Down-Regulation of Phosphoglucose Isomerase/Autocrine Motility Factor Results in Mesenchymal-to-Epithelial Transition of Human Lung Fibrosarcoma Cells

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Abstract

Phosphoglucose isomerase (PGI) is one of the glycolytic enzymes and is a multifunctional enzyme that functions in glucose metabolism inside the cell while acting as a cytokine outside the cell, with properties that include autocrine motility factor (AMF) regulating tumor cell motility. Although there are many studies indicating that PGI/AMF has been implicated in progression of metastasis, no direct studies of the significance of exogenous PGI/AMF on tumor progression have been reported. Here, we report on the mesenchymal-to-epithelial transition (MET), which is the reverse phenomenon of the epithelial-to-mesenchymal transition that is associated with loss of cell polarity, loss of epithelia markers, and enhancement of cell motility essential for tumor cell invasion and metastasis. Mesenchymal human fibrosarcoma HT1080 cells, which have naturally high levels of endogenous and exogenous PGI/AMF, were stably transfected with PGI/AMF small interfering RNA (siRNA). The siRNA targeting human PGI/AMF down-regulated the endogenous PGI/AMF expression and completely extinguished the secretion of PGI/AMF in a human fibrosarcoma HT1080, whereas the control siRNA showed no effects. The PGI/AMF siRNA caused cells to change shape dramatically and inhibited cell motility and invasion markedly. Suppression of PGI/AMF led to a contact-dependent inhibition of cell growth. Those PGI/AMF siRNA-transfected cells showed epithelial phenotype. Furthermore, tumor cells with PGI/AMF deficiency lost their abilities to form tumor mass. This study identifies that MET in HT1080 human lung fibrosarcoma cells was initiated by down-regulation of the housekeeping gene product/cytokine PGI/AMF, and the results depicted here suggest a novel therapeutic target/modality for mesenchymal cancers. [Cancer Res 2007;67(9):4236–43]

Introduction

It is now accepted that the conversion from epithelial to mesenchymal cells is an essential mechanism for developmental processes (1, 2). Epithelial-to-mesenchymal transition (EMT) is reflected in changes from a tightly organized cobblestone-like structure, whereby the cells adhere to each other and manifest apical basal polarity to spindly migratory elongated cells with disrupted cell-cell/cell-substratum contacts. During cancer progression, EMT has been implicated in the regulation/initiation of tumor cell invasion and metastasis (1–3). Several growth factors and cytokines, such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and tumor necrosis factor-α (TNF-α), have shown to regulate EMT (3–6). Although numerous studies have been examined the molecular mechanisms of EMT, very little is known on the reverse mesenchymal-to-epithelial transition (MET), especially in cancer.

Phosphoglucose isomerase (PGI, EC 5.3.1.9) is a housekeeping cytosolic enzyme of sugar metabolism that plays a key role in both glycolysis and gluconeogenesis pathways, catalyzing the interconversion of glucose 6-phosphate and fructose 6-phosphate (7), which behaves extracellularly as a cytokine. Molecular cloning and sequencing have identified PGI as an autocrine motility factor (AMF) found to be a major cell motility–stimulating factor associated with cancer development and progression (7–9). PGI was found to be a neuroleukin that promotes growth of embryonic spinal and sensory neurons (10), a maturation factor mediating differentiation of human myeloid leukemia cells (11), sperm antigen-36 (12), and a myofibril-bound serine proteinase inhibitor (13). Of note, aberrations in PGI expressions or activities due to mutations or deletions in PGI are of significant clinical importance because mutations in PGI lead to hereditary nonspherocytic hemolytic anemia disease (14–16). In clinical cancer pathology, the presence of PGI/AMF in the serum and urine is of prognostic value indicating cancer progression (17–19). The levels of PGI/AMF and its cell surface receptor gp78/AMFR expressions are associated with the pathologic stage, grade, and degree of tumor penetration to surrounding tissues marking a poor prognosis (17–21). AMF-AMFR is thought to be involved in EMT because their up-regulation in epithelial cancers is concomitant with the down-regulation of E-cadherin and up-regulation of its repressor, the Snail zinc finger transcription factor (22, 23). Thus, here, we examined the biological consequences of the down-regulation of PGI/AMF expression in an aggressive mesenchymal human cancer cell line, the human lung fibrosarcoma HT1080.

Materials and Methods

Reagents and antibodies. Erythrose 4-phosphate (E4P), 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-β-actin were obtained from Sigma. Anti-vimentin, anti-cytokeratin (AE1/AE3), and anti-thy1thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-β-actin were obtained from Sigma. Anti-vimentin, anti-cytokeratin (AE1/AE3), and anti-thy1

[Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).]

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©2007 American Association for Cancer Research. doi:10.1158/0008-5472.CAN-06-3935
Cancer Foundation) and maintained in DMEM-F12 supplemented with 0.1 μg/mL chola toxin, 0.02 μg/mL epidermal growth factor, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5% horse serum. Cultures were maintained at 37°C in an air–5% CO2 incubator.

RNA interference. To design specific small interfering RNA (siRNA) targeting PGI/AMF, several sequences from different parts of the human PGI/AMF gene were selected using siRNA Target Finder available at the Ambion Web site.3 The siRNA duplexes targeted against PGI/AMF were synthesized by Dharmacon, Inc. Twenty-four hours after inoculation of HT1080 cells, siRNA duplex transfection was done using Oligofectamine reagent (Invitrogen) according to the manufacturer's protocol. The efficiency of PGI/AMF silencing was analyzed after 24 h of transfection by reverse transcription-PCR (RT-PCR) and immunoblot, and the best siRNA target site for the PGI/AMF gene was selected for generating stable siRNA cell lines. The target sequence for the PGI/AMF siRNA was 5′-AATTGCC-TACCCGAGACCAGCGT-3′. The specificity of the sequence was verified by a BLAST search of the public databases. pSilencer 3.1-H1 neo expression vector (Ambion) that produces siRNA targeted against PGI/AMF (named siPGI/AMF) was also prepared according to the manufacturer's protocol. In addition, pSilencer 3.1-H1 neo vector, HT1080 cells were transfected using the LipofectAMINE 2000 reagent (Invitrogen) with PGI/AMF siRNA. Stably expressed single clones were established by G418 selection (50 μg/mL). The specificity of the sequence was verified by a BLAST search of the public databases. pSilencer 3.1-H1 neo expression vector (Ambion) that produces siRNA targeted against PGI/AMF (named siPGI/AMF) was also prepared according to the manufacturer's protocol.

RT-PCR analysis. RT-PCR analysis was done as described previously (24). Total RNA was extracted with Trizol reagent (Invitrogen). The cDNA for PCR template was generated by using First-Strand cDNA Synthesis kit (Amersham Biosciences) as recommended in the manufacturer's protocols. For quantitative evaluation of the amplified product, PCR encompassing 20 to 40 cycles was preliminarily done to determine the most suitable number of amplifications for each reaction. Each PCR cycle consisted of 1 min at 95°C, 1 min at 52°C, and 2 min at 72°C for Snail and Slug; 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C for PGI/AMF; and 1 min at 95°C, 1 min at 60°C, and 2 min at 72°C for β-actin. PCR-amplified products were electrophoresed in 1% or 1.5% agarose gel and stained with ethidium bromide. Each expression was standardized using GAPDH and β-actin as housekeeping genes.

Western blot analysis. Western blot analysis was done as described previously (24). Equal amounts of the proteins were loaded in each lane. All protein samples were separated on 4%, 8%, or 12.5% SDS-PAGE gels and transferred to 0.2-μm polyvinylidene fluoride membrane (Osmonics, Inc.) at 15 V, 30 mA overnight at 4°C. Then, the membrane was blocked with 0.1% casein solution in 0.2% PBS for 1 h at room temperature. The blocked membrane was incubated with diluted primary antibody for 1 to 3 h at room temperature. After extensive washing to remove the excess antibody from the membrane, appropriate secondary antibodies conjugated with fluorophores [IRDye 800 antibodies (Rockland Immunochemicals) or Alexa Fluor 680 antibodies (Molecular Probes)] were added (1:5,000) and incubated for 1 h at room temperature. Blots were visualized by using the LI-COR Bioscience Odyssey Infrared Imaging System (LI-COR Biosciences). Density of each band was quantitated with NIH Image software.

Phagokinetic track assay. Random cell motility was measured by phagokinetic track assay (25). Briefly, coverslips were coated with a uniform layer of 1.0% bovine serum albumin (BSA) by means of fixing with 100% ethanol and warm air blowing. The treated coverslips were then embedded with colloidal gold particles and placed onto six-well plates with complete medium. Then, 2×105 cells were seeded on each coverslip. After 24 h of incubation, phagokinetic tracks were visualized under a microscope at a magnification of ×200. The area produced by at least 50 locomoted cells was measured using NIH Image software.

Invasion assay. The in vitro invasion assay was done using Transwell cell culture chambers (Corning Costar No. 3422). Briefly, polycarbonate filters with 8.0-μm pore size were precoated with 5 μg Matrigel (Collaborative Biomedical Products) on the upper surface. Cells were resuspended in DMEM supplemented with 0.1% FBS. The cell suspensions (1×105 cells) were added to the upper compartment of the chamber. The bottom chamber was filled with DMEM supplemented with 10% FBS. After 2 h of incubation, the top side of the insert membrane was scrubbed with a cotton swab to remove the noninvasive cells and the bottom side was fixed with methanol and stained with 1% eosin and hematoxylin. Viable cells were counted under a microscope at a magnification of ×400. Each assay was done in triplicate.

MTT assay. For cell proliferation, a MTT assay was done. Briefly, 1×103 cells per well were plated in 96-well plates and cultured for different times indicated. At the end of the assay time, 10 μL MTT (5 mg/mL) was added to each well and then incubated at 37°C for 4 h. After removing the medium completely, solubilizing solution (acidic isopropanol) was added at 100 μL/well. Plates were read at 570 nm on a spectrophotometric plate reader with a reference wavelength at 650 nm.

Proliferation assays. Cell proliferation was determined by using the bromodeoxyuridine (BrdUrd) proliferation assay kit (Calbiochem) according to the manufacturer's instructions. Briefly, cells were seeded onto 96-well plates. After incubation, cells were labeled with BrdUrd for 4 h, fixed, incubated with anti-BrdUrd antibodies, and developed using a colorimetric reaction. Absorbance at 460 nm was measured using a spectrophotometric plate reader.

Immunofluorescence analysis. Immunofluorescence analysis was done as described previously with slight modification (24). Cells seeded on coverslips were fixed with 4% paraformaldehyde/PBS for 15 min at room temperature. The fixed cells were washed thrice with PBS and permeabilized with 0.1% Triton X-100/PBS (pH 7.2) for 15 min. The cells were blocked with 3.0% BSA/PBS for 30 min and then labeled with primary antibody in 0.1% BSA/PBS overnight at 4°C. After incubation, the cells were washed with 0.05% Triton X-100/PBS and incubated with FITC-conjugated secondary antibody in 0.05% Triton X-100/PBS for 1 h at room temperature in the dark. After washing with PBS, the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI; targeting DNA in the cell nucleus) for 5 min. After extensive washing, the cells were mounted on a glass slide with 80% glycerol and fluorescent images were analyzed in an Olympus fluorescence microscope using a ×400 lens.

Anchorage-independent growth assay. Six-well plates were coated in 1% SeaPlaque agarose (BioWhittaker Molecular Applications) dissolved in complete growth medium. Cells (1×103) were suspended in complete medium containing 0.5% agarose and plated in six-well plates over a basal layer. Colonies (>10 cells) developed in the agar suspension were counted under microscopic visualization at 21 days. The clonogenicity was determined in three independent experiments.

http://www.ambion.com
**In vivo tumor growth.** HT1080 cells, vector control, and two stable siPGI/AMF clones were assayed for tumorigenicity in 6-week-old athymic nude mice. Cells (4 × 10^6) were suspended in PBS, and the suspension was inoculated s.c. into the right flanks of NCR nu/nu mice on day 0. The animals were monitored for tumor formation every 3 days. Tumors were measured by slide calipers, and the tumor volume was calculated according to the following formula: volume = (width)² × length / 2. Growth curves for tumors were plotted from the mean volume ± SE of tumors from 10 mice.

**Statistical analysis.** Data are expressed as mean ± SD. Comparisons between the groups were determined using unpaired t-test. P < 0.05 was considered statistically significant.

**Results**

**Suppression of PGI/AMF expression in tumor cells by siRNA.**

The human fibrosarcoma HT1080, well known as a highly aggressive tumor-forming cell line, was transfected with the constructed plasmid encoding siRNA targeting to PGI/AMF and with control plasmid, respectively. The stable transfectants with PGI/AMF siRNA plasmid (siPGI/AMF) and control plasmid (mock) were established by G418 screening. To examine the efficiency for silencing PGI/AMF, we detected the level of mRNA and protein expression in the transfectants (Fig. 1A). The RT-PCR data showed that the level of PGI/AMF mRNA was apparently decreased in two clones, siPGI/AMF-1 and siPGI/AMF-2, compared with HT1080 parental cells, but there was no difference between the parental and mock cells (Fig. 1A). siPGI/AMF-1 and siPGI/AMF-2 exhibited decreased expression of endogenous PGI/AMF at protein level, ~70%, respectively, by Western blot analysis. Interestingly, downregulation of endogenous PGI/AMF by siRNA led to the complete depletion of PGI/AMF secretion (Fig. 1A).

**Effect of PGI/AMF gene silencing on tumor cell locomotion and invasion.** The *in vitro* phagokinetic track assay was designed to test whether transfection of siRNA PGI/AMF altered the locomotive potential of tumor cells. After 24 h of incubation, reduction of the PGI/AMF protein resulted in an 80% decrease in cell migration (Fig. 1B).

To study the effect of siRNA PGI/AMF transfection on invasion, parental and transfected cells were seeded on Matrigel-coated Transwell chambers. The ability of siPGI/AMF cells to invade through Matrigel was decreased by 50% compared with control cells (Fig. 1C). Both motility and invasive potential of PGI/AMF knockdown cells seemed significantly reduced.

**Reduction of PGI/AMF expression induces dramatic change in cell morphology.** Next, we investigated whether the above changes in phenotypes were associated with any morphologic changes. The cells were plated and cultured, and morphologies were documented during progression from sparse monolayers to confluences (Fig. 1D). No obvious morphologic changes were observed when the mock cells were compared with the parental cells at either sparse or confluent density, and all of the cells maintained their mesenchymal elongated spindle-shaped fibroblast-like morphology. In sharp contrast, the siPGI/AMF cells underwent a significant morphologic transformation whereby, under sparse culture conditions, the cells acquired a flattened and less elongated shape. On reaching confluence, the cell culture acquired a cobblestone-like appearance typical of epithelial monolayer. Furthermore, the cells seemed to be contact inhibited as opposed to the control cells that continued at confluence to pile up in a typical mesenchymal growth fashion.

**Inhibition of cell growth.** To investigate the effect of knockdown of PGI/AMF on cell proliferation, cell proliferation assay was done. The cells were seeded at low density and grown for 8 days. Fresh medium was provided everyday. The results showed that silencing of the PGI/AMF gene caused a stagnation of the number of viable cells, whereas control cells continued proliferating normally (Fig. 2A). Knockdown of PGI/AMF slowed down the growth of the cells significantly (Fig. 2A).

**Inhibition of cell migration and invasion.** To verify the effect of PGI/AMF knockdown on cell adhesion, Boyden chamber assay was conducted. The results showed that knockdown cells seemed significantly reduced (Fig. 2B). Both motility and invasive potential of PGI/AMF knockdown in human fibrosarcoma HT1080 cells. A, HT1080 cells were stably transfected with plasmid containing PGI/AMF-specific siRNA (siPGI/AMF) or control plasmid (mock) as described in Materials and Methods. Cells were then analyzed by RT-PCR and immunoblot analysis for PGI/AMF and -actin expression. Bottom, quantificational analysis of PGI/AMF expression. Representative results of three different experiments. B, phagokinetic track assay. Cells were seeded onto the colloidal gold cover slides in culture medium. After 24 h, each motile area of cells was calculated by NIH image software. C, in vitro invasion assay. Cells were seeded onto the upper surface of Matrigel-coated Transwell chambers. After 24 h of incubation, the cells invading to the lower surface were visually counted. *, P < 0.05; **, P < 0.01; ***, P < 0.001, compared with control cells. D, dramatic morphologic changes in PGI/AMF knockdown cells. HT1080 parental cells (top), mock cells (middle), and siPGI/AMF-1 cells (bottom) were cultured sparsely (left) or densely (right) on the plastic dishes and taken photographs under phase-contrast microscopy. Bar, 15 μm. Similar images were obtained using siPGI/AMF-2 cells (data not shown).
proliferation rate in cells, resulting in a reduction of cell numbers (0.8-fold at day 6, 0.75-fold at day 7, and 0.7-fold at day 8). When the medium was not changed and the cells were grown for 5 days, cell numbers were inhibited more markedly (0.7-fold at day 4 and 0.65-fold at day 5; data not shown).

Moreover, we assessed G1-S transition in the cell cycle by measuring the BrdUrd incorporation in sparse and confluent control and siPGI/AMF cells. As shown in Fig. 2B, knockdown of PGI/AMF cells decreased BrdUrd incorporation in sparse cells to 70% compared with control cells and decreased in confluent cells to 40%. There was no significant difference between sparse and confluent cultured control cells, but confluent control cells tended to increase BrdUrd incorporation compared with sparse cells (data not shown). Confluent siPGI/AMF cells behaved like epithelium, showing a markedly lower DNA synthesis than sparse cells (Fig. 2B).

Effect of PGI/AMF inhibitor on tumor cell growth and DNA synthesis. PGI/AMF is neutralized by specific PGI/AMF inhibitors, including E4P and mannose 6-phosphate (carbohydrate phosphates; ref. 8). It has been reported that the carbohydrate phosphates bind to the enzymatically and cytokine functionally active site of PGI/AMF, which is considered to be the significant domain to recognize and bind to PGI/AMF receptor (9, 26). Indeed, cytokine functions of PGI/AMF are inhibited by the carbohydrate phosphates (8). Therefore, we first examine whether carbohydrate phosphates can inhibit PGI/AMF protein expression in HT1080 cells. In the presence of E4P, we observed a slightly decreased expression of endogenous PGI/AMF in both sparse and confluent cultured cells (Fig. 3A). The expression of secreted PGI/AMF protein levels in confluent cells was increased by 1.3-fold relative to the levels in sparse cells; moreover, E4P had a suppressive effect on secreted PGI/AMF levels in both sparse and confluent cultured cells (Fig. 3A). These results imply that E4P could not only bind to exogenous PGI/AMF and inhibit the cytokine activity but also decrease PGI/AMF expression in tumor cells.

Next, we used MTT assays to assess the growth of the tumor cells treated with or without the carbohydrate phosphates. As shown in Fig. 3B, HT1080 cells continued proliferating normally, and siPG/ AMF-1 cells used as a positive control stopped proliferating on reaching confluence. Addition of 1 or 10 µmol/L of E4P caused growth inhibition of HT1080 cells similar to siPG/AMF–1–positive control cells, whereas 0.1 µmol/L E4P had a small tendency to inhibit.

The effect of E4P on G1-S transition in the cell cycle was determined with BrdUrd incorporation. When cells were treated with the indicated concentrations of E4P, S-phase entry was dose dependently inhibited (Fig. 3C). E4P has a greater suppressive effect on DNA synthesis in control cells (HT1080 parental and mock cells) than those in siPGI/AMF cells; moreover, it has a clearly suppressive effect in confluent cultured cells than those in sparsely cultured cells.

Although E4P affects tumor cell growth and proliferation, cell morphology was not changed by E4P treatment (data not shown). PGI/AMF silencing completely suppresses tumorigenicity of human fibrosarcoma cells. Next, we examined whether the above phenotypic changes were also associated with functional changes by exploring in vitro anchorage-independent growth in soft agar and in vivo tumor growth in athymic nude mice. As shown in Fig. 4A, both control cells effectively grew and developed into tumor colonies in soft agar, whereas the siPGI/AMF cells lost the in vitro cancer hallmark and failed to grow in an anchorage-independent fashion, suggesting loss of tumorigenicity.

As we showed that stable PGI/AMF siRNA clones showed completely inhibited colony formation on soft agar, we further tested their ability to induce tumorigenicity in nude mice. We injected control HT1080 cells and PGI/AMF siRNA clones into nude mice s.c., and the mice were monitored for 24 days, during which tumor growth was assessed periodically. These results are shown in Fig. 4B, whereas insets show an example of a tumor induced by injection of either clone of mock or PGI/AMF siRNA at day 24. As expected, mice injected with control cells (parental and mock) developed visible solid tumors by week 1, but there was no sign of tumor formation on mice injected with PGI/AMF siRNA clones. These studies highlight the critical function of PGI/AMF expression during tumor growth and support prior results showing that overexpression of PGI/AMF in NIH-3T3 fibroblasts leads to a gain of tumorigenic properties in vivo (27). Down-regulation of PGI/AMF leads MET in tumor cells. Growth factors and cytokines, such as TGF-β, FGF, HGF, and TNF-α, can regulate EMT (3–6). The morphologic changes and the loss of tumor abilities in siPGI/AMF cells prompted us to examine whether they are associated with loss of mesenchymal and/or gain of epithelial markers. Thus, the expression of the intermediate filaments vimentin (a mesenchymal marker) and cytokeratin (an epithelial marker) was tested. The expression and subcellular distribution of vimentin were examined by immunofluorescent staining using the human breast epithelial cell line MCF10A as a negative control (Fig. 5A). Vimentin was prominently expressed throughout the cytoplasm of the parental HT1080 and the mock cells, whereas a significantly weaker vimentin signal was detected.
in the siPGI/AMF cell clone. As expected, the MCF10A cells were vimentin null. Western blot analysis confirmed that the expression of vimentin protein was indeed reduced in the siPGI/AMF cells compared with control cells (Fig. 5B). Next, we tested whether the reduced vimentin expression was associated with a gain of epithelial markers. Thus, the cells were immunostained for the presence of the mesenchymal intermediate filament marker (i.e., cytokeratin). Neither the parental HT1080 cells nor the mock-transfected cells expressed cytokeratin proteins (Fig. 5A). In contrast, cytokeratins were expressed in the siPGI/AMF cells, albeit at a lower level than expressed by a bona fide epithelial cells (Fig. 5A). Western blot analysis detected a cytokeratin protein band in the siPGI/AMF cells that comigrated with some of the cytokeratin proteins expressed by MCF10A epithelial cells (Fig. 5B).

We also examined whether PGI/AMF inhibitor affected these EMT-related protein expressions. We exposed HT1080 cells to various concentrations of E4P and immunoblotted cell lysates. However, E4P did not affect protein expressions in HT1080 cells (data not shown).

In analyzing the expression of E-cadherin, an adhesion molecule characteristic of epithelial cells (28), we could not detect its expression in the siPGI/AMF cells under either sparse or confluent culture conditions (data not shown). Thus, we proceeded to examine the expression of Snail and Slug that are transcription repressors that play a central role in EMT during both embryonic development and tumor progression (28, 29). Of note, in EMT, Snail genes lead to the loss of epithelial markers as well as changes in cell shape and the expression of mesenchymal markers (28, 29) and we therefore tested whether their expressions are affected by the down-regulation of PGI/AMF expression. Expression of Snail and Slug was examined by RT-PCR and Western blot analysis. Significant reductions were observed in the siPGI/AMF cells compared with the control cells (Fig. 5C). To corroborate the link between Snail genes and blockade of PGI/AMF-induced MET, we examined the expression of fibronectin, a mesenchymal marker downstream target of Snail genes (30). The fibronectin protein level was decreased in siPGI/AMF cells compared with the control cells similar to the reduction in the level of vimentin expression (Fig. 5D).

Discussion

PGI is the second enzyme in the glycolytic pathway and catalyzes the interconversion of glucose 6-phosphate and fructose 6-phosphate during glycolysis and gluconeogenesis. Thus, it is highly conserved in bacteria and eukaryotes (15). Mutations in PGI are a cause of hereditary nonspherocytic hemolytic anemia, and PGI
deficiency can be associated with hydrops fetalis and immediate neonatal death (14–16). According to these knowledge, PGI plays an important role in the cellular metabolism. Meanwhile, outside the cell, PGI has been found to function both as a cytokine and as a growth factor. AMF, one of the cytokines, causes tumor cells to migrate and seems to be involved in tumor invasion and metastasis. Its presence in the serum and urine is of prognostic value associated with cancer progression (17–19). Thus, exogenous AMF plays an important role in tumor progression. In attempt to better understand how PGI/AMF is contributing to tumor progression, stable RNA interference–mediated knockdown of PGI/AMF was established in human fibrosarcoma HT1080 cells. We could reduce the cytoplasmic level of PGI/AMF, and remarkably, we could extinguish the PGI/AMF secretion by RNAi. Because all tumor cell lines we have investigated secrete PGI/AMF protein to some extent (24), these stable lines with PGI/AMF secretion knocked out can be useful for understanding tumor progression. Indeed, we found dramatic changes on varied cell functions in PGI/AMF knockdown cell lines.

In HT1080 cells stably transfected with PGI/AMF siRNA, expression of PGI/AMF mRNA was down-regulated, endogenous PGI/AMF was decreased, and exogenous PGI/AMF was completely diminished. Down-regulation of PGI/AMF markedly changed cell shapes and behaviors in both single cells and sheets of cells, and the cells with siRNA significantly inhibited their migration and invasion abilities. Suppression of exogenous PGI/AMF stopped synthesizing DNA and ceased proliferating cells once the cells became confluent (i.e., exogenous expression of PGI/AMF regulates contact inhibition of cell growth). Finally, HT1080 cells with PGI/AMF secretion knocked out lost their ability to form tumors in mice. All those changes were proportional to attenuated amount of exogenous PGI/AMF by siRNA. Collectively, these data clearly indicate that exogenous expression of PGI/AMF regulates tumorigenesis besides tumor metastasis.

The EMT plays an important role during early development at the gastrulation stage and in the ontogeny of the neural crest in vertebrates and is also an essential component of tissue remodeling and wound repair (1, 2). During EMT, the epithelial phenotype, which is bound together tightly, exhibiting polarity and attached to the underlying extracellular matrix, is replaced by a mesenchymal phenotype, with losing apical basal polarity, elongated cell shape, and migrating as separate cells into the matrix. In this process, loss of cadherin expression is required for losing epithelial cell–cell adherens junctions (6). The EMT is also implicated in the regulation of most cancer metastasis (4). Differentiated mesenchymal cells can spread into tissues surrounding the original tumor as well as separate from the tumor, invade blood and lymph vessels, and travel to new locations where they divide and form additional tumors. PGI/AMF silencing resulted in MET as judged by reduction of mesenchymal cell marker proteins, detection of epithelial cell marker proteins, marked changes in cell morphology, and loss of tumorigenicity. Our results of this study also confirmed the former hypothesis that AMF signaling system is thought to be involved in EMT (22, 23). It should be noted that others have suggested that metastatic cells derived from EMT-mediated primary tumor must be followed by a reverse MET to form a secondary tumor, and several studies have identified a hybrid cell showing both epithelial and mesenchymal phenotypes (3, 31, 32). Furthermore, coexpression of both properties within the same cell may be consistent with the stem cell–like profiles of tumor cells (33). The results presented here may suggest that PGI/AMF can regulate, in part, such plasticity found in progenitor cells in various organs during tumor development, mainly affecting the mesenchymal phenotype. Future studies should explore PGI/AMF silencing because PGI/AMF is largely associated with tumor metastasis, and a recent publication suggested that MET facilitates tumor metastasis in bladder carcinoma (34). Moreover, our results suggest that the conversion between epithelial and mesenchymal cells or morphologic change requires endogenous PGI/AMF: on the other hand, cell growth and proliferation require exogenous PGI/AMF. This hypothesis is also supported by our data that there is no difference in AMFR expression between mesenchymal control tumor cells and epithelial PGI/AMF knockout cells (data not shown). Further investigation is still necessary to address the phenomenon.

The Snail family members Snail and Slug trigger EMT during embryonic development and tumor progression. Because Snail is able to induce a complete EMT, it must have many targets (28–30). Indeed, Snail is upstream of molecules involved in the gain of the mesenchymal markers, repression of epithelial markers, changes in cell shape, cell migratory and invasive properties, cell proliferation, or cell survival (28–30). Among these phenotypic changes, the one would be unexpected is that cell proliferation is attenuated by Snail.
Tumor development is usually associated with increased cell proliferation, but the change to invasive phenotype is related to tumor malignancy, not with its formation or growth. Further evidence about this observation can be seen in the low proliferation in the invasive front of carcinomas (36) and the link between high p21 expression and poor prognosis in breast carcinomas (37). Therefore, Snail might favor invasion over tumor growth. Although Snail genes up-regulate a cyclin-dependent kinase inhibitor expression, our PGI/AMF knockout cells showed its high expression, whereas Snail genes were attenuated. Our results suggest that PGI/AMF strongly increases in cell proliferation by overcoming Snail function and also support our prior report (38).

The siRNA we designed for PGI/AMF was a very efficient suppressor of the expression of the target protein. We observed similar changes in protein expressions and cell morphology by using an alternative target sequence siRNA against PGI/AMF (Supplementary Fig. S1), suggesting that the suppression of PGI/AMF is not due to an off-target effect of the siRNA. Because PGI/AMF seems to promote the malignancy of several cancers, PGI/AMF siRNA may represent an approach to the prevention or
therapy of malignant tumors in vivo. Thus far, only very few studies describe the in vivo use of siRNAs (39, 40). Although further investigation is required to identify the effective strategies for therapy using PGI/AMF siRNA approaches because PGI/AMF plays an important role in normal tissue, it will be important to address this point in the near future. Alternatively, small-molecule antagonists against PGI/AMF would be useful as antitumor or metastasis agents (41, 42).

In conclusion, because elevated expression of PGI/AMF was noted in various targeting, it might be a beneficial therapeutic approach, although further investigation is required to identify effective therapeutic strategies using either siRNA approaches or small PGI/AMF antagonist molecules as useful antitumor or metastasis agents. Our data provide the first in vitro/in vivo data showing the complete suppression in tumor growth due to the MET conversion, suggesting a new therapeutic target/modality for treatment of mesenchymal cancer, and may further the understanding of MET during embryogenesis.

Acknowledgments

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Received 10/24/2006; revised 2/13/2007; accepted 2/22/2007.

Grant support: NIH/National Cancer Institute grant R01 CA51714 (A. Raz).

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We thank G. Brush and Y. Xie for the valuable comments on the manuscript and V. Powell for her editing of the manuscript.
Down-Regulation of Phosphoglucone Isomerase/Autocrine Motility Factor Results in Mesenchymal-to-Epithelial Transition of Human Lung Fibrosarcoma Cells

Tatsuyoshi Funasaka, Huankai Hu, Takashi Yanagawa, et al.