G206D Mutation of Presenilin-1 Reduces Pen2 Interaction, Increases Aβ42/Aβ40 Ratio and Elevates ER Ca2+ Accumulation

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Abstract Early-onset familial Alzheimer’s disease (AD) is most commonly associated with the mutations in presenilin-1 (PS1). PS1 is the catalytic component of the γ-secretase complex, which cleaves amyloid precursor protein to produce amyloid-β (Aβ), the major cause of AD. Presenilin enhancer 2 (Pen2) is critical for activating γ-secretase and exporting PS1 from endoplasmic reticulum (ER). Among all the familial AD-linked PS1 mutations, mutations at the G206 amino acid are the most adjacent position to the Pen2 binding site. Here, we characterized the effect of a familial AD-linked PS1 G206D mutation on the PS1-Pen2 interaction and the accompanied alteration in γ-secretase-dependent and -independent functions. We found that the G206D mutation reduced PS1-Pen2 interaction, but did not abolish γ-secretase formation and PS1 endoproteolysis. For γ-secretase-dependent function, the G206D mutation increased Aβ42 production but not Notch cleavage. For γ-secretase-independent function, this mutation disrupted the ER calcium homeostasis but not lysosomal calcium homeostasis and autophagosome maturation. Impaired ER calcium homeostasis may due to the reduced mutant PS1 level in the ER. Although this mutation did not alter the cell survival under stress, both increased Aβ42 ratio and disturbed ER calcium regulation could be the mechanisms underlying the pathogenesis of the familial AD-linked PS1 G206D mutation.

Keywords Alzheimer’s disease · Presenilin-1 · Presenilin enhancer 2 · G206D mutation · Amyloid beta · Calcium

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Introduction

Alzheimer’s Disease and Amyloid-Beta (Aβ) Peptide

Alzheimer’s disease (AD) is the most common form of age-dependent dementia [1]. One of the pathological hallmarks of AD is amyloid plaque, which mainly consists of aggregated amyloid-β (Aβ), a 38–49-amino acid peptide. Aβ is produced through the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases [2]. Among the secreted Aβs, Aβ40 is the predominant species, but Aβ42 has higher tendency to form neurotoxic aggregates. These Aβ aggregates are believed to be the initiator of AD [3, 4].

Presenilin and γ-Secretase

γ-Secretase complex consists of presenilin (PS), nicastrin (Net), anterior pharynx-defective 1 (Aph1), and presenilin enhancer 2 (Pen2) [5, 6]. PS is the catalytic component of γ-secretase that cleaves APP and other proteins such as Notch [7, 8]. Vertebrates have two presenilin genes, named PSEN1 that encodes presenilin 1 (PS1) and PSEN2 that encodes presenilin 2 (PS2). PS1-associated γ-secretase displays higher activity than PS2 [9]. PS1 resides at the membrane of endoplasmic reticulum (ER) and endosomal system [10–12]. Binding of Pen2 regulates PS1 export from ER to endosomal system and other compartments [13]. The residue in PS1 essential for the Pen2 binding is the polar amino acid N204 [14–16]. Pen2 binding induces PS1 endoproteolysis to generate the active form of γ-secretase [6]. In the process of PS1 endoproteolysis, the 50 kDa PS1 holoprotein cleaves itself to produce a 30 kDa N-terminal fragment (PS1-NTF) and a 20 kDa C-terminal fragment (PS1-CTF), both of which are essential to the catalytic site.

γ-Secretase has at least two different enzymatic activities, ε- and γ-cleavages [17–19]. Following the β-secretase cleavage of APP to produce C99, ε-cleavage activity acts on C99 to produce long Aβs (Aβ48 and Aβ40). These long Aβs are further processed by γ-cleavage activity to release a mixture of Aβ species [17]. Apart from APP, γ-secretase cleaves many other transmembrane proteins such as Notch. ε-Cleavage on Notch yields notch intracellular domain (NICD) [19, 20], which modifies gene expression in many cell types. The PS1 knockout mice lead to an embryonic lethal phenotype similar to the mice lacking Notch [21, 22].

PS1 Function Independent of γ-Secretase Activity

Independent of γ-secretase activity, PS1 has diverse biological roles [23] in the regulation of intracellular calcium homeostasis [24] and the proteolysis through autophagy-lysosome system [25]. Deficiency of PS leads to ER Ca2+ accumulation and excess intracellular Ca2+ signals [24, 26–29], which is important for regulating neurotransmission and synaptic plasticity [30]. The disturbed ER Ca2+ homeostasis by PS1 mutations is linked to the early presynaptic deficits in AD [30–33].

Deficiency of PS also leads to autophagosome accumulation [25, 34], which might be resulted from the failure of lysosomal acidification [25] or lysosomal Ca2+ release [35, 36]. Lysosomal fusion with autophagosome is necessary for efficient proteolysis and neuron survival. However, the mechanism on whether PS1 directly or indirectly regulates lysosomal Ca2+ homeostasis remains controversial [37–39]. In addition, PS1 regulates many signaling pathways essential for cell survival. Several PS1 mutants fail to activate pro-survival signaling and reduce cell survival under stress conditions [40].

Discovery of the PS1 G206D Mutation

Early onset (<65 years of age) familial AD (FAD) usually associates with three genes: APP, PSEN1, and PSEN2 [41]. Previously, we reported a Taiwanese family of early-onset AD characterized by a rapid deterioration course, appearance of cotton wool-like plaque, and seizure, with a G→A substitution in the position 617 at exon 7 of PSEN1 gene causing a glycine to aspartate acid substitution of PS1 protein at amino acid 206 (G206D) [42]. This G206D mutation has also been reported in the other family [43], but the detailed pathogenic mechanism is still unclear. Among all the FAD PS1 mutations, the G206D mutation is the one most adjacent to the Pen2 binding site (N204). In this study, we explore the effects of the G206D mutation on γ-secretase-dependent and -independent PS1 functions. This study would provide the genetic evidence for the contribution of Pen2 in the pathogenesis of AD.

Methods

Cell Culture

Wild-type, PS1-null, and PS1/2-null mouse embryonic fibroblast (MEF) [9] were maintained in Dulbecco’s modified Eagle’s medium (DMEM, 12100–046, Gibco, CA, USA). Human neuroblastoma SH-SY5Y-APP (stably overexpressing APP695) cells were maintained in Minimum Essential Medium (MEM, 41500–034, Gibco, CA, USA), F-12 nutrient mixture (21700–075, Gibco, CA, USA), and 400 μg/ml puromycin. Human embryonic kidney (HEK293T) cells and COS7 cells were maintained in DMEM. All culture media were supplemented with 10 % fetal bovine serum (FBS, SH3007, HyClone, UT, USA), 1 % L-glutamine (GLL01, Caisson laboratory, UT, USA), and 100 μg/ml penicillin-streptomycin (PSL01, Caisson laboratory, UT, USA), and maintained at 37 °C in 5 % CO2. Cells were transiently transfected with either PS1 variants or control
vector using Lipofectamine 2000 (Invitrogen, CA, USA) or Turbofect (R0531, Thermo Scientific, MA, USA; only for Fig. 5).

cDNA Constructs The constructs we applied include the HA-tagged human APh1 cDNA, or FLAG-tagged human PEN2 cDNA in pcDNA3.1/Zeo(+) plasmid [44], the wild-type human PS1 cDNA in pcDNA3.1/Zeo(+) plasmid [45], the DsRed-GFP-LC3 cDNA in pDsRed-Monomer-C1 plasmid [46], the NotchΔmyc or V/LΔmyc constructs [47], the EGFP-Sec61β or EGFP-Rab5 constructs [48], and the pEGFP-C1 construct (GenBank Accession no. U55763, Catalog no. 6084-1, Clontech, CA, USA). For the EGFP-PS1 construct, the PS1 cDNA was clipped out by KpnI and XbaI restriction enzymes, acting at 5′ and 3′ end of the human PS1 cDNA containing pcDNA5/TO plasmid [45], respectively. The PS1 cDNA variants were then inserted into the KpnI-XbaI site of pEGFP-C1. The GFP-PS1 variants are endoproteolytically processed [Electronic Supplementary Material (ESM) Fig. 1], indicating the generation of active form of γ-secretase, but full functionality has not been completely tested.

**Immunoprecipitation (IP) Analysis** Twenty-eight hours after transfection, HEK293T cells were directly extracted with IP buffer (5 mM HEPES, 1 % CHAPSO and the complete protease inhibitor cocktail tablet, Roche, CA, USA) for 1 h on ice. After centrifugation (3,000× g, 4 °C, 10 min), 100 μg of total protein from supernatant was pre-cleared by protein G beads (LSKMagXX, Millipore, MA, USA). For IP-Aph1, lysates were incubated with anti-PS1 loop antibodies (1:1000, MAB5232, Millipore, MA, USA). For IP-Pen2, lysates were incubated with anti-PS1 NTF antibodies (1:1000, MAB1563, Millipore, MA, USA). The IP reactions were performed at 4 °C overnight with gentle rotation and then precipitated by protein G beads at room temperature for 1 h. After washing three times with washing buffer (1 % Tritone X-100 and 3 % BSA in PBS), proteins were eluted with 1X SDS sample buffer at 37 °C for 20 min. Western blotting result was quantified and the interactions were calculated as: (IP-APH1/total-APH1) or (IP-PEN2/total-PEN2)/(IP-PS1/total-PS1).

**Gel Electrophoresis and Western Blotting Analysis** Proteins were separated by 10 % Tris-Glycine SDS-polyacrylamide gel electrophoresis (PAGE), except the proteins from IP were separated by 10 and 15 % Tricine-SDS PAGE, and transferred to nitrocellulose membranes. Membranes were probed by primary antibodies: mouse anti-APP (6E10, NE1003, Millipore, MA, USA), mouse anti-PS1 loop (MAB5232, Millipore, MA, USA), rat anti-PS1 NTF (MAB1563, Millipore, MA, USA), mouse anti-myc (9E10, 05-419, Millipore, MA, USA), mouse anti-Flag (F3165, Sigma, MO, USA), mouse anti-actin (MAB1501, Millipore, MA, USA), and mouse anti-GAPDH (GTX100118, Genetex, CA, USA). Membranes were washed and probed with HRP-conjugated affinity-purified secondary antibodies: goat anti-mouse IgG, goat anti-rat IgG, and goat anti-rabbit IgG (12–349, AP136P, AP132P, Millipore, MA, USA). Protein signals were developed with chemiluminescent substrate ECL detection system (WBKLS0500, Millipore, MA, USA) and quantified by luminescence imaging system (LAS-4000, FujiFilm, Japan).

**Cellular Organelle Fractionation** The ER isolation kit (ER0100, Sigma, MO, USA) was applied. Briefly, HEK293T cells transfected with PS1wt or PS1G206D were mildly broken by pestle for 15 strokes. After differential centrifugation, the ER-enriched fraction was precipitated by 7 mM CaCl2 in the post mitochondria fraction. The ER-enriched fractions were either directly visualized by Western blotting or further separated by 15–30 % Optiprep™ (ER0100, Sigma, MO, USA) density gradient (0.5 ml of 30 %, 1 ml of 25 %, 1 ml of 20 % with samples, 1.5 ml of 15 %). After ultracentrifugation (rotor SW55 Ti, 3 h at 4 °C, 35,000 rpm, 150,000×g), eight fractions were collected from the top to the bottom and analyzed by Western blotting. Result is quantified and calculated as: PS1 in ER-enriched fraction/PS1 in total lysate/ER marker.

**Immunocytochemistry** COS7 cells were co-transfected PS1 with either GFP-Sec61β or GFP-Rab5. Cells were fixed in 4 % paraformaldehyde in PBS at 4 °C overnight and then blocked and permeabilized by 10 % FBS containing 0.3 % Triton X-100 for 1 h at room temperature. Fixed cells were incubated with anti-PS1-loop antibody (MAB5232, Millipore, MA, USA) overnight at 4 °C and next with Cy3-conjugated goat anti-mouse IgG (115-165-003, Jackson ImmunoResearch, PA, USA) for 2 h at room temperature. Images were captured by confocal microscope (FV1000, Olympus, Japan). Co-localization was analyzed by image analysis software (MetaMorph Premier, Molecular Devices, CA, USA).

**Enzyme-Linked Immunosorbent Assay (ELISA)** The levels of Aβtotal and Aβ42 were measured by human amyloid-β (1–42) and amyloid-β (1–x) assay kit (IBL). Forty-eight hours after transfection, conditioned media were incubated with capture antibody overnight and followed by the HRP conjugated anti-human Aβ (11–28) antibody for 1 h at 4 °C. Color was developed by tetramethylbenzidine chromogen and read at 450 nm by microplate reader (Sunrise, TECAN, Switzerland).

**Fura-2-AM Ca2+ Imaging Experiments** Intracellular Ca2+ concentration was measured by a dual wavelength ratiometric method [49]. Fura-2-AM stock was dissolved in pluronic F-127 (20 % solution in DMSO, P-3000MP, Life technologies,
CA, USA). MEFs were cultured on poly-L-lysine-coated coverslips and transfected with GFP-PS1wt or GFP-PS1G206D. The transfected cells were identified by GFP imaging. Forty-eight hours after transfection, cells were stained with 5 μM Fura-2-AM (Fl221, Invitrogen, CA, USA) in HEPES buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 10 mM HEPES, pH 7.3) containing 2 mM CaCl2 for 1 h at room temperature. Immediately before imaging, cells were shifted to Ca2+ free HEPES buffer containing 100 μM EGTA. Fura-2-AM signals were excited by polychrome V monochromator (TILL Photonics, Germany) at 340- and 380-nm wavelengths with 200-ms exposure time. Images were collected every 5 s using an EM-CCD camera (QuantEM 512SC, Photometrics, AZ, USA) mounted on an upright microscope (BX51WI, Olympus, Japan). Ionomycin (Asc-370, Ascent, UK), thapsigargin (Asc-286, Ascent, UK), and gly-Phe-β-naphthylamide (sc-252858, Santa cruz, TX, USA) were applied to induce Ca2+ signals. Region of interest was defined by the margin of the transfected cell. After background subtraction, fluorescence intensity was quantified by MetaFluor Fluorescence Ratio Imaging Software (Molecular Device, CA, USA).

The absolute value of cytosolic Ca2+ concentration ([Ca2+]i) was determined by the equation [49]:

\[
[Ca^{2+}] = K_d \frac{[R-R_{\text{min}}]}{[R_{\text{max}} - R]} S_{380} \frac{S_{380}}{S_{380}}
\]

where the Fura-2 affinity (K_d) to Ca2+ is 140 nM, R is the experimentally determined 340/380 ratio, R_{max} is the 340/380 ratio of Ca2+-saturated Fura-2 (addition of 20 mM Ca2+ and 10 μM ionomycin), R_{min} is the 340/380 ratio of Ca2+-free Fura-2 (addition of 10 mM EGTA), and S_{380}/S_{380} is the ratio of fluorescence intensity of Ca2+-free and Ca2+-bound form of Fura-2 at 380 nm.

**Autophagy Assay** The autophagosome accumulation or lysosomal dysfunction is measured by the ratio of GFP to DsRed fluorescence intensity in DsRed-GFP-LC3 reporter transfected cells [50]. Twenty-four hours after transfection, cells were collected and fixed by 1 % PFA. Fluorescence intensity of GFP or DsRed was measured by flow cytometry and analyzed by Cell Quest software. Quantified result is shown as the normalization of the ratio of GFP to DsRed to that of the wild-type control.

**Apo-BrdU-RedTM In Situ BrdU (TUNEL) Labeling Assay** DNA fragmentation was labeled using Apo-BrdU-RedTM in situ DNA Fragmentation assay kit (Biovision, CA, USA). Twenty-four hours after transfection, HEK293T cells expressing GFP or GFP-PS1 variants were treated with 1,500 μM H2O2 for 24 h and then fixed with 4 % parformaldehyde. Fixed cells were incubated in DNA labeling solution for 1 h at 37 °C, stopped the reaction with rinse buffer, and then incubated with anti-BrdU-Red antibodies for 30 min at room temperature. Labeled signals were analyzed by flow cytometry within 3 h after staining.

**Propidium Iodide Staining for Cell Cycle Analysis** Propidium iodide (PI, Sigma, MO, USA) was used to stain DNA content [51]. Twenty-four hours after transfection, HEK293T cells were treated with 1500 μM H2O2 for 24 h and fixed by 70 % ice-cold ethanol. Fixed cells were resuspended in 0.5 ml PBS and incubated in 0.5 ml DNA extraction buffer (192 mM NaHPO4, 0.004 % Triton X-100, pH 7.8) for 5 min at room temperature and centrifuged for 5 min. Cell pellets were re-suspended in 1 ml DNA staining solution (20 μg/ml propidium iodide, 200 μg/ml DNase-free RNase) for 30 min in the dark at room temperature and analyzed by flow cytometry within 3 h after staining.

**Flow Cytometry** Samples were analyzed by flow cytometry (FACScalibur, BD, NJ, USA) using Cell Quest software. Fluorescence was excited by 488-nm argon-laser and detected through 530/30 nm (FL1 channel) and 585/42 nm (FL2 channel) band pass filter. Non-transfected cells without staining were used to define the threshold and to amplify adjustment. Transfected cells were used to compensate fluorescence crosstalk. For TUNEL assay, DNA fragmentation was labeled with BrdUTP, probed by anti-BrdU antibody conjugating with Cy3 (Em, 576 nm), and detected by FL2-H channel. For PI staining, after filtering out single cells by area-width plot, the DNA content of single cells was detected by FL2-A channel. Cell cycle was analyzed by FlowJo version 7.6.1 under appropriate constraints employing Dean-Jett-Fox model [52].

**Result**

The G206D Mutation Reduces the PS1-Pen2 Interaction But Does Not Abolish γ-Secretase Activation

The G206D mutation is adjacent to the amino acids N204 crucial for PS1 interacting with Pen2, which is a required step for γ-secretase activation [6, 14, 15]. To examine γ-secretase formation, we co-transfected Pen2 or Aph1 with PS1wt or PS1G206D into HEK293T cells and analyzed their interaction by co-immunoprecipitation (Fig. 1a). The levels of Aph1 co-immunoprecipitated with PS1wt (set as 1) or PS1G206D (0.936 ±0.167) had no significant difference, but the levels of Pen2 co-immunoprecipitated with PS1G206D (0.331±0.135) were significantly lower than that with PS1wt (set as 1) (Fig. 1b). This result indicates that the G206D mutation partially
reduces PS1-Pen2 interaction but does not abolish the γ-secretase formation.

PS1 endoproteolysis is required for the γ-secretase activation. To test PS1 endoproteolysis, we measured the levels of PS1 holoprotein and endoproteolytic fragments (PS1-CTF and PS1-NTF) in PS-null MEF transfected with PS1wt or PS1G206D (Fig. 1c). The proportion of endoproteolytic fragments over total PS1 in PS1wt (set as 1) or PS1G206D (CTF, 0.921±0.046; NTF, 1.24±0.028) had no significant difference, suggesting normal endoproteolytic activity of γ-secretase (Fig. 1d). Thus, the G206D mutation did not abolish the PS1 endoproteolysis.

The G206D Mutation Increases the Production of Aβ42 But Not NICD

PS1 mutations could alter the γ-secretase activity toward APP and Notch, producing more Aβ42 but less NICD [53, 54]. Thus, we measured the levels of Aβ and NICD in PS1wt- or PS1G206D-expressing cells.

To examine the effect of the G206D mutation on levels of Aβ42 and Aβtotal, SHSY-5Y cells stably expressing APP (SHSY-5Y-APP) were transfected with PS1wt or PS1G206D. Secreted Aβ level in the culture media was measured by ELISA, and expression levels of PS1wt- and PS1G206D-expressing cells were monitored by Western blotting. Although there was no significant difference in Aβtotal level among all groups, the Aβ42 level in the untransfected control (0.529±0.205 ng/ml) was significantly higher than that of the untransfected control (0.529±0.205 ng/ml) (Fig. 2a, b). The Aβ42/Aβtotal ratio in PS1G206D-expressing cells (2.13±0.347-fold higher than control) was significantly higher than that in PS1wt-expressing cells (1.541±0.3-fold higher than control) (Fig. 2c), suggesting that the G206D mutation promotes the production of Aβ42.
To explore whether the G206D mutation alters γ-secretase activity toward Notch, truncated Notch sequence fused with 6x myc tags (Notch Δmyc) was used as a reporter for NICD production. The same construct containing a V1742L mutation (V/L Δmyc) to disrupt the γ-secretase cleavage was used as a negative control [47, 55]. The Notch reporters were co-transfected with PS1wt or PS1G206D into PS1-null MEF cell, and the Notch and NICD levels were measured by Western blotting (Fig. 2d). There was no difference in NICD/Notch ratio between PS1wt- and PS1G206D-expressing cells (1.014±0.047) (Fig. 2e). Thus, γ-secretase activity toward Notch is similar between PS1G206D and PS1wt. Together, our result shows that the G206D mutation increases the ratio of Aβ42/Aβtotal without affecting NICD levels.

Apart from the role in γ-secretase, PS1 also regulates the homeostasis of Ca2+ in ER [24, 26, 27]. To measure the NICD production, Notch Δmyc or V/L Δmyc was co-transfected with PS1 variants into PS1-null MEF. Representative Western blotting images show the levels of NICD and PS1 holoprotein. V/L Δmyc construct that could not be cleaved by γ-secretase was used as a negative control. The ratio of NICD to Notch (Notch Δmyc) was normalized to mock-transfected control (CT, Notch Δmyc expression only) group (set as 1). There was no difference between the two groups. Data are presented as the mean ± SD (n=3 independent transfection, *p<0.05, by ANOVA).

Application of 5 μM IO or 1 μM TG in the absence of extracellular Ca2+ evoked larger areas under curves in the PS-null MEF compared with the control MEF (Fig. 3b). The average area under curve induced by IO- or TG-sensitive Ca2+ pools in PS-null MEF (IO, 82.97±13.65; TG, 133.3±17.86 μM×s) was significantly larger than the control MEF (IO, 33.51±8.45; TG, 42.04±6.2 μM×s) (Fig. 3c), which is consistent with previous reports on the essential role of PS1 in preventing ER Ca2+ over-accumulation [24, 26, 27]. To examine whether PS1G206D impairs ER Ca2+ homeostasis, we transfected GFP, GFP-PS1wt, or GFP-PS1G206D into PS-null MEF. The IO or TG-evoked Ca2+ signals were larger in GFP-PS1G206D-expressing cells (IO, 109.22±22.15; TG, 55.46±6.87 μM×s) compared with GFP-PS1wt-expressing cells (IO, 45.75±9.97; TG, 27.78±2.97 μM×s) (Fig. 3d, f). Our result suggests that PS1G206D loses the ability to regulate ER Ca2+ homeostasis, leading to abnormally high levels of Ca2+ stored in ER.

Furthermore, we examined whether the effects of PS1 on ER Ca2+ storage are γ-secretase activity dependent. Applying 1 μM L-685,458, a γ-secretase inhibitor, did not change the ER Ca2+ levels in PS-null MEF expressing GFP, GFP-PS1wt, or GFP-PS1G206D as analyzed by two-way ANOVA (Fig. 3e, f). Together, our result indicates that the G206D mutation reduces the ability of PS1 to maintain ER Ca2+ homeostasis and this effect is independent of γ-secretase activity.

The G206D Mutation Disrupts the ER Ca2+ Homeostasis

Apart from the role in γ-secretase, PS1 also regulates the homeostasis of Ca2+ in ER [24, 26, 27]. To examine ER Ca2+ level, we depleted ER Ca2+ storage in MEF by ionomycin (IO) or thapsigargin (TG) (Fig. 3a). IO is an ionophore that induces formation of Ca2+-permeable pores. Because ER Ca2+ is the major intracellular Ca2+ pool, IO-induced signal is frequently used to implicate the calcium storage of ER [24, 56, 57]. TG is a specific inhibitor of the smooth ER Ca2+-ATPase (SERCA) pump, thereby blocking Ca2+ pumped into ER while allowing the passive Ca2+ leak.
The G206D Mutation Alters PS1 Localization in ER and Early Endosome

One of the potential factors dysregulating ER Ca\textsuperscript{2+} in PS1\textsuperscript{G206D}-expressing cells is the reduction of PS1 in ER. To examine whether the G206D mutation affects the localization of PS1 in ER, we analyzed the percentage of co-localization of PS1 with Sec61β, an ER marker [58]. Same as other report [12], PS1 expression was mainly overlapped with ER markers. Ratio of Sec61β overlapping with PS1\textsuperscript{G206D} (0.89±0.082) was significantly lower than that with PS1\textsuperscript{wt} (set as 1) (Fig. 4a), suggesting that the G206D mutation reduces PS1 localization in ER.

γ-Secretase processing of APP is highlighted in the endosome-lysosome system [12, 59]. To evaluate whether the G206D mutation affects the localization of PS1 in endosome, we analyzed the percentage of co-localization of PS1 with Rab5, an early endosome marker [60]. Ratio of Rab5 overlapping with PS1\textsuperscript{G206D} (1.43±0.433) was significantly higher than that with PS1\textsuperscript{wt} (set as 1) (Fig. 4b), suggesting that the G206D mutation promotes the PS1 localization in early endosome. Together, our results indicate that the G206D mutation affects the cellular sorting of PS1, which is reduced in ER and increased in early endosome.

To further confirm our immunostaining result, we performed cellular organelle fractionation to compare the level of PS1 in different cellular compartments. In the ER-enriched fraction, the PS1-NTF level in PS1\textsuperscript{G206D}-expressing cells (0.84±0.029) was lower than that in PS1\textsuperscript{wt}-expressing cells (set as 1) (Fig. 4c). In addition, the ER-enriched fraction was further separated by Optiprep\textsuperscript{TM} density gradient. The distribution of PS1\textsuperscript{wt} was similar to the ER marker, ERp72, while the distribution of PS1\textsuperscript{G206D} spread out to the fractions with ERp72 or with early endosome marker, Rab5 (Fig. 4d). The cellular organelle fractionation data further confirm our result that the G206D mutation decreases PS1 levels in ER but increases that in early endosome.

The decrease of PS1\textsuperscript{G206D} in the ER may due to that this mutation causes PS1 misfolding and degraded by ER-associated degradation (ERAD). Thus, we compared the stability of PS1 holoprotein. Transfected cells were treated with cycloheximide (CHX), a protein synthesis inhibitor, and lysed after 1, 2, 4, 8, 12 h (ESM Fig. 2a). The levels of PS1 holoprotein were normalized to
GAPDH and the levels of that at 0 h in each group were set as 1. PS1<sub>G206D</sub> holoprotein was less stable than PS1<sup>wt</sup> at 2 h after CHX treatment, but overall stability between these two proteins did not have significant difference (ESM Fig. 2b).

**Effect of the G206D Mutation on Lysosomal Calcium and Autophagy**

Deficiency of PS1 also leads to the accumulation of autophagosome and lower lysosomal Ca<sup>2+</sup> storage [25, 34, 38]. Here, we dissected the autophagosome maturation process by using DsRed-GFP-LC3 reporter [50]. Green fluorescence reflects autophagosomes that did not fuse with lysosomes while red fluorescence reflects all autophagosomes and autolysosomes. The higher ratio of GFP to DsRed fluorescence indicates more autophagosome accumulation. Our result that the relative change of GFP to DsRed fluorescence in PS-null MEF (1.26±0.072) was significantly higher than that in control MEF (set as 1) (Fig. 5a), indicating the accumulation of autophagosome in the PS-deficient cells. To examine whether the G206D mutation affects autophagosome maturation process, we co-transfected PS1<sup>wt</sup> or PS1<sub>G206D</sub> with DsRed-GFP-LC3 reporter into PS-null MEF. The GFP to DsRed fluorescence ratio showed no difference between PS1<sup>wt</sup>-expressing cells (set as 1) and PS1<sub>G206D</sub>-expressing cells (0.87±0.057) (Fig. 5b), indicating that the G206D mutation does not affect the autophagosome maturation in the PS1 overexpression system.

The failure fusion of lysosome with autophagosome in PS1-deficient cells might be caused by the low lysosomal Ca<sup>2+</sup> storage [36]. Therefore, we monitored the lysosomal Ca<sup>2+</sup> release by treating Fura-2-loaded MEFs with Gly-Phe-β-naphthylamide (GPN), which causes osmotic lysis of cathepsin C-positive lysosomes. Application of 500 μGPN evoked larger areas under curves in the control MEF compared with the PS-null MEF (Fig. 5c). The average size of GPN-sensitive Ca<sup>2+</sup> pools in control MEF (13.84±1.561 μM×s) was significantly larger than PS-null MEF (9.21±1.361 μM×s) (Fig. 5d), which is consistent with previous reports on the decreasing of lysosomal Ca<sup>2+</sup> in the PS-deficient cells [38, 39].

To examine whether PS1<sub>G206D</sub> lowers lysosomal Ca<sup>2+</sup> level, we transfected GFP-PS1<sup>wt</sup> or GFP-PS1<sub>G206D</sub> into PS-null MEF. Because we found that Lipofectamine™ may interfere with lysosomal Ca<sup>2+</sup>, we used the TurboFect™ transfection reagent to examine the GPN-evoked Ca<sup>2+</sup> signals. The GPN-evoked Ca<sup>2+</sup> signal in the GFP-PS1<sup>wt</sup>-expressing cells (10.00±1.745 μM×s) was similar with that in the GFP-PS1<sub>G206D</sub>-expressing cells (8.08±1.405 μM×s) (Fig. 5e, f). Our result suggests that PS1<sub>G206D</sub> does not affect lysosomal Ca<sup>2+</sup> homeostasis.
The G206D Mutation Did Not Affect Cell Death Rate Under Oxidative Stress

Some of the PS1 FAD mutants increase cell death under oxidative stress [61, 62]. Thus, we measured cell death by DNA fragmentation and induced oxidative stress by H2O2. The DNA fragmentation was measured using the TUNEL assay and PI staining in GFP or GFP-PS1-transfected HEK293T cells. For TUNEL assay, after 24-h treatment of 1,500 μM H2O2, the percentage of transfected cells with TUNEL-positive signal (Q2/(Q2+Q3)) had no significant difference between GFP-PS1wt (0.593±0.0274-fold of GFP control) and GFP-PS1G206D (0.721±0.0016-fold of GFP control) transfected cells (Fig. 6a, b). This result indicates that the G206D mutation does not affect the percentage of TUNEL-positive cells under oxidative stress.

For PI staining, the presence of sub-G1 phase indicates DNA fragmentation and is defined as dead cells [63]. After treatment of H2O2, the sub-G1 population was increased in all groups compared to each untreated control (Fig. 6c, d), but there was no difference between GFP-PS1wt (2.79±1.288-fold higher than untreated control) and GFP-PS1G206D (2.56±0.462-fold higher than untreated control) transfected cells. This result indicates that H2O2 can induce cell death, but the G206D mutation does not affect the rate of H2O2-induced cell death.

Interestingly, under oxidative stress, GFP-PS1-transfected groups had more cells in G1 phase (PS1wt, 1.051±0.051; PS1G206D, 1.112±0.011) (Fig. 6e) and less cells in G2 phase (PS1wt, 0.784±0.216; PS1G206D, 0.745±0.255) (Fig. 6g) and no difference in S phase (Fig. 6f) compared to the GFP-transfected group (G1, 0.819±0.182; G2, 1.113±0.113). This result indicates that overexpression of either GFP-PS1wt or
GFP-PS1G206D induces cell cycle arrest under oxidative stress. Together, our results suggest that the G206D mutation does not affect cell death under oxidative stress.

**Discussion**

In this study, we demonstrated that the G206D mutation reduced the PS1-Pen2 interaction but did not alter PS1 endoproteolysis, suggesting that γ-secretase could be assembled and activated. PS1G206D increased the Aβ42 to Aβtotal ratio, which might promote the formation of toxic Aβ42 oligomers. Furthermore, PS1G206D increased the ER Ca²⁺ storage but did not affect lysosomal function, indicating that the PS1 regulation in ER Ca²⁺ homeostasis might not directly link to that in lysosome.

Although PS1G206D did not directly contribute to cell death, both the increase of Aβ42 proportion and the dysregulation of ER Ca²⁺ have been implicated in the etiology of AD.

**Effects of the G206D Mutation on γ-Secretase Formation and Activity**

The formation and activation of γ-secretase are tightly controlled by ER-Golgi recycling regulators and ER retention signals to ensure that only fully assembled γ-secretase leaves ER [64–66]. PS1 contains at least two ER retention signals: one in the proximal end of PS1 CTF [64] and the other in the transmembrane domain 4 (TMD4) of PS1 NTF [13], in which the G206D mutation is located [14–16]. The interaction between Pen2 and PS1TMD4 is required for fully assembled γ-
secretase to leave ER [13]. In our findings, the reduced PS1\textsuperscript{G206D}-Pen2 interaction and less PS1\textsuperscript{G206D} level in ER suggest that the G206D mutation might only contribute to minor conformational changes, but not abolishes the \( \gamma \)-secretase formation and ER export. The normal PS1\textsuperscript{G206D} endoproteolysis also indicates the \( \gamma \)-secretase activation.

The rate of ER export and protein degradation could contribute to the reduced PS1\textsuperscript{G206D} level in ER. The PS1\textsuperscript{G206D} has an additional polar transmembrane residue, which may regulate both ER export and protein degradation [67]. A single mutation in polar amino acid N204 is sufficient to disrupt ER export, PS1-Pen2 interaction and \( \gamma \)-secretase activation [16]. However, the G206D mutation increased ER export, partially reduced the PS1-Pen2 interaction, and had no effect on \( \gamma \)-secretase activation. This difference may be due to that the G206D contributes an additional polar residue located at the opposite position to the N204 in TMD4.

In our findings, the PS1 G206D mutation increased the ratio of \( \alpha_3 \beta_{42} / \alpha_3 \beta_{\text{total}} \) but had no influence on the ratio of NICD/Notch, indicating that this mutation has effects on \( \gamma \)-cleavage but not \( \epsilon \)-cleavage. The increased \( \alpha_3 \beta_{42} \) proportion could be due to the PS1 conformational changes [68, 69]. In previous reports, PS1 conformational changes induced by elongation of Pen2 N terminus shows effects on \( \gamma \)-cleavage but not \( \epsilon \)-cleavage, thus causes \( \alpha_3 \beta_{42} \) overproduction [70]. Also, the majority of PS1 mutations have independent effects on \( \gamma \)-cleavage and \( \epsilon \)-cleavage [18, 53, 54]. Loss of \( \epsilon \)-cleavage on Notch, Erb4, N-Cadherin, and APP is less observed among PS1 FAD mutants, but the impairment of the consecutive \( \gamma \)-cleavage on \( \alpha_3 \beta \) and increase of \( \alpha_3 \beta_{42} \) ratio is conserved in PS1 FAD mutants [18].

The G206D Mutation Increased ER \( Ca^{2+} \) Storage

Deficiency of PS1 results in ER \( Ca^{2+} \) overload, leading to the excess intracellular \( Ca^{2+} \) release from ER after cellular signaling stimulation [24, 26, 27]. Presence of excess intracellular \( Ca^{2+} \) from ER induces synaptic dysfunction [30–33], and perturbation in \( Ca^{2+} \) homeostasis affects A\( \beta \) generation [57, 71]. Therefore, ER \( Ca^{2+} \) overload has been implicated as a causative factor for the early pathological changes in AD [31–33], and normalization of intracellular \( Ca^{2+} \) homeostasis could be a strategy for the development of effective disease-modifying therapies.

Autophagosome accumulation is involved in the pathogenesis of AD [72]. How could PS deficiency lead to autophagosome accumulation? Autophagosome accumulation could be caused by the disruption of lysosomal acidification or the failure of lysosomal \( Ca^{2+} \) release during the fusion of lysosome and autophagosome. One possibility is that PS1 acts as a chaperon of the V0a1 ATPase and thus directly affects the lysosomal acidification [25, 73]. However, several groups did not observe the lysosomal acidification defects [34, 38, 74]. The other possibility is that lysosomal \( Ca^{2+} \) storage may be indirectly affected by the PS-induced impairment of ER \( Ca^{2+} \) homeostasis [37]. More investigations are needed to elucidate the mechanism of the higher autophagosome accumulation in the PS-null cells. However, the G206D mutation only affects ER \( Ca^{2+} \), but not lysosomal \( Ca^{2+} \) and autophagy maturation process in our system. Our result indicates that the PS1 regulation in ER and lysosomal function might not be fully correlated.

Subcellular Distribution of PS1 Altered by the G206D Mutation

The alteration of PS1\textsuperscript{G206D} subcellular distribution might help to explain the \( \gamma \)-secretase-dependent and -independent deficits of this mutant. Intracellular PS1 is distributed in two pools: a major pool in ER and a minor pool in trans-Golgi network, plasma membrane, and endosomes [10, 11]. Our result indicates that the ER \( Ca^{2+} \) overload might be due to the decreased level of PS1\textsuperscript{G206D} in ER (Fig. 5). We also found the increased level of PS1\textsuperscript{G206D} in early endosome (Fig. 5), where the active form of \( \gamma \)-secretase for APP processing is found [75], that might correlate with the increased ratio of \( \alpha_3 \beta_{42} \) in PS1\textsuperscript{G206D}-expressing cells. However, due to the approach we used, it is hard to distinguish whether the holoprotein or NTF is responsible for these changes.

The G206D Mutation Did Not Alter Cell Survival Under Oxidative Stress

Although PS1\textsuperscript{G206D} increased ER \( Ca^{2+} \) storage, the G206D mutation did not accelerate cell death under oxidative stress. Nonetheless, effect of PS1 FAD mutations on cell survival is dependent on experimental system, amino acid substitution, and stress type. Under different experimental systems, the same PS1 mutant could show diverse consequences. For example, the PS1 P264L mutation had no effect on tunicamycin-induced cell death in the P264L knock-in mice [76], but it promoted caspase 3 signaling when expressed in primary neurons [40]. Moreover, the stress type plays an important role in the consequence of cell survival. For example, the PS1 L286V mutant rendered neuronal vulnerability to isoflurane toxicity, but not sevoflurane or desflurane toxicity [77]. Therefore, we cannot exclude the possibility that the G206D mutation could alter the cell survival in the other experimental systems.

Overexpressing PS1\textsuperscript{wt} or PS1\textsuperscript{G206D} leads to cell cycle arrest at G1 phase under oxidative stress (Fig. 5e) [78, 79]. A possible mechanism of PS1-induced arrest is mediated through the abnormal activation of Notch, which arrests cells in the G1 phase by preventing Wnt-induced G2 arrest [80]. Because the PS1 G206D mutation did not alter \( \gamma \)-secretase
activity toward Notch (Fig. 2d, e), PS1-induced G1 arrest might be due to the over-activation of Notch via high level of PS1\(^{Wt}\) or PS1\(^{G206D}\).

**Conclusion**

There are three other mutations found at this location including G206A [81], G206S [82], and G206V [83], implying that mutations at this G206 position may have a great impact on AD pathogenesis. According to bioinformatics analysis on the FAD-linked PS1 mutations, the amino acid G206 is a highly conserved amino acid among 14 species and mutation from glycine into aspartic acid has more potential to damage PS1 storage are the pathogenic mechanisms underlying the FAD-linked PS1 G206D mutation. Our finding also promotes the understanding of PS1-Pen2 interaction in the etiology of AD.

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**Conflict of Interest** The authors declare that they have no competing interests.

**Author Contributions** I.H.C. designed research and analyzed data; W.T.C., Y.F.H., Y.J.H., C.C.L., and Y.T.L. performed research and analyzed data; Y.C.L. and C.C.L. helped with \(\text{Ca}^{2+}\) imaging analysis; and W.T.C. and I.H.C. wrote the paper.

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