Establishing the Freestyle 293 Expression System as a Tool to Generate Recombinant Apolipoprotein E in Mammalian Cells

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Establishing the Freestyle 293 Expression System as a Tool to Generate Recombinant Apolipoprotein E in Mammalian Cells

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Submitted in Partial Completion of the Requirements for Departmental Honors in Biological Sciences

Bridgewater State University

May 10, 2022

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ABSTRACT

Alzheimer’s disease (AD) is a devastating form of dementia with 5 million people suffering from the disease in the United States alone. Thus, there is a need to understand the cause of AD so that therapeutics can be developed to treat it. The greatest risk factor for AD is the *APOE* gene, which encodes the apolipoprotein E protein. Three predominant *APOE* alleles exist in humans, which give rise to three isoforms, called apoE2, E3, and E4. Individuals with apoE4 are ten times more likely to develop AD. In contrast, the apoE2 isoform has a neuroprotective effect and is associated with a 2-fold lower risk of developing AD. A growing body of evidence has demonstrated that apoE plays a major role in AD and cognitive aging in an isoform-specific manner, through either contributing to or counteracting AD pathogenesis. Our lab is interested in studying the molecular mechanisms of apoE in AD, but we first needed to establish a system to produce apoE protein in mammalian cells. Here, I present the culmination of the work I conducted in the Adams Lab establishing a system to evaluate apoE isoform-specific effects in mammalian cells, focusing on the establishment of the 293 FreeStyle Expression system, a serum-free, high-density HEK cell culture system, to generate recombinant apoE in mammalian cells. From the generation of apoE expression constructs to the quantification of secreted apoE protein levels, this project establishes the tools necessary to study apoE in the context of AD in mammalian cell culture.
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INTRODUCTION

Dementia is the 7th leading cause of death worldwide [1]. Alzheimer’s disease (AD) is the most common form of dementia, accounting for 60-80% of all cases [2]. Over 5 million Americans are living with AD and the number is predicted to triple by 2060 [3]. Currently, no effective therapeutics exist to treat or prevent AD and the cause of the disease is still not well understood. AD is an especially difficult disease to treat because the disease progression is slow and insidious. Pathophysiological changes in the brain begin years, if not decades, before the presentation of cognitive decline [4]. Despite the fact that it was characterized over a century ago, it wasn’t until the development of recent technology that scientists have been able to investigate the molecular basis of AD [5]. Since these advancements, research in neuroscience has erupted, and we are beginning to understand what is happening to the brains of AD patients at a cellular and molecular level.

AD was first characterized by Dr. Alois Alzheimer (1894-1915), after whom the disease is named [6]. Alzheimer was a renowned German physician known for his work in neurosyphilis and vascular dementia [7]. While working at the Frankfurt mental hospital, Alzheimer was presented with Auguste Deter, a 51-year old woman experiencing memory impairment, mania, insomnia, and agitation [8]. Alzheimer oversaw Deter’s care and studied her symptoms for several years, and after her death, conducted an autopsy on her brain. It was here that he first characterized three distinct abnormalities in Deter’s brain that are now identified as the hallmark features of AD [6]. The first striking observation made by Alzheimer when studying Deter’s brain was profound brain atrophy. We now know this atrophy to be the result of neurodegeneration (i.e., death of neurons). Neurons control and execute virtually all aspects of human behavior, including those affected by
AD (memory, motor function, language, emotional regulation) [6]. Alzheimer also identified two hallmarks at the microscopic level, now known as amyloid plaques and neurofibrillary tangles.

Neurofibrillary tangles form from the deposition of hyperphosphorylated tau protein. Tau is a microtubule-associated protein that is especially abundant in neurons, where it plays a central role in the dynamic assembly and stabilization of microtubule bundles in axons [9]. Microtubules are one of the principal components of the cytoskeletal system where they provide structural support and serve as tracks for motor proteins to transport cargo through the cell. Synapses are especially reliant on cellular trafficking, so impairment to microtubules and microtubule-associated proteins often lead to synaptic degeneration [10]. In healthy cells, tau binding to microtubules is regulated by phosphorylation of the microtubule-binding domain of tau, which dissociates tau from the microtubule during cytoskeletal reassembly [9]. However, in diseased brains, tau can become hyperphosphorylated, causing it to aggregate into large neurofibrillary tangles (NFTs) in the cell bodies of neuronal cells, which leads to cell death [11] (Figure 1). This hyperphosphorylated tau is capable of propagating, or “seeding”, to other cells in a prion-like manner [12]. Hyperphosphorylated tau spreads in a highly specific manner through the brain as the disease progresses and has been characterized as a series of distinct stages, known
as Braak stages [11]. Braak staging is associated with severity of AD symptoms and has shown recent applications in monitoring disease severity in living humans through PET imaging [13].

Amyloid plaques occur as a result of amyloid-β (Aβ) accumulation. Aβ is produced from the cleavage of amyloid precursor protein (APP) (Figure 2). While the function of APP itself is not well understood, the mechanism of enzymatic cleavage by β-secretase and γ-secretase is well characterized, as well as the oligomerization and accumulation of Aβ in the extracellular space in the brain, referred to as the parenchyma [14]. Aβ deposition is one of the earliest pathological mechanisms observed in pre-clinical AD [15], and the development of clinical symptoms is believed to be due to the deficient brain clearance of the peptide [16] leading to the development of the amyloid cascade hypothesis of AD [17]. This hypothesis is supported by the fact that mutations in APP and 1 and -2, the catalytic subunits of γ-secretase, are sufficient to cause early-onset autosomal dominant AD, also known as familial AD (FAD) [18]. However, this hypothesis is becoming increasingly controversial as new evidence surfaces. For example, individuals can present with substantial amyloid burdens without any indications of accompanying cognitive decline [19, 20]. Additionally, therapies targeting Aβ have generally not been effective, suggesting that the pathological mechanism of AD is much more complicated than Aβ accumulation [21].

Figure 2. β-Amyloidogenic cleavage of APP by β-secretase and γ-secretase. Aβ is formed from two cleavage events of APP, resulting in a 40-42 amino acid peptide highly prone to oligomerization. Created with Biorender.com.
The *APOE* gene, which encodes the apolipoprotein E apoE protein, is the strongest known risk factor for AD [22, 23]. ApoE belongs to a family of lipoproteins (Figure 3), which are secreted by cells and incorporated into a complex ball of lipids, or fats, that function to carry lipids to growing or damaged neurons [24]. The *APOE* gene exists in the population as three alleles that encode three protein variants (or, isoforms)--apoE2, apoE3, and apoE4--that are each 299 amino acids in length. The apoE isoforms are identical with the exception of two amino acids at positions 112 and 158. ApoE2 has the amino acid cysteine at both positions, apoE3 has cysteine at 112 and arginine at 158, and apoE4 has arginine at both positions (Figure 4). Importantly, individuals who inherit the apoE4 isoform are 10 times more likely to develop AD with an age of onset 10 years younger compared to individuals with apoE3 (the most common isoform). In contrast, the apoE2 isoform has a neuroprotective effect and is associated with a 2-fold lower risk of developing AD [23].

**Figure 3. Apolipoprotein E (apoE) in lipoprotein complex.** The apoE gene encodes the apoE protein, which is secreted from cells and incorporated into a complex of lipids and other proteins known as a lipoprotein complex. Created with Biorender.com

**Figure 4. Linear schematic depiction of apoE protein.** The apoE protein is 299 amino acids in length. The isoforms differ in positions 112 and 158.
A growing body of evidence has demonstrated that apoE plays a major role in AD and cognitive aging in an isoform-specific manner, through either contributing to or counteracting to AD pathogenesis [25]. A recent case study reported the identification of a rare mutation in apoE3 (R136S), known as apoE Christchurch, which protected an individual with a PSEN1 FAD mutation from cognitive decline well into her 70s [26]. Similarly, a rare mutation in apoE3 (V236E), known as apoE Jacksonville, was found to reduce Aβ oligomerization by preventing apoE self-oligomerization [27]. The mechanisms of apoE in AD pathogenesis are so impactful that a new hypothesis – the apoE cascade hypothesis – is now being entertained as a promising therapeutic target for AD [28].

I began my research at Bridgewater State University with Dr. Ken Adams in the summer of 2019 and spent the first 8 months studying the little-known transcriptional coregulator NAB2. Dr. Adams spent time working with Dr. Bradley Hyman at Massachusetts General Hospital during his sabbatical in the fall of 2019 and brought back with him a new motivation to start studying AD in our lab. Eager to start contributing to a cause in research that truly matters, I scrambled to write a research proposal to study apoE isoform-specific effects on neurite outgrowth in PC12 cells with the Adrian Tinsley Program Summer Research grant. PC12 cells have long been a popular choice in studying neuronal mechanisms in cell culture, as treatment with nerve growth factor will trigger a cascade of cellular events promoting differentiation into a phenotype comparable to sympathetic neurons [29] (Figure 5). As a continuation of the work I conducted in the spring 2020 semester establishing conditions to evaluate neurite outgrowth in PC12 cells, I planned to transfect PC12 cells with apoE2, apoE3, or apoE4 and determine if apoE affects neuritogenesis in PC12 cells.
I was thrilled to be rewarded the Adrian Tinsley Program Summer Research grant in March of 2020 and immediately began working to establish the tools necessary to complete my project. However, within a week of receiving apoE expression constructs from the Hyman Lab, the COVID pandemic had consumed our lab, and I joined scientists around the world in the long, frustrating wait for lab access. Once conditions were safe enough to conduct research while practicing social distancing, I was able to finally begin my project. Little did I know that the project I had so ambitiously proposed in 2020 would present its own unexpected obstacles.

Here, I present the culmination of the work I conducted in the Adams Lab establishing a system to evaluate apoE isoform-specific effects in mammalian cells, focusing on the establishment of the 293 FreeStyle Expression system, a serum-free, high-density HEK cell culture system, to generate recombinant apoE in mammalian cells.
MATERIALS & METHODS

Molecular cloning

ApoE2, E3, and E4 cDNAs were subcloned from pCMV4-apoE2, E3, and E4 plasmid constructs (provided from Dr. Bradley T. Hyman, Massachusetts General Hospital) into pcDNA3 plasmid vector (provided by Dr. Geoffrey M. Cooper, Boston University). Each plasmid was subjected to double digest in a 50 µl reaction containing 1 µg of plasmid DNA, 20 units of XbaI (New England Biolabs), 20 units of KpnI (New England Biolabs) and 5 µl of 10X NEBuffer r2.1 (New England Biolabs). The reactions were mixed and incubated at 37°C for 4 hours. 10 µl of 6X gel loading dye (New England Biolabs) were added per reaction before electrophoresis through 1% agarose gels containing ethidium bromide. Gels were visualized over a UV gel dock and bands containing the desired DNA fragments (apoE2, E3, and E4 inserts and linearized pcDNA3) were excised with a razor blade. DNA was extracted from each agarose gel slab using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. In the final step, the DNA fragments were eluted in 30 µl of nuclease-free ultrapure water (Ambion).

Ligation reactions were conducted using T4 DNA ligase (New England Biolabs). In brief, 20 µl reactions were prepared containing 2 µl of 10X T4 DNA ligase buffer, 800 units of T4 DNA ligase, and the maximum amount of apoE2, or E3, or E4 insert + pcDNA3 vector to acquire a 3:1 molecular ratio. To calculate 3:1 ratios, concentrations for each DNA fragment were estimated by assuming 100% recovery during the gel extraction procedure because the actual concentrations were too low to accurately quantify by spectrophotometry. The reactions were mixed and incubated at room temperature for approximately 18 hours.

One Shot TOP10 chemically competent E. coli (Thermo Fisher Scientific) were transformed with each ligation reaction by heat shock. In brief, 5 µl of ligation reaction was added to a vial of
One Shot TOP10 *E. coli* and then incubated on ice for 10 minutes with gentle swirling once every 2 minutes. The cells were then subjected to heat shock by incubating in a 42°C water bath for 30 seconds and then incubated on ice for 2 minutes. 500 µl of SOC medium (Thermo Fisher Scientific) were added to each transformation before incubation at 37°C in a shaker at 250 rpm for 1 hour. The transformations were then distributed evenly onto two agar plates supplemented with 100 µg/ml ampicillin, which were incubated at 37°C for approximately 18 hours to isolate colonies containing recombinant clones of pcDNA3-apoE2, E3, or E4.

For each ligation reaction transformation (pcDNA3 + apoE2, E3, or E4), several clones were screened to identify true recombinant plasmids. In brief, for each clone, 6 ml of Luria's Broth (LB) supplemented with 100 µg/ml of ampicillin were inoculated with a bacterial colony and incubated for 8–12 hours at 37°C in a shaker at 250–300 rpm. Cells were then collected by centrifugation at 4000 rpm for 15 minutes at 4°C and subjected to plasmid mini prep using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. In the final step, plasmid DNA was eluted in 30 µl of nuclease-free water and quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

Each clone recovered by mini prep was subjected to double restriction digest with XbaI + KpnI followed by agarose gel electrophoresis, as described above, to confirm the presence of an apoE insert + pcDNA3 vector. Clones with insert and vector of expected sizes were then subjected to Sanger sequencing (Eurofins Genomics) using T7 and SP6 primers, for which pcDNA3 has priming sites located upstream and downstream, respectively, of its multiple cloning site. The sequencing results were then examined to confirm the presence of the full apoE allele—E2, E3, or E4—sequence. One clone for each apoE isoform was then selected for plasmid maxi prep, for which transformed *E. coli* were expanded 200 ml of LB supplemented with 100 µg/ml ampicillin
for 18 hours. Plasmid maxi prep was then conducted using the QIAGEN Plasmid Maxi Kit (Qiagen) and final plasmid concentrations determined with a NanoDrop One spectrophotometer.

**Site-directed mutagenesis**

Site-directed mutagenesis (SDM) was conducted to mutate arginine-158 to cysteine in pcDNA3-apoE3 using the QuickChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. Mutagenic primers were generated with a melting temperature ($T_m$) of 78-83°C using the following equation:

$$T_m = 81.5 + 0.41(\% \text{ GC}) - (675/N) - \% \text{ mismatch}$$

for which $N$ equals the primer length, % GC and % mismatch were both included as whole numbers. The following primers were designed and ordered as custom primers from Integrated DNA Technologies:

(sense) 5'-GCC GAT GAC CTG CAG AAG *TGC* CTG GCA GTG TAC C-3’

(antisense) 5’-G GTA CAC TGC CAG GC *TCT* CTG CAG GTC ATC GGC-3’

Once received, the primers were suspended to in Tris-EDTA (TE), pH 8.0 buffer at 1 mg/ml concentration and stored at -20°C.

SDM reactions were prepared with a total volume of 50 µl and containing 5 µl of 10x reaction buffer (provided with the kit), 10 ng of the appropriate pcDNA3-apoE plasmid, 125 ng of each mutagenic primer, 1 µl of dNTP mix (provided with the kit), 3 µl of QuickSolution (DMSO, provided with the kit), and 1 µl of *Pfu Turbo* DNA polymerase (2.5 units). The reactions were mixed and then subjected to polymerase chain reaction (PCR) in a thermal cycler with the following parameters:
<table>
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<th>Step</th>
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<tr>
<td>1</td>
<td>1</td>
<td>95°C</td>
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<td>2</td>
<td>18</td>
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<td>60°C</td>
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<td>4</td>
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<tr>
<td>5</td>
<td>1</td>
<td>68°C</td>
<td>7 minutes</td>
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*The step 4 time was based on the manufacturer's recommendation for 1 minute/kilobase of plasmid. The pcDNA3-apoE plasmids are approximately 6500 base pairs, thus step 4 was carried out for 7 minutes.

Following PCR, 10 units of DpnI restriction endonuclease were added to each reaction to digest, which was mixed and incubated at 37°C for 1 hour to digest the parental DNA.

To recover mutant clones, XL-10 Gold Ultracompetent *E. coli* (Agilent Technologies) were transformed with the DpnI-treated PCR product. To do so, XL-10 Gold cells were thawed gently on ice and 45 µl of the cell suspension was transferred to a pre-chilled 14-ml BD Falcon polypropylene round-bottom tube. 2 µl of β-mercaptoethanol mix (provided with the *E. coli*) were added to the cells, which were then mixed and incubated on ice for 10 minutes with gentle swirling every 2 minutes. 2 µl of the DpnI-treated PCR product were added and the suspension was gently mixed by swirling and incubated on ice for 30 minutes. The mixture was then subjected to heat shock at 42°C for 30 seconds and placed back on ice for 2 minutes. 500 µl of pre-warmed NZY+ broth was added to each transformation and the suspensions were incubated at 37°C for 1 hour in a shaker at 250 rpm. Each transformation was then distributed evenly between two agar plates supplemented with 100 µg/ml ampicillin, which were incubated at 37°C for 18–20 hours to grow and isolate transformed colonies with mutant clones. For each SDM reaction,
several colonies were then grown, subjected plasmid mini prep using QIAprep Spin Miniprep Kit according to the manufacturer's instructions. In the final step, plasmid DNA was eluted in 30 µl of nuclease-free water and quantified using a NanoDrop One spectrophotometer.

Recovered clones were subjected to Sanger sequencing via Eurofins Genomics using two primers, T7 (5'-TAATACGACTCACTATAGGG-3') and a custom primer named ApoEF1 (5'-TTTGGGATTACCTGCGCTGG-3'). As previously noted, pcDNA3 vector has a T7 priming site upstream of its multiple cloning site. The ApoEF1 primer was designed to anneal within the apoE cDNA insert, 216 base pairs upstream of the codon for cysteine/arginine-112, 354 bp upstream of the codon for cysteine/arginine-158, and 783 bp from the stop codon. These two primers yielded complete coverage of the entire apoE insert sequence, enabling identification of clones containing the intended mutation and lacking unintended mutations, deletions, or insertions. Positive clones were selected for plasmid maxi prep, for which transformed E. coli were expanded 200 ml of LB supplemented with 100 µg/ml ampicillin for 18 hours. Plasmid maxi prep was then conducted using the QIAGEN Plasmid Maxi Kit and final plasmid concentrations determined with a NanoDrop One spectrophotometer.

**PC12 and HEK cell culture and plasmid transfection**

PC12 cells were grown in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 5% horse serum (HS), and penicillin and streptomycin (P/S) at 37°C incubator and 10% CO₂. HEK 293 cells were maintained in DMEM supplemented with 10% FBS and P/S also at 37°C incubator and 10% CO₂. Transfections of both cell lines were conducted using TransIT-LT1 transfection reaction (Mirus). One day before transfection, 1-1.5 x 10⁶ cells were plated on 60 mm tissue culture dishes in 4 ml of growth medium. The following day, for each culture, transfection mixtures were prepared consisting of 500 µl of serum-free
DMEM, 2 µg of plasmid DNA, and 10 µl of TransIT-LT1, which were incubated 15-30 minutes at room temperature before applying to the appropriate cell culture in a drop-wise manner. The transfections were then incubated at 37°C at 10% CO₂ for 18-24 hours before the medium was replaced with either full-serum growth medium or low-serum medium, as discussed in the Results section.

**FreeStyle 239-F cell culture and transfection**

FreeStyle™ 293-F cells (Invitrogen R790-07) were maintained following recommendations from the manufacturer. Cells were incubated in a 37°C incubator containing a humidified atmosphere of 8% CO₂ on an orbital shaker platform rotating at 125 rpm in disposable, sterile, Erlenmeyer flasks with vented caps. For cell line maintenance, 293-F cells were grown in FreeStyle 293 Expression Medium (Invitrogen 12338-026) supplemented with P/S. Cells were subcultured every 3-5 days once the cell density reached 1-3 x 10⁶ cells/ml. To count cells, a small aliquot of cells was vortexed briefly and combined with PBS at a 1:1 ratio. The PBS/cell suspension mixture was combined with Trypan Blue Solution (Gibco 15250061) at a 1:1 ratio and counted using a hemocytometer. Cells were then seeded at a density of 3-5 x 10⁵ cells/ml in fresh expression medium. Transfections were conducted using 293fection transfection reagent (Invitrogen, 12347-019). For each transfection culture, 30 µg of plasmid DNA were diluted in 1 ml of either FreeStyle 293 Expression Medium or Opti-MEM I Reduced-Serum Medium (Invitrogen 31985-062) and 60 µl of 293fectin were diluted in a separate 1 ml of either FreeStyle 293 Expression Medium or Opti-MEM I Reduced-Serum Medium. The mixtures were incubated separately for 5 minutes and then combined and incubated for an additional 30 minutes before applied to 293-F cells at 1 x 10⁶ cells/ml in 30 ml total volume of FreeStyle 293 Expression Medium.
Collecting and concentrating conditioned medium from 293-F transfections

To collect conditioned medium for time-course experiments, 400 μl of cell suspension were centrifuged at 7,900 x g in a table-top microcentrifuge for 5 minutes. Supernatant or cell pellets were combined at a 1:1 ratio with 2x Laemmli buffer (Bio-Rad 15250061) and boiled for 5 minutes. For concentrating conditioned medium, 20-30 ml of cell suspensions were centrifuged at 200 x g for 5 minutes. 15 ml of conditioned medium were applied to Amico Ultra-15 Centrifugal Filter Units with a 10 kDa molecular weight cutoff (Millipore, UFC901024). The filter units were centrifuged 5000 x g at 4ºC 10 min several times until the volume was reduced to approximately 1 ml. 100 μl of concentrated medium was combined with 100 μl of 2x Laemmli buffer and boiled for 5 minutes.

SDS-PAGE, general protein staining, and Western blot

20 μl of conditioned medium samples and cell lysates were electrophoresed through 10% SDS-polyacrylamide gels alongside E. coli-derived recombinant apoE (produced by Dr. Adams) to use as standard. Gels were then either incubated in InstantBlue Coomassie Protein Stain (Abcam ab119211) for 15 minutes or transferred to nitrocellulose membrane. Membranes were blocked in 5% nonfat dried milk in tris buffered saline (TBS) + 0.2% Tween-20 (blocking buffer) for 1 hour at room temperature. Blocking buffer was then removed and replaced with primary antibody diluted in blocking buffer to detect apoE (Novus, NPBI-31123, 1:5,000 dilution) or β-actin (Sigma, A5441, 1:20,000 dilution) to incubate for 1 hour at room temperature. Membranes were washed three times for 15 minutes in TBS + 0.2% Tween-20 at room temperature. Secondary antibody (goat anti-rabbit HRP-conjugate, Bio-Rad, 172–1019, 1:3000 dilution for apoE Western blots; goat anti-mouse HRP-conjugate, Bio-Rad, 170–6516, 1:20,000 dilution for β-actin Western blots) was diluted in fresh blocking buffer and placed on the membranes to incubate for 1 hour at room
temperature. Membranes were washed three times for 15 minutes in TBS + 0.2% Tween-20 at room temperature and imaged with C-Digit Blot Scanner (LI-COR) by chemiluminescence (Western Lightning Plus ECL, Enhanced Chemiluminescence Substrate, Perkin Elmer, NEL105001).

**Quantification of apoE protein levels**

For quantification of conditioned medium samples, standards were prepared from recombinant apoE produced in *E. coli* diluted in 2X Laemmli sample buffer. 15 μl of standards and conditioned medium samples were subjected to SDS-PAGE through a 10% SDS-polyacrylamide gels. Gels were then incubated in Instant Blue Coomassie protein stain for 15 minutes and scanned with a standard desktop scanner. Images were then analyzed with ImageJ (NIH) Gel Analyzer. Images were converted to 8-bit and a rectangular selection about 1/3 the width of the protein band of the first lane was drawn and marked as the first lane. This selection was then dragged to the next lane and marked as “next lane” until all lanes had been selected (Figure 6A). The lanes were then plotted to produce a profile for each lane and the area under the peak was measured (Figure 6B).

![Figure 6](image_url)

**Figure 6.** Representative screen displays of Gel Analyzer ImageJ software.

Data for each gel were imported into GraphPad Prism 9 software for linear regression analysis. ApoE standard concentrations (in μg) were plotted against area under the peak (in AU). Concentration of apoE in conditioned medium samples were interpolated from the linear
regression. Given that each lane of the gel contains 7.5 µl of conditioned medium (15 µl per lane, 1:1 Laemmli to conditioned medium), interpolated values can be converted to µg/mL concentrations as follows:

\[
\frac{\mu g \text{ apoE (interpolated)} \times 1000}{7.5 \mu l} = \text{apoE (ng/µl)} = \text{apoE (µg/mL)}
\]

For quantification of concentrated apoE fractions, µg/mL concentrations were calculated as above and multiplied by the associated dilution factor. These results were averaged to approximate the concentration of the concentrated preps.
RESULTS

In order to express human apoE in mammalian cells, pCMV4-apoE2, E3, and E4 constructs were acquired from Dr. Bradley T. Hyman (Massachusetts General Hospital). However, generating sufficient supplies of the pCMV4-apoE constructs by plasmid maxi prep proved challenging for reasons that are unclear. KpnI and XbaI restriction enzyme sites were identified flanking the apoE cDNAs in the pCMV4 vector and also present in the multiple cloning site in the pcDNA3 vector in the needed orientation. (Figure 7). pcDNA3 vector and pCMV4-apoE2, apoE3, and apoE4 plasmids were subjected to double digest with KpnI and XbaI restriction enzymes. Digest reactions were electrophoresed through a 1% agarose gel and visualized on UV transilluminator, which confirmed digested fragments of the sizes expected for the linearized pcDNA3 vector and apoE cDNA inserts (Figure 8A). Linearized pcDNA3 vector and apoE cDNAs were excised from the gel, purified, and combined in ligation reactions. E. coli were transformed with the

Figure 7. Subcloning of apoE cDNA from pCMV4 into pcDNA3 vector schematic. Schematic showing the subcloning protocol for digesting pCMV4-ApoE and pcDNA3 vector with KpnI and XbaI restriction enzymes to generate an apoE cDNA insert compatible with the pcDNA3 vector.
ligation reactions to recover recombinant clones, which were subjected to double digest with KpnI and XbaI and screened through agarose gel electrophoresis, confirming the successful insertion of apoE cDNAs (Figure 8B). *E. coli* were transformed with the ligation reactions to recover recombinant clones, which were subjected to double digest with KpnI and XbaI and screened through agarose gel electrophoresis, confirming the successful insertion of apoE cDNAs (Figure 8B). The clones were also screened by Sanger sequencing to confirm the presence of the correct apoE isoform insert—E2, E3, or E4. Unexpectedly, the Sanger sequencing results indicated a 20 bp deletion was upstream of the apoE insert in the pcDNA3-apoE2 clones (Figure 9). Although the deletion was not within the apoE2 coding sequence, we were concerned that the deletion could impact expression levels of apoE2 versus apoE3 and apoE4 from their respective constructs,
Perhaps complicating interpretation of experimental results. Thus, pcDNA3-apoE2 was generated from pcDNA3-apoE3 through site-directed mutagenesis to introduce C112R. pcDNA3-apoE2, pcDNA3-apoE3, and pcDNA3-apoE4 sequences were then finally confirmed through Sanger sequencing (Figure 10).

As previously discussed, the original goal of my thesis project was to evaluate apoE isoform-specific effects on neuritogenesis, using PC12 cells as a model system. PC12 cells respond to nerve growth factor (NGF) by differentiating into neuronal-like cells, but in order to elicit a robust differentiation response, the cells must be maintained in low-serum medium [31]. However, exposure to reduced growth factors also inhibits protein synthesis [31] so we needed to confirm that PC12 cells would continue to
express apoE in the absence of growth factors. PC12 cells were plated in collagen-coated 6-well plates and transfected with pcDNA3 vector or pcDNA3-apoE3 in full-serum medium. Cells were maintained in full-serum medium for 24 hours, after which medium was replaced with full-serum medium or low-serum medium. Cells in low-serum medium were treated with NGF 24 hours after the medium change. Conditioned medium was collected over the course of five days in Laemmli buffer and subjected to SDS-PAGE and Western blot to detect apoE protein. ApoE was not detected in the conditioned medium of the transfected cells grown in low-serum medium compared to cells kept in full-serum medium. (Figure 11). Unfortunately, these data indicated that our experimental system to evaluate isoform-specific effects of apoE on neuritogenesis was not technically feasible.

**Figure 11. ApoE expression in PC12 cells.** PC12 cells were transfected with pcDNA3-apoE3 and grown in either full-serum medium or Low-serum media. Conditioned medium samples were subjected to SDS-PAGE and Western blot to detect apoE.

In December of 2020, I gave a journal club presentation on a recent publication from Dr. Thomas C. Südof’s lab [32], in which they demonstrated apoE isoform-specific activation of the ERK/MAP kinase pathway in neurons upon treatment with conditioned medium from HEK-293 cells transfected with apoE expression constructs. They reported that apoE in the conditioned medium was usually 50-80x more concentrated than their desired treatment dose (10 μg/mL). They also reported a simple method for quantification of apoE using a recombinant protein standard, which can be used to generate a densitometric standard curve to interpolate the concentrations of
apoE in the conditioned medium [33]. If HEK-293 cells could produce such a high concentration of apoE, the medium could be diluted enough to significantly reduce the concentration of serum proteins, thus providing a source of recombinant apoE that we could introduce to PC12 cells without introducing large amounts of serum proteins. To evaluate this approach as an alternative to PC12 transfections, HEK293 cells were transfected with pcDNA3-apoE3 and conditioned medium was collected over a course of six days. Huang et al. (2017) conducted densitometry on Coomassie-stained SDS-PAGE gels run with conditioned medium from HEK-293 cells. Coomassie true-blue staining of SDS-PAGE gels run with conditioned medium collected from our HEK-293 cells revealed a strikingly abundant amount of serum proteins in the medium, which made discerning the apoE protein from the rest of the proteins in the medium impractical (Figure 12A). Therefore, Western blot was conducted on the conditioned medium samples alongside E. coli-derived recombinant apoE standards (Figure 12B). The apoE concentration in the medium was significantly below the concentration used in the standards, but we approximated that the concentration of apoE in the conditioned medium was about 25% of the lowest concentration used in the standard (10 ng/lane). If we were to treat PC12 cells with the same concentration of apoE as Huang et al. (2017) used in their experiments when treating neurons, we would need to treat the cells with 10 μg/mL apoE.

Given that each lane of the gel contains 7.5 μl of conditioned medium (15 μl per lane, 1:1 Laemmli to conditioned medium), the amount of conditioned medium required to treat a 60 mm tissue culture plate with PC12 cells grown in 4 mL of medium could be approximated as follows:

\[
40 \mu g \text{ apoE} \left( \frac{1000 \text{ ng}}{1 \mu g} \right) \left( \frac{7.5 \mu l}{10 \text{ ng}} \right) = 30 \text{ mL of conditioned media per plate}
\]

Since this treatment dose would be physically impossible to administer, we were forced to reconsider our methodology.
Figure 12. ApoE expression in HEK-293 cells. HEK-293 cells were transfected with pcDNA3-apoE3 and monitored over six days. To ensure cells remained viable through the six-day timecourse, HEK-293 cells were plated at two different densities. (A) Coomassie staining of HEK-293 conditioned medium. (B) Western blot with *E. coli* apoE standards probing for apoE.
Huang et al. (2019) referred to a specialized line of HEK-293 cells, FreeStyle 293-F cells, which are cultured in suspension in serum-free medium. Based on the abundance of serum proteins present in medium conditioned by standard HEK-293 cell, we considered the use of FreeStyle 293-F cells as a source of mammalian-derived recombinant apoE protein. Thus, 293-F cells were acquired and transfected with pcDNA3-apoE3 or pcDNA3 vector and monitored over the course of four days. At each time point, a 500 µl fraction of each culture was collected and subjected to centrifugation at 7,900 x g in a table-top microcentrifuge for 5 minutes. The resulting cell pellet and supernatant—the conditioned medium—were then separated. The cell pellet was suspended in 200 µl of Laemmli buffer, the conditioned medium was combined 1:1 with Laemmli buffer, and the samples were boiled for 5–10 minutes to lyse the cell pellet and denature the proteins in all samples. Equal volumes of each sample were then subjected to SDS-PAGE and Western blot to detect apoE and β-actin (Figure 13A). As expected, apoE was detected in the conditioned medium of pcDNA3-apoE3–transfected cells and accumulated through the course of 4 days, whereas apoE was not detected in conditioned medium 4 days after transfection with with pcDNA3 empty vector. ApoE3 was also detected in the lysates of pcDNA3-apoE3–transfected cells, although its levels were largely constant throughout 4 days, reflecting the fact that newly synthesized apoE3 was continuously secreted, yielding a steady-state level of apoE3 in the cells over time. We also failed to detect β-actin in the conditioned medium samples, confirming that the cells were separated from the conditioned medium successfully. Total protein staining with Coomassie of SDS-PAGE gel with E. coli apoE standards revealed that apoE was the predominant protein in the conditioned medium (Figure 13B), a shocking contrast from the total protein-staining of the full-serum HEK-293 medium (see Figure 12A). 293-F cells were then transfected with pcDNA3-apoE2, apoE3, or apoE4 and conditioned medium was collected over five days for SDS-PAGE and Western blot analysis (Figure 13B). ApoE2, apoE3, and apoE4 accumulated in a time-dependent manner, peaking at 4 days post transfection.

In order to produce as much apoE in the conditioned media as possible, we needed to determine the optimum transfection conditions for protein expression. Cell viability of HEK-293-
F cells cultured in FreeStyle Expression Medium supplemented with penicillin + streptomycin (P/S) was stable during cell line maintenance, but once transfected with pcDNA3 vector, crude analysis by Trypan Blue staining suggested that cell viability decreased significantly. Thus, to quantify whether P/S affects cell viability during transfections, we then assessed whether culturing cells in the absence of P/S would rescue cell viability during transfection. Cells transfected with pcDNA3 in the presence of P/S exhibited a 20% decrease in viability, while cells grown in the absence of P/S were more than 95% viable following transfection (Figure 14A). These data indicated that, while 293-F cells appear to withstand the presence of P/S in their medium under standard culturing conditions, the inclusion of P/S during transfection causes undesired cytotoxic effects. Not only would this cytotoxicity likely reduce yields of apoE, it could also result in the release of cellular factors from dying cells that could complicate the effects of apoE treatments. Thus, these data provided strong incentive to exclude P/S from the cultures during our transfection experiments.

The manufacturer of 293-F cells (Invitrogen) recommends that transfection mixtures be prepared in Opti-MEM low-serum medium for optimal transfection efficiency, so we also compared transfection efficiency of cells transfected with pGFP using FreeStyle Expression Medium or Opti-MEM low-serum medium in the transfection mixtures. Transfection efficiency increased from 67% to 77% in the presence of Opti-MEM (Figure 14B), so we decided to continue using Opti-MEM for future transfections.

A significant challenge in working with recombinant apoE is quantifying its concentration, as ELISA often yields unreliable data (unpublished communications with Dr. Bradley T. Hyman’s laboratory, Massachusetts General Hospital) and serum proteins can obscure other protein quantification methods such as BCA and Bradford assays. Huang et al. (2017) used Coomassie staining of conditioned media from 293-F cells alongside a series of protein standards to produce
Figure 13. Characterization of apoE expression in FreeStyle cells. (A) 293F cells were transfected with pcDNA3 vector or pcDNA3-ApoE3. Conditioned medium and cell lysates were collected over four days and subjected to SDS-PAGE and Western blot alongside 100 ng His-ApoE purified from E. coli, CM=Vector Day 3 conditioned medium, CL=Vector Day 3 cell lysates; (B) 293F cells were transfected with apoE2, apoE3, or apoE4 and conditioned medium was collected over five days. Conditioned medium samples were subjected to SDS-PAGE alongside 100 ng His-ApoE purified from E. coli. Gels were then stained with Coomassie protein stain or subjected to Western blot.
Figure 14. Determination of transfection efficiency. (A) 293F cells were transfected with pcDNA3 in the presence or absence of P/S and monitored over the course of three days. Cell counts were collected with a hemocytometer using Trypan Blue as a viability marker (B) 293F cells were transfected with pGFP and subjected to flow cytometry. Transfection efficiency was determined as a measure of FITC absorption by positively transfected cells expressing pGFP.

a densitometric standard curve from which the concentration of the conditioned media can be approximated. To test this strategy, conditioned media produced through apoE2, apoE3, and apoE4 transfections were subjected to SDS-PAGE alongside apoE produced from *E. coli* to use as standards, which had been successfully quantified by BCA assay. Gels were stained with Coomassie Instant Blue stain and scanned using a desktop office scanner. Linear regression of the concentration of apoE in the protein standards plotted against the area under the peak (AU)
Figure 15. Quantification of apoE2, apoE3, and apoE4 protein levels. (A) 293F cells were transfected with (A) pcDNA3-ApoE2, (B) pcDNA3-ApoE3, and (C) pcDNA3-ApoE4. Conditioned medium was collected over the course of five days and subjected to SDS-PAGE alongside a His-ApoE protein standard. A densitometric standard curve was generated for each gel and used to interpolate the concentration of apoE in the conditioned medium samples. (D) Violin plot for E. coli apoE standard curves from apoE2, apoE3, and apoE4 quantification gels.
collected through ImageJ GelAnalyzer produced a remarkably well-fit regression lines ($r^2 = 0.986$, $r^2 = 0.999$, $r^2 = 0.980$) (Figure 15A-C). AU for the protein standards was generally consistent between gels, but became more variable at higher concentrations (Figure 15D). Interpolation of apoE concentrations from the densitometric standard curve revealed surprisingly high concentrations of apoE, reaching 200-300 μg/mL four days post-transfection.

Protein is generally more stable during storage at higher concentrations and generating concentrated sources of apoE is beneficial for conducting experimental treatments. Thus, experiments were conducted to concentrate apoE secreted from HEK 293-F cells. To do so, HEK 293-F cells were transfected with pcDNA3-apoE2, apoE3, apoE4, or pcDNA3 vector and conditioned media was collected after 5 days. Conditioned medium was concentrated using 10 kDa molecular weight cutoff centrifugal filter units and then subjected to SDS-PAGE. ApoE was not detected in the flowthrough fractions, but concentration of the medium revealed the presence of extraneous proteins undetected in the unconcentrated medium (Figure 16A). Quantification of the concentrated medium demonstrated that the concentrations were 1-3 mg/mL, indicating a 5-10-fold increase from unconcentrated medium (Figure 16B).
Figure 16. Concentrating FreeStyle apoE. Conditioned medium from FreeStyle cells transfected with pcDNA3-ApoE2, ApoE3, apoE4 or vector was collected on D5 and concentrated with Millipore filter units with a 10 kDa molecular weight cutoff. (A) Undiluted fractions of unconcentrated (UC), concentrated (C), or flowthrough (FT) from the filter units. (B) Concentrated apoE was diluted and subjected to SDS-PAGE alongside His-ApoE standard. (C) Standard curve generated from densitometric analysis of E. coli apoE standard. (D) ApoE was then quantified as described previously. The mean concentration interpolated from each dilution was calculated.
DISCUSSION

From the generation of apoE expression constructs to the quantification of secreted apoE protein levels, this project establishes the tools necessary to study apoE in the context of AD in mammalian cell culture. Conditioned medium produced by HEK 293-F cells contains minimal protein aside from secreted recombinant apoE, which is highly concentrated and easily quantifiable. Additionally, we observed a distinct banding pattern in recombinant apoE secreted by HEK-293-F cells that was not observed in apoE produced from *E. coli* indicative of post-translational modifications (see Figure 15 and Figure 16). Post-translational modifications have an established role in regulating protein function, and apoE has been reported to be subject to methylation [34], S-nitrosylation [35], glycosylation [36], sialylation [37], as well as other modifications, often in an isoform-specific manner [38]. Evidence of post-translational modifications in apoE produced from 293-F cells provides increased confidence in the potential applications of the FreeStyle System. Recent studies in our lab (Durant, unpublished) show promising results in the use of apoE produced from 293-F cells in thermal shift assays, and experiments treating PC12 and N2A cells with apoE conditioned medium are ongoing. The groundwork has been laid, and I am thrilled to begin using this system for my Master’s thesis at Bridgewater State University, where I will (finally) begin studying apoE isoform-specific effects in mammalian cell culture.
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