Cross-regulation between protein L-isoaspartyl O-methyltransferase and ERK in epithelial mesenchymal transition of MDA-MB-231 cells

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Aim: Protein L-isoaspartyl O-methyltransferase (PIMT) regulates cell adhesion in various cancer cell lines through activation of integrin αv and the PI3K pathway. The epithelial mesenchymal transition (EMT) enables epithelial cells to acquire the characteristics of mesenchymal cells, and to allow them to migrate for metastasis. Here, we examined the relationship between PIMT and EMT with attached or detached MDA-MB 231 cells.

Methods: Human breast cancer cell line MDA-MB-231 cells were maintained in a suspension on poly-HEMA in the presence or absence of PIMT siRNA or ERK inhibitor PD98059. The mRNAs and proteins were analyzed using RT-PCR and immunoblotting, respectively.

Results: During cellular incubation under detached conditions, PIMT, integrin αv and EMT proteins, such as Snail, Slug and matrix metalloproteinase 2 (MMP-2), were significantly increased in correlation with the phosphorylation of ERK1/2. The ERK inhibitor PD98059 (25 µmol/L) strongly suppressed the expression of the proteins and PIMT. Interestingly, PIMT siRNA blocked the phosphorylation of ERK and the expression of the EMT proteins. Additionally, PIMT and ERK phosphorylation were both co-activated by treatment with TGF-β (10 ng/mL) and TNF-α (10 ng/mL).

Conclusion: A tight cross-regulation exists between ERK and PIMT in regards to their activation and expression during the EMT.

Keywords: protein L-isoaspartyl O-methyltransferase (PIMT); breast cancer; metastasis; epithelial mesenchymal transition; extracellular signal-regulated kinase (ERK); siRNA

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Because the role of PIMT in the EMT and metastatic processes remains unclear, in this study, we explored the involvement of PIMT in the regulation of the detachment and attachment of the anoikis-resistant cell line MDA-MB-231, an aggressive breast cancer cell line with a highly invasive, migrative, and metastatic character,[10] by culturing the cells in poly-HEMA (2-hydroxyethylmethacrylate)-coated dishes and introducing siRNA specific for PIMT.

**Materials and methods**

**Materials**
LiCl and poly-HEMA were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin solution (10,000 unit/mL and 10 mg/mL, respectively), fetal bovine serum (FBS) and Dulbecco’s phosphate-buffered saline (DPBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). MDA-MB 231 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Moloney murine leukemia virus (M-MLV) reverse transcriptase, polymerase chain reaction (PCR) premix, and Sapphire Super Taq were purchased from Rexgene Biotech Co, Ltd (Ochang, Korea). All primers used for PCR were purchased from Bioneer (Taejeon, Korea). A mixture of Stealth[39]/siRNA duplex oligoribonucleotides against PIMT and Lipofectamine[70] RNAiMAX was purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-PIMT antibody was produced against recombinant PIMT proteins of porcine brain as previously described (Koh and Hong, unpublished data). Antibodies against MAPK, MMP-2, MMP-9, N-cadherin, integrin αv, phospho-GSK3 (Tyr279/216), phospho-ERK1/2 (Thr202/Tyr204), phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226), phospho-Akt1/PkBα (Ser473) and phospho-p90RSK (Ser479) were obtained from Millipore (Bedford, MA, USA). β-actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ERK kinase inhibitor PD98059 was purchased from CalBiochem (La Jolla, CA, USA).

**Cell culture**
MDA-MB 231 cells (poorly differentiated adenocarcinoma, grade III), a human breast cancer cell line, appearing phenotypically as spindle shaped cells exhibiting highly invasive phenotype,[10, 11] and differentiation markers such as E-cadherin and keratin, β-catenin, and vimentin,[12] were grown in DMEM, penicillin/streptomycin solution (10,000 unit/mL and 10 mg/mL, respectively), fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Polymers were obtained from 3 to 6 replicate cultures.

**Quantitative analysis of EMT**
Detached (d) MDA-MB-231 cells (10^6 cells/well × 6 wells) were maintained in suspension on poly-HEMA or were allowed to attach (a) to culture dishes in serum-free media for 24 h. The cells were then treated with 0.025% trypsin/0.02 mmol/L EDTA and re-attached to the culture plates. The images were taken by a digital camera at the indicated time after seeding.

The numbers of round and total cells were counted in a hemocytometer, as reported previously.[13] The percentage of cells morphologically changed was determined by the following equation: % of cells in aggregates=([total cells−round cells]/total cells)×100. All values, expressed as mean±SEM, were obtained from 3 to 6 replicate cultures.

**Reverse transcriptase-polymerase chain reaction analysis**
Cells were plated at a density of 1×10^6 cells/well in 6-well tissue culture plates. After treatment with each of the indicated reagents, total RNA was obtained with an assay-BLUE[34] total RNA extraction reagent (Invitrogen, Sangnam, Korea). After determination of the amounts of the total RNA in each sample followed by electrophoresis on a formaldehyde-agarose gel, cDNA synthesis was performed using M-MLV reverse transcriptase (Rexgene, Ochang, Korea) as reported previously[14]. Polymerase chain reaction (PCR) was then conducted using Premix (Rexgene, Ochang, Korea) in a 20 μL reaction that contained 2 μL of reaction buffer, 1 μL of 5 mmol/L dNTPs, 1 μL of forward and reverse primer and 1 μL of cDNA as the template, as reported previously[15]. The primer sequences are shown in Table 1.

**Immunoblotting**
Cells were lysed in PRO-PREP (Invitrogen, Sangnam, Korea), after which the protein concentrations were quantified using a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Next, the lysates were boiled for 5 min, after which 20 μg of each total protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 12% slab gels. The proteins were then transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA), which were subse-

| Table 1. The sequence of primers used. |
|--------------------------------------|
| **Gene** | **Sequences** |
| GAPDH | 5′-AAGGAGTCATCATCTCTGCCC-3′ |
| | 5′-GTGATGGCATGACTGTGTT-3′ |
| PiMT | 5′-TCAGAAAGGAGATCCACA-3′ |
| | 5′-TCTCCGGGCTTTAAACTGAT-3′ |
| Fibronectin | 5′-ATTCGGGTGAAATAGAGC-3′ |
| | 5′-CTGTCAGGGAACACAAAT-3′ |
| TGF-β1 | 5′-CGAGGGAAGATGAGGCT-3′ |
| | 5′-CGTGGATGAAACCGTTGAC-3′ |
| MMP-2 | 5′-AATGCGATCCCGGATAACC-3′ |
| | 5′-AAATTCGACGTCCTTCAGAC-3′ |
| MMP-9 | 5′-GAGGTGATCATAGGCTCATAGGT-3′ |
| | 5′-AGGGAGCTGGTTAGAGGAGA-3′ |
| Snail | 5′-GGCCAGGTATGAGGAGGA-3′ |
| | 5′-GCTTCTGGCTCTGACTCTGC-3′ |
| Slug | 5′-CGTTGTCAGAAGACTTCTAC-3′ |
| | 5′-GCCCAAAATGGGAGGTA-3′ |
| Integrin αv | 5′-CCATGCGATGAGGAGGAGA-3′ |
| | 5′-ACACAACGGCATGCGAACA-3′ |
| E-cadherin | 5′-CAGCAGTACACACAGCTT-3′ |
| | 5′-GCCAAGCTGGTCAAAGTCCC-3′ |
quently blocked for 2 h in TBS containing 0.5% Tween-20 and 5% (v/v) dry skim milk powder and then incubated overnight with primary antibodies as reported previously[16, 17]. The membranes were then washed with TBS-T and incubated for 2 h with HRP-linked anti-rabbit IgG (Millipore, Bedford, MA, USA) or HRP-linked anti-mouse IgG (Millipore, Bedford, MA, USA) secondary antibody. For chemiluminescence detection, the probed blots were incubated for 5 min with Immobilon Western HRP substrate (Millipore, Bedford, MA, USA) as reported previously[18, 19].

siRNA transfection
To conduct PIMT siRNA transfection, 4×10⁶ cells per well were seeded in 6-well plates and then incubated for 48 h at 37 °C in medium containing serum, which allowed the cells to become approximately 80% confluent. A mixture of PIMT siRNA and Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) was prepared prior to treatment as reported previously[20]. Briefly, the Lipofectamine™ RNAiMAX was incubated with siRNA in OPTI-MEM (Gibco, Gaithersburg, MD, USA) at room temperature for 30 min. The cells were then incubated in this mixture for 48 h at 37 °C in a fresh culture medium containing serum.

Gelatin zymography
Gelatin zymography was performed for semiquantitative analysis of the gelatinase activities of conditioned media secreted from detached human breast cancer cells as previously described by Gottschall and Yu[21], with slight modifications[22]. Briefly, samples of the culture media were mixed with non-reducing sample buffer [5% w/v SDS, 20% glycerol, 0.5 mol/L Tris-HCl (pH 6.8) and 1% bromophenol blue] and then separated by electrophoresis for 1 h at 150 V on 7.5% to 10% polyacrylamide gel containing 0.1% SDS and 1.5 mg/mL gelatin. Next, the gels were washed twice with 2.5% Triton X-100 for 30 min to remove the SDS and then incubated for 24 h at 37 °C with developing buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, pH 7.6). The gels were then stained with Coomassie Brilliant Blue R-250 (0.1% Coomassie Brilliant Blue, 40% (v/v) methanol, and 10% (v/v) acetic acid) for 30 to 60 min, after which the gels were destained with 10% (v/v) acetic acid /20% (v/v) methanol. The gelatinolytic activities were then visualized as a white band against the uniformly stained background.

Wound healing assay
The method for our wound closure assay was modified as reported previously[23, 24]. Briefly, cells were grown to confluence in 24-well-plates. A control group was cultured in DMEM supplemented with 10% FBS, and the experimental group was transfected with PIMT siRNA. After the cells grew into full confluence, the medium was aspirated, and a wound was scraped on the cell monolayer using a 200 µL micropipette tip. The wounded culture plates were then incubated for 24 h at 37 °C. The migration of cells into the scraped area was evaluated using a bright field microscope and then photographed by digital photography (Canon, Japan).

Statistical analysis
A student’s t-test and a one–way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data are expressed as mean ± standard errors (SEM) and the results are taken from at least three independent experiments performed in triplicate. P values of 0.05 or less were considered to be statistically significant.

Results
Cell detachment leads to EMT-like changes of MDA-MB 231 cells
Cell detachment from the ECM suppresses anoikis, allowing these tumor cells to survive and proliferate in the body; however, these cells must eventually resettle in the substratum at a different site[25]. Indeed, detached cells (d) reattached to the culture dish at a much faster rate than attached cells (a) and exhibited a spindle-like morphology (Figure 1A). Indeed, detached-reattached cells exhibited morphologically changed pattern at 1 h incubation up to 4-fold (Figure 1A, right panel). To determine if the change in the morphology of the detached cancer cells represents EMT, the mRNA and protein levels of several EMT-specific proteins were evaluated. The expression levels of integrin αv, fibronectin, TGF-β1, and MMP-2 were all strikingly increased (Figure 1B and 1C). Furthermore, the transcription factors Snail and Slug, known to repress E-cadherin, a suppressor of invasion during carcinoma progression[26], were highly upregulated in the detached cells up to 3 to 10 folds (Figure 1B). In agreement with expression level tests, MMP-2 activity (72 kDa) occurred prominently only in detached cells grown in conditioned media (CM).

ERK is a critical enzyme involved in the regulation of EMT proteins in MDA-MB 231 cells
To evaluate a regulatory pathway controlling EMT and EMT protein expression, the activated (phosphorylated) forms of several enzymes were investigated using detached cells. Of tested proteins, the levels of phospho-ERK1/2 and phospho-MEK1/2 were increased in a time-dependent manner, while phospho-AKT1 appeared at 1 h and was maintained at similar levels for up to 6 h. Additionally, the levels of phospho-GSK3 decreased in a time-dependent manner (Figure 2A, left panel).

To confirm this pattern, selective inhibitors of ERK kinase (PD98059, 25 µmol/L) and the activator of GSK3 (LiCl, 2 µmol/L) were employed, and the mRNA levels of EMT proteins or EMT-regulatory proteins were then determined. As Figure 2B shows, LiCl enhanced phospho-GSK3 levels, and PD98059 blocked the phosphorylation of ERK1/2, indicating that the drugs were functioning. As expected, PD98059 suppressed the mRNA expression of the EMT proteins. In contrast, the induction effect by LiCl was only observed in PIMT expression assays. PD98059 also suppressed the activity of MMP-2 (Figure 2D).
PIMT is a critical enzyme involved in the regulation of EMT proteins in MDA-MB 231 cells

To examine the importance of PIMT in the EMT, the expression of PIMT was measured. As Figure 3A shows, the mRNA and protein levels of PIMT were markedly increased in cells cultured on poly-HEMA-coated dishes compared to cells adhered to uncoated plastic dishes. Interestingly, levels of PIMT were clearly reduced by PD98059 treatment, whereas LiCl enhanced PIMT mRNA and protein levels (Figure 3B). These data suggest that ERK acts as a positive regulator, while GSK3 acts as a negative one, as reported previously[27].

Knockdown of PIMT is linked to the suppression of EMT protein expression in MDA-MB 231 cells

To determine whether PIMT influences the expression of EMT proteins, siRNA targeting PIMT was introduced to detached MDA-MB-231 human breast cancer cells. Intriguingly, knockdown of PIMT led to blockade of the EMT-like transformation as well as suppression of the mRNA levels of PIMT, integrin αv, fibronectin, and TGF-β1 (Figure 4A). Conversely, RT-PCR revealed that E-cadherin expression occurred in PIMT-knockdown MDA-MB-231 cells (Figure 4A). Interestingly, PIMT depletion also induced the suppression of phospho-ERK levels and the phosphorylation of p90RSK, a downstream enzyme of ERK, indicating that there may be a cross-regulation between the PIMT and ERK pathways that affects the expression of these proteins in EMT.

Finally, to determine if PIMT is important for the conversion of stationary cells into motile cells capable of invasion through the ECM[2], a wound healing migration assay was employed. As Figure 4C shows, PIMT siRNA-treated cells displayed a clear reduction in cell migration, suggesting that PIMT is indeed linked to cancer cell migration and invasion.

PIMT is up-regulated during the EMT of MDA-MB-231 cells triggered by cotreatment with TGF-β and TNF-α

During siRNA analysis, we found that PIMT expression was required for the EMT phenotype of MDA-MB-231 breast cancer cells in poly-HEMA-coated dishes. As such, we sought to determine if PIMT expression is up-regulated during the induction of EMT by TGF-β and TNF-α. Previously, treatment with a combination of TGF-β and TNF-α was shown to induce EMT in some cell types[38]. Indeed, when MDA-MB-231 breast cancer cells were treated with TGF-β/TNF-α, a morphological change from an epithelial-like to a mesenchymal-like appearance was induced (Figure 5A). Additionally, TGF-β/
TNF-α-treated MDA-MB-231 cells showed increased mRNA expression of EMT proteins such as integrin αv, fibronectin, Snail, Slug, MMP-2, and MMP-9 as well as PIMT, up to 2 to 5 folds (Figure 5B). The protein levels of PIMT, integrin αv, MMP-2, N-cadherin, phospho (p)-ERK, and p-GSK3 were also significantly upregulated by 2 to 5 folds during TGF-β/TNF-α-induced EMT (Figure 5C).

Discussion
At the cellular level, one requirement of metastasis is that cells detach from their primary site and then migrate to the lymphatic and circulatory systems. In the absence of ECM attachment, cells undergo a form of apoptosis known as anoikis[29]. It is thought that anoikis-resistant cells contribute to the epithelial-mesenchymal transition (EMT), which is one of the metastatic components that lead to a more flexible and migratory phenotype[30]. In an attempt to gain further insight into the biochemical features underlying the EMT, we selected anoikis-resistant cells by culturing the breast cancer cell line MDA-MB-231 on poly-HEMA-coated tissue culture plates. We found that detached MDA-MB-231 cells exhibited spindles and a lengthened morphology compared to attached cells (Figure 1A). During this transition, mesenchymal cells acquire a morphology that is appropriate for both migration in an extracellular environment and interactions between epithelial and mesenchymal cells[31]. Our results also showed that detached MDA-MB-231 cells expressed increased levels of metastatic factors such as integrin αv and EMT proteins such as TGF-β1, Snail, Slug, and MMP-2 (Figure 1B and 1C). All of these factors are important for tumor progression and metastasis, as they facilitate cell migration[32].

It has been reported that ERK plays a critical role in the
process of EMT\cite{33, 34}. During the course of our assays, phosphorylation of MEK1/2 and ERK was found to be increased in detached MDA-MB-231 cells (Figure 2A). The critical role of ERK in EMT process has been illustrated by treating cells with PD98059, a specific ERK inhibitor (Figure 2B). This compound strongly inhibited the expression of EMT proteins such as integrin αv, fibronectin, Snail, Slug, and MMP-2 (Figure 2C), suggesting that ERK is crucial for the EMT. Although it is well known that ERK is functionally important for the survival of detached cells\cite{35}, our results and those of others suggest that this enzyme plays a critical role throughout the entire EMT, promoting morphological changes, migration, and adhesiveness. In addition, ERK inhibition has been reported to strongly suppress the migration of various cancer cells and the homotypic cell-cell adhesion induced by integrins\cite{13, 36, 37}.

Under our conditions, PIMT expression was also markedly increased in detached cells compared to attached MDA-MB-231 cells (Figure 3A). Similar findings have been reported in other nonattached HUVEC, Caki-1, and U-87 cells\cite{9}. Interestingly, the protein and mRNA levels of PIMT were reduced upon exposure to the ERK inhibitor PD98059 (Figure 3B), indicating that ERK is biochemically important for PIMT synthesis. Interestingly, siRNA targeting PIMT suppressed various EMT proteins in addition to ERK phosphorylation, suggesting that PIMT possesses a critical function in the biochemical regulation of EMT proteins. Furthermore, PIMT siRNA treatment also resulted in diminished cell migration, as assessed by a wound healing assay (Figure 4C). Up-regulation of PIMT was also observed in TGF-β/TNF-α-stimulated EMT.
conditions exhibiting enhanced levels of integrin αv, Snail, Slug, MMP-2, and MMP-9 (Figure 5), up to 2 to 5 folds, as reported previously.[38] Additionally, it has been reported that PIMT possesses a potential anti-apoptotic function in Bax-overexpressing cells[39]. Considering these findings, enhanced levels of PIMT appear to be essential for the survival of detached cells. This protein likely influences the early events of anoikis and migration, both of which are regulated by EMT proteins and ERK activity. We cannot, however, rule out the possibility that PIMT is functioning as a repair enzyme[40], although the involvement or appearance of aged proteins or isoaspartyl residues in the signaling cascade required for EMT protein synthesis was not verified. Surprisingly, it has been observed that valproic acid and lithium, used for the treatment of epileptic seizures and mania in bipolar disorder, have both been shown to simultaneously upregulate the ERK pathway and PIMT induction[27, 41]. These results suggest that the activation and expression of ERK pathway members and PIMT could be tightly associated. As it remains unclear how ERK and PIMT co-regulate the EMT, our future experiments will focus on understanding this cross-regulation mechanism.

Numerous lines of evidence indicate that EMT is able to impose cancer cells into self-renewal cancer stem-like cells to spawn macroscopic metastases[32, 43]. EMT-based tumor metastatic events include cell migration requiring for embryonic development and wound healing managed by adult stem cells[44]. Therefore, it could be interesting to determine whether our EMT conditions are capable of transforming MDA-MB-231 cells into highly metastatic stem-like cells. To do this, phenotype of these cells will be determined by measuring the surface levels of CD44/CD24[45].

Taken together, the results presented here provide in vitro support for the idea that PIMT and ERK are tightly expressed and activated in EMT conditions generated during the detachment of MDA-MB-231 cells from poly-HEMA coated dishes and by TGF-β/TNF-α stimulation, as summarized in Figure 6. These findings suggest that the development of specific inhibitors of PIMT may lead to a novel route of inhibition of the EMT and metastasis.

**Abbreviations**

- ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ERK1/2, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PIMT, protein L-isoaspartyl O-methyltransferase; PI3K, phosphoinositide 3-kinase; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha.

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**Author contribution**

Jiyeon Ryu and Sungyoul HONG designed research; Jiyeon Ryu, Jihyeok Song, Jieun Heo and Yongwoo Jung performed research; Jiyeon Ryu, Sang-Jin Lee, Jayoul Cho, and Sungyoul HONG analyzed data; Jayoul Cho and Sungyoul HONG wrote the paper.

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**Figure 6.** Schematic diagram of EMT process of MDA-MD-231 cells induced by detachment and reattachment conditions.
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