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Targeted over-expression of mPGES-1 and elevated PGE_2 production is not sufficient for lung tumorigenesis in mice

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There is a significant body of evidence suggesting that enzymes involved in arachidonic acid metabolism and their eicosanoid products play a role in various cancers, having both pro- and antitumorigenic effects. The goal of this study was to further define the role microsomal prostaglandin E synthases (mPGES-1) play in lung tumorigenesis. Transgenic mice were created with targeted over-expression of human mPGES-1 in the alveolar and airway epithelial cells using an SP-C promoter driven construct. Transgene positive (mPGES-1^+) mice were shown to significantly over-express functional mPGES-1 in the lung and more specifically in alveolar type II cells. To study the effects of mPGES-1 over-expression in lung tumor formation, mice were exposed to a complete carcinogen protocol with a single injection of urethane or an initiation/promotion model with a single injection of 3-methylcholanthrene (MCA) followed by multiple injections of butylated hydroxytoluene (BHT). mPGES-1^+ mice did not show a significant difference in tumor multiplicity or tumor size at 10, 16, 19 or 30 weeks after urethane injection compared with mPGES-1^- mice. No significant difference was seen in tumor incidence, multiplicity or size at 19 weeks after treatment with MCA/BHT. Western blots verified that mPGES-1 expression was increased in tumors versus uninvolved tissue of both mPGES-1^+ and mPGES-1^- mice with overall expression being significantly higher in mPGES-1^+ mice. Cyclooxygenase-2 levels were elevated in tumors in both groups. From these studies we conclude that over-expression of mPGES-1 and highly elevated PGE_2 production are not sufficient to induce lung tumors.

Introduction

Lung cancer is the leading cause of cancer-related deaths in both men and women in the United States (1). Defining the molecular mechanisms leading to lung tumor formation is currently an intense area of research. Numerous studies indicate that the enzymes involved in arachidonic acid metabolism and their products play a potential role in various cancers, including lung cancer. Cytosolic phospholipase A_2 ($cPLA_2$) represents the rate-limiting enzyme in eicosanoid production by preferentially hydrolyzing membrane phospholipids at the *sn-2* position to release arachidonic acid (AA) (2). Cyclooxygenases (COX) convert free AA to the unstable endoperoxide PGG_2 and then PGH_2 by reduction. Two forms of cyclooxygenase have been identified (3). COX-1 is constitutively expressed in most cell types and involved in maintaining cellular homeostasis, while COX-2 is an immediate early response gene (4) induced by mitogenic stimuli and associated with inflammation. PGH_2 is further isomerized into the various prostaglandins by terminal synthases including prostaglandin E synthase (PGES) that produces PGE_2 and prostacyclin synthase (PGIS) that forms prostacyclin (PGI_2).

A large body of evidence indicates that increased prostaglandin production contributes to tumorigenesis. COX-2 has been shown to be up-regulated in various cancers including those of the colon (5–7), breast (8) and lung (9–11), and targeted over-expression of COX-2 is sufficient to cause mammary tumorigenesis in transgenic mice (12). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX isoforms, block the growth of cancer cells *in vitro*, and inhibit tumor growth *in vivo* (13,14). Constitutively high levels of prostaglandin production are observed in established non-small cell lung cancer (NSCLC) as a result of elevated expression of $cPLA_2$ and COX-2 (15,16). Up-regulation of COX-2 is associated with increased production of PGE_2 in tumor tissue and established tumor cell lines (17,18). In addition, elevated levels of prostanoids are found in lung cancer tissue compared with normal lung tissue with consistently higher production in adenocarcinomas (19). The mechanisms whereby COX-2-derived PGE_2 promotes tumorigenesis and progression are not well understood but may involve stimulating growth (20,21), preventing apoptosis (21,22), increasing cell motility and adhesion (20,22), inducing angiogenesis (23) and inhibiting immune surveillance (24). Gene disruption of the various PGE_2 receptors has been shown to suppress carcinogen-induced colon cancer (25–27).

On the other hand, evidence suggests that PGI_2 plays an anti-neoplastic role in carcinogenesis by suppressing inflammation (28), inhibiting platelets (29) or preventing metastasis (30). Studies have shown that prostacyclin and its stable analogs inhibit both hematogenous and lymphatic metastasis as well as growth of established micrometastases in several animal models and tumor types. These effects may be associated more with effects on tumor cell–host interactions rather than a direct inhibition of primary tumor growth (30). Studies from our laboratory have demonstrated that transgenic mice selectively over-expressing PGIS in the lung are protected from carcinogen-induced lung cancer (31). These studies suggest that in terms of tumorigenesis, PGI_2 may be the 'good' prostaglandin while

Abbreviations: BHT, butylated hydroxytoluene; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; EIA, enzyme immunoassay; MCA, 3-methylcholanthrene; mPGES, microsomal prostaglandin E synthase; NSAIDs, non-steroidal antiinflammatory drugs; NSCLC, non-small cell lung cancer; PGI_2, prostacyclin; PGIS, prostacyclin synthase.

PGE_2 is the 'bad' prostaglandin, and shifts in the balance of production of these prostanoids may play a role promoting tumor progression.

PGESs are the terminal synthases responsible for converting COX-derived PGH_2 into PGE_2. Two isoforms of PGES have been identified, termed cytosolic PGES (cPGES) and microsomal PGES (mPGES). cPGES is a glutathione-dependent enzyme that is expressed constitutively in a wide variety of tissues and is linked with COX-1 in immediate prostaglandin production (32). Jakobsson *et al*. (33) first identified a known protein, human microsomal GST-1-like 1, as microsomal prostaglandin E synthase-1 (mPGES-1), a member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily. mPGES-1 is an inducible, glutathione-dependent, membrane-bound form of PGES that is functionally linked to COX-2 in delayed PGE_2 production (33–35). Recent studies support that it is also linked with COX-1 in basal PGE_2 synthesis (36,37). Expression of mPGES-1 is induced by pro-inflammatory stimuli, frequently with concordant up-regulation of COX-2, and is down-regulated by anti-inflammatory glucocorticoids (33,34,38,39). Recently, a second, distinct mPGES, termed mPGES-2, has been purified from bovine heart (40) and cloned from sequences of human and monkey enzymes (41).

Although many studies provide support for a role for COX-2 and elevated PGE_2 levels in tumorigenesis, only a few studies have looked at the involvement of the terminal prostaglandin synthases. In this study, we evaluated the role of mPGES-1 in carcinogen-induced lung cancer using transgenic mice with targeted over-expression of mPGES-1 in the type II alveolar epithelial cells of the lung.

Materials and methods

Construction of SP-C/mPGES-1 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-kb flanking sequence of the human SP-C promoter in addition to the SV-40 small T intron as a polyadenylation signal (42,43). The mPGES-1 expression plasmid contains a full-length cDNA for the human mPGES-1 (0.6 kb, GenBank accession no. NM_004878) subcloned into the *Xho*I/*Eco*RI site of pcDNA-3. The full-length human mPGES-1 cDNA was cut and cloned into the *Sal*I/*Eco*RI site of the hSP-C/SV-40 plasmid by blunt end ligation, creating the SP-C promoter-mPGES-1 cDNA fusion gene (Figure 1A). The proper cloning orientation of our construct was confirmed by direct sequence analysis.

Development and genotyping of transgenic mPGES-1 over-expressing mice

Transgenic mice were developed in a FVB/N strain using the SP-C-mPGES-1 construct described above. Pronuclear injections were performed, which yielded five founding lines. Transgenic mice were genotyped by analyzing genomic DNA isolated from tail biopsies with the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen, Santa Clarita, CA). PCR 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′) (Figure 1B). All mice were propagated as heterozygous transgenic mice by breeding with wild-type FVB/N mice.

Carcinogenesis protocols

FVB/N mice 8–12 weeks of age were maintained on a standard, antioxidant-free laboratory chow (Lab Diet; PMI Nutrition, 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, and subjected to one of the following experimental protocols.

(i) Urethane carcinogenesis. A single urethane (Sigma Chemical) dose (1 mg/g mouse weight), dissolved in normal saline, was administered i.p., and animals were killed 10, 16, 19 and 30 weeks later.

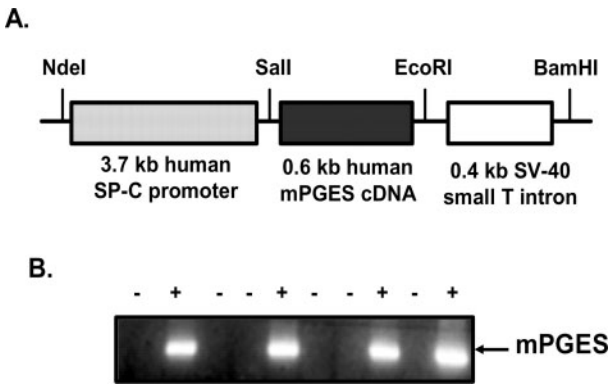

Fig. 1. Genotyping of transgenic mice. (**A**) Transgenic mice were developed using a construct consisting of the full-length human mPGES-1 cDNA and SV-40 small T intron downstream of the human SP-C promoter. The SP-C promoter allows targeted expression to the distal epithelium of the lung. (**B**) Transgenic mice were genotyped by performing PCR on genomic DNA isolated from tails using primers spanning a segment of the SV-40 small T intron. Each line was propagated as heterozygotes. mPGES-1^+ mice were always bred with wild-type FVB/N mice to produce experimental mPGES-1^+ mice as well as mPGES-1^- littermates, which we used as controls in all of the experiments.

(ii) 3-Methylcholanthrene (MCA)/butylated hydroxytoluene (BHT) carcinogenesis. A single dose of 3-methylcholanthrene (MCA: 15 μg/g mouse weight) was administered i.p. followed by eight weekly i.p. doses of butylated hydroxytoluene (BHT; the first dose was 150 μg/g mouse weight, and subsequent doses were 200 μg/g mouse weight) dissolved in corn oil. Mice were killed 19 weeks after the MCA dose was administered.
(iii) MCA carcinogenesis. A single dose of i.p. MCA (15 μg/g mouse weight) was administered dissolved in corn oil. Mice were killed after 19 weeks.

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 (mPGES-1^- or mPGES-1^+) was not revealed until after tumor multiplicities and sizes were determined. Several lungs were inflated with 10% buffered formalin to be paraffin embedded and sectioned for H&E staining and immunohistochemistry.

Immunoblotting

For immunoblotting, excised whole lungs, dissected tumors and uninvolved lung tissue were homogenized in 1× Earles Balanced Salts Solution (Sigma Chemical). Extracts were centrifuged at 10 000 *g* to remove tissue debris, and supernatants were matched for protein. Extracts (40 μg protein for mPGES, COX-2, $cPLA_2$, PGIS; 20 μg for SP-C) were separated by SDS–polyacrylamide gel electrophoresis and transferred to Immobilon-P polyvinylidine fluoride membranes (Millipore, Bedford, MA). Blots were blocked for 1 h at room temperature with 5% non-fat dry milk in TTBS, and incubated overnight at 4°C with either a rabbit polyclonal antibody against mPGES-1 (16 kDa) at 1:1000 dilution (Cayman Chemical, Ann Arbor, MI), a rabbit polyclonal antibody against PGIS (56 kDa) at 1:1000 dilution (generous gift from Dr David Dewitt, Department of Biochemistry, Michigan State University, East Lansing, MI), a goat polyclonal antibody against COX-2 (72 kDa) at a 1:500 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA), a mouse monoclonal antibody against $cPLA_2$ (110 kDa) at a 1:300 dilution (Santa Cruz), or a rabbit polyclonal antibody against pro-SP-C (21, 16 and 26 kDa) at a 1:500 dilution (Research Diagnostics, Flanders, NJ). Blots were then incubated for 1 h at room temperature with alkaline phosphatase conjugated secondary antibodies (Santa Cruz) and visualized using the Lumi-Phos system (Pierce Biotechnology, Rockford, IL) and exposure to Hyperfilm™ ECL (Amersham Biosciences UK, Buckinghamshire, UK). Bands were quantified by densitometry using Scion Image software (Scion, Frederick, MD).

Type II cell isolation

Type II pneumocytes were isolated from untreated transgenic or wild-type mice as described previously (44,45). Briefly, mice were anesthetized with an i.p. 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 dispase for 45 min at room temperature. Lungs were then transferred to a 60-mm culture dish containing 7 ml HEPES-buffered DMEM and 100 U/ml DNase I; 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 pre-washed 100-mm culture dishes that had been pre-coated with 42 μg anti-CD45 antibody and 16 μg anti-CD32 antibody in PBS for 24–48 h at 4°C, and incubated for 1.5 h at 37°C. The media 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, protease inhibitors), centrifuged at 10 000 *g* for 10 min, and matched for protein. For enzyme immunoassay (EIA) and LCMS 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 media (DMEM plus 10% fetal calf serum) and incubated at 37°C for 30 min. The media was collected and centrifuged at 10 000 *g* for 10 min. The supernatant was diluted 1:3 in methanol for analysis of PGE_2 levels and the cell pellets were processed for immunoblotting as described above.

Determination of PGE_2 and 6-keto $PGF_{1\alpha}$ levels by EIA

PGE_2 and 6-keto $PGF_{1\alpha}$ levels were determined by EIA according to the manufacturer's instructions (Cayman Chemical). For whole lung homogenate samples, untreated mPGES-1$^-$ and mPGES-1$^+$ littermates were killed by i.p. injection of a lethal dose of phenobarbital, whole lungs were removed and homogenized in 1× Earles Balanced Salts Solution (Sigma). The samples were diluted 1:3 with methanol and centrifuged at 10 000 *g* for 10 min to remove tissue debris.

Statistical analysis

All values are represented as means ± SEM. Unpaired Student's *t*-tests were performed to determine differences between groups. In all cases $P < 0.05$ was considered significant.

Results

Generation of mice with targeted over-expression of mPGES-1

A SP-C/human mPGES-1 DNA construct (Figure 1A) was used to establish five transgenic lines over-expressing mPGES-1 targeted to the distal epithelium of the lung. Previous studies have shown that the SP-C promoter directs expression predominately in alveolar epithelial cells, but expression is also present in bronchial epithelial cells (46). The founders were bred with wild-type FVB/N mice and the transgene was passed to offspring following Mendelian rules (Figure 1B). Western blot analysis was performed (Figure 2) on lysates from whole lung (A) and isolated type II pneumocytes (B) from transgene positive (mPGES-1$^+$) and wild-type (mPGES-1$^-$) control mice. mPGES-1$^+$ mice showed a significant over-expression of mPGES-1 protein compared with wild-type controls with both lysates (7.3-fold in whole lung, 4.8-fold in type II cells) (Figure 2C). The level of mPGES-1 expression was similar among all founding lines (data not shown). Steady-state levels of $cPLA_2$, COX-2 and PGIS in whole lung were unchanged in mice carrying the transgene (see Figure 6). *In vivo* mPGES-1 enzyme activity was measured by determining PGE_2 levels in whole lung homogenates (Figure 3A). mPGES-1$^+$ mice produced 12.2-fold higher levels of PGE_2 than mPGES-1$^-$ littermates. To look at the effects of over-expression on the production of other eicosanoids, PGI_2 production was measured by determining the levels of 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, in the same whole lung homogenates. There was no significant difference in 6-keto $PGF_{1\alpha}$ levels in mPGES-1$^+$ mice compared with mPGES-1$^-$ littermates (Figure 3B). Lung architecture was not grossly different in any of the mice.

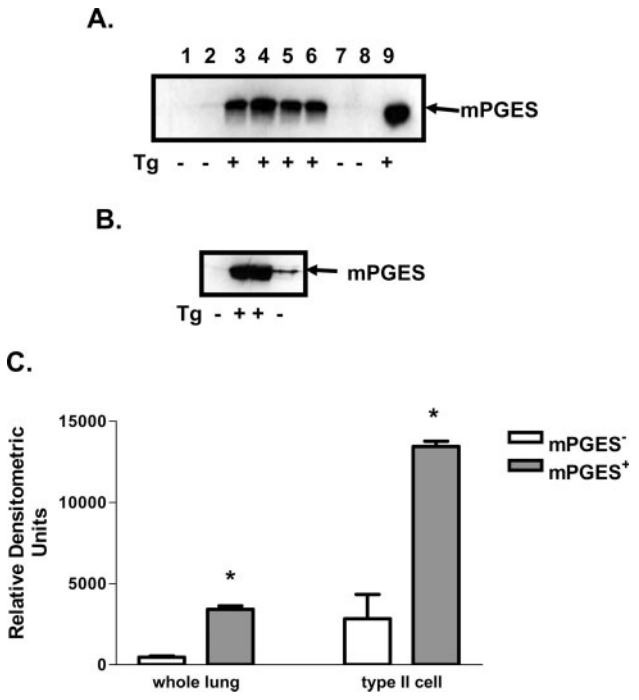

Fig. 2. Characterization of transgenic mice with targeted over-expression of mPGES-1 in the lung. Forty micrograms of protein homogenates from either whole lung tissue (**A**, lanes 1–8) or isolated type II cells (A, lane 9 and **B**) from individual mPGES-1$^+$ and mPGES-1$^-$ littermates were immunoblotted for mPGES-1 to confirm expression of the transgene. (**C**) Bands were quantified by densitometry using Scion Image software and shown as an average for all mPGES-1$^+$ and mPGES-1$^-$ samples, respectively. $^*P < 0.02$ versus mPGES$^-$ ($n = 4$ for whole lung, $n = 2$ for type II cell).

To confirm that mPGES-1$^+$ mice were producing more active PGE_2, LC/MS analysis was performed on media collected from isolated type II cells from untreated mPGES-1$^+$ mice and wild-type littermates. A 2.5-fold increase in PGE_2 was seen in mPGES-1$^+$ mice versus mPGES-1$^-$ controls (Figure 3C and D). Similar results were seen with EIA analysis of the same samples confirming specificity of our EIA analysis (data not shown). A minor peak corresponding to the inactive 15-keto-PGE_2 metabolite was detected in all of the samples. However, the major peak in both groups of mice corresponded to authentic PGE_2, indicating that the active form of PGE_2 is the major product being produced in these mice (data not shown).

Over-expression of mPGES-1 is not sufficient for the promotion or progression of lung tumors in mice

Seventy-five littermates (37 mPGES-1$^-$ and 38 mPGES-1$^+$, from three founding lines) were subjected to a complete carcinogen protocol employing a single i.p. injection of urethane (1 mg/g) at 7–8 weeks of age. Mice were killed at 10, 16, 19 and 30 weeks after injection, and tumors were enumerated in fresh lungs. Adenomas were visible by eye after 10 weeks. The gross (Figure 4A) and microscopic (Figure 4B) appearance of the tumors is characteristic of adenomas produced by both the urethane and MCA/BHT carcinogenesis protocols in other studies (31). Tumor multiplicity was not significantly different in mPGES-1$^+$ versus mPGES-1$^-$ littermates at any of the time points examined though an overall increase in multiplicity was seen between 19 and 30 weeks (Figure 4C). Tumor diameter

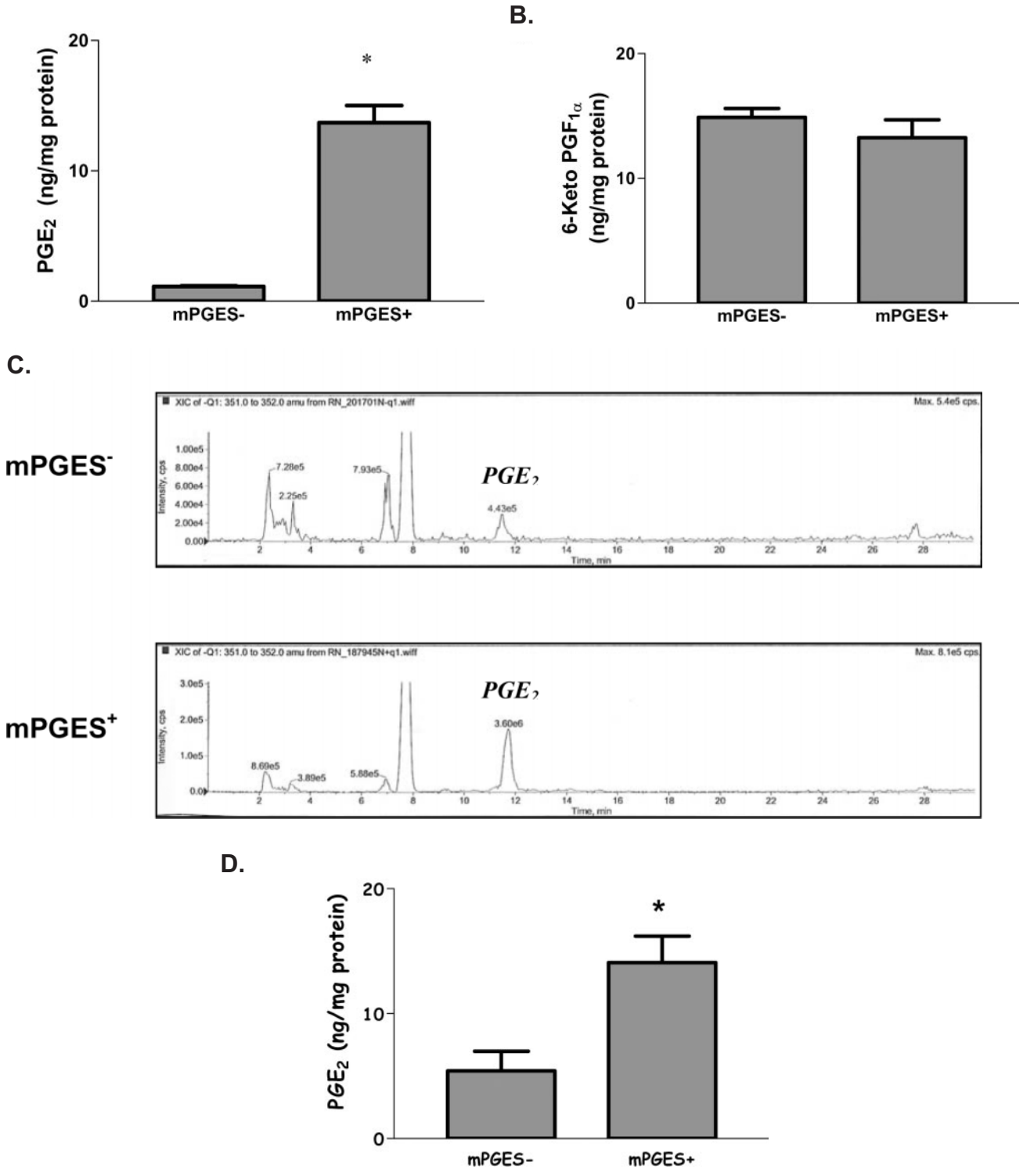

Fig. 3. PGE_2 levels are elevated in mPGES-1^+ mice while PGI_2 levels remain unchanged. Whole lung homogenates were prepared from mPGES-1^+ and mPGES-1^- littermates to measure *in vivo* enzyme activity. PGE_2 (**A**) and 6-keto $PGF_{1\alpha}$ (**B**) levels were determined by EIA. $^*P = 0.0005$ versus mPGES$^-$ ($n = 2$). Media was collected from isolated type II cells and assayed for PGE_2 using LC/MS. (**C**) Representative chromatograms. PGE_2 levels were quantified compared with a standard (**D**). $^*P < 0.02$ versus mPGES$^-$ ($n = 4$). Under the chromatographic conditions used PGE_2 co-eluted consistently at 11.6 min. Subsequent analysis of all samples in full scan mode over the mass range m/z 250–800 showed no evidence for the presence of 13,14-dihydro-15-keto PGE_2 in any of the samples.

was measured at the 19- and 30-week time points using digital calipers. No significant difference was seen in average tumor size between mPGES-1^+ and mPGES-1^- littermates though an overall increase in tumor size was seen at 30 weeks (Figure 4D). Tumor incidence was 100% in both mPGES-1^+ and mPGES-1^- mice (data not shown). H&E staining showed no difference in morphology between tumors from mPGES-1^+ and mPGES-1^- mice (data not shown).

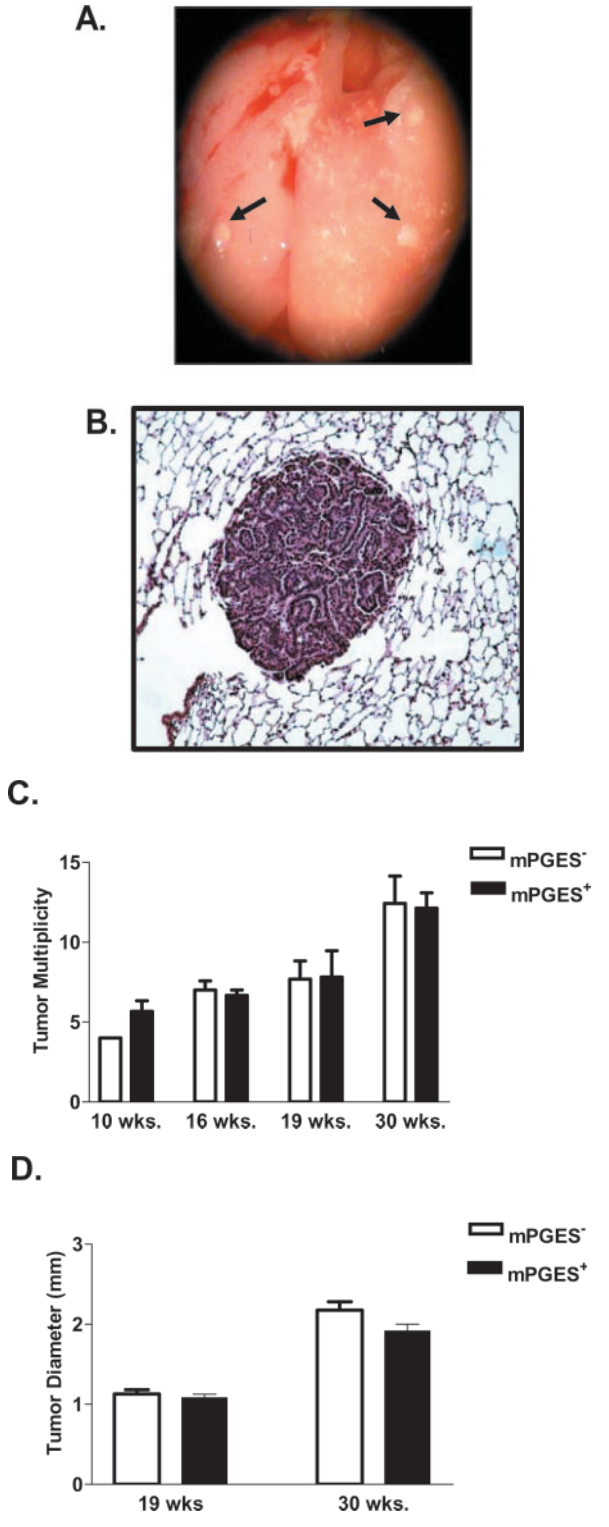

Fig. 4. Over-expression of mPGES-1 does not promote urethane-induced lung tumor formation in FVB/N mice. (**A**) Gross appearance of adenomas (arrows) on pleural surface under 10× magnification in lungs 19 weeks after treatment with MCA/BHT. (**B**) Microscopic appearance of a lung adenoma, stained with H&E, 19 weeks after treatment with urethane under 40× magnification. (**C**) FVB/N mice 6 weeks of age were maintained on a standard, antioxidant-free laboratory chow and given food and water *ad libitum*. They were kept on cedar-free bedding with a 12-h light/dark cycle in a climate-controlled animal facility. Animals were given a single urethane dose (1 mg/g mouse weight), dissolved in normal saline and administered i.p. Animals were killed at 10, 16, 19 and 30 weeks. Tumors were enumerated in fresh lungs under a dissection microscope. (**D**) All tumors were dissected from the lung parenchyma and measured using digital calipers.

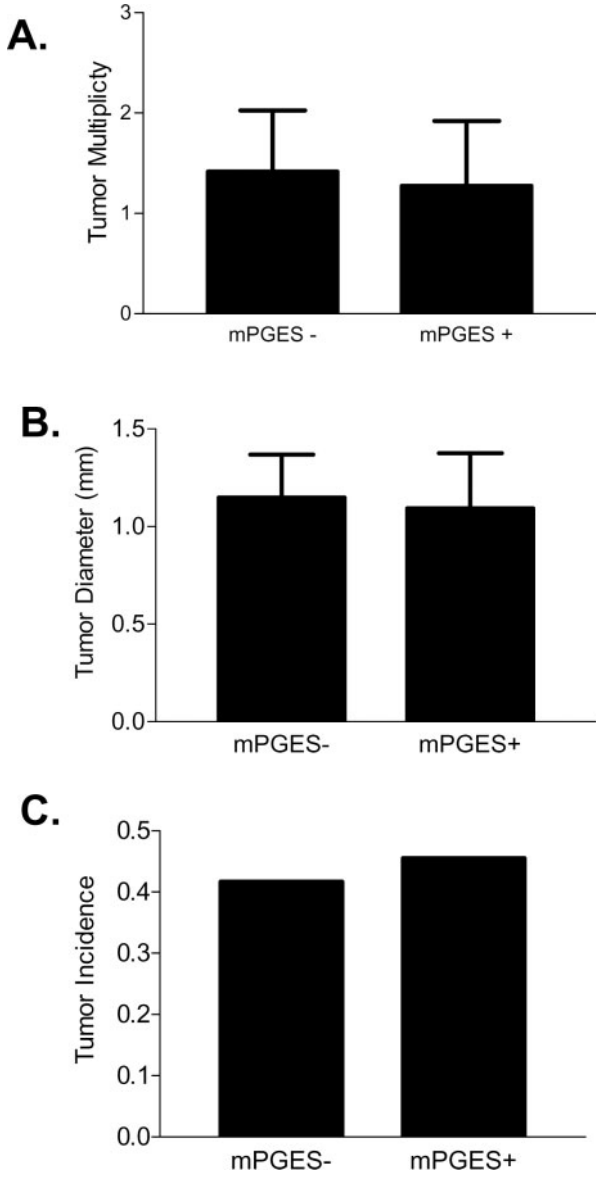

Fig. 5. Over-expression of mPGES-1 does not promote MCA/BHT-induced lung tumor formation in FVB/N mice. (**A**) Animals were given a single MCA dose (15 μg/g mouse weight), followed by eight weekly doses of BHT (first dose 150 μg/g mouse weight, subsequent doses 200 μg/g mouse weight), dissolved in corn oil, and administered i.p. Animals were killed at 19 weeks and tumors were enumerated in fresh lungs under a dissection microscope. (**B**) All tumors were dissected from the lung parenchyma and measured using digital calipers. (**C**) Tumor incidence measures the relative percentage of mice that developed tumors under this protocol.

We employed a second model of mouse lung tumorigenesis in which 30 littermates (14 mPGES-1$^-$ and 16 mPGES-1$^+$, from a single founding line) were subjected to a two-step initiation/promotion carcinogenesis protocol and administered a single i.p. dose of MCA (15 μg/g) followed by eight weekly i.p. doses of BHT (initial dose 150 μg/g and subsequent doses 200 μg/g) at 7–8 weeks of age. Mice were killed at 19 weeks of age, and tumors were enumerated in fresh lungs. mPGES-1$^+$ mice showed no significant difference in either tumor multiplicity or tumor incidence as compared with mPGES-1$^-$ littermates (Figure 5A and C). In addition, mPGES-1$^+$ mice showed no significant difference in tumor size when compared with mPGES-1$^-$ littermates (Figure 5B). A group

of 21 littermates (seven mPGES-1$^-$ and 14 mPGES-1$^+$, from two founding lines) were treated with only a single injection of MCA to see if over-expression of mPGES-1 alone was sufficient as a promoter of MCA initiated lung adenoma formation. No tumors were found in either mPGES-1$^+$ or mPGES-1$^-$ mice after 19 weeks (data not shown).

Expression of mPGES-1, COX-2 and cPLA$_2$ are up-regulated in urethane and MCA/BHT-induced tumors. Protein lysates were prepared from tumors and uninvolved lung tissue from mPGES-1$^-$ and mPGES-1$^+$ mice in both carcinogenesis protocols at the time of death. Western blot analysis was performed to determine the level of expression of enzymes in the eicosanoid pathway. mPGES-1 expression was high in both normal and tumor tissue in mPGES-1$^+$ mice at all time points examined. Figure 6A and B shows the results at 19 weeks. The data suggest that the tumors in mPGES-1$^+$ mice are arising from cells expressing the transgene (Figure 6A and B). mPGES-1 was up-regulated in tumors from mPGES-1$^-$ mice (Figure 6A), but overall expression is significantly lower than that of mPGES-1$^+$ animals. COX-2 expression was increased in tumors versus uninvolved lung to the same extent in both mPGES-1$^-$ and mPGES-1$^+$ mice at both 16 and 19 weeks (Figure 6A). cPLA$_2$ was expressed in both tumors and uninvolved tissue to approximately the same level in all of the mice. Expression of prostacyclin synthase in uninvolved tissue was comparable in mPGES-1$^+$ and mPGES-1$^-$ animals, and was low in the tumors of all mice at either time point (Figure 6A). Expression of SP-C was enriched in the tumors from both mPGES-1$^-$ and mPGES-1$^+$ after treatment with both urethane and MCA/BHT confirming that the tumors are likely arising from type II alveolar epithelial cells and that these cells are the progenitor cells of lung adenomas (Figure 6A). PGE$_2$ levels were increased in tumor versus uninvolved tissue from both mPGES-1$^+$ (6.5-fold) and mPGES-1$^-$ (15.4-fold) mice 19 weeks after urethane treatment with higher amounts overall in mPGES-1$^+$ mice (Figure 6B). PGE$_2$ levels were increased by ~1.7-fold in tumors and 4-fold in uninvolved tissue in mPGES-1$^+$ versus mPGES-1$^-$ mice (Figure 6B). 6-Keto PGF$_{1\alpha}$ levels in both mPGES-1$^+$ and mPGES-1$^-$ mice were lower in tumors than in uninvolved tissue, consistent with our earlier findings (31), with no statistical difference between the two groups of animals (Figure 6C). Levels in uninvolved tissue were similar to what was observed in whole lung of untreated animals.

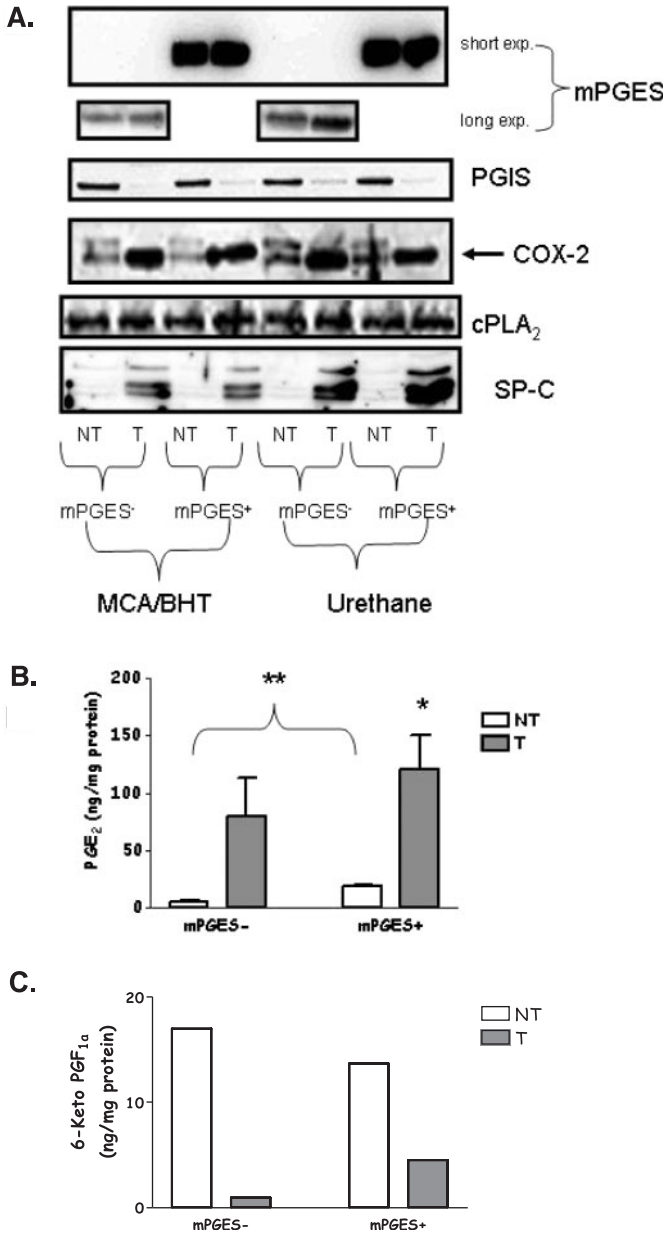

Fig. 6. Expression of mPGES-1, COX-2 and cPLA$_2$ is elevated in urethane and MCA/BHT induced lung adenomas. (**A**) All tumors were dissected from the lung parenchyma. Protein homogenates (40 μg) prepared from both tumor (T) and uninvolved lung tissue (NT) from mPGES-1$^-$ and mPGES-1$^+$ littermates 19 weeks after treatment with either MCA/BHT or urethane were immunoblotted for expression of mPGES-1, PGIS, COX-2 and cPLA$_2$. (**B** and **C**) Homogenates were prepared from dissected tumor (T) and uninvolved (NT) lung tissue from mPGES-1$^+$ and mPGES-1$^-$ littermates treated with urethane for 19 weeks. All tumors from an individual mouse were combined. PGE$_2$ (B) and 6-keto PGF$_{1\alpha}$ (C) levels were determined by EIA. (B) $^*P < 0.05$ versus NT ($n = 4$), $^{**}P = 0.0004$ versus mPGES$^-$ NT ($n = 4$).

Discussion

The low 5-year survival rate following diagnosis (15%) (1) highlights the urgent need for the development of new lung cancer chemotherapies. Many studies provide evidence that high levels of PGE$_2$ resulting from elevated expression and activity of cPLA$_2$ and COX-2 are associated with increased lung tumorigenesis (9–11,15–17,19,47,48). Most studies have focused on the inhibition of COX-2, but recent data suggest that the distal enzymes in the prostaglandin pathway may prove to be more promising targets for lung cancer chemotherapy than COX-2 alone. mPGES-1 has potential as a novel drug target in the treatment of cancer and inflammatory diseases by allowing for more selective regulation of the prostaglandin pathway.

Yoshimatsu *et al.* (49), found that mPGES-1 is over-expressed in 80% of human NSCLC, and we have observed similar results using a tissue microarray containing lung tumors from NSCLC patients (S.A.Blaine, manuscript in preparation). As we reported previously for both cPLA$_2$ and COX-2 (13), mPGES-1 mRNA and protein levels are increased in established NSCLC cell lines that contain activating mutations in the *ras* oncogene (49).

To test the specific role of PGE$_2$ in mouse models of lung cancer, we used two established models of lung carcinogenesis

to evaluate the generality of any effect of mPGES-1 over-expression on lung tumor formation. In the first model, administration of urethane acts as a complete carcinogen leading to both initiation and promotion of tumorigenesis (50) while in the second model, MCA, a polycyclic aromatic hydrocarbon found in tobacco smoke, acts only as an initiator and BHT promotes the development of lung tumors (51). The choice of these models was based on previous studies demonstrating that modulation of the eicosanoid pathway is critical for tumor formation. Treatment of mice with indomethacin inhibited tumor formation using both models (52). Surprisingly, similar experiments using the COX-2-specific inhibitor celecoxib, failed to inhibit tumor formation, suggesting that both COX-1 and COX-2 may contribute to lung carcinogenesis (53). More recently we have also shown that mice, which are deficient in $cPLA_2$, which represents the most proximal step in eicosanoid biosynthesis, are protected against developing lung tumors (54). Our current results show that targeted over-expression of mPGES-1 leading to significantly elevated production of PGE_2 (Figures 2 and 3) is not sufficient for the promotion of carcinogen-induced lung tumorigenesis in either model (Figures 4 and 5). These findings were somewhat unexpected based on the evidence presented above suggesting that elevated PGE_2 production and mPGES-1 expression are important in lung cancer (34,49,55,56). We found no evidence that mPGES-1^+ mice compensate for the hugely elevated levels of PGE_2. Steady-state levels of $cPLA_2$, COX-2 and PGIS were unchanged in mPGES-1^+ mice (Figure 6) and preliminary microarray analysis of RNA isolated from untreated whole lungs showed no significant change in the expression of any of the EP receptors in mPGES-1^+ mice (data not shown). LC/MS analysis also showed that type II cells from mPGES-1^+ mice are secreting higher amounts of active PGE_2 (Figure 3C) and that the levels of the inactive 15-keto metabolite represent a small fraction of the active compound. The inflammatory response of the animals, as measured by quantifying macrophage recruitment following a single BHT injection, was also not different between mPGES-1^+ and wild-type littermates (data not shown).

Interestingly, the level of over-expression of mPGES-1 in tumors from mPGES-1^+ mice did not correlate with the level of PGE_2 produced. While in uninvolved tissue significant over-expression of mPGES-1 in mPGES-1^+ mice leads to an increase in PGE_2 production, in tumors the large increase in mPGES-1 expression in mPGES-1^+ mice only results in a modest increase in PGE_2. The difference in PGE_2 production between mPGES-1^+ and mPGES-1^- mice is ~4-fold in both uninvolved lung tissue and whole lung from untreated mice (Figure 3A), but that difference is $<$2-fold in tumors (Figure 6B). Both mPGES-1^+ and mPGES-1^- mice show an increase in PGE_2 levels in tumor versus uninvolved tissue but the fold change is greater in mPGES-1^- mice (15.4- versus 6.5-fold). For one, this implies the presence of an upper threshold or 'ceiling' for increased PGE_2 production to play a role in lung tumorigenesis, above which no additional effects are seen. In our studies, treatment with either urethane or MCA/BHT resulted in a modest increase in expression of mPGES-1 in lung adenomas from mPGES-1^- as well as mPGES-1^+ mice (Figure 6A), so it is possible that elevated levels of PGE_2 production caused by the carcinogens themselves are enough to promote lung tumor formation, and expression of the transgene has no additive effect once PGE_2 levels have reached this threshold.

The data suggest a model where in normal lung PGE_2 production is coupled to COX-1 through either cPGES or mPGES. Under these conditions PGES is limiting, and over-expression of the enzyme, as achieved in the transgenic animals, leads to increased levels of PGE_2 production, which correlate with levels of mPGES expression. In tumors however, COX-2 is induced and there is an induction of prostaglandin production. We propose that under these conditions, mPGES is selectively coupled to COX-2 and that COX-2 is now limiting for PGE_2 production. Therefore, marked increases in mPGES expression do not result in comparable increases in PGE_2 production. Since COX-2 is induced to the same extent in both mPGES-1^+ and mPGES-1^- mice, the increased expression of mPGES-1 seen in mPGES-1^+ mice has little effect in further increasing PGE_2 production. Recent studies support that while mPGES-1 may couple more efficiently and preferentially with COX-2, it is capable of functionally coupling with COX-1 in basal, non-inflammatory PGE_2 synthesis (31,34,36,37). Also in support of this model, Boulet *et al*. (36), found that in macrophages, while COX-2 and mPGES-1 work together to regulate PGE_2 production COX-2 is predominantly responsible for differences in PGE_2 release seen in resident or elicited macrophages. Based on this proposed mechanism, our data indicate that increased PGE_2 levels do not affect early tumor development. However, if mPGES-1 and PGE_2 play a role in lung tumor progression our transgenic mice would not be the optimal model to study the effects of mPGES-1 in lung cancer.

The fact that frequent co-expression of COX-2 and mPGES occurs in NSCLC suggests that these enzymes may mediate tumor progression. However, there is evidence that mPGES-1 may be expressed as a consequence of transformation since NSCLC cells but not normal human bronchial cells express mPGES-1 and cytokine treatment causes induction of mPGES-1 levels only in NSCLC cells (49). Ongoing studies using recently developed mPGES-1 knockout mice (57) will help elucidate a role for mPGES-1 in tumor progression.

The idea that there may be 'good' and 'bad' prostaglandins in the process of lung tumorigenesis is intriguing and warrants further exploration. The action of COX-2 provides a common substrate for the production multiple prostaglandins that potentially have opposing effects on tumorigenesis. Overall, blocking prostaglandin production by inhibiting COX-2 is anti-tumorigenic, but it has been postulated that shifts in the balance of these opposing prostaglandins may play an integral role in tumorigenesis. We and others (11) have shown that while mPGES-1 is highly expressed in human NSCLC, PGIS is virtually undetectable in tumors. A recent *in vivo* study showing that PGI_2 has a protective effect against lung tumor formation suggests that blocking pro-tumorigenic prostaglandins while simultaneously boosting antitumorigenic prostaglandins may prove to be therapeutically beneficial (31). This study along with the data presented here also support that any carcinogenic effects of PGE_2 are independent of PGI_2 and vice versa.

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