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RESEARCH ARTICLE

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Quantitative comparison of presenilin protein expression reveals greater activity of PS2-γ-secretase

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Abstract

γ-secretase processing of amyloid precursor protein (APP) has long been of interest in the pathological progression of Alzheimer's disease (AD) due to its role in the generation of amyloid-β. The catalytic component of the enzyme is the presenilins of which there are two homologues, Presenilin-1 (PS1) and Presenilin-2 (PS2). The field has focussed on the PS1 form of this enzyme, as it is typically considered the more active at APP processing. However, much of this work has been completed without appropriate consideration of the specific levels of protein expression of PS1 and PS2. We propose that expression is an important factor in PS1- and PS2-γ-secretase activity, and that when this is considered, PS1 does not have greater activity than PS2. We developed and validated tools for quantitative assessment of PS1 and PS2 protein expression levels to enable the direct comparison of PS in exogenous and endogenous expression systems, in HEK-293 PS1 and/or PS2 knockout cells. We show that exogenous expression of Myc-PS1-NTF is 5.5-times higher than Myc-PS2-NTF. Quantitating endogenous PS protein levels, using a novel PS1/2 fusion standard we developed, showed similar results. When the marked difference in PS1 and PS2 protein levels is considered, we show that compared to PS1-γ-secretase, PS2-γ-secretase has equal or more activity on APP and Notch1. This study has implications for understanding the PS1- and PS2-specific contributions to substrate processing, and their potential influence in AD pathogenesis.

KEYWORDS

amyloid-β, APP, Notch1, presenilin-1, presenilin-2, quantitative presenilin expression, γ-Secretase

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aph1, anterior pharynx-defective 1; Aβ, amyloid-β; CTF, C-terminal fragment; en, endogenous; ex, exogenous; Nct, nicastrin; NTF, N-terminal fragment; Pen-2, presenilin enhancer 2; PS-Std, presenilin fusion standard; PS1, presenilin-1; PS2, presenilin-2.

1 | INTRODUCTION

Presenilin (PS) is the catalytic component of γ-secretase, a tetrameric enzyme that cleaves type I transmembrane proteins. The two PS homologues, PS1 and PS2, share approximately 67% amino acid sequence similarity, and form active γ-secretase complexes when incorporated with nicastrin (Nct), anterior pharynx defective-1 (Aph1), and presenilin enhancer-2 (Pen-2).[1] The γ-secretase enzyme has been shown to cleave a large repertoire of substrates,[2] the most well investigated of which are amyloid precursor protein (APP)[3] and Notch1.[4] The cleavage of APP has received the most attention as it ultimately results in the generation of amyloid-β (Aβ) peptides, accumulation of which contributes to Alzheimer's disease (AD) pathogenesis.[5] Consequently, γ-secretase has been proposed as a therapeutic target, with the development of inhibitors of PS-related γ-secretase activity.[6–10] However, these molecules have failed in clinical trials due to off-target effects, which are thought to be caused by the inhibition of substrates other than APP, particularly Notch1,[7,11,12] and may be influenced by differences in affinity for PS1- and PS2-γ-secretase.[13–15] More recently, the focus has shifted to the development of γ-secretase modulators (GSMs), small molecules that modulate the type of Aβ peptides released, while still maintaining cleavage of the intracellular domain of substrates.[16–18] However, there is still a need for improved understanding of γ-secretase activity and insight into the differing roles of PS1- and PS2-γ-secretase enzymes.

γ-secretase cleaves its substrates via a process termed regulated intramembrane proteolysis (RIP), where type I transmembrane proteins undergo multiple cleavages as part of the signaling or degradation processes. The first step in RIP is the shedding of the substrate ectodomain by proteases, in particular ADAM (a disintegrin and metalloproteinase) family enzymes and the aspartyl proteases BACE1 (β-APP cleaving enzyme) and BACE2.[19,20] The second step is performed by γ-secretase, where multiple intramembrane cleavages of the substrate transmembrane domain lead to release of the intracellular domain (ICD) and secreted peptides.[3,21] APP processing can be initiated by either ADAM or BACE cleavage;[22,23] however, it is BACE1 cleavage that initiates the amyloidogenic pathway, leading to the generation of Aβ peptides. γ-secretase is known to successively "trim" APP after the initial cleavage by tri- and tetrapeptide cleavages until the Aβ peptide is released from the luminal membrane.[24,25] It must be acknowledged that much of how APP is cleaved has been determined via investigations of PS1-γ-secretase, with little understanding of whether this process differs for PS2-γ-secretase.

The focus on PS1 appears to be largely a result of the significantly greater number of familial AD causing missense mutations in *PSEN1* (200+) compared to *PSEN2* (20+) (retrieved from www.alzforum.org/mutations September 2022).[26] While both *PSEN1* and *PSEN2* mutations generally cause increased Aβ42:Aβ40 ratios, *PSEN1* mutations have an earlier average age of onset and are typically more aggressive.[27] However, the recent identification of a *PSEN2* variant in the 3′ UTR that mutates a miRNA-binding region suggests PS2 protein expression may influence AD pathology.[28,29] This mutation has been shown to cause upregulated PS2 protein expression and subsequently increased Aβ42:Aβ40 ratio.[29]

While there is considerable functional overlap between PS1- and PS2-γ-secretase, there are several key differences. Subcellular localization has been shown to differ, with PS2-γ-secretase localized to late endosomal and lysosomal compartments,[30–32] while the localization of PS1-γ-secretase predominately resides within the plasma membrane.[30,31] As ectodomain shedding by BACE1 is a prerequisite for Aβ formation, its localization in intracellular organelles, including endosomal compartments,[33] links the PS2-γ-secretase complex to Aβ generation. PS2 has been shown to generate significantly more intracellular Aβ[31,34] and produce a higher Aβ42:Aβ40 ratio,[32,35–37] supporting its greater activity within the endosomal-lysosomal cellular compartment.

One aspect of PS1- and PS2-γ-secretase activity, which is not often considered, is expression within cells and tissues. Evidence derived from post-mortem tissues and in vivo studies suggest that PS expression levels vary with age and other AD-associated changes. Lee et al.[38] show that transcript expression of PS1 is significantly higher than PS2 in human fetal cortex, and that following birth and with age, a concomitant decrease in PS1 and increase in PS2 leads to approximately equal PS1 and PS2 expression. A similar PS expression profile has been observed during terminal differentiation of iPSC-derived neurons, where PS1 expression decreases and PS2 expression increases.[32] Interestingly, PS1 protein expression is decreased in human AD cortex and hippocampus,[39] and in aged murine cortex, there is a concomitant decrease in PS1 and increase in PS2 protein expression.[40] These observations are suggestive of a PS2 role in neuronal maturation and, considered together with the role of PS2-γ-secretase in generating intracellular Aβ and increased Aβ42 product, may indicate that PS2 contributes more to AD pathology than previously credited.

In vitro studies comparing PS1- and PS2-γ-secretase activity have typically shown PS1 complexes to be more active at processing APP and Notch. However, a major

limitation of this work is the assumption that PS1 and PS2 expression levels are equal. A comparable assessment of PS1 and PS2 expression is difficult, as there are no common PS antibodies that detect both PS1 and PS2. To address the inability to assess PS1 and PS2 endogenous expression directly, activity is often determined in cells, where both PS1 and PS2 have been ablated and the PS is exogenously re-introduced to the cell. This presents an opportunity to tag the exogenous PS enabling equi-detection. However, to our knowledge, this approach has only been presented twice; firstly for determining the cellular localization of PS1 vs. PS2,[31] and secondly for use in PS quantitation, after which it was determined that, when PS expression was considered, there was no significant difference in γ-secretase activity.[41] Other studies using the relative levels of mature Nct to normalize for exogenous PS expression have shown discordant results; no difference in APP and Notch ICD generation,[42] or reduced Aβ generation by PS2-γ-secretase.[43] Lastly, the only evidence we are aware of, where endogenous PS expression has been compared, utilized radioactive methionine labeling to correlate PS1 and PS2 antibody detection and showed that in murine blastocyte-derived membranes and cells, PS1-γ-secretase generated more Aβ than PS2-γ-secretase.[44]

Given the observed differences in tissues/cells, it is crucial to resolve the limitations of directly comparing cellular expression of PS1 and PS2 protein units and understand how this relates to γ-secretase activity. In this study, we investigated the activity of PS1 and PS2 in relation to the expression levels of these proteins, with an overarching hypothesis that PS1 does not have greater activity than PS2. We address this hypothesis using two approaches: (1) Myc-tagging of the PS N-terminus to allow for detection of exogenous PS1 and PS2 via the same antibody, and (2) development of a novel PS1/2 fusion standard to enable, for the first-time, absolute quantitative assessment of endogenous PS1 and PS2 protein using specific antibodies. Our results demonstrate that in both the exogenous and endogenous PS expression systems, PS1 and PS2 are not equally expressed, and when PS expression is accounted for, PS2 is at least as active as PS1 at processing APP and Notch in HEK cells.

2 | MATERIALS AND METHODS

2.1 | Mammalian cell culture

All cell lines were cultured in Dulbecco's Modified Eagle Medium (Sigma D5671) supplemented with 1 mM sodium pyruvate (Sigma S8636), 1 mM L-glutamine (Sigma G7513), 100 units/mL penicillin, 0.1 mg/mL streptomycin (Sigma P4333), and 10% v/v fetal bovine serum (Serana FBS-Au-015). Cells were incubated at 37°C with 5% v/v atmospheric carbon dioxide.

2.2 | CRISPR presenilin knockout in HEK-293

To generate HEK PS2+ cells, PS1 was knocked out of HEK-293 cells using presenilin 1 CRISPR/Cas9 KO plasmid in conjunction with presenilin 1 HDR Plasmid from Santa Cruz Biotechnology (sc-401227 and sc-401227-HDR), as per the supplier protocol. Briefly HEK-293 cells were plated in 6-well plates (1.0×10^6 cells/well), 24 h prior to transfection. When cells were approximately 80% confluent, 1.25 μg each of the CRISPR/Cas9 KO and HDR plasmids were transfected into cells, using Lipofectamine 3000 (Invitrogen L3000015) as per the manufacturer's instructions. Cells were then incubated overnight, after which the medium was changed. At 48 h post transfection, cells were sorted for GFP-positive and RFP-positive cells and cultured in puromycin selection medium (0.25 μg/mL). Cells were selected for 8 days with medium replacement every 48 h.

PS2 knockout was completed using the pSp-Cas9-(BB)-2A-GFP vector and methodology previously described by Ran et al.[45] Guide RNA sequences were designed using ChopChop.com.au, and two guide sets were used in combination to generate the PS2KO in the cells. The guide sequences used were 5′GCTCC CCTACGACCCGGAGA3′ and 5′ACGATCATGCACAG AGTGAC3′. 10 μM each of sense and antisense synthesized oligonucleotides with appropriate flanking sequences[45] were phosphorylated using T4 PNK (NEB M0201) in a 10 μL reaction, as per the manufacturer's protocol. The reaction was incubated at 37°C for 30 min, before heating to 95°C for 5 min followed by a temperature ramp of 1°C per min until reaching 25°C to anneal the oligonucleotides. Oligonucleotides were ligated into pSp-Cas9-(BB)-2A-GFP plasmid that had been linearized by digestion with BbsI-HF (NEB R3539) and gel purified (Bioline BIO-52060), as per the supplier protocols. Briefly, 2 μmol of dsDNA guide was ligated into 10 ng of pSp-Cas9-(BB)-2A-GFP using 400 units T4 DNA ligase (NEB M0202) and incubated for 16 h at 16°C. 5 μL of ligation product was transformed into chemically competent *Escherichia coli* XL10 cells and grown overnight on agar plates supplemented with 100 μg/mL ampicillin. Individual colonies were selected and cultured overnight in 5 mL Luria Broth supplemented with 100 μg/mL ampicillin. Plasmids were extracted (Bioline BIO-52057) and correct insertion of the guide RNA sequence was confirmed by sequencing.

To generate HEK-293 PS1+ and HEK-293 PSnull cells, we seeded HEK-293 PS1+PS2+ and HEK-293 PS2+ cells in 6-well plates at 1.0×10^6 cells per well, 24 h prior to transfection. 1.25 μg each of the two guide plasmids were prepared using Lipofectamine 3000 and cells transfected as per the manufacturer's instructions. Cells were incubated for 24 h, after which they were sorted using a BD FACSJazz cell sorter at 1 cell per well into 96-well plates, gated for medium GFP intensity and no/minimal propidium iodide intensity. Monoclonal populations were expanded and screened for PS1 and PS2 protein expression, and selected clones were further screened for substrate processing (Figure S1). One representative clone was selected for subsequent experiments.

2.3 | Plasmid construct generation

All plasmid constructs used for transient transfection of PS and substrate proteins were generated in the backbone vector pIRES2-AcGFP1 (Takarabio). Human PS1 and PS2 cDNA sequence with Myc N-terminal tags, human APP695Swe and human Notch1 (lacking the extracellular domain (21-1713 bp) termed ΔEhNotch1) sequences were cloned into pIRES2-AcGFP1 vector linearized via digestion by restriction enzymes at sites EcoRI and BamHI (PS1, PS2, and ΔEhNotch1) and SalI and XmaI (hAPP695Swe). After sequence confirmation, 50 mL cultures were grown in Luria Broth and plasmids extracted (BioRad 7326120).

2.4 | Whole-genome sequencing and copy number variation analysis

Genomic DNA was extracted from HEK 293 PS1+PS2+, HEK 293 PS1+, HEK 293 PS2+, and HEK 293 PSnull cells using the Monarch Genomic DNA Purification Kit (NEB T3010S) as per manufacturer instructions and eluted in nuclease free H_2O. DNA was assessed for quality and concentration using Nanodrop ND-1000 spectrophotometer and Qubit 4 Fluorometer respectively. 4 μg of genomic DNA from each cell line was sheared to a target size of 8 kb, using g-TUBEs (Covaris 520079) as per manufacturer instructions. Sample concentrations were again measured, and libraries subsequently prepared for Oxford Nanopore Technologies (ONT) sequencing using Native Barcoding Kit 24 V14 (SQK-NBD114.24) as per manufacturer instructions. Briefly, 1 μg of DNA from each cell line was individually end repaired and dA-tailed using NEBNext Ultra II End Repair/dA-Tailing Module (NEB E7546), then, ligated to unique ONT barcodes. Barcoded samples were pooled and purified using AMPure XP beads. Native adapters were ligated, after which DNA was enriched for fragments >3 kb using the long fragment buffer and purified again with AMPure XP beads. The concentration of the resultant library was measured and 20 fmol library prepared in elution buffer. The multiplexed DNA library was loaded onto a PromethION R10.4.1 flow cell (FLO-PRO114M) and run on P2 Solo sequencing device. During the 72-h sequencing run, the flow cell was twice washed and reloaded with the same library to maximize output.

Raw Pod5 files were basecalled and aligned to the GRCh38 reference using Dorado v0.3.1 with the high-accuracy model "dna_r10.4.1_e8.2_400bps_hacv4.1.0" into a single aligned BAM. Demultiplexing was performed using guppy_barcoder with default parameters. The wf-human-variation workflow (https://github.com/epi2me-labs/wf-human-variation) was implemented with genomic bins size set to 10 kb to obtain a copy number variation (CNV) output for each individual barcode. Gene edits were confirmed at target loci by viewing of sequence data using Integrative Genomics Viewer (IGV_2.16.2).[46]

2.5 | Transient transfection

Transient transfection experiments were performed in 6-well plates, with cells seeded at 4.0×10^5 per well. Prior to plating cells, plates were coated overnight with 50 μg/mL poly-L-lysine (Sigma P9155) to improve adherence of PS knockout cell lines. The medium was replaced with antibiotic-free medium 24 h after plating and cells transiently transfected. Lipofectamine 3000 was used for co-transfection of PSnull cells (Figures 2 and 4). PEI Max (Polysciences 24765), using 3 μg PEI per 1 μg DNA, was used for substrate only transfections for investigation of endogenous PS (Figures 6 and 8). Where substrate (pIRES2-hAPP695Swe-AcGFP1 or pIRES2-ΔEhNotch1-AcGFP1) and presenilin (pIRES2-Myc-PS1-AcGFP1 or pIRES2-Myc-PS2-AcGFP1) co-transfection was undertaken in PSnull cells, the vectors were used in a PS:Substrate per unit ratio of 1:3, such that the total amount of DNA transfected was 500 ng. For transfection of substrate only, to investigate endogenous PS activity, the same amount of substrate vector was transfected as per the co-transfection assays. Cells were incubated for 24 h, after which conditioned media and whole-cell lysates were collected. Conditioned media were collected, for all hAPP695Swe transfections, in microfuge tubes, centrifuged at 17 000 *g* for 5 min, the supernatant transferred into a clean tube and snap frozen in liquid nitrogen and stored at −80°C. For lysate collection, the medium was aspirated, and plates washed with cold PBS and aspirated. Cells were scrapped into 100 μL of RIPA lysis buffer (Astral Scientific 786-490) supplemented with protease inhibitor cocktail (Roche 11697498001) and transferred to microfuge tubes.

TABLE 1 qPCR primer sequences.

Gene target	Forward primer	Reverse primer
PSEN1	5′CCAGAGGAAAGGGGAGTAAAACTT3′	5′ACAGGCTATGGTTGTGTTCCA3′
PSEN2	5′TCATCTGCCATGGTGTGGAC3′	5′GTCTTCTTCCATCTCCGGGT3′
APH1a	5′GGTGTTTTTCGGCTGCACTT3′	5′CAGAAAAATGCCCCTGCGAC3′
APH1b	5′CTGCGCCTTCATTGCCTTC3′	5′GAAGAAAGCTCCGGCGATGA3′
NCSTN	5′ACTAGCAGGTTTGTGCAGGG3′	5′TCTGATGAGTGGCGTTGAGC3′
PEN2	5′TGCCTTTTCTCTGGTTGGTCA3′	5′CGCCAGACATAGCCTTTGAT3′
UBC	5′CCGGGATTTGGGTCGCAG3′	5′TCACGAAGATCTGCATTGTCAAG3′
GAPDH[47]	5′CTGCTTTTAACTCTGGTAAAGT3′	5′GCGCCAGCATCGCCCCA3′

Lysate samples were incubated for 1 h at 4°C with rotation and centrifuged at 14 000 *g* for 10 min at 4°C, and the supernatant collected and stored at −20°C.

2.6 | Quantitative PCR

Cells were grown to confluency in 6-well plates and harvested for mRNA extraction. Briefly, plates were washed twice with cold PBS and cells scrapped and collected. Cells were centrifuged at 300 *g* for 5 min, after which supernatant was aspirated. RNA was extracted using ISOLATE II RNA Mini kit as per the manufacturer's instructions (Bioline BIO-52072) and RNA concentration and quality determined by Nanodrop (Thermo Fisher). To generate cDNA using Tetro cDNA synthesis kit (Bioline BIO-65043), 0.5 μg of RNA was used in a 10 μL reaction with a 1:1 ratio of random hexamer and oligo $(dT)_{18}$ primer mix, as per the manufacturer's instructions. Resultant cDNA samples were diluted to a final volume of 100 μL for use in qPCR. GoTaq qPCR master mix (Promega A6001) was used in a final reaction volume of 20 μL. For all genes, diluted cDNA solution (2 μL) was used in 20 μL reactions. Primer details are listed in Table 1 and were designed using NCBI Primer-BLAST,[47] except *GAPDH* primers.[48] Each biological replicate was run in technical triplicate using the Applied Biosystems Viia7 real-time PCR system, and average Ct values determined for each gene. Human *UBC* and *GAPDH* reference genes were used for normalization. Gene expression levels were calculated using the Pfaffl method,[49] and expression relative to PS1+PS2+ cells determined.

2.7 | Immunoblotting

Total protein concentration of cell lysates was determined using micro-BCA kit (Thermo Fisher 23235). Presenilin and APP proteins were separated with 12% v/v acrylamide, tris-tricine gel chemistry. Notch, Pen-2, Aph1a, and Nct proteins were separated with 8%–10% v/v acrylamide, bis-tris gel chemistry (Invitrogen Surecast system). Samples were prepared using either 4x tris-tricine sample buffer (16% w/v SDS, 200 mM tris, 48% v/v glycerol, 0.5% w/v Coomassie G-250) or 4x LDS sample buffer (Thermo Fisher B0007) as appropriate, reducing agent and treatment conditions vary dependent on the protein of interest (see Table 2). Samples were vortexed for 30 s, heated for 10 min (as per temperature in Table 2), centrifuged at 17 000 *g* for 5 min, and then electrophoresed at 100 V for 1 h 45 min. Proteins were transferred to 0.2 μm nitrocellulose membrane (BioRad 1620112), via wet transfer method using tris-glycine buffer (19.2 mM glycine, 2.5 mM tris, 20% v/v methanol) at 150 mA for 16 h at 4°C. Membranes were stained with Ponceau S (1% w/v Ponceau S, 5% v/v acetic acid) for 5 min to assess transfer quality before destaining with boiled TBS (2 mM tris, 1.5 mM NaCl). Membranes were subsequently incubated in blocking buffer, as appropriate for the primary antibody used, for 1 h at room temperature with agitation. Membranes were incubated in primary antibody (all antibody conditions and details are available in Table 2) overnight at 4°C. Membranes were subsequently washed three times in TBS-tween (0.05% v/v tween) for 10 min with agitation. Membranes were then incubated with appropriate secondary antibody, anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibody (Thermo Fisher 31430, 31460), diluted at 1:20 000 in 0.5% w/v non-fat dry milk in TBS-tween, for 1 h at room temperature with agitation. Membranes were then washed again three times in TBS-tween, followed by a 5-min wash with TBS. Membranes were incubated in either Clarity ECL (BioRad 1705061) or Prime ECL (Cytiva GERPN2232) (see Table 2) for 5 min as per the manufacturer's instructions and then imaged on a BioRad ChemiDoc MP system.

2.8 | PS1/2 fusion standard and absolute PS1 and PS2 quantitation

The presenilin fusion standard (PS-Std) (see results section, Figure 5A) was recombinantly generated in *E. coli*

TABLE 2 Antibody conditions for immunoblotting.

Protein target	Antibody	Reducing agent	Treatment temp	Blocking/Antibody diluent[a]	Antibody dilution	ECL
Myc-tag	Myc-Tag 9B11 CST 2276S	None	37°C	3%BSA/3%BSA	1/1000	Clarity
PS1 NTF	PS1 NT1 Biolegend 823401	None	37°C	5%NFDM/0.5%NFDM	1/2000	Clarity
PS1 CTF	PS1 D39D1 CST 5643S	None	37°C	5%NFDM/0.5%NFDM	1/1000	Clarity
PS2 NTF	PS2 Biolegend 814204	DTT	37°C	5%NFDM/0.5%NFDM	1/2000	Prime
PS2 CTF	PS2 EP1515Y Abcam ab51249	DTT	No Heat	5%NFDM/0.5%NFDM	1/20000	Clarity
Aph1a	In-house provided by PE Fraser	DTT	No Heat	5%NFDM/0.5%NFDM	1/500	Prime
Nicastrin	Nicastrin Sigma-Aldrich N1660	BME	70°C	5%NFDM/0.5%NFDM	1/1000	Clarity
Pen-2	Pen-2 Sigma-Aldrich P5622	BME	55°C	5%NFDM/0.5%NFDM	1/500	Clarity
APP-FL & CTF	APP C1/6.1 Biolegned	DTT	75°C	5%NFDM/0.5%NFDM	1/2000	Clarity
ΔEhNotch1	Notch1 Origene TA500078	DTT	75°C	5%NFDM/0.5%NFDM	1/1000	Clarity
NICD	Notch Val1744 CST 4147	DTT	75°C	3%BSA/3%BSA	1/1000	Clarity
GAPDH	GAPDH CST 5174	As per initial sample, blots stripped and reprobed		3%BSA/3%BSA	1/5000	Clarity
GAPDH	GAPDH Abclonal A19056			5%NFDM/0.5%NFDM	1/10000	Clarity

Abbreviations: BME, β-mercaptoethanol [10% v/v]; BSA, bovine serum albumin; DTT, dithiothreitol [50 mM]; NFDM, non-fat dried milk powder.

[a]All blocking and antibody diluent concentrations provided are % w/v.

and purified by GenScript. The final size of the protein including tags is 30.7 kDa, which equates to 5.10×10^{-11} ng per protein unit. In order to use the standard to quantify the number of endogenous PS protein units, a 5- to 6-point standard curve was generated on every PS immunoblot. The actual range used was determined empirically and is dependent on the total protein of sample used, the protein fragment being detected, that is, PS1 or PS2, NTF or CTF, and the specific antibody used. The ng of PS-Std is converted to protein units of PS-Std as follows; protein units PS-Std = [(ng PS-Std) × 90%]/5.10×10^{-11}—note the PS-Std purity was determined to be 90% in quality control report from supplier. This value was then plotted against the corresponding densitometry units quantitated from the immunoblot for the corresponding PS-Std band, to generate a standard curve, using multiple replicates. The standard curve line of best fits equation was used to determine the PS1 or PS2 protein units in the sample. Therefore, the total PS protein units in PS1+PS2+ cells will be the sum of PS1 and PS2 protein units.

2.9 | ELISA

ELISA kits were used to detect Aβ40 (Invitrogen KHB3482) and Aβ42 (Invitrogen KHB3442) in conditioned media as per the manufacturer's instructions. For detection of Aβ40 from conditioned media, endogenous PS activity samples were diluted 1/6, while exogenous PS activity samples were diluted 1/3. No dilution was necessary for detection of Aβ42 in conditioned media.

2.10 | Statistics

All statistical analyses were completed using GraphPad Prism 9.5.0. Three to six experimental replicates were completed for all assays. Statistical significance was determined via unpaired *t*-test, where only two groups were examined. For comparisons of more than two groups, one-way ANOVA or two-way ANOVA, with Holm–Šidák's multiple comparison were used where appropriate.

3 | RESULTS

3.1 | Presenilin knockout cell line generation and characterization

To evaluate and compare the influence of exogenous and endogenous PS1 or PS2 on γ-secretase, presenilin knockout cell lines derived from HEK-293WT (PS1+PS2+) were generated. The cell lines included those lacking PS1 but retaining PS2 expression, lacking PS2 but retaining PS1 expression, or lacking both PS1 and PS2 protein expression. These cell lines were referred to as PS2+, PS1+, and PSnull, respectively. Six to eight monoclonal populations of each cell line were assessed for PS1 and PS2 expression as well as γ-secretase processing of APP and Notch1 substrates (Figure S1). The clone representative of average substrate processing was selected for use in subsequent assays. As genomic copy number variations (CNV) have been observed in genetically altered HEK-293 cells,[50] and as PS1 and PS2

were ablated using different CRISPR vectors, we used low coverage long-read whole-genome sequencing to assess the structural landscape of each of the monoclonal cell lines to be used for subsequent experiments. These sequencing data were also leveraged to confirm that successful gene edits of *PSEN1* or *PSEN2* in the appropriate cell lines occurred at the CRISPR gRNA targeted sites (Figure S2). We found similar CNV architecture between the CRISPR cell lines and their HEK-293WT counterpart (Figure S3), indicating that any observed expression changes were likely the result of the loss of PS1 or PS2 protein expression.

The expression of the components of γ-secretase is regulated by the formation of the enzyme complex.[1,51–53] Therefore, to assess the effect of the absence or presence of PS on the expression of γ-secretase components and to further characterize the cell lines, expression of PS1, PS2, Aph1, Pen-2, and Nct was assessed at transcript (Figure 1A) and protein levels (Figure 1B–F). As expected, compared to the wildtype, *PSEN1* and *PSEN2* mRNA expression is absent or markedly reduced in cells, where the specific PS has been ablated. In the single PS knockout cell lines, an increase in mRNA expression was observed for the alternate homologue, indicating that loss of one PS homologue is causing a compensatory increase in transcript expression of the alternate homologue. For all other components of γ-secretase, mRNA expression was significantly reduced in the PS2+ and PSnull cell lines, while no significant changes were observed in the PS1+ cell line.

PS1 and PS2 protein expression (Figure 1B,C,E) is commensurate with observed mRNA expression. No PS1 or PS2 protein was detected in cells, where the respective PS had been knocked out. Increases in PS1 and PS2 protein were observed in the PS1+ and PS2+ cell lines respectively, in line with the observed mRNA results. Aph1a protein levels similarly aligned with the mRNA results (Figure 1D,E). However, Pen-2 protein levels differed from the mRNA expression profile, with significant reduction in protein levels evident in both the PS1+ and PS2+ cell lines, and no protein detected in PSnull cells (Figure 1D,E).

In all cell lines except for PSnull, Nct was detected as two protein bands, representing immature and mature protein (Figure 1D,F). In the PSnull cells, Nct was detected as immature protein only, as has been previously reported in double knockout cells[36,42] and consistent with the requirement of presenilin for maturation of Nct.[53] Notably, mature vs. immature Nct levels vary in a PS-dependent manner, consistent with previous reports.[31,36] Where PS1+PS2+ cells have approximately 4 times more mature Nct, PS2+ cells have approximately equal levels of mature and immature Nct, and PS1+ cells have approximately 4 times more immature Nct levels.

3.2 | Exogenous PS expression highlights difference in PS levels and subsequently higher PS2 activity

To directly compare exogenously expressed PS1- and PS2-γ-secretase processing of hAPP695Swe and hNotch1, PS1 and PS2 constructs N-terminally tagged with Myc were transfected into PSnull cells. N-terminal tagging has been previously used for exogenous PS expression,[31,41] while C-terminal tagging would be unsuitable, as this region interacts with the Aph1 component of γ-secretase.[54] Exogenous, Myc-tagged, PS1 (exPS1), or PS2 (exPS2) was co-transfected with either hAPP695Swe or ΔEhNotch1 at a ratio of 1:3 (PS:Substrate). The amount of exPS used in the transfections was titrated to reduce the amount of unincorporated full-length protein, while retaining maximum PS-NTF levels (Figure S4).

APP processing was assessed via immunoblotting (Figure 2A) of whole-cell lysates APP full length (APP-FL) and APP C-terminal fragment (APP-CTF) levels were quantitated and expressed as the ratio of APP-CTF/APP-FL as an initial indicator of γ-secretase activity. APP-CTF protein accumulation was significantly reduced with the co-expression of either exPS1 or exPS2 compared to control; notably, APP-CTF accumulation was 2.0-fold lower with exPS2 compared to exPS1 (Figure 2B). In contrast, the Aβ40 and Aβ42 levels in conditioned media detected by ELISA were significantly higher when hAPP695Swe was co-expressed with exPS1 (Figure 2C,D).

It was observed that Myc-PS2 NTF expression levels were dramatically lower than Myc-PS1 NTF expression, consequently the total protein loaded had to be adjusted for appropriate detection of Myc-PS NTF, and 3-times more protein was loaded for exPS2 cell lysates (Figure 2E). Normalized quantitation of the Myc-PS-NTF showed that in the absence of hAPP695Swe expression, exPS1-NTF expression was 5.5-fold higher than exPS2-NTF. In the presence of hAPP695Swe, exPS1-NTF expression significantly increased, and exPS2-NTF expression trended toward an increase (Figure 2F). Having directly quantitated exPS1- and exPS2-NTF expression levels by detecting the Myc-tag, we normalized the hAPP695Swe substrate processing products to determine the specific contributions of exPS1- and exPS2-γ-secretase. Consequently, the normalized results showed that exPS1-γ-secretase accumulated 8.1-fold more APP-CTF than exPS2-γ-secretase (Figure 2G). Additionally, after normalization for the levels of exPS1-NTF and exPS2-NTF there were no significant differences in Aβ40 or Aβ42 levels observed between exPS1- and exPS2-γ-secretase (Figure 2H,I). Schematic representation of these results is presented in Figure 3. Furthermore, no significant difference in the Aβ42:Aβ40

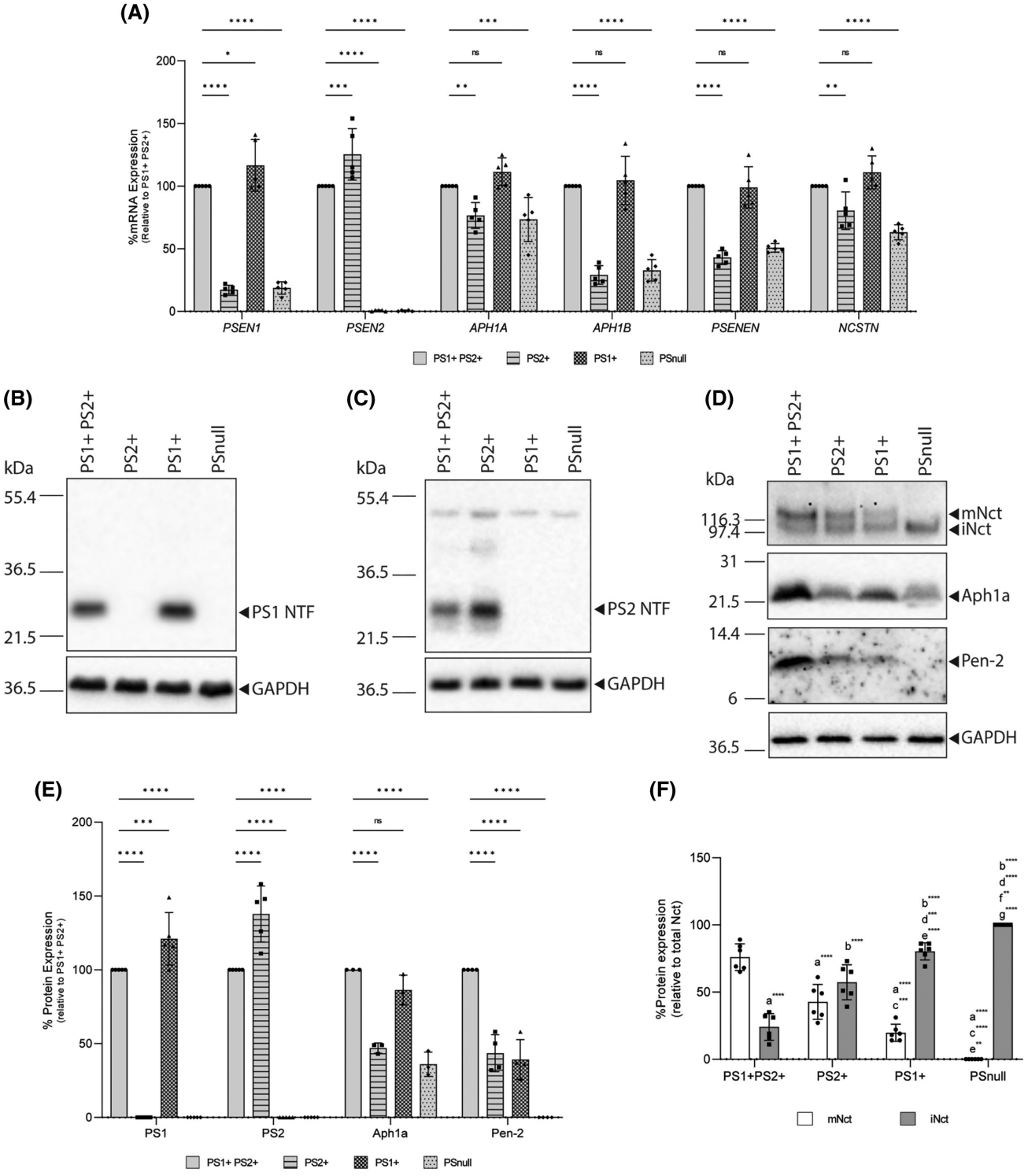

FIGURE 1 Characterization of HEK-293 PS1+PS2+, PS1+, PS2+, and PSnull cell lines. Cell lines were generated by CRISPR-Cas9 knockout of PSEN1 and/or PSEN2 from HEK-293 cell lines. Representative clone of each cell line selected for further analysis by mRNA expression of γ-secretase subunits PS1, PS2, Aph1a, Aph1b, Pen-2, and Nct and presented relative to PS1+PS2+ cell line expression (A). Whole-cell lysates were analyzed by immunoblot for detection of PS1 protein (B) PS2 protein (C) and Aph1a, Pen-2, and Nct proteins (D). Protein expression levels were quantitated by densitometry analysis and are presented relative to PS1+PS2+ cell line expression for PS1, PS2, Aph1a, and Pen-2 (E). Both mature Nct (mNct) and immature Nct (iNct) were quantitated and the percentage of each relative to total Nct calculated (F). Values shown are mean ± SD of $n = 3–6$ independent experiments. Two-way ANOVA with Holm–Šidák's multiple comparison was completed for (A, E, F). For (F) a = significantly different to mNct PS1+PS2+, b = significantly different to iNct PS1+PS2+, c = significantly different to mNct PS2+, d = significantly different to iNct PS2+, e = significantly different to mNct PS1+, f = significantly different to iNct PS1+, g = significantly different to mNct PSnull. For all quantitated data; $^{ns}p > .05$, $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$.

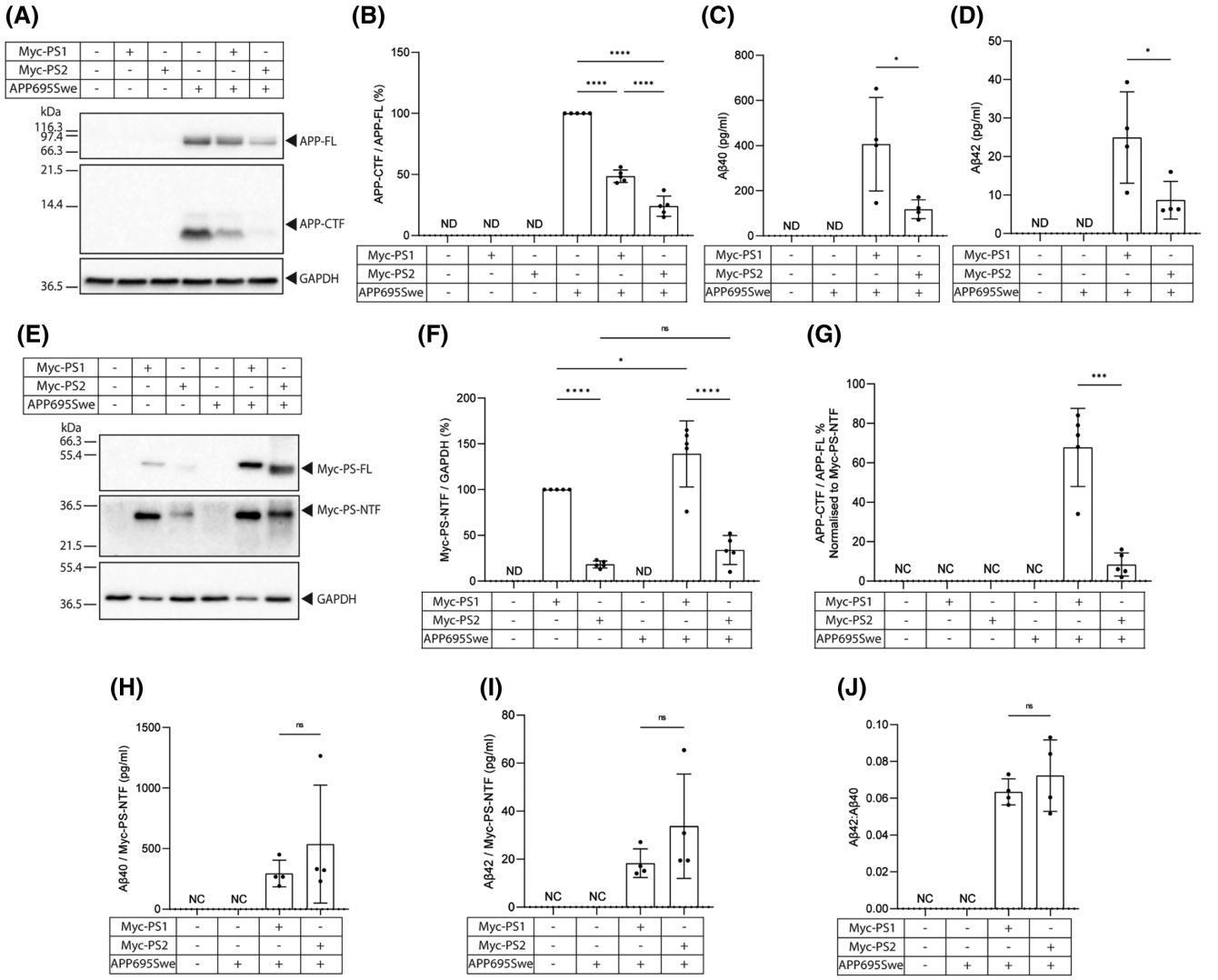

FIGURE 2 APP processing by exogenous Myc-tagged PS1 and PS2 in PSnull cells. Both hAPP695Swe and Myc-tagged PS were transiently co-expressed in PSnull cells to assess APP processing and directly compare PS1 and PS2 expression to enable the effect of variable expression to be considered. Whole-cell lysates were assessed via immunoblotting to determine APP-FL and APP-CTF protein levels (A) and the accumulation of APP-CTF/APP-FL quantitatively determined by densitometry assessment and presented relative to PSnull cells transfected with hAPP695Swe only (B). Conditioned media was collected concurrently with whole-cell lysates for analysis of Aβ40 (C) and Aβ42 (D) levels (pg/mL) by ELISA. Exogenous PS1 and PS2 expression was directly compared by immunoblotting using antibody directed against the Myc-tagged N-terminus of exPS1 and exPS2 (E). Note that to enable simultaneous detection of exPS1 and exPS2 the total protein loaded was adjusted, for exPS1 transfected lysates 10 μg total protein was loaded, while for exPS2 transfected lysates 30 μg of protein was loaded. Myc-PS-NTF levels were quantitated by densitometry analysis and normalized for GAPDH to account for the different amounts of total protein loaded between exPS1 and exPS2 samples (F). APP-CTF, Aβ40, and Aβ42 were subsequently normalized for Myc-PS-NTF levels to account for variable PS1 and PS2 expression (G–I). Aβ42:Aβ40 ratio was calculated (J). Values shown are mean ± SD of $n = 4–5$ independent experiments. Statistical tests applied were unpaired *t*-test for (C, D, G–J) and ordinary one-way ANOVA with Holm–Šidák's multiple comparison for (B, F) where $^{ns}p > .05$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

ratios between exPS1 and exPS2 co-expression systems was evident (Figure 2J).

The effect of presenilin expression on Notch1 cleavage was similarly examined. The Notch1 ICD (NICD), which directly reflects γ-secretase activity, and ΔEhNotch1 proteins were detected via immunoblotting of whole-cell lysates, from exPS1 or exPS2 co-transfection with ΔEhNotch1. The NICD/ΔEhNotch1 ratio was assessed, initially without consideration of exPS-NTF expression. As expected, no NICD product was detected in the absence of PS expression, and exPS1 co-transfection led to a 2.7-fold higher NICD generation than exPS2 co-transfection (Figure 4A,B). Myc-PS-NTF levels in the absence or presence of ΔEhNotch1 expression were quantitated and used to normalize the NICD levels (Figure 4). In the absence of substrate overexpression, 4.5-fold more exPS1-NTF was

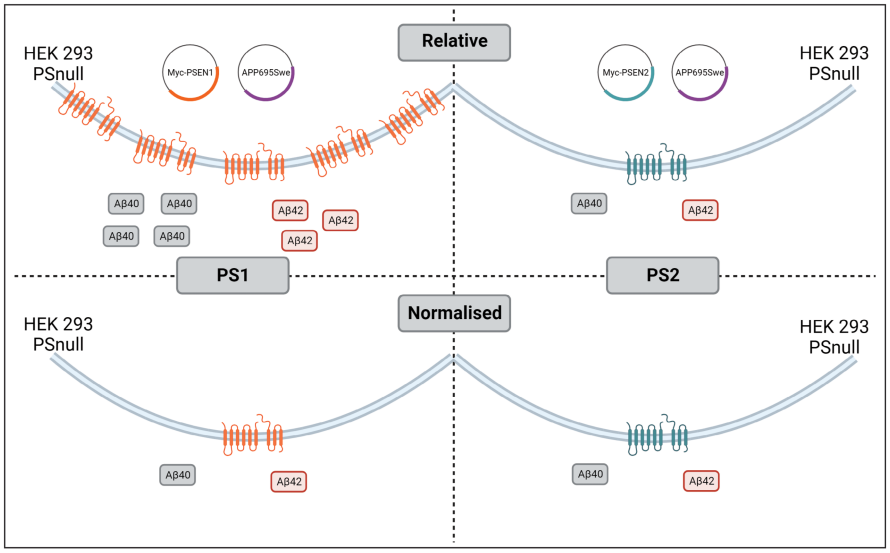

FIGURE 3 Effect of exogenous PS normalization on Aβ generation. Exogenous expression of PS1-NTF and PS2-NTF was directly compared using the Myc-tag and the relative exPS1:exPS2 expression ratio was calculated to be 5.5:1. Subsequently the Aβ40 and Aβ42 levels were normalized to determine the amount of Aβ generated by a single PS1- or PS2-γ-secretase enzyme. Consequently, the levels of Aβ generated by an individual exPS1-γ-secretase or exPS2-γ-secretase enzyme were not significantly different. Note Aβ40 and Aβ42 changes are represented relatively, and are not a direct comparison, for absolute Aβ40 and Aβ42 levels refer to Figure 2. Created with BioRender.com.

expressed than exPS2-NTF (Figure 4C,D). ΔEhNotch1 co-expression with exPS1 increased Myc-PS1-NTF levels, while no change was observed in Myc-PS2-NTF levels with co-expression (Figure 4C,D). These results indicate that exPS1 but not exPS2 is upregulated when Notch substrate is expressed. Subsequent normalization of ΔEhNotch1 processing with the Myc-PS expression data, to account for the different expression levels, showed that exPS2-γ-secretase generated 2.8-fold more NICD exPS1-γ-secretase (Figure 4E).

Overall, the findings indicate that exogenous expression of PS1 and PS2 does not lead to comparable expression of the γ-secretase incorporated Myc-PS-NTF proteins. Interestingly, when the difference in expression was accounted for, exogenously expressed PS2 was more active than PS1 at cleaving hAPP695Swe and ΔEhNotch1.

3.3 | PS1/2 fusion standard: A method for absolute quantitation of endogenous PS1 and PS2

Recognizing that significantly more PS1 was expressed than PS2 in an exogenous system, we sought to understand whether this PS expression profile (and effects on γ-secretase activity) was an artifact or recapitulated the endogenous expression profile. The ability to quantitatively compare endogenous PS1 (enPS1) and PS2 (enPS2) expression levels remains a challenge, as PS1 and PS2 are detected by different antibodies, with no commercially available antibody able to detect both homologues. To facilitate this, we designed a presenilin fusion standard (PS-Std). The PS-Std incorporates residues from the N-terminal sequence and the cytoplasmic loop of human PS1 and PS2. These regions are hydrophilic, non-transmembrane regions that contain the epitopes for several commercially available antibodies (Figure 5A and Table S1).

PS1 and PS2 antibodies that detect either the N-terminal fragment (NTF) or C-terminal fragment (CTF) were used to probe for the PS-Std via immunoblot (Figure 5). All antibodies detected a single band on a tris-tricine gel under denatured conditions across the mass range of PS-Std used. The theoretical size of the PS-Std is 30.7 kDa; however, the standard migrates at approximately 37 kDa, likely due to it being relatively acidic (pI = 4.15).[55,56] When probed with unrelated antibodies, anti-GAPDH and anti-GSK3β, no bands were detected in the PS-std samples (Figure S5), confirming that the PS-std contains PS epitopes specific to PS1 and PS2 antibodies. Having validated antibody detection of the standard, we set out to quantitate endogenous PS expression levels in the HEK presenilin knockout cell lines generated.

To quantitatively assess expression, varying amounts of the PS-Std underwent SDS-PAGE along with whole-cell lysate samples from the cell lines and were subsequently detected via immunoblotting using PS1-NTF, PS1-CTF, PS2-NTF, and PS2-CTF antibodies (Figure 5B–E). The densitometry results for the PS-Std were used to generate standard curves for each antibody and set of immunoblot replicates. Due to protein size differences between

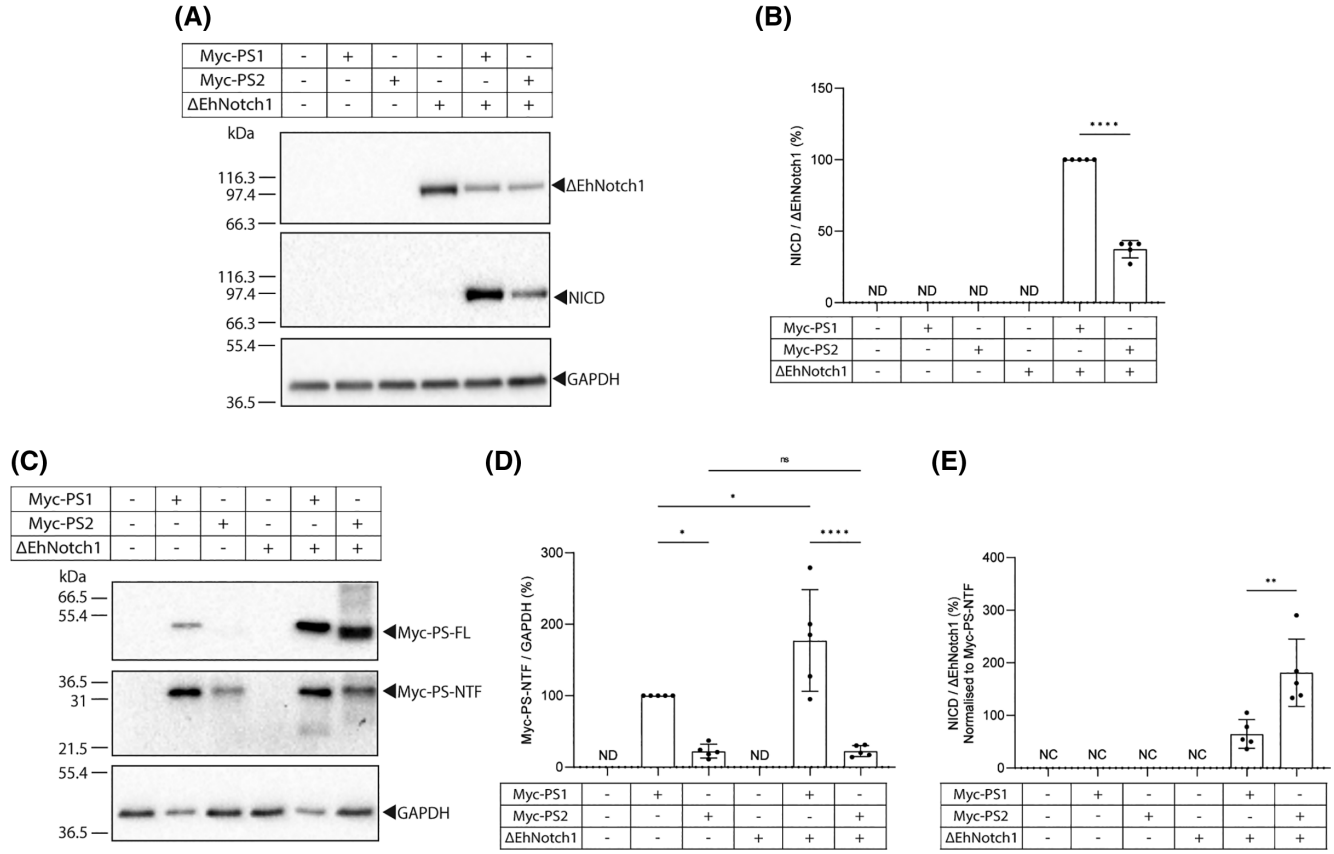

FIGURE 4 Notch1 processing by exogenous Myc-tagged PS1 and PS2 in PSnull cells. Both ΔEhNotch1 and Myc-tagged PS were transiently co-expressed in PSnull cells to assess Notch1 processing and directly compare PS1 and PS2 expression to enable the effect of variable expression to be considered. Whole-cell lysates were assessed via immunoblotting to determine ΔEhNotch1 and NICD protein levels (A) and the level of NICD/ΔEhNotch1 quantitatively determined by densitometry assessment and presented relative to exPS1 transfection with ΔEhNotch1 (B). Exogenous PS1 and PS2 expression was directly compared by immunoblotting using an antibody directed against the Myc-tagged N-terminus of exPS1 and exPS2 (C). Note that to enable simultaneous detection of exPS1 and exPS2 the total protein loaded was adjusted, for exPS1 transfected lysates 10 μg total protein was loaded, while for exPS2 transfected lysates 30 μg of protein was loaded. Myc-PS-NTF levels were quantitated by densitometry analysis and normalized for GAPDH to account for the different amounts of total protein loaded between exPS1 and exPS2 samples (D). NICD was subsequently normalized for Myc-PS-NTF levels to account for variable PS1 and PS2 expression (E). Values shown are mean ± SD of $n = 5$ independent experiments. Statistical tests applied were unpaired *t*-test for (B, E) and ordinary one-way ANOVA with Holm–Šidák's multiple comparison for (D) where $^{ns}p > .05$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

the PS-Std, and the NTF and CTF of PS1 and PS2, we did not simply determine the equivalent mass of standard, but rather, determined the number of PS protein units. This was achieved by calculating the number of PS-Std units of protein per ng of PS-Std (given one PS-Std unit is 5.10×10^{-11} ng) and plotting against the corresponding densitometry values (Figure S6). The equations from the resultant standard curves were used to convert the densitometry results to the number of PS1 or PS2 protein units. This value was subsequently normalized for total protein loaded on the PAGE, to determine the PS protein units per μg total protein.

Importantly, no significant differences were observed in any of the cell lines between the expression levels of the NTF and CTF proteins for either PS1 or PS2 (Figure 5F). This result further supports the use of the PS-Std for quantitation, as γ-secretase is known to contain components in a stoichiometric ratio of 1:1:1:1,[57] and PS NTF and CTF fragments are tightly regulated at a 1:1 ratio.[58] We calculated the difference in PS1 and PS2 expression, and determined that in the wildtype (PS1+PS2+ cells), PS1 expression was 5.2-fold higher than PS2 expression, which closely aligns with the exogenous expression profile determined above (see results section *Exogenous PS expression highlights difference in PS levels and subsequently higher PS2 activity*), reflecting that PS expression is tightly regulated at a homologue-specific level.

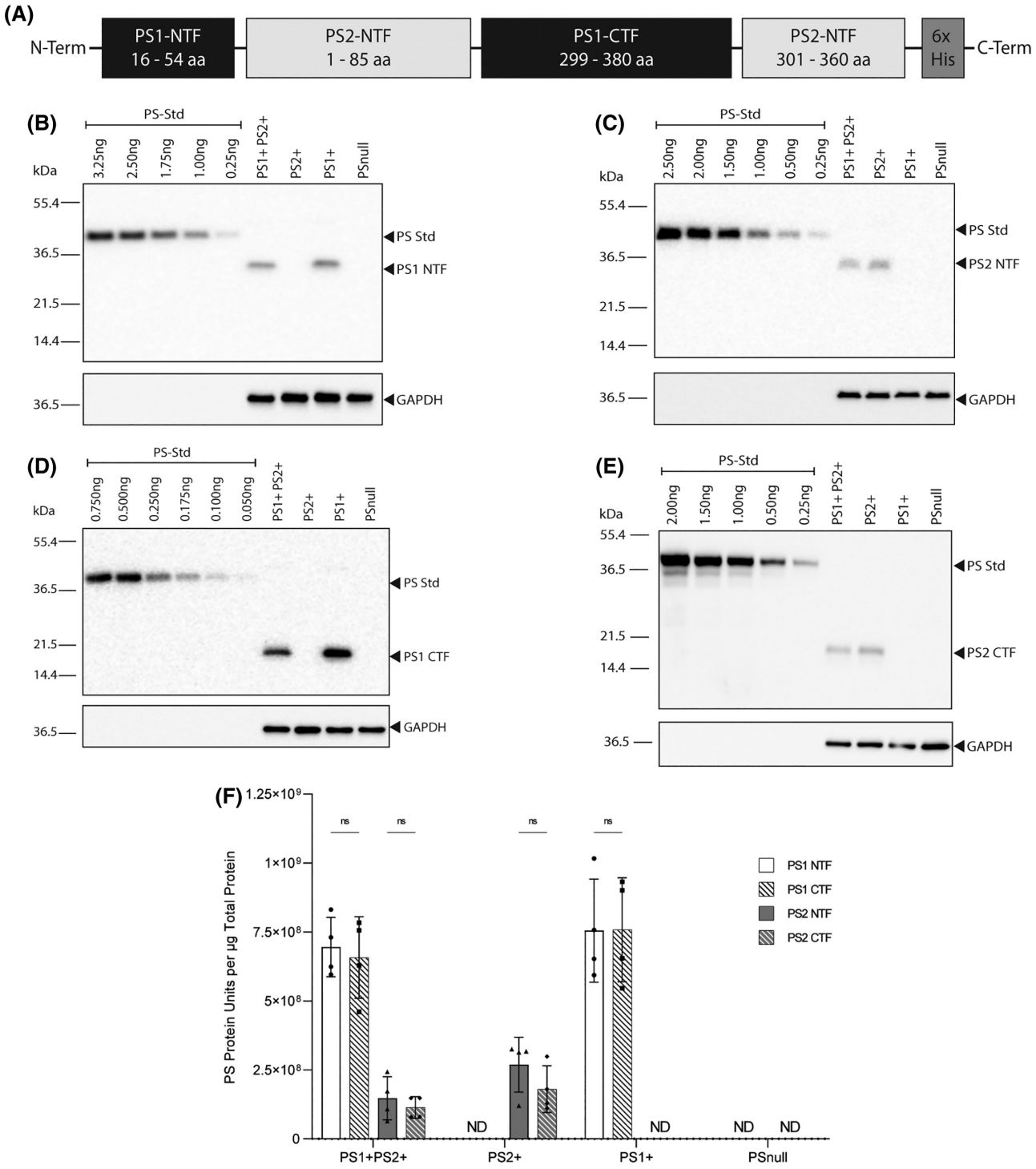

FIGURE 5 Validation of novel method to directly compare endogenous PS1 and PS2 expression. No commercially available antibody detects both PS1 and PS2. Thus, to enable direct quantitation of endogenous PS1 and PS2 expression, we developed a synthetic PS1/2 fusion protein (PS-Std) containing multiple epitope regions for several commercially available antibodies for use as a standard to enable comparative quantitation. The PS-Std contains protein sequences for the N-terminus and the cytoplasmic loop regions of PS1 and PS2 as shown schematically (A). A range of amounts of the PS-Std were immunoblotted alongside whole-cell lysates from the PS1+PS2+, PS1+, PS2+, and PSnull cells to generate a standard curve using the same experimental conditions and probed with antibodies directed against the PS1 NTF (B), PS2 NTF (C), PS1 CTF (D), and PS2 CTF (E). Immunoblot bands underwent densitometry assessment for quantitation and the PS-Std densitometry results were used to generate a standard curve for each replicate set of immunoblots relative to the number of PS-Std protein units $[(\text{ng PS-STD}) \times 90\%]/5.10 \times 10^{-11}$ (Figure S6). The resultant standard curve was used to calculate the amount of PS protein units detected in the whole-cell lysate samples for each cell line (F). Values shown are mean ± SD of $n = 4$ independent experiments and analyzed using two-way ANOVA with Holm–Šidák's multiple comparison, where $^{ns}p > .05$.

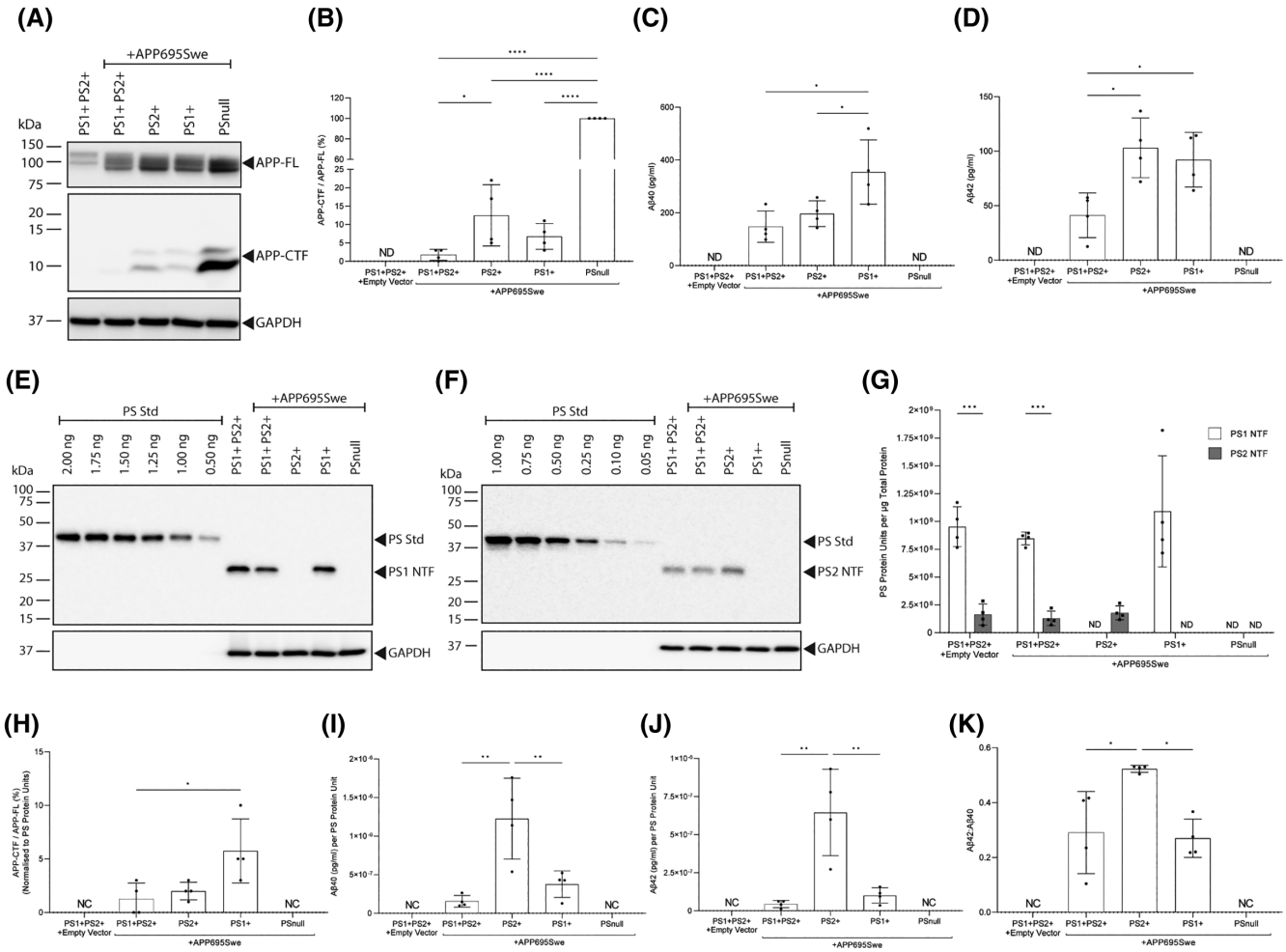

FIGURE 6 APP processing by endogenous PS1 and PS2 assessed in PS1+ and PS2+ cells. hAPP695Swe was transiently expressed in PS1+PS2+, PS1+, PS2+, and PSnull cells to assess APP processing by endogenous PS1 and PS2 and our novel PS-Std used to quantitatively determine PS1 and PS2 expression to enable the effect of variable expression to be considered. Whole-cell lysates were assessed via immunoblot to determine APP-FL and APP-CTF protein levels (A) and the accumulation of APP-CTF/APP-FL quantitatively determined by densitometry assessment and presented relative to PSnull cells (B). Conditioned media was collected concurrently with whole-cell lysates for analysis of Aβ40 (C) and Aβ42 (D) levels (pg/mL) by ELISA. Endogenous PS1 and PS2 expression was determined by immunoblotting using antibodies directed against the PS1 NTF and PS2 NTF (E, F). PS1 NTF and PS2 NTF levels were quantitated by densitometry analysis and the PS protein units determined using standard curves generated alongside the whole-cell lysates (G). APP-CTF, Aβ40, and Aβ42 were subsequently normalized for PS protein units to account for variable PS1 and PS2 expression (H–J). Aβ42:Aβ40 ratio was calculated (K). Values shown are mean ± SD of $n = 4$ independent experiments. Statistical tests applied were ordinary one-way ANOVA with Holm–Šidák's multiple comparison (B–D, H–K) and two-way ANOVA with Holm–Šidák's multiple comparison for (G) where $^{ns}p > .05$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

3.4 | Endogenous PS2 demonstrates greater activity in cleaving APP and equivalent activity in cleaving Notch

Having validated the method for quantitating endogenous PS protein levels, we next sought to investigate γ-secretase substrate processing by endogenous PS1- and PS2-γ-secretase in the HEK presenilin knockout cell lines. hAPP-695Swe was transiently transfected into the cell lines, and whole-cell lysates were harvested for immunoblotting 24 h after transfection; additionally, conditioned media was collected for the determination of Aβ generation.

APP-FL and APP-CTF levels were detected via immunoblotting and quantitated (Figure 6A,B). Levels of overexpressed APP-FL were noticeably variable between the cell lines (particularly in the absence of PS1), despite equal levels of GAPDH loading control. Consequently, APP-CTF accumulation was represented as a ratio of APP-FL, to measure PS-specific γ-secretase activity. In the PSnull cells, considerable APP-CTF accumulated as a result of lack of γ-secretase activity, due to the absence of PS. Comparatively, less than 2% APP-CTF was detected in PS1+PS2+ cells. While the accumulation of APP-CTF protein in both the PS2+ and PS1+ cells was significantly

less than the PSnull cells, significantly higher APP-CTF levels were detected in PS2+ cells compared with the PS1+PS2+ cells. Conditioned medium samples collected from hAPP695Swe transfections were analyzed via ELISA. Aβ40 levels are significantly higher in the PS1+ cells compared to both the PS1+PS2+ and PS2+ cells, whereas both PS1+ and PS2+ cells generate increased Aβ42 compared to PS1+PS2+ cells (Figure 6C,D). These results are indicative of PS2-γ-secretase preferentially initiating the Aβ42 generation pathway, consequently leading to a higher Aβ42:Aβ40 ratio (Figure 6K).

Endogenous PS1 and PS2 levels were detected via immunoblotting and quantitated against the PS-Std to determine PS protein units (Figure 6E–G). The expression level of enPS1 was greater than enPS2 in PS1+PS2+ cells (5.9-fold higher). When hAPP695swe was overexpressed in the PS1+PS2+ cells, enPS1 was 6.5-fold higher than enPS2. No significant difference was observed in the level of either enPS1 or enPS2 in the absence of the alternate PS homologue. We next assessed substrate processing at a per PS unit level to allow for a more accurate assessment of γ-secretase activity. Prior to normalization, it was observed that more APP-CTF accumulated in PS2+ cells, compared to PS1+ cells, which is indicative of less processing (Figure 6B). Normalizing for PS units reveals that, when the lower levels of PS2 were considered, PS2+ cells show less APP-CTF compared to PS1+ cells, indicative of increased processing (Figure 5H). Taken together with the results of Aβ40 and Aβ42 (Figure 6I,J), these findings suggest that PS2γ-secretase is more active at processing hAPP695swe (Figure 6I,J), and is schematically presented in Figure 7.

Furthermore, endogenous PS processing of Notch1 was investigated by transiently transfecting cell lines with the ΔEhNotch1 vector and collecting whole-cell lysates after 24 h. NICD levels and ΔEhNotch1 levels were detected via immunoblotting, and the NICD/ΔEhNotch1 levels determined (Figure 8A,B). As expected, no NICD was detected in either the PS1+PS2+ cells transfected with the vector control or in the PSnull cells. Prior to any consideration of PS expression levels, there was no significant difference observed between the levels of NICD generated by PS1+PS2+ and PS1+ cells. The PS2+ cells, however, generated 3.0-fold less NICD than either the PS1+PS2+ or PS1+ cells. On quantitating PS expression levels, significantly higher expression of enPS1 than enPS2 expression (4.8-fold higher enPS1) was observed in PS1+PS2+ cells (Figure 8C–E). No significant differences between enPS1 or enPS2 levels were observed in either PS1+ or PS2+ cells, compared with PS1+PS2+ cells. Subsequently, after normalizing NICD generation for the PS protein units expressed, no difference was observed between any cell lines (Figure 8F).

4 | DISCUSSION

The study of the specific contributions of PS1 and PS2 proteins to γ-secretase substrate processing, be it using endogenous PS or exogenous overexpression of PS, typically does not consider PS1 vs. PS2 expression levels. While only a handful of studies have directly compared presenilin expression, showing higher PS1 expression than PS2,[41,44] there is evidence that PS2 expression increases with age,[38,40] is associated with ADAD[29] and increases in response to mutant PS1 expression,[37] suggesting the importance of PS2 in AD pathogenesis. The current study has developed and applied the use of new methods for the direct quantitation of both exogenous and endogenous PS expression. Our findings highlight the importance of considering PS expression when interpreting γ-secretase activity. We show that in HEK-293WT (PS1+PS2+) cells, there is 5.2-times more PS1 than PS2 expression, possibly as a result of the embryonic origin of this cell line.[38] Importantly, we identify that this expression profile is retained when exogenous PS1 and PS2 are expressed after PS ablation. Finally, we have demonstrated that when PS expression is considered, PS2-γ-secretase processes at least equal amounts, if not more, APP and Notch1 than PS1-γ-secretase, depending upon the experimental system in use.

Our novel method for quantitating endogenous PS demonstrates that in HEK-293 cells, PS1 expression is significantly higher than PS2. Interestingly, the endogenous PS1:PS2 profile is maintained when PS is exogenously expressed. This contrasts with the generally accepted rationale that ectopic gene expression using a constitutive promoter, such as CMV used in this study, will result in comparable protein levels of homologous proteins in the same cell lines. The differential PS1 and PS2 expression is likely the effect of post-translational protein regulation influenced by PS-specific localization[30,31,59] and the requisite involvement of other proteins for stable protein retention, namely Nct, Aph1, and Pen-2.[1,51–53,60–62] The transfection of exogenous PS into cells has shown that PS holoprotein is quickly degraded, while the endoproteolysed PS heterodimer fragments, by virtue of being incorporated into γ-secretase, are more stable.[63] γ-secretase incorporation of exogenously expressed PS is limited by the normal cellular regulation of the other complex components, and it appears from our data that the innate cellular regulation of PS1 and PS2 is likely driven by the specific PS homologues. Equal ectopic expression of PS1- and PS2-γ-secretase could presumably be achieved by simultaneous overexpression of all γ-secretase components, such as that employed by Meckler and Checler.[30] However, the extent to which PS expression is regulated by subcellular localization, and organelle compartment size, remains unclear, and may be potentiated by the use of a variety of cell models.

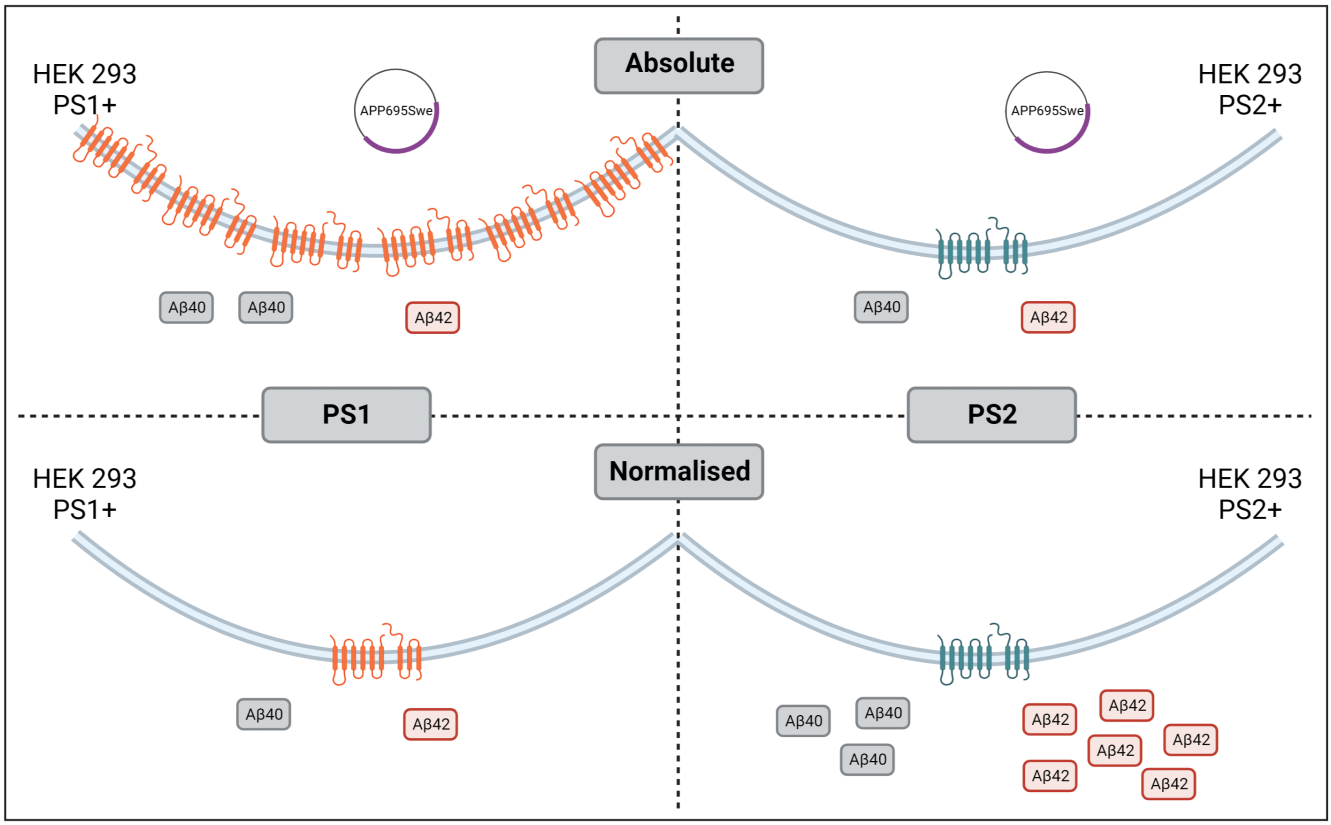

FIGURE 7 Effect of endogenous PS normalization on Aβ generation. Absolute levels of endogenous PS1-NTF and PS2-NTF were determined using the PS-Std, enabling direct comparison of enPS1 and enPS2 levels. This facilitated normalization of the Aβ40 and Aβ42 levels produced from enPS1-γ-secretase or enPS2-γ-secretase. Consequently, the levels of Aβ generated by an individual enPS2-γ-secretase enzyme are significantly higher than that generated by an enPS1-γ-secretase enzyme. Notably, this result does not align with the exogenous PS system, demonstrating that exPS does not recapitulate the endogenous system. Note Aβ40 and Aβ42 changes are represented relatively, and are not a direct comparison, for absolute Aβ40 and Aβ42 levels refer to Figure 6. Created with BioRender.com.

Despite similar PS1:PS2 ratios, we show that there is variation in APP processing between the exogenous (ex) and endogenous (en) PS expression systems, used in this study, prior to normalizing for PS expression. A notable example is the difference in APP-CTF levels observed between expression systems (Figures 2 and 6). Similarly, differences in the Aβ40 and Aβ42 levels generated between exogenous and endogenous systems result in different Aβ42:Aβ40 ratios between PS1 and PS2. Specifically, we observed that enPS2 generated a higher Aβ42:Aβ40 ratio, driven by lower Aβ40 production, compared with enPS1, while no difference was observed between exogenous PS. Our results for Notch1 processing, however, are recapitulated between the exogenous and endogenous system prior to normalization of PS expression. There is no consensus in the literature regarding the Aβ42:Aβ40 ratio[30–32,34,36,37] or NICD[21,31,34,64] generation by PS1- vs PS2-γ-secretase, which is likely the result of the variety of expression systems, cell types, and activity assays used. However, the differences observed between enPS and exPS processing of APP in this study may be the result of exogenous PS overexpression leading to alterations in the normal subcellular distribution,[31] causing an increase in cell surface PS2 expression. Interestingly PS1 and PS2 localization does not affect NICD production but does influence Aβ generation.[31] Thus, the differences observed in APP processing in this study may be a function of altered PS localization, although further experiments are required to confirm this. An additional difference we observed between experimental systems was that exPS1-NTF levels significantly increase in response to co-expression with either APP or Notch1, while exPS2-NTF levels do not differ. This is not observed in the endogenous PS system and is further evidence that exogenous PS is not a faithful mimic of endogenous PS. The use of endogenous PS systems reduces the potential introduction of experimental artifacts and should be the preferred system, particularly given the current availability of CRISPR-Cas9 technology for cell line development.

Similar to other recent studies, we used CRISPR-Cas9 technology to develop HEK-293 cell lines that retain enPS1 or enPS2, allowing us to study PS-specific γ-secretase function. While these recent studies use a variety of cell lines,[31,32,36] the most comparable cell line to those used

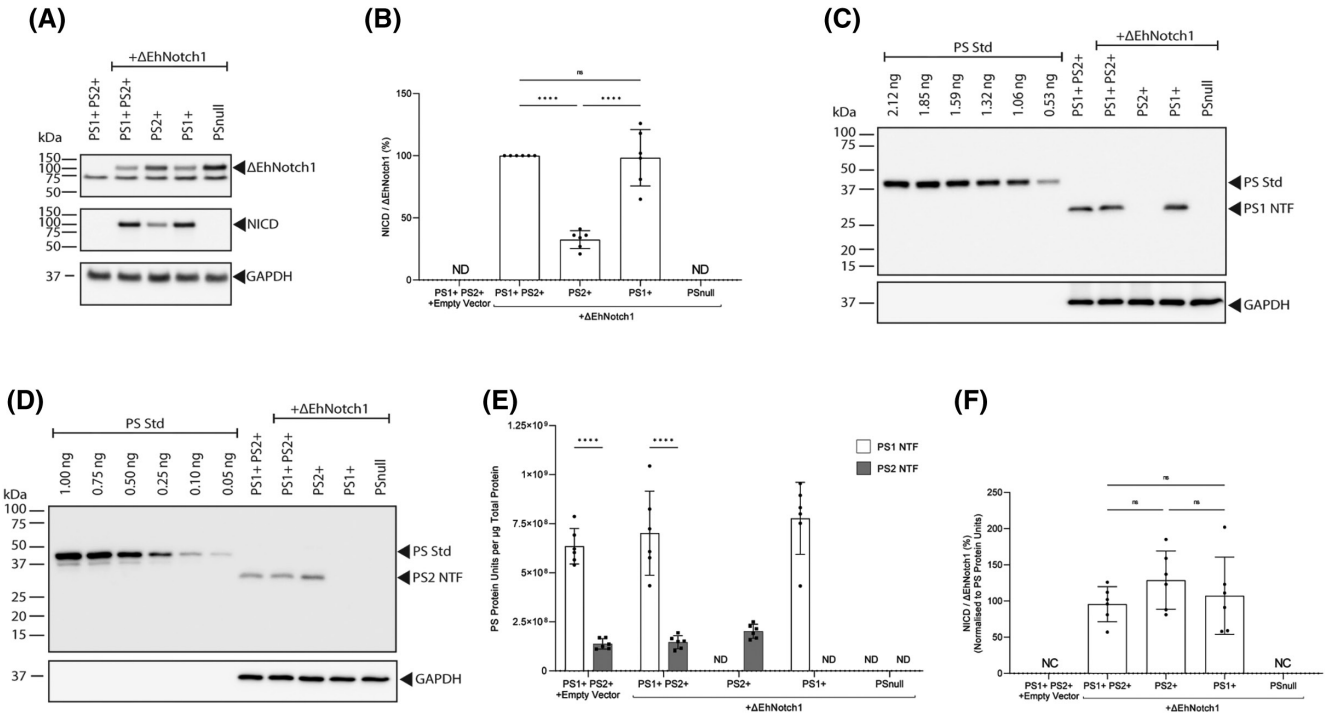

FIGURE 8 Notch1 processing by endogenous PS1 and PS2 assessed in PS1+ and PS2+ cells. ΔEhNotch1 was transiently expressed in PS1+PS2+, PS1+, PS2+, and PSnull cells to assess Notch1 processing by endogenous PS1 and PS2 and our novel PS-Std used to quantitatively determine PS1 and PS2 expression to enable the effect of variable expression to be considered. Whole-cell lysates were assessed via immunoblot to determine ΔEhNotch1 and NICD protein levels (A) and the generation of NICD/ΔEhNotch1 quantitatively determined by densitometry assessment and presented relative to PS1+PS2+ cells (B). Endogenous PS1 and PS2 expression was determined by immunoblotting using antibodies directed against the PS1 NTF and PS2 NTF (C, D). PS1 NTF and PS2 NTF levels were quantitated by densitometry analysis and the PS protein units determined using standard curves generated alongside the whole-cell lysates (E). NICD was subsequently normalized for PS protein units to account for variable PS1 and PS2 expression (F). Values shown are mean ± SD of $n = 6$ independent experiments. Statistical tests applied were ordinary one-way ANOVA with Holm–Šidák's multiple comparison (B, F) and two-way ANOVA with Holm–Šidák's multiple comparison for (E) where $^{ns}p > .05$, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

in this study is the HEK-293T cells developed by Lessard et al.[34] These authors present both intracellular and extracellular levels of total Aβ, Aβ40, and Aβ42 using the HEK-293T cell line, and show that PS2-expressing cells generate significantly more intracellular total Aβ, Aβ40, and Aβ42.[34] While we successfully measured extracellular Aβ, several attempts to detect intracellular Aβ were unsuccessful. We found that the amount of exogenous hAPP695Swe required for detection of intracellular Aβ by the ELISA assay used caused considerable cell death in the enPS1+ cells (Figure S7). This may be due to the use of the HEK-293 cell line in this study rather than HEK-293T used by Lessard et al. While we similarly show no significant difference in the absolute amount of secreted Aβ40 and Aβ42 between the PS1+ and PS2+ cell lines, we did show a significant increase in the Aβ42:Aβ40 ratio associated with enPS2 expression, due to accompanying reduction in Aβ40 generation. These findings are consistent with results reported in murine N2a cells lacking PS1[36] and iPSC cells, where PS1 and/or PS2 were conditionally knocked out and differentiated into neurons.[32] Watanabe et al.[32] further showed that during extended neuronal maturation of iPSCs, there is a concomitant decrease in PS1 expression and increase in PS2 expression. Combined with our findings, these studies highlight the importance of PS2 in AD pathogenesis and the need to directly compare PS1 and PS2 expression to enable appropriate interpretation of PS-specific γ-secretase contributions across multiple experimental systems.

There have only been a handful of studies that consider PS expression, or mature Nct as a measure of γ-secretase expression. Yonemura and colleagues[41] used Myc-tagged exogenous PS in a yeast system, and demonstrated that PS1-NTF levels are approximately 28-times higher than PS2, concluding that, after normalizing for expression, there was no difference in overall activity. Lai et al.[44] used radioactive labeling to determine PS1- and PS2-specific antibody sensitivity in order to calculate endogenous PS expression levels; in doing so, they identified that PS1 expression is approximately 1.4-times higher than PS2 in a murine blastocyte model. While Lai et al. ultimately concluded that PS1 generates more Aβ than PS2,

they interestingly observed that while PS1 is similarly active in both membrane-enriched cell-free γ-secretase assays and in-cell assays, PS2 is significantly less active in the membrane-based assay compared with the in-cell assay—an additional confounding feature to consider when interpreting the many γ-secretase activity studies. The use of mature Nct as a measure of active γ-secretase has also been used to normalize for activity between PS1 and PS2.[42,43] These studies conclude that PS1-γ-secretase generates more Aβ,[42,43] and similar levels of AICD and NICD[42]; however, the use of cell-free membrane-based assays,[43] and poor evidence of Nct maturation,[42] potentially confound interpretation. To our knowledge, our findings provide the first reported absolute quantitative measure of endogenous presenilin expression and demonstrate that PS2-γ-secretase processes equal amounts of Notch1 and more APP, compared to PS1-γ-secretase, when considered at an enzymatic unit level.

It should be acknowledged that our findings in HEK-293 cells cannot be generalized to other cell types, which may show different presenilin expression profiles related to their developmental origin and function. Additionally, it must be noted that substrate-specific localization will likely affect the accessibility of PS1- and PS2-γ-secretase to certain substrate pools, influencing overall substrate processing capability.[31,32] Nonetheless, we have developed useful tools that allow us to address this in future studies. Another potential limitation of this study is the use of incorporated PS expression as a measure of γ-secretase levels, which does not consider evidence suggesting that there is a pool of in-active γ-secretase. This evidence is from pulldown studies using modified γ-secretase inhibitors to capture "active" γ-secretase complexes.[37,65,66] The L685,458 inhibitor used as the basis for these studies does not, however, have equal affinity for PS1 and PS2.[13,15] Additionally, these experiments were performed using membrane extractions, which reduce PS2—but not PS1 activity,[44] potentially resulting in biased capture of PS1 complexes. Future experiments should consider the use of the BMS708163-derived γ-secretase capture tools,[67,68] which has comparable affinity for both PS1 and PS2.[6] Additionally, these experiments should be developed in cell-based systems for accurate reflection of the active γ-secretase pool and can be used in conjunction with the PS quantitation method developed in this study for a more robust understanding of PS1 and PS2 specific γ-secretase expression and activity.

With the recognition that both PS1 expression and PS2 expression are important in the context of AD pathogenesis,[29,39] that PS1 and PS2 are differentially expressed throughout development,[38,40] and vary between cell types,[31,41,44] the field must look to methods that enable direct comparison of PS1 and PS2 expression when interpreting data. We acknowledge investigating individual endogenous PS in the absence of the homologous counterpart is not reflective of the physiological environment, and may belie the realities of the dynamic interplay between PS1 and PS2 for the other components of γ-secretase.[37,69,70] However, we believe that coupling the use of an endogenous PS model, with a method to quantitate PS expression, presents a suitably representative experimental system to assess γ-secretase activity and the specific contributions of PS1 and PS2. To achieve this, we have developed and validated a PS1/2 fusion standard that, when used in conjunction with appropriate standard curves, calculates the number of PS protein units. This represents a novel approach to the absolute quantitation of presenilin levels in a cellular system that can be extended to tissues. Furthermore, we have demonstrated that endogenous PS expression is recapitulated in an exogenous expression system. While the resultant substrate processing is not in complete agreement, we have shown that PS2-γ-secretase is at least as active, if not more, than PS1-γ-secretase at processing both APP and Notch substrates.

There is a growing body of evidence that suggests that PS2 has greater implications for Aβ-related pathogenesis in AD than previously considered. In particular, PS2-γ-secretase generates higher Aβ42:Aβ40 ratios shown in this study and others,[32,36,37] and more intracellular Aβ.[31,34] Furthermore, PS2 expression increases with neuronal maturation,[32,71] age,[38,40] in response to PS1 mutations,[37] and a rare autosomal dominant AD mutation.[28,29] We have presented tools here that enable accurate, direct comparison between PS1 and PS2 expression, and demonstrate how these can be used to improve our understanding and interpretation of the effect of PS expression on γ-secretase activity.

AUTHOR CONTRIBUTIONS

Melissa K. Eccles and Giuseppe Verdile conceived and designed the research. Melissa K. Eccles, Nathan Main, Rodrigo Carlessi, Miheer Sabale, and Brigid Roberts-Mok performed experiments and acquired the data. Melissa K. Eccles, Paul E. Fraser, and Giuseppe Verdile analyzed and interpreted data. All authors were involved in manuscript preparation.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the results and/or supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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