Silencing of Autocrine Motility Factor Induces Mesenchymal-to-Epithelial Transition and Suppression of Osteosarcoma Pulmonary Metastasis

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Abstract

Phosphoglucose isomerase (PGI) is a multifunctional enzyme that functions in glucose metabolism as a glycolytic enzyme catalyzing an interconversion between glucose and fructose inside the cell, while it acts as cytokine outside the cell, with properties that include autocrine motility factor (AMF)–regulating tumor cell motility. Overexpression of AMF/PGI induces epithelial-to-mesenchymal transition with enhanced malignancy. Recent studies have revealed that silencing of AMF/PGI resulted in mesenchymal-to-epithelial transition (MET) of human lung fibrosarcoma cells and breast cancer cells with reduced malignancy. Here, we constructed a hammerhead ribozyme specific against GUC triplet at the position G390 in the human, mouse, and rat AMF/PGI mRNA sequence. Mesenchymal human osteosarcoma MG-63, HS-Of-1, and murine LM8 cells were stably transfected with the ribozyme specific for AMF/PGI. The stable transfectant cells showed effective downregulation of AMF/PGI expression and subsequent abrogation of AMF/PGI secretion, which resulted in morphologic change with reduced growth, motility, and invasion. Silencing of AMF/PGI induced MET, in which upregulation of E-cadherin and cytokeratins, as well as downregulation of vimentin, were noted. The MET guided by AMF/PGI gene silencing induced osteosarcoma MG-63 to terminally differentiate into mature osteoblasts. Furthermore, MET completely suppressed the tumor growth and pulmonary metastasis of LM8 cells in nude mice. Thus, acquisition of malignancy might be completed in part by upregulation of AMF/PGI, and waiver of malignancy might also be controlled by downregulation of AMF/PGI. Cancer Res; 70(22); OF1–11. ©2010 AACR.

Introduction

Epithelial-to-mesenchymal transition (EMT) is an essential mechanism for the development of malignant cells for invasion and metastasis (1). EMT is a well-documented phenomenon occurring during embryonic development and wound healing, as well as tumorigenesis (2). EMT was defined as the process that produces a complete loss of epithelial traits by the former epithelial cells accompanied by the acquisition of mesenchymal characteristics in vitro, i.e., loss of epithelial markers and gain of mesenchymal markers (3, 4). Normally, epithelial cells are polarized and tightly connected to each other; in contrast, mesenchymal cells do not establish stable intercellular contacts (3, 4). During EMT, epithelial cells lose intercellular junctions causing dissociation from the original mass (5).

Several growth factors and cytokines such as transforming growth factor-β (TGF-β) are known to initiate EMT (6). In the center of E-cadherin repression, a zinc finger transcription factor Snail functions as a molecular organizer by down-modulating epithelial genes and up-modulating mesenchymal genes, is activated by most pathways triggering EMT, and is negatively regulated by GSK-3β (7). TGF-β acts through serine/threonine kinase receptors to phosphorylate the cytoplasmic Smads that activate the Snail family. The serine/threonine integrin-linked kinase (ILK) is a signaling protein stimulated by both integrins and growth factor receptors. Activated ILK can directly phosphorylate downstream target GSK-3β, thus resulting in the inhibition of GSK-3β activity, which stimulates the Wnt-β-catenin pathway and upregulates Snail, that in basal conditions is suppressed by active GSK-3β.

Phosphoglucose isomerase (PGI) is a housekeeping cytosolic enzyme of sugar metabolism that plays a key role in both glycolysis and gluconeogenesis pathways (8); extracellularly,
it behaves as a cytokine that includes autocrine motility factor (AMF). Partial amino acid sequencing of fragment peptides (9) and molecular cloning and sequencing of AMF have identified PGI (10). AMF is originally identified as a major cell motility-stimulating factor associated with cancer development and progression (11). Independently, PGI was found to be a neuroleukin promoting growth of embryonic spinal and sensory neurons (12) and a maturation factor mediating differentiation of human myeloid leukemia cells (13). The presence of aberrations in the serum and urine is of prognostic value associated with cancer progression (14–16). Overexpression of AMF/PGI induced transformation and survival of NIH-3T3 fibroblast through EMT with enhanced malignancy (17). In contrast, downregulation of AMF/PGI sensitized fibrosarcoma cells to oxidative stress to cellular senescence (18) and resulted in mesenchymal-to-epithelial transition (MET) with reduced malignancy (19, 20).

Ribozymes are RNA molecules with highly specific intrinsic enzymatic cleavage activity against target RNA sequence, which can discriminate mutant sequences differing by a single base from their wild-type counterparts (21). After binding to the RNA substrate by base pair complementation, the ribozyme cleaves the target RNA irreversibly. A hammerhead ribozyme has been identified as the smallest ribozyme composed of ~30 nucleotides with a conserved catalytic domain in the single-stranded region and variable stem regions formed by base-pairing with basically any desired target sequences (22). The characteristic site-specific cleavage of a phosphodiester bond after uridine of the triplet GUC sequence (23) provides a very valuable tool for gene therapy as well as experimental gene targeting. A gene therapy trial using anti-HIV ribozyme, the first randomized, double-blind, placebo-controlled, phase II autologous cell-delivered gene transfer clinical trial, was carried out and indicated cell-delivered gene transfer to be safe and biologically active (24). Thus, a ribozyme might be one of the most reliable tools for gene therapy, of which safety and feasibility of the approach were already proved.

In the present report, we constructed a hammerhead ribozyme specific against GUC triplet at the position G390 in the AMF/PGI mRNA conserved sequence for the gene targeting. Then, we introduced the ribozyme into human and murine osteosarcoma cells to knock down the translation and secretion of AMF/PGI.

Materials and Methods

Plasmid construction and transfection

The AMF-targeted hammerhead ribozyme RzG390 was designed from a comparison of AMF cDNA sequences as shown in Fig. 1. In addition, a control ribozyme mRz with a single base pair mutation (A-G exchange) was prepared. Each pair of complementary oligonucleotides was artificially synthesized (Operon), annealed together, and ligated into the BamHI/EcoRI sites of pBK-CMV (Stratagene). Osteosarcoma cells were transfected with 8 µg of pBK-CMV ligated with or without Rz390G or mRz using 20 µL of Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer’s protocols. Stable transfectants were selected by 800 µg/mL of G418 (Sigma) for 1 week and maintained with 100 µg/mL of G418.

Cells and cell culture

The human osteosarcoma cell line MG-63 was obtained in 2007 from the cell bank Riken Bioresource Center (routinely authenticated by short tandem repeat profiling ref. 25) and frozen as original stocks in 2007. The human HS-Os-1 and metastatic murine osteosarcoma cell line LM8 (26) were obtained in February 2010 from the cell bank Riken Bioresource Center. A human oral squamous cell carcinoma (SCC) cell line HSC-3 was established in 1989 (27), authenticated by...
short tandem repeat profiling in 2005, and frozen as original stocks in 2005 at Maxillofacial Surgery, Tokyo Medical and Dental University. All cells were grown in DMEM (Sigma) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma). Cultures were maintained at 37°C in an air–5% CO₂ incubator at constant humidity. To ensure maximal reproducibility, cultures were grown for no longer than six passages after recovery from original frozen stocks and monitored to prevent Mycoplasma contamination (10).

Antibodies and chemicals

The recombinant human AMF (rhAMF) was affinity-purified by anti-AMF antibody conjugated with sepharose beads, and polyclonal antibodies including anti-rhAMF were prepared by immunization of rabbit (Takara Shuzo) as described previously (10, 28). Mouse monoclonal antibodies AE1/AE3 to the human cytokeratins (Dako), mouse monoclonal antibody VIM-13.2 to the human vimentin (Sigma), and mouse monoclonal antibody 4A2C7 to E-cadherin (Invitrogen) were all purchased. The secondary antibodies horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG antibody and HRP-conjugated goat anti-mouse IgG antibody were purchased from Sigma.

Preparation of cell lysate and conditioned medium

After the cells reached their 70% to 80% confluence, the medium was removed. The cells were washed twice with PBS and lysed in 1 mL/100 mm dish of cell lysis buffer [25 mmol/L Tris–HCl (pH 7.8), 150 mmol/L NaCl, 10 mmol/L EDTA, 1% deoxycholate–Na, 1% Triton X-100, 1% aprotinin, 1% leupeptin, 0.1 mmol/L phenylmethylsulfonyl fluoride for cell lysis] or incubated with 5 mL/100 mm dish of serum-free DMEM for 48 hours to obtain conditioned medium. Cell supernatants were concentrated up to 100-fold using Amicon Ultra (30 kDa cutoff; Millipore). Protein concentrations of each sample were determined using Bio-Rad protein assay reagent (Bio-Rad; ref. 10).

SDS-PAGE and Western blotting

Aliquots of the cell lysates (25 μg) and the conditioned medium (25 μg) were separated by 7.5% SDS-PAGE and blotted onto polyvinylidene difluoride plus membranes (MSI). The blots were blocked with 5% nonfat dry milk in PBS overnight and incubated with polyclonal antibody (1:3,000) or monoclonal antibodies (1:1,000) for 1 hour at room temperature, followed by appropriate secondary antibodies (1:2,500) for 1 hour at room temperature. The labeled bands were revealed by chemiluminescence using enhanced chemiluminescence Western blotting detection reagents (Amersham) and exposed to Kodak X-Omat film. Density of each band was quantitated with NIH Image software (10).

Phagokinetic track assay and invasion assay

Random cell motility (chemokinetics) was measured by phagokinetic track assay as described previously (10, 28). Briefly, 2.0 × 10³ cells were seeded on a coverslip coated with 1.0% bovine serum albumin and colloidal gold particles. After 24 hours of incubation, phagokinetic tracks were visualized under a microscope, and the area cleared by at least 25 locomotive cells was measured using NIH Image software (http://www.rsb.info.nih.gov/jij/). The in vitro invasion assay was done using Transwell cell culture chambers (Corning Costar) separated by 8.0-μm pore filter precoated with Matrigel (Collaborative Biomedical Products) on the upper surface. The bottom chamber was filled with DMEM supplemented with 10% FBS, and cell suspensions of 1 × 10⁵ cells per well were added onto the upper chamber. After 15 hours of incubation at 37°C, the top side of the insert membrane was scrubbed with a cotton swab, and the bottom side was fixed with methanol and stained with 1% H&E. Viable cells were counted under a microscope. Each assay was done in triplicate.

MTT assay

For cell proliferation, an MTT assay was done using Vybrant MTT cell proliferation assay kit (Invitrogen) according to the manufacturer’s instructions. Briefly, 1 × 10⁵ cells per well were plated on 96-well plates and cultured for different times indicated in 100 μL of culture medium. At the end of the assay time, 10 μL of 12 mmol/L MTT were added to each well and then incubated at 37°C for 4 hours. Solubilizing solution (SDS-HCl) was added and mixed thoroughly using pipette. The microplate was incubated in air at 37°C for 4 hours in a humidified chamber. Plates were read at 570 nm on a spectrophotometric plate reader with a reference wavelength at 650 nm.

Reverse transcription-PCR analysis

Reverse transcription-PCR (RT-PCR) analysis was done as described previously (10). Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s protocols. The cDNA was generated by using random hexamer primers and reverse transcriptase SuperScriptIII (Invitrogen). For quantitative evaluation of the amplified product, PCR encompassing 20 to 35 cycles was preliminarily done to determine the most suitable amplifications for each reaction. PCR consisted of appropriate cycles of denaturing at 93°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 60 seconds with initial denaturation at 93°C for 5 minutes and final extension at 72°C for 10 minutes, was carried out using PC320 (Astek). PCR-amplified products were electrophoresed in 0.75% agarose (H14 agarose, Takara) and stained with ethidium bromide. The PCR product size and the sequence of oligonucleotide primers were as shown in Table 1.

Pulmonary metastatic assay in vivo

LM8parental, LM8mRz, and LM8Rz cells were inoculated s.c. (1 × 10⁶ per mouse) into the back space of eight female BALB/c-nu/nu mice at 6 weeks of age, respectively. The tumor size was measured every week, and tumor volume was calculated with the following formula: tumor volume (mm³) = 0.5XY² (X and Y being the longest and shortest diameters of the tumor, respectively). Five weeks later, the s.c. tumor and lungs were removed to make frozen specimens. The specimens were cut at their maximum dimensions and fixed with 4% paraformaldehyde in PBS and stained either by H&E or

Waiver of Malignancy in Osteosarcoma

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Table 1. List of PCR primers used in this study

<table>
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(Continued on the following page)
suppression of AMF/PGI expression in MG-63 cells by RzG390

According to the cDNA sequence of human AMF/PGI, there are 22 GUC triplet sites, which are the target accessible for ribozymes to cleave (21–24). As shown in Fig. 1, comparison of human, mouse, and rat cDNA for AMF/PGI indicated a well-conserved sequence around the GUC triplet at G390. Here, a ribozyme specific against GUC at the position G390 was synthesized and constructed in pBK-CMV (RzG390). In addition, a control ribozyme with point mutation (mRz) was introduced. Similarly forward and reverse primers of AMF/PGI were designed in the conserved sequence to use for human and mouse (Table 1). Human osteosarcoma MG-63 cells with mesenchymal origin were transfected with RzG390, mRz, or vector only (mock). The stable transfectants were established by G418 selection; finally, six clones of each were obtained. To examine the efficiency for AMF/PGI silencing, we detected the level of mRNA by RT-PCR and the level of protein expression by Western blot. Therefore, we further analyzed the expression of vimentin and concomitant broad and cytokeratin was tested by Western blot analysis. Reduction of cell motility and morphologic changes in Rz cells prompted us to examine whether they are associated with loss of mesenchymal and/or gain of epithelial markers.

Effect of AMF/PGI gene silencing on tumor cell behaviors

We further analyzed whether the complete abrogation of AMF/PGI secretion influenced cell growth, motility, and invasion. As shown in Fig. 2B, cell growth of Rz was slightly inhibited without significance. However, phagokinetic track assay representing intrinsic chemokinesis in Rz was drastically reduced to 20% compared with its parental counterpart and controls. Therefore, we also carried out in vitro invasion assay to depict whether the complete abrogation of AMF/PGI secretion influenced cell invasion. As shown in Fig. 2C in vitro invasion assay, gene silencing of AMF/PGI also reduced invasiveness to <50% compared with parental and control cells. Thus, the depletion of extracellular AMF/PGI reduced intrinsic cell motility and invasion in an autocrine manner. The cells were plated and cultured, and morphologies were monitored during progression from sparse monolayer (Fig. 2D, top) to confluency (Fig. 2D, bottom). The parental MG-63 cells maintained their mesenchymal elongated spindle-shaped fibroblast-like morphology. In sharp contrast, the Rz cells underwent a significant morphologic transformation, whereby, under sparse conditions, the cells acquired a rounded flattened shape, without an elongated shape (Fig. 2D, top). On reaching confluency, the Rz cells acquired a paving stone-like appearance, which is typical to epithelial monolayer (Fig. 2D, bottom). Furthermore, the Rz cells seemed to be tightly contacted and be contact-inhibited as opposed to the control cells that continued at confluence to pile up in a typical mesenchymal growth pattern (data not shown).

Silencing of AMF/PGI leads MET in osteosarcoma cells

It is known that growth factors and cytokines such as TGF-β, fibroblast growth factor, epidermal growth factor, and tumor necrosis factor-α can regulate EMT (1, 2). The reduction of cell motility and morphologic changes in Rz 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 and cytokeratin was tested by Western blot analysis. Reduction of vimentin expression and concomitant broad and strong signals of lower molecules of cytokeratins were observed in Rz cells (data not shown); however, we could not address which cytokeratins are expressed in Rz cells by Western blot. Therefore, we further analyzed the expression of cytokeratins K1 to K8, known as basic (Type II), and K13 to K20, known as low molecular acidic (Type I), using RT-PCR. As shown in Fig. 3A, mesenchymal markers N-cadherin, vimentin, ILK, and α-smooth muscle actin (α-SMA) were all downregulated. In contrast with mesenchymal antibody. The number of lung metastatic nodules was counted microscopically, and the occupancy was also microscopically calculated by NIH image.

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

Table 1. List of PCR primers used in this study (Cont’d)

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Table 1. List of PCR primers used in this study (Cont’d)
markers, an epithelial marker E-cadherin was upregulated. Similarly, most cytokeratins were upregulated in Rz cells except for K13, K14, and K15. Thus, we concluded that silencing of AMF/PGI leads MET in osteosarcoma MG-63 cells.

Silencing of AMF/PGI changes signal transduction to MET

We proceeded to examine the expression of molecules related to signal transduction. Snail, Slug, Twist1, and Twist2 are transcription repressors that have a key role in EMT both during development and tumor progression (1, 2, 4). Of note, E-cadherin repressor Snail leads to the loss of polarity and morphologic change by repressing E-cadherin (7). The Wnt/β-catenin pathway participates in EMT by activating snail (7). TGF-β is well known to induce EMT, in which cooperation of TGF-β and other signaling pathways is required. TGF-β initiates receptor phosphorylation and activates Snail family (29). Activated snail induces downregulation of E-cadherin, which is negatively regulated by GSK-3β (7). We, therefore, performed RT-PCR analysis of EMT-related molecules, including these molecules. As shown in Fig. 3B, Snail expression was downregulated in Rz cells, whereas the expressions of Slug, ZEB-1/δEF-1, and ZEB-2/SIP1 were not altered. Twist1 was not expressed in those cells, and Twist2 was, rather,
downregulated in Rz cells. TGF-β1 was not expressed in those cells, whereas TGF-β2 and TGF-β3 were downregulated in Rz cells. Interestingly, the expression level of GSK-3β was not altered. Thus, results obtained by RT-PCR confirmed that gene silencing of AMF/PGI induced MET.

**Silencing of AMF/PGI induces MG-63 cells to differentiate into osteoblasts**

MG-63 has been reported to differentiate into osteoblast (29) and to express E-cadherin, which maintained the activity and mRNA levels of alkaline phosphatase and osteocalcin (OC; ref. 30). We therefore, investigated the possibility for MG-63 cells to differentiate into osteoblasts by RT-PCR. As shown in Fig. 3B, silencing of AMF induced MG-63 cells to express OC and osteopontin (OP). To differentiate into osteoblast transcription factor, runt-related transcription factor 2 (Runx2) is essential (30), which induces to produce type I collagen, OC, OP, and other proteins to form bone tissue. Osterix is a transcription factor with zinc-finger inducing to produce OC inevitable for calcification. OC is a bone-specific protein

![Figure 3. RT-PCR. Silencing of AMF leads MET in osteosarcoma MG-63 cells. A, PCR for mesenchymal and epithelial markers. Mesenchymal markers: N-cadherin, vimentin, ILK, and α-SMA. Epithelial markers: E-cadherin and cytokeratins (K1 to K8 as basic; K13 to K20 as acidic). B, PCR for EMT-related molecules, osteoblast differentiation-related genes, and Wnt genes. Silencing of AMF induced MET in MG-63 cells and terminal differentiation into osteoblasts. B, Wnt2b, Wnt5a, and Wnt9a are suppressed, and Wnt3, Wnt10b, Wnt11, and Wnt16 are enhanced by AMF gene silencing.](image-url)
and is produced by mature osteoblast (31). In the process of osteoblast differentiation, Runx2, Osterix, and canonical Wnt signaling molecules inhibit mesenchymal stem cells from differentiating into chondrocytes and adipocytes (30). After the commitment to osteoblast lineage, Runx2 maintains the osteoblasts in an immature stage, and Runx2 must be suppressed for immature osteoblasts to become fully mature osteoblasts (31). As shown in Fig. 3B, the expression of Osterix was upregulated in Rz cells, although the Runx2 was slightly suppressed in the Rz390G cells compared with other lineage. Lack of Twist gene expression in OC- and OP-expressing cells strongly suggested maturation of osteoblasts (31). To date, 19 different Wnt genes are recognized in humans (32, 33). As shown in Fig. 3B, Wnt2b, Wnt5a, and Wnt9a were downregulated in Rz cells; on the other hand, Wnt3, Wnt9b, Wnt10b, Wnt11, and Wnt16 were upregulated.

Silencing of AMF/PGI induces osteosarcoma cells to reduce malignancy through MET

These data showed that AMF/PGI gene silencing induced MET in osteosarcoma MG-63 cells. Therefore, we further introduced the ribozyme into the other osteosarcoma cell lines, i.e., murine metastatic osteosarcoma LM8 and human osteosarcoma HS-Os-1. As shown in Fig. 4A, the ribozyme downregulated the expression of AMF/PGI and abrogated the secretion of extracellular AMF, concomitant with downregulation of vimentin and upregulation of E-cadherin in those cells. The growth of both LM8 and HS-Os-1 was slightly inhibited as shown in Fig. 4B. Random cell motility and in vitro invasion of both LM8 and HS-Os-1 were also significantly suppressed by silencing of AMF/PGI (Fig. 4C and D). These data were very similar to the behaviors observed in MG-63 cells. Therefore, we further performed RT-PCR on HS-Os-1 cells to characterize MET in relation to osteoblastic differentiation (as was observed in MG-63 cells). Silencing of AMF/PGI upregulated E-cadherin and downregulated vimentin, ILK, and N-cadherin. OC, OP, Wnt10b, and Wnt11 were upregulated, whereas Wnt2b and Wnt5a were commonly downregulated (data not shown). Consequently, we concluded that silencing of AMF/PGI might induce MET and osteoblast differentiation in osteosarcoma cells. Thus, these data also suggested that silencing of AMF might result in loss of malignancy in osteosarcoma cells. Therefore, we further carried out experiments using nude mice to investigate whether silencing of AMF/PGI influences tumor growth and pulmonary metastasis of LM8. The murine osteosarcoma LM8 cells, with high metastatic potential to the lung (26), were inoculated s.c. into the back space.
of eight female BALB/c-nu/nu mice at 6 weeks of age. The tumor size was measured every week. Five weeks later, the s.c. tumor and lungs were removed to make frozen specimens. In contrast to the LM8parental and LM8mRz cells, LM8Rz cells neither formed tumor mass s.c. nor metastasized to the lung as shown in Fig. 5A and B, respectively.

Discussion

Overall gene silencing of AMF/PGI and the following abrogation of AMF/PGI secretion resulted in reduction of cell growth, motility, invasion, and pulmonary metastasis. Intra-cellular enzymatic activity of intrinsic PGI is more essential for an individual cell to survive, which seems to be a minimum requirement for the cell, than to secret AMF as an extracellular form of AMF/PGI. PGI is observed in all cells ubiquitously, whereas secretion of AMF is observed in only tumor cells or activated T cells (10). Secretion pathway of AMF is not classic because PGI lacks signal sequences. AMF/PGI is secreted extracellularly by phosphorylation at serine 185 by casein kinase II (34). Additionally, calcium-dependent mechanism or calcium ionophores may be important for the AMF secretion, because calcium ionophores enhanced AMF secretion and EDTA-inhibited AMF secretion (data not shown). Secreted AMF/PGI functions as a growth factor as well as a motility factor (35). As shown in Figs. 2B and 4B, knockdown of AMF/PGI affected the growth of osteosarcoma cells, especially in murine metastatic osteosarcoma cells LM8 (Fig. 5A). Knockdown of AMF/PGI also reduced intrinsic chemokinesis and in vitro invasion (Figs. 2C and 4C and D). Funasaka and colleagues showed that the knockdown of AMF/PGI expression in fibrosarcoma HT1080 cells using small interfering RNA (siRNA) reduced cell growth, motility, and cell invasion. Moreover, knockdown of PGI/AMF inhibited anchorage-independent growth of tumor cells and completely suppressed growth of tumor xenograft in nude mice (18). Furthermore, gene silencing of AMF/PGI completely suppressed tumor growth and pulmonary metastasis in nude mice (Fig. 5A and B). These accumulated data suggested that knockdown of AMF/PGI induced MET and that the MET resulted in loss of malignancy or waiver of malignancy, although it was suggested that cancer cells probably use this MET process during the later stages of metastasis (36, 37). Ribozyme provided a very valuable tool for gene targeting for the induction of MET, suggesting that gene therapy by manipulation of EMT/MET using ribozyme targeting AMF/PGI might be efficient, at least in this case.

N-cadherin, vimentin, ILK, and α-SMA were all downregulated in MG-63Rz cells, and E-cadherin and cytokeratins were upregulated (in Fig. 3A). Thus, silencing of AMF/PGI in osteosarcoma MG-63 cells resulted in MET. TGF-β is known to activate Snail through phosphorylation of Smads, and Snail is known to suppress E-cadherin expression (36). TGF-β also signal ILK, mitogen-activated protein kinase, and phosphoinositide 3-kinase pathway, and activated ILK can directly phosphorylate downstream target GSK-3β to inactivate (7). MG-63 cells do not express TGF-β1, but TGF-β2 and TGF-β3 (Fig. 3B). Silencing of AMF/PGI reduced the TGF-β2 and TGF-β3 production and secretion, resulting in downregulation of Snail, which was released from the basal stage of suppression of E-cadherin. In addition, GSK-3β, a negative regulator of Snail, was stably expressed in MG-63 cells, and the activity of GSK-3β was also free of inhibition, because of shortage of ILK and snail as shown in Fig. 3. Funasaka and colleagues showed that the knockdown of AMF/PGI using siRNA induced MET in fibrosarcoma HT1080 cells, in which vimentin was downregulated and cytokeratin 23 was upregulated (18–20). Similarly, knockdown of AMF/PGI using siRNA induced MET in breast cancer MDA-MB-231 cells, in which E-cadherin and GSK-3β were upregulated, while fibronectin, Snail, and β-catenin were downregulated (20). These accumulated data strongly suggested that knockdown of AMF/PGI might release the course of E-cadherin suppression through upregulation of GSK-3β and downregulation of TGF-β, Snail, and ILK.

Loss of malignancy in osteosarcoma cells may be involved in the process of differentiation as well as MET, because it is well known that differentiated cells are less malignant in general (37). Wnt signals are also known to induce EMT, which
is also negatively regulated by GSK-3β (7). Knockdown of AMF/PGI in osteosarcoma cells MG-63 and HS-Os-1 cells commonly reduced expression of Wnt2b and Wnt11. Among them, Wnt2b functions as the stem cell factor for neural or retinal progenitor cells during embryogenesis and also for gastric cancer, esophageal cancer, and skin basal cell carcinoma during carcinogenesis (38). Thus, Wnt2b might be involved in progression of cancer via EMT. Similarly, upregulation of Wnt15a expression concomitant with downregulation of Wnt4 was observed in mesenchymal phenotype of SCC. Moreover, EMT through overexpression of Snail in SCC resulted in upregulation of Wnt5a and ZEB-1 (6E1) and downregulation of Wnt4 (39). However, MET through the knockdown of PG1/AMF failed to downregulate ZEB-1 and upregulate Wnt4 (Fig. 3B). Silencing of AMF/PGI induced osteosarcoma MG-63 cells to upregulate Wnt10b and Wnt11 gene expression, which might be important in differentiation because upregulation of Wnt10b was reported to increase bone mass via enhancing osteoblast differentiation (32) and Wnt11 signals through both canonical (β-catenin) and noncanonical pathways and was upregulated during osteoblast differentiation and fracture healing (40). Moreover, temporal and spatial expression of AMF/PGI was observed in bone and cartilage cells during postnatal development and fracture repair, in which immature osteocytes had further differentiated toward mature osteoblasts, and the expression of AMF/PGI was decreased markedly (41). Thus, silencing of AMF might be involved in loss of malignancy through differentiation via MET.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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