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PGE2-Driven Expression of c-Myc and OncomiR-17-92 Contributes to Apoptosis Resistance in NSCLC

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Abstract

Aberrant expression of microRNAs (miRNA) with oncogenic capacities (oncomiRs) has been described for several different malignancies. The first identified oncomiR, miR-17-92, is frequently overexpressed in a variety of cancers and its targets include the tumor suppressor PTEN. The transcription factor c-Myc (*MYC*) plays a central role in proliferative control and is rapidly upregulated upon mitogenic stimulation. Expression of c-Myc is frequently deregulated in tumors, facilitating proliferation and inhibiting terminal differentiation. The c-Myc–regulated network comprises a large number of transcripts, including those encoding miRNAs. Here, prostaglandin E2 (PGE2) exposure rapidly upregulates the expression of the *MYC* gene followed by the elevation of miR-17-92 levels, which in turn suppresses PTEN expression, thus enhancing apoptosis resistance in non–small cell lung cancer (NSCLC) cells. Knockdown of *MYC* expression or the miR-17-92 cluster effectively reverses this outcome. Similarly, miR-17-92 levels are significantly elevated in NSCLC cells ectopically expressing COX-2. Importantly, circulating miR-17-92 was elevated in the blood of patients with lung cancer as compared with subjects at risk for developing lung cancer. Furthermore, in patients treated with celecoxib, miR-17-92 levels were significantly reduced. These data demonstrate that PGE2, abundantly produced by NSCLC and inflammatory cells in the tumor microenvironment, is able to stimulate cell proliferation and promote resistance to pharmacologically induced apoptosis in a c-Myc and miR-17-92–dependent manner.

Implications: This study describes a novel mechanism, involving c-Myc and miR-17-92, which integrates cell proliferation and apoptosis resistance. *Mol Cancer Res; 12(5); 765–74. ©2014 AACR.*

Introduction

Among the fundamental hallmarks of cancer, the capacity to sustain cancer cell proliferation is critical (1). Chronic proliferation is mediated by several mechanisms, including deregulated growth factor production, abnormal surface receptor expression, loss of negative regulators of cell proliferation due to mutagenesis and transcriptional or epigenetic silencing of tumor suppressors. Importantly, mitogenic signaling by prostaglandin E2 (PGE2)–dependent cross-activation of mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (Erk) pathway has been recently shown to promote such hallmarks of cancer as enhanced cell proliferation and apoptosis resistance (2–5). The c-Myc transcription factor plays a central role in the control of cellular proliferation (6, 7), and is rapidly upregulated upon mitogenic stimulation (8). In contrast, antiproliferative signals have been shown to trigger prompt reduction of c-Myc levels due to the transcriptional block and mRNA turnover (9, 10). *MYC* is among the most frequently deregulated proto-oncogenes in a variety of human malignancies (11). The causal role of *MYC* gene deregulation in cancer development and metastasis has been established in early seminal studies (12, 13). Recently, c-Myc has been identified as a driver of gene signatures associated with poor prognosis in different cancers, including lung cancer, and a necessary component of metastatic cell behavior (14–16). In keeping with these findings, c-Myc overexpression has been reported to be a constituent of a stem cell–like signature in aggressive poorly differentiated tumors (15).

Interestingly, a relationship between c-myc and PTEN has been recently established (17). PTEN antagonizes phosphoinositide 3-kinase (PI3K) signaling that is necessary for the activation of Akt kinase involved in cell survival, and thus increases sensitivity of cells to apoptotic stimuli (18). Lack of functional redundancy makes *PTEN* a central (and

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haploinsufficient) tumor suppressor in various tumor types (19). PTEN expression is regulated at multiple levels, including transcription, mRNA stability, microRNA (miRNA) targeting, translation, and protein stability. *PTEN* is one of the most frequently inactivated tumor-suppressor genes (19, 20), and loss of its expression drives tumor development through deregulation of PI3K signaling, thus attributing the cells to the second hallmark of cancer, resistance to cell death (1). Similarly, in murine models, inactivation of one allele of *PTEN* gene increased the incidence of cancer, including lung cancer (21). Recent studies demonstrate that PTEN is also critical for maintaining the stem cell phenotype, and loss of PTEN can contribute to malignant transformation (22, 23).

miRNAs are small regulatory RNAs that control gene expression at the posttranscriptional level and thus serve important roles in a variety of normal and pathologic processes in a wide range of organisms (24, 25). Recent studies indicate that deregulation of miRNAs is implicated in the pathogenesis of cancer and metastasis (26, 27). Various miRNAs can function as either tumor suppressors or oncogenes; the latter are often referred to as oncomiRs. The first characterized oncomiR, miR-17-92 cluster, encodes seven mature miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a, and is overexpressed in various cancers, and is a direct transcriptional target of c-myc (28, 29). Recent studies have shown that ectopic expression of the miR-17-92 cluster attenuates differentiation and promotes proliferation of lung progenitor cells in transgenic mice (30). Importantly, *PTEN* has been identified as one of the main targets of the miR-17-92 cluster, specifically, of miR-19 (31). Studies of the individual contribution of each miRNA to the oncogenic properties of the miR-17-92 cluster identified miR-19, and to a lesser extent miR-18, as the predominant oncogenic miRNA of the cluster (32, 33).

Recent studies demonstrate that miRNAs are present not only intracellularly but also in the peripheral blood as circulating miRNA and may reflect those in tumor tissue extracts (34). miRNA profiling in both solid tumors and peripheral blood has been used successfully for cancer detection (35, 36). miRNAs have been shown to be more robust biomarkers of disease processes than mRNA-based biomarkers (37).

In the current study, we describe a novel mechanism of PGE2-dependent apoptosis resistance that includes c-myc–mediated miR-17-92 upregulation and repression of *PTEN* expression. We report that PGE2 treatment rapidly induces c-myc expression in non–small cell lung cancer (NSCLC) cells. In accord with the previously reported data suggesting that miR-17-92 cluster expression is regulated by c-Myc, our studies reveal that this cluster is also rapidly upregulated upon PGE2 treatment in a time-dependent manner. Concomitantly, we discovered that the levels of *PTEN* expression are reduced and the pattern of this reduction concurs with upregulation of the miR-17-92 cluster. Finally, we demonstrated that the levels of the circulating miR-17-92 were elevated in plasma of patients with lung cancer compared with subjects at risk for developing lung cancer. In patients with lung cancer treated with the COX-2 inhibitor celecoxib, the miR-17-92 levels were significantly reduced following 8 weeks of treatment compared with the baseline. Our studies identify a novel mechanism integrating cell proliferation and apoptosis resistance mediated by PGE2 in lung cancer.

Materials and Methods

Reagents

16,16-Dimethyl-PGE2 was purchased from Cayman Chemical. Other reagents were purchased from Sigma Chemicals unless otherwise specified.

Cell culture

The human squamous cell carcinoma NSCLC cell line H157, adenocarcinoma H460 (American Type Culture Collection, ATCC), and RH2 (previously established in our laboratory; ref. 38) were grown in RPMI supplemented with 10% FBS (Gemini Bio-Products). H157 cells with ectopic expression of COX-2 were generated as previously described (39). Cells used in these studies were passaged no more than eight times. The cells were genotyped regularly (usually, every 3 months) using the Promega Cell ID system (Promega). All cell lines were tested and found negative for mycoplasma contamination (MycoAlert Mycoplasma Detection Kit; Lonza).

Western blotting

Cells were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce). Western blotting was performed as previously described (40). Briefly, proteins were separated by SDS-PAGE and transferred to the nitrocellulose membrane (Amersham Biosciences). Membranes were blocked in 5% Blotto (Santa Cruz Biotechnology) in TBS + 0.1% Tween-20, incubated with the primary antibodies (Cell Signaling Technology) diluted 1:1,000 in blocking solution, followed by the incubation with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz; 1:10,000). The blots were developed using the SuperSignal West Pico Kit (Pierce).

Human plasma samples

This study used plasma samples from the University of California, Los Angeles (UCLA) Lung Cancer SPORE biorepository and samples collected in a randomized phase II trial evaluating the efficacy of erlotinib plus celecoxib combination in advanced NSCLC (41). Samples were collected at UCLA or City of Hope, Duarte, CA, respectively, in accordance with the Institutional Review Board requirements. Blood was centrifuged in Vacutainer tubes at 3,000 rpm for 15 minutes within 1 hour of collection, and plasma was aliquoted and stored at −80°C.

PGE2 assay

PGE2 levels in NSCLC cell culture supernatants were assessed using the PGE2 EIA Kit (Cayman Chemical) and normalized to the total RNA concentrations.

Circulating miRNA isolation and analysis

Circulating miRNA was isolated from 400 μL of plasma using the miRNeasy Kit (Qiagen). After addition of the lysis reagent, 2.5 fmol of synthetic *Caenorhabditis elegans* miRNAs (cel-39, cel-54, and cel-238) were added to the samples as spike-in normalization controls, as previously described (37). Circulating miRNA levels were assessed using (i) the miR-ID platform (Somagenics; ref. 42) for the data represented in Fig. 3A and B) and (ii) TaqMan assays (Life Technologies) in phase II trial patients. Data were normalized using spike-in controls or RNA concentration (determined using the Quant-iT RiboGreen Kit; Life Technologies), respectively. Relative expression was calculated using the delta–delta Ct (ΔΔCt) method (43).

Cellular RNA isolation and analysis

Total RNA was isolated from NSCLC cells using the miRNeasy Kit. For mRNA, reverse transcription (RT) was performed using iScript kit (Bio-Rad) and 2 μL of the cDNA were used for subsequent PCR. For miRNA, RT was performed using the TaqMan MicroRNA RT Kit. Quantitative reverse transcription PCR (qRT-PCR) was performed using TaqMan assays in the conditions recommended by the manufacturer. Normalization controls were used as follows: for mRNA, glucuronidase (GUS) or beta-2-microglobulin (B2M); for miRNA, Z30, RNU44, or RNU6B small nucleolar RNAs. All reactions were performed in triplicates. Relative expression was calculated using the ΔΔCt method.

Next-generation RNA sequencing

RNA sequencing (RNA-seq) was performed on circulating miRNA isolated from pooled 400 μL aliquots of plasma from 30 high-risk control subjects and 30 patients with advanced-stage lung cancer from the UCLA Lung Cancer SPORE biorepository. The libraries were prepared using Illumina's TruSeq Small RNA Sample Preparation kit as described by the manufacturer. Each sample was sequenced on one lane of an Illumina HiSeq instrument to generate approximately 60 million 35-bp reads. Initial data processing was done using Illumina Pipeline SCS 2.9. Adapters were trimmed from the reads using FASTX clipper 0.0.13, and reads were aligned to hg19 using Bowtie 0.12.7. BEDTools 2.9.0 was used to obtain expression counts and the data were reads-per-million normalized.

C-Myc knockdown

Stable c-Myc knockdown was performed using Mission lentiviral short hairpin RNA (shRNA) constructs in pLKO.1 vector (Sigma). Three constructs (TRCN0000010389, TRCN0000039640, and TRCN0000174055) targeting different regions of c-myc transcript were used. The RNAi Consortium (TRC) nonhairpin and scrambled shRNA constructs in pLKO.1 vector were used as negative controls. Briefly, the viral particles were produced in genotyped and mycoplasma-negative 293T cells (ATCC) by cotransfection of the shRNA construct, pMD2 viral envelope, and pSPAX2 packaging plasmids (Addgene) using the Calcium Phosphate Transfection Kit (Clontech). Next day, the medium was replaced with the fresh growth medium and the cells were incubated for another 48 hours before the viral supernatants were collected, filtered through 0.45 μm filters, and used for transduction of H157, H460, and RH2 NSCLC cells. The cells were plated at 10^5 cells/well in 12-well plates. The following day, viral supernatant + 10 μg/mL polybrene were added to the cells for 24 hours, followed by selection with 1 μg/mL puromycin. C-Myc knockdown was confirmed by Western blotting analysis (Supplementary Fig. S1).

miRNA inhibition by anti-miR

Anti-miR miRNA inhibitors and Anti-miR Negative Control #1 were purchased from Life Technologies. Transfection was performed using Lipofectamin 2000 reagent (Invitrogen). Briefly, (i) the day before the transfection, 2×10^5 cells were plated in 6-well plates; (ii) on the day of transfection, the medium was changed to 1.6 mL of Opti-MEM; (iii) 80 pmol of anti-miR was diluted in 200 μL of Opti-MEM; (iv) 4 μL of Lipofectamin 2000 was diluted in 200 μL of Opti-MEM; and (v) Anti-miR and Lipofectamin suspensions were mixed together, incubated for 20 minutes at room temperature, and added to the cells for 24 hours. The efficacy of transfection was monitored by the fluorescent microscopy using Cy3 dye–labeled Negative Control #1 (Life Technologies).

Apoptosis assay

Apoptosis was induced by staurosporine treatment. For the apoptosis assays, the cells were plated in T25 flasks at 3.5×10^5 cells and allowed to adhere overnight. The next day, the cells were transfected with Anti-miR miRNA inhibitors or Anti-miR Negative Control #1 for 4 hours, as described above (all volumes were scaled up 2-fold). Four hours later, PGE2 was added at 5 μg/mL where applicable without changing the medium, and the cells were incubated overnight. On the following day, apoptosis was induced where applicable by adding 1 μmol/L (H157) or 0.4 μmol/L staurosporine (H460 and RH2 cells) for 60 minutes. The control cells were treated with the equivalent concentration of the diluent (dimethyl sulfoxide, DMSO). Following the treatment, the cells were washed twice with prewarmed PBS and the fresh culture medium was added. Cells were incubated overnight and the next morning, the apoptosis was assessed using Annexin V/PI kit (BD Biosciences) according to the manufacturer's instructions. Both the culture mediums containing the floating cells and the adherent cells were collected and combined for the apoptosis analysis. The analysis was performed using the FACScan flow cytometer and CellQuest software (BD Biosciences). All experiments included the nonstained and single-stained controls and were performed in triplicates.

Statistical analysis

All statistical analyses were performed using SAS v.9.3 (SAS Institute) and SPSS v.21 (IBM Corp.). For circulating miRNA, we tested the celecoxib arm for differences between pre- and posttreatment Ct values for both miR-19b and

miR-92a using a paired samples *t* test. Differences between groups were compared using a two-sample *t* test. Differences were considered significant for *P* values < 0.05. Significance values indicated in the figures are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Graphed data represent mean ± SEM.

Results

PGE2 exposure upregulates c-Myc expression in NSCLC cells

To elucidate the molecular mechanisms of PGE2-dependent NSCLC cell proliferation demonstrated in our previous studies (5), we evaluated the expression of several key positive regulators of the cell cycle in a subset of NSCLC cells that were previously shown to be responsive to PGE2 treatment. We found that treatment of H157, H460, and RH2 NSCLC cells with physiologically relevant concentrations of PGE2 induced rapid upregulation of c-Myc expression (Fig. 1). An increase in c-myc protein was also detected by Western blotting analysis after 1 hour of treatment, and the elevated protein levels were present throughout the 3-hour exposure (Fig. 1A). Similarly, an increase of *CMYC* gene expression was detected by qPCR 60 minutes following PGE2 exposure and the c-Myc mRNA levels remained elevated following 3 hours of treatment (Fig. 1B). C-Myc is a short-lived protein and our findings indicate that PGE2 induces its expression at the transcriptional level. Among other cell cycle–related proteins, we found similar patterns of time-dependent cyclin D1 and c-Fos upregulation in response to PGE2 treatment (data not shown). Such upregulation of c-Myc, as an immediately early responder to mitogenic signals, as well as other positive regulators of the cell cycle, is consistent with our previously reported findings demonstrating prolonged Erk kinase phosphorylation upon PGE2 treatment (5).

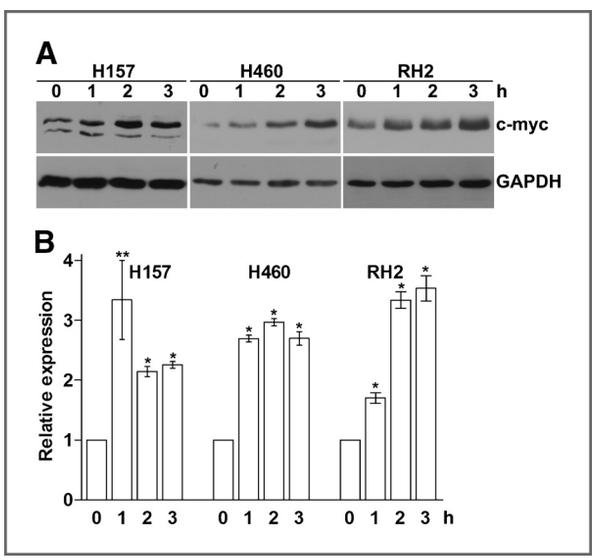

Figure 1. Induction of c-Myc expression in NSCLC cells by PGE2. H157, H460, and RH2 NSCLC cells were serum deprived for 3 hours and treated with 5 μg/mL PGE2 in the fresh serum-free medium for 0, 1, 2, or 3 hours. The cells were then harvested and total RNA and proteins were isolated. Upregulation of c-Myc following the PGE2 treatment was detected at transcriptional and protein levels starting at 60 minutes after treatment and for the entire 3 hours of treatment. A, proteins were separated by SDS-PAGE and c-Myc was detected by immunoblotting. Representative of two independent experiments is shown. B, c-Myc mRNA expression was assessed by TaqMan qRT-PCR. *GUS* (H157 and H460) or *B2M* (RH2) expression was used as internal control. Expression was normalized to that observed in the untreated group (0 hour). *, $P < 0.05$; **, $P < 0.01$.

PGE2 upregulates the miR-17-92 cluster in a c-Myc–dependent manner

Given the previously established association between c-Myc and multiple miRNAs involved in cell cycle control and apoptosis resistance, we hypothesized that PGE2 may affect the expression of the miR-17-92 cluster. We assessed the expression of several key members of the miR-17-92 cluster to determine if the polycistron was upregulated upon PGE2 exposure. Previous studies suggested that the predominant mediators of the oncogenic properties of miR-17-92 are miR-19a and 19b (32, 33), for which sequences differ by one nucleotide. Therefore, we assessed the PGE2-dependent expression of miR-19b and another member of the cluster, miR-18a (data not shown), in H157, H460, and RH2 NSCLC cell lines. The expression of both miR-19b and miR-18a followed the upregulation of c-Myc and was rapidly elevated following the PGE2 exposure in a time-dependent manner (Fig. 2A). Because the dynamics of the expression of different cluster members were very similar, in our subsequent studies we evaluated miR-19b as a representative member of the miR-17-92 cluster. To verify that the expression of the miRNA cluster was c-Myc dependent, we stably knocked down c-Myc expression in NSCLC cells using the shRNA technique. Three different knockdown constructs that expressed shRNAs targeting different regions of c-Myc transcript were used. Only the constructs that demonstrated a substantial inhibition of c-Myc expression were used in the subsequent experiments. After the stable c-Myc knockdowns were established, we compared their basal levels of miR-18a and miR-19b with the cells transduced with the nonsilencing control constructs. We found that both miR-18a and miR-19b basal levels were significantly reduced in c-Myc knockdown cells (Fig. 2B). Then, we assessed miR-19b expression with and without PGE2 treatment. Knockdown of c-Myc expression reversed the PGE2-driven miR-17-92 cluster upregulation (Fig. 2C). These data support the notion that the miR-17-92 cluster is a transcriptional target for c-Myc and interference with the c-Myc expression in NSCLC cells leads to significant reduction in the level of these oncomiRs.

Ectopic expression of COX-2 leads to an increase in miR-17-92 expression

To assess the importance of COX-2 expression and PGE2 production in regulating miR-17-92 expression, we used H157 NSCLC cell line ectopically expressing COX-2, which produces nearly 10-fold higher levels of PGE2 than do the vector control cells. Exposure of the H157-COX-2 cells to celecoxib led to a significant reduction in PGE2 production (Fig. 2D). We found that compared with the

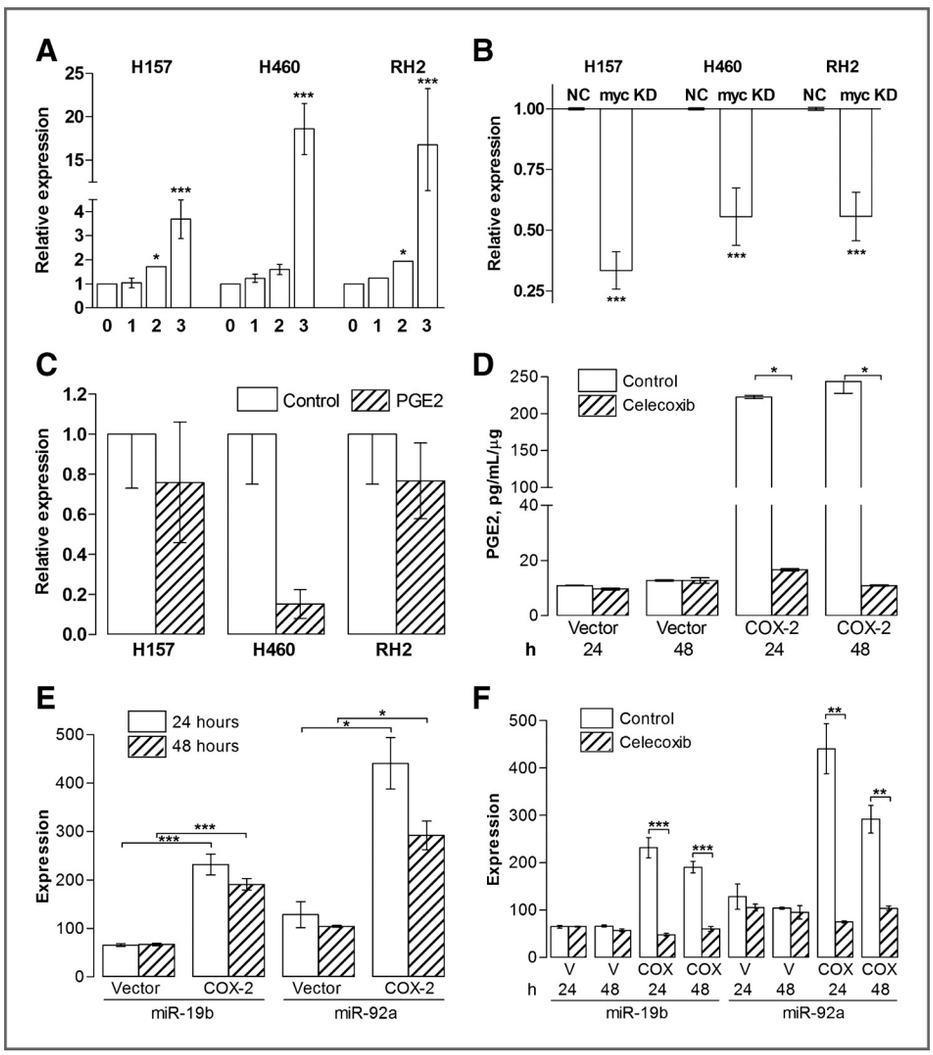

Figure 2. Expression of miR-19b is upregulated by PGE2. A, miR-19b is upregulated by PGE2 treatment in a time-dependent manner in NSCLC cells. H157, H460, and RH2 cells were serum deprived for 3 hours and treated with 5 μg/mL PGE2 in the fresh serum-free medium for 1, 2, or 3 hours followed by total RNA isolation and TaqMan qRT-PCR for miR-19b and RNU44 (internal control). B, basal miR-19b levels are reduced in c-Myc knockdown (KD) H157, H460, and RH2 cells compared with nonsilencing construct-transduced control cells (NC). C, c-Myc knockdown abolishes PGE2-driven upregulation of miR-19b. C-Myc knockdown cells were serum deprived and treated with the diluent control or 5 μg/mL PGE2 in the fresh serum-free medium for 3 hours. D, PGE2 levels are elevated in H157 cells with ectopic COX-2 expression compared with vector control cells and are significantly reduced by incubation with 2.5 μmol/L celecoxib for 24 or 48 hours. PGE2 data were normalized to the total RNA concentration. E, ectopic COX-2 expression leads to the upregulation of the miR-17-92 cluster. qRT-PCR was performed on RNA from vector control and COX-2 overexpressing H157 cells. F, celecoxib treatment (2.5 μmol/L for 24 or 48 hours) leads to the miR-17-92 downregulation in COX-2–overexpressing cells (COX) compared with vector control (V) as determined by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

vector control cells, H157-COX-2 cells had significantly elevated levels of miR-19b and miR-92a (Fig. 2E). When the cells were cultured in the presence of celecoxib (2.5 μmol/L for 24 or 48 hours), the levels of the miR-17-92 expression were significantly reduced (Fig. 2F). These results further corroborate that the miR-17-92 cluster expression is mediated by PGE2 in NSCLC.

The levels of the miR-17-92 cluster are elevated in patients with NSCLC

Using our own studies and the published work of others, we developed a panel of miRNAs comprising 17 miRNAs highly relevant to lung carcinogenesis, including two members of the miR-17-92 cluster, that is, miR-17a and miR-20a. In our initial study using this miRNA panel, we assessed circulating miRNA levels in plasma of five subjects at high risk for developing lung cancer and 5 patients with advanced-stage lung cancer from the UCLA Lung Cancer SPORE biorepository by qRT-PCR. Although the number of samples used in the study was insufficient to achieve a significant statistical power, there was an obvious trend towards elevated levels of both miR-17a and miR-20a in the blood of patients with lung cancer (data not shown). Then, we performed RNA-seq of circulating miRNA isolated from pooled plasma samples from 30 high-risk control subjects and 30 patients with advanced-stage lung cancer. RNA-seq yielded a high number of reads with approximately 87% of reads aligning. Out of the aligned reads, approximately 29% aligned to miRNA. Our analysis revealed a number of miRNAs that were differentially expressed in cancer compared with high-risk controls. Among these, we found that the expression of the miR-17-92 cluster was elevated by greater than 2-fold in the advanced-stage cancer sample pool compared with the high-risk control pool (Fig. 3A).

Celecoxib reduces circulating miR-17-92 levels in patients with advanced-stage NSCLC

To further investigate the effect of celecoxib treatment on the levels of circulating miR-19b and miR-92a, we assessed plasma samples collected from a randomized phase II trial that evaluated treatment with erlotinib or erlotinib plus celecoxib in advanced-stage NSCLC (Supplementary Table S1; ref. 41).

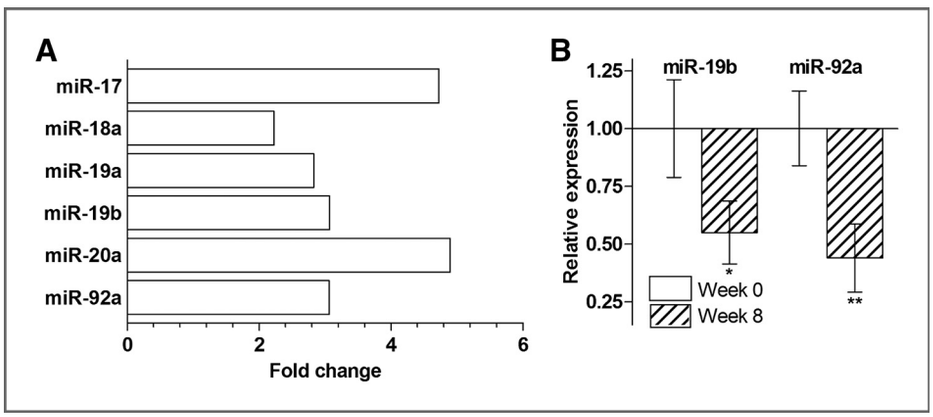

Figure 3. miR-17-92 cluster is upregulated in circulating miRNA from serum of patients with lung cancer. A, miR-17-92 is highly elevated in patients with cancer compared with subjects at high risk for developing lung cancer. Analysis of miR-17-92 expression in plasma of 30 high-risk controls and 30 patients with advanced-stage NSCLC by RNA-seq. Results are fold change in advanced-stage lung cancer vs. high-risk controls. B, celecoxib treatment reduces the levels of the circulating plasma miR-19b and miR-92a in patients with NSCLC. Results are mean relative expression ± SEM, *P* values computed by a paired samples *t* test. *, $P < 0.05$; **, $P < 0.01$.

We measured miR-19b and miR-92a levels by qPCR in plasma collected before therapy and following 8 weeks of treatment from 30 patients. We found that celecoxib significantly reduced circulating miR-17-92 expression in plasma (Fig. 3B).

PTEN expression is downregulated by PGE2

PTEN functions as a key negative regulator of the PI3-K/Akt/mTOR axis, thus affecting cell growth, proliferation, and survival. Previous studies in human fibroblast cell culture suggested that PTEN might be a target of miR-19 members of the miR-17-92 cluster (32). However, the causal relationship between PGE2, c-Myc, and PTEN expression has not yet been established. To define the effect of PGE2 on PTEN levels, we treated H157, H460, and RH2 NSCLC cells with PGE2 for up to 3 hours and assessed PTEN mRNA and protein levels. We found that PTEN transcription was sharply reduced upon PGE2 treatment (Fig. 4A). This is in accord with our finding of c-Myc and miR-17-92 cluster upregulation and supports the previously reported studies suggesting that PTEN is a miR-19b target. We did not detect a reduction of PTEN after 3 hours of PGE2 treatment at the protein level (data not shown), presumably because PTEN is a relatively stable protein. We first compared PTEN levels in the nonsilencing construct transduced control cells and c-myc knockdown cells. PTEN levels in the knockdown cells were slightly higher than in the controls (data not shown). We then evaluated the ability of PGE2 to reduce PTEN expression in control and c-Myc knockdown cells and found that while in the control cells PGE2 treatment sharply reduced PTEN expression, in c-Myc knockdowns this effect was not evident (Fig. 4B). These results suggest that in NSCLC cells, c-Myc contributes to PTEN downregulation upon mitogenic stimuli, whereas the baseline PTEN expression is controlled by the other mechanisms. To elucidate the role of miR-17-92 cluster members in PTEN downregulation, we transiently knocked down the expression of miR-19b with anti-miR antisense RNA. The control cells were treated with the Anti-miR Negative Control #1. Then, the controls and cells with knocked down miR-19 expression were treated with PGE2 and the levels of PTEN transcripts were assessed. We found that while in the negative control cells, PTEN expression was significantly reduced by PGE2 treatment, knocking down miR-19b expression reversed this effect (Fig. 4C). These results clearly indicate that PTEN is a miR-19b target and provided a rationale for the use of miR-19b inhibition in our subsequent functional experiments.

Suppression of miR-19b sensitizes NSCLC cells to apoptosis

Our previous studies demonstrated that PGE2 treatment renders NSCLC cells resistant to pharmacologically induced apoptosis by the modulation of antiapoptotic proteins levels (4, 40). Our current findings indicate that PGE2 is also able to modulate PTEN expression through upregulation of the miR-17-92 cluster, suggesting a novel mechanism contributing to apoptosis resistance in lung cancer cells. Therefore we determined if PTEN suppression is functionally relevant in these experimental settings. miR-19b expression was knocked down by anti-miR oligonucleotides in H157, H460, and RH2 cell lines. Four hours later, the control and miR-19b knockdown cells were treated with PGE2 for 16 hours, followed by apoptosis induction where applicable as described in Materials and Methods. The apoptosis levels were assessed by flow cytometry using Annexin V/PI method. In accord with our previously published studies, PGE2 treatment rendered cells resistant to apoptosis. Knockdown of miR-19b expression, however, reversed the PGE2 effect and upon the apoptosis induction, there was no significant change in levels of cell death in PGE2-treated miR-19b knockdowns as compared with the parental cells (Fig. 5).

Discussion

Our results demonstrate that PGE2 is able to initiate a sequence of events including c-Myc upregulation that

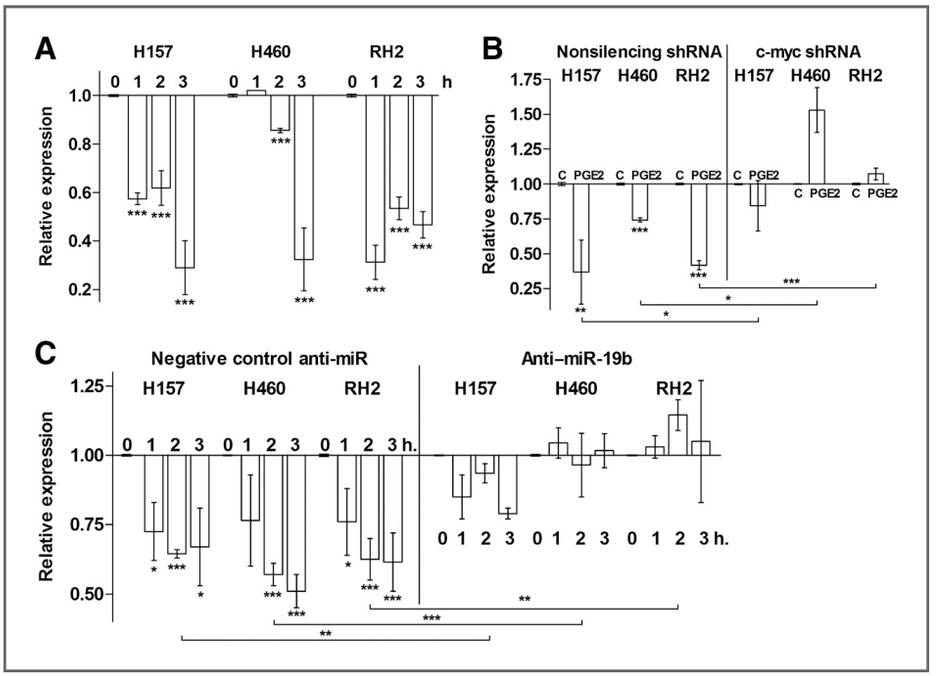

Figure 4. PTEN is negatively regulated by PGE2. Total RNA was isolated from H157, H460, and RH2 cells and probed for PTEN expression using the TaqMan system. Expression was normalized to that observed in the control or untreated groups. A, PGE2 reduces PTEN mRNA levels. The parental H157, H460, and RH2 cells were serum deprived and treated with the diluent control or 5 μg/mL PGE2 in the fresh serum-free medium for 0, 1, 2, or 3 hours. B, c-Myc knockdown reverses PGE2-driven PTEN downregulation. Nonsilencing shRNA construct–transduced control cells and c-myc knockdown cells were plated in 6-well plates and incubated overnight. The cells were then serum deprived and treated with the diluent control (C) or 5 μg/mL PGE2 (PGE2) in the fresh serum-free medium for 3 hours. PGE2 treatment significantly reduced PTEN expression in control cells but not in c-Myc knockdown cells. C, miR-19b knockdown blocks c-Myc–dependent downregulation of PTEN. miR-19b expression was knocked down with anti-miR antisense RNA in H157, H460, and RH2 cells. The control cells were transfected with the Negative Control anti-miR. Twenty-four hours after the transfection, the cells were serum deprived and treated with the diluent or 5 μg/mL PGE2 for 0, 1, 2, or 3 hours. Negative control- but not anti-miR-19b–transfected cells demonstrated significant downregulation of PTEN by PGE2. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

induces miR-17-92 cluster expression, which in turn dampens the expression of PTEN, ultimately promoting apoptosis resistance in NSCLC cells. Moreover, we found that this PTEN downregulation and augmented apoptosis resistance can be reversed by knocking down c-Myc or miR-17-92 cluster expression.

We have previously demonstrated that a subset of approximately 50% NSCLC cell lines respond to PGE2 by the growth factor receptor–independent activation of the MAPK/Erk pathway, induction of cell proliferation, and augmented apoptosis resistance (5, 40). *CMYC* belongs to a set of immediate early response genes, whose expression is activated early during the transition of cells from a resting to a growing state by a variety of mitogenic stimuli that are usually conveyed through MAPK/Erk cascade activation (8, 44). Our results suggest that PGE2-driven MAPK/Erk activation is able to induce c-Myc expression even in the absence of growth factor signaling, thus promoting myc-dependent cellular responses. A c-Myc–dependent network of genes, including miRNA genes, has been previously described (7, 32, 45). In accord with these data, we found that the expression of the miR-17-92 cluster was elevated upon PGE2-driven c-Myc upregulation in NSCLC cells. Similarly, the miR-17-92 levels in NSCLC cells ectopically expressing COX-2 were significantly elevated compared with the vector control cells and were markedly reduced by the COX-2 inhibitor celecoxib. The miR-17-92 cluster, known as oncomiR-1, produces six individual mature miRNAs that are often elevated in various cancers due to the transcriptional activation or genomic amplification (32). The miR-17-92 cluster has been shown to play a role in embryonic development and to be implicated in tumorigenesis, predominantly through the repression of apoptosis (28). Subsequent studies revealed that different members of the cluster had differing tumorigenic properties with miR-19 possessing the most potent oncogenic capacities and being absolutely required for the entire cluster activity (28, 31, 33).

The same aforementioned studies that dissected the input of the cluster member identified *PTEN* as a primary target of miR-19 and a central mediator of miR-17-92 tumorigenicity. Here, we report that PGE2-dependent upregulation of c-myc leads to the reduction of PTEN levels via the miR-17-92 cluster upregulation, leading to apoptosis resistance in NSCLC cells. This finding may partially explain why PTEN loss is frequently found in NSCLC despite somatic *PTEN* mutations occurring at a low frequency in lung cancer. For example, one study demonstrated that 24% of early-stage NSCLC tumors lacked PTEN expression due to promoter methylation (46). In later reports, PTEN protein expression

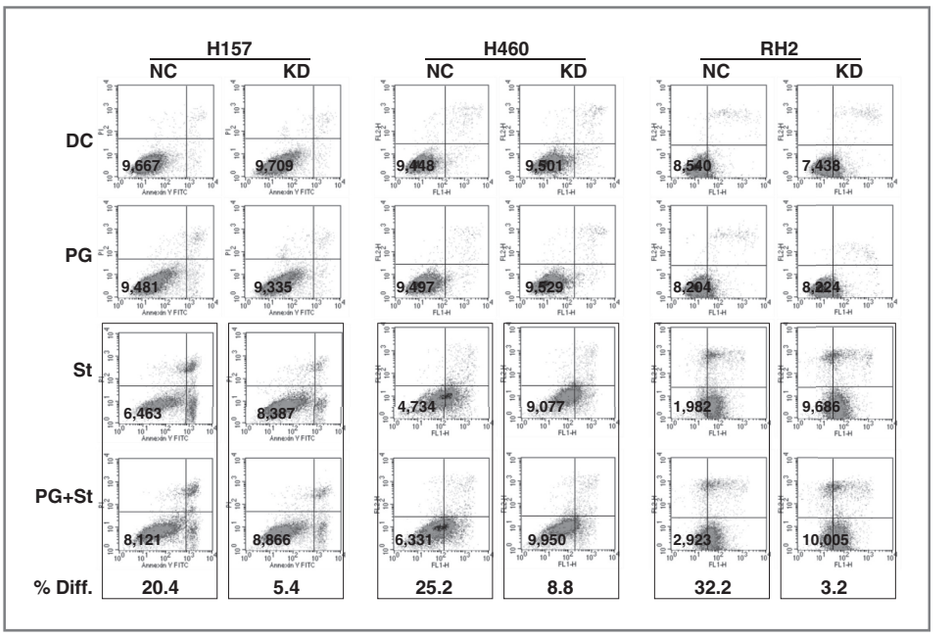

Figure 5. miR-19b knockdown abolishes the antiapoptotic effect of PGE2 in NSCLC cells. PGE2 protects the cells with intact miR-19b expression from apoptosis and this effect is reversed by miR-19 knockdown. The expression of miR-19b was knocked down with anti-miR antisense RNA in H157, H460, and RH2 cells (KD). Negative control anti-miR was used to transfect the control cells (NC). The cells were then pretreated with 5 μg/mL PGE2 (PG) overnight followed by the induction of apoptosis by staurosporine (St) where applicable. The control cells were treated with the equivalent concentrations of the diluents (diluent control, DC). The cells were allowed to recover overnight (5 μg/mL PGE2 was added to the PGE2 groups), and were then harvested. The apoptosis levels were evaluated by flow cytometry using Annexin V/PI staining. Percentage difference (% diff.) indicates the difference in the total number of nonapoptotic cells per 10,000 gated cells (shown in the lower left quadrants) between cells treated with staurosporine alone or in combination with PGE2.

was reduced or lost in 74% of advanced-stage lung cancers (47, 48). Moreover, the level of PTEN expression is a strong prognostic indicator in lung cancer and may determine treatment outcome. Similarly, PTEN loss has been described as one of the mechanisms of acquired resistance to targeted therapies in patients with lung cancer (20). Thus, our results indicate that PGE2 is able to negatively regulate PTEN via c-Myc–mediated expression of the miR-17-92 cluster. This effect contributes to the loss of PTEN expression in mutation-independent manner and increases apoptosis resistance of lung cancer cells. We determined that knocking down miR-19, the most potent suppressor of PTEN expression, restored PTEN levels and susceptibility to apoptosis in PGE2-treated NSCLC cells. Consistent with these data, PGE2-induced miR-17-92 cluster upregulation and PTEN repression were abolished in c-Myc knockdown NSCLC cells.

Recent studies suggest that circulating miRNAs can serve as robust and tissue-specific noninvasive biomarkers of lung cancer (49). Using microarrays and next-generation sequencing techniques, elevated levels of the miR-17-92 cluster members have been found in the circulating miRNA isolated from serum of patients with colon, gastric, ovarian, and prostate cancers (50). Our results indicate that the miR-17-92 is also highly upregulated in circulating miRNA in lung cancer, suggesting that its deregulation may also play a role in the pathogenesis of lung cancer. Furthermore, in patients with lung cancer who received celecoxib, the miR-17-92 expression was significantly reduced compared with the baseline. Importantly, the variations in the cluster members' copy numbers within the samples might reflect the differences in the stability of the individual cluster miRNAs or their transfer rate into the bloodstream. This, in turn, may suggest that using some miR-17-92 members as biomarkers might be advantageous compared with others. However, further studies are needed to test this hypothesis. Finally, from the functional point of view, one may speculate that the exosomal transfer of miR-19 from the tumor to the noninvolved cells might lead to PTEN downregulation in these cells and thus contribute to their malignant transformation.

In conclusion, our studies integrate elements of the inflammatory tumor microenvironment and promotion of aggressive tumor traits, including lack of response to apoptotic stimuli and enhanced proliferation. These findings identify a novel PGE2-dependent network operative in a subset of NSCLC cells that contributes to apoptosis resistance. Additional lung cancer studies will be required to (i) define the utility of the miR-17-92 cluster for early detection, prognosis, and response to therapy; (ii) assess the correlation between the loss of tumor PTEN expression and the levels of circulating miR-19; and (iii) determine if interference with miR-17-92 cluster expression could be beneficial in clinical settings by helping to restore PTEN expression in NSCLC and to improve response to therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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