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A Cellular Model of Amyloid Precursor Protein Processing and Amyloid- β Peptide Production

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Abstract

Background—A hallmark pathologic feature of Alzheimer's disease (AD) is accumulation of neuritic senile plaques in the brain parenchyma. Neurotoxic plaque cores are composed predominantly of amyloid- β (A β) peptides of 40 and 42 amino acids in length, formed by sequential cleavage of amyloid precursor protein (APP) by β -, and γ -secretases. There is great interest in approaches to modulate A β peptide production and develop therapeutic interventions to reduce A β levels to halt or slow the progression of neurodegeneration.

New Method—We characterized and present the BE(2)-M17 human neuroblastoma cell line as a novel *in vitro* model of the APP-cleavage cascade to support future 1) functional studies of molecular regulators in A β production, and 2) high-throughput screening assays of new pharmacotherapeutics.

Results—In BE(2)-M17 cells, both RNA (i.e., RT-PCR, RNA Sequencing) and protein analyses (i.e., Western blots, ELISA), show endogenous expression of critical components of the amyloidogenic pathway, APP-cleavage intermediates CTF83 and CTF99, and final cleavage products A β 40 and A β 42. We further report effects of retinoic acid-mediated differentiation on morphology and gene expression in this cell line.

Comparison with Existing Method(s)—In contrast to primary isolates or other cell lines reported in current literature, BE(2)-M17 not only sustains baseline expression of the full contingent of APP-processing components, but also remains stably adherent during culture, facilitating experimental manipulations.

Conclusions—Our evidence supports the use of BE(2)-M17 as a novel, human, cell-based model of the APP processing pathway that offers a potential streamlined approach to dissect molecular functions of endogenous regulatory pathways, and perform mechanistic studies to identify modulators of A β production.

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Keywords

Alzheimer's; BE(2)-M17; amyloid-beta; *in vitro*; secretase; TNF

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder and its incidence in the global population continues to rise as average life expectancy increases. Despite efforts to develop effective medical and lifestyle interventions, the etiology of AD remains elusive (Mucke, 2009) and there are currently no preventive or curative treatments available to AD patients.

Neuropathological hallmarks of AD include the extracellular brain deposits of amyloid- β peptide (A β) aggregates forming senile plaques (Hampel et al., 2010; Selkoe, 1997), and the intracellular neurofibrillary tangles of hyperphosphorylated tau protein (Iqbal et al., 2010). Additionally, underlying metabolic disorders and neuroinflammatory conditions accompany many neurodegenerative diseases such as AD, Parkinson's, multiple sclerosis, and amyotrophic lateral sclerosis (Glass et al., 2010; Sutinen et al., 2012). In the case of AD, excessive accumulation of nonfibrillar, soluble A β oligomers and tangles, elevated levels of neuroinflammatory cytokines (e.g., tumor necrosis factor- α (TNF α), interleukin-1 (IL-1), IL-6, and IL-18), and activated microglia and astrocytes are considered to contribute to the pathologic processes sustaining synaptic dysfunction, neuronal death, and the progressive cognitive decline.

Neurons are a major source of A β peptides, with A β 42 and A β 40 isoforms comprising the main components of the dense-core senile plaques in the AD brain parenchyma (Gouras et al., 1998). A β peptides are produced by cleavage of the type I transmembrane amyloid precursor protein (APP) in a stepwise process mediated by β - and γ -secretases (Chow et al., 2010; Kandalepas and Vassar, 2012). β -secretase provides the first cleavage/commitment event in the amyloidogenic pathway (Dislich and Lichtenthaler, 2012; Vassar et al., 2009) that defines the N-terminus of A β peptides. The resulting carboxy-terminal fragment of APP, a membrane-associated 99 aa carboxy-terminal fragment, CTF99, is then a direct substrate for γ -secretase transmembrane cleavage, consequently releasing A β peptide isoforms of 38–43 aa residues in length (Zhang et al., 2012). Alternatively, an α -secretase-mediated cleavage of APP can occur within the precursor protein's A β peptide sequence, precluding A β production (Kuhn et al., 2010). Proteolysis of APP or the CTF99 intermediate (Flammang et al., 2012) by α -secretase generates a carboxy-terminal fragment, CTF83, that is subsequently cleaved by γ -secretase to yield a non-amyloidogenic, 23–25 aa peptide called P3.

Based on the amyloid cascade hypothesis and the central role ascribed to A β deposition in AD brain pathology (Masters and Selkoe, 2012), designs for effectual drug modulation of β - or γ -secretase, two enzymes involved in A β peptide production, remain promising therapeutic strategies for improving the clinical outcome of AD (DeStrooper et al., 2010). The major β -secretase in the brain is β -site APP-cleaving enzyme-1 (BACE1). And while many BACE1 and γ -secretase inhibitor compounds have been developed, the field lacks a singularly robust cellular model expressing high levels of endogenous APP and processing components for mechanistic screening purposes.

Neuroblastoma is the most common extracranial solid tumor of infancy and in childhood. Short-term cultures of neuroblastoma tumors were initially used for clinical diagnoses, but long-term *in vitro* culture of the cells soon became experimentally significant, due in large

part to their susceptibility to differentiation (Thiele, 1998). The capacity for neuroblastoma cells to be differentiated to more neuron-like phenotypes by retinoic acid (RA) treatment (Lee et al., 2009) has been intensively investigated for anti-cancer therapeutic options (Oppenheimer et al., 2007) and for establishing improved experimental models for neurobiological studies.

The human neuroblastoma cell line used in this study, BE(2)-M17, hereafter also referred to as M17, and its associated RA-induced morphological and functional characterizations from neuroblastic to more mature neuron-like phenotype have been reported (Andres et al., 2013). M17 cells are multipotential and have been assessed, with and without RA-treatment, for expression of neuronal markers and genes including corticotrophin, tyrosine hydroxylase (Ciccarone et al., 1989), acetylcholinesterase, and dopamine- β -hydroxylase. In this study, we report our characterization of BE(2)-M17 cells for expression of amyloidogenic genes involved in production of the A β peptide isoforms associated with AD brain pathology, and uniquely examined whether the RA-induced effects alter M17 expression of genes associated with the APP-processing pathway.

1. Materials and methods

1.1 Culture of Human Neuroblastoma BE(2)-M17 Cells

BE(2)-M17 human neuroblastoma cells were purchased as a frozen aliquot from American Type Culture Collection (ATCC) (Manassas, VA). They are described as an adherent neuroblastic (N-type) subclone isolated by limiting dilution of the SK-N-BE(2) parental cell line (Crestini et al., 2006; Fuso et al., 2005; Korner et al., 1994) which is a mixed adherent and suspension neuroblastoma cell line established from a marrow biopsy of a 2 year old male. BE(2)-M17 cells were grown in plastic tissue culture dishes (Greiner Bio-One, Frickenhausen, Germany) and maintained in a 1:1 mixture of Eagle's Minimum Essential Medium with L-Glutamine (ATCC, Manassas, VA) and Ham's F12 Medium (Corning Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C, in a 5% CO₂ and humidified atmosphere. M17 cells have a reported saturation density of greater than 10⁶ cells/cm². For differentiation assays, cells at 60% confluence received 10 μ M all-trans-retinoic acid (RA) (Sigma-Aldrich, St. Louis, MO) in growth medium. The 10 μ M RA-medium was replaced every 24 hrs for 4 days. Cultured in parallel, untreated M17 controls were fed fresh growth medium every 24 hrs. Phase contrast microscopic images of cell monolayers were captured by Infinity Analyze, Version 5.0 software (Lumenera, Ottawa, Ontario, Canada). BE(2)-M17 for our studies were subcultured using split ratios of 1:30 or greater, for no more than 20 continuous passages, at which time a low passage, liquid nitrogen stored aliquot of cells was seeded to fresh culture dishes.

1.2 RNA Isolation and cDNA Preparation

For total RNA isolation, M17 cells were grown in 10 cm dishes (55 cm²/dish) or 6-well plates (9.4 cm²/well) to approximately 85% confluence prior to harvest. The adherent cell layers were washed with cold 1 $\times$ Hanks' Balanced Salt Solution (HBSS) without Ca²⁺, Mg²⁺, and phenol red (Corning Cellgro, Manassas, VA), then lysed directly in the tissue culture dish using the Absolutely RNA Miniprep Kit (Agilent Technologies, La Jolla, CA). Subsequent RNA isolation was performed as recommended by the manufacturer. Total RNA yields and RNA Integrity Numbers (RIN) were measured by Agilent RNA 6000 Nano Kit chip analysis (Agilent Technologies, Waldbronn, Germany). cDNA was synthesized in a 20 μ l reaction volume including 2.0 μ g of total RNA, 500 nM dNTP mix (Finnzyme, Thermo Scientific, Pittsburgh PA), 25 ng/ μ l anchored Oligo(dT)₂₀ (Invitrogen, Life Technologies, Grand Island, NY), 5 units ANTI-RNase (Ambion, Life Technologies, Grand Island, NY),

and 50 units/ μ l M-MuLV Reverse Transcriptase (RNase H⁻) (New England Biolabs, Ipswich, MA). After incubation at 42°C for 90 min, each cDNA sample was adjusted, based on starting RNA concentration, to 20 ng/ μ l with ddH₂O.

1.3 Endpoint Reverse Transcriptase PCR (RT-PCR)

Conventional RT-PCR was performed in a BioRad T100 thermal cycler (Hercules, CA). Primer pairs were purchased from OriGene Technologies (Rockville, MD) for human APP, BACE1, TNF α , TNF receptor-1 (TNFR1), TNFR2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. GAPDH was included as an endogenous housekeeping gene control. Reactions included 10 ng cDNA template, 400 nM primers and Choice Taq Blue DNA Polymerase Mastermix (Denville Scientific, South Plainfield, NJ) in a 25 μ l volume. Cycling conditions were: 95°C, 10 min; 42 cycles of (95°C, 15 sec; 60°C, 10 sec); 72°C, 5 min. Amplicons were electrophoretically separated through 2% agarose/0.5 $\times$ Tris-Borate-EDTA (TBE) gels, visualized with Molecular Probes SYBR Safe dye (Invitrogen, Carlsbad, CA) and gel images captured using a Multimager III and AlphaView Software, Version 3.1.1.0 (Alpha Innotech, Santa Clara, CA). Molecular size markers, 100 bp DNA Ladder (New England Biolabs, Ipswich, MA), were loaded adjacent to sample lanes on each agarose gel.

1.4 Real Time Quantitative RT-PCR

Quantitative RT-PCR (*qRT-PCR*) was performed using a Stratagene MX3000P real-time PCR system with analysis software MxPro Version 4.0.1, and SYBR GreenER qPCR SuperMix Universal (Stratagene, Agilent Technologies, La Jolla CA). The OriGene primers, listed in Subsection 1.3 above and used here, are validated by the manufacturer for SYBR Green based qPCR experiments and used at 250 nM in 20 μ l reaction volumes. Optimized reaction conditions included 10 ng cDNA for APP, BACE1 and GAPDH PCR and 20 ng cDNA for TNFR1, TNF α , and TNFR2 PCR. “No Template” control reactions replaced cDNA sample volume with ddH₂O and confirmed the specificity of predicted qRT-PCR amplicons and absence of DNA contamination. Technical replicates were measured for all samples. An additional level of correction was introduced to minimize interassay variation by including a passive fluorescent reference dye, 50 nM ROX, to normalize potential well-to-well differences. The calibrator control for this study was untreated M17, and GAPDH served as the normalizer reference control. GAPDH-normalized gene expression was presented in the relative quantity charts of Figure 3C as fold-change by the ratio RA/M17. The calibrator control ratio M17/M17 was therefore defined as 1.0. In the comparative quantitation chart data, error bars are derived from threshold cycles, fluorescence measurements at each cycle, and software-based estimates of imprecision of the normalizer. Cycling conditions used: 50°C, 2 min; 95°C, 10 min; 40 cycles of (95°C, 15 sec; 60°C, 60 sec); followed by melting curve analysis: 95°C, 1 min; 60°C, 30 sec; 95°C, 30 sec.

1.5 RNA Sequencing and Analysis

RNA sequencing was performed using 1.0 μ g of total RNA quantified via Nanodrop (Thermo Scientific, Pittsburgh, PA). A sequencing library was prepared with Illumina's Truseq RNA Sample Preparation Kit v2 (Illumina Inc, San Diego, CA) following the manufacturer's protocol. In brief, poly-A containing mRNA molecules were purified using poly-T oligo attached magnetic beads. The mRNA was then thermally fragmented and converted to double-stranded cDNA. The cDNA fragments were end-repaired, a single ‘A’ nucleotide was incorporated, sequencing adapters were ligated, and fragments were enriched with 15 cycles of PCR. Final PCR-enriched fragments were validated on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and quantified by qPCR using Kapa's Library Quantification Kit (Kapa Biosystems, Woburn, MA) on the 7900HT (Applied Biosystems,

Foster City, CA). The final library was sequenced by 50 bp paired-end sequenced on a HiSeq2000 (Illumina, San Diego, CA).

Raw reads passing Illumina quality filters were converted to FASTQ format in Phred33 scale with CASAVA 1.8.3. RNA-Seq reads were aligned with TopHat (v2.0.8) (Trapnell et al., 2009) which first utilizes Bowtie (v2.1.0.0) (Langmead and Salzberg, 2012) to map reads with ‘splice-aware’ alignments to the Homo Sapiens build GRCh37 from Ensembl (Flicek et al., 2012). To estimate the library fragment size for TopHat we initially mapped a subset of 1 million reads with bwa (v0.6.1) to the human genome, followed by picard version 1.80 (“<http://picard.sourceforge.net>,” n.d.) module CollectInsertSizeMetrics and provide these values to TopHat options `--mate-inner-dist 87 --mate-std-dev 86`. Additional TopHat flags utilized were --transcriptome-index (to Ensembl GRCh37.70), --no-coverage-search, --b2-sensitive and --keep-fastq-order. Next, we calculated gene expression values expressed as fragments per kilobase pair of exon per million fragments mapped (FPKM) using cufflinks version 2.1.1 (Trapnell et al., 2012). We used the --GTF option in cufflinks to annotate to human gene models GRCh37.70. Additionally we used the --multi-read-correct and --frag-bias-correct options in cufflinks and masked tRNAs, rRNAs and mtRNAs as suggested in the cufflinks documentation.

1.6 Western Blots

For protein lysate preparation for immunoblot assays, M17 cells were harvested in exponential growth phase at approximately 85% confluence in 10 cm dishes. Spent medium was discarded and adherent cells washed with cold 1× HBSS. Cells were overlaid with fresh, cold 1× HBSS and collected using a rubber policeman scraper. Collected cells were pelleted at 4°C by a 5 min centrifugation step at 1,500×g. Supernatants were discarded and the cells lysed by resuspension in one packed cell volume of lysis buffer (1× Phosphate Buffered Saline/1% TritonX100/0.1% SDS/0.05% NP40) supplemented with the Complete Mini proteinase inhibitor cocktail (Roche, Nutley, NJ), then incubated for 30 min on ice and stored at −80°C. Samples were thawed on ice and following centrifugation at 14,000×g at 4°C for 120 min, clarified supernatants were quantitated using the colorimetric DC Protein Assay (BioRad, Hercules, CA). For reference material, human frontal lobe tissue from Alzheimer’s disease (AD) or non-demented (ND) brains were processed as reported recently (Decourt et al., 2013). After electrophoretic separation of either 25, 50, or 100 µg of protein lysate/lane through Tris-Glycine 9% or 12% SDS-PAGE gels, proteins were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes, 0.45 µm (EMD Millipore, Billerica, MA). Every gel included prestained Precision Plus molecular weight standards (BioRad, Hercules, CA). Membranes were blocked with 5% (w/v) non-fat milk in Tris-Buffered Saline (TBS) for 1 hr at room temperature (RT), and primary antibodies were diluted in TBS/0.05% Tween20 plus 0.1% to 5% non-fat milk for overnight incubation at 4°C. Horseradish peroxidase (HRP)-conjugated, species-specific secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas) were diluted in 0.1% milk/TBS/0.05% Tween20/0.01% SDS and incubated for 1 hr at RT. Membranes were washed 3 times between antibody steps using TBS, followed by 3 washes in TBS/0.1% Tween20. The antibodies used in this study were: mouse anti-Aβ, 6E10 (Covance, Princeton, New Jersey); rabbit anti-BACE1 N-terminus (46–62), #B0681 (Sigma-Aldrich, St. Louis, MO); mouse anti-BACE1 C-terminus, #MAB5308 (EMD Millipore, Billerica, MA); rabbit anti-TNFR1, #MAB3736 (Cell Signaling Technologies, Danvers, MA); and mouse anti-β-actin #A1978 (Sigma-Aldrich, St. Louis, MO). For the anti-Presenilin 1 (PSEN1) Western blot, we used the same procedure and lysates as above with the following modifications: 18% acrylamide gel, blocking and antibody buffer containing bovine serum albumin instead of milk, and detection with the Odyssey system (Li-Cor) as described previously (Decourt et al., 2009).

The monoclonal antibody used was rabbit anti-PSEN1, #5643 (Cell Signaling, Danvers, MA).

For immunoblot detection of the APP-cleavage intermediates, CTF99 and CTF83, 50 µg of M17 protein lysate was separated on 12% Tris-Tricine gels and transferred to Immobilon-PSQ PVDF, 0.2 µm, membranes. PSQ membranes were boiled for 3 min in TBS then blocked in 5% non-fat milk dissolved in TBS/0.05% Tween20 for 2 hrs at RT, shaking. Mouse anti-APP C-terminus, SIG39152 (Covance, Princeton, New Jersey) was diluted in 1% non-fat milk/TBS/0.05% Tween20 for overnight incubation at 4°C, and secondary antibody goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Dallas, Texas) was diluted in 1% non-fat milk/TBS/0.05% Tween20/0.1% SDS and incubated for 1 hr at RT. Antibody-specific staining of proteins on the Western blots was detected by enhanced chemiluminescence (ECL) (EMD Millipore, Billerica, MA) and exposure to X-ray films (Research Products International Corp., Mount Prospect, IL).

1.7 Aβ Enzyme Linked-Immuno-Sorbent Assays and β-Secretase Activity Assay

Human Aβ42 and Aβ40 production was quantified for M17 cells by solid phase sandwich Enzyme Linked-Immuno-Sorbent Assays (ELISAs) using Novex ELISA kits (Invitrogen Corporation, Camarillo, CA). Conditioned media (CM) were collected from independent passages of confluent M17 cells grown in 10 cm tissue culture dishes either 48 hrs or 120 hrs after final feeding, quick-frozen on dry ice, then stored at -80°C until assay. Thawed CM samples were heated at 50°C for 10 min and clarified for 10 min at 10,000×g. ELISAs were performed as recommended by the manufacturer. Samples were measured in quadruplicate and the Aβ42 and Aβ40 assays repeated in independent experiments. The Aβ42 and Aβ40 ELISA kits were manufacturer tested to have no cross-reactivity with each other, 7 alternative Aβ variants, or APP (50–250 ng/ml).

To demonstrate M17 cells possess β-secretase cleavage capability, we carried out a β-secretase activity assay in two independent experiments, performed as recommended by the kit manufacturer (#ab65357, Abcam, Burlingame, CA). The BACE1 inhibitor provided in the kit was also used on the cell lysate to determine to which extent BACE1 contributes to the substrate consumption. For statistical analysis, we used the Conover-Inman post-hoc method which is Fisher's least significant difference method performed on ranks.

2. Results

2.1 BE(2)-M17 cells and baseline expression of AD-associated mRNAs and proteins

A phase contrast photomicrograph shows the BE(2)-M17 cells as they appear in culture, growing as adherent multilayering, neuroblastic cells with occasional short neuritic processes (Fig. 1A). To evaluate M17 cells as a potential model for APP processing, we assessed the expression pattern for several AD-related genes-of-interest by reverse transcriptase-PCR (RT-PCR) and corresponding proteins by Western blot. APP, BACE1, and presenilin 1 (PSEN1) genes were selected as representing the substrate and enzymes, respectively, necessary for the amyloidogenic pathway, and TNFα, TNFR1, and TNFR2 genes were examined as mediators of inflammation that can influence Aβ production (He et al., 2007). GAPDH was evaluated as an endogenous control to be utilized in subsequent quantitative measurements. Conventional endpoint RT-PCR analysis of M17 mRNA revealed relatively high expression levels of APP, BACE1, and GAPDH mRNAs, as well as the presence of TNFα, TNFR1, and TNFR2 mRNAs (Fig. 1B). To validate the mRNA expression, we used Western blots to confirm the expression of APP, BACE1, PSEN1, and TNFR1 proteins in M17 cells (Fig. 1C). Included on the immunoblots were positive reference controls, human brain homogenates prepared from Alzheimer's (AD) and non-

demented (ND) brains (Decourt et al., 2013). The M17 protein samples run alongside AD and ND brain protein lysates showed uniform migration distances with human brain APP (110–130 kilodaltons (kD)), BACE1 (N-terminus 60–70 kD, 55 kD, or 45 kD); (C-terminus 70 kD), TNFR1 (55 kD), and PSEN1 (20, 22, 23 kD carboxy-terminal fragments) (Fig. 1C) suggesting that the M17 cells process AD proteins similarly to endogenous human brain proteins.

2.2 Detection of APP-cleavage products in BE(2)-M17 cells

In addition to detecting APP processing proteins by Western blot, we tested whether M17 cells possess β -secretase activity by carrying out an activity assay (see Methods for details). Different dilutions of M17 cell lysate were used to assess the linearity of the cleavage reaction (Fig. 2C). We observed, linearity for concentrations ranging 3.5–28 μ g total protein ($R^2=0.997$). Above 28 μ g total protein, the slope of the reaction decreased as expected in enzymatic reactions. To assess the relative contribution of BACE1 to the substrate cleavage, we used the inhibitor provided with the activity assay kit for 28 and 56 μ g total protein (note that the inhibitor is not identified by the manufacturer). In both cases, we observed a significant 13% decrease in β -secretase activity ($p<0.05$). Therefore, part of the substrate consumption is due to BACE1 activity. Altogether, our data suggest that M17 possess all enzymatic components and activities to process APP by the amyloidogenic pathway.

We investigated the occurrence of APP-cleavage products in M17 cells by Western blots of M17 cell protein lysates as well as ELISAs of M17 conditioned media. Immunoblots using antibody directed against the carboxy-terminal 20 aa of APP, detected not only full-length APP (110 – 130 kD) in M17 cells, but also the APP cleavage intermediates, CTF99 (12 kD) and CTF83 (10 kD) (Fig. 2A). The presence of the CTF99 proteolytic product provides evidence of BACE1-mediated cleavage activity of APP, while detection of the CTF83 fragment demonstrates α -secretase activity is also present in M17 cells.

Next, as a measure of γ -secretase expression in M17 cultures, we performed anti-A β 40 and A β 42 ELISAs on M17 conditioned media (CM). ELISA results represented in the chart in Figure 2B, indicate that M17 cells produce both the A β 40 and A β 42 peptides. The presence of A β 40 and A β 42 in the condition media suggests that γ -secretase is expressed and active in M17 cells, cleaving CTF99 to produce both A β 40 and A β 42. ELISA measurements further demonstrated M17 cells produce A β peptides at an A β 40:A β 42 ratio of about 7:1, independent of absolute A β isoform concentrations, at either 48 or 120 hrs of harvest (Fig. 2B). For example, for the data presented in Fig. 2B, the 48 hr CM mean concentration of A β 40 was 334.5 pg/ml (± 12.0) and A β 42 was 51.4 pg/ml (± 2.9), producing an A β 40:A β 42 ratio of 6.5. The mean concentration of A β 40 in the 120 hr CM was 271.8 pg/ml (± 18.6) and A β 42 was 38.0 pg/ml (± 2.9), making the A β 40:A β 42 ratio 7.1.

Detection of CTF99 and CTF83 by Western blots (Fig. 2A), combined with detection of A β 40 and A β 42 peptides by ELISA (Fig. 2B), provides evidence that the expression and proteolytic activities of β -, α -, and γ -secretases needed for APP processing and A β production are present in M17 cells.

2.3 RNA Sequencing of the BE(2)-M17 cell line transcriptome

High-throughput RNA sequencing was applied to this study to capture the complete RNA expression profile of the BE(2)-M17 cell line. The approach both identified genome-wide expression of RNA species and quantified the relative expression levels of the RNAs and their isoforms. Of the more than 62,000 lines of M17 RNA sequence data produced, a representative sampling of the M17 transcriptome, shown in Table 1, is focused on genes of interest to neurodegenerative processes of AD and related to the APP cleavage pathway. The

fragments per kilobase pair of transcript per million fragments mapped (FPKM) column in Table 1 presents gene expression levels as a relative ranking compared to other expressed transcripts in the M17 cell. Notably, we observed that the APP, BACE1, TNF α , TNFR1, and TNFR2 transcripts detected by this technique show good agreement with the relative abundance we observed by gene-specific qRT-PCR analyses (Ct values, data not shown). For example, GAPDH, the highest expressing, and TNF α , one of the lowest expressing genes of the M17 cells detected by qRT-PCR assays, received FPKM ranking values of 4399.93 and 0.00, respectively, by RNA sequencing analysis. The active γ -secretase enzyme is a multi-protein complex and involves expression of presenilin (PSEN) 1 or 2, anterior pharynx defective (APH1), presenilin enhancer 2 (PSENEN/PEN2), and nicastrin (NCSTN) genes. Presented with the sequencing results shown in Table 1, we show that all the γ -secretase subunit mRNAs, except nicastrin, were detected by this sequencing platform. However, despite falling below the sensitivity of this assay, nicastrin mRNA is likely expressed in M17 as ELISA-specific detection of A β peptide production (see Fig. 2B) implies nicastrin protein, required for multimeric γ -secretase activity, is present in M17 cells. Likewise, the strong anti-CTF83 signal on protein immunoblots (Fig. 2A), suggestive of proteolytic activity and α -secretase gene expression in M17 cells, was confirmed by the corresponding detection and FPKM rankings for the mRNAs of the α -secretase gene family members of “a disintegrin and metalloproteinase” (ADAM) domain proteins, listed in Table 1 as ADAM9, ADAM10, and ADAM17. The RNA sequence data provides evidence for M17 expression of additional signaling intermediates such as receptors, ligands, transcription factors, inhibitors, and enzymes (Donmez and Outeiro, 2013; Hoe et al., 2007; Ly et al., 2013) as shown in the partial list in Table 1.

Truncated and N-terminally modified forms of A β peptides are found in diffuse and dense core plaques and are of great current interest for their likely contribution to A β -associated toxicity. We searched our RNA Sequencing M17 transcriptome data tables for evidence of RNA expression of several of the known A β processing enzymes, including: glutamyl cyclase (QPCT) which catalyzes a critical N-terminal glutamate conversion, generating N-modified A β peptide with pyroglutamyl at residue 3 (De Kimpe et al., 2012) and correlates to severity of dementia (Valenti et al., 2013); meprin A, a beta (MEP1B) metalloprotease that produces N-terminal truncated A β peptides independent of BACE1 (Bien et al., 2012); and aminopeptidase A/glutamyl aminopeptidase (ENPEP) which can contribute to potential pathogenic A β modifications by removing the first two residues and exposing Glu3 (Sevalle et al., 2009). We detect RNA expression for each of these three genes in the M17 cell line, shown in Table 1.

The M17 Transcriptome FPKM data tables are hosted at the Translational Genomics Research Institute, Neurogenomics Data site: <https://www.tgen.org/research/research-divisions/neurogenomics/supplementary-data/supplementary-dataa-cellular-model-of-amyloid-precursor-protein-processing-and-amyloid-beta-peptide-production.aspx>

2.4 Retinoic acid-mediated differentiation of BE(2)-M17 cells

The distinct morphological effects of RA-treatment on M17 cells growing in culture are evident in Figure 3A (right panel) when compared to untreated M17 cells by phase contrast microscopy (Fig. 3A, left panel), and include 1) decreased proliferation, 2) increased number of neuritic processes, and 3) increased length of neuritic processes.

The RA-treated cells were collected and the mRNAs processed for RT-PCR analyses. In Fig. 3B, endpoint RT-PCR analysis revealed that expression patterns for APP, BACE and GAPDH appear unchanged, while expression of TNF α , TNFR1, and TNFR2 genes appear to be altered in the RA-treated compared to untreated M17 cells. Quantitation of the RA-induced effects on gene expression by real-time qRT-PCR (Fig. 3C) compared to untreated

M17 cells as the calibrator control (1.0), confirmed the apparently stable expression of APP (1.07), and BACE1 (1.26), whereas TNF α levels were decreased (0.328), and both TNFR1 and TNFR2 levels were increased, 17.5-fold and 33.0-fold, respectively, with RA-treatment. Western blots comparing total protein from untreated and RA-treated cell lysates were consistent with the RNA changes observed for RA-treated M17 cells (Fig. 3D), that is, expression of APP and BACE1 proteins were stable and apparently unchanged, while protein expression of TNFR1 was concomitantly increased.

3. Discussion

We report in this study, the characterization of BE(2)-M17 cells for the expression profile of AD-associated genes and the proteolytic products involved in A β peptide production. Based on our findings, we propose M17 cells could serve as a cell-based model for APP processing studies and a valuable tool for high-throughput screening of new therapeutic agents modulating A β production. Along with this initial report of the potential of M17 cells to model amyloidogenic processes, we make publicly available the RNA sequencing data for the M17 cell transcriptome.

Various *in vitro* cell culture systems have been published, addressing a specific activity of the amyloid-processing pathway (Andrau et al., 2003; Murayama et al., 2005). Cell systems have included human or rodent, primary cells or cell lines of neuronal and non-neuronal origin, such as SH-SY5Y, HeLa, HEK293, and COS, many of which required stable or transient transfection of expression and/or reporter DNA constructs (Andrau et al., 2003; Lahiri and Ge, 2004; Lahiri and Nall, 1995; Pluta et al., 2013). Advantages we believe the human M17 cells offer over other cell-based assay systems for studies of A β production include 1) the endogenous expression of all necessary enzymes and substrates to model complete processing of the amyloid protein, from precursor to final A β peptide product, 2) the human origin of the cell line, 3) the high metabolic, neuroblastoma nature of the cells, 4) its capacity to be conditionally differentiated to a more mature neuron-like phenotype, and 5) strong adherence properties during culture that permit demanding experimental manipulations without cell detachment. It has been reported that M17 cells have been successfully transfected, for example with a human APP “Swedish” mutation expression construct (Durkin et al., 1999), offering a proven experimental option when other than wild-type molecular targets are to be assayed.

We have demonstrated here the mRNA and corresponding protein expression of APP, β - and γ -secretase or cleavage products of the amyloid cleavage cascade (see Figs. 1 and 2, and Table 1). The M17 cell-based model system has great potential value for studying baseline transcriptional regulation of APP, BACE1, α -secretase, and γ -secretase components, and as well for evaluating downstream effects of exogenous stimulatory or inhibitory compounds on AD-associated gene promoter regulation. The RNA sequence data indicating expression of several genes involved in producing pathogenic truncated and N-terminal modified forms of A β peptides (Table 1), suggest the M17 cells could also be useful for studies of these additional processing pathways.

Promising new AD drugs in development include anti-A β immunotherapeutics, anti-inflammatory, anti-angiogenic, immunomodulatory drugs, designed to decrease A β 42 production (Weggen et al., 2001), reduce A β aggregation, enhance A β clearance from the brain, or alternatively, increase non-amyloidogenic α -secretase activity (Yiannopoulou and Papageorgiou, 2013). Our results suggest the M17 model can be used to investigate the molecular mechanisms directing the β - versus α -secretase cleavage site-selection of APP. Modulatory effects on α - versus β -secretase activity by pharmaceutical agents added to M17

cultures could be quantified as associated change in the CTF99 and CTF83 cleavage product ratio distinguished in M17 lysates by Western blot analysis (see Fig. 2A).

In AD, A β 40 and A β 42 are the most common of the A β peptide variants, of which A β 42 monomers readily aggregate to form neurotoxic oligomers (Jarrett et al., 1993). Differing by 2 amino acids at the carboxy-terminus, the choice of A β 40 and A β 42 isoform production is determined by γ -secretase cleavage site selection (Carter et al., 2008; Durkin et al., 1999). Notably, while A β 40 accounts for approximately 90% of secreted A β and is the most abundant species recovered from cerebrospinal fluid, it is A β 42 that is the predominant insoluble amyloid isoform deposited in the brain (Iwatsubo et al., 1994). Molecular mechanisms that regulate total A β production or the relative proportions of the A β isoforms deposited in AD brains (Rajadas et al., 2013), are not well characterized. Our ELISA quantitation of A β 40 and A β 42 products in conditioned media (see Fig. 2B) revealed the 7:1 ratio of A β 40:A β 42 peptides produced by M17 cells is similar to the 10:1 ratio found in the CSF of non-familial AD patients (Seubert et al., 1992), and suggests the M17 *in vitro* system provides an approach to investigate the regulation of the A β intramembrane cleavage site selection. Interestingly, familial AD (FAD)-linked PSEN mutations alter γ -secretase cleavage of CTF99 to favor A β 42 production and thus, decrease A β 40/A β 42 ratios (Sato et al., 2012). Using the M17 cell-based system, a measurable experimental outcome of drug treatments (i.e., γ -secretase modulators) could include ELISA quantitation of increased, or decreased, A β 42 production in drug-treated or untreated M17 cell medium to help identify molecular determinants of the A β 40/A β 42 ratio observed in homeostasis and in AD.

The high depth of unbiased genome-wide coverage generated in this study by the RNA sequencing platform, has quantified the expression of known and novel RNA species in the BE(2)-M17 cell transcriptome. This powerful dataset, now made available in entirety at open access online data sites (see Results, subsection 2.3) enables informed design of future hypotheses and functional assays to be based on comprehensive expression information presented for additional compelling M17 cellular processes, for example regulated phosphorylation of an expressed target substrate such as MAPT/TAU.

In conclusion, we present evidence of characteristics of BE(2)-M17 cells that support our view of its value as a novel, human cell-based model for mechanistic studies of the AD APP-cleavage pathway. Endogenous amyloidogenic properties of the M17 cells, from baseline APP gene transcription, to β - and γ -secretase-mediated release of A β peptides, consequently underscore its potential to serve as a high-throughput screening tool for new drug discovery.

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Highlights

We validate BE(2)-M17 as a new model for studies of APP cleavage and A β production.

Endogenous APP-processing events in BE(2)-M17 produce C83, C99, A β 40 and A β 42.

BE(2)-M17 could potentially support high-throughput screens for β - or γ -secretase modulators.

Fig. 1. BE(2)-M17 cells

(A) Phase contrast micrograph of the neuroblastoma cell line (100 $\times$ magnification). (B) Endogenous RNA gene expression in BE(2)-M17 cells by RT-PCR analysis. M17 RNA was either reverse transcribed (RT +) or for negative controls, not reverse transcribed (RT -) and the amplicons from RT-PCR visualized on 2% agarose/0.5 $\times$ TBE gels: APP 141 bp; BACE1, 122 bp; TNFR1, 134 bp; TNF α , 135 bp; TNFR2, 102 bp; GAPDH, 131 bp. Amplicon positions are indicated by arrowheads to the right of each panel. 100 bp Ladder and marker sizes are shown on the left. (C) Western blots of BE(2)-M17 cell lysates (M17), Alzheimer's (AD) and Non-Demented (ND) human brain homogenates detect expression of

proteins associated with AD neuropathology. On 9% Tris-Glycine gels, 25 μ g (left 3 panels) or 50 μ g (center 3 panels), and on an 18% Tris-Glycine gel, 30 μ g (right 2 panels) samples were electrophoretically separated and transferred to PVDF membranes for immunoblotting. Anti- β -actin blots are shown as sample loading controls. Molecular weights of Precision Plus protein standards (kilodaltons) are given to the left of each panel.

A **B**

Fig. 2. Amyloid Precursor Protein (APP) cleavage pathway products in M17 cell lysates and conditioned media

(A) Tris-Tricine Gel Western blotting detects the presence of both β - and α -secretase-mediated APP-cleavage products, carboxy-terminal fragments CTF99 and CTF83, respectively. 100 μ g of total M17 lysate was electrophoretically separated and transferred to Immobilon-PSQ PVDF membrane for immunoblot detection using an anti-APP carboxy terminal 20aa primary antibody, SIG39152. Precision Plus and Kaleidoscope molecular weight standards (left) are given for size references in kilodaltons (kD). (B) ELISA quantitation of A β production by BE(2)-M17 cells. Using Novex ELISA kits specific for human A β 40 and A β 42, levels of secreted A β 40 and A β 42 were measured neat in the conditioned medium of BE(2)-M17 cultures at either 48 hrs (48-M17) or 120 hrs (120-M17) post-feeding. (C) β -secretase activity in M17 cell lysates. The level of secretase enzymatic

activity measured on an Abcam kit-provided substrate, is proportional to the level of fluorescent signal intensity shown on the y-axis. Total protein quantity of M17 lysate per assay is shown on the x-axis (μg).

A

**Untreated BE(2)-M17
(day 4)**

**10 μ M RA-treated BE(2)-M17
(day 4)**

B

C

Fig. 3. Four day treatment of BE(2)-M17 cells with 10 μ M retinoic acid (RA)
(A) Phase contrast microscopic images (100 $\times$ magnification) of untreated M17 neuroblastoma cells (left panel) and retinoic acid-treated cells (right panel). The RA-treated cells exhibit the morphological changes, increased number of neuritic extensions, and increased length of neurite outgrowths associated with RA-mediated neuroblastoma differentiation. **(B)** Endpoint RT-PCR analysis of altered gene expression in untreated BE(2)-M17 (M17) and RA-treated cells (RA). Total RNAs purified from M17 or RA-treated cells were reverse transcribed (RT +) or not (RT –) for RT-PCR assays and the products separated on 2% agarose/0.5 $\times$ TBE gels. Names of target genes are indicated above, and

amplicon sizes indicated below, each panel. Migration of the 100 bp Ladder size standards are shown to the left.

C Real-time quantitative RT-PCR (QRT-PCR) Relative Quantity Charts of 10 μ M Retinoic Acid (RA)-Treated BE(2)-M17 Cells (day 4). The changes in expression of the genes-of-interest in RA-treated M17 cells, relative to untreated controls, are given in parentheses above each chart.

D Western Blots of M17 lysates from untreated and retinoic acid-treated (day 4) cells. Electrophoretic separation of 100 μ g of total protein of M17 or RA-treated (RA) cell lysate through 12% Tris-Glycine SDS-PAGE gels was followed by immunoblotting and ECL detection of the effects of RA-treatment on protein expression. Consistent with increased TNFR1 RNA by RT-PCR (Fig. 3B and 3C) is increased TNFR1 protein expression in RA-treated cell lysates, indicated by an arrowhead to the right of the TNFR1 panel. The anti- β actin blot is shown as a sample loading control. Positions of the Precision Plus molecular weight standards in kilodaltons (kD), are shown to the left.

Table 1

Partial listing of genes reported from the RNA sequenced transcriptome of BE(2)-M17 cells. These 27 target genes (27/62K) were selected for their putative roles in amyloidogenesis or AD neuropathology. Shown are the Ensembl Gene IDs, Gene name (symbol), and the determined Fragments Per Kilobase of transcript per Million mapped reads (FPKM).

GENE ID	GENE SHORT NAME	FPKM
ENSG00000142192	APP	201.54
ENSG00000186318	BACE1	29.49
ENSG00000232810	TNF	0.00
ENSG00000067182	TNFRSF1A	0.47
ENSG00000028137	TNFRSF1B	0.05
ENSG00000111640	GAPDH	4399.93
ENSG00000080815	PSEN1	12.89
ENSG00000143801	PSEN2	12.66
ENSG00000117362	APH1A	158.48
ENSG00000138613	APH1B	0.76
ENSG00000205155	PSENEN (PEN2)	103.71
ENSG00000162736	NCSTN	0.00
ENSG00000137845	ADAM10	19.42
ENSG00000168615	ADAM9	14.35
ENSG00000151694	ADAM17	8.78
ENSG00000096717	SIRT1	13.98
ENSG00000068903	SIRT2	20.19
ENSG00000182240	BACE2	0.15
ENSG00000140564	FURIN	7.10
ENSG00000117020	AKT3	37.10
ENSG00000082701	GSK3B	12.59
ENSG00000100030	MAPK1 (ERK)	12.69
ENSG00000109320	NFKB1	7.91
ENSG00000186868	MAPT (TAU)	2.26
ENSG00000141434	MEP1B	0.01
ENSG00000115828	QPCT	0.32
ENSG00000138792	ENPEP	0.02