Individual and combined presenilin 1 and 2 knockouts reveal that both have highly overlapping functions in HEK293T cells

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Presenilins 1 and 2 (PS1 and 2) are the catalytic subunits of γ-secretase, a multiprotein protease that cleaves amyloid protein precursor and other type I transmembrane proteins. Previous studies with mouse models or cells have indicated differences in PS1 and PS2 functions. We have recently reported that clinical γ-secretase inhibitors (GSIs), initially developed to manage Alzheimer’s disease and now being considered for other therapeutic interventions, are both pharmacologically and functionally distinct. Here, using CRISPR/Cas9-based gene editing, we established human HEK 293T cell lines in which endogenous PS1, PS2, or both have been knocked out. Using these knockout lines to examine differences in PS1- and PS2-mediated cleavage events, we confirmed that PS2 generates more intracellular β-amyloid than does PS1. Moreover, we observed subtle differences in PS1- and PS2-mediated cleavages of select substrates. In exploring the question of whether differences in activity among clinical GSIs could be attributed to differential inhibition of PS1 or PS2, we noted that select GSIs inhibit PS1 and PS2 activities on specific substrates with slightly different potencies. We also found that endoproteolysis of select PS1 FAD-linked variants in human cells is more efficient than what has been previously reported for mouse cell lines. Overall, these results obtained with HEK293T cells suggest that selective PS1 or PS2 inhibition by a given GSI does not explain the previously observed differences in functional and pharmacological properties among various GSIs.

γ-Secretase is a multi-component aspartyl protease that cleaves amyloid protein precursor (APP)3 and many other type I transmembrane proteins within their transmembrane domains. Presenilin 1 and presenilin2 (PS1 and PS2) form the catalytic core of γ-secretase (1, 2), and three accessory proteins, anterior pharynx-defective 1 (APH-1), nicastrin, and PEN2 (presenilin enhancer protein 2), are required for the γ-secretase complex to mature (3–5). Human γ-secretase has a high degree of heterogeneity because of the differential inclusion of two PSEN and two APH-1 proteins: APH-1A and APH-1B. Because each complex exists in a stoichiometry of 1:1:1:1 for each of each component (6), at least four different “base” γ-secretase complexes can be formed, and even more may be generated by inclusion of alternatively spliced subunits (7, 8).

γ-Secretase inhibitors (GSIs) were initially developed as therapeutic agents for Alzheimer’s disease (AD); because they inhibit APP processing into Aβ, GSIs do attenuate the accumulation of Aβ in the brain of preclinical models (9). However, GSIs have largely been abandoned in the context of therapeutic development for AD, because of toxicity and lack of efficacy in symptomatic AD (10–12). Indeed, the only GSI to advance to a phase 3 trial for AD showed negative effects on cognition. Additionally a number of dose-limiting toxicities were also observed, most of which are thought to be on-target (13). GSIs have now been repurposed for various cancers, largely based on the premise that they act as inhibitors of Notch1 signaling (14, 15). However, γ-secretase cleavage mediates complex signaling events through cleavage of multiple transmembrane proteins on both tumor cells and normal cells, and there remain large gaps in our knowledge regarding how GSIs alter specific physiologic and pathophysiologic signaling events.

We have recently reported that clinical GSIs are both pharmacologically and functionally distinct (16). The clinical and preclinical GSIs evaluated showed differential potencies and profiles of inhibition of NOTCH1–4 substrates, with several even enhancing cleavage of NOTCH3 at concentrations where NOTCH1 cleavage is inhibited. Several GSIs were also shown to be potent inhibitors of select signal peptide peptidase (SPP/...
SPPL) family members, whereas others are not. These data demonstrated that the clinical GSIs studied were not functionally equivalent and that potency or substrate specificity did not appear to account for these differences. Although they provide an important framework to evaluate results from ongoing and completed human trials with these compounds, our previous studies did not provide a mechanistic basis for why the different GSIs have different profiles of inhibition when evaluated against a panel of substrates.

Here we explored whether the differences in clinical GSI activity could be attributed to differential inhibition of PS1 or PS2. Although differences in PS1 and PS2 function have been reported, these studies were largely conducted in mouse models or cells (17–21). Further, differences in select GSI potency for PS1 versus PS2 have also been reported but primarily from data generated in mouse knockout cells or reconstituted complexes (22, 23). Here we report the generation of human HEK 293T cell lines in which endogenous PS1, PS2, or both have been knocked out using CRISPR-Cas 9 gene editing. We characterize these cell lines and use them to explore differences in PS1- and PS2-mediated cleavage events and potential for differential inhibition by various GSIs, including those in clinical cancer trials. These studies reveal (i) subtle differences in PS1- and PS2-mediated cleavage for select substrates, (ii) slightly different potencies of PS1 versus PS2 inhibition for select GSIs on select substrates, and (iii) that endoproteolysis of select PS1 FAD-linked mutants in human cells is more efficient than what has been reported in mouse cell lines. However, they suggest that selective inhibition of PS1 or PS2 by a given GSI does not explain the previously observed differences in functional and pharmacologic properties of various GSIs (16). Nevertheless, the development of the human cell lines lacking PS1, PS2, or both provides novel tools for the field. Indeed, we demonstrate the utility of the PS null cell 293T line for detecting whether rare variants are likely to be associated with AD-risk based on alterations in Aβ42:Aβ40 levels.

Results

Generation and initial characterization of human presenilin knockout HEK 293T cells

Most previous studies designed to understand differences in PS1 and PS2 function used mouse knockout cells. Multiple human cell lines we examined have roughly similar ratio of PS1 and PS2 (Fig. 1A), although mouse cells have been reported to have higher relative levels of PS1 (19). To create human cells lacking PS1 or PS2, we used CRISPR-Cas 9 gene editing, and generated HEK 293T cell lines that expressed only PS1 (PSEN1+/−, PSEN−/−; referred to as PS1 lines) or expressed...
only PSEN2 (PSEN1\(^{-/-}\), PSEN2\(^{+/+}\); referred to as PS2) (Fig. 1B). Gene editing was confirmed by genomic sequencing (Fig. S1). Based on distinct mutations within each allele, three independent clonal lines for PS1 and PS2 were selected, and A\(\beta\) was evaluated following transient transfection with APPsw. Although PS1 or PS2 knockout causes a compensatory regulation of the other PS homolog slightly (Fig. S2), these data show that all of the clonal 293T lines expressing only PS1 or PS2 produce similar levels of A\(\beta\) total, A\(\beta\)40, and A\(\beta\)42 (Fig. 1, C–E). Given the similarities between these lines, we focused our further studies only on two lines: PS1 #1 and PS2 #1. Because simultaneous editing of PS1 and PS2 did not enable us to establish a de novo DKO line, PS1 line #1 was subject to a second round of editing with CRISPR-Cas9 targeting PSEN2 to generate the DKO line (PSEN1\(^{-/-}\), PSEN2\(^{-/-}\); referred to as DKO) (Fig. 1B). The DKO was validated by comparing the levels of total A\(\beta\) following treatment with a \(\gamma\)-secretase inhibitor. As shown in Fig. S3A, the levels of total A\(\beta\) in the DKO are equivalent to the levels form WT HEK following inhibitor treatment, indicating that these are indeed complete knockouts with the signal equivalent to background from the ELISA. When the DKO cell was co-transfected with APPsw and either PSEN1, PSEN2, or both, the total A\(\beta\) levels were also equivalent (Fig. 1F). These data suggest that human PS1 and PS2 in 293T cells are quite similar functionally with respect to generation of secreted A\(\beta\). Because a previous report had shown that in mouse cells PS2 generates more intracellular A\(\beta\) than PS1, we evaluated intracellular A\(\beta\) levels by ELISA. In these 293T cells, we also observe that PS2 alone generates more intracellular total A\(\beta\), A\(\beta\)40, and A\(\beta\)42 than PS1 (Fig. 1, G–J). We also observed that PS1 has relatively higher processivity than PS2. As shown in Fig. S3 (B and C), PS1 generated more A\(\beta\)38 in both cell-based assay and cell-free assay.

The DKO line potentially provides an excellent tool to study individual PSEN mutations on a PSEN1/2 null human background. To validate this assertion, we first co-transfected the DKO line with either PS1wt, three FAD-linked PSEN1 mutants (M139V, R278I, \(\Delta E\)x9) or the catalytically inactive dominant negative mutant D385A with APPsw into DKO cells. In the Western blotting using a PS1 CTF antibody (Fig. 2A, top panel), CTF of PS1wt, M139V, and R278I were clearly detected. As expected, PS1 \(\Delta E\)x9 is not endoproteolyzed and does not generate a CTF (24). A faint CTF-like band from PS1 D385A is detected. This CTF-like fragment might result from a caspase cleavage or a “presenilinase” activity (25, 26). Notably, in mouse cells PS1 R278I undergoes limited endoproteolysis (27, 28), but in the human DKO cell line, it is efficiently endoproteolized. Western blotting for APP with a C-terminal antibody (Fig. 2A, bottom panel) shows that APP CTFs accumulate in the DKO cell and that expression of PS1 D385A or R278I does not appreciably reduce the level of the CTF, as opposed to clear reductions observed with PS1wt, M139V, or \(\Delta E\)x9. IP-MS analysis of conditioned media shows the expected effects on A\(\beta\) peptide generation. PS1 wt transfection results in A\(\beta\) profiles very similar to the parent 293T cell line, PS1 D385A generates no A\(\beta\), PS1 M139V and \(\Delta E\)x9, shift profiles to favor increased production of A\(\beta\)42 and R278I almost exclusively generates A\(\beta\)43 (Fig. 2B) (27).

We evaluated whether the HEK293T DKO line would be useful to screen for subtle effects of PS1 variants not previously associated with AD. Whole-exome sequencing has revealed that there is a large number of rare PSEN1/2 variants that could potentially be associated with AD if they alter \(\gamma\)-secretase processivity (http://exac.broadinstitute.org/, https://www.biorxiv.org/content/10.1101/030338v1; and Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/), Accessed May, 2016) (38). We generated expression plasmids encoding cDNAs of 16 rare PSEN1 variants identified from the NHLBI Exome Sequencing Project including 6 that were annotated by PolyPhen 2 to have potential harmful functional effects, as well as a more common variant (E318G) previously demonstrated not to alter A\(\beta\) production (29). These constructs were co-transfected into the DKO cell line with APPsw. WT PS1, PS2, PS1 M139V, and PS1 \(\Delta E\)x9 served as controls (Fig. 2C). These data show that the 16 rare variants did not significantly alter A\(\beta\)42:A\(\beta\)40, suggesting they have little effect on \(\gamma\)-secretase activity and are likely to be benign.

**PS1 \(\gamma\)-secretase and PS2 mediated \(\gamma\)-secretase cleavage of other substrates**

We have recently developed quantitative cell-based cleavage assays for a number of other \(\gamma\)-secretase substrates. These cell-based assays use chimeric truncated fusion protein constructs for NOTCH1–4, CD44, and VEGFR1 and allow us to use an ELISA for A\(\beta\) as a surrogate for \(\gamma\)-secretase cleavage (16). For these studies, the expression plasmids encoding the various fusion protein substrates were transfected into PS1 #1 or PS2 #1 lines and A\(\beta\) release into the media measured. Although the overexpression levels in PS1 and PS2 cells were similar (data not shown), for five of the six substrates PS1 cells produced more A\(\beta\) than PS2 cells, suggesting that except for NOTCH4, all of the substrates are cleaved slightly more efficiently by PS1. Although the differences in PS1- or PS2-mediated \(\gamma\)-secretase cleavage as assessed by A\(\beta\) levels are all highly statistically significant, the differences are not extremely large (ranging from 20 to 32%) (Fig. 3A).

To more closely examine whether processive cleavage distinguishes PS1 from PS2, we performed IP-MS from media of the PS1 and PS2 cells co-transfected with the various chimeric fusion protein constructs. We were only able to obtain reliable MS spectra in these cell-based studies from cNOTCH1sub- and cNOTCH3sub-transfected cells. Nevertheless, these data revealed subtle difference in processivity. For cNOTCH1sub, PS1 appeared to be more processive with higher levels of His-1735 and Ala-1741, whereas PS2 had higher levels of Val-1745 (Fig. 3B). All MS peaks were numbered according to the amino acid sequences from the Uniprot data bank. For cNOTCH3sub the opposite was observed. PS2 cells produced more of a shorter peptide cleaved at Pro-1645. Further, another shorter peptide

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cleaved at Leu-1646 could also be identified in the PS2 spectra but not in the PS1 spectra.

To complement and extend these cell-based assays, we performed a cell-free assay using recombinant substrates rNOTCH1sub, rNOTCH2sub, rNOTCH3sub, rNOTCH4sub, and rCD44sub, which we have previously characterized (16). Cell membranes from either DKO cell or WT cells treated with 1 μM of the GSI LY411575, showed similar spectra and enabled identification of nonspecific peaks (data shown only for rNOTCH1) (Fig. 4A). Overall, these data showed only subtle differences between PS1 and PS2 in terms of processivity of different substrates. rNOTCH1 was consistently more efficiently cleaved in the cell-free assay by PS1 compared with PS2; thus, peak heights were lower in the PS2 spectra, although the relative levels of each peak were similar. PS2 showed only subtle differences in the spectra compared with PS1 or WT cell membranes (Fig. 4B). rNOTCH3sub in the PS2 cell membranes showed higher levels of short peptide Pro-1645 and Leu-1646.
Differences in potency for PS1- and PS2-mediated cleavages does not explain the pharmacological and functional differences among GSIs

In our previous study, we had observed unexpected variance in the overall potency, potency of inhibition of select substrates, and inhibition of proliferation triple negative breast cancer lines in mammosphere assays (16). Having developed the PS1 and PS2 cells lines, we probed whether these differences could be explained by differential effects of the GSI on PS1 or PS2. The clinical GSI PF-308414 reduces growth of two different

Figure 3. PS1 and PS2 process NOTCH receptor and other substrates. A, Aβ-like peptide levels in conditioned media show PS1 process NOTCHs slightly more efficiently than PS2 (n = 3), except NOTCH4. Aβ-like peptides were assayed using AB5 and HRP-conjugated 4G8 as capture and detection antibody, respectively. B, mass spectra of Aβ-like peptide generated from cNOTCH1sub and cNOTCH3sub in cell-based assay. Intens., intensity.

Figure 4. Mass spectra of Aβ-like peptide from different recombinant substrates in cell free assay. A, mass spectra of NOTCH1 Aβ-like peptide in WT, PS1, PS2, and DKO HEK cell membrane. B, mass spectra of NOTCH2, NOTCH3, NOTCH4, and CD44 Aβ-like peptide in PS1 and PS2 HEK cells. All experiments were repeated three times. Intens., intensity.
triple negative breast cancer lines more potently than other clinical and nonclinical GSIs and shows selectivity for inhibition of Notch2 cleavage. We therefore tested PF-308414 for inhibition of PS1- versus PS2-mediated cleavage of several substrates in cell-based assays. As seen previously this PF-308414 is an extremely potent, low picomolar IC50 of cNOTCH2sub relative to other substrates, but it inhibits PS1- and PS2-mediated cleavages of all substrates tested equivalently (Fig. 5A, Table 1, and Table S1). In our previous study, multiple GSIs showed reduced potency for inhibition of cleavage of cNOTCH3sub compared with other substrates, and this is again observed in both the PS1 and PS2 lines. Notably, some subtle differences in potency for PS1 and PS2 are observed. The GSIs, BMS-906024, Semagacestat, DAPT, and R04949097 all show slightly increased potency for inhibition of cleavage of PS2 versus PS1, whereas both MK-0752 and PF-3084014 show roughly equivalent inhibition of PS1 and PS2 (Fig. 5B, Table 2, and Table S2).

Previous reports also suggested that several GSIs show differential potencies against PS1 or PS2. The sulfonamide-based GSI BMS299897 has been reported to selectively inhibit APP processing conducted by PS1 with IC50 = 8 nm and by PS2 with IC50 = 308 nm (23). Using these HEK293T cell lines, we saw only very minor differences in inhibition of PS1- or PS2-mediated cleavages of several substrates (Fig. 6A, Table 3, and Table S3). Another sulfonamide-based GSI MRK560 has been reported to inhibit PS1 with 30–37 times greater efficiency than PS2 (22, 30). Here using our cell-based assay, we found it does indeed show higher potency for inhibition of PS1-mediated cleavage of two substrates (cC100sub and cNOTCH4sub) (Fig. 6B and Table 3).

### Figure 5. PS1’s and PS2’s responses to GSIs with different substrates in cell-based assay.

A, PS1 and PS2 have similar dose responses to GSI PF-3084014 with cNOTCH2–4sub and cCT100sub. B, dose response of PS1 and PS2 on NOTCH3 with other clinical trial GSIs. IC50 values were calculated using Prism (version 7) software and are summarized in Tables 1 and 2. 95% confidence intervals of IC50 values are summarized in Tables S1 and S2.

### Table 1

| IC50 values of PF-3084014 on different substrates |
|---------------------------------|
|       | WT  | PS1 | PS2 |
| cC100sub  | 1.95 | 0.33 | 0.34 |
| cNOTCH2sub | 0.002 | 0.002 | 0.001 |
| cNOTCH3sub | 15.07 | 0.69 | 1.66 |
| cNOTCH4sub | 10.77 | 5.68 | 5.05 |

### Table 2

| IC50 values of GSIs on cNOTCH3sub |
|---------------------------------|
|       | WT  | PS1 | PS2 |
| BMS-906024  | 0.32 | 0.12 | 0.04 |
| MK-0752    | 146.3 | 40.06 | 72.94 |
| PF-3084014 | 15.07 | 0.69 | 1.66 |
| RO4929079 | 28.31 | 9.52 | 1.77 |
| Semagacestat | 523.3 | 276.6 | 38.52 |
| DAPT       | 552.4 | 215.6 | 25.45 |

### Discussion

We have developed and characterized human 293T cell lines expressing only PS1, expressing only PS2, or lacking both PS1 and PS2. Our data show that these are highly useful tools for probing PS1- or PS2-mediated cleavages in a human background, as well as for exploring effects of novel variants on a null background. These cell lines enabled us to rigorously test our hypothesis that functional and pharmacological differences between select GSIs might be attributable to differences in targeting of PS1 or PS2 or selective PS1 or PS2 cleavage of select substrates. Although we do note subtle differences in PS1- and PS2-mediated cleavages of various and subtle differences in
potency of select GSIs for PS1- or PS2-mediated cleavages, we believe that our data strongly refute our initial hypothesis. Thus, it remains enigmatic why various GSIs exhibit pharmacologic and functional differences in various assays. Differential inhibition of PS1 and PS2 does not account for the functional differences or differences in GSI inhibitor profiles we and others have observed with clinical and preclinical GSI; so what might account for such differences? One possibility is that γ-secretase complexes, regardless of the subunit composition, can adopt multiple conformations, either independently of or upon substrate engagement. If these conformers have preferences for different substrates and are differentially targeted by various GSIs, then such a model could account for the differential effects on substrate cleavage and inhibition by GSIs. Although there is ample evidence from structural studies that γ-secretase can adopt different structures (31), linking conformational differences to the functional differences we and others report will be very challenging. Alternatively, it is possible that different GSIs may differentially target γ-secretase complexes in different subcellular compartments (plasma membrane, endosomes, mitochondria) because of differences in solubility, pKa, or sensitivity to drug transporters. These and other studies indicate that the path forward for improving or identifying an optimal GSI for a given indication (outside of potency and pharmacokinetic properties) is going to be challenging and likely empirical in nature. The mechanistic basis for differences in functional effects of the GSIs has not been elucidated. One could speculate that profiling GSI activity against a much broader panel of substrates or structural studies such as those described above might provide this mechanistic

Table 3
IC50 values of BMS299897 and MRK560 on different substrates

| Substrate | IC50 (nM)    |
|-----------|-------------|
| WT PS1 PS2 | WT PS1 PS2 |
| cC100sub  | 263.4 45.51 124.7 12.69 1.14 15.37 |
| cNOTCH2sub| 2.3 2.16 4.12 |
| cNOTCH3sub| 206.7 187.7 261.6 |
| cNOTCH4sub| 1468 2138 1663 1.73 0.45 4.7 |

Figure 6. PS1’s and PS2’s dose responses to “selective” inhibitors in cell-based assay (n ≥ 3). A, BMS299897 shows very minor differences in inhibition of PS1- or PS2-mediated cleavages of cNOTCH2–4sub and cC100sub. B, MRK560 show higher potency for inhibition of PS1-mediated cleavage of cNOTCH4sub and cC100sub. IC50 values were calculated using Prism (version 7) software and are summarized in Table 3. 95% confidence intervals of IC50 values are summarized in Table S3.
guidance. However, such studies would require extensive resources, would likely take many years to accomplish, and still may not yield the desired mechanistic insight that would help guide GSI development.

Sulfonylurea-based GSIs have been reported to have higher potency for PS1 using different assay systems (22, 23, 30). The results we present in this study show some complexity because MRK560 did show PS1 selection, but BMS299897 did not. These data further suggest we have a long way to go to understand the mechanism of GSIs.

One of the surprising findings from these data is just how similar human PS1 and PS2 are with respect to their enzymatic activities and response to a panel of inhibitors. As previous studies of PS1 and PS2 show, they are distinct in terms of their subcellular localization, with PS2 containing a targeting motif that directs γ-secretase to the late endosome/lysosome (32, 33), such data together with the current data, would indicate that differences in functionality and substrate cleavage is likely much more dependent on nonenzymatic factors such as subcellular localization. Notably, we do confirm the finding that PS2 produces more intracellular Aβ than PS1 (33).

CRISPR-Cas9–based gene editing to develop cell lines lacking select proteins is a valuable tool that can facilitate both target identification and validation studies. Here, we have used CRISPR-Cas9 gene-edited 293T cells to enable rigorous pharmacologic studies of GSI in human cell lines lacking PS1 and PS2 and generated PS1 and PS2 null cell lines. These cell lines represent novel tools for the field and have already provided novel insights. Indeed, we demonstrate that they can be used to rapidly screen novel PS variants for possible pathogenic activity. For cell-based γ-secretase cleavage assay, each substrate was transiently transfected into HEK cells using PEI. After incubate 16 h, fresh media with DMSO or GSIs were added. Conditioned media were collected after 24 h and assayed by ELISA and MS. For the assay of PS1 mutants, pcDNA encoding D385A, M139V, R278I, and ∆EEXon9 were co-transfected with APPsw at ratio of 1:3 using PEI. For GSI assay, PF-3084014, RO4929079, MK-0752, Semagacestat (all purchased from MedChem Express, Monmouth Junction, NJ), BMS-906024 (Maplewood, NJ), MRK560 (Tocris, Minneapolis, MN), BMS299897, DAPT, and LY411575 (Sigma) were tested at the range of 1 μM to 10 μM.

**Cell free γ-secretase cleavage assay**

CHAPSO-solubilized CHO cell, HEK cell membrane, and recombinant γ-secretase substrates rNOTCH1sub, rNOTCH2sub, rNOTCH3sub, rNOTCH4sub, rCD44sub, and rVEGFR1sub were prepared as described in a previous report (16). 25 μg/ml of each substrate was incubated with the membrane (100 μg/ml total protein) in sodium citrate buffer (150 mM, pH 6.5, Roche Complete protease inhibitor added) for 2 h at 37 °C. The reaction was terminated by placing tubes on ice until immunoprecipitation.

**Immunoprecipitation and MS**

Immunoprecipitation and MS of Aβ and Aβ-like peptides in cell free assay or conditioned media were performed as previously described (36). Briefly, the peptides were immunoprecipitated using anti-Aβ Ab5 antibody bound to sheep anti-mouse IgG magnetic Dynabeads (Life Technologies) and eluted with 0.1% TFA in water. CTFs were immunoprecipitated with anti-FLAG M2 magnetic beads (Sigma). Eluted samples were mixed 2:1 with saturated α-cyano-4-hydroxycinnamic acid matrix (Sigma) in a mixture of acetonitrile (60%) and methanol (40%) and loaded onto a α-cyano-4-hydroxycinnamic acid-pretreated MSP 96 target plate (Bruker, Billerica, MA). The samples were analyzed using a Bruker Microflex LRF-MALDI-TOF mass spectrometer.

**ELISA and Western blotting**

Sandwich ELISAs used for Aβ detection were performed as previously described (36, 37). Briefly, Aβ and Aβ-like peptides in conditioned media were captured with Ab5 antibody. Aβ40 and Aβ42 were captured with Ab13.11 and Ab2.13 antibody, respectively. All plates were detected with horseradish peroxidase–labeled mAb 4G8 (Biolegend). Synthetic Aβ1–40 was used as a standard. For intracellular Aβ, APPsw-transfected WT, PS1, and PS2 cells in 15 cm dishes were washed twice with ice-cold PBS and then resuspended in 500 μl of PBS,
supplied with Complete Protease Inhibitor and 1% Triton-100. The cells were sonicated 10 s followed by centrifugation at 18,000 × g for 10 min to remove debris. The supernatant were diluted to 1 ml and loaded to ELISA plates. Total protein concentration of each sample was measured with BCA method to make sure equal amounts of supernatant were loaded. All ELISAs were developed with TMB substrate (KPL, Gaithersburg, MD). Bis-Tris precast gels (Bio-Rad) were used for all SDS-PAGE. Monoclonal anti-FLAG M2 antibody (Sigma) and Aβ1–16 antibody 6E10 (Covance) were used for Western blotting.

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