Phosphoglucone Isomerase/Autocrine Motility Factor Mediates Epithelial-Mesenchymal Transition Regulated by miR-200 in Breast Cancer Cells

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Abstract
Phosphoglucone isomerase/autocrine motility factor (PGI/AMF) plays an important role in glycolysis and gluconeogenesis and is associated with invasion and metastasis of cancer cells. We have previously shown its role in the induction of epithelial-mesenchymal transition (EMT) in breast cancer cells, which led to increased aggressiveness; however, the molecular mechanism by which PGI/AMF regulates EMT is not known. Here we show, for the first time, that PGI/AMF overexpression led to an increase in the DNA-binding activity of NF-κB, which, in turn, led to increased expression of ZEB1/ZEB2. The microRNA-200s (miR-200a, miR-200b, and miR-200c) are known to negatively regulate the expression of ZEB1/ZEB2, and we found that the expression of miR-200s was lost in PGI/AMF overexpressing MCF-10A cells and in highly invasive MDA-MB-231 cells, which was consistent with increased expression of ZEB1/ZEB2. Moreover, silencing of PGI/AMF expression in MDA-MB-231 cells led to overexpression of miR-200s, which was associated with reversal of EMT phenotype (i.e., mesenchymal-epithelial transition), and these findings were consistent with alterations in the relative expression of epithelial (E-cadherin) and mesenchymal (vimentin, ZEB1, ZEB2) markers and decreased aggressiveness as judged by clonogenic, motility, and invasion assays. Moreover, either reexpression of miR-200 or silencing of PGI/AMF suppressed pulmonary metastases of MDA-MB-231 cells in vivo, and anti-miR-200 treatment in vivo resulted in increased metastases. Collectively, these results suggest a role of miR-200s in PGI/AMF-induced EMT and thus approaches for upregulation of miR-200s could be a novel therapeutic strategy for the treatment of highly invasive breast cancer.

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Introduction
Phosphoglucone isomerase (PGI) is a housekeeping cytosolic enzyme that brings the interconversion of glucose-6-phosphate and fructose-6-phosphate (1), playing key role during glycolysis and gluconeogenesis. In addition, PGI serves several other functions—as tumor-secreted cytokine, autocrine motility factor (AMF), it stimulates cell motility, migration, invasion, and metastasis (2, 3); as neurotrophic factor, neuroleukin, it supports the survival of embryonic spinal neurons, skeletal motor neurons, and sensory neurons (4); and, as maturation factor, it mediates differentiation of human myeloid leukemic cells (5). Other functions of PGI include its role as sperm antigen-36 (6) and a serine proteinase inhibitor (7). PGI/AMF and its receptor are also associated with cancer progression and poor prognosis (8–10).

Recent evidences have suggested that PGI/AMF plays an important role during the intraconversion between EMT and MET (11–13). The phenomenon of EMT is associated with acquisition of invasive phenotype by cancer cells (14, 15) and, in particular, an aggressive behavior of breast cancer cells in vitro (16) and in vivo (17). The role of PGI/AMF in the progression of breast cancer is well established (10, 18, 19), which is consistent with our recent observation (12), showing induction of EMT by PGI/AMF in MCF-10A nontumorigenic human breast epithelial cells. Conversely, we found reversal of EMT leading to mesenchymal-epithelial transition (MET) on silencing of PGI/AMF in aggressive MDA-MB-231 cells. These findings suggested an importance of PGI/AMF in the regulation of EMT; however, the molecular mechanism is yet to be established.

Loss and gain of specific microRNAs (miRNA) is associated with invasion and metastasis, and miRNAs are known to regulate the acquisition of mesenchymal phenotype of cells (20–22). However, nothing is known as to the interrelationships between PGI/AMF, miRNAs, and EMT–MET, which prompted the current investigation. Here we report for the
first time that PGI/AMF overexpression could lead to increased DNA-binding activity of NF-κB, which transcriptionally upregulates the expression of ZEB1 and ZEB2, resulting in the induction of EMT, associated with the loss of miR-200s expression.

Materials and Methods

Cell lines and reagents

MCF-10A human breast epithelial cells were maintained in DMEM-F12 medium supplemented with 0.1 μg/mL cholera toxin, 0.02 μg/mL epidermal growth factor, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 100 U/mL penicillin, 100 μg/mL streptomycin, and 5% horse serum (12). Human breast cancer cell lines MDA-MB-231 and BT-549 were cultured in DMEM and RPMI media, respectively, with 10% FBS and penicillin/streptomycin. All cells were cultured in 5% CO2-humidified atmosphere at 37°C. The cell lines have been tested and authenticated in core facility (Applied Genomics Technology Center at Wayne State University) by short tandem repeat profiling, using the PowerPlex 16 System from Promega. Antibodies were purchased from following sources; vimentin (Abcam), MMP-9 (R&D Systems), E-cadherin, ZEB1, and uPAR (Santa Cruz Biotechnology Inc.), and ZEB2 and β-actin (Sigma-Aldrich).

Real-time reverse transcriptase PCR

Total RNA was isolated using TRizol reagent (Invitrogen) according to the manufacturer’s instructions. Real-time PCR was used to quantify mRNA expression. Sequences of primers for E-cadherin, vimentin, ZEB1, ZEB2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were same as reported earlier (23), and the amount of RNA was normalized to GAPDH expression. For miRNA analysis, total RNA was isolated using the mirVana miRNA isolation kit (Ambion). The levels of miRNAs were determined using miRNA-specific TaqMan MGB probes from the TaqMan MicroRNA Assay (Applied Biosystems). The relative amounts of miRNA were normalized to RNU6B.

Transfection experiments

Detailed methodology for the generation of stably transfected MCF-10A cells with full-length PGI/AMF cDNA and MDA-MB-231 cells with specific siRNA targeting PGI/AMF has been described (12). Cell clones were maintained by adding 300 μg/mL zeocin (Invitrogen) to the culture medium.

Preparation of nuclear lysates and electrophoretic mobility shift assay

Nuclear protein extract was prepared and subjected to electrophoretic mobility shift assay (EMSA) for assessing the DNA-binding activity of NF-κB (24). EMSA was done by incubating 4 μg of nuclear protein extract with IRDye-700-labeled NF-κB oligonucleotides (LI-COR, Inc.). Incubation mixture included 2 μg of poly(dI)–poly(dC), a copolymer of polydeoxyinosinic polydeoxyctydilic acid, in the binding buffer. DNA–protein complex formed was separated from free oligonucleotide on 8% native polyacrylamide gel and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1 (LI-COR, Inc.).

Wound healing assay

Monolayer cultures of MCF-10A and MDA-MB-231 (control and PGI/AMF-transfected/silenced), seeded in 6-well plates, were wounded with 200-μL pipette tips once they were 80% confluent. Pictures were taken at 0 and 24 hours time points under phase-contrast microscope to monitor the migration of cells into the open space (wound). Quantification of wound healing was done using NIH Image software. The outlines of wound were marked by 2 diagonal parallel lines in each image. Lines within the wound mark the progress of cells that migrated into the wound. The migratory distances (in microns) of all cells that migrated into the wound were totaled and are represented as bar graphs.

Clonogenic assay

To test the survival of breast cells, clonogenic assay was done (25). Briefly, cells were plated in 6-well plates, incubated overnight, appropriately transfected, grown for 48 hours, trypsinized, and the viable cells counted (trypsin blue exclusion) and plated in 100-mm Petri dishes in a range of 100 to 1,000 cells per plate. The cells were then incubated for 14 to 21 days at 37°C in a 5% CO2/5% O2/90% N2 incubator, and colonies were stained with 2% crystal violet, counted, and photographed.

Western blot analysis

For Western blot analysis, cells were lysed in RIPA (radioimmunoprecipitation assay) buffer containing complete mini EDTA-free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich; ref. 24). After resolution on 12% polyacrylamide gels under denaturing conditions, proteins were transferred to nitrocellulose membranes, incubated with appropriate primary/horseradish peroxidase–conjugated secondary antibodies, and visualized using chemiluminescence detection system (Pierce).

Cell invasion and migration assay

Cell invasion assay was done using 24-well Transwell permeable supports with 8-μm pores (Corning; ref. 25). Cells were suspended in serum-free medium and seeded into the Transwell inserts coated with growth factor–reduced Matrigel (BD Biosciences). Bottom wells were filled with media containing complete media. After 24 hours, cells were stained with 4 μg/mL calcein AM (Invitrogen) in PBS at 37°C for 1 hour and photographed under a fluorescent microscope. The cells were detached from inserts by trypsinization, and fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN).

Experimental pulmonary metastasis assay

The animal experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Wayne State University Institutional Users of Animal Care Committee. Two million MDA-MB-231 cells transected with either empty vector or PGI/AMF-specific siRNA (PGI/AMF-silenced
cells) were injected through tail vein of female ICR-SCID mice (5–6 weeks old) obtained from Taconic Farms. Effect of pre-miR-200s was studied by transfections of expression plasmids with pre-miR-200s (Origene Technologies) in MDA-MB-231 vector control cells prior to injection in mice. To study the effect of anti-miR-200s in vivo, PGI/AMF-silenced MDA-MB-231 cells were injected in mice via tail vein, and starting with the next day, animals were treated with PBS-formulated, locked nucleic acid (LNA)-modified oligonucleotide (LNA-anti-miR200b; obtained from Exiqon; ref. 26) administrated intravenously (25 mg/kg) 3 times a week, for a total of 5 weeks. All mice were euthanized at the end of week 5. Lungs were removed, formalin fixed, and stained with hematoxylin and eosin (H&E). Lung metastatic nodules were counted microscopically. Histologic and microscopic analysis was carried out by a pathologist, and the data were analyzed by a statistician.

Data analysis
The experimental results presented in the figures are representative of 3 or more independent observations. The data are presented as the mean values ± SE. For in vivo group comparison tests, we used log-transformed data with continuity correction. The normality assumption in each group was checked using the Kolmogorov–Smirnov test. The pairwise 2-sample t test is used if normality assumption holds. The pairwise Wilcoxon test is used otherwise if normality assumption fails. Values of $P < 0.05$ were considered to be statistically significant.

Results
Expression of PGI/AMF correlates with ZEB1/ZEB2 and NF-κB activity
It has been reported that ectopic expression of PGI/AMF in a nontumorigenic cell line MCF-10A results in an increase in the expression of mesenchymal marker vimentin, accompanied by a decrease in epithelial marker E-cadherin (12), suggesting the acquisition of EMT phenotype. Here we conducted reverse transcriptase (RT-PCR) assessing the mRNA transcripts of these markers and other mesenchymal markers, ZEB1 and ZEB2. In addition to the verification of earlier results with E-cadherin and vimentin (Fig. 1A), we found that PGI/AMF transfection resulted in a significant increase in the expression of ZEB1 (4-fold increase) and ZEB2 (60% increase; Fig. 1A; $P < 0.01$). Downregulation of PGI/AMF expression in MDA-MB-231 resulted in an increase in E-cadherin mRNA expression, concomitant with a decrease in vimentin expression (Fig. 1A). A significant decrease in the expression of ZEB1 (almost 100% inhibition) and ZEB2 (62% decrease; Fig. 1A) in these cells ($P < 0.01$) was also noted. To rule out any cell line–specific effects, we carried out similar studies using aggressive BT-549 breast cancer cells and found that the silencing of PGI/AMF resulted in a significant increase in E-cadherin and decrease in vimentin/ZEB1/ZEB2 expression (Fig. 1B), which was consistent with the data obtained from MDA-MB-231 cells.

It is known that ZEB1/ZEB2 can be regulated by NF-κB (27) and that cells undergoing EMT have elevated NF-κB activity (28, 29). Therefore, we evaluated DNA-binding activity of NF-κB in our models (Fig. 1C). The characteristic p65 NF-κB bands were quantitated (identified by the arrows in the figure) and increased NF-κB activity (more than 4-fold) was observed in MCF-10A cells transfected with PGI/AMF as well as in control MDA-MB-231 and BT-549 cells that have natural mesenchymal phenotype. We also observed a significant decrease in the DNA-binding activity of NF-κB in MDA-MB-231 and BT-549 cells (47% and 50%, respectively) silenced for PGI/AMF expression (Fig. 1C). These observations suggested a plausible involvement of NF-κB signaling in EMT induction by PGI/AMF. To confirm this, we evaluated the expression of uPAR and MMP-9, 2 NF-κB downstream target genes, and found that ectopic expression of PGI/AMF in MCF-10A cells led to increased expression of uPAR and MMP-9 whereas silencing of PGI/AMF in MDA-MB-231 and BT-549 cells led to a significant downregulation of their expression (Fig. 1D). uPAR (30) and MMP-9 (31) have been linked with the induction of EMT in breast cancer cells, which further implicates the role of NF-κB signaling in PGI/AMF-induced EMT. Silencing of PGI/AMF in MDA-MB-231 and BT-549 cells showed similar effects on markers of EMT and NF-κB signaling. Therefore, we chose MDA-MB-231 cells for further detailed mechanistic studies.

Increased PGI/AMF expression reduced miR-200s, and reexpression of miR-200s restored epithelial phenotype in MCF-10A cells
In addition to the activation by NF-κB, ZEB1 and ZEB2 are also regulated by miR-200 family (22, 32, 33). Therefore, we questioned whether the levels of miR-200 family could be affected in PGI/AMF-expressing MCF-10A cells. Analysis of basal levels of miR-200 family in the paired cell lines revealed that the overexpression of PGI/AMF caused a significant decrease (miR-200a 60%; miR-200b 38%; and miR-200c 20%) in the levels of all 3 miR-200s (Fig. 2A). To assess functional implications of these findings, we transfected PGI/AMF-overexpressing MCF-10A cells with a cocktail of pre-miRNAs (pre-miR-200a + pre-miR-200b + pre-miR-200c). Transfection of pre-miRNAs is a standard technique to induce the expression of target miRNAs (23). Reexpression of miR-200s resulted in increased expression of E-cadherin (2.5-fold increase; Fig. 2B; $P < 0.01$) and decreased expression of vimentin (56% decrease; Fig. 2C; $P < 0.01$), ZEB1 (79% decrease; Fig. 2D; $P < 0.01$), and ZEB2 (25% decrease; Fig. 2E; $P < 0.05$). The levels of miRNAs in pre-miRNA–transfected cells approached those in native cells, thus verifying the efficiency of reexpression of miRNAs (Fig. 2A).

PGI/AMF silencing–induced MET could be reversed by suppressing the expression of miR-200s
Induction of MET by silencing of PGI/AMF in aggressive MDA-MB-231 cells has been previously shown (12). Here we found that such silencing results in an increased expression of all miR-200s (miR200a, 3.5-fold; miR200b, 5.8-fold; and miR200c, 2.7-fold; Fig. 3A; $P < 0.01$). Among the 3 miR-200s, miR-200b levels were found to be particularly elevated. Suppression of these upregulated miR-200s, using a cocktail of...
specific anti-miR-200s (anti-miR-200a + anti-miR-200b + anti-miR-200c), resulted in derepression of vimentin mRNA transcripts (Fig. 3B; \( P < 0.01 \)). Levels of miRNAs in anti-miRNA–transfected cells approached those in native cells, thus verifying the efficiency of downregulation of miRNAs (Fig. 3A).

**Role of miR-200s in cell motility and clonogenicity of breast cells**

In MCF-10A cells transfected with PGI/AMF, significant migration of cells into open space (wound) was observed after 24 hours, which was found to be inhibited by reexpression of miR-200s (Fig. 4A). In MDA-MB-231 cells, wound closure was observed in the control cell cultures after 24 hours but not in PGI/AMF-silenced cells (Fig. 4B). Expression of miR-200s in control cells inhibited wound healing, whereas suppression of miR-200s, by anti-miR200s, in PGI/AMF-silenced cells stimulated cell motility (Fig. 4B).

Next, we tested the effect of PGI/AMF and miRNA-200s on the clonogenic potential of MCF-10A and MDA-MB-231 cells. PGI/AMF-transfected MCF-10A cells formed more colonies as revealed by crystal violet staining, and reexpression of miR-200s suppressed this effect (82% decrease; Fig. 4C). Silencing of PGI/AMF in MDA-MB-231 cells, on the other hand, significantly decreased the number of colonies (57% decrease), and this inhibition was reversed by the suppression of miR-200s (~3-fold increase in the number of colonies; Fig. 4C).

**miR-200s reverse the effects of PGI/AMF on markers of EMT and invasion of breast cells**

Reexpression of miR-200s in PGI/AMF-transfected MCF-10A cells resulted in reexpression of E-cadherin and suppression of vimentin/ZEB1/ZEB2, whereas suppression of miR-200s caused a downregulation of E-cadherin and upregulation of vimentin/ZEB1/ZEB2 in PGI/AMF-silenced MDA-MB-231 cells (Fig. 5A). Because PGI/AMF is involved in the invasion of
Figure 2. Reexpression of miR200s reverses EMT induction by PGI/AMF in MCF-10A cells. A, basal expression of miR-200a, miR-200b, and miR-200c was evaluated by real-time RT-PCR in MCF-10A (vector control vs. PGI/AMF-expressing cells ± pre-miRNAs). B–E, effect of reexpression of miR200s (miR-200a + miR-200b + miR200c) in PGI/AMF-expressing MCF-10A cells on the expression of indicated EMT markers was also evaluated by real-time RT-PCR. N, nonspecific pre-miRNA; PM or M, specific pre-miRNAs (pre-miR200a + pre-miR200b + pre-miR200c). *, P < 0.05 and **, P < 0.01 versus vector/nonspecific control.

Figure 3. Suppression of miR200s negates PGI/AMF silencing–induced effects on EMT in MDA-MB-231 cells. A, basal expression of miR-200a, miR-200b, and miR-200c was evaluated by real-time RT-PCR in MDA-MB-231 (vector control vs. PGI/AMF-silenced cells ± anti-miRNAs). B–E, effect of suppression of miR200s (miR-200a + miR-200b + miR200c) in PGI/AMF-silenced MDA-MB-231 cells on the expression of EMT markers was also evaluated by real-time RT-PCR. N, nonspecific anti-miRNA; AM or M, specific anti-miRNAs (anti-miR200a + anti-miR200b + anti-miR200c). **, P < 0.01 versus vector/nonspecific control.
breast cancer cells, we carried out invasion assays using Matrigel-coated Transwell inserts (Fig. 5B). In MCF-10A cells, PGI/AMF-induced increase in invasion was inhibited by reexpression of miR-200s, whereas in MDA-MB-231 cells, PGI/AMF silencing–induced inhibition of invasion was eased by suppression of miR-200s.

Role of miR-200s in experimental pulmonary metastases of breast cancer cells

Because our in vitro results suggested a mechanistic role of miR-200s in PGI/AMF-induced invasion, we carried out further investigations in vivo by injecting metastatic MDA-MB-231 cells via tail vein in SCID mice, which led to spontaneous lung metastases (Fig. 6), whereas either reexpression of miR-200 or silencing of PGI/AMF by siRNA led to significant decrease in pulmonary metastases (Fig. 6). The Wilcoxon tests comparing reexpression of miR-200 to control and silencing of PGI/AMF to control resulted $P$ values of 0.0048 and 0.0043, respectively. Our aforementioned in vitro experiments suggested that loss of miR-200 is associated with increased tumor aggressiveness and that reexpression of miR-200 reduced tumor aggressiveness. Because PGI/AMF-silenced MDA-MB-231 cells showed reduced pulmonary metastases consistent with overexpression of miR-200, we hypothesized that anti-miR-200 could increase the pulmonary metastases of PGI/AMF-silenced MDA-MB-231 cells in vivo. We found that the treatment of animals, inoculated with PGI/AMF-silenced MDA-MB-231 cells, with anti-miR-200b (by intravenous injection with a special formulation that combines high-affinity LNA-anti-miR with phosphorothioate modifications to achieve an efficient delivery and silencing of target miRNA (26) led to increased pulmonary metastases; Fig. 6), suggesting a molecular link between PGI/AMF, miR-200, and metastases. The Wilcoxon test comparing nonspecific anti-miRNA to specific anti-miRNA resulted $P$ value of 0.0154.

Discussion

The major conclusions from our current study are as follows: (i) ectopic expression of PGI/AMF in nontumorigenic MCF-10A breast epithelial cells results in the downregulation of epithelial marker, E-cadherin, with concomitant upregulation of mesenchymal markers vimentin, ZEB1, ZEB2, and NF-$k$B activity. Silencing of PGI/AMF in MDA-MB-231 cells, on the other hand, results in upregulation of E-cadherin and downregulation of vimentin, ZEB1, ZEB2, and NF-$k$B activity; (ii) expression of PGI/AMF correlates negatively with the expression of miRNAs miR-200a, miR-200b, and miR-200c; (iii) reexpression of these miRNAs in MCF-10A cells abrogates the effects of PGI/AMF overexpression and restores epithelial...
phenotype, whereas their silencing in MDA-MB-231 cells overcomes the changes brought about by PGI/AMF silencing; and (iv) miRNAs play a key role in the PGI/AMF-mediated EMT in breast cells. A, Western blot analysis for the expression of indicated EMT markers. β-Actin protein was used as a protein loading control. PM, specific pre-miRNAs (pre-miR200a + pre-miR200b + pre-miR200c); AM, specific anti-miRNAs (anti-miR200a + anti-miR200b + anti-miR200c); P/A, PGI/AMF-transfected cells; sP/A, PGI/AMF-silenced cells. B, invasion of breast cells MCF-10A (top) and MDA-MB-231 (bottom) was assayed by plating cells in Matrigel-coated inserts. The cells that invaded through the Matrigel were stained and photographed using a fluorescence microscope: a, MCF-10A control cells; b, PGI/AMF-transfected MCF-10A cells; c, PGI/AMF-transfected MCF-10A cells + pre-miR200a (pre-miR200a + pre-miR200b + pre-miR200c); d, MDA-MB-231 control cells; e, PGI/AMF-silenced MDA-MB-231 cells; f, PGI/AMF-silenced MDA-MB-231 cells + anti-miR200a (anti-miR200a + anti-miR200b + anti-miR200c).

Figure 5. miR-200s modulate PGI/AMF-mediated EMT and invasion of breast cells. A, Western blot analysis for the expression of indicated EMT markers. β-Actin protein was used as a protein loading control. PM, specific pre-miRNAs (pre-miR200a + pre-miR200b + pre-miR200c); AM, specific anti-miRNAs (anti-miR200a + anti-miR200b + anti-miR200c); P/A, PGI/AMF-transfected cells; sP/A, PGI/AMF-silenced cells. B, invasion of breast cells MCF-10A (top) and MDA-MB-231 (bottom) was assayed by plating cells in Matrigel-coated inserts. The cells that invaded through the Matrigel were stained and photographed using a fluorescence microscope: a, MCF-10A control cells; b, PGI/AMF-transfected MCF-10A cells; c, PGI/AMF-transfected MCF-10A cells + pre-miR200a (pre-miR200a + pre-miR200b + pre-miR200c); d, MDA-MB-231 control cells; e, PGI/AMF-silenced MDA-MB-231 cells; f, PGI/AMF-silenced MDA-MB-231 cells + anti-miR200a (anti-miR200a + anti-miR200b + anti-miR200c).
evidence in support of the involvement of miR-200s in PGI/AMF-mediated effects on aggressive behavior of MDA-MB-231 cells. We show using a reciprocal model in MCF-10A cells that PGI/AMF-mediated induction of invasion can be effectively blocked by reexpression of miR-200s. Our results with the expression levels of EMT markers (Fig. 5) in the 2 cell lines confirm the key regulatory role of miR-200s in the modulation of EMT by PGI/AMF.

On the basis of existing literature, together with our current findings, a model for the regulation of EMT by PGI/AMF involving miR-200s, NF-κB, and mesenchymal markers ZEB1/ZEB2 is emerging as represented by a hypothetical diagram (Fig. 7). There seems to be a complex reciprocal relationship between miR-200s and ZEB1/ZEB2. It has been suggested that there are 3 putative binding sites for miR-200a in the 3′-UTR (untranslated region) region of ZEB1 and ZEB2 whereas miR-200b/c have 5 binding sites in the 3′-UTR region of ZEB1 and 6 sites in ZEB2 (22), respectively. The miR-200s repress the expression of ZEB1 and ZEB2 by direct binding to these sites (22). Reciprocally, ZEB1 and ZEB2 negatively regulate miR-200a/miR-200b by binding to paired CACCTG (E-box) sites (45). Such repression has been shown in several mesenchymal breast cancer cell lines, including those used here (MDA-MB-231 and BT-549; ref. 45). Another report showed direct repression of miR200s by ZEB1 and a significant upregulation of miR-200b/c following a stable knockdown of ZEB1 in MDA-MB-231 cells (46). Collectively, these reports indicate a reciprocal relationship between miR-200s and ZEB1/ZEB2 as summarized in Figure 7. A direct relationship between miR-200s and NF-κB has not been clearly established; however, there is evidence to suggest the regulation of ZEB1/ZEB2 by NF-κB (27). In this report, ectopic expression of NF-κB (p65) in MCF-10A cells was shown to induce ZEB1/ZEB2 expression and p65 was observed to regulate ZEB1 via direct binding to its promoter. Our results clearly show upregulation of NF-κB (Fig. 1C) and downregulation of miR-200s (Figs. 2 and 3) by PGI/AMF, and both these events could result in the upregulation of ZEB1/ZEB2 leading to EMT and aggressiveness of cancer cells (Fig. 7), thus explaining the mechanism of PGI/AMF action.

Because metastasis of breast cancer is directly related to poor prognosis, thus molecular markers of invasion and metastasis, such as PGI/AMF, offer attractive targets for therapy. PGI/AMF-mediated induction of EMT provides a broad mechanism through which it influences the invasion of breast cells. Here, we provided further insight into the individual regulator, the miR-200 family, in mediating the effects of PGI/AMF signaling at the molecular level. These in vitro and in vivo results provide mechanistic evidence, for the first time, linking miR-200 with the biological activity of
PGI/AMF, further suggesting that innovative approaches by which miR-200s could be upregulated can potentially serve as a novel therapeutic strategy for the treatment of highly invasive breast cancer in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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