The Heparan Sulfate Proteoglycan GPC3 Is a Potential Lung Tumor Suppressor

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Recently, we used gene expression profiling of lung adenocarcinoma and paired normal tissue from smokers and nonsmokers to identify genes and molecular pathways associated with cigarette smoking and lung carcinogenesis. The gene encoding Glypican 3, a glycosylphosphatidylinositol-linked heparan sulfate proteoglycan, was decreased in lung adenocarcinoma. Within nonmalignant lung, GPC3 expression was decreased in smokers compared with nonsmokers; indicating that expression is associated with cigarette smoking. Microarray results were confirmed using an independent cohort of tumors and nonmalignant lung tissues. Immunohistochemical studies localized Glypican 3 protein expression to the apical surface of lung bronchiolar epithelial cells, potential cells of origin for adenocarcinoma. Northern blot analysis demonstrated expression was absent in all tested non–small cell lung carcinoma lines. Pharmacologic treatment of lung cell lines indicated that GPC3 expression was epigenetically silenced by promoter hypermethylation. Human lung carcinoma tumor cells ectopically expressing GPC3 demonstrated increased apoptosis response when exposed to etoposide and growth inhibition when implanted in nude mice. These findings suggest that GPC3 is a candidate lung tumor suppressor gene whose expression may be regulated by exposure to cigarette smoke and functions to modulate cellular response to exogenous damage.

Gene expression profiling of tumors is increasingly contributing to investigations of carcinogenesis and pathogenesis. For example, microarray-based studies have demonstrated that gene expression profiling can enhance the classification of non–small cell lung cancer (1, 2), identify novel genes important in prostate carcinogenesis (3), and distinguish molecular pathways associated with mutations of BRCA1 in women with hereditary breast cancer (4). Recently, we used gene expression profiling of lung adenocarcinoma and paired normal tissue from smokers and nonsmokers to identify genes and molecular pathways associated with cigarette smoking and lung carcinogenesis (5).

Gene lists (a complete list of genes can be found on the website http://www.bubipulmatics.org/) were interrogated to identify candidate lung tumor suppressor genes with expression in normal lung tissue that was associated with cigarette smoking. These genes would potentially identify novel pathways that promote the transition of bronchial epithelial cells toward clonal transformation and lung tumor formation upon exposure to cigarette smoke. Among these genes was GPC3, which encodes Glypican 3, a 580–amino acid heparan sulfate proteoglycan. GPC3 expression was decreased in lung adenocarcinoma and in the normal lung of smokers compared with the normal lung of nonsmokers.

The Glypicans, a family with six known mammalian members, are linked to the cell surface by a glycosylphosphatidylinositol (GPI) anchor and are thought to mediate morphogen signaling (6, 7). Glypican 3 mutations have been identified as the genetic defects associated with Simpson-Golabi-Behmel syndrome (SGBS), which is characterized by overgrowth, dysplasia, and multiple congenital anomalies as well as by an increased prevalence of Wilms tumor, nephroblastoma, and hepatoblastoma (8, 9). The role of GPC3 in cell growth regulation is demonstrated by overgrowth and organ dysgenesis associated with altered gene expression in mouse models (10–13). Interestingly, examination of ovarian cancer cell lines, mesotheliomas, and breast tumors demonstrated decreased expression of GPC3 compared with normal tissue (14–18).

The phenotype of altered GPC3 expression suggests that Glypican 3 inhibits cell proliferation and may suppress tumor growth. Microarray experimental data suggests that this heparan sulfate proteoglycan is associated with lung carcinogenesis and that loss of expression may be an early step in tobacco-associated lung carcinogenesis. In this study, we characterize GPC3 expression and function in lung carcinoma.

Materials and Methods

Patients and Tissue Specimens

Primary lung adenocarcinomas and adjacent nonmalignant lung tissue were collected from six smokers and six clinically matched nonsmokers with adenocarcinoma undergoing lung cancer resection as described previously (5). All nonsmokers were lifetime nonsmokers. Among smokers, cigarette pack-years ranged from 15–80, with two patients smoking at the time of resection. An additional set of lung tumors and nonmalignant lung tissue samples were independently obtained from the Tumor Bank of Columbia University Comprehensive Cancer Center. These specimens included

Abbreviations: 5-aza-2′-deoxycytidine, 5Aza-dC; green fluorescent protein, GFP; loss of heterozygosity, LOH; phosphate-buffered saline, PBS; Simpson-Golabi-Behmel syndrome, SGBS; Trichostatin, TSA.


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11 adenocarcinomas and nonmalignant lung specimens from 13 smokers and 4 nonsmokers with lung carcinoma. Similar to the original cohort, cigarette pack-years ranged from 10–120, with four patients smoking at the time of resection.

Microarray Data Acquisition
A total of 3–6 μg of total RNA from each of 22 samples was prepared and hybridized onto the Affymetrix (Santa Clara, CA) HuGeneFL array, which provides expression data for ∼5,600 full-length genes, as described earlier. Additional information on the statistical methods, microarray protocol, and expression levels for all the genes is available at http://www.bubiopulmatics.org.

Cell Culture and Tumor Growth
Lung cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. As cells reached 80–90% confluence, total RNA was extracted with Trizol. Tumor growth studies in nude mice were conducted as described (19), except 8 × 10^6 cells resuspended in phosphate-buffered saline (PBS) were injected into the dorsal subcutaneous tissue.

Northern Analysis
GPC3 expression in total RNA from non–small cell lung carcinoma cells was assessed by Northern analysis using standard procedures (20). RNA was probed with a GPC3 cDNA PCR fragment (base pairs 1262–1758 NM004484).

Production of Anti-GPC3 Mouse Monoclonal Antibody, 8H5
BALB/c mice were immunized with an intraperitoneal injection containing 50 mg of a His-tagged carboxyl-terminal GPC3 fragment emulsified with Titermax Gold adjuvant (Cedarlane, Hornby, ON, Canada). The selected hybridoma was cloned twice, its isotype determined (IgG1, K) using Isostrip (Roche, Mannheim, Germany), and was purified from hybridoma conditioned media using protein A column chromatography Affi-Gel Protein A MAPS II kit (Bio-Rad, Hercules, CA).

Immunostaining
Tissue sections from paraffin-embedded tumor and nonmalignant lung and placenta samples (obtained from the Columbia University Tumor Bank) were deparaffinized and rehydrated. Nonenzymatic unmasking was accomplished by boiling the slides in a microwave oven using Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA). Anti-GPC3 antibody 8H5 (50 ng/ml) with 3% serum was incubated with the sections overnight. Detection was by an avidin–biotin complex (ABC)-based method (Vector). 3,3’-diaminobenzidine was used as a substrate for peroxidase complexes yielding a brown reaction product at the site of the target antigen. Sections were counterstained with methyl-green.

Ectopic Expression of GPC3
The hemagglutinin A (HA)–tagged Rat GPC3 cDNA (15) was cloned into a bicistronic retroviral expression vector pMSCV-IRES-GFP (21) containing an enhanced green fluorescence protein (GFP) as an expression marker. The expression construct was packaged into retrovirus by cotransfection into 293T cells (22, 23). Two lung carcinoma cell lines (A549 and NCI-H460) were incubated with viral supernatant, and infected (GFP+) cells were sorted by a FACSScan flow cytometer (Becton Dickinson, San Jose, CA). The ectopic expression of GPC3 was confirmed by Western blotting using a rabbit polyclonal HA antibody (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA), and by cell immunostaining following standard procedures. For immunostaining, cells were seeded on coverslips in a 24-well plate and were incubated with HA probe (Y-11) and with Alexa Fluor 546 goat anti-rabbit IgG (H+L) (Molecular Probes, Eugene, OR) for 30 min each. Clones with highest GPC3 expression as detected by immunoblot (clone 1 and clone 5 in A549 and H460 cells, respectively) were selected for xenotransplantation studies. Cells infected with the empty expression vector (GFP alone) were controls for all experiments.

Cell treatment Annexin V FACS Assay
Both A549 and NCI-H460 cell lines and clones isolated by limiting dilution were used in the apoptosis studies. Cells were maintained in Dulbecco’s modified Eagle’s medium with 0.29 mg/ml L-glutamine and 10% fetal calf serum and were exposed to pro-apoptosis stressors including etoposide, serum starvation, and ultraviolet irradiation (24). They were seeded at a density of 5 × 10^6 cells/well, and were harvested after 48 h of continuous exposure to etoposide (Sigma, St. Louis, MO) solubilized in dimethyl sulfoxide. Adherent cells were removed after trypsinization, collected in PBS, centrifuged at 610 × g for 5 min, washed twice with cold PBS, and resuspended in 0.5 ml PBS. Phosphatidylinerse translocation was measured using the Annexin V-PE Apoptosis Detection Kit (BD Biosciences, PharMingen, San Diego, CA) for fluorescence labeling and the FACScan for measurement of the resulting fluorescence. Cells were counterstained with 7-Amino-actinomycin D to confirm that Annexin V staining was specific for early apoptotic events.

Pharmacologic Unmasking of GPC3 Expression
Lung cell lines A549 and NCI-H460, and the breast cell line MDA-231 (positive control for re-expression after demethylation), were treated with 5 μM 5-aza-2′-deoxycytidine (5Aza-dC, Sigma) for 4 d or with 1 μM Trichostatin (TSA; Wako, Richmond, VA) for 2 d with daily replenishment of medium. GPC3 expression before and after treatment was detected by RT-PCR using conditions and primers reported previously (16). In brief, total RNA was isolated with Trizol and used to make cDNA using the Superscript II system (Invitrogen, Baltimore, MD). PCR was performed using GPC3 specific primers (forward: 5′-CCACAATGCTGCTCAAGAAA GATGGGAGG-3′; reverse: 5′-TGACGGGGTCAACCACACTG TGCCCATCTA-3′) for 35 cycles. To normalize for starting total RNA, GAPDH was used as the primers (5′-CCAT GGGGAGGTAGAGTCGGAGTGC-3′; reverse: 5′-GGTGGT GCCAGAGGCATTGCTGATG-3′).

Quantitative RT-PCR
GPC3 was detected using the LightCycler System (Roche). Primers were: forward, 5′-TGCCTCTATGCCAGGGACT-3′; reverse, 5′-GCTTTCTGGAGATTTCTCGT-3′. The product was quantified using a standard curve that correlated each cycle number at which the amplification of the product was in the linear phase with a value. This value was then normalized to the value of the internal standard B2M (Roche).
Statistics
Quantitative RT-PCR data was analyzed with the Mann Whitney test, one-tailed, to confirm microarray data showing GPC3 expression was higher in nonsmokers compared with smokers. All other statistical comparisons utilized the Student’s t test.

Results
**GPC3 Expression Is Decreased in Adenocarcinoma and in the Nonmalignant Lung of Smokers Compared with Nonsmokers**

We have previously identified distinct molecular profiles for adenocarcinomas and nonmalignant lung tissue in smokers and nonsmokers (5). We hypothesized that gene lists generated by comparing differences in gene expression in these samples could identify lung tumor suppressor genes associated with cigarette smoking. Seventy genes were found to have at least a 2-fold decreased expression in tumors compared with nonmalignant lung tissue and unique expression in the nonmalignant tissue of nonsmokers compared with smokers ($P < 0.05$). Among these genes was GPC3, which demonstrated 10-fold decreased expression in tumors compared with nonmalignant lung. Furthermore, expression was decreased by 60% in the nonmalignant lung of smokers compared with the nonmalignant lung of nonsmokers (Figure 1A). Microarray data were confirmed by quantitative RT-PCR of the original samples (5). Additional validation of GPC3 silencing in adenocarcinoma was provided by examination of expression data obtained from a recently published gene-expression profiling study (1). In this large data set of 203 non-small carcinomas and 17 nonmalignant lung tissues (smokers and nonsmokers), mean normalized GPC3 expression was lower in adenocarcinoma and squamous cell carcinoma compared with nonmalignant lung tissues ($P < 0.05$). Data regarding the smoking status of subjects were not provided.

To further validate the association of GPC3 expression with cigarette smoking and neoplasia, we examined GPC3 expression in an independently obtained set of adenocarcinomas and nonmalignant specimens obtained from smokers and nonsmokers (Figure 1B). Quantitative real-time PCR indicated that expression is lower in tumors and confirmed that within normal tissues; GPC3 expression is lower in smokers ($P < 0.05$).

**GPC3 Expression Is Absent in all Subtypes of Non–Small Cell Lung Carcinoma and Is Normally Expressed in Respiratory Bronchiolar Epithelial Cells**

We performed Northern analysis of lung cancer cell lines to examine GPC3 expression in lung cancer cell types other than adenocarcinoma (Figure 2A). GPC3 expression was not detected in any of the non–small cell lines tested. GPC3 is normally expressed in the placenta, ovary, mammary gland, kidney, and lung in humans (16, 25). To determine the lung cell types that constitutively express Glypican 3, we performed immunohistochemistry on nonmalignant lung tissue and tumors from smokers and nonsmokers with adenocarcinoma (Figure 2B). Placenta was used as positive control and as reported previously, Glypican 3 expression was localized to trophoblasts (26). Protein expression was not detectable in lung tumors. In the nonmalignant lung of nonsmokers, Glypican 3 protein was localized to the apical cell membrane surface of the respiratory bronchiolar epithelium. As Glypican 3 has been shown by others to be secreted, it is unclear whether staining identifies cells expressing GPC3 or cells harboring paracrine secreted protein (27). In either instance, the anatomic localization of Glypican 3 to the bronchiolar epithelium is significant because of its proximity to Clara cells, alveolar type 2 cells, and cells of the bronchiolar–alveolar junction, all of which are potential cells of origin of adenocarcinoma (28, 29).

**Ectopic Expression of GPC3 Inhibits Lung Tumor Cell Growth and Is Associated with Apoptosis**

To examine Glypican 3 function in lung carcinoma, we infected two lung cancer cell lines (NCI-H460-large cell and A549-adenocarcinoma) with a retrovirus that co-expressed GPC3 and GFP. We used Western analysis to confirm that the HA-tagged Glypican 3 protein was present in the cell lines and we used immunocytochemistry to demonstrate that the protein was appropriately localized to the cell surface (Figures 3A–3B). Colony formation assays did not demonstrate a difference in cell proliferation or apoptosis in GPC3-infected cells compared with control cells (data not shown). To determine if GPC3 could affect lung tumor growth in vivo, we injected tumor cells containing control...
vector or GPC3 into nude mice and monitored the animals for tumor growth. Ectopic expression of GPC3 was associated with decreased growth of both A549 and NCI-H460 cells compared with cells infected with vector alone (Figures 4A–4C). These data demonstrate that ectopic Glypican 3 expression inhibits lung tumor growth in vivo with a more pronounced effect in NCI-H460 cells compared with A549 cells. Increased growth inhibition in NCI-H460 compared with A549 cells may possibly be attributed to increased expression of Glypican 3, as shown in the Western analysis.

To determine if enhanced apoptosis was a mechanism of tumor growth inhibition, we used the TUNEL assay to detect apoptotic cells in tumor xenografts (data not shown). In NCI-H460 cells, ectopic expression of GPC3 was associated with enhanced apoptosis (P < 0.05). However, in A549 cells, although there was more apoptosis in tumors transfected with GPC3 compared with controls, the difference was small (P > 0.05). To confirm that GPC3-expressing cells are more susceptible to apoptosis, we studied the effect of GPC3 on apoptosis induced in cultured cells by various insults. We examined Annexin V staining, which is a marker of early apoptotic events, in lung cells exposed to proapoptotic stressors of serum starvation, ultraviolet irradiation, and etoposide (30). Although no effect on apoptosis was seen in serum starvation or ultraviolet irradiation, GPC3 expression was associated with increased apoptosis in cells exposed to etoposide. This effect was seen in individual clones and derivative lines of both A549 and H460 cells, supporting the generalizability of the effect. Etoposide, a topoisomerase II inhibitor, is a DNA damaging agent that causes double strand breaks. Our results suggest that GPC3 may function to modulate cellular apoptosis in response to exposure to exogenous DNA damaging agents.

**GPC3 Re-Expression after Treatment with 5Aza-dC and TSA**

The mechanisms underlying the potential association of GPC3 expression with cigarette smoking are unclear. In mesothelioma, and in breast and ovarian carcinoma, GPC3 silencing in cancers is attributable to epigenetic silencing (14, 16–18). CpG island hypermethylation attracts histone deacetylases, which leads to chromatin condensation and transcriptional silencing (31). To determine if expression is epigenetically silenced in lung carcinoma A549 and NCI-

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**Figure 2.** Expression is decreased in non–small cell cancer cells and is detected in normal respiratory bronchiolar epithelial cells. (A) Total RNA (18 μg) from lung carcinoma cell lines was hybridized with a GPC3 probe. Nonmalignant lung from a nonsmoker and placenta RNA (2 μg) were used as positive controls. GAPDH was detected as a control for RNA loading. Lane 1, A-549; lane 2, NCI-H23; lane 3, NCI-H322; lane 4, NCI-H522; lane 5, SK-MES-1; lane 6, NCI-H441; lane 7, NCI-H460; lane 8, SK-LU; lane 9, NCI-H1435; lane 10, nonmalignant lung tissue from a non-smoker; lane 11, placenta. (B) Paraffin-embedded tissue sections were stained with 8H5, an anti-GPC3 monoclonal antibody. Staining was detected in the nonmalignant lung from non-smokers (a), with expression localizing to the luminal surface of bronchiolar epithelial cells (arrow, inset). No staining was detected in comparable sections of the nonmalignant lung of smokers (b) or in lung adenocarcinoma tumor cells (arrows in c). Placenta (d) was used as a positive control; staining localized to trophoblasts (arrow). The results presented are representative of duplicate experiments. Magnification is ×100.

**Figure 3.** Ectopic expression of GPC3 in lung carcinoma cells. GFP gene expression constructs containing HA-tagged GPC3 cDNA or empty vector control (GFP alone) were transduced into A549 and NCI-H460 cells. (A) Immunoblot of Glypican 3 protein in GPC3-infected cells (+) and in control (−) cell lysates. The 69-kD band indicates detection of Glypican 3 core protein (arrow). (B) Immunofluorescence detection of GPC3 (b, e, h, and k) and GFP (c, f, i, and l) in transduced A549 (a–f) and NCI-H460 (g–l) cells. a–c and g–i are cells infected with GPC3 construct; d–f and j–l are cells infected with the GFP vector alone. Ectopically expressed GPC3 localizes to the cell surface (arrow), as expected for GPI-linked proteins; GPC3 is not detected in negative controls transduced with empty vector control (e and k). Magnification is ×400.
H460 cells, we examined GPC3 expression after treatment with the demethylating agent 5Aza-dC and with TSA, a histone deacetylase inhibitor.

A549 cells demonstrated re-expression of GPC3 only after inhibition of histone deacetylase, and NCI-H460 cells demonstrated re-expression after demethylation and after TSA treatment, with an apparent additive effect after both treatments (Figure 5, Table 1). GPC3 could be re-expressed after demethylation alone in NCI-H23 cells, and after inhibition of histone deacetylation alone in A549 cells, and after both treatments in NCI-H460 and NCI-H522 cells. Re-expression could not be demonstrated in SK-MES-1 or NCI-H1435 cells. These data suggest that epigenetic transcriptional silencing of GPC3 is important in lung carcinogenesis, but that the mechanisms of repression are cell line–dependent. Cell line dependence of GPC3 promoter hypermethylation has been reported in other tumor types (16).

Discussion

Evidence gathered from epidemiologic, whole animal and human tissue studies indicate that GPC3 may function as a tumor suppressor. Epidemiologic studies of patients with SGBS, for which there have been a total of 20 identified GPC3 deletions/mutations thus far, indicate that GPC3 regulates growth and cell survival (9). Common clinical features of SGBS include macrosomia, macroglossia, nephromegaly, polydactyly, supernumerary nipples, and cystic dysplasia of the kidneys. Patients with SGBS are at increased risk for embryonal tumors such as Wilms tumor, neuroblastoma, and testicular gonadoblastoma (32). There are no reports of lung tumors, but there are few reports of SGBS features in the occasional patient that survives into adulthood. This reporting bias is significant because lung carcinoma is rare in individuals under age 40 (33). Gpc3-null mice demonstrate pneumomegaly as well as macrosomia and renal dysplasia. No lung tumors or other cancers have been reported in null mice thus far, suggesting that additional genetic alterations or different genetic background might be required for elaboration of tumors or other phenotypes. This is supported by a recent report of postaxial polydactyly and rib malformations that was apparent only upon mating Gpc3-deficient mice.
mice with mice heterozygous for Bone morphogenetic protein 4 (Bmp4) and was absent in mice with exclusive Gpc3 or Bmp4 alterations (12). These data suggest that in animal models and in humans, some GPC3 phenotypes require genetic interaction with other co-factors.

In this study, we have shown that GPC3 expression is decreased in lung adenocarcinoma and in the nonmalignant lung of smokers compared with nonsmokers. The expression profile of GPC3 in human lung tissues and its association with cigarette smoking, the leading cause of lung cancer, suggest GPC3 may be a lung tumor suppressor and that decreased GPC3 expression may represent an early step in cigarette smoke–associated lung carcinogenesis. To our knowledge, this is the first report implicating GPC3, a member of the Glypican heparan sulfate proteoglycan family, in the pathogenesis of lung carcinoma. This hypothesis is supported by recent studies that have implicated GPC3 as a tumor suppressor in other human tumors. Studies using differential display and CpG island microarrays have identified decreased expression in mesotheliomas and breast carcinomas (14, 17). Other investigators have directly examined GPC3 expression in human tumors and observed decreased expression in ovarian cancer cells and in breast tumors (16, 18). We did not detect an inhibition of colony formation in lung A549 or NCI-H460 cells upon ectopic expression of GPC3. This result suggested that GPC3 function in lung tumors might require the presence of other proteins, matrix/epithelial interactions, or stressors that were absent in vitro. Indeed, in nude mouse xenograft experiments, we were able to demonstrate that ectopic expression of GPC3 decreased tumor cell growth in both lung cell lines tested.

Our results indicate that GPC3 expression may inhibit tumor growth by increasing susceptibility to early apoptotic events in cells exposed to DNA-damaging agents. Apoptosis as detected by Annexin V staining was significantly increased in GPC3-transduced NCI-H460 and A549 cells that were treated with etoposide, a topoisomerase II inhibitor that induces DNA double strand breaks. Interestingly, GPC3 expression was not associated with apoptosis in serum-deprived cells or in cells treated with ultraviolet irradiation. This result suggests that the apoptotic response modulated by GPC3 may be specific to the mechanism of DNA damage in these cells. Other inducers of double strand DNA breaks include γ irradiation and volatile hydrocarbons such as 1,3 butadiene, the latter of which is a cigarette smoke carcinogen (34, 35). These results suggest GPC3 silencing in bronchial epithelial cells, which may also be associated with tobacco exposure, can facilitate lung carcinogenesis by promoting apoptosis resistance in genetically damaged cells. These cells in the cancerized lung epithelium may subsequently accumulate additional genetic alterations and eventually undergo neoplastic transformation.

Our gene expression data, although limited by a small sample size, suggest that cigarette smoking is associated with decreased expression of GPC3. The association of lung GPC3 expression with smoking will need to be confirmed in larger cohorts of smokers and nonsmokers with and without lung cancer. The mechanisms for the interaction between GPC3 and cigarette smoking are unclear, but investigations of other lung cancer tumor suppressor genes indicate that loss of heterozygosity (LOH) and promoter hypermethylation are mechanisms that can be involved in the regulation of tumor suppressor genes (36). Importantly, in contrast to other tumor suppressor genes such as p53 and p16, which require inactivation of two alleles for elaboration of a tumor suppressor phenotype, GPC3 silencing requires a single event because it is mono-allelically expressed on Xq26.

Widespread allelic loss at chromosomal loci harboring tumor suppressor genes is frequently detected in lung carcinoma as well as in the nonmalignant bronchial epithelium of smokers with and without lung cancer (37, 38). Specific allelic loss at Chromosome Xq22–23, near the location of GPC3 at Xq26, has recently been reported in non–small cell and small cell lung carcinomas (39), suggesting that LOH may be a mechanism of inactivation for GPC3 in cancer. Similar to LOH, epigenetic silencing of lung tumor suppressor genes (i.e., p16) and other genes associated with lung cancer (i.e., RARB, TIMP-3), is commonly detected in primary tumors and in the nonmalignant bronchial epithelium of smokers (40, 41).

Several investigators have demonstrated that GPC3 transcription in tumors is epigenetically silenced by promoter hypermethylation (14, 16–18). We have shown that CpG island methylation represses expression in lung cell lines by demonstrating re-expression with 5Aza-dC and/or TSA. However, we were unable to demonstrate re-expression in all lines tested. Based on these data and those derived from other tumor types, it is likely that GPC3 CpG epigenetic silencing is not the sole mechanism regulating GPC3 expression in the lung. The role of alternative mechanisms such as LOH, mutation, and interaction with other transcription factors remains to be established.
In conclusion, oligonucleotide microarray analysis of lung adenocarcinomas from smokers and nonsmokers identified GPC3 as a candidate lung tumor suppressor gene with expression associated with cigarette smoking. Functional studies demonstrated growth inhibition in tumor explants and enhanced apoptosis response in etoposide-treated lung cancer cells upon ectopic expression of GPC3. Our data suggest that GPC3 downregulation is an early event in the molecular pathogenesis of lung carcinoma in smokers and may be important in facilitating neoplastic transformation and tumor growth in cells genetically damaged upon exposure to chronic cigarette smoking.

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