Prostacyclin and EMT Pathway Markers for Monitoring Response to Lung Cancer Chemoprevention

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Abstract

Lung cancer is the leading cause of cancer death worldwide and global burden could be reduced through targeted application of chemoprevention. The development of squamous lung carcinoma has been linked with persistent, high-grade bronchial dysplasia. Bronchial histology improved in former smokers in a chemoprevention trial with the prostacyclin analogue iloprost. Prostacyclin acts through peroxisome proliferator-activated receptor gamma (PPARγ) to reverse epithelial to mesenchymal transition and promote anticancer signaling. We hypothesized that the prostacyclin signaling pathway and EMT could provide response markers for prostacyclin chemoprevention of lung cancer. Human bronchial epithelial cells were treated with cigarette smoke condensate (CSC) or iloprost for 2 weeks, CSC for 16 weeks, or CSC for 4 weeks followed by 4 weeks of CSC and/or iloprost, and RNA was extracted. We measured potential markers of prostacyclin and iloprost efficacy in these models. We identified a panel of markers altered by tobacco carcinogens and inversely affected by prostacyclin, including PPARγ, 15PGDH, CES1, COX-2, ECADHERIN, SNAIL, VIMENTIN, CRB3, MIR34c, and MIR221. These data introduce a panel of potential markers for monitoring interception of bronchial dysplasia progression during chemoprevention with prostacyclin. Chemoprevention is a promising approach to reduce lung cancer mortality in a high-risk population. Identifying markers for targeted use is critical for success in future clinical trials of prostacyclin for lung cancer chemoprevention. Cancer Prev Res; 11(10): 643–54. © 2018 AACR.

Introduction

There are more cancer-related deaths from lung cancer than any other malignancy worldwide (1). Lung cancer chemoprevention aims to reduce mortality from lung cancer by treating patients at high risk of developing lung cancer while they are still at the premalignant stage. Prostacyclin and its downstream target peroxisome proliferator-activated receptor gamma (PPARγ) play an important role in lung cancer and in chemoprevention. Decreased expression of PPARγ in lung tumors is associated with poor prognosis, whereas a retrospective clinical study showed a 33% reduction in lung cancer among patients with diabetes using rosiglitazone, a PPARγ agonist (2, 3). Several preclinical studies have shown a similar protective effect against the development of lung cancer from increased pulmonary prostacyclin and the prostacyclin analog iloprost (4, 5). A phase II clinical trial of oral iloprost in high-risk patients demonstrated regression of endobronchial premalignant histology in former smokers (6). Histologic regression of dysplastic lesions is an accepted endpoint for effective chemoprevention, however intermediate biomarkers of response (potentially measured in noninvasive specimens) could indicate if prostacyclin is generating a response independent of visible histologic changes.

The loss of epithelial features and gain of mesenchymal properties, known as the epithelial-to-mesenchymal transition (EMT), has been associated with an invasive or metastatic phenotype in lung cancer and poor clinical outcomes, including increased cancer recurrence and decreased survival (7). EMT is also relevant at the earliest stages of lung cancer development (8). Cigarette smoke alters markers of EMT, including ECADHERIN, VIMENTIN, and FIBRONECTIN (9). Conversely, activation of PPARγ in non-small cell lung cancer inhibits EMT by increasing...
expression of ECADHERIN and inhibiting expression of COX-2 and the transcription factor SNAIL (10–12). Early stages of lung premalignancy are also regulated by miRNA, making them potentially relevant to prostacyle chemoprevention (8, 13). Although very little is known about prostacyclin or PPARg and miRNA, we demonstrated that reduction of MIR31 occurs with prostacyle treatment and may be linked to expression of the presumed iloprost receptor in the lung epithelium (14, 15).

We hypothesized that downstream targets of prostacyclin are affected by cigarette smoke and could act as markers of response to prostacyle chemoprevention. We tested this hypothesis using several models, including in vitro in human bronchial epithelial cells (HBEC), in vivo in murine models of lung cancer carcinogenesis, and in cultured primary dysplastic bronchial epithelial cells. Across these models, we identified markers that are altered by cigarette smoke carcinogens and are inversely affected by prostacyclin. These markers could be used to monitor response to iloprost chemoprevention and their identification is a critical step towards providing effective, personalized lung cancer chemoprevention.

Materials and Methods

Cell culture

HBEC (a gift in 2013 from Dr. John Minna, University of Texas Southwestern, Dallas, TX) were cultured in Keratinocyte Serum Free Medium (GIBCO) at 37°C in a humidified 5% CO2 incubator and passaged twice per week. Morphology of cells lines was verified twice weekly. Cells were tested for mycoplasma and fingerprinted upon receipt. All long-term HBEC cultures were passaged once after thawing before beginning experiments and were grown and handled in a dedicated incubator. Independently passaged, biologic triplicates were performed for each HBEC experiment.

Drug and cigarette smoke condensate exposure

Cigarette smoke condensate (CSC; Murty Pharmaceuticals) was applied to HBEC at 5 µg/mL and iloprost (Cayman Chemicals) to HBEC or primary cells at 10 µmol/L in cell growth media. DMSO or methyl acetate were the vehicle controls. Cells were allowed 24 hours for recovery after plating before drug or carcinogen application. In the 8-week smoke model, HBEC were treated with CSC or vehicle every 72 hours for 4 weeks, then CSC was removed or continued with or without iloprost treatment every 72 hours for another 4 weeks (Fig. 1B). HBEC with CSC and iloprost exposure were carried as individual cell lines in triplicate with twice a week passaging for 2 to 16 weeks.

Mouse models

All procedures were approved by the Denver Veterans Administration Medical Center Institutional Animal Care and Use Committee. Mouse tissues used in this study were frozen in RNALater (Qiagen). For urethane studies in wild-type and prostacyclin synthase transgenic (Pgis) FVB mice, one 1 mg/g urethane intraperitoneal injection was given, and mice were sacrificed 20 weeks later (5). Each group included five animals. Tumors and visible adenomas were dissected from the lungs and uninvolved tissue was saved for RNA extraction. For the 1-week smoke exposure study, wild-type and Pgis FVB mice were exposed to whole body cigarette smoke for 6 hours/day for 5 days/week in a TE-10 smoking machine (Teague Enterprises) at 80 mg/m3 (4). Each group included five mice. Mice were sacrificed after 1 week and whole lung tissue was removed for RNA extraction. All experiments were conducted in accordance with AAALAC International guidelines and the United State Animal Welfare Act.

Primary dysplastic cells

The University of Colorado Cancer Center SPORE in Lung Cancer Tissue Bank and Biomarkers Core (Tissue Bank) provides publicly available, deidentified samples for small studies. The Colorado Multiple Institutional Review Board approves all the human experimental protocols that generate specimens for the Tissue Bank and collects written, informed consent from all subjects. Three primary dysplastic lung cell cultures were isolated from squamous dysplasia biopsies at the University of Colorado Cancer Center and were obtained from the Tissue Bank in 2018. Cultures were taken from tissue adjacent to the biopsy site used for histologic grading. After thawing, the cultures were grown in Bronchial Epithelial Basal Media (Lonza) at 37°C in a humidified 5% CO2 incubator for 2 weeks and treated every 48 hours with 10 µmol/L iloprost or vehicle. Cells were treated with iloprost in triplicate, but typically only two replicates survived to the end of treatment for RNA extraction.

Quantitative PCR

RNA was extracted from HBEC and primary cells using the AllPrep Universal Kit (Qiagen) and from mouse tissue using the AllPrep DNA/RNA Kit (Qiagen) on the Qiacube automated processor (Qiagen). mRNA was reverse transcribed using the ABI HC cDNA Kit (Thermo Fisher Scientific) and miRNA using the miScript II RT Kit (Qiagen). qPCR primers for human and mouse included: PPARg, 15PGDH, CES1, COX-2, (PTGS2), SNAIL, VIMENTIN, ECADHERIN, CRB3, 18sRNA, and RPL13 (Bio-Rad) GAPDH and MIR34c, MIR221, and RNU6 (Qiagen). qPCR was conducted using standard protocols for Sso Advanced SYBR Green Master Mix (Bio-Rad) or the miScript SYBR Green PCR Kit (Qiagen) on a CFX96 Touch (Bio-Rad). Genes for normalization of mouse model PCR were determined by screening eight genes with the Mouse Housekeeping Genes PCR Array (Qiagen; PAMM-0002Z). 18sRNA was used for the urethane study and RPL13 for the smoke studies. Unless indicated, PCR analysis was conducted in triplicate.
Statistical analysis
For single comparisons, a two-sided t test was used to calculate P values. For multiple comparisons, ANOVA was used. P values are indicated in figure legends. All statistical analysis was done using GraphPad Prism software.

Results
Expression changes in prostacyclin targets and EMT genes are associated with CSC and iloprost exposure in HBEC cells. We used HBEC to investigate mRNA markers of CSC exposure that are reversed by iloprost treatment as potential markers of iloprost response during a chemoprevention treatment protocol. In HBEC exposed to 16 weeks of continuous CSC, PPARγ, 15PGDH, and CES1 expression decreased, whereas Cox-2 expression increased, suggesting that these targets of prostacyclin in the lung epithelium could be affected by exposure to tobacco smoke (Fig. 1A). In HBEC, we modeled current and former smokers to parallel the oral iloprost clinical trial. We exposed cells to 4 weeks of CSC or control and then removed or continued CSC for another 4 weeks (Fig. 1B). We added iloprost exposure alone at 4 weeks or after CSC was removed (former smoke with iloprost) (Fig. 1B). At 8 weeks, RNA was extracted from all cells for qPCR analysis.

PPARγ expression increased with iloprost compared with control and was lower than control with current or former CSC exposure (Fig. 1C). When iloprost was added after CSC was removed (former smoke with iloprost), PPARγ level increased over any CSC exposure, almost reaching control level. 15PGDH is a prostaglandin pathway enzyme that degrades tumor-promoting PGE2, is inversely associated with COX-2, and decreased in lung cancer (16). 15PGDH expression increased with iloprost alone or with iloprost after CSC exposure but remained at control level with continued CSC exposure (Fig. 1C). CES1 is an enzyme involved with drug metabolism shown to have increased expression in prostacyclin synthase transgenic mice compared with wild type (17, 18). In our HBEC model, CES1 increased with iloprost but remained low with any CSC exposure, suggesting increased length of iloprost treatment may be necessary to overcome CSC induced repression (Fig. 1C). COX-2 expression is reduced by PPARγ activation and has been associated with worse outcome in for NSCLC, whereas its inhibition in combination with chemotherapy increases overall survival for advanced NSCLC (19, 20). COX-2 expression decreased with iloprost and significantly increased with continued CSC exposure. When CSC was...
removed, COX-2 expression decreased, an effect that was not significantly augmented by the addition of iloprost (Fig. 1C). 

EMT changes occur early in the development of lung cancer and EMT is targeted by PPARγ, so we also measured a panel of EMT-related genes (11, 21). After 16 weeks of CSC exposure in HBEC, mesenchymal markers SNAIL and VIMENTIN increased compared with control whereas epithelial markers ECADHERIN and CRB3 decreased (Fig. 2A). PPARγ inhibits EMT in part by suppressing SNAIL and VIMENTIN expression and increasing Ecadherin in NSCLC (10, 11, 22). CRB3 is essential for normal epithelial polarity, is destabilized by Snail, and suppresses epithelial tumor progression (23, 24). In the 8-week HBEC model (depicted in Fig. 1B), 4 weeks of iloprost decreased SNAIL expression and increased ECADHERIN and CRB3 (Fig. 2B). Current smoke increased SNAIL and VIMENTIN, while decreasing ECADHERIN and CRB3. When CSC was removed (former smoke), expression of SNAIL and VIMENTIN returned to control levels, whereas ECADHERIN and CRB3 remained low. When 4 weeks of iloprost was added to former smoke cells, SNAIL and VIMENTIN remained at control levels, whereas ECADHERIN and CRB3 increased over former smoke.

Prostacyclin and tobacco carcinogens lead to changes in prostacyclin targets and EMT gene expression in murine models of lung cancer chemoprevention. Preclinical models are essential to generating evidence for chemoprevention clinical trials and we interrogated two models for prostacyclin marker data. In the urethane mouse model (depicted in Fig. 1B), mRNA expression was measured by qPCR in triplicate, normalized to GAPDH, and compared with vehicle control. In the HBEC smoke model (depicted in Fig. 1), mRNA expression was measured by qPCR in triplicate, normalized to GAPDH, and compared with vehicle control and is represented as fold change. "", vs. control, P-value ≤ 0.05. mRNA expression was measured by qPCR in triplicate, normalized to GAPDH, and compared with vehicle control.

Figure 2. EMT markers are altered by CSC and iloprost exposure in HBEC. A, HBEC cells were treated in triplicate with 5 μg/ml CSC or vehicle for 16 weeks. *, vs. control, P-value ≤ 0.05. B, in the HBEC smoke model (depicted in Fig. 1), mRNA expression was measured by qPCR in triplicate, normalized to GAPDH, and compared with vehicle control and is represented as fold change. *, vs. control, P-value ≤ 0.05.

whether early lesion development resulted in marker changes. With urethane exposure, ECADHERIN, CRB3, PPARγ, CES1, and 15PGDH decreased whereas VIMENTIN, SNAIL, and COX-2 increased, suggesting a switch toward a mesenchymal state and progression toward malignancy (Fig. 3A–H). The level of prostacyclin in the Pgistg mice resulted in rescue of urethane induced changes in ECADHERIN, PPARγ, CRB3, 15PGDH, COX-2, SNAIL, and VIMENTIN (Fig. 3A–H). In the 1-week smoke exposed model, mice are harvested after 1 week of smoke exposure or ambient air. After 1 week of smoke, FVB mice have early gene expression changes associated with lung cancer but do not yet have lesions (14). With smoke exposure, SNAIL, VIMENTIN, and COX-2 increase, whereas ECADHERIN, CRB3, and PPARγ decrease (Fig. 3A–H). Pgistg mice with smoke exposure reversed this trend for ECADHERIN, PPARγ, CRB3, 15PGDH, CES1, VIMENTIN, SNAIL, and COX-2 (Fig. 3A–H). Gene expression analysis in whole lung from these mouse models of chemoprevention revealed a trend toward decreased epithelial markers, increased mesenchymal markers, and decreased PPARγ signaling with carcinogen exposure. Increased prostacyclin reversed this trend.

Tobacco carcinogens and prostacyclin alter expression of MIR34c and MIR22 in vitro and in vivo. We investigated MIR34c and MIR221 as potential markers of iloprost activity based on our initial screens and previous studies linking their dysregulation to lung cancer (14, 25–29). In HBEC treated for 2 weeks, MIR34c expression increased with iloprost but did not change with CSC (Fig. 4A). Longer CSC exposure was required to observe a decrease in MIR34c expression (Fig. 4B). MIR221 expression increased with CSC at both 2-week and 16-week time points and decreased with 2 weeks of iloprost treatment (Fig. 4A and B). In our 8-week HBEC model of iloprost and smoke exposure (depicted in Fig. 1B), iloprost alone increased
MIR34c expression while current exposure to smoke decreased expression (Fig. 4C). Removing CSC increased MIR34c expression and adding iloprost to former smoke cells led to an additional small increase. There was a large increase in MIR221 with CSC exposure that decreased with removal of CSC and further decreased with iloprost treatment (Fig. 4D).

We also examined MIR34c and MIR221 expression in our in vivo lung cancer models using PGIStg mice. A very small decrease in MIR34c expression occurred with urethane in the tumor model, but a significant increase occurred with Pgis overexpression (Fig. 5A). However, in urethane exposed mice, Pgis overexpression did not increase MIR34c expression, mir-34c decreased with 1 week of smoke exposure, increased in Pgis t mice, and was slightly elevated with increased prostacyclin over smoke exposure alone (Fig. 5B). Reinforcing the observed inverse expression pattern compared with MIR34c, MIR221 increased with urethane and smoke exposure and was lower with increased prostacyclin (Fig. 5C and D). Results from both in vitro and in vivo models indicate MIR34c and MIR221 may be markers for prostacyclin chemopreventive activity.

Gene expression changes in human primary dysplastic cells parallel in vitro and in vivo models. To expand marker analysis to primary human samples, we identified three dysplastic cell bronchial epithelial cultures from biopsy sites with high grade dysplasias. These types of higher grade biopsies have been associated with an increased risk of developing invasive squamous lung carcinoma (30).
To determine the effects of iloprost on dysplastic cells, we cultured the biopsies and treated them with iloprost for 2 weeks. We then used qPCR to measure expression of our panel of iloprost activity markers. In general, the three iloprost treated dysplastic cultures demonstrated similar expression patterns to our in vitro and in vivo models. Fig. 6A–C show expression data with SEM for the replicate treatments within each culture, whereas Fig. 6D shows average expression data of the three dysplasias for each marker. Expression of prostacyclin targets associated with antitumor signaling increased with iloprost, while COX-2 decreased (Fig. 6A). EMT markers trended toward decreased mesenchymal marker expression and increased epithelial marker expression with iloprost (Fig. 6B). Agreeing with the inverse expression pattern identified in our other models, MIR34c increased slightly with iloprost whereas MIR221 decreased (Fig. 6C). In Fig. 6D, the data from dysplasias is displayed in aggregate for each target to compare average expression across all markers and to highlight those with the strongest marker potential. Data from these primary cultures suggest that iloprost reverses gene expression changes associated with cigarette smoke exposure, likely leading to interception of dysplasia progression, and support the potential of these markers for clinical application.

**Discussion**

The prostacyclin analogue iloprost is an agent with strong potential as lung cancer chemoprevention. Significant preclinical data supported moving this agent to clinical trials (4, 5, 14, 15, 31). Former smokers treated with iloprost in a phase II clinical trial had improved endobronchial dysplasia, a premalignant lesion linked with development of squamous cell carcinoma (6, 30). This trial demonstrated that intermediate endpoints in the lung can measure the effectiveness of chemoprevention. Of the 48 patients who received iloprost in the chemoprevention trial, 23 had regressive histology and 25 had stable or progressive histology, pointing to the potential for a precision medicine approach (6). Identification of predictive markers could improve future clinical trials of iloprost by limiting patients enrolled to those likely to respond, thereby reducing the trial cost, sample size, duration,
and improving detection of a clinical effect. Reducing the invasiveness of the treatment protocol by replacing bronchoscopies with blood or sputum iloprost activity marker assays would improve interest, compliance, and cost of future chemoprevention trials. Even though prostacyclin is the subject of one of very few successful phase II lung cancer chemoprevention trials, iloprost as a chemoprevention agent requires refinement of its application and improved understanding of its mechanisms. This refinement may come in the form of delivery (oral vs inhaled), drug choice (iloprost vs. next-generation prostacyclin analogues), mechanism (manipulating drug receptors or targets), or biomarkers (response predicting or treatment monitoring).

Prostacyclin activates PPARγ in lung epithelial cells (15, 31). PPARγ has been investigated for its role in many aspects of lung cancer, including as chemoprevention or treatment (32). PPARγ inhibits lung cancer proliferation, promotes sensitivity to targeted therapy, and sensitizes cells to chemotherapy (33–35). In lung epithelial cells, PPARγ is activated by WNT7a binding to its receptor FZD9, leading to decreased transformed growth, a pathway also stimulated by iloprost (15, 22). FZD9 is the presumed receptor for iloprost in the lung epithelium and its expression is reduced in HBEC by 1 week of CSC exposure (14, 15). We identified FZD9 as a potential marker for predicting response to iloprost in high-risk subjects and are investigating its role in the mechanism of iloprost activity (14, 15). Although we found decreased PPARγ expression in response to tobacco carcinogens in this study, some lung tumors retain expression of PPARγ (36, 37). Another study found increased PPARγ expression in response to tobacco carcinogens in this study, some lung tumors retain expression of PPARγ (36, 37). Another study found increased PPARγ expression with KRAS/p53 alterations premalignant HBEC in vitro, but decreased expression when tumorigenic clones of these cells were grown in vivo (20). In addition, activation of PPARγ in macrophages may stimulate metastasis after primary tumor development (38). PPARγ expression during lung cancer progression is likely context and time dependent, so it will be important to establish its expression and activity in the earliest stages of lung lesions for chemoprevention approaches that activate PPARγ.

With our in vitro and in vivo chemoprevention models and primary cells, we tested markers across a range of tobacco-carcinogen and prostacyclin exposures. In a targeted approach, we selected activity markers to test based

Figure 5. MIR34c and MIR221 expression changes with exposure to prostacyclin and tobacco carcinogens in vivo. qPCR was used to measure gene expression in RNA extracted from murine whole lung samples. A and C, 20-week urethane mice were exposed to one injection of urethane or saline and sacrificed 20 weeks later (N = 5/group). B and D, Mice were exposed to 1 week of mainstream cigarette smoke before lungs were harvested (N = 5/group). A and B, MIR34c expression. C and D, MIR221 expression. Targets were measured in triplicate, normalized to RNU6 and compared with saline controls. *, vs. control; #, vs. PGIS; ^, vs. smoke P-value <0.05.
on known prostacyclin pathways. PPARγ expression increased with iloprost treatment, suggesting it could be an activity marker for iloprost and that iloprost may use a positive feedback loop to increase response. 15pgdh, COX-2, and CES1 are all known targets of prostacyclin and we demonstrated that their expression is also altered by iloprost (4). EMT pathways are targeted by prostacyclin through PPARγ, offering another set of potential activity markers (11). We found that SNAIL, VIMENTIN, ECADHERIN, and CRB3 are altered by iloprost after exposure to tobacco carcinogens, suggesting that reversal of EMT may be an important mechanism for iloprost chemoprevention activity.

As biomarkers, miRNA have strong potential because they are highly stable and exhibit tissue specificity. They have been proposed as markers in tissue and liquid biopsies for diagnosis, prognosis, and therapeutic approaches (39). miRNA expression has been associated with smoke exposure, stages of lung cancer progression, lung tumor aggressiveness, and treatment resistance (8, 39, 40). Animal models have demonstrated that chemopreventive agents, such as N-acetyl-l-cysteine or phenethyl isothiocyanate, can modulate miRNA in vivo in the context of smoke exposure (41). However, little is known about miRNA targets of prostacyclin or PPARγ. We previously identified MIR31 as associated with iloprost binding to FZD9, suggesting that miRNA do play an important role in iloprost signaling (14). A growing understanding of these master regulators in lung cancer and their suitability as biomarkers warrants a thorough investigation of miRNA in lung cancer chemoprevention.

Low expression of lung or circulating MIR34 family members is associated with poor prognosis, metastasis, and relapse (26, 27). MIR34 directly targets PD1, suggesting potential interactions between iloprost chemoprevention and current immunotherapies (42). MIR221 is included in profiles for lung cancer detection and has an oncogenic role in lung tumor progression (29, 43). Also, MIR221 expression is inhibited by prostacyclin in vascular cells (44). In this study, we found MIR34c expression was decreased with exposure to tobacco...
carcinogens and increased with iloprost or prostacyclin overexpression, whereas MIR221 demonstrated inverse changes. In our mouse models, we observed that increased prostacyclin led to increased MIR34c only in a model of very early lesions and not in a tumor model. These data suggest that after carcinogen exposure, prostacyclin may only increase MIR34c in the context of early lesions and that MIR34c may be resistant to prostacyclin-induced changes once tumors develop. Changes in MIR221, however, were observed in both the tumor and premalignant mouse models, suggesting that MIR221 may be involved continuously in lesion progression and could reflect prostacyclin activity throughout progression. Decreases in MIR221 in urethane or smoke exposed PGIS mice were modest but might be stronger in a smoke model with longer exposure.

In a previous qPCR study, expression of MIR34c progressively decreased from normal epithelium of non-smokers to invasive squamous bronchial lesions of smokers (25). Examination of MIR34c in biopsies from the oral iloprost chemoprevention trial also found that expression was inversely correlated with histologic grade and with current smoke exposure (45). This supports our observation of a significant decrease in MIR34c expression in HBEC after 16 weeks of CSC and a slight increase with removal of CSC. MIR34c was associated with response in both treatment and placebo arms in this study on oral iloprost clinical trial samples, so the authors did not find that MIR34c predicts response to iloprost or function as an intermediate endpoint marker (45). Because of design limitations from the previous study and based on our preclinical data, further studies are warranted to determine whether MIR34c could serve as an activity marker for iloprost chemoprevention. This also points to the importance of confirming that chemoprevention drugs target the lesions most likely to progress to carcinoma, rather than lesions that would have regressed on their own. In small clinical studies, molecular testing results could be confounded by patients with low-grade lesions more likely to spontaneously regress. Ongoing studies are characterizing bronchial dysplasias as likely to progress or regress and this data can be combined with predictive markers to precisely apply chemoprevention (30, 46).

Our current murine chemoprevention studies are limited by lack of access to oral iloprost, however, similarities in mechanism between prostacyclin and iloprost, identified by comparing cells treated with iloprost to Pgdstg mice, support continued studies using the Pgdstg mouse. We were unable to directly associate marker changes with reduced dysplasia in our models, although in the mouse urethane model, increased prostacyclin and changes in marker expression are associated with decreased tumor burden (18). Significance of our in vitro experiments was limited, however, the data does fit a trend supporting the concept that in HBEC, iloprost activity is associated with increased expression of anti-cancer markers and decreased expression pro-cancer markers. Dysplastic cultures could only be treated with iloprost for 2 weeks, which also may not be enough time to see significant changes across all markers in these premalignant cells. Ongoing work will measure potential response prediction and activity markers in the oral iloprost chemoprevention trial, which can be validated in samples from the ongoing inhaled iloprost chemoprevention trial.

Over half of lung cancers are diagnosed in former smokers, patients who have already made the most important change for lung cancer prevention but remain at high risk (1, 47). In this population, likely to have multiple areas of premalignant, mildly altered lung cells, interception of progression with chemoprevention may have a stronger impact on mortality than treatment of highly mutated, established lung cancers. High-grade bronchial dysplasias associated with squamous carcinoma are most likely to be identified by bronchoscopy and biopsy. However, patients may develop carcinomas at sites distant from biopsies, highlighting the need for systemic chemoprevention, as opposed to localized therapy (30). Implementation of chemoprevention for lung cancer has been challenged by multiple negative trials and by the economic and health risks inherent in treating large, at-risk populations for extended periods of time. Improved identification of the highest risk populations, and the highest risk lesions, will allow for more effective clinical trial design and targeted chemoprevention. A recent search of Clinicaltrials.gov revealed that for every one lung cancer chemoprevention trial there are over 150 lung cancer therapy trials. Even with this focus on treatment, 5-year survival for lung cancer has remained around 15% for decades (1). To significantly reduce lung cancer mortality, we must improve application of existing strategies, such as prostacyclin, and increase the development of new prevention approaches.

Disclosure of Potential Conflicts of Interest
R.L. Keith has ownership interest (including stock, patents, etc.) in U.S. Patent No. 8,623,917 (issued January 7, 2014). Uses of prostacyclin analogs for cancer chemoprevention. No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.L. New, P. McGonigle, R.L. Keith, M.A. Tennis
Analysis and interpretation of data (e.g., statistical analysis, bios- statistics, computational analysis): M.L. New, C.M. White, L.D. Dwyer-Nield, R.L. Keith, M.A. Tennis
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