smoke-exposed Tg^- animal. The lesions consisted of pigment-laden macrophages within bronchioles and alveoli, along with peribronchiolar infiltration of lymphocytes. In humans, respiratory bronchiolitis is a smoking-related interstitial lung disease characterized by a mononuclear inflammatory infiltrate of the membranous and respiratory bronchioles (13). A subset of respiratory bronchiolitis patients develop respiratory bronchiolitis-associated interstitial lung disease with fibrous scarring extending from the bronchioles to the surrounding alveoli (21). Microscopic examination of the lungs failed to reveal a quantitative difference in the degree of bronchiolitis between Tg^+ and Tg^- littermates.

Prostacyclin Synthase-Overexpressing Mice Develop Significantly Fewer Lung Tumors. Transgenic mice overexpressing prostacyclin synthase develop significantly fewer lung tumors when subjected

to the tobacco smoke exposure model developed by Witschi *et al.* (5). Tg^+ mice had a significant reduction in tumor multiplicity when compared with Tg^- littermates (0.40 ± 0.50 versus 1.21 ± 0.86 tumors/mouse; * $P < 0.0001$; Fig. 2A). All of the pulmonary adenomas were grossly apparent at the time of sacrifice with the aid of a dissection microscope ($\times 5$ magnification), and tumors were reviewed microscopically to confirm that they represented pulmonary adenomas. Serial sections were also performed on paraffin-embedded lungs and failed to reveal additional tumors not detected at the time of sacrifice.

Prostacyclin Synthase-Overexpressing Mice Had a Decreased Incidence of Lung Tumors. In addition to having decreased tumor multiplicity, prostacyclin synthase overexpressors demonstrated a significant decrease in tumor incidence, indicating chemoprevention from the development of lung cancer. After smoke and ambient air exposure, 60% of the transgenics (9 of 15) failed to develop lung tumors compared with 16% of the wild-type littermates (3 of 19; $P = 0.012$; Fisher's exact test). Fig. 2B contains the individual tumor incidence data.

Prostaglandin I_2 and PGE_2 Production in Alveolar Type II Cells from Prostacyclin Synthase Overexpressors Showed Maintenance of the Baseline Phenotype. Our previous studies have demonstrated that the balance of pulmonary prostaglandins, particularly prostaglandin I_2 and possibly PGE_2 , may be very important in explaining the observed chemoprevention in prostacyclin synthase overexpressors (12). To investigate whether prostaglandin production in transgenics and wild-types was altered by smoke exposure we determined the pulmonary levels of these prostaglandins in isolated alveolar type II cells. The surfactant protein C promoter is activated in alveolar type II cells (15), meaning isolated type II cells will be an excellent method to evaluate prostaglandin production. At the time of sacrifice, both 6-keto prostaglandin $F_{1\alpha}$ (the stable breakdown product of prostaglandin I_2) and PGE_2 were measured in Tg^+ and Tg^- littermates. Significant elevations in 6-keto prostaglandin $F_{1\alpha}$ (corrected for protein levels) persisted at the time of sacrifice in prostacyclin synthase overexpressors ($n = 4$) compared with wild-type littermates ($n = 5$; 237.5 ± 35.20 versus 33.50 ± 8.60 pg/g protein; $P = 0.0013$). These results (illustrated in Fig. 3A) represent a >7 -fold

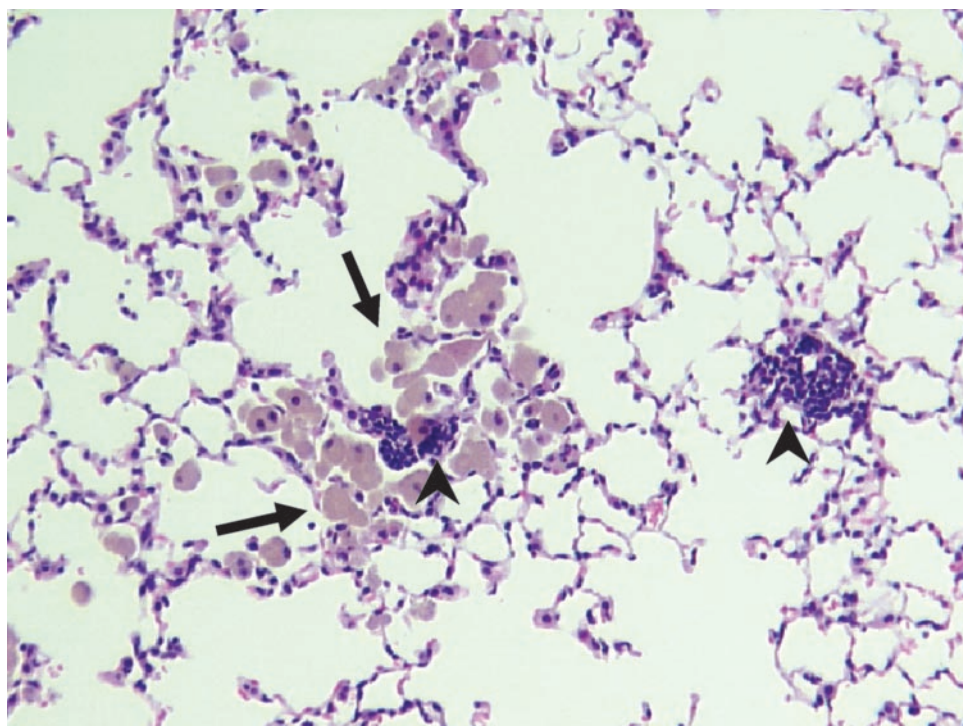


Fig. 1. Representative hematoxylin and eosin specimen from a wild-type animal exposed to tobacco smoke illustrating the pathological lesion terminal bronchiolitis (magnification, $\times 40$). Arrows indicate pigment-laden macrophages, and arrowheads indicate the mononuclear inflammatory infiltrate surrounding terminal bronchioles.

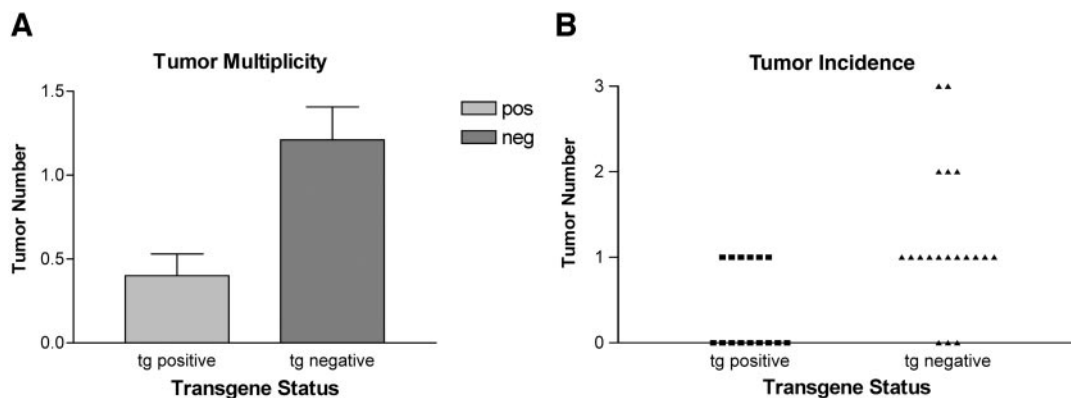


Fig. 2. *A*, lung tumor multiplicity and incidence after smoke exposure. Transgenic animals ($n = 15$) had a significant reduction in tumor number compared with transgene-negative littermates ($n = 19$; 0.40 ± 0.50 versus 1.21 ± 0.86 tumors/mouse; * $P < 0.0001$). *B*, prostacyclin synthase overexpression reduces tumor incidence. The individual data from the sacrificed animals are shown. There was a significant reduction in tumor incidence; 9 of the 15 transgenic animals failed to develop tumors, whereas only 3 of the 19 wild-type littermates developed tumors ($P = 0.012$; Fisher's exact test).

increase in prostaglandin I₂ production in type II cells from Tg⁺ and are consistent with increases in prostaglandin I₂ production seen in nonsmoke-exposed prostacyclin synthase overexpressors.

PGE₂ levels were also measured in isolated type II cells. Elevated PGE₂ levels have been associated with decreased immune surveillance of tumors and have been implicated a variety of epithelial cancers (22). Our previous studies have shown that the chemoprevention observed in prostacyclin synthase-overexpressing mice requires elevated prostaglandin I₂ and is not completely explained by decreases in PGE₂ (12). However, our Tg⁺ mice do exhibit decreased PGE₂ levels at baseline, and this decrease is maintained after treatment with urethane but not after chemical carcinogenesis with MCA/BHT (12). At the time of sacrifice, Tg⁺ mice ($n = 4$) did have significantly lower PGE₂ levels when compared with wild-type littermates ($n = 5$; 2.73 ± 1.76 versus 9.32 ± 1.57 ng/g protein; $P = 0.0266$; Fig. 3B).

Microarray Analysis. Microarray analysis of type II cell RNA isolates identified several genes that clearly distinguished Tg⁺ from Tg⁻ mice. The genes are expressed as fold change in signal intensity as detailed in Table 1. As expected, Tg⁺ mice demonstrated a near 13-fold increase in prostacyclin synthase gene expression, and this was confirmed in a Western blot for prostacyclin synthase (Fig. 4A). The decrease in cytochrome p450 2e1 seen in Tg⁺ animals was also confirmed with Western blot analysis (Fig. 4B). Equal loading and transfer of protein for Western analysis was confirmed by Ponceau staining (data not shown). Quantitative reverse transcription-PCR

confirmed the gene expression changes for PPAR γ , transforming growth factor- β , and carboxylesterase (Fig. 5). The genes identified can be classified into several categories, including those involved in antioxidation, immune response, cytokine activity, and cell cycle control.

DISCUSSION

Pulmonary specific prostacyclin synthase overexpression (and resulting elevations in pulmonary prostaglandin I₂ levels) significantly decreases both tumor incidence and multiplicity in a smoke exposure model involving 22 weeks of exposure to a smoke mixture, followed by 20 weeks of recovery in ambient air. The observed chemoprevention in the smoking model complements our previous report showing prostacyclin synthase overexpression prevents lung tumorigenesis in multiple chemical induction models (12). The results additionally support that prostaglandin I₂ likely plays a pivotal role in preventing lung tumorigenesis (the overwhelming majority of which is tobacco smoking associated) and makes prostaglandin I₂ an attractive agent for human chemoprevention trials.

Using inhaled tobacco smoke as a mouse lung carcinogen represents an advanced method in murine modeling of lung cancer and will allow for the evaluation of potential chemopreventive and chemotherapeutic agents. There are several published reports examining the effects of smoke exposure on a lung tumorigenesis in inbred mouse strains (4–6), and similar to chemical carcinogen-induced lung tu-

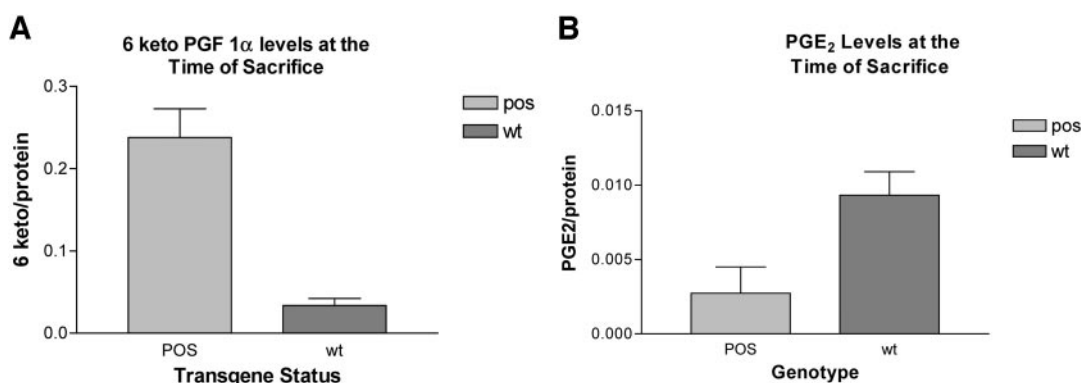


Fig. 3. Determination of prostaglandin levels after the smoke exposure model. *A*, at the conclusion of the smoke exposure protocol, alveolar type II cells isolated from Tg⁺ animals ($n = 4$) maintained their baseline phenotype with significant elevations in 6-keto prostaglandin F_{1α} (PGF_{1α}) levels when compared with type II cells isolated from Tg⁻ littermates ($n = 5$; 2.375 ± 0.0352 versus 0.0335 ± 0.0086 ng/g protein; $P = 0.0013$). All of the samples were corrected for protein. *B*, at the conclusion of the smoke exposure protocol, alveolar type II cells isolated from Tg⁺ animals ($n = 4$) maintained their baseline phenotype with significant decreases in prostaglandin E₂ (PGE₂) levels when compared with type II cells isolated from Tg⁻ littermates ($n = 5$; 0.00273 ± 0.00176 versus 0.00932 ± 0.00157 ng/g protein; $P = 0.0266$). All of the samples were corrected for protein; bars, \pm SE.

Table 1 Direct comparison of gene expression in alveolar type II cells from *PGIS* overexpressors and wild-type littermates

	Probe ID	Accession no.	Fold change
Increased genes			
Oxidative stress			
Gstm1	1448330_at	NM_010358.1	1.4
Glutathione peroxidase 2	1449279_at	NM_030677.1	1.3
GST, $\pi 2$	1449575_a_at	NM_013541.1	1.3
Cell-cell adhesion			
PPAR γ	1420715_a_at	NM_011146.1	1.8
Drug hydrolysis			
Carboxylesterase 1	1449486_at	NM_021456.1	2.7
Fatty acid biosynthesis			
Prostaglandin I ₂ (prostacyclin) synthase	1448816_at	NM_008968.1	12.9
Other			
Fos-like antigen	1417487_at	NM_010235.1	1.6
Wrch-1	1449028_at	AF378088.1	1.5
Decreased genes			
Oxidative stress			
Cytochrome P450, 2e1, ethanol inducible	1415994_at	NM_021282.1	10.4
Cell-cell adhesion			
Syndecan 1	1415943_at	BC010560.1	1.3
Fatty acid biosynthesis			
Stearyl-coenzyme A desaturase 1, mRNA	1415965_at	NM_009127.1	2.6
Immune response and cytokine activity			
Tumor necrosis factor (ligand) superfamily, member 9	1422924_at	NM_009404.1	2.5
TGF β -induced	1456250_x_at	BB533460	2.1
Surfactant associated protein A	1422334_a_at	NM_023134.1	1.3
Growth-related transcriptional control			
Jun oncogene	1448694_at	NM_010591.1	1.4

NOTE. Gene expression analysis revealed significant differences between Tg⁺ and - with the fold change being listed for all of the genes identified.

mors, there exists a great variability depending on the strain of mouse used. Witschi (23) has shown that continuous exposure of strain A mice to smoke for 9 months produces significantly fewer tumors than mice exposed to smoke for 5 months and then allowed to recover in air for 4 months. The exact mechanisms accounting for this observation have yet to be determined. Some have postulated that the cytotoxic constituents of tobacco smoke induce *in vivo* apoptosis and necrosis (24), and this may effectively inhibit tumor growth.

This lung carcinogenesis model, unlike those that only administer selective chemicals found in tobacco smoke, allows for study of the full admixture of elements contained in the tobacco smoke and may give a better representation of the chemopreventive effects of potential agents. Additionally, smoke exposure models can add to the current preclinical chemoprevention studies examining efficacy that should be completed before agents are translated to larger clinical trials. The experience with β -carotene, retinoic acid derivatives, and *N*-acetylcysteine highlight the need for extensive preclinical support with animal testing (25–28), as β -carotene supplementation studies done in murine models failed to show chemoprevention (28). However, the animal studies with β -carotene did not suggest that there would be an increased lung cancer rate in current smokers as was observed in the human trial (28).

Our data represent the first report on smoke exposure in FVB/N mice and the first report evaluating the effects of prostacyclin synthase overexpression in a smoke exposure model. Extensive studies examining various inbred murine strains have revealed that there exist considerable differences in the sensitivities of strains to lung tumor induction. For example, C57/BL6 mice are extremely resistant to lung tumor induction and can require up to 10 doses of urethane to successfully induce pulmonary adenomas (29). Our observed tumor incidence and multiplicity are similar to those seen in other sensitive strains such as A/J, BALB/c, and SWR (5, 6) and would suggest that

FVB/N mice are an appropriate strain for modeling the effects of tobacco smoke exposure. To date, murine chemoprevention studies have been most extensively conducted in strain A/J mice, with green tea, phenethyl isothiocyanate, acetylsalicylic acid, 1,4-phenylenebis-[methylene]selenocyanate, D-limonene, and *N*-acetylcysteine all failing to show reductions in tumor incidence or prevalence in a similar tobacco smoke exposure model (23). Studies evaluating a mixture of dietary myo-inositol and dexamethasone in A/J mice did show significant reductions in multiplicity and incidence in animals exposed to tobacco smoke (23), although our reduction in incidence with prostacyclin synthase overexpression was greater.

The development of bronchiolitis in this tobacco smoke exposure model mirrors the respiratory bronchiolitis commonly seen in current and former smokers. The finding of a mononuclear cell infiltration in the distal lung of smoke exposed mice has been demonstrated previously. Prior animal models of cigarette exposure have demonstrated similar inflammatory changes in distal airways (30–32). In a murine model of chronic cigarette smoke inhalation, lymphocytes and vacuolated macrophages accumulated around the distal airways (32). In our model of smoke exposure, prostacyclin synthase overexpression did not abrogate the mononuclear cell bronchiolitis, suggesting that manipulation of this prostaglandin had little modulatory effect on the macrophage influx and distal airway inflammation caused by cigarette smoke.

Another interesting finding in our mice exposed to cigarette smoke is the presence of brown-pigmented macrophages in the distal airways. In human smokers, respiratory bronchiolitis is universally seen and is characterized by the accumulation of brown-pigmented macrophages and increased connective tissue deposition in the respiratory bronchioles (13). It has been postulated that this lesion may be the precursor of emphysema. In a small number of smokers, the inflammation leads to a pronounced bronchiolocentric fibrosis producing respiratory bronchiolitis-interstitial lung disease (21). The pigmented macrophages noted in our animal model localized in the terminal airways parallel the characteristic inflammatory lesion of human smoking. Again, this inflammatory lesion was universal to our smoking mice whether prostacyclin synthase overexpressors or not. One of the key microscopic features of respiratory bronchiolitis is fibrosis that extends from the bronchioles to the alveoli. There exists an increased risk for lung cancer in patients with pulmonary fibrosis (33), an association that may be explained by chronic inflammation seen in patients with pulmonary fibrosis secondary to diseases like usual interstitial pneumonia (33). The association between respiratory bronchiolitis and lung cancer remains unclear.

To better understand the effects of this tobacco smoke exposure model on prostaglandin production, we measured both prostaglandin I₂ and PGE₂ levels at the time of sacrifice. Prostaglandins can have distinct, and often opposite, effects on tumorigenesis. The role of prostaglandin I₂ in colon carcinogenesis is one example. Transfection of prostacyclin synthase into colon cancer cell lines has been shown to slow growth and decrease tumor vascularity (34), whereas stromal prostaglandin I₂ production markedly reduces butyrate-induced apo-

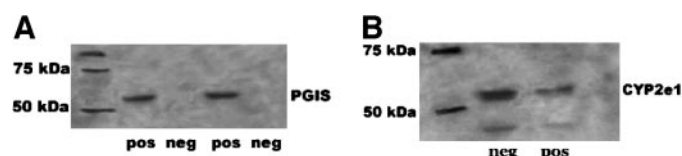


Fig. 4. Western blots of isolated type II pneumocytes from prostacyclin synthase (*PGIS*) overexpressors and wild-type littermates confirm gene expression findings. A, *PGIS* overexpressors (*pos*) type II cells have increased levels of *PGIS* (56 kDa) when compared with transgene-negative wild-type littermates (*neg*). B, *PGIS* overexpressors (*pos*) type II cells have decreased levels of cytochrome p450 2e1 (57 kDa) when compared with transgene negative littermates (*neg*).

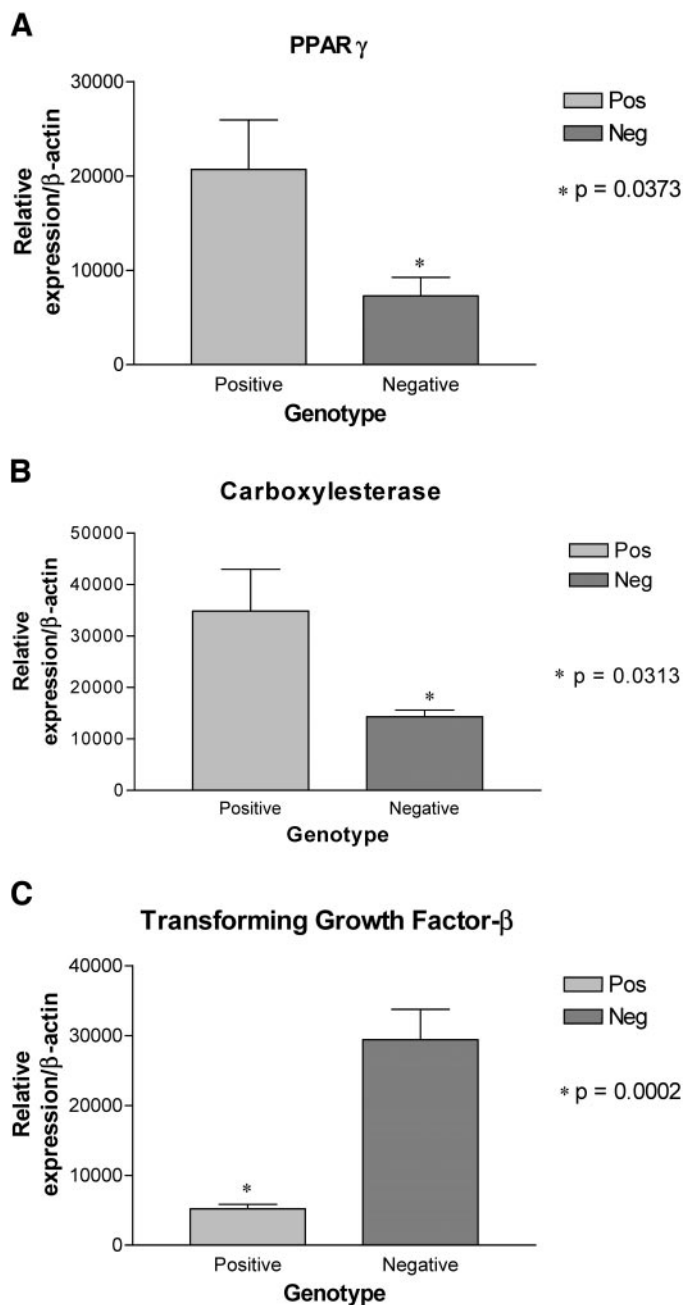


Fig. 5. Quantitative PCR performed on RNA isolated from type II pneumocytes confirms gene expression findings. *A*, prostacyclin synthase overexpressors (*pos*) type II pneumocytes have significantly increased peroxisome proliferator-activated receptor (*PPAR*) γ expression (corrected for β -actin) compared with wild-type littermates (*neg*; $P < 0.05$). *B*, prostacyclin synthase overexpressors type II cells have significantly increased carboxylesterase expression (corrected for β -actin) compared with wild-type littermates ($P < 0.05$). *C*, prostacyclin synthase overexpressors type II cells have significantly decreased transforming growth factor- β expression (corrected for β -actin) compared with wild-type littermates ($P < 0.001$); bars, \pm SE.

ptosis of a different human colorectal cell line (35). Additionally, PGE₂ occupies a critical role in colon carcinogenesis, and anti-inflammatory drugs that inhibit the COX enzymes have been shown to decrease colonic polyp size and number (36). Decreased PGE₂ levels may also explain the observed decrease in lung cancer rates after chronic administration of COX inhibitors (37). Our results show that Tg⁺ animals have significantly higher levels of prostaglandin I₂ metabolite and significantly reduced levels of PGE₂. These results are consistent with prostaglandin measurements performed after the urethane tumorigenesis model (12).

Whereas the underlying chemoprotective mechanism remains unclear, the gene expression analysis does aid our understanding. Prostacyclin synthase overexpression may have the following effects: direct or indirect effects on angiogenesis; cell proliferation; tumor immunity; oxidation; and cell-cell adhesion. Table 1 lists several genes involved in oxidative stress, immune response, and cytokine activity and classified them according to their known functions. As anticipated, microarray analysis identified a near 13-fold increase in prostacyclin synthase expression, which was confirmed by Western blot analysis (Fig. 4A). The observed gene alterations will be addressed in the following discussion.

Oxidative Stress

Cytochrome P450 2E1. The association between lung cancer and exposure to cigarette smoke is clear. In particular, several tobacco smoke constituents including polycyclic aromatic hydrocarbons and nitrosamines have been identified as being procarcinogenic (38). The cytochrome P450 family of enzymes has been implicated in xenobiotic and endobiotic metabolism. Polycyclic aromatic hydrocarbons in tobacco smoke are metabolized to reactive DNA binding epoxides by both Phase I (cytochrome P450 1A1) and phase II enzymes [including glutathione *S*-transferases (GSTs); Ref. 38]. Cigarette smoke is a known inducer of murine cytochrome P450 isoforms Cyp1A1, Cyp3A, and Cyp2E1 (39). In turn, hepatic and extra-hepatic cytochrome P450 can activate procarcinogens in cigarette smoke (38). CYP 2E1 has been localized in liver, brain, and lung tissue and catalyzes oxidation of many low molecular weight procarcinogens such as ethanol, benzene, styrene, butadiene, and urethane (39). Furthermore, CYP2E1 may be integral in tobacco-induced DNA damage and subsequently in the etiology of many human cancers. Villard *et al.*, in a murine model of cigarette smoke exposure, identified a temporal induction of CYP2E1 expression in both liver and lung (38). Our microarray analysis identified a 10-fold reduction in CYP 2E1 expression among Tg⁺ mice, which was confirmed by Western blot analysis (Fig. 4B).

Glutathione *S*-Transferase (μ 1 and P1). Tobacco smoke induce Phase II detoxifying enzymes, including GSTs (40). GSTs function as detoxifiers of both mutagens and carcinogens and confer protection from oxidative stress (40). The GST family is additionally divided into five isoenzymes with tissue-specific expression. GST expression is crucial in determining sensitivity to certain toxins and may represent an adaptive response to chronic smoke exposure (40). GST μ 1 (GSTm1) is polymorphic, with deficient activity in \sim 50% of the Caucasian population (41). Researchers have identified a correlation between the GSTm1 null genotype and increased risk for squamous cell carcinomas of the lung, susceptibility to mutagen-induced cytogenetic damage, asbestos damage, smoking-related bladder, and lung cancer (41). GSTP1 has been identified in alveoli, alveolar macrophages, and respiratory bronchiole (42). Murine GST P2 has only recently been identified and carries an \sim 83% homology with human GSTP1 (42). GSTP1 is expressed in various tumors including breast, esophageal, and hepatocellular carcinomas and been implicated in drug resistance of cancer cells (40). Recently, authors have demonstrated a protective effect of GSTP1 on cigarette smoke-induced apoptosis and necrosis in human lung fibroblasts (42). Tg⁺ animals exhibited a 1.4-fold increase in GSTm1 and a 1.3-fold increase in GSTP1.

Immune Response and Cytokine Activity

Tumor Necrosis Factor Superfamily 9. Our microarray analysis revealed a 2.5-fold decrease in tumor necrosis factor Superfamily 9

expression. Tumor necrosis factor superfamily nine (4–1BB ligand) is involved in cell proliferation, differentiation, and death (43). 4–1 BB ligand interacts with 4–1 BB receptor and delivers a costimulatory signal for T-cell activation and growth (43). Like other members of the tumor necrosis factor superfamily, 4–1BB ligand has been shown to signal both through a cellular receptor and to signal the cell originally expressing the ligand (44). Human 4–1BB ligand is expressed on several cell types including antigen-presenting cells and most recently on several human carcinoma cell lines (44). Reverse signaling of 4–1BB results in apoptosis, decreased cell proliferation, and increased expression of Fas on lymphocyte interleukin 8 by macrophages (44). Interleukin 8 is a potent neutrophil and basophil chemoattractant involved in the acute immune response and is produced in colonic, bronchial epithelial, and gastric carcinoma cells (44). Cancer cell lines including ovarian, colon, and lung have all been reported to express functional 4–1BB ligand and produce interleukin 8 in response to 4–1 BB immunoglobulin (44). This suggests a likely role for 4–1BB ligand in tumor/T-cell interaction that has yet to be clarified.

Transforming Growth Factor β -Induced. Microarray analysis revealed a 2.1-fold decrease in transforming growth factor β (TGF- β) expression, a finding confirmed by quantitative PCR (Fig. 5C). TGF- β functions as both a potent inducer of angiogenesis and inhibitor of immune function (45). Furthermore, TGF- β has been reported to promote tumor growth by inhibition of tumor immunity (45). The role in lung carcinomas is less defined than in other malignancies such as breast, hepatocellular, and prostate. In lung cancer cell lines TGF- β is highly expressed, and plasma TGF- β levels may be a potential tumor marker (46).

Peroxisome Proliferator Activated Receptor γ . PPAR γ is a subtype of ligand-activated transcription factors that have been identified recently as potential targets for COX-independent mechanisms of nonsteroidal anti-inflammatory and antitumorigenic effects (47). PPAR γ has a direct role in adipocyte differentiation and is expressed in the lung, colon, ovary, and breast (48). PPAR γ receptor activation by established ligands, such as synthetic thiazolidinediones, results in growth arrest in human lung adenocarcinoma cell lines (A549; Ref. 48). In the present study, prostacyclin synthase overexpressing mice exhibited a near 2-fold induction in PPAR γ gene expression (confirmed by quantitative PCR; Fig. 5A) suggesting that the antitumorigenic properties of prostacyclin synthase overexpression may be partially mediated through PPAR γ activation.

Cell Cycle Control

Jun. The proto-oncogene cJun is a constituent of the AP-1 transcription factor and is involved in the control of cellular growth and differentiation (49). Increased cJun expression is observed early in human lung carcinogenesis and may possibly serve to mediate growth factor signaling in non-small cell lung cancer (49). Down-regulation of cJun has been associated with improved survival in non-small cell lung cancer patients (50).

In summary, FVB/N mice with pulmonary-specific prostacyclin synthase overexpression are chemoprotected from developing lung tumors in a smoke-exposure model. The transgenics exhibit significant decreases in both tumor multiplicity and incidence. The animals also develop a pathological lesion (terminal bronchiolitis) that is similar to a smoke-induced human lung disease (respiratory bronchiolitis). Gene expression analysis defines a protective milieu induced in the lungs by overexpression of prostacyclin synthase and suggests potential targets that warrant additional investigation.

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