

Original Article

Knock-down of protein *L*-isoaspartyl *O*-methyltransferase increases β -amyloid production by decreasing ADAM10 and ADAM17 levels

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Aim: To examine the role of protein *L*-isoaspartyl *O*-methyltransferase (PIMT; EC 2.1.1.77) on the secretion of A β peptides.

Methods: HEK293 APPsw cells were treated with PIMT siRNA or adenosine dialdehyde (AdOX), a broad-spectrum methyltransferase inhibitor. Under the conditions, the level of A β secretion and regulatory mechanism by PIMT were examined.

Results: Knock-down of PIMT and treatment with AdOX significantly increased A β ₄₀ secretion. Reductions in levels of PIMT decreased the secretion of soluble amyloid precursor protein alpha (sAPP α) without altering the total expression of APP or its membrane-bound C83 fragment. However, the levels of the C99 fragment generated by β -secretase were enhanced. Moreover, the decreased secretion of sAPP α resulting from PIMT knock-down seemed to be linked with the suppression of the expression of α -secretase gene products, α -disintegrin and metalloprotease 10 (ADAM10) and ADAM17, as indicated by Western blot analysis. In contrast, ADAM10 was not down-regulated in response to treatment with the protein arginine methyltransferase (PRMT) inhibitor, AMI-1.

Conclusion: This study demonstrates a novel role for PIMT, but not PRMT, as a negative regulator of A β peptide formation and a potential protective factor in the pathogenesis of AD.

Keywords: Alzheimer's disease; β -amyloid protein; *L*-isoaspartyl *O*-methyltransferase; soluble amyloid precursor protein alpha; ADAM10; ADAM17

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Introduction

Alzheimer's disease (AD), the most common neurodegenerative disease, is characterized by progressive memory loss and other cognitive impairments^[1, 2]. Neuropathological hallmarks of AD include the deposition of amyloid beta (A β) peptides, which are organized in senile plaques. In addition, AD is characterized by the accumulation of phosphorylated tau proteins, which are arranged in neurofibrillary tangles (NFTs)^[2]. A β peptides are generated through the proteolysis of the amyloid precursor protein (APP). In the amyloidogenic pathway, β -secretase cleaves APP to produce soluble amyloid precursor protein beta sAPP β and a C99 fragment. Membrane-bound C99 can be further processed by γ -secretase to produce A β peptides^[3, 4]. As an alternative, non-amyloidogenic pathway, α -secretase can cleave within the A β region to produce a

sAPP α fragment and a C83 fragment^[4].

High levels of homocysteine (HCY) can lead to increased concentrations of *S*-adenosylhomocysteine (SAH), a strong methyltransferase inhibitor^[5]. This up-regulation of SAH results in an overall decrease in the activity of *S*-adenosylmethionine (SAM)-dependent methyltransferases. Increased SAH levels in the brain tissue of patients with AD has been associated with the inhibition of catechol-*O*-methyltransferase (COMT) and phenylethanolamine-*N*-methyltransferase (PNMT), two enzymes that are widely distributed throughout the human brain^[6]. Treatment of Neuro-2a neuroblastoma cells with SAH has been shown to inhibit protein phosphatase 2A methyltransferase (PPMT), resulting in decreased methylation of protein phosphatase 2A^[7]. SAH treatment has also been associated with the increased accumulation of APP and phosphorylated tau and with increased A β secretion^[8]. Protein *L*-isoaspartyl methylation is also essential for the maintenance of neural activity in the central nervous system (CNS). Deficiency of protein *L*-isoaspartyl *O*-methyltransferase (PIMT,

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EC2.1.1.77), an enzyme that catalyzes the transfer of an active methyl group from SAM to *L*-isoaspartate and *D*-isoaspartate, leads to fatal progressive epileptic disease^[9]. Alterations in the SAM/SAH ratio, which is relevant to the overall excitatory state of neurons, have been reported in PIMT-deficient mice^[10]. Previous studies have identified deposits of A β peptides with isoaspartates in brain tissue isolated from AD patients and PIMT knock-out mice, suggesting a potential pathophysiological role in progressive neurodegeneration^[10, 11]. In patients with AD, PIMT is up-regulated in degenerating neurons and is localized in NFTs^[10]. Despite the increasing evidence supporting a role for PIMT in neurodegeneration, the mechanism by which PIMT modulates A β peptide generation in AD pathogenesis remains unclear. To uncover the mechanism whereby PIMT exerts its effects, we examined the ability of PIMT to regulate A β secretion *in vitro*.

Materials and methods

Antibodies and reagents

Adenosine dialdehyde (AdOX), SAM, and mouse anti- β -actin antibodies were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA). AMI-1 was obtained from Calbiochem (La Jolla, CA, USA). Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, Dulbecco's phosphate buffered saline (DPBS), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Calbiochem (La Jolla, CA, USA). Moloney Murine Leukemia virus (M-MLV) reverse transcriptase and polymerase chain reaction (PCR) premix was purchased from Rexgene Biotech Co, Ltd (Ochang, Korea). All of the primers used for PCR were purchased from Bioneer (Daejeon, Korea). A mixture of StealthTM/siRNA duplex oligoribonucleotides against PIMT and LipofectamineTM RNAiMAX were purchased from Invitrogen (Carlsbad, CA, USA). Monoclonal mouse anti-APP (6E10) antibody was obtained from Signet Laboratories (Dedham, MA, USA). Polyclonal rabbit antibodies to ADAM9, ADAM10, and ADAM17 were obtained from Chemicon International (Temecula, CA, USA). Monoclonal mouse BACE1, monoclonal anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies, and anti-rabbit HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-PIMT antisera was produced against recombinant porcine PIMT proteins as described^[12]. The Genbank nucleotide sequence database accession number of the nucleotide sequence of the clone is AF239700.

Cell culture, drug treatment, and protein preparation

HEK293 APPsw and SH-SY5Y cells were plated on 100-mm culture dishes (Corning Incorporated, Corning, NY, USA). The dishes were filled with DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin. The cultures were maintained at 37 °C with 5% CO₂ under humidified conditions. Cells were treated with drugs (SAM, AdOX, and AMI-1) for indicated times. Vivaspin20 centrifugal filter

devices (Satorius, Goettingen, Germany) were used to concentrate conditioned media (CM) from HEK293 APPsw and SH-SY5Y cells collected after drug or siRNA treatments. Cells were washed with DPBS and lysed in Pro-PrepTM protein extraction buffer for 20 min on ice. The protein concentration of each sample was quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA).

Cell viability assay

To determine cell viability, cells were plated on 96-well plates at a density of 2×10^4 cells per well. The original media was then replaced with media containing MTT at a final concentration of 0.5 mg/mL^[13]. Four hours later, the medium was discarded, and DMSO was added for the colorimetric assay. Absorption values were determined using an E_{\max} microplate reader from Molecular Devices (Union City, CA, USA) with a 540-nm filter.

Reverse transcriptase-polymerase chain reaction

RNA was isolated from HEK293 APPsw cells treated with or without PIMT siRNA using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. For each RT-PCR reaction, 1 μ g of RNA was used. Each sample was preheated to 60 °C with oligo (dT)₁₈ primers for 10 min. One unit per milliliter of M-MLV reverse transcriptase was added. The reaction was then performed at 37 °C for 60 min with the following primers: PIMT, forward 5'-TCAGGAAGGACGATCAACA-3', reverse 5'-TCCTCCGGGCTTTAACTGAT-3'; and GAPDH, forward 5'-AAGGGTCATCATCTCTGCCC-3', reverse 5'-GTGATGGCATGGACTGTGGT-3'. Amplification was carried out for 20 to 30 cycles with the following parameters: 94 °C for 30 s, 55–57 °C for 40 s, and 72 °C for 30 s. These steps were followed by a final 5 min extension step at 72 °C.

siRNA transfection

To conduct the PIMT siRNA transfection, 500 000 cells were seeded onto 100-mm culture plates. Cells were cultured for 48 h at 37 °C in culture medium containing serum, which allowed the cells to be approximately 80% confluent. Immediately prior to transfection, lipofectamine RNAiMAX was incubated with the siRNA of interest in OPTI-MEM (Gibco) at room temperature for 10 min. The cells were then incubated in this mixture for 48 h at 37 °C in fresh medium containing serum.

Immunoblotting

Twenty micrograms of protein mixed with 5 \times loading buffer [0.313 mol/L Tris-HCl (pH 6.8), 10% SDS, 0.05% bromophenol blue, 50% glycerol], and 20 \times reducing agent (2 mol/L DTT: Fermentas, Hanover, MD, USA) were boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in 20 mmol/L Tris-HCl (pH 7.4) containing 150 mmol/L NaCl and 0.1% Tween 20 (TBS-T). They were then incubated overnight at 4 °C with

primary antibodies (1:2000 for 6E10, 1:1000 for 22C11, 1:3000 for β -actin, 1:2000 for ADAM10, 1:1000 for BACE1, 1:3000 for PIMT) in non-fat milk. The membranes were washed for 10 min in TBS-T and then incubated for 2 h in non-fat milk at room temperature with horseradish peroxidase-conjugated anti-mouse/rabbit secondary antibodies. Bound antibodies were visualized with an enhanced chemiluminescence detection kit (Amersham Bioscience, Pittsburgh, PA, USA).

Cell surface biotinylation

HEK 293 APPsw cells were surface biotinylated by incubation with 2 mg/mL Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA) in ice-cold PBS. After 30 min, the cells were washed and quenched with PBS containing 100 mmol/L glycine. Cells were lysed in 1% NP-40 buffer and incubated with Neutra-vidinTM immobilized onto 6% cross-linked beaded agarose (Pierce). The beads were washed in NP-40 buffer, boiled in sample buffer, separated using SDS-PAGE, and immunoblotted with the indicated antibodies.

A β ₄₀ ELISA assay

A variety of A β peptides, ranging from 38 to 43 amino acids in length, have been shown to be secreted in response to γ -secretase activation^[14, 15]. A β ₄₂ is the peptide most widely implicated in AD pathogenesis^[16]; however, the antibody for A β ₄₀ was selected based on its reproducibility and accuracy in the A β ₄₀ ELISA kit. The CM was cleared of debris, and the secreted A β ₄₀ was measured using a sandwich ELISA kit (Signet Laboratory, Dedham, MA, USA) according to the manufacturer's instructions.

Statistical analysis

Quantitative analysis of Western blotting was performed by calculating the relative density of immunoreactive bands. The data are expressed as a percentage of the control values. Data are presented as the mean \pm SD. Each procedure was performed in three to five independent experiments. A Student's *t*-test analysis was used to evaluate statistical significance.

Results

PIMT siRNA and AdOX induce A β secretion in HEK293 APPsw cells

As shown in Figure 1A and 1B, PIMT siRNA transfection and AdOX treatment increased the secretion of A β ₄₀ in HEK293 APPsw cells approximately two fold. Compared to controls, PIMT siRNA induced a 35.6% \pm 8.0% reduction in mRNA and protein levels in HEK293 APPsw cells 48 h after transfection (Figure 1C). Importantly, the concentration of AdOX used was not cytotoxic (Figure 1D).

PIMT siRNA transfection and AdOX decreases sAPP α secretion and increases C99 in HEK293 APPsw cells

To understand the molecular mechanism of PIMT siRNA-mediated A β ₄₀ secretion, we evaluated whether PIMT played a role in the processing of APP. To do this, we measured the levels of the APP cleavage products: sAPP α , C99, and C83.

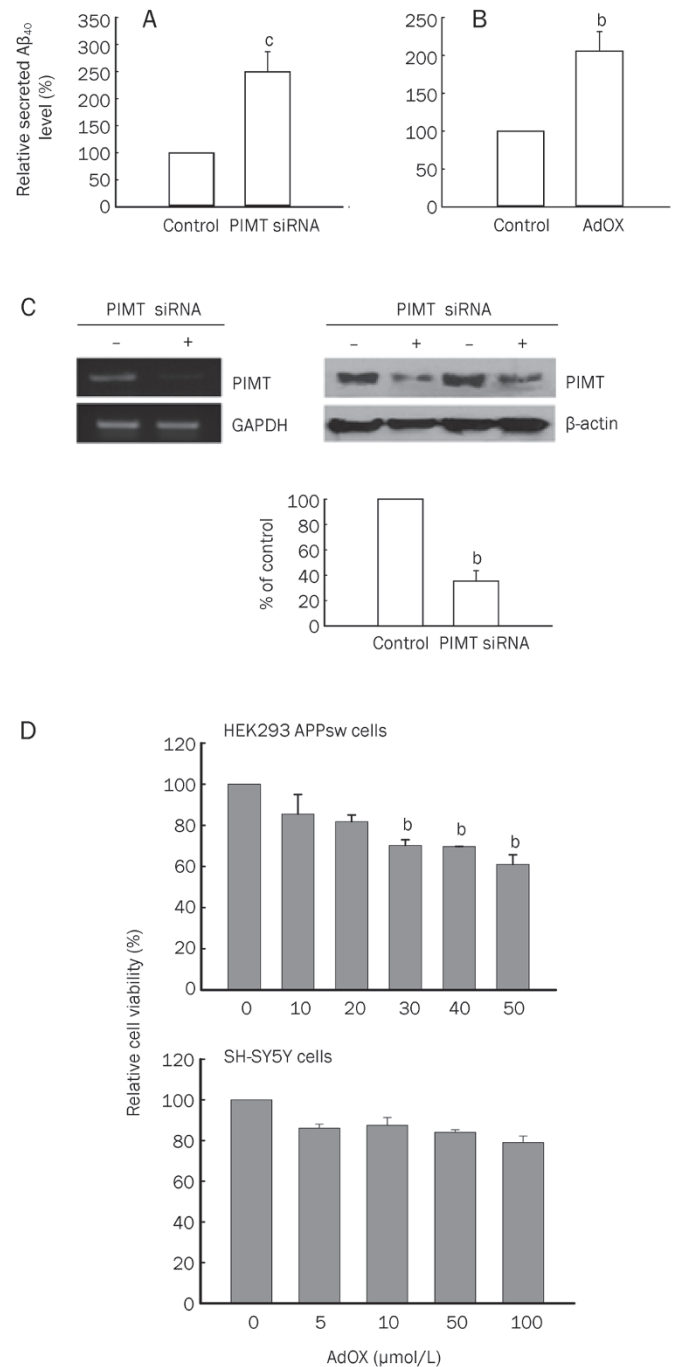


Figure 1. The effects of PIMT siRNA and AdOX treatments on A β production. (A and B) HEK293 APPsw cells were treated with or without PIMT siRNA and AdOX. The levels of A β ₄₀ peptide were analyzed using ELISA. (C) HEK293 APPsw cells were transiently transfected with PIMT siRNA at a concentration of 30 nmol/L. RT-PCR of RNA isolated at 48 h shows PIMT mRNA levels were reduced by PIMT siRNA. As a loading control, GAPDH mRNA levels were examined in HEK293 APPsw cells. Expression levels of PIMT protein were analyzed using a Western blot with rabbit anti-PIMT antiserum. As a loading control, levels of β -actin were also determined. (D) HEK293 APPsw cells and SH-SY5Y cells were treated with AdOX. Cell viability was determined using an MTT assay. Results are expressed as the mean \pm SD for three independent experiments. Mean \pm SD. ^b*P*<0.05, ^c*P*<0.01 compared to untreated or control group.

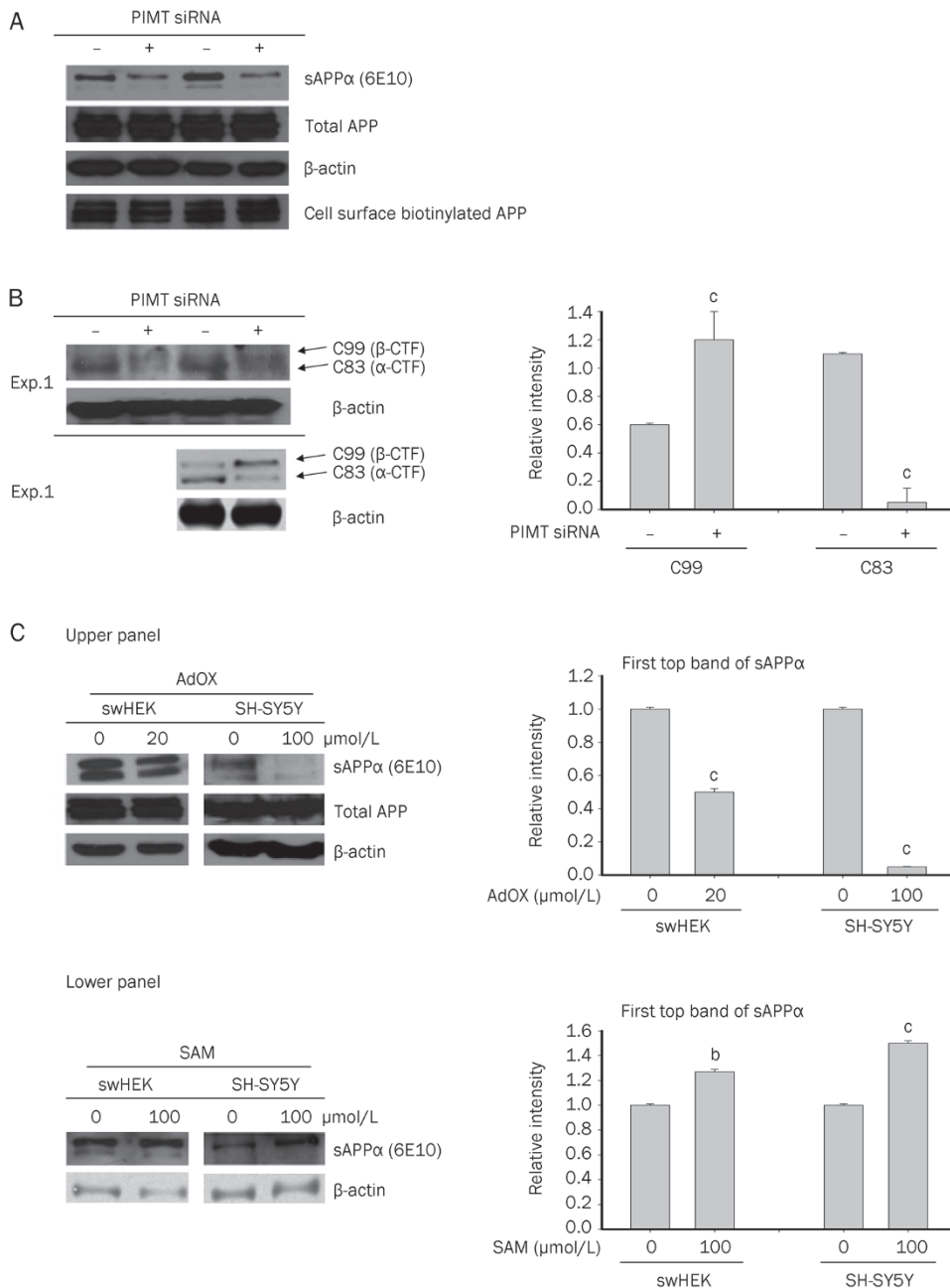


Figure 2. The effects of PIMT siRNA, AdOX, and SAM treatments on levels of membrane bound APP, total APP, and sAPPα. (A) The accumulation of sAPPα in the concentrated media, total cellular and membrane-bound levels of APP, C83, and C99 were analyzed by Western blot analysis. (B) HEK293 APPsw cells were transiently transfected with PIMT siRNA at a concentration of 30 nmol/L. The levels of C99 and C83 proteins were analyzed using Western blotting. As a loading control, levels of β-actin were also determined. (C) HEK293 APPsw (swHEK) and SH-SY5Y cells were incubated without or with AdOX (20 μmol/L) and SAM (100 μmol/L) for 24 h. The levels of sAPPα in the concentrated media, total cellular APP, and membrane-bound APP were analyzed by Western blot analysis. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 compared to normal group.

Western blotting for sAPPα in the CM revealed that PIMT siRNA decreased the secretion of sAPPα by 67.4%±3.4%. However, the overall expression of total APP and biotin-labeled membrane APP remained unchanged (Figure 2A), suggesting that the amount of sAPPβ, a critical component for secretion of Aβ₄₀, might be increased. Indeed, the C99 fragment, the cleavage product of β-secretase^[17], was increased in PIMT siRNA-treated cells. At the same time, C83 was decreased in PIMT transfected cells (Figure 2B). Similar patterns of sAPPα and total APP were also observed in the AdOX treatment group (Figure 2C, upper panel). In contrast, the induction of transmethylation with 100 μmol/L SAM, a methyl donor^[5], increased sAPPα in both cell types (Figure 2C lower panel), demonstrating a critical role of transmethylation

in APP processing.

PIMT siRNA transfection decreases ADAM10 and ADAM17 expression

A decrease in sAPPα without a corresponding reduction in total APP expression suggests a potential alteration in the expression or activity of APP processing enzymes. To investigate whether PIMT siRNA altered the expression of APP processing enzymes, we measured the expression levels of several α-secretase candidates: ADAM9, ADAM10, and ADAM17; and the β-secretase candidate, BACE1. As shown in Figure 3A, PIMT siRNA reduced the expression of both the mature and the immature forms of ADAM10 by 45% to 55% compared to controls. In contrast, PIMT siRNA did not significantly

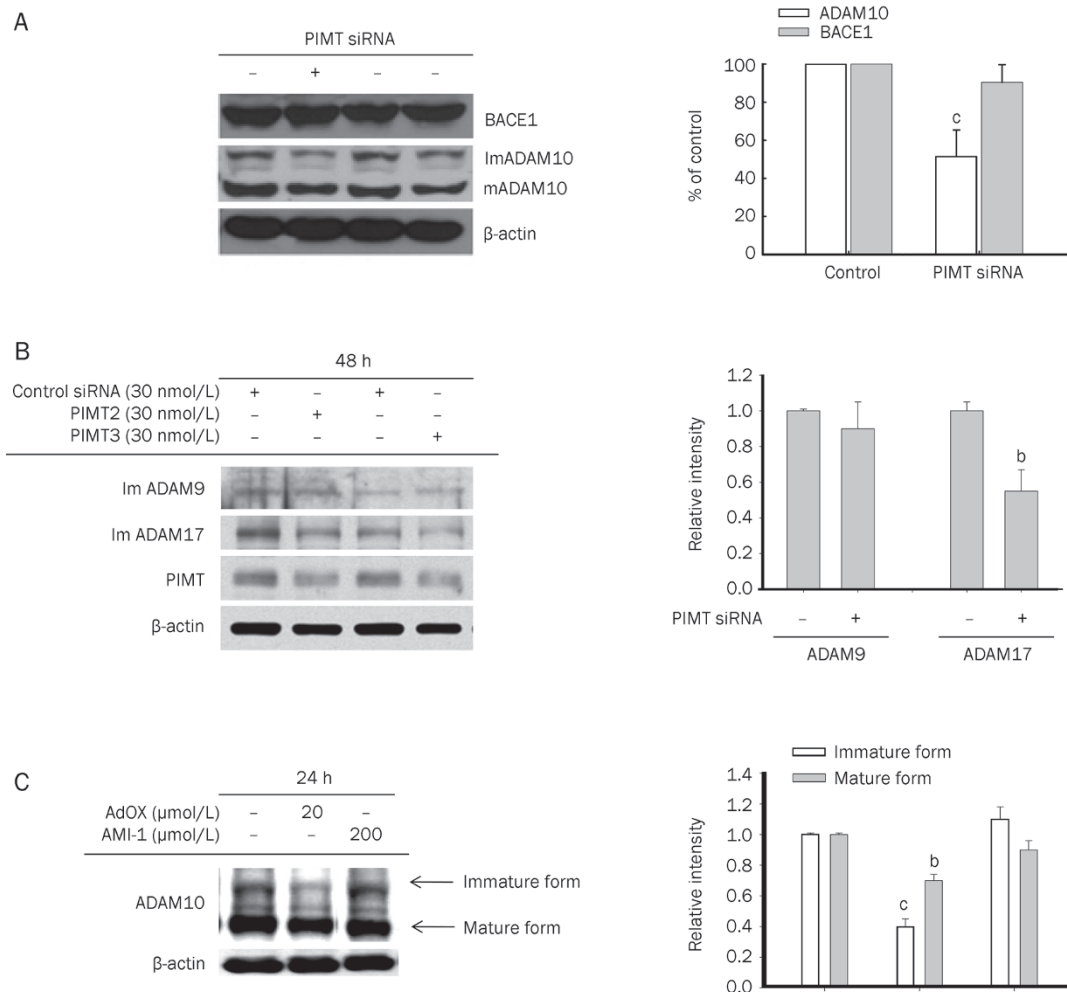


Figure 3. The effects of PIMT siRNA on ADAM10 and BACE1 expression levels. (A) HEK293 APPsw cells were treated with PIMT siRNA for 48 h. Cells were then harvested, and the protein levels of BACE1 and immature (ImADAM10) and mature (mADAM10) forms of ADAM10 were analyzed by Western blot analysis. Densitometric analysis was performed to determine the relative protein level of immature ADAM10 and BACE1. Results are expressed as the mean±SD for three independent experiments. (B) HEK293 APPsw cells were treated with PIMT siRNA for 48 h. Cells were then harvested, and the protein levels of immature ADAM9 and ADAM17, as well as PIMT were analyzed by Western blot analysis. Densitometric analysis was performed to determine the relative protein level of immature ADAM9 and ADAM17. (C) HEK293 APPsw cells were treated with AdOX or AMI-1 for 24 h. Cells were then harvested and the protein levels of immature and mature ADAM10 were analyzed by Western blotting analysis compared to control. Densitometric analysis was performed to determine the relative level of immature and mature ADAM10. Mean±SD. ^c*P*<0.05, ^b*P*<0.01 compared to normal group.

alter the levels of BACE1 (90.4%±14% compared to control). PIMT siRNA treatment down-regulated the total protein levels of ADAM17, but not ADAM9 (Figure 3B). In agreement with these data, AdOX, but not AMI-1, a PRMT inhibitor, also strongly reduced ADAM10 levels (Figure 3C). These results suggest that PIMT, but not PRMT, selectively modulates the protein levels of ADAM10 and ADAM17.

Discussion

In this study, we used HEK293 APPsw cells and SH-SY5Y cells to investigate the effects of protein *L*-isoaspartyl methylation on APP processing. HEK293 APPsw cells express high levels of Aβ^[18], and SH-SY5Y human neuroblastoma cells express considerable levels of APP and secrete non-toxic, non-amyloidogenic sAPP^[17]. Because of this, these cell lines have been

widely used to study the regulation of APP processing related to the pathogenesis of AD^[19]. Therefore, we used these cells in our study to examine the regulatory role of transmethylation on APP processing. Interestingly, treatment of either cell type with PIMT siRNA and AdOX, a well-known inhibitor of transmethylation^[20], remarkably induced the release of Aβ₄₀ peptides (Figure 1), indicating the involvement of PIMT-mediated methylation in APP cleavage. Because numerous papers have shown that the secretion of Aβ₄₀ is accompanied by the release of additional γ-secretase-generated Aβ peptides, such as Aβ₃₈, Aβ₄₂, and Aβ₄₃, it is likely that the production of these peptides would also be regulated by PIMT siRNA treatment.

To investigate the molecular mechanism underlying this phenomenon, the levels of the enzyme that generate Aβ₄₀ peptides were first determined. Figures 2 and 3 reveal that PIMT

knock-down modulates both the secretion and cleavage of sAPP α . In response to PIMT siRNA treatment, sAPP α secretion was dramatically diminished, but the levels of total APP and membrane-bound biotinylated APP remained unchanged (Figure 2A). These results suggest that sAPP β might be relatively enhanced by PIMT siRNA. The C99 fragment, a β -secretase cleavage product of APP, was increased by PIMT knock-down, whereas C83 was dramatically diminished (Figure 2B). Overall, our results suggest that the PIMT-mediated A β ₄₀ production pathway might be primarily associated with the sAPP α cleavage pathway. It has been reported that green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) exerts a beneficial role in reducing brain A β levels by promoting the cleavage of the C99 fragment of APP. The corresponding elevation of sAPP α ^[21] and G-protein coupled signaling, a major excitatory signal transduction pathway in neuronal cells, is known to activate a sAPP α generation pathway^[22]. Therefore, PIMT knock-down could contribute to the down-regulation of sAPP α during A β ₄₀ production.

The improper production of APP isoforms or aberrant APP trafficking during AD pathogenesis is believed to favor the amyloidogenic pathway^[23]. In addition, recent reports have shown that A β production is influenced more by the location of APP cleavage than the total amount of secretase present within the cell^[24]. However, in our study, neither the total amount of APP nor the amount of membrane-associated APP (Figure 2A) was altered in response to PIMT siRNA treatment. These results imply that PIMT does not regulate the trafficking of APP to the cell membrane or its synthesis. Instead, the protein levels of A β ₄₀ generating enzymes (Figure 3) clearly reveal an involvement of proteolytic processing in the observed decrease in sAPP α and increase in A β ₄₀ peptides in response to PIMT siRNA. Indeed, the expression of the α -secretase gene products, α -disintegrin and metalloprotease 10 (ADAM10) and ADAM17^[21], was reduced after PIMT siRNA transfection in HEK293 APP_{sw} cells according to Western blot analysis (Figure 3). The facts that BACE1 expression was not altered (Figure 3A) and that the PRMT inhibitor, AMI-1, did not affect ADAM10 levels (Figure 3C) seem to highlight the specificity of this pathway leading to A β ₄₀ generation. However, we cannot exclude the possibility that PIMT knock-down leads to the direct activation of BACE1 despite not affecting its expression level. Indeed, previous work has shown that BACE1-inducible cells exhibit increased production of A β ₄₀ peptides^[25]. To date, there is no experimental evidence suggesting that PIMT can regulate the enzyme activity of BACE1. However, several studies have reported that BACE1 can be modified by S-palmitoylation^[26] and ubiquitination^[27], indicating the importance of post-translational modifications of BACE1. Future studies will determine whether PIMT-induced methylation of BACE1 at aspartyl residues increases its enzyme activity.

In conclusion, we have demonstrated that knock-down of PIMT increased A β production via the inhibition of the non-amyloidogenic α -secretase pathway, an effect that is linked to a decrease in sAPP α and ADAM10/17 levels as summarized in Figure 4. Therefore, our study suggests a novel protective

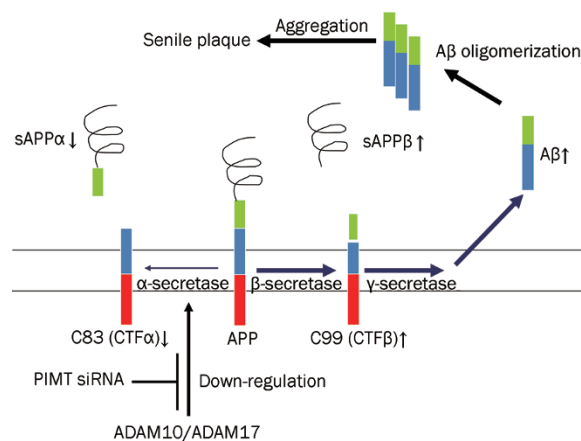


Figure 4. Proposed mechanism of action for PIMT in the regulation of A β ₄₀ peptide generation. A β peptides are generated from APP by β - and γ -secretase activity. PIMT seems to modulate the levels of α - and β -secretases synthesized from ADAM10- and -17.

role for PIMT in the pathogenesis of AD as a negative regulator of A β ₄₀ peptide formation.

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Author contribution

Narkhyun BAE, Jae Youl CHO, and Sungyoul HONG designed research; Narkhyun BAE, Se Eun BYEON, Jihyuk SONG, and Sang-Jin LEE performed research; Moosik KWON and Inhee MOOK-JUNG analyzed data.

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