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Antitumorigenic Effects of Peroxisome Proliferator-Activated Receptor-γ in Non–Small-Cell Lung Cancer Cells Are Mediated by Suppression of Cyclooxygenase-2 via Inhibition of Nuclear Factor-κB[S]

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ABSTRACT

Pharmacological activators of peroxisome proliferator-activated receptor-γ (PPARγ) inhibit growth of non–small-cell lung cancer (NSCLC) cell lines in vitro and in xenograft models. Because these agents engage off-target pathways, we have assessed the effects of PPARγ by overexpressing the protein in NSCLC cells. We reported previously that increased PPARγ inhibits transformed growth and invasiveness and promotes epithelial differentiation in a panel of NSCLC expressing oncogenic K-Ras. These cells express high levels of cyclooxygenase-2 (COX-2) and produce high levels of prostaglandin E_2 (PGE_2). The goal of these studies was to identify the molecular mechanisms whereby PPARγ inhibits tumorigenesis. Increased PPARγ inhibited expression of COX-2 protein and promoter activity, resulting in decreased PGE_2 production. Suppression of COX-2 was mediated through increased activity of the tumor suppressor phosphatase and tensin homolog, leading to decreased levels of phospho-Akt and inhibition of nuclear factor-κB activity. Pharmacological inhibition of PGE_2 production mimicked the effects of PPARγ on epithelial differentiation in three-dimensional culture, and exogenous PGE_2 reversed the effects of increased PPARγ activity. Transgenic mice overexpressing PPARγ under the control of the surfactant protein C promoter had reduced expression of COX-2 in type II cells and were protected against developing lung tumors in a chemical carcinogenesis model. These data indicate that high levels of PGE_2 as a result of elevated COX-2 expression are critical for promoting lung tumorigenesis and that the antitumorigenic effects of PPARγ are mediated in part through blocking this pathway.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear-hormone-receptor superfamily and was initially demonstrated to have important roles in lipid metabolism and adipose differentiation (Berger and Moller, 2002). PPARγ binds as a heterodimer with the retinoic acid X receptor. These heterodimers, complexed with either coactivators or corepressors, bind to specific PPAR response elements in the promoter regions of their target genes. PPARγ is activated by polyunsaturated fatty acids and eicosanoids and by the class of thiazolidinediones (TZDs). Activation of individual PPAR isoforms has been implicated in many types of cancer, including breast, colon, and prostate (Feige et al., 2006). In lung cancer, several studies, including work from our laboratory, have demonstrated that pharmacological activation of PPARγ can inhibit growth of lung cancer cells (Wick et al., 2002). In samples from human lung tumors, decreased expression of PPARγ was correlated with poor prognosis (Sasaki et al., 2002). In a separate study, well-differentiated adenocarcinomas exhibited a greater

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ABBREVIATIONS: PPARγ, peroxisome proliferator-activated receptor-γ; NSCLC, nonsmall cell lung cancer; TZD, thiazolinedione; COX-2, cyclooxygenase-2; PGE_2, prostaglandin E_2; NF-κB, nuclear factor κB; PTEN, phosphatase and tensin homolog; AA, arachidonic acid; mPGES, microsomal prostaglandin E_2 synthase; IKK, IκB kinase complex; DMSO, dimethyl sulfoxide; 3-D, three dimensional; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; ELISA, enzyme-linked immunosorbent assay; bp, base pair; IL, interleukin; DN, dominant-negative; Luc, luciferase; PGDH, 15-hydroxyprostaglandin dehydrogenase; β-gal, β-galactosidase; LNCX, empty vector; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PI-3, phosphatidylinositol-3; T007, T0070907, 2-chloro-5-nitro-*N*-4-pyridinyl-benzamide; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride.

frequency of PPARγ-positive cells compared with poorly differentiated samples (Theocharis et al., 2002). Prostaglandins are a family of bioactive lipid mediators, which are derived through metabolism of arachidonic acid (AA). Release of AA from membranes is mediated through the action of phospholipase A_2, primarily the cytosolic intracellular form designated as $cPLA_2$. Cyclooxygenases (COXs) convert free AA to prostaglandin H, which is the precursor for other prostaglandins and thromboxane. Two isoforms of COX have been described. COX-1 is constitutively expressed in most cell types and is believed to be responsible for prostaglandin production involved in maintenance of vascular tone. COX-2, normally expressed at low or undetectable levels in most cells, is rapidly induced by mitogenic or inflammatory signals through transcriptional activation. Conversion of prostaglandin H to downstream eicosanoids is mediated by the expression of specific terminal synthases. In non–small-cell lung cancer (NSCLC), the major eicosanoid produced is prostaglandin E_2 (PGE_2) produced through microsomal PGE_2 synthase (mPGES). A large body of evidence indicates that increased prostaglandin production contributes to tumorigenesis. COX-2 has been shown to be constitutively upregulated in various cancers, including colon, breast, and lung, and targeted overexpression of COX-2 is sufficient to cause mammary tumorigenesis in transgenic mice (Liu et al., 2001). Elevated levels of PGE_2 are found in lung cancer tissue compared with normal lung tissue, with consistently higher production in adenocarcinomas (McLemore et al., 1988). Constitutively high levels of PGE_2 production are observed in a subset of NSCLC and correlate with expression of oncogenic K-Ras (Heasley et al., 1997). This is a consequence of expression of $cPLA_2$ and COX-2. nonsteroidal antiinflammatory drugs, which inhibit COX isoforms, block the growth of cancer cells in vitro and inhibit tumor growth in vivo (Heasley et al., 1997; Hida et al., 1998). The mechanisms whereby COX-2-derived PGE_2 promotes tumorigenesis and progression are not well understood but may involve stimulating growth, preventing apoptosis, increasing cell motility and adhesion, inducing angiogenesis, and inhibiting immune surveillance.

Because TZDs have been reported to engage off-target effectors, it has been difficult to define the specific pathways regulated by PPARγ. We have used a molecular approach to overexpress PPARγ in NSCLC, resulting in levels of activity comparable with stimulation of untransfected cells with TZDs (Bren-Mattison et al., 2005). Overexpression of PPARγ in human NSCLC cell lines did not have a major effect on cell proliferation but had selective inhibitory effects on anchorage-independent growth and metastasis and promoted epithelial differentiation in three-dimensional cultures (Bren-Mattison et al., 2005). However, the mechanisms underlying these effects have not been identified. The goal of the present study was to determine whether the antitumorigenic effects of PPARγ were mediated through regulation of the COX-2 pathway in vivo and in NSCLC. We report here that increased PPARγ activity inhibits COX-2 expression through inhibition of Akt signaling, resulting in reduced NF-κB activity.

Materials and Methods

Materials. T0070907 (PPARγ inhibitor) was from Cayman Chemical Co. (Ann Arbor, MI). Sulindac sulfide and IKK-inhibitor VII were from Calbiochem (San Diego, CA). NF-κB assay kit (TransAM NF-κB Chemi kit) was from Active Motif (Carlsbad, CA).

Cell Culture and Three-Dimensional Culture. H2122 and A549 cells, both human lung adenocarcinoma cell lines, were obtained from the University of Colorado Health Science Center Tissue Culture Core. Cells were maintained in RPMI containing 10% fetal bovine serum. H2122-LNCX and H2122-PPARγ stable clones have been described previously (Wick et al., 2002). Cells were grown in three-dimensional basement membrane cultures according to Debnath et al. (2003) with the following modifications: growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) was combined in a 1:1 ratio with full serum medium (RPMI + 10% fetal calf serum). Eighty microliters was added to each well of an eight-well glass slide chamber (LAB-TEK; Nalge Nunc International, Rochester, NY) and allowed to solidify for 2 h in a 37°C incubator. Cells were trypsinized, counted, and diluted to 25,000 cells/ml. A 20% Matrigel solution was prepared in full serum medium. The cell suspension was combined in a 1:1 ratio with the 20% Matrigel solution, and 200 μl of this mixture was added to each well for a final concentration of 5000 cells/well in 10% Matrigel. Cells were fed with 4% Matrigel (in full serum medium) every other day for a term of 8 to 10 days of culture. For experiments with exogenous PGE_2, cells were incubated with 5 μM; this higher concentration was chosen for longer experiments. NSCLC express 15-hydroxyprostaglandin dehydrogenase (PGDH), which rapidly degrades PGE_2 (Hazra et al., 2007). A similar range of concentrations has been used by other investigators (Huang and Cabot, 1990; Casibang et al., 2001), even for acute stimulation of lung cancer cells. Control cells received vehicle (0.1% DMSO).

Promoter Transfections. Cells were plated in 60-mm dishes at approximately 600,000 cells per dish. Promoter transfections were performed using 1 μg each of the indicated luciferase promoter construct and CMV-β-gal using Lipofectamine (Invitrogen, Carlsbad, CA) as described previously (Wick et al., 2002). Total DNA content was matched for each sample using the appropriate empty vector. Cells were harvested in reporter lysis buffer (Promega, Madison, WI), and luciferase normalized to β-galactosidase was determined.

Indirect Immunofluorescence. Cells grown in 3-D culture were immunostained as described previously with some modifications (Debnath et al., 2003). In brief, cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized with 0.5% Triton X-100, and rinsed three times with 100 mM glycine. The cells were then incubated in primary block (7.7 mM NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20, and 10% goat serum in PBS) for 1 h at room temperature. After a 30-min incubation in secondary block (primary block plus 20 μg/ml goat anti-mouse $F(ab')_2$ fragment; Jackson ImmunoResearch, West Grove, PA), cells were incubated with primary antibodies, cleaved Caspase-3 (at 1:50; Cell Signaling Technology, Danvers, MA), and E-cadherin (at 1:100; BD Biosciences) overnight at room temperature. Cells were rinsed three times before incubation with secondary antibodies (Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, and Alexa Fluor 568 goat anti-rabbit, all used at 1:250; Invitrogen). Cells were mounted using Vectashield fluorescent mount medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). At least 20 aggregate structures were examined in each of three independent experiments; representative images are shown. Structures grown in 3-D matrices were imaged and acquired on a Zeiss 510 Meta NLO confocal microscope (Carl Zeiss Microimaging, Thornwood, NY) and analyzed using Zeiss Image Examiner software.

Immunoblotting. Cells grown in two-dimensional culture were lysed in ice-cold RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS) containing protease inhibitors, centrifuged at 10,000*g* for 5 min, and the supernatant was saved. Cells grown in 3-D Matrigel were first isolated using BD Cell Recovery Solution (BD Biosciences) according to the manufacturer's instructions. This involves dissociation of the Matrigel matrix by enzymatic digestion with Dispase. These cells were then processed as the cells grown in two-dimensional culture. Pro-

tein was quantitated using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA). Protein samples were run on precast 4 to 20% Gradient Tris-HCl gels (Bio-Rad Laboratories). Antibodies against COX-2 and PPARγ were from Cayman Chemical. Antibodies against Akt were from Cell Signaling. Blots were visualized by enhanced chemiluminescence, and changes in expression normalized to β-actin expression were determined by densitometry using at least three independent experiments.

Prostaglandin E_2 Assay. PGE_2 levels were measured by ELISA as per the manufacturer's instructions (Prostaglandin E_2 EIA Kit-Monoclonal; Cayman Chemical). In brief, cells were grown in 10- cm plates to 90% confluence. Medium was removed, plates were washed with Hanks' buffered saline, and new medium was added. Medium (1 ml) was immediately removed and assayed for PGE_2 levels to establish the "zero" time point. One hour later, a second 1-ml aliquot of medium was removed and assayed for PGE_2 levels. All samples were analyzed according to the manufacturer's recommended protocol.

PTEN Activity Measurements. PTEN was immunoprecipitated from cell lysates using anti-PTEN antibody from Cell Signaling Technology (Danvers, MA) and Protein A-Sepharose. Activity was measured in the immunoprecipitate using PIP_3 as a substrate for 45 min at 37°C by following the procedure described in the Echelon kit, and phosphate generated in the supernatant was quantified using the generic phosphatase assay kit from Echelon (Salt Lake City, UT).

Construction of SP-C/PPARγ Transgene. The 3.7 hSP-C/SV-40 plasmid was a generous gift from Dr. Jeffrey A. Whitsett (Children's Hospital Medical Center, Cincinnati, OH). This pUC 18 plasmid contains the 3.7-kilobase flanking sequence of the human SP-C promoter in addition to the SV-40 small T intron as a polyadenylation signal. The full-length human PPARγ1 cDNA was cut and cloned into the SalI/EcoRI site of the hSP-C/SV-40 plasmid by blunt end ligation, creating the SP-C promoter-PPARγ cDNA fusion gene. The proper cloning orientation of our construct was confirmed by direct sequence analysis. Transgenic mice were developed in an FVB/N strain by pronuclear injections of the linearized construct. Transgenic mice were genotyped by analyzing genomic DNA isolated from tail biopsies with the DNeasy Tissue Kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA). Polymerase chain reaction was performed on genomic DNA using primers spanning the 400-bp segment of the SV-40 small T intron to detect the presence of the transgene (sense primer, 5′-TGTGAAGGAACCTTACTTCTGTGG-3′; antisense primer, 5′-TGGACAAACCACAACTAGAATGCAC-3′). All mice were propagated as heterozygous transgenic mice by breeding with wild-type FVB/N mice.

Mouse Carcinogenesis Protocols. FVB/N mice 8 to 12 weeks of age were maintained on a standard, antioxidant-free laboratory chow (Lab Diet; PMI Nutrition International, St. Louis, MO) and given food and water ad libitum. Animals were kept on cedar-free bedding with a 12-h light/dark cycle in a climate-controlled animal facility. A single urethane (Sigma Chemical Co., St. Louis, MO) dose (1 mg/g mouse weight), dissolved in normal saline, was administered intraperitoneally, and animals were sacrificed 20 weeks later. Tumors were enumerated in fresh lungs under a dissection microscope. All tumors were dissected from the lung parenchyma. The diameter of individual tumors was measured using digital calipers. The genetic identity of the mice ($PPAR\gamma^-$ or $PPAR\gamma^+$) was not revealed until after tumor multiplicities and sizes were determined. All procedures were performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Health Sciences Center.

Type II Cell Isolation. Type II pneumocytes were isolated from untreated transgenic or wild-type mice as described previously (Corti et al., 1996). In brief, mice were anesthetized with intraperitoneal injection of phenobarbital (200 μl). The abdominal cavity was opened, and mice were exsanguinated by severing the renal artery. The trachea was isolated and cannulated with a 20-gauge Luer lock cannula. The diaphragm was cut, and the anterior chest wall and thymus were removed. Lungs were perfused with 10 ml of 0.9% saline via the pulmonary artery using a 21-gauge needle fitted on a 10-ml syringe. Dispase (3 ml) was rapidly instilled through the trachea cannula followed by 0.5 ml of 45°C agarose. The lungs were covered with ice for 2 min to harden the agarose. After this incubation, the lungs were dissected out from the animals and incubated in a culture tube with 2 ml of dispase for 45 min at room temperature. Lungs were then transferred to a 60-mm culture dish containing 7 ml of HEPES-buffered Dulbecco's modified Eagle's medium and 100 U/ml DNaseI; lung tissue was gently teased apart and minced until only connective tissue was visible, and the cell suspension was filtered through progressively smaller cell strainers (100 and 40 μm) and nylon gauze (20 μm). Cells were collected by centrifugation at 130*g* for 8 min at 4°C, placed on prewashed 100-mm culture dishes that had been precoated with 42 mg of anti-CD45 antibody and 16 mg of anti-CD32 antibody in PBS for 24 to 48 h at 4°C, and incubated for 1.5 h at 37°C. The medium containing type II cells was gently removed from the plates, and the cells were collected by centrifugation. Cell pellets were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, and protease inhibitors), centrifuged at 10,000*g* for 10 min, and matched for protein. For enzyme immunoassay and liquid chromatography/mass spectrometry analysis of PGE_2 levels, the collected cells were washed several times with 1× PBS. The cells were then suspended in 1 ml of complete medium (Dulbecco's modified Eagle's medium plus 10% fetal calf serum) and incubated at 37°C for 30 min. The medium was collected and centrifuged at 10,000*g* for 10 min. The supernatant was diluted 1:3 in methanol for analysis of PGE2 levels, and the cell pellets were processed for immunoblotting as described above.

Results

PPARγ Inhibits COX-2 Expression and PGE_2 Production in NSCLC. H2122 cells are from a human adenocarcinoma cell line, and they express elevated levels of COX-2 and produce high levels of PGE_2 (Heasley et al., 1997). We have shown previously that increased PPARγ activity promotes epithelial differentiation of these cells in 3-D culture (Bren-Mattison et al., 2005). To examine whether these effects of PPARγ are mediated through changes in prostaglandin pathways, we compared the expression of enzymes in this pathway in H2122 cells stably overexpressing PPARγ (H2122-PPARγ) or control cells transfected with empty vector (H2122-LNCX). Expression of $cPLA_2$ and COX-2 was significantly decreased in H2122-PPARγ cells compared with H2122-LNCX cells (Fig. 1A) or untransfected H2122 cells (data not shown); mPGES levels were not significantly changed. Densitometry of three independent experiments showed a 50% decrease in $cPLA_2$ expression and a 72% reduction in COX-2 expression. We have also performed expression profiling of these cells using Affymetrix microarrays. Both $cPLA_2$ and COX-2 were called as "decreased" on the arrays ($cPLA_2$ by 60% and COX-2 by 75%), whereas expression of mPGES was predicted to be moderately increased (20%; data not shown). Consistent with these data, H2122-PPARγ cells exhibited a marked decrease in production of PGE_2 compared with H2122-LNCX cells (Fig. 1B). This effect was reversed by a specific pharmacological inhibitor of PPARγ, T0070907 (T007) (Lee et al., 2002). We tested whether the effects of PPARγ on COX-2 were mediated at the transcriptional level by using a COX-2-luciferase (COX-2-Luc) reporter construct that contains approximately 500 bp of the human COX-2 promoter ligated to a luciferase reporter. Activity of this promoter construct is strongly induced by oncogenic Ras in lung epithelial cells (Van Putten et al., 2001). PPARγ-H2122 showed a 70% decrease in promoter activity compared with control cells (Fig. 1C). This

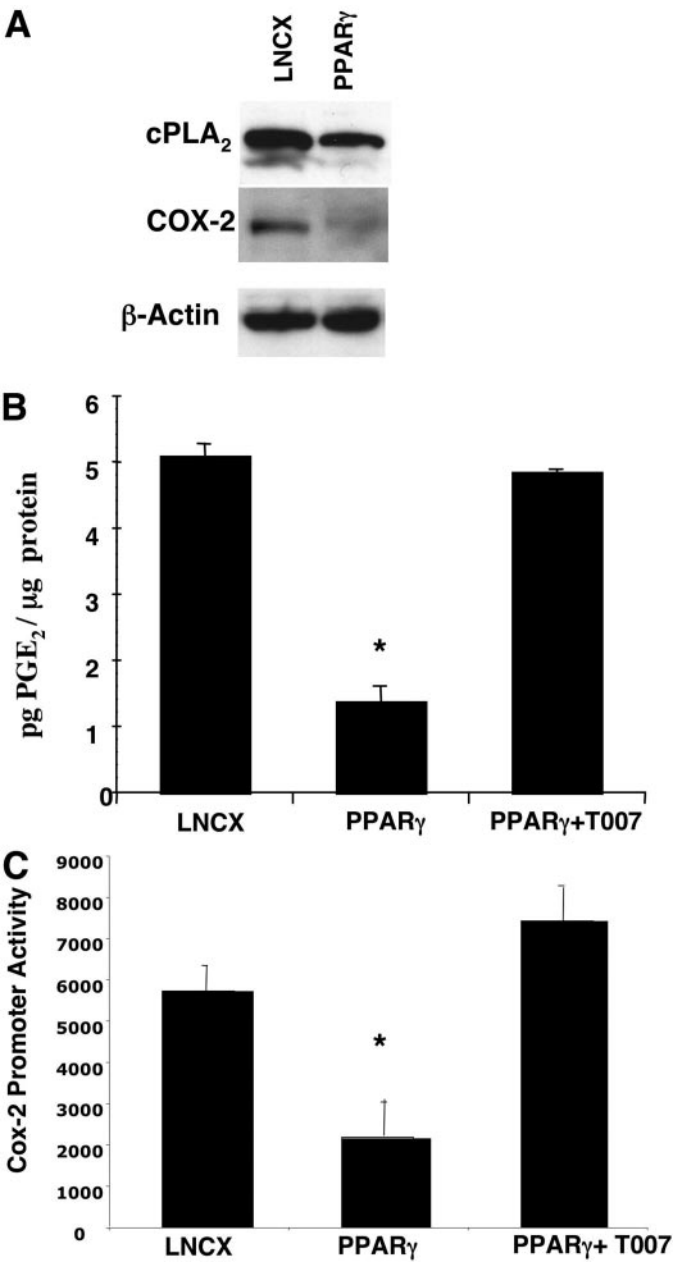

Fig. 1. Effect of PPARγ on COX-2 expression and PGE_2 production. H2122-LNCX (LNCX) or H2122-PPARγ (PPARγ) cells were grown under standard conditions. A, cell lysates were prepared in RIPA buffer, and equal amounts of protein were immunoblotted for $cPLA_2$, COX-2, and mPGES. Levels of both COX-2 and $cPLA_2$ were decreased in H2122-PPARγ cells. Data shown are a representative blot of three independent experiments. B, conditioned medium from 90% confluent dishes of H2122-LNCX or H2122-PPARγ treated overnight with either vehicle or 10 μM T007 was collected after a 1-h incubation and assayed for PGE_2 production by ELISA. The cells on the dish were harvested, and total protein was determined. PGE_2 production was normalized to cell protein. Results represent the mean of three independent experiments with the S.E.M. indicated. C, cells were transfected with a construct encoding 500 bp of the human COX-2 promoter linked to a luciferase reporter along with a construct encoding β-galactosidase under the control of the CMV promoter to normalize for transfection efficiency. Five hours after transfection, cells were treated with 10 μM T007 or vehicle (DMSO); final concentration, 0.1%. Cells were harvested after 24 h, and luciferase activity normalized to β-gal was determined. Results represent the mean of three independent experiments with the S.E.M. indicated. $*$, $P < 0.05$ versus LNCX.

inhibition of COX-2 promoter activity was also completely reversed by the PPARγ inhibitor. Similar results were found in a second NSCLC adenocarcinoma cell line, A549, which also expresses high levels of COX-2 (Heasley et al., 1997) (Fig. 5).

Effects of PPARγ on Epithelial Differentiation in Three-Dimensional Culture Are Mediated by Inhibition of PGE_2. We have shown previously that PPARγ promotes an epithelial-like cell morphology in NSCLC when grown in three-dimensional Matrigel culture (Bren-Mattison et al., 2005). Nontransformed epithelial cells form hollow, polarized acinar structures, in which cells not in contact with the matrix undergo apoptosis (Debnath et al., 2003). To determine the role of PGE_2 production in this process, cells grown in 3-D culture were exposed to sulindac sulfide, a COX inhibitor. The concentration of sulindac sulfide used in these experiments (5 μM) inhibits PGE_2 production more than 90% but does not activate PPARγ (Wick et al., 2002) (Supplementary Fig. S1). Polarized acinar structures were defined by positive E-cadherin staining in the basolateral membrane, and luminal cell death was indicated by positive cleaved caspase 3 staining. Consistent with our previous findings (Bren-Mattison et al., 2005), H2122-LNCX cells formed disorganized aggregates, (Fig. 2, A and E), whereas H2122-PPARγ cells form ordered structures (Fig. 2, B and F). In contrast to nontransformed epithelia, cells in the center do not undergo apoptosis, suggesting that although these cells are more differentiated, they are still structurally distinct from nontransformed epithelial cells. Exposure of H2122-LNCX to low concentrations of sulindac sulfide resulted in the formation of ordered acinar structures similar to those seen in H2122-PPARγ cells (Fig. 3, C and G). In fact, regions in the center of the structures stained positive for activated caspase 3 (red color in 3G). On the other hand, addition of exogenous PGE_2 (5 μM) to H2122-PPARγ cells caused these cells to revert to a disordered aggregate (Fig. 2, D and H), similar to what is seen in H2122-LNCX cells. These data strongly suggest that inhibition of PGE_2 production is critical for the formation of differentiated structures in 3-D by PPARγ.

PPARγ Inhibits COX-2 Expression via an NF-κB-Dependent Mechanism. Several regions of the COX-2 promoter have been shown to be critical for regulation, including cAMP response element, NF-IL-6, and NF-κB consensus binding sites (Reddy et al., 2000). Because previous reports have demonstrated the effects of PPARγ on NF-κB (Keshamouni et al., 2005), we examined whether regulation of COX-2 expression in H2122 cells was mediated through the control of NF-κB. The effects of PPARγ on NF-κB were assessed using a plasmid containing three consensus NF-κB binding sights upstream of a luciferase reporter. Overexpression of PPARγ in H2122 cells decreased NF-κB activity by approximately 50% (Fig. 3A). This decrease was reversed by exposure to the PPARγ inhibitor T007. Changes in NF-κB activity were confirmed by quantitation of nuclear NF-κB protein by ELISA (see *Materials and Methods*). Nuclear extracts were prepared from H2122-PPARγ and H2122-LNCX cells and NF-κB binding quantitated using the TransAM NF-κB Chemiluminescence kit from Active Motif. Nuclear NF-κB activity was decreased by approximately 70% in H2122-PPARγ cells compared with N2122-LNCX (Fig. 3B).

To directly assess the role of PPARγ on NF-κB activity in

control of the COX-2 promoter, H2122 cells were cotransfected with the COX-2 promoter construct along with an expression plasmid encoding a dominant-negative IκB (DN-IκB), in which the amino terminal containing regulatory phosphorylation sites has been deleted. Expression of DN-IκB decreased NF-κB activity by more than 90% (Fig. 4A, left). It is noteworthy that COX-2 promoter activity was decreased by 50% in the presence of DN-IκB (Fig. 4A, right). These findings were confirmed using a pharmacological inhibitor of NF-κB, which acts by inhibiting Iκ-B kinase, IKK (IKK inhibitor VII; Calbiochem). Exposure of H2122 cells to this agent decreased NF-κB activity in a dose-dependent manner, with an IC_{50} value of less than 5 μM (Fig. 4B). At a concentration of drug that inhibited NF-κB activity by ~70%, COX-2 protein expression was decreased by approximately 70% (Fig. 4C). Thus, regulation of NF-κB is a dominant signaling pathway controlling the expression of COX-2 in H2122 cells. We confirmed the regulation of NF-κB in a second NSCLC line, A549. Similar to what was observed in H2122 cells, overexpression of PPARγ decreased NF-κB activity (Fig. 5A) and decreased COX-2 promoter activity (Fig. 5B).

PPARγ Effects on NF-κB and COX-2 Are Mediated through Increased Activity of PTEN and Inhibition of Phospho-Akt. Several studies have demonstrated that constitutive activation of NF-κB in tumor cells is mediated through activation of Akt, although the precise mechanisms have not been well defined (Romashkova and Makarov, 1999;

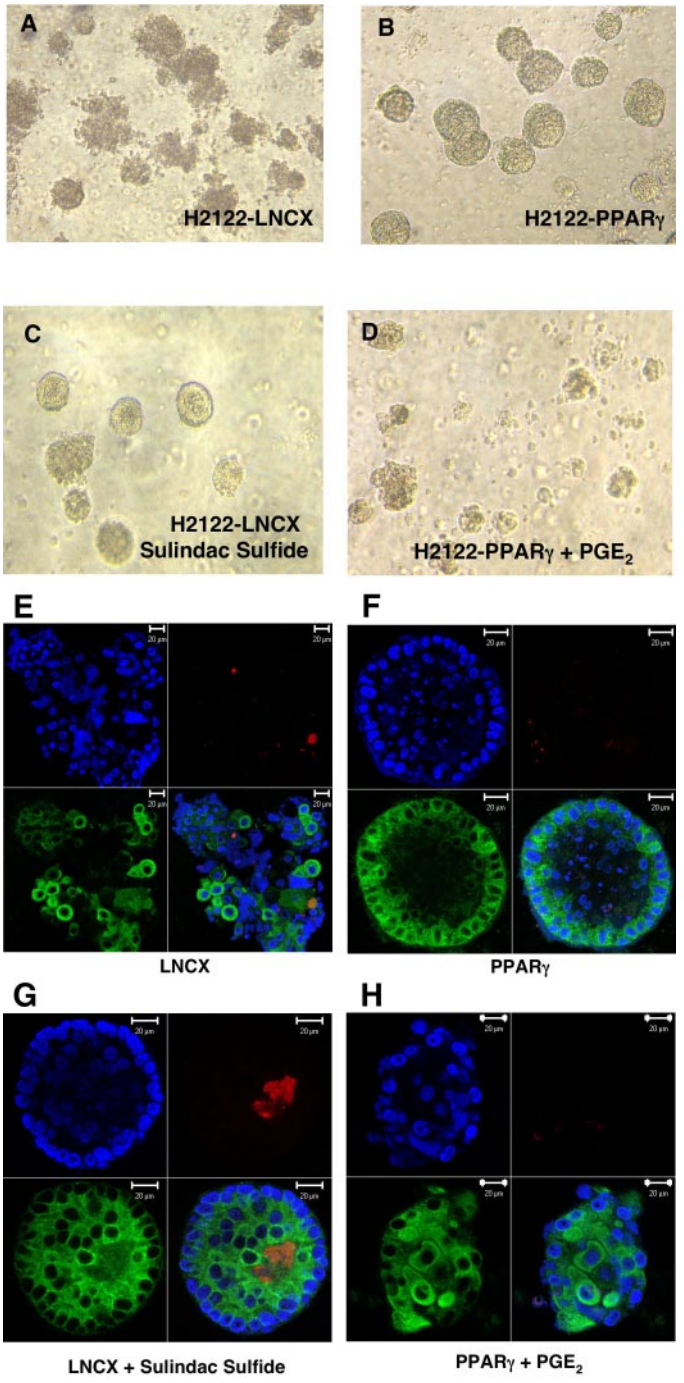

Fig. 2. PGE_2 reverses the effects of PPARγ in 3-D culture. H2122-LNCX or H2122-PPARγ cells were grown in 3-D Matrigel culture as described under *Materials and Methods* for 8 to 10 days. For cells treated with either sulindac sulfide or PGE_2, drugs were added to the Matrigel at the time of plating (5 μM of each drug) and were replaced with each feeding of the cells. Cells were visualized under phase at low power (A–D). Cells were then fixed and stained for E-cadherin expression (green), activated caspase (red), or 4,6-diamidino-2-phenylindole (blue) as described under *Materials and Methods*. Representative acinar structures are shown in E to H. H2122-PPARγ (B and F) cells formed regular acinar structures compared with H2122-LNCX(A and E). Sulindac sulfide caused LNCX cells to adopt a more highly differentiated morphology (C and G). On the other hand, the addition of exogenous PGE_2 caused PPARγ cells to adopt a more dedifferentiated morphology.

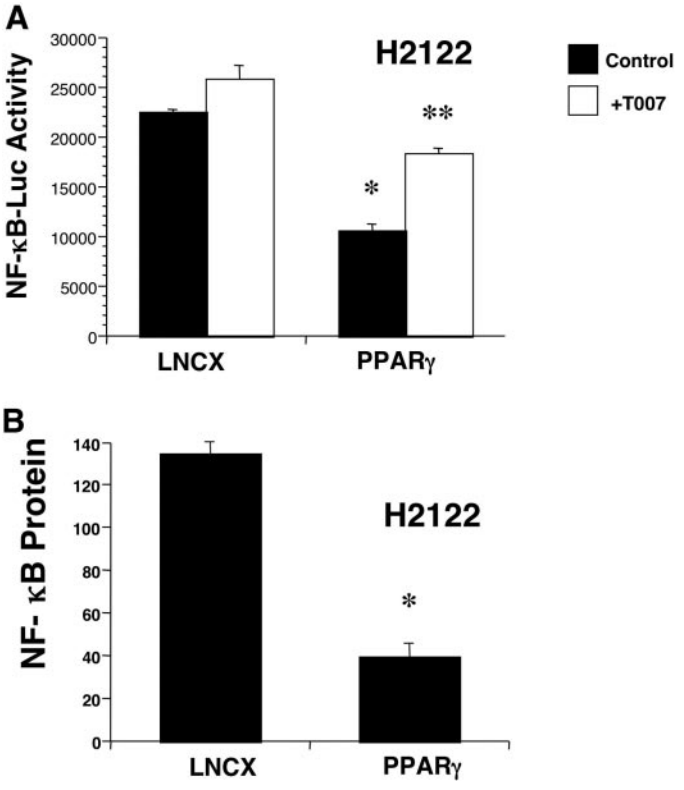

Fig. 3. PPARγ inhibits NF-κB activity. A, H2122-LNCX and H2122-PPARγ cells were transiently transfected with an NF-κB-luciferase construct along with CMV-β-gal to normalize for transfection efficiency. Cells were then treated with 10 μM T007 or vehicle and incubated for 24 h. Extracts were assayed for luciferase and β-gal activity. Luciferase activity was decreased in H2122-PPARγ cells, and this effect was blocked in the presence of the PPARγ inhibitor. Results represent the mean of four independent experiments. B, NF-κB activity was assayed in H2122-LNCX and H2122-PPARγ cells by ELISA. Nuclear extracts were prepared from each cell type using the Active Motif nuclear extract kit and assayed for activity with the TransAM NF-κB chemiluminescence kit from Active Motif. Results represent the mean of two independent experiments. *, $P < 0.05$ versus LNCX cells.

Sizemore et al., 1999; Madrid et al., 2000). We therefore examined the role of Akt in mediating the effects of PPARγ. NSCLC expressing oncogenic K-Ras mutations had constitutively high levels of activated phospho-Akt (p-Akt). In H2122 cells, overexpression of PPARγ resulted in marked inhibition of steady state p-Akt levels, with no change in total Akt levels (Fig. 6A). Treatment of cells with LY294002 (10 μM), a PI-3 kinase inhibitor, decreased levels of p-Akt (data not shown). In H2122-LNCX cells, LY294002 reduced NF-κB-Luc activity by approximately 50%, comparable with activity seen in H2122-PPARγ cells (Fig. 6B); LY294002 had no further inhibitory effect in H2122-PPARγ cells.

Decreased phospho-Akt could be a result of inhibiting PI-3 kinase activity or increasing the activity of PTEN, which dephosphorylated PIP_3, opposing the actions of PI-3 kinase. Because several reports have indicated that PTEN may be a downstream effector of PPARγ (Lee et al., 2006; Teresi et al., 2006), we assessed the effects of PPARγ on PTEN activity in H2122 cells. No consistent effect of PPARγ on PTEN expression was detected by immunoblotting. However, PTEN enzymatic activity as measured by hydrolysis of PIP_3 was significantly increased in the H2122-PPARγ cells compared with H2122-LNCX (Fig. 6C). We propose that decreased phospho-Akt levels in H2122-PPARγ cells is a result of lower PIP_3 levels secondary to increased PTEN activity.

Transgenic Mice Overexpressing PPARγ Have Decreased COX-2 Expression and Are Protected against Developing Lung Tumors. To test the relationship between PPARγ and COX-2 expression in vivo, we developed transgenic mice with targeted overexpression of PPARγ in the type II alveolar epithelial cells of the lung, using the surfactant protein C promoter (Keith et al., 2002). Expression of PPARγ in type II cells isolated from Tg+ animals was markedly increased compared with wild-type litter mates (Fig. 7A). Consistent with our observations in H2122 cells, increased PPARγ expression was correlated with decreased expression of COX-2 in these cells (Fig. 7A). The Tg+ mice seemed to have normal lung architecture and did not display any other abnormalities (data not shown). The role of in-

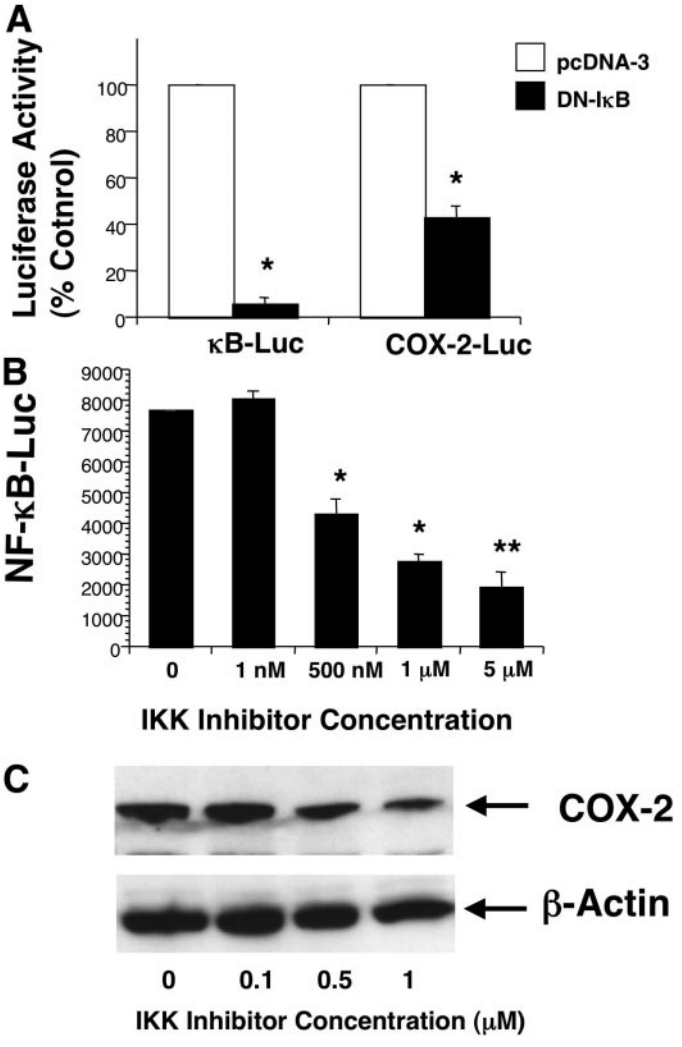

Fig. 4. PPARγ inhibition of NF-κB blocks COX-2 expression in NSCLC. A, H2122 cells were transiently transfected with either the NF-κB-Luc reporter (left bars) or the COX-2-promoter construct (right bars) along with an expression plasmid encoding DN-IκB or empty vector (pcDNA-3). B, H2122 cells were transfected with the NF-κB-luciferase reporter. Five hours after transfections, cells were exposed to the indicated concentration of the IKK inhibitor. After 24 h, cells were harvested, and luciferase activity normalized to β-gal was determined. Results for both A and B represent the mean of three independent experiments with the S.E.M. indicated. *, $P < 0.05$ versus no drug; **, $P < 0.01$ versus no drug. C, H2122 cells were exposed for 24 h to the indicated concentration of the IKK inhibitor or vehicle (0.1% DMSO). Cell lysates were prepared and immunoblotted for COX-2 expression. Lysates were blotted for β-actin as a loading control. Results are representative of three independent experiments.

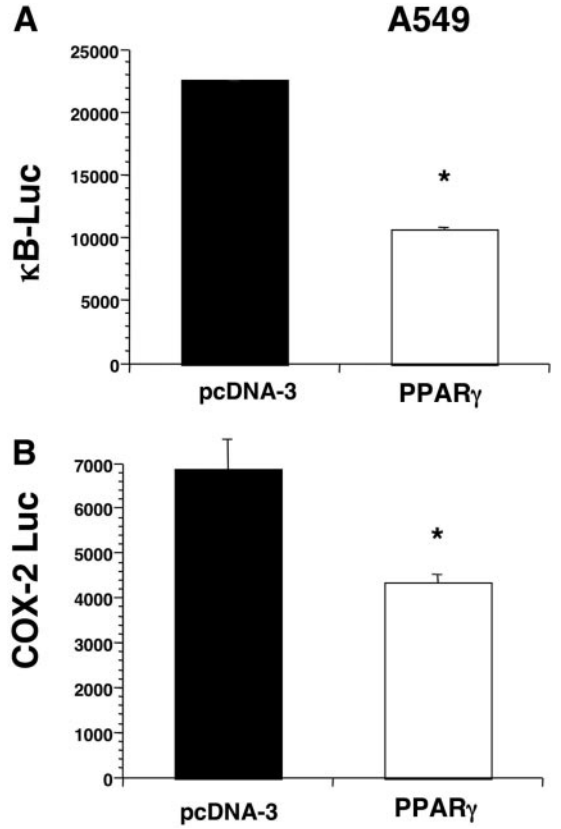

Fig. 5. PPARγ expression inhibits NF-κB activity and COX-2 promoter in A549 cells. A549 cells were transiently transfected with either the NF-κB-Luc reporter (A) or the COX-2-promoter construct (B) along with either an expression plasmid encoding full-length PPARγ or a control plasmid (pcDNA-3). Expression of PPARγ decreased both NF-κB-Luc and COX-2 Luc activity. All experiments represent the mean of at least three independent experiments with the S.E.M. indicated. *, $P < 0.05$ versus pcDNA-3.

creased PPARγ in lung tumorigenesis was determined using a well-characterized chemical carcinogenesis protocol, in which mice were injected with a single dose (1g/kg body weight) of urethane, a complete carcinogen (Horio et al., 1996; Malkinson, 1998; Blaine et al., 2005). After 18 weeks, animals were sacrificed, and tumors were evaluated as described previously (Blaine et al., 2005). Tg+ animals had a 75% decrease in tumor number compared with Tg− litter mates (Fig. 7B); no difference in mean tumor size was detected (data not shown). Thus, increased *PPARγ* expression inhibits tumorigenesis and COX-2 expression both in vitro and in vivo. Examination of tumors indicated that there were no significant differences in the histology of tumors from the two groups of mice (Fig. 7C).

Discussion

Data from a number of laboratories, including ours, have demonstrated that activation of PPARγ inhibits the growth of NSCLC in vitro and in vivo (Wick et al., 2002; Bren-Mattison et al., 2005; Han and Roman, 2006). Likewise, studies over the past 10 years have shown that inhibition of prostaglandin production has similar inhibitory effects (Rigas and Kashfi, 2005). From the current studies, we propose that these two pathways are linked. Specifically, the antitumorigenic effects of PPARγ are mediated, at least in part, through decreasing production of protumorigenic prostaglandins such as PGE_2. This is mediated through decreased expression of enzymes required for PGE_2 synthesis, specifically $cPLA_2$ and COX-2. In NSCLC in vitro, this decrease in PGE_2 production is both necessary and sufficient for the increased epithelial differentiation observed in the setting of increased PPARγ activity. Pharmacological inhibition of PGE_2 production mimicked the effects of PPARγ on epithelial differentiation in 3-D culture, and on the other hand, adding exogenous PGE_2 was sufficient to reverse the effects of PPARγ on epithelial differentiation. In vivo, targeted overexpression of PPARγ to type II cells also resulted

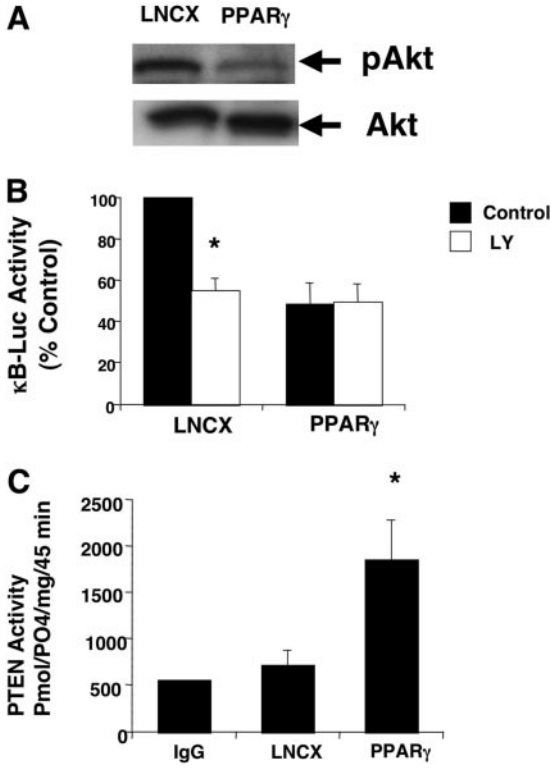

Fig. 6. PPARγ effects on NF-κB are mediated through activation of PTEN and lead to inhibition of Akt. A, cell lysates were prepared from H2122-LNCX and H2122-PPARγ cells and immunoblotted for p-Akt or total Akt. A representative blot is shown. B, H2122-LNCX and H2122-PPARγ cells were transiently transfected with the NF-κB-luciferase reporter along with CMV-β-gal encoding plasmid to normalize for transfection efficiency. After 16 h, cells were treated with 10 μM LY294402 or vehicle (0.1% DMSO) for an additional 48 h. Luciferase activity normalized to β-gal was determined. Data represent the mean of three independent experiments with the S.E.M. indicated. C, cell lysates from H2122-LNCX or H2122-PPARγ cells were immunoprecipitated with anti-PTEN antibody. Extracts were also immunoprecipitated with normal IgG as a negative control. Phosphatase activity against PIP_3 was determined in the immunoprecipitate and quantitated using a phosphatase assay kit from Echelon. Data represent the mean of two independent experiments with the S.E.M. indicated.

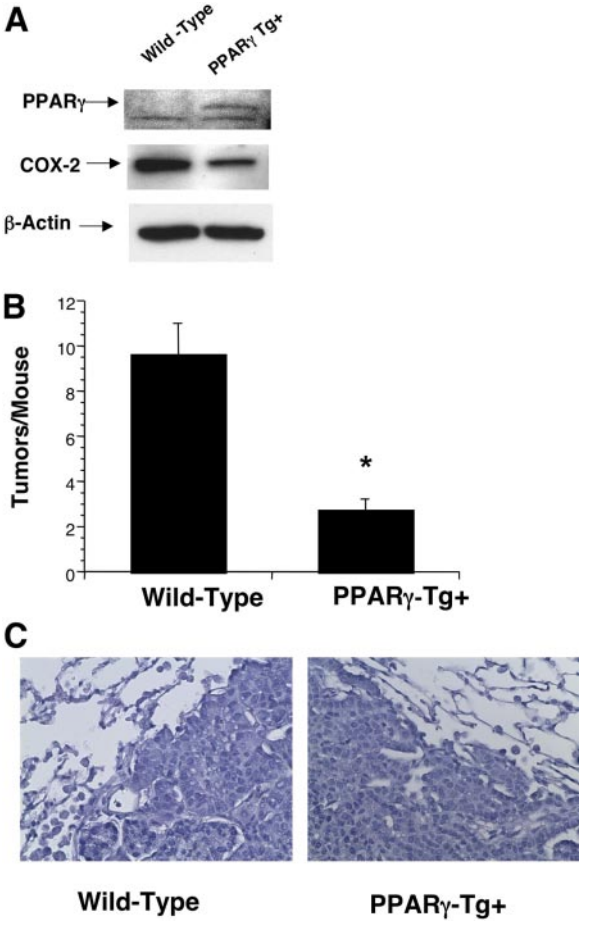

Fig. 7. Mice with targeted overexpression of PPARγ are protected against lung tumorigenesis. Transgenic mice overexpressing PPARγ under the control of the surfactant protein C promoter were developed as described under *Materials and Methods*. Mice were genotyped as described previously (Blaine et al., 2005) and propagated as heterozygotes in the FVB strain. A, type II pneumocytes were isolated from Tg+ mice and wild-type litter mates. Cell lysates were immunoblotted for PPARγ and COX-2. PPARγ expression was markedly increased in the Tg+ mice; the lower band in the immunoblot is a nonspecific band. COX-2 levels were decreased in extracts of type II cells. B, equal numbers of PPARγ-Tg+ mice and wild-type litter mates (10/group) were injected with urethane as described previously (Blaine et al., 2005). After 18 weeks, animals were sacrificed, lungs were removed, and tumor number and size were quantitated using a dissecting microscope. PPARγ-Tg+ mice had 75% fewer tumors. *, $P < 0.05$. C, sections showing urethane-induced tumors after 18 weeks at 40× magnification: right, wild-type mice; left, PPARγ-Tg+ mice.

in decreased COX-2 expression in the type II cells, and this was associated with a marked decrease in lung tumors. The level of protection observed in our study is similar to what has been observed in urethane-injected mice exposed to indomethacin (Moody et al., 2001). These data suggest that PGE_2 production is critical for the initiation of lung tumorigenesis in mice. Further support for that hypothesis comes from studies showing that $cPLA_2$-deficient mice also develop significantly fewer lung tumors in response to the same chemical carcinogenesis model (Meyer et al., 2004). However, data from our laboratory indicate that mice with targeted overexpression of mPGES fail to show alterations in lung tumor formation using the urethane model (Blaine et al., 2005). In that study, increased levels of PGE_2 were observed in the transgenic mice both under basal conditions and after urethane administration. However, wild-type mice had elevated levels of PGE_2, which was probably mediated by increased COX-2 expression without any change in mPGES. We therefore concluded that the increased PGE_2 production by mPGES is necessary but not sufficient to promote tumorigenesis. On the other hand, increased PGE_2 production mediated by COX-2 induction is sufficient to promote tumor formation, even without changes in mPGES.

Although the COX-2 promoter contains multiple regulatory elements, our data indicate that the inhibitory effects of PPARγ are mediated through NF-κB. Expression of DN-IκB decreased COX-2 promoter activity, and a pharmacological IKK inhibitor decreased COX-2 protein in these cells. Induction of COX-2 expression by growth factors is largely mediated through the cAMP response element and involves phosphorylation of c-Jun (Xie and Herschman, 1995). However, other studies have demonstrated a role for NF-κB binding in control of COX-2 transcription. In macrophages, PPARγ ligands inhibited the induction of COX-2 in response to LPS (Inoue et al., 2000), and this involved NF-κB. These effects can be mediated through PPARγ or potentially through off-target effects of these agents. However, other studies have indicated that PPARγ-mediated inhibition of COX-2 expression involves other regulatory elements, suggesting that there will be different pathways operative in a cell-specific fashion. The ability of PPARγ to inhibit NF-κB is likely to have wider implications for the biology of these cells. NF-κB has been identified as a critical transcription factor controlling production of a family of cytokines, specifically ELR + CXC cytokines such as IL-6 and IL-8. This family of cytokines has been implicated in angiogenesis and metastasis in several types of cancer, including lung cancer, through direct effects on the cancer cells, or signaling to stromal fibroblasts, endothelial cells, and innate immune cells (Strieter et al., 2004). We have observed decreased production of both IL-6 and IL-8 in NSCLC-overexpressing PPARγ and have identified several other cytokines whose expression is decreased on Affymetrix gene arrays (V. Van Putten, L. E. Heasley, and R. A. Nemenoff, unpublished observations). These data are consistent with studies using pharmacological activators of PPARγ, which also decrease production of these cytokines (Keshamouni et al., 2005). The role of individual ELR ± CXC cytokines on the transformed growth and differentiation of NSCLC and on specific tumor-stromal interactions is an area of active investigation.

Based on our data, we propose that inhibition of NF-κB is mediated through activation of PTEN and subsequent inhibition of Akt activation. Two PPARγ consensus sites have been identified approximately 10 kilobases upstream of the minimal promoter region of the PTEN gene (Patel et al., 2001), and increased PTEN expression has been observed in response to pharmacological PPARγ activators (Patel et al., 2001). Although our data do not support that PTEN is a direct transcriptional target of PPARγ, we propose that the increased PTEN activity in the setting of PPARγ activation is a critical mediator of the antitumorigenic effects of PPARγ in lung cancer cells. Further studies demonstrating direct binding of PPARγ to these elements using chromatin immunoprecipitation and mutational analysis of these regions are needed to define the precise mechanism of PTEN regulation. However, it is also possible that PPARγ may regulate PTEN activity through post-transcriptional mechanisms. PTEN activity has been reported to be regulated by phosphorylation/dephosphorylation (Gericke et al., 2006). Specifically, dephosphorylation of the protein is associated with increased enzymatic activity and decreased stability. Increased PPARγ activity could regulate PTEN activity by modifying the expression of protein kinases and/or phosphatases, which act on PTEN. Activation of PTEN and subsequent inhibition of Akt activation will also have additional effects on NSCLC. Finally, it should be noted that decreased levels of Akt activation could also be a result of inhibition of PI-3 kinase by PPARγ. Future studies using siRNA approaches will be required to better define the role of PTEN in the responses of NSCLC.

A recent study has demonstrated that pharmacological activators of PPARγ also decrease PGE_2 production in NSCLC (Hazra et al., 2007). However, these authors determined that this was not a result of alterations in COX-2 expression but rather involved induction of PGDH, the enzyme that degrades PGE_2. One important difference in these two studies is the use of pharmacological agents to activate PPARγ. These drugs have off-target effects, and thiazolidinediones have been shown to activate PGDH (Backlund et al., 2005), which would be predicted to decrease PGE_2 production. In our study, we have used a molecular approach by overexpressing PPARγ to achieve levels of activity comparable with what is achieved by thiazolidinedione stimulation (Bren-Mattison et al., 2005). Whether the effects in the study by Hazra et al. (2007) are mediated through off-target mechanisms or whether it reflects differences in the conditions used remains to be determined.

In conclusion, our data identify a link between PPARγ and COX-2, mediating the anti-tumorigenic effects of PPARγ. Furthermore, the activation of PPARγ will not only have direct effects on growth and transformation of NSCLC but through inhibition of NF-κB will also disrupt tumor-stromal interactions required for progression and metastasis. We propose that specific activators of PPARγ, such as thiazolidinediones, will have both chemopreventive and chemotherapeutic effects in the treatment of NSCLC. Unfortunately, pharmacological PPARγ activators such as rosiglitazone and pioglitazone have been associated recently with increased cardiovascular risk (Lincoff et al., 2007). However, it is unclear whether the adverse effects are mediated through PPARγ-dependent pathways or through off-target effects of these drugs. Further studies comparing molecular overexpression of PPARγ with thiazolidinediones in NSCLC and other cell types are therefore required to determine whether

second-generation PPARγ activators may represent effective safer therapies.

Acknowledgments

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