Mammalian Cell Line Development Platform for Recombinant Protein Production: Expanding the Protein Expression Toolbox for Research and Drug Discovery Applications

Elizabeth Del Greco
Dominican University of California

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Mammalian Cell Line Development Platform For Recombinant Protein Production:
Expanding the Protein Expression Toolbox for Research and Drug Discovery

Applications

By
Elizabeth Anne Del Greco

A culminating thesis submitted to the faculty of Dominican University of California in partial fulfillment of the requirements for the degree of Master of Science in Biology

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This thesis, written under the direction of candidate’s thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree of Master of Science in Biology at Dominican University of California. The written content presented in this work represent the work of the candidate alone.

Elizabeth Del Greco 6/26/2017
Candidate

Vishal Agrawal 6/28/2017
Thesis Advisor

Ekaterina Kalashnikova 6/28/2017
Second Reader

Maggie Louie 6/28/2017
Program Director
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Abstract

Recombinant proteins have revolutionized the biomedical industry, providing therapeutics for life-threatening diseases and protein reagents for research applications. BioMarin Pharmaceutical Inc. develops recombinant protein therapeutics to treat rare diseases including lysosomal storage disorders (LSDs), a group of about 50 individually rare disorders together affecting 1 in 8,000 live births. With an increase in the number of novel therapeutics in our drug discovery pipeline, there is a high demand to produce a variety of recombinant proteins for early-stage drug development projects. In order to equip our protein production process with the tools and capability for diverse protein expression, it is valuable to expand our expression toolbox with high-expressing platforms. The goal of this project is to expand to our current expression platforms by developing a murine myeloma based expression system with SP2/0 cells as a host. Since the SP2/0 cell line is amongst the most commonly used cell lines for therapeutic and reagent protein production, developing a SP2/0 expression system may offer additional benefits to our recombinant protein production needs including: expression of difficult-to-express proteins, improving titers, and extending recombinant cell line stability. A lysosomal enzyme therapeutic candidate is expressed in the SP2/0 cells as a proof-of-concept for developing this protein expression platform. To this end, we have shown that SP2/0 cells can be grown to a high density in commercially available serum-free media with a doubling time of less than twenty four hours. A clone isolation strategy was used to pick the top clone expressing high levels of recombinant protein. Using the highest expressing clone, we developed a high yielding bioprocess at a two liter scale to
demonstrate the utility of this system for generating recombinant proteins at large scale. Furthermore, the therapeutic properties of the recombinant protein expressed in SP2/0 cells are similar to the recombinant protein expressed in CHO cell lines, demonstrating similar uptake into diseased cells ($K_{\text{uptake}}$ values) and binding affinity to the receptor responsible for drug mediated cellular uptake. Thus, the SP2/0 expression system proves to be a valuable addition to our expression toolbox for the production of research-grade protein therapeutics for cell-based assays.
1.0 INTRODUCTION

Development of recombinant proteins for therapeutic and research purposes represent one of the fastest expanding branches of the biotech industry. BioMarin Pharmaceutical Inc. utilizes recombinant protein technology to develop protein treatments for patients with rare genetic disorders including lysosomal storage diseases (LSDs). Although each LSD is individually rare, the entire group of LSDs affect 1 in 8,000 live births and make up a significant fraction of diseases caused by inborn errors of metabolism (Platt, Boland, & van der Spoel, 2012; M. Xu et al., 2016). LSDs are characterized by disruption of lysosomal homeostasis, due to the deficiency of a lysosomal enzyme, lysosomal membrane protein, or other proteins necessary for lysosomal biogenesis. Since the lysosome is essential for degrading and recycling macromolecules from autophagy, endocytosis, and phagocytosis, cell metabolism is significantly impacted when the lysosome malfunctions (Appelqvist, Wäster, Kågedal, & Öllinger, 2013). For example, lysosomal disruption eliminates downstream recycled products necessary for cellular functions, causing cellular problems including mitochondrial dysfunction, inflammation, altered calcium homeostasis, and accumulation of toxic waste products (Appelqvist et al., 2013). Patients with LSDs display a range of clinical symptoms depending on the type of LSD. Generally, clinical phenotypes of LSDs include damage to the central nervous system, bone abnormalities, and organomegaly (Meikle, Hopwood, Clague, & Carey, 1999). The severity of the symptoms from LSDs vary depending on the type of accumulating substrate, the amount of functioning hydrolase activity, and the tissue affected by the accumulating substrate.
Currently, there are limited options to improve or restore the enzymatic activity of the missing lysosomal hydrolase in patients with LSDs. Current therapeutic approaches include hematopoietic stem cell transplantation, enzyme replacement therapy, gene therapy, substrate reduction therapy, and chaperone therapy (Bruni, LoSchi, coppa, & Bruni, 2007). Enzyme replacement therapy (ERT) is currently the most widely-used therapy to treat LSDs especially in the cases where lysosomal enzyme production is affected (Parenti, Pignata, Pietro, & Salerno, 2013). As the name suggests, ERT is the therapeutic approach to replace or restore deficient enzymatic activity by delivering the functional recombinant lysosomal enzymes into the cell and its lysosome. This critical step of delivering the drug to the lysosomal compartment is achieved by endocytosis of the recombinant protein through the binding with mannose-6-phosphate (M6P) receptors. In 1990, the first successful ERT used recombinant galactocerebrosidase to treat patients with Gaucher’s disease (Neufeld, 2006). As of 2013, commercially available ERT treatments are available for six LSDs (Ratko, Marbella, Godfrey, & Aronson, 2013).

A key component to producing an effective ERT is to ensure that the recombinant enzyme contains M6P residues on N-glycans, glycans that are covalently bound to asparagine residues on a protein (Stanley, Schachter, & Taniguchi, 2009). The mannose-6-phosphate residues serve as a recognition marker for the transport of newly-synthesized enzymes into the lysosome (Figure 1). In healthy cells, lysosomal enzymes are synthesized and glycosylated on the N-linked oligosaccharide residues in the endoplasmic reticulum. Then, lysosomal enzymes are modified with M6P residues during a two-step reaction process in the Golgi apparatus. During the first step, a
phosphotransferase, UDP-N-acetylglucosamine 1-phosphotransferase (GlcNAc-1-
phosphotransferase) catalyzes a reaction allowing the transfer of a GlcNAc-1-phosphate residue from UDP-GlcNAc to specific mannose sites on the N-linked oligosaccharides on the lysosomal enzymes (Coutinho, Prata, & Alves, 2012). The second step requires an uncovering enzyme, N-acetylglucosamine-1-phosphodiester α-N-acetyl-glucosaminidase (UCE), which removes the GlcNAc residue, uncovering the M6P residue (Coutinho et al., 2012). At the trans-face of the Golgi, the lysosomal enzymes with the M6P recognition marker are separated from other newly-synthesized proteins by binding to M6P receptors. Next, the lysosomal enzymes bound to the M6P receptors are packaged in clathrin-coated vesicles and bud off from the Golgi apparatus. The clathrin-coated vesicles containing the lysosomal enzyme then fuse with late endosomes (Coutinho et al., 2012). As the internal pH of the endosome lowers during lysosomal maturation, the lysosomal enzymes become active and can function to degrade macromolecules within the cell.
Figure 1. Transport of lysosomal enzymes to the lysosome

Newly synthesized lysosomal proteins are transported to the lysosome by the mannose-6-phosphate pathway.

Source: Coutinho et al., 2012

In addition to the transport of newly synthesized enzymes to the lysosome, M6P residues are also important for the internalization of extracellular lysosomal enzymes, for example ERTs (Figure 2). Two types of mannose-6-phosphate receptors, the cation-independent M6P receptor (CI-M6PR) and the cation-dependent M6P receptor (CD-M6PR) are scattered along the surface of the plasma membrane. Upon binding of an extracellular M6P-marked enzyme, to the CI-M6PR, the enzyme is endocytosed and can be transported to the lysosome. Although the CD-M6PR and the CI-M6PR both mediate
trafficking of newly-synthesized endogenous lysosomal enzymes to the lysosome, extracellular M6P-marked lysosomal enzymes are only internalized into the cell upon binding to the CI-M6PR (Saftig, 2006). Another key component of a successful ERT is to manufacture the lysosomal enzyme in its active conformation. ERTs need to be active to degrade accumulating macromolecules within diseased cells. Due to the critical role of M6P residues, all ERT production host candidates must have complex glycosylation capabilities.

Figure 2. Cellular uptake of recombinant enzymes by M6P pathway

Extracellular uptake of extracellular recombinant enzymes including ERTs, are mediated by the M6P. Binding of extracellular M6P-marked lysosomal enzymes to the M6P receptors on the plasma membrane of the cell initiates enzyme delivery to the lysosome by the endocytic route (dotted arrows).

Source: Parenti et al., 2013
Most commonly, *Escherichia coli* (*E. coli*), mammalian cells, yeast, or baculovirus-infected insect cells are used as hosts to produce biologics. While producing biologics in bacteria is cheaper, faster to grow, and yield higher titers compared to mammalian cells, bacteria lack appropriate machinery to post-translationally modify proteins with glycoproteins, a critical component to biologic drug efficacy and uptake efficiency (Jayapal, Wlaschin, Hu, & Yap, 2007). Most biologics require human glycosylations, usually N-glycans, where the glycosylation site is on the asparagine residue (e.g. ERTs), and sometimes O-glycans, where the glycosylation site is on the serine or threonine residues (Ghaderi, Zhang, Hurtado-Ziola, & Varki, 2012; Solá & Griebenow, 2010). Variation in glycan branching pattern, length, and the arrangement of monosaccharides also add complexity to N-glycan structure (Solá & Griebenow, 2010). All N-glycans fall into one of the three categories: high-mannose, complex, or hybrid (Figure 3). The type of N-glycan structure added to a recombinant protein is dependent on the cellular machinery of the cell host. M6P residues are usually added to complex N-glycans on human lysosomal enzymes. Furthermore, human proteins have additional structural variation since the terminal ends of the glycan contain variations of chemically-charged glycans, such as sialic acid. These chemically-charged glycans are important for drug efficacy because they affect the isoelectric point (pI) and the surface charge of the protein therapeutic (Solá & Griebenow, 2010). Ideally, protein therapeutics should mimic human protein glycosylations to improve drug efficacy and minimize immunogenicity to the drug. Recently, new genetic modifications in yeast and insect cells have improved the cell’s capability of post-translationally modifying proteins. However, post-translational
modifications (PTMs) from yeast and insect cells are still limited to simple PTMs or do not have the complex human glycosylation patterns, impairing drug quality (Figure 4).

**Figure 3. Types of N-glycans**

The structures of the three major categories of N-glycans: high mannose, complex, and hybrid. All N-glycan types are added to proteins on the asparagine residues. All structures have two N-acetylglicosamine (GlcNAc) residues followed by three mannose residues.

Source: Higel, Seidl, Sorgel, & Friess, 2016
Above are examples of N-glycan structures added to biologics in different eukaryotic expression platforms during protein production. The first step of N-glycan synthesis occurs in the endoplasmic reticulum (ER), where glycosylation machinery in all species are highly conserved, generating the common Man$_3$GlcNAc$_2$ structure. Glycosylation in the Golgi apparatus varies greatly among different species, contributing to N-glycan variation. Varying residues between species include N-glycolylneuraminic acid (Neu5Gc), galactose (Gal), fucose (Fuc), N-Acetylneuraminic acid (Neu5Ac), xylose (Xyl), and mannose (Man). Since animal (mammalian) N-glycan structures are most similar to humans, mammalian cell platforms are the system of choice for the production of complex protein therapeutics or reagents. Humans generate antibodies to the Neu5Gc and Galα1-3Galβ1-(3)4GlcNAc (alpha-Gal) epitopes (shown by black arrows), making biologics with these structures more immunogenic.

Source: Ghaderi et al., 2012

Owing to the need for complex PTMs, ERTs are usually produced in mammalian cell lines. Currently, only a few mammalian cells have the ability to produce biologics with human-like N-glycans, including but not limited to, Chinese hamster ovary (CHO) cells, SP2/0 (murine myeloma), and NS0 cells (murine myeloma). However, selecting
host cells for biologic production requires more consideration than choosing between a platform with or without complex PTM capabilities. Human-like glycans produced by animal cells still exhibit differences that are immunogenic to humans. Specifically, humans produce antibodies to two structures commonly present on non-human mammalian cells, the Galα1-3Galβ1-(3)4GlcNAc (alpha-Gal) epitope and N-glycolylneuraminic acid (Neu5Gc), a sialic acid (Ghaderi et al., 2012) (Figure 4). Over the past few years, research efforts have been put into engineering cell lines that mimic human glycosylation patterns, and reduce alpha-Gal and Neu5Gc. For example, the enzyme, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH), is responsible for catalyzing N-acetylneuraminic acid (Neu5Ac) to Neu5Gc and is active and highly expressed in mammals but deficient in humans (Xu et al., 2012). Gene editing can be utilized to knockout CMAH, removing the Neu5Gc epitope (Beaton et al., 2015). In addition, the gene, glycoprotein alpha-galactosyltransferase 1 (GGTA1), can also be knocked-out to remove the presence of the alpha-Gal epitope (Beaton et al., 2015).

Currently, CHO cells are the workhorse of the biopharmaceutical industry, producing about 70% of the FDA-approved biologics on the market (Jayapal et al., 2007). The first commercial therapeutic protein, tissue plasminogen activator (tPA) was produced in CHO cells (Wurm, 2004). Ever since, CHO cells have gained increasing popularity for biologic production due to its FDA regulatory approval rate, ability to produce protein with human-like N-glycans, high protein production yields, and can quickly grow in serum-free media, high cell densities and large-scale bioreactors (Ghaderi et al., 2012). Proteins expressed in CHO expression systems are more
compatible with the human immune system compared to other mammalian cell lines due to their ability to modify proteins with human-like N-glycan residues (Ghaderi et al., 2012). Although more compatible than most mammalian systems, CHO cells add the Neu5Gc and the alpha-Gal epitope to the recombinant protein which can result in an immunogenic response in humans (Ghaderi et al., 2012). CHO cells are also limited by phenotypic drift, affecting desired phenotypic properties of a clonal cell line including product quality, cell growth, and viability during the production process (Bandaranayake & Almo, 2014).

Myeloma cells, such as SP2/0 and NS0, naturally secrete high amounts of antibodies, making them a logical host for protein manufacturing. Murine myeloma cells still make up the second most popular choice of mammalian expression platforms due to high regulatory approval of antibody therapeutics. Due to high regulatory approval, murine myeloma cells are known to produce some of the top-selling blockbuster therapeutics, including Remicade (infliximab), generating over $8.37 billion annually (Walsh, 2014). Myeloma cells originate from immunoglobulin-producing tumor cells, but were genetically engineered to eliminate the production of its native immunoglobulin (Markusen & Robinson, 2013). As a result, the required machinery for protein production remains available for the manufacturing process of recombinant proteins. Together, SP2/0 and NS0 cells are the most popular antibody production platform due to their ability to produce high protein titers, add human α2-6 sialyl linkages, and have much higher sialylation compared to CHO cells (Ghaderi et al., 2012). In addition, murine myeloma cells are the fusion partner for antibody-producing hybridoma cell lines.
However, recombinant proteins expressed in myeloma cells are limited by higher levels of alpha-Gal and Neu5Gc compared to CHO, making myeloma products more immunogenic (Ghaderi et al., 2012). To prevent immunogenic responses, careful dosing of a myeloma-produced biologic is required (Ghaderi et al., 2012). With the high protein productivities and gene editing technology to remove or minimize immunogenicity, murine myeloma cell lines remain a top choice for recombinant protein production and still have the capability of producing high quality proteins for research applications.

The traditional method to produce protein therapeutics is to express the therapeutic protein in a stable cell line. The protein therapeutic is usually encoded in genetic material with the sequences for the protein of interest and a selectable marker encoding a resistance gene. Due to increasing regulatory requirements, most biologic-producing cell lines are usually selected using metabolic selection methods instead of antibiotic selection. The use of antibiotic resistant markers is discouraged for biologic production in order to avoid horizontal genetic transfer of antibiotic resistant genes to bacteria that may be present in the normal microflora or the environment (Mignon, Sodoyer, & Werle, 2015). The genetic material is transfected into the host cell and is subjected to rounds of selection pressure, giving a survival advantage to cells successfully transfected with the selectable marker (Wurm, 2004). A stable pool of successfully transfected cells will grow, expressing the protein of interest. Next, a single clonal cell expressing the recombinant protein is isolated into its own cell culture, usually performed in individual wells within a 96-well cell culture plate. Clonal cells are then expanded into larger culture volumes, where they can then be screened by various assays
to ensure that the recombinant protein is expressed. After confirmation that the clonal cell expresses the recombinant protein, the clone will be scaled up to liter-scale volumes in bioreactors. The cells and media are harvested days later after the start of a production run in the bioreactor. The collected harvest is later purified and stored for future use.

One of the most commonly used metabolic selection methods to isolate a high-expressing clone is Lonza’s glutamine synthetase (GS) system, which is the selection method for the manufacture of 29 FDA-approved therapeutics (Lai, Yang, & Ng, 2013). Glutamine is essential for cell growth and metabolism. The glutamine synthetase (GS) selection method utilizes mammalian cell’s dependence on glutamine for survival. In glutamine-free culture conditions, cells rely on GS, an enzyme which catalyzes the synthesis of glutamine from glutamate and ammonia. The method works best using host cells with low or no endogenous GS expression (e.g. murine myeloma cells), promoting survival of the cells successfully transfected with a genetic construct encoding glutamine synthetase and the recombinant protein of interest. The GS system can also be used to amplify recombinant protein expression by using methionine sulfoximine (MSX), an inhibitor of GS. The overexpression of the recombinant protein is the result of cellular survival mechanisms for adaptation to increasing selection stringency. Adaptation to selection pressure can be accomplished by gene amplification of the integrated vector, or more efficient transcription (Zhang, 2010). Usually, the GS selection marker and the recombinant protein are closely linked in the genetic construct allowing the recombinant protein to be co-amplified with GS. This is an effective selection method to use while the cells are recovering after transfection and during the cloning process.
With an increase in the number of novel therapeutics in our drug discovery pipeline, there is a high demand to produce a variety of recombinant proteins, including biologics and reagent proteins, for early-stage drug development projects. Currently, the main challenges of biotherapeutic and protein reagent production are the high cost, low production yields, and lengthy process of cell line development. For these reasons, most research efforts in protein production are focused on improving the speed of the production process and boosting the recombinant protein yield. Furthermore, some proteins are considered difficult-to-express because they may aggregate, demonstrate protein instability, or they could be modified without critical PTMS for proper functioning. Some proteins are also considered difficult-to-express if the expressed protein is toxic to the host cell. Lysosomal enzyme recombinant therapeutics fall under the difficult-to-express protein category for several reasons. First, it is estimated that only 10-20% of the overexpressed lysosomal enzyme expressed is secreted, with the rest of the lysosomal enzymes localizing to the host cells’ lysosomes (Migani, Smales, & Bracewell, 2017). This reduces the secreted protein yield available for harvesting and potentially causes a LSD within the production host itself. Various techniques are currently available to overcome some of the factors that cause a protein to be difficult-to-express. For example, a common issue during the protein production process is that a cell line can undergo cell death early during a production as a result of shear stress or nutrient deprivation, lowering the amount of cells available for protein production. To increase cell productivity, the cells can be co-transfected with the gene encoding the recombinant protein with an anti-apoptotic gene such as, Bcl-XL or Bcl-2, extending cell line viability.
However, the production of some difficult-to-express proteins may pose several different problems involving many cellular pathways that need to be adjusted. Instead of finding ways to implement many changes in the host cells’ biochemistry, it is faster and usually more effective to change expression platforms. Having multiple expression platforms is invaluable during cell line development process for efficient production of recombinant proteins.

The goal of this project is to expand to our current expression systems for difficult-to-express proteins by developing a protein production platform using the SP2/0 myeloma cell line as a host. By expanding our expression toolbox with high-expressing platforms, we can equip our protein production process with the tools and capability for diverse protein expression. Since the SP2/0 cell line is amongst the most commonly used cell lines for therapeutic and reagent protein production, developing a SP2/0 expression system may offer additional benefits to our recombinant protein production needs including: expression of difficult-to-express proteins, improving titers, and extending recombinant cell line stability. A lysosomal enzyme therapeutic candidate, a difficult-to-express protein, was expressed in SP2/0 cells as a proof-of-concept for developing this protein expression platform. Using high-resolution imaging in conjunction with a GS selection method, we isolated a high-yielding SP2/0 clone. The bioprocess for the high-yielding clone was optimized to establish a working SP2/0 protein production method, boost production titers, and maintain high cell viability during protein production. Using the optimized SP2/0 expression platform, milligram amounts of the ERT was generated for a series of assays for product quality characterization including its glycosylation
profile and cellular uptake.

## 2.0 RESEARCH DESIGN AND METHODS

### 2.1 Cell Culture

SP2/0 cells were maintained in a proprietary myeloma medium (referred to in this paper as SP2/0-BMN media) supplemented with 8 mM GlutaMAX (Life Technologies, Grand Island, NY) in shake flasks. The SP2/0 cells were incubated at 37°C, 125 RPM, and 8% CO₂. Cell cultures were passaged 2-3 times a week, at a seeding density of 0.5 to 2 x 10⁶ cells per mL. This maintenance culture was used for early experiments and transfections for this project where wild-type SP2/0 cells were needed. The cell culture was kept under 20 passages for the experiments.

Primary human fibroblast cells with the lysosomal storage disease of interest (Coriell, Camden, NJ), were grown in DMEM with L-glutamine (ThermoFisher Scientific, Rockford, IL) supplemented with 15% fetal bovine serum (VWR, Radnor, PA). Cell cultures were passaged when cells reached 90% confluence, and were maintained in 150-cm² tissue culture flasks (Corning, Manassas, VA) at incubation conditions of 37°C and 5% CO₂. All cells were kept under 20 passages for the experiments.

### 2.2 Total Protein Extraction from Cell Lysates

Total protein was extracted from cell lysates using the manufacturer’s instructions included with the Pro-Prep Protein Extraction Solution. The cell pellet was washed once in 1 X Dulbecco’s phosphate buffered saline (DPBS) (Corning) and removed after
centrifugation at 250 x g for 5 minutes. Excess DPBS wash was removed after a second round of centrifugation at 13,000 RPM for 30 seconds. The cell pellet was resuspended in 400 µl prep protein extraction solution for approximately every 5 x 10^6 cells. The cell and lysis buffer solution was then incubated at -20 °C for 20 minutes. After incubation, the cells were spun down at 13,000 RPM for 10 minutes. The supernatant, which contains the cell lysates, was carefully removed and stored in a separated microcentrifuge tube.

2.3 BCA Assay

A BCA assay (Pierce, Rockford, IL) was performed for each sample, using the manufacturer’s protocol, to estimate total protein concentration. Each sample was done in duplicate at three different dilutions, 2X, 5X, and 10X, plated at a volume of 10 µl per well in a 96 well plate. 24.5 ml Reagent A and 0.5 ml Reagent B were mixed and immediately added to each of the wells at a volume of 200 µl per well. Plates were incubated for 30 minutes at 37°C then were immediately read using a plate reader at a wavelength of 562 nm. Each sample concentration was estimated using the average of the duplicate wells which were within range of the standard curve.

2.4 Western Blots and Ponceau S Staining

Protein samples, cell lysates, and media samples with secreted proteins were treated with NuPAGE 1X LDS buffer (Life Technologies, Carlsbad, CA). The protein samples were then denatured by heating at 95˚C for 10 minutes, and separated in NuPAGE 1.0 mm 4-12% Bis-Tris Gel (Life Technologies). The protein was transferred from the gel onto a nitrocellulose membrane using the iBlot 2 device (Life Technologies)
at 20V, for 7 minutes. Following the transfer, the membrane was stained using one 5 minute wash of 1X Ponceau S stain in 5% acetic acid followed by two washes in 5% (v/v) acetic acid for 5 minutes each, then two washes in distilled water for 5 minutes each. The membrane was imaged using a photo scanner to provide an image of total protein, demonstrating equal loading. After imaging, the membrane was then washed in StartingBlock T20 (TBS) blocking buffer (Thermo Fisher Scientific) for 1 hour. The primary antibodies (listed per experiment, with the exception of proprietary antibodies) were diluted at 1:1,000 in StartingBlock T20 (TBS) blocking buffer. The secondary antibodies (listed per experiment) were diluted to 1:5000 in StartingBlock T20 (TBS) blocking buffer. Blots were visualized by adding Western blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI) or if using an IRDye 800CW antibody, imaged using the Odyssey CL Imaging system (LI-COR Lincoln, Nebraska).

### 2.5 Development of a SP2/0 Cell Line Expressing an ERT as a Proof-of-Concept Model

#### 2.5.1 Testing the Glutamine Synthetase (GS) Selection System in SP2/0 Cells

In order to determine whether expressing the GS gene can be used as an appropriate metabolic marker for successfully transfected SP2/0 cells, SP2/0 cell growth and viability were monitored in the presence and absence of glutamine. Three other cell lines were also tested for comparison. Each cell line was seeded into shake flasks with different proprietary basal media, each of which is optimal for cell viability and growth specific to each cell line. SP2/0 cells were seeded in SP2/0-BMN media. In total, four
different cell lines were tested: SP2/0, wild-type CHO, GSKO CHO cells, and SP2/0 cells transfected with a vector encoding GS. The flasks were seeded at a cell density of $5 \times 10^5$ cells per ml in 30 ml of basal media supplemented with 8 mM GlutaMAX and another flask with the same basal media but lacking glutamine. 1 ml sample was collected daily for 8 days to track viable cell density (viable cells/ml) and viability using the Vi-Cell XR cell counter (Beckman Coulter, Indianapolis, IN). In addition, 400 µl of the culture was pelleted by centrifugation at 250 x g for 5 minutes and stored at -80°C until the all of the time point samples were collected and were ready for further experimentation. Protein from cell lysates were extracted using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology Inc., Korea) and the western blot protocol as described previously. For the western blot, the gel was loaded with 15 µg protein per well. The glutamine synthetase was probed using the glutamine synthetase AB1055 monoclonal antibody (Novus Biologicals, Littleton, CO) diluted at 1:1,000 in StartingBlock T20 (TBS) blocking buffer. The primary antibodies were probed using anti-mouse IgG (H+L) AP Conjugate (Promega) diluted 1:5,000 in StartingBlock T20 (TBS) blocking buffer, and visualized by adding Western blue stabilized substrate for alkaline phosphatase (Promega).

### 2.5.2 Stable Transfection of SP2/0 Cells

100 µg plasmid DNA encoding the recombinant lysosomal enzyme and a glutamine synthetase (GS) selection marker was linearized at 37°C overnight in 1X cutsmart buffer (New England Biolabs, Ipswich, MA) and 20U PvuI-HF (New England Biolabs). The linearized DNA was cleaned up using QiaQuick PCR cleanup kit (Qiagen,
Valencia, CA) using the spin column and centrifuge method detailed in the manufacturer's instructions. This linearized DNA was used for the stable transfection. One day before transfection, SP2/0 cells were counted using the Vi-Cell XR and were passaged to a viable cell density of \(1 \times 10^6\) cells/ml in SP2/0-BMN media supplemented with 8 mM GlutaMAX. The day of transfection, the culture cell density was counted using the Vi-Cell XR to calculate the volume of cell culture required for \(2 \times 10^6\) total cells, the amount of cells needed for transfection. \(2 \times 10^6\) cells were pelleted by centrifugation at 250 x g for 5 minutes. The cell pellet was resuspended in Hyclone Buffer EP (GE Healthcare, Gaithersburg, MD) and 20 µg of the linearized plasmid DNA to a final volume of 400 µl. The cell-DNA mixture was transferred into an OC-400 processing assembly (Maxcyte, Gaithersburg, MD) then electroporated using the MaxCyte STX Scalable Transfection System and its SP2/0 electroporation program, the optimal voltage for SP2/0 cells pre-programed into the MaxCyte STX Scalable Transfection System. After electroporation, the cells were placed into an empty shake flask and recovered in a stationary incubator at 37°C and 5% CO₂ for 30 minutes. The cells were then resuspended in 13 ml of SP2/0-BMN media+8 mM GlutaMAX and incubated in a 37 °C stationary incubator overnight. One day after transfection, cells were pelleted by centrifugation at 250 x g for 5 minutes, then resuspended in a different proprietary media, SP2/0-BMN-2 glutamine-free media. The transfected cells were maintained in the stationary incubator until cell viability increased to 80%. The transfected cell population was maintained in glutamine-free SP2/0-BMN-2 media + 8 mM GlutaMAX in volumes of 10 mL or less.
2.5.3 Screening the Transfected Pool and Clones for ERT Expression Using Western blots

The transfected SP2/0 cells were tested to ensure that the cell population expressed the recombinant lysosomal enzyme. The transfected SP2/0 cell population was counted using the Vi-cell XR. 4 x 10^6 cells were pelleted using the centrifuge at 250 x g for 5 minutes. The supernatant, containing the culture media and the secreted recombinant lysosomal enzyme was separated from the cell pellet and stored at -80°C until needed for the enzyme activity assays or western blots. A western blot was performed to screen for the presence of the secreted recombinant enzyme using the protocol described previously. For this western blot, the gel was loaded with 15 µl of the supernatant and LDS mixture per well, each well containing a different clone sample. The secreted recombinant enzyme was probed using a proprietary antibody specific to the enzyme, which was diluted at 1:1,000 in StartingBlock T20 (TBS) blocking buffer. The primary antibody was probed using anti-rabbit IgG (Fc) AP Conjugate (Promega) diluted 1:5,000 in StartingBlock T20 (TBS) blocking buffer, and visualized by adding Western blue stabilized substrate for alkaline phosphatase (Promega). The membrane was imaged using a photo scanner.

2.5.4 Single Cell Isolation to Collect High-Expressing Clones

Clones were isolated from the transfected cell population by limiting dilution. The clones were seeded and expanded in increasing volumes using an optimized post-transfection method for SP2/0 cells which requires two different types of media (Supplementary Figure 1, Supplementary Figure 2, and Supplementary Figure 3). The
cells were diluted to a low cell concentration in SP2/0-BMN-2 glutamine-free media, and were seeded into each well in 96 well plates at one cell per well or 0.5 cells per well (one cell per two wells). In order to track and document the growth from a single cell, a high-resolution imaging instrument, the Cell Metric CLD (Solentim, UK), was used to image and monitor the growth of single cells after 2 hours, 24 hours, 4 days, 5 days, 7 days, 14 days, and 19 days after plating. Wells that contained more than one cell per well were excluded from the clonal selection.

The fastest growing clones were chosen for expansion. These 24 clones were expanded in increasing volumes in stationary incubation settings at 37°C and 5% CO₂. In small volumes of 2 mL or less, SP2/0-BMN-2 media was used. When a clonal colony was expanded to volume larger than 2 mL, SP2/0-BMN media was used. Clones were expanded in stationary incubation conditions until the cells reached a cell density of 2 x 10⁶ cells per ml in 40 ml volume. Viable clones were then transferred to 25 ml shake flasks and were maintained at a cell density of 0.5-2 x 10⁶ cells/ml in SP2/0-BMN media. The viable clones were used for further experiments after a minimum of three rounds of passaging in with 96% viability or higher in shake flasks.

2.5.5 Screening for the Top-Producing Clone

All clones that were able to scale up in volume and survive in shake flasks were seeded into separate flasks at 5 x 10⁵ cells/ml in 25 ml SP2/0-BMN media for an initial production run. 1 ml was taken from each cell culture at seven time points over the course of 10 days to monitor cell growth, viability, and lysosomal enzyme production. At the peak of cell growth and viability, day 8, a lysosomal enzyme activity assay was used
to quantify the amount of lysosomal enzyme produced by each clone.

2.5.6 Recombinant Enzyme 4-Methylumbelliferone (4-MU) Activity Assay

To quantify the amount of active recombinant lysosomal enzyme produced from the transfected SP2/0 cells, a fluorescent plate-based 4-methylumbelliferone (4-MU) assay was used. Cell samples were pelleted in the centrifuge at 250 x g for 5 minutes. The supernatant, containing the secreted lysosomal enzyme, was separated into a fresh tube. The enzyme-media mixture was diluted 10 fold and 20 fold in a dilution buffer (5 mM sodium phosphate, 150 mM NaCl, 0.005% Tween 80, 0.1% BSA, pH=6.5) and plated in duplicate within a black flat-bottom 96-well plate. 90 µM of 4-MU conjugated substrate specific to the recombinant lysosomal enzyme, mixed with an assay buffer (50 mM sodium citrate, 125 mM NaCl, 0.5% Triton X 100, 0.1% BSA, 2 mM 4-MU, pH=4.5) then added to the plated samples. The plate was incubated at 37˚C for 40 minutes. After incubation, the reaction was stopped by adding 200 µl/well stop buffer solution (0.5 M glycine, 0.3M NaOH, pH 10.3). 4-MU-conjugated substrate cleavage by the lysosomal enzyme released fluorescent 4-MU which was measured using a fluorescent plate reader (Molecular Devices, Sunnyvale, CA) using excitation wavelength, 355 nm and emission wavelength 460 nm. In addition, fluorescent 4-MU was serially diluted to generate a standard curve in known concentrations to convert relative fluorescent units (RFU) to 4-MU concentrations. A known concentration of a control lysosomal enzyme sample was used to convert the 4-MU concentrations to concentrations of the enzyme.

2.5.7 Production Run of the Top Five Clones Expressing the Lysosomal Enzyme

The top five clones went through a second production run to ensure consistent
results with the previous production run. The top five clones were seeded into separate flasks at 5 x 10^5 cells/ml in 25 ml SP2/0-BMN media. 1 ml sample of cell culture was collected daily for 8 days to monitor cell growth, viability, and lysosomal enzyme production. The clone capable of maintaining high viability and producing the highest amount of the lysosomal enzyme was considered the top clone from this experiment. This clone will be used for further characterization experiments.

2.6 SP2/0 Cell line Characterizations of the Top-Producing Clone

2.6.1 Screening Basal Media for the SP2/0-2H3 Clone

A seven-day production run of the SP2/0 clone with the highest expression of the lysosomal enzyme, clone SP2/0-2H3 was used to screen a panel of five proprietary serum-free basal media. Over the course of seven days in different media, cell growth, viability, and protein yields were observed for SP2/0-2H3. Clone SP2/0-2H3 was seeded at 5 x10^5 cells per ml in a total volume of 25 ml of each of the different basal medium, Media A, Media B, Media C, Media D, or Media E. Each 25 ml culture was cultured in 125 ml shake flasks and were incubated at 37°C, 8% CO₂, and 125 RPM. One ml culture sample was taken daily for seven days to count viable cell density (viable cells per ml) and cell viability using the Vi-Cell XR cell counter. The sample was also used to determine protein production yields by the enzyme activity assay described previously. This condition was done in two independent experiments with two technical replicates per independent experiment.

2.6.2 Production Runs of Clone SP2/0-2H3 using Temperature Shifting
A popular approach to improving protein yields involves using a temperature shift, a method which slows cell growth to extend the cultivation process. During a temperature shift, mild hypothermia is induced to the cell cultures by shifting incubation temperature from 37°C to a temperature a few degrees lower. To determine whether the induction of mild hypothermia improves protein production yields for clone SP2/0-2H3, six 250 ml shake flasks of the clone were seeded at 5 x 10^5 cells per ml in a total culture volume of 50 ml using the top performing media, determined previously. Each flask was incubated at 37°C, 8% CO₂, and 125 RPM, and later temperature shifted to 32°C, 8% CO₂, and 125 RPM on different days during the production run. Specifically, of the six flasks, one flask was not shifted and remained at 37°C, one flask was temperature shifted day 0, and the remaining four flasks were temperature shifted either days 1, 2, 3, or day 4. 1 ml samples were collected daily for 7 days to observe changes in cell growth, viability and protein yields in response to temperature shifting.

2.6.3 Screening Myeloma Supplements for Improved Protein Production

A seven day production run of the SP2/0-2H3 clone was used to screen five commercial serum-free nutrient supplements formulated for improved protein production from myeloma cell hosts. Clone SP2/0-2H3 will be seeded at 5 x 10^5 cells per ml in a total volume of 50 ml in the best basal media as determined previously. The cultures were incubated in 250 ml shake flasks at 37°C, 8% CO₂, and 125 RPM. For feed strategy 1, cell cultures were fed with 5% volume feed on day 0, 10% volume feed on day 3, and 10% volume feed on day 5 with three commercial supplements. For feed strategy 2, cell cultures were fed with 10% volume feed on day 3 and 10% volume feed on day 5 with
five commercial supplements. Cell culture samples were taken daily for seven days to measure viable cell density, cell viability, and protein yield. Each condition was done in two independent experiments.

2.6.4 Gene Amplification of SP2/0-2H3 Clone Using Methionine Sulphoximine (MSX) Selection Pressure

To further boost protein yields for clone SP2/0-2H3, methionine sulphoximine, an inhibitor of GS, was added to the cell cultures to amplify the copy number of the integrated plasmid encoding the protein of interest. The top performing clone, SP2/0-2H3, was seeded into five 250 ml shake flasks, in a 50 ml volume with a cell density of 5 x 10^5 cells per ml in the top performing media determined previously. Each flask contained a different concentration of MSX: no MSX, 5 µM, 10 µM, 20 µM, or 50 µM MSX. The cells were passaged every 2-3 days to 5 x 10^5 cells per ml for three weeks. Passaging was performed by pelleting cells by centrifugation at 250 x g for 5 minutes, and resuspending the cell pellet in fresh Media A supplemented with the same MSX concentration. Gene amplification was checked by western blot after three weeks using the protocol described previously. Each well was loaded with 6 µg total protein, determined by a BCA using the protocol described previously. The nitrocellulose membrane was probed with the glutamine synthetase AB1055 monoclonal antibody and detected using the anti-mouse IgG (H+L) AP conjugate antibody (Promega).

After three weeks of selection pressure under different concentrations of MSX, the SP2/0-2H3 cells were seeded at a cell density of 5 x 10^5 cells per ml in 50 ml fresh MSX-free media for a production run. Over the course of the seven day production run,
cell culture samples were taken daily to measure viable cell density, cell viability and protein yield. Each production run was done in two independent experiments, with two technical replicates per independent experiment.

2.7 Large Scale Recombinant Protein Production Run

The SP2/0-2H3 clone was scaled up to two liters with the goal of producing at least milligrams to gram quantities of the recombinant lysosomal enzyme. To produce the recombinant enzyme, a production run was done using the 50 µM MSX-pressured SP2/0-2H3 cell line and the optimal bioprocess conditions determined previously including: basal media, supplement, and temperature. The 50 µM MSX-pressured SP2/0-2H3 clone was scaled up to 2 liters in Media A and seeded at 5 x 10⁵ cells per ml in a 5L shake flask. On days 3 and 5, 10% volume Feed 2 was added to the culture. On day 6, the protein was harvested from the cell culture by centrifugation at 250 x g for 15 minutes. The supernatant containing the recombinant protein was filtered using a vacuum filter, HarvestMax-100 (Marin Scientific Development Company, Greenbrae, CA), to remove the remaining cells and cell debris for fluid suitable for chromatography.

2.8 Recombinant Protein Purification

The filtered supernatant containing the recombinant protein was thawed from -80˚C storage. The recombinant protein was diluted with the half the volume of 20 mM Tris and was adjusted to pH 7.3. The recombinant protein was loaded into a GigaCap Q-650M column (Sigma) and washed with 20 mM Tris. The purified samples were eluted with 20 mM Tris 1M NaCl buffer. The recombinant protein was further concentrated using Amplicon Ultra 0.5 ml Centrifugal Filters (Millipore, Billerica, Massachusetts) and
the manufacturer instructions.

2.9 ELISA Quantification of the Recombinant Protein

One day before performing the sandwich ELISA, wells of 96-well plates were coated with 15 µg/ml of antibody specific to the recombinant enzyme, diluted in coating buffer (0.05 M carbonate, pH9.6) and incubated at 4°C overnight. Approximately 16 hours later, the wells were washed three times with TBST, then incubated in blocking buffer (6% BSA in TBST) for 1 hour. After blocking, and after each step of the ELISA, the wells were washed three times with TBST. The wells were then incubated with 3125 pg/well of recombinant protein produced from CHO cells or SP2/0 cell hosts, diluted in blocking buffer for 2 hours. The recombinant protein was probed in a 1 hour room temperature incubation with 50 µL 1 µg/ml of a biotin-conjugated antibody diluted in blocking buffer. The biotin was bound by 100 µL Streptavidin-HRP (R and D Systems, Minneapolis, MN), which was diluted 1:200 in blocking buffer and incubated for 1 hour. Binding of the biotin-conjugated antibody was detected using a QuantaBlu Fluorogenic Peroxidase Substrate kit (ThermoFisher Scientific) according to manufacturer instructions.

2.10 Protein Quality Comparison Studies

2.10.1 Passaging to Determine SP2/0-2H3 Cell Line Stability

The top five SP2/0 clones with the highest recombinant enzyme production were determined by the enzyme activity assay. The top clone was passaged 2-3 times a week. Cells were passaged down to a viable cell density of 5 x10^5 cells per ml in a total volume of 25 ml. Four days after passaging, one ml of each cell culture was kept for the 4-MU
enzyme activity assay. Enzyme activity was tracked over the course of 30 passages. These cultures were maintained in Media A, the best basal media determined by the media screen.

2.10.2 Uptake Assays

Primary human fibroblast cells were seeded into 24-well culture plates (Corning) at 150,000 cells/well and were grown for 24 hours at 37°C and 5% CO₂ to reach approximately 90% confluency. To generate a dose-response curve, the cells were then dosed with six concentrations of recombinant protein produced from CHO cells or the same recombinant protein expressed in SP2/0 cells. Doses of the recombinant protein were diluted with uptake medium (DMEM with L-glutamine supplemented with 0.5 mg/ml BSA and 1 mM HEPES). In addition, 5 mM M6P (Sigma) was added to some wells as a competitive inhibitor to the recombinant protein drug. Each well containing the recombinant protein therapeutic and for some wells, M6P, were incubated at 37°C and 5% CO₂ for 4 hours. After incubation, the cells were washed twice with DPBS, and lysed by shaking for 10 minutes at room temperature in 100 μL Mammalian Protein Extraction Reagent (M-PER) (ThermoFisher Scientific) supplemented with 1x protease inhibitor cocktail (Sigma). The cell debris from the lysates was removed by centrifugation at 14,000 x g, 10 minutes, and 4 °C. The recombinant enzyme activity in the cell lysates were measured using the recombinant enzyme 6-HMU assay.

2.10.3 6-HMU Enzyme Assay

Assay standards, 6-HMU, and recombinant enzyme controls, were diluted in M-
PER. 30 µL of lysate samples from the uptake assays, controls, and standards were plated per well. The enzyme reaction was initiated by adding 30 µL mixture of 31.25 µM 6-HMU conjugated substrate in assay buffer (0.1/0.2 M citrate phosphate buffer, sodium taurocholate, and sodium oleate, pH 4.4) to each sample. The reactions were incubated at 37˚C for one hour. The reaction was stopped with 200 µL stop buffer (0.5 M glycine, 0.3 M NaOH, pH 10.3). Fluorescence was measured with a plate reader (Molecular Devices, Sunnyvale, CA), using excitation wavelength, 355 nm and emission wavelength 460 nm. Dose response curves were fit using Michaelis-Menten in Prism version 7.02 (Graphpad Software Inc., La Jolla, CA), where $K_M$ and $V_{max}$ values were calculated using that curve.

2.10.4 CI-M6PR Binding Assay

Cation-independent mannose-6-phosphate receptor (CI-M6PR) was diluted to 4 µg/mL in coating buffer (100 mM Carbonate/bicarbonate buffer). 100 µL of CI-M6PR at µg/mL was immobilized to the wells of a black FluoroNunc MaxiSorp 96 well plate. The plate was incubated at 4˚C overnight. Wells of the plate were washed three times with wash buffer (PBS + 0.05% Tween 20), then blocked with 100 µL per well of SuperBlock T20 (PBS) Blocking Buffer (ThermoFisher Scientific) for one hour. Blocking buffer was removed by washing three times with wash buffer. CHO-produced ERT and SP2/0-produced ERT were diluted to 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM, 1.56 nM, and 0 nM. 50 µL of each sample was added to the plate, and incubated for two hours at room temperature. After incubation, the plate was washed three times with wash buffer. 4-MU conjugated ERT substrate was diluted to 1.8 mM in assay buffer (50mM Citrate / 125mM NaCl / 0.5% Triton X-100, pH 4.5). 50 µL 4-MU substrate solution was

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added to the receptor coated plate and incubated at 30 minutes for 37°C. At the end of the incubation period, 200 µL stop buffer (0.5M Glycine, 0.3 M NaOH, pH 10.5) was added per well. 4-MU fluorescence was released upon cleavage of the 4-MU-conjugated substrate by the ERT. Fluorescence was measured using a plate reader (Molecular Devices) using excitation wavelength, 366 nm and emission wavelength 446 nm.

2.10.5 Comparison of ERT Half-life Using the 6-HMU Enzyme Activity Assay and Western Blot

Primary human fibroblast cells were seeded into 24-well culture plates (Corning) at 150,000 cells/well and were grown for 24 hours at 37°C and 5% CO₂ to reach approximately 90% confluency. The CHO and SP2/0-produced ERTs were diluted to 50 nM in uptake medium (DMEM with L-glutamine (ThermoFisher Scientific) supplemented with 0.5 mg/ml BSA and 1 mM HEPES). The fibroblasts were dosed with 500 µL of the 50 nM ERT produced from CHO or SP2/0 hosts. For each time point, the SP2/0-produced ERT, CHO-produced ERT and uptake media were dosed to the fibroblasts in duplicate. Four hours after ERT dosing, the ERT was removed from the fibroblasts by washing wells once with DPBS. One mL DPBS media with L-glutamine and 15% FBS was added to the wells then incubated in a stationary incubator at 37°C and 5% CO₂. The 0 hour time point sample was taken immediately after the four hour incubation dose. Five additional time points were collected at 24 hours, 48 hours, 72 hours, 120 hours, and 168 hours. Each sample was harvested by washing cells twice with DPBS, then lysing using 100 µL M-PER supplemented with 1X protease inhibitor cocktail and shaking for 10 minutes. Samples were stored at -80°C until needed for
analysis by the 6-HMU enzyme activity assay and western blot. The western blot was performed using the methods described previously. A proprietary monoclonal antibody specific to the ERT was used as a primary antibody, diluted 1:1,000 in blocking buffer. The primary antibody was probed using a secondary antibody, IRDye 800CW Goat anti-mouse (LI-COR) diluted 1:5,000 in blocking buffer and detected using the Odyssey Imaging System (LI-COR).

3.0 RESULTS

3.1 Development of a SP2/0 Expression Platform for ERT Production

The first goal of this project was to build a model SP2/0 expression platform for difficult-to-express proteins. For this project, a difficult-to-express human lysosomal enzyme that could potentially be used as an enzyme replacement therapy (ERT), was expressed in the SP2/0 cells as a proof-of-concept model. Compared to CHO cells, SP2/0 cells have low endogenous GS expression, making the GS selection system (Lonza) ideal for selection of successfully transfected cells (Yazaki et al, 2004). To build a stable cell line, SP2/0 cells were transfected with a linearized plasmid encoding the GS and ERT gene. To select for successfully transfected cells, glutamine was removed from the media, forcing cells to rely on the incorporation of the transfected GS gene to convert glutamate and ammonia into glutamine.

To verify that the endogenous levels of GS in SP2/0 cells is insufficient for cell division, cell growth, and viability, SP2/0 cell cultures were tracked over the course of 6 days in glutamine supplemented and glutamine-free conditions (Figure 5). In addition to
the SP2/0 cells, other cell lines with varying levels of endogenous GS expression were tested in glutamine-free and glutamine supplemented conditions. In glutamine supplemented conditions, cell growth was constant and maintained high percent viability (over 80 percent viability) until it reached maximum cell capacity within the culture vessel, seen on day 5. In the absence of glutamine, a cell line without endogenous GS, the GS knockout CHO (GSKO) cells, and the cell line with low endogenous GS levels, SP2/0 cells, were unable to survive, dropping below 50% viability by day 6, and halted cell growth immediately after removing glutamine (Figure 5). The only cell lines capable of healthy cell division and maintenance of high viability in glutamine-free media were the wild-type CHO cells, which are known to express higher levels of endogenous GS compared to SP2/0, and the SP2/0 cell line transfected with the plasmid encoding the ERT and selectable marker (SP2/0-GS-ERT). Furthermore, after only four passages of SP2/0 cells in glutamine-free media, cell viability dropped below 4% (data not shown). These results are consistent with literature review, demonstrating that low level of endogenous GS is not sufficient for survival in glutamine-free conditions, making the GS selection system a logical choice for selection of successfully transfected cells.

![Graphs showing viability vs culture duration for different cell lines under different glutamine conditions.](image-url)
Figure 5. The effect of glutamine-free media on cell growth and viability

Suspension adapted CHO cells, SP2/0 cells, GS knockout CHO cells, and SP2/0-GS-ERT cells (transfected with a plasmid encoding the GS selection marker and a gene encoding the ERT) were tested in glutamine-free media or the same media supplemented with 8 mM GlutaMAX. 1 ml sample from each cell culture was collected daily to determine cell growth and viability over the course of 6 days. Error bars represent a variability of two biological replicates.

To confirm the GS phenotype of the CHO, GSKO CHO, SP2/0, and SP2/0-GS-ERT cell lines, cell lysates from the four cell lines and NS0 cells were run on a western blot to observe GS expression levels. The western blot results demonstrate that the CHO cells and SP2/0-GS-ERT express high levels of GS, whereas SP2/0 cells have lower endogenous levels of GS compared to CHO, and the GSKO CHO and NS0 cells did not express GS (Figure 6). Together, the western blot along with the growth and viability data indicate that the low endogenous GS levels in SP2/0 cells are insufficient for cell division or healthy metabolism in the absence of glutamine. To rescue this phenotype, exogenous GS is required to increase GS levels in SP2/0 cells for survival in glutamine-free conditions. This confirms that the GS selection method can be used to isolate successfully transfected clones which express the ERT.
Figure 6. Endogenous glutamine synthetase in different cell types

15 μg total protein from cell lysates of SP2/0, SP2/0-GS, NS0, CHO-S, and GS knockout CHO cells (GSKO CHO) cells were loaded into each well. A Ponceau staining of the membrane (left) was used to demonstrate equal loading. The western blot (right) displays endogenous expression levels of glutamine synthetase (GS) of various cell lines.

To verify that the ERT is expressed in the transfected SP2/0 cells, the presence of the lysosomal enzyme was tested by western blot. Since the recombinant lysosomal enzyme is secreted from the cell, enzyme was harvested by removing the cell pellet from the media. Results demonstrate that the lysosomal enzyme was expressed in the transfected SP2/0 cell population. The SP2/0 cells continued to express the lysosomal enzyme after passaging the transfected SP2/0 cells in glutamine-free media for over two weeks, indicating that the plasmid was successfully integrated into the host genome, and was not expressed transiently.

After verifying that the transfected SP2/0 population stably expresses the transfected plasmid, single cells from the transfected population were isolated to limit genetic variation, a factor which could affect recombinant protein titers after several
passages. To do this, single cells were isolated by limiting dilution, and were plated 1 cell per well or 0.5 cells per well (one cell every other well) in 96-well plates. To eliminate wells that contained more than one cell per well, the plated cells were checked using the Cell Metric high-resolution imaging system. Wells with more than one cell per well were eliminated from clonal analysis. Of the wells which contained one cell per well, photo images were collected to demonstrate monoclonality (Figure 7). The most critical time points for clonal imaging are at 2-3 hours and 24 hours post-seeding. Imaging 2 hours post-seeding allows cells to settle into the well, reducing any cell migration while suspended in the media. The 24-hour time point is also an important time point as it provides photo evidence of the first cell division.
Transfected SP2/0 cells capable of surviving in glutamine-free media were plated into 96-well plates and monitored using a high-resolution imaging system. Cell growth was monitored for all clones for 19 days. An overview of the entire well is imaged by the imaging system (top image, well timeline) as well as a 10X magnification image of the entire well for each time point. Upon identification of a single cell in a well, the cell was marked clonal on the imaging program, allowing the software to compile photos of the same spot in the well for the duration of the cloning procedure (bottom images).

Out of the clones that derived from a single cell, the 24 fastest growing clones were screened for recombinant protein expression. The 24 clones were expanded in
increasing volumes, maintaining the viable cell density between 0.5 million cells per ml and two million cells per ml. Of the 24 clones, 7 clones were not able to expand to a 25 ml culture volume, due to low viability or growth impairment. The remaining 17 clones were screened for expression of the recombinant enzyme by western blot, indicating successful integration of the transfected plasmid into the host genome. Out of the 17 clones, four clones, 2F1, 257, 2D6, and 1H7, did not express the recombinant enzyme, and two clones 1C7 and 1B8 expressed low levels of the recombinant enzyme (Figure 8).

![Figure 8. Screening SP2/0 clones for expression of the lysosomal enzyme](image)

Culture media samples were harvested from each clone and were screened for the secretion of the recombinant lysosomal enzyme by western blotting. The lysosomal enzyme is expressed at 68 kDa, as seen by the positive control sample. Out the clones screened, clones 257, 2F1, 2D6, and 1H7 did not express the lysosomal enzyme.

The remaining 13 clones were passaged about four times to restore normal cell growth and improve viability to a minimum of 90%. Although the western blot confirmed that the clones express the recombinant enzyme, its analysis is limited since it does not provide any information about the enzymatic activity or quantity of the enzyme.
produced. To determine which of the 13 clones have the highest production yield of the active enzyme, small scale production runs of the 13 clones were used to produce the recombinant protein. While the clones showed varying rates of cell growth, most clones maintained at least 70 percent viability by day 8 (Figure 9). To quantify the amount of active recombinant enzyme produced by each clone, a plate-based 4-MU enzyme activity assay was used. This production screen identified clone 2H3 as the highest producing clone and the clone capable of maintaining at least 80 percent viability until day 8.
Figure 9. First clone screen: Recombinant enzyme production run using the GS-ERT transfected SP2/0 clones
Shake flasks were seeded at 5 x 10^5 cells/ml in a total volume of 25 ml. At seven different time points during the production run, a sample from each cell culture was collected to monitor cell viability (A) and growth (B) during the production run. On day 8, the day of highest cell density, a sample was collected to quantify the amount of lysosomal enzyme produced by each clone using a 4-MU enzyme activity assay (C).
To confirm that clone 2H3 is the top producing clone, a second production run was done using the top five clones with the best production yields determined by the initial production run. The same procedure was used as the first production run except that 10% volume of supplemental nutrient feed was added to the clones on day 3. Adding the cell supplement improved the rate of cell growth for all clones, maximizing cell growth at day 5 instead of day 8 (Figure 10). The culture media from each flask was analyzed to quantify the amount of active lysosomal enzyme produced. Consistent with the previous experiment, clone 2H3 yielded a higher amount of active lysosomal enzyme and was considered the best production clone (Figure 10).
Figure 10. Second clone screen: Lysosomal enzyme production run using the 5 top-producing clones

A seven-day production run was used to confirm which clones yield the highest recombinant protein titers. Using the top five producing clones from the previous screen, shake flasks were seeded at $5 \times 10^5$ cells/ml in a total volume of 25 ml. A sample of each cell culture was collected daily to monitor cell viability (A), growth (B) and production titer of the active recombinant enzyme (C). Each point represents one independent experiment.
3.2 Bioprocess Studies for the SP2/0 Cell Line

To establish a working protein production protocol for the top producing SP2/0 clone, a few characterization studies were done to optimize media, incubation temperature, and nutrient feed for improved protein productivity.

3.2.1 Basal Media Screen

Generally, basal media is one of the most important components of cell culture optimization process since it is key source of nutrients to the cells, directly affecting cell viability and growth. The first cell line characterization study for this project screened a panel of five serum-free myeloma or hybridoma specific media for optimal viability, cell growth, and productivity. Shake flasks were seeded at $5 \times 10^5$ cells per ml in a 50 ml volume, each with different media. Out of the five different media types, Media A was able to support rapid cell growth and maintain high cell density during the production run up to day 6 (Figure 11A and B). In addition to supporting fast growth and cell health, using Media A yielded the highest amount of the recombinant lysosomal enzyme (Figure 11C). Since Media A was the best performing media to maintain cell health and recombinant protein production, this media will be used for the rest of the characterization experiments for the SP2/0-2H3 clone.
A production run for the lysosomal enzyme was done using SP2/0 clone 2H3. Samples were collected daily for 7 days to track cell viability (A) and growth (B). On day 5, the day of the highest cell density and percent viability (over 80% viable), the active recombinant enzyme was quantified (C). Each bar represents the mean of two
independent experiments. Error bars represents range in variability of two biological replicates, each of which represent the average of two technical replicates.

3.2.2 Temperature Shifting with SP2/0 Clone

A widely used technique to boost protein productivity in CHO cells during the production process is temperature shifting, where mild-hypothermia is induced to suppress cell growth and glucose consumption while enhancing protein productivity (Furukawa & Ohsuye, 1998; Kumar, Gammell, Meleady, Henry, & Clynes, 2008) and extending cell viability (Moore et al., 1997). When cell cultures are temperature shifted, the incubation temperature is reduced from 37°C to a few degrees lower. The effect of temperature shifting is different per cell line and can also have varying effects between different clones (Yoon, Hwang, & Lee, 2004). Unlike CHO cells which generally have a positive response to temperature shifting, there are varying reports on how temperature shifting affects productivity and metabolism in hybridoma cells (myeloma cell fused with a B-cell) (Furukawa et al, 1998, Mason, 2014). To observe whether production of our recombinant enzyme from the SP2/0-2H3 clone can be improved using a temperature shift, shake flasks at a 50 ml volume were seeded at the same cell density in Media A. Shake flasks were either temperature shifted and incubated at 32°C a specific day during the production run or remained at 37°C. When temperature shifting the SP2/0-2H3 cells line early during the production run (days 0, 1, or 2), cell growth increased at a steady rate and maintained high percent viability through the entire duration of the production run (Figure 12A and B). In comparison, the flasks that were temperature shifted later in the production run (days 3 and 4) reached the maximum cell density, resulting in a drop in percent viability. These results were reflected in protein productivity. By temperature
shifting early into the production run, clone 2H3 was able to reach over 18 mg/L, compared to the shifting late into the production run, which was only able to yield between 11 and 14.9 mg/L (Figure 12C). These results suggest that temperature shifting at day 0 can be used to maintain high viability and higher titers.
Figure 12. The effects of temperature shifting on clone SP2/0-2H3

Shake flasks were seeded at $5 \times 10^5$ cells/ml and were sampled and counted daily for 7 days to track viability (A) and cell growth (B). Samples were collected day 0 and days 3-7 to quantify lysosomal protein production during the production run (C). Each data point represents one biological replicate.
3.2.3 *Nutrient Feed Optimization*

Developing a feeding strategy is one of the main optimizations for fed-batch processes. In a fed batch process, the basal media supports initial cell growth, and additional nutrient feed is added to replenish the consumed nutrients, maximize productivity and extend the production phase. A general feed strategy method for fed-batch cultures, feed strategy 1, was previously used method for fed batch cultures in the lab. In feed strategy 1, 5% volume feed was administered to the cultures on day 0, and 10% volume feed on days 3 and 5. Cell cultures were assessed for improved productivity using three different nutrient feeds. When feeding strategy 1 was used, productivity was improved slightly (Figure 13). Using feed strategy 1, the culture without feed reached an average titer of 15.4 mg/L, while adding feed 2 improved the titer to an average of 23.1 mg/L. Although this is an improvement in titer, it is also known that protein production from fed-batch processes can be limited when the cultures are over supplied with nutrients (Gorfien, Paul, Judd, Tescione, & Jayme, 2003). When a culture has excess nutrients, metabolic byproducts can accumulate, increasing cellular toxicity and stunting cell growth (Gorfien et al., 2003). If this was the case for feed strategy 1, a new feeding strategy needed to be employed to overcome that limitation. To create a strategy that reduces supplementation, feed strategy 2 was used. For feed strategy 2, cultures were fed with 10% volume feed on days 3 and 5 without the day 0 feed. As a result of changing feed strategy, productivity increased from an average titer of 17.7 mg/L to 37.8 mg/L and 23.1 mg/L to 46.9 mg/L for feed 1 and feed 2 respectively. Overall, feed 2 was capable of reaching the highest product yield using feed strategy 2, reaching an average of 46.9
mg/L compared to feed 2, feed, 3 and the control culture without feed which reached an average titer of 37.8 mg/L, 0.771 mg/L, and 16.9 mg/L respectively. These results indicate that both the nutrient feed and the strategy are important optimization parameters for improving protein titers.

![Figure 13](image)

**Figure 13. The effect of feeding strategy on protein productivity**

Three different nutrient feeds were administered to SP2/0 clone 2H3 using either feed strategy 1 (5% volume feed on day 0, and 10% volume feed on days 3 and 5) or feed strategy 2 (10% volume feed on days 3 and 5). On the last day of the production run, day 7, the recombinant protein activity was measured. Error bars represent two biological replicates.

Since feed strategy 2 had the most success in boosting protein productivity, strategy 2 was used to screen two additional supplements to identify other nutrient feed candidates at a 50 ml scale. All nutrient feed, with the exception of feed 3, was able to extend high viability past day 5 compared to the control (Figure 14A). In addition, all
feed with the exception of feed 3, increased viable cell density (Figure 14B). As a result of extending high viability during the production run and increasing the cell density, feed 1, 2, 4, and 5 yielded higher titers than the control. Of the four supplements that improved protein production, two feeds were high performing, feed 2 and 5. Compared to the control which yielded an average titer of 16.9 mg/L by day 7, feed 2 and 5 increased the average titer to 46.9 mg/L and 47.1 mg/L respectively (Figure 14C). It should also be noted that recombinant protein titer for each nutrient feed may reach its maximum titer at different days during the production run. For example, where feeds 1, 2 and 5 reached maximum titer at day 7, feed 4 reached its maximum titer at day 6 yielding 41.9 mg/L.

When optimizing nutrient feed, the protein titers should be measured the last few days of the production run to ensure that the recombinant proteins are harvested on their peak day during the production run. Using this optimization method, two nutrient feeds that doubled average protein production were quickly identified.
Figure 14. Screening glutamine-free nutrient feed for SP2/0 clone 2H3
Ten percent volume of five nutrient feeds was administered to SP2/0 clone 2H3 production cultures on days 3 and 5. Over the course of 7 days, culture viability (A), cell density (cell growth) (B), and protein production (enzyme activity) (C) were measured to
identify nutrient feeds that support robust growth and improved production. Error bars represent the range of two biological replicates.

### 3.2.4 Amplification of the Expression Vector using GS-based MSX Selection

A widely-used method to amplify protein production from industrial cell lines is to amplify copy number of the gene encoding the recombinant protein. The GS selection marker is known to be a useful target for gene amplification (Wilson, 1993). Adding MSX to the cell culture acts as a selective pressure, requiring the cells to overexpress or amplify the GS gene along with the recombinant protein in the same vector construct. Cell colonies resistant to the MSX selective pressure are known to amplify the GS gene copy number up to 10 copies in a single round of selection (Wilson, 1993). The GS-MSX selection pressure method was used to increase expression of the GS along with the recombinant lysosomal enzyme stably integrated into the SP2/0-2H3 clone. The SP2/0 clone 2H3 was pressured in four different concentrations of MSX for three weeks. As seen in the western blot images in Figure 15, GS expression of SP2/0 clone 2H3 increased, along with the recombinant protein, as the concentration of MSX increased. Results of this experiment indicate that endogenous GS expression levels were successfully increased in response to MSX selection pressure. Since the recombinant lysosomal enzyme in the same expression vector was also co-amplified, these results indicate that the GS-MSX selection can be successfully used to increase protein production within three weeks.
Figure 15. Amplification the recombinant protein expression in clone 2H3 by adding MSX

SP2/0 clone 2H3 was pressured in methionine sulfoximine (MSX) concentrations at either 5 µM, 10 µM, 20 µM, 50 µM, or no MSX for three weeks. 5.5 µg protein from MSX-pressured cells lysates were separated on an SDS-PAGE gel and were blotted onto a nitrocellulose membrane. Total protein load is visualized with a Ponceau-S stain (left) while GS and the recombinant protein were probed with either an anti-glutamine synthetase antibody or an antibody specific to the recombinant (right). The asterisk (*) indicates a nonspecific band.

Although an increase in recombinant enzyme expression was previously shown by western blot, the western blot analysis cannot provide any information about enzyme activity or cellular health during protein production. To determine the quantity of the active recombinant enzyme that can be harvested from the MSX-pressured SP2/0 clone, a seven-day production run of MSX-pressured SP2/0 clone was performed. During the production run, percent viability was not dependent on MSX selection pressure (Figure 16A). By day 5 all cell cultures started to decrease in viability regardless of MSX
concentration. When a higher concentration of MSX was used to pressure the cells, the maximum cell density was reduced (Figure 16B). Although cell density was lowered as a result of higher MSX concentration, this did not translate to a lower amount of recombinant protein. The production titers increased incrementally with MSX concentrations up to 50 µM. On day 5, the day before the viability and cell density dropped, each sample pressured with MSX was assayed to determine the recombinant protein titers. The cells pressured with 50 µM MSX were capable of reaching the highest average active recombinant protein titer, 35.9 mg/L, compared to the control, which reached 8.1 mg/L (Figure 16C). Using this method to increase protein productivity took three weeks, but was effective and would not require many supplements for large scale production.
Figure 16. MSX-amplified GS-ERT expression vector in clone 2H3 raises productivity

SP2/0 clone 2H3 was pressured in methionine sulfoximine (MSX) concentrations at either 5 µM, 10 µM, 20 µM, 50 µM, or no MSX for three weeks. Shake flasks were seeded at 0.5 million cells per ml and sampled daily to monitor cell health including
viability and growth (A and B). On day 5 of the production run, the last day before a decrease in cell viability, protein production titers were measured by an enzyme activity assay (C). As concentrations of MSX selection pressure increased, protein titers increased. Error bars represent variation of two biological replicates, each of which was done in duplicate.

Stable cell lines are characterized as cells that have the ability to maintain constant protein production over a long period of time. However, literature reports demonstrate that recombinant protein production can be affected by long-term culture of the stable cell lines. After a number of cell generations, cell line characteristics including cell morphology and growth rates are altered due to genetic variation. Changes in stable cell line characteristics ultimately lower recombinant protein expression and affect protein quality. The number of passages, or number of subculturing cells from one vessel to another vessel in fresh growth medium, is commonly kept under 20 passages to ensure cell line stability. To ensure that the top clone can maintain long-term cell line stability, recombinant protein expression was measured after four day production runs for each passage up to 20 passages. In addition, the recombinant protein expression was detected in cell lysates by western blot to visualize any changes in protein over several passages. Western blots display consistent expression up to passage 20 (Figure 17A). At passage 25, the recombinant protein seems to be expressed at a slightly lower molecular weight. Although the protein appeared to be expressed differently, normal molecular weight returned by passage 30, but with lower expression. The protein titer decreased at passage 25 but was still higher than the titer from passage 1, indicating that the SP2/0 clone is stable at least up to 20 passages (Figure 17B). In addition, the method used to transfect SP2/0 cells and isolate high-yielding clones is capable of establishing cell lines with
protein stability up to 20 passages.

**Figure 17. Determining SP2/0 clonal cell line stability**

Recombinant protein titers were quantified over 30 passages to determine long-term recombinant protein production in a stable SP2/0 cell line, clone 2H3. A) Western blot
bands display expression of the recombinant lysosomal enzyme (top) along with a Ponceau S stain to demonstrate equal loading (bottom). B) Enzyme activity titers after a 4 day production run for over 30 passages.

3.3 Quality and Functionality of SP2/0 Products

Comparison of ERT Drug Uptake from CHO cells and SP2/0 cells

SDS-PAGE reveals that the ERT produced in CHO and SP2/0 cells have the same molecular weight of 65 kDa (Figure 18). To verify that the recombinant proteins produced by SP2/0 cells can be used for research applications, the recombinant proteins were tested for function in two cell-based experiments. Purified SP2/0 and CHO produced ERT were administered to diseased lysosomal storage disorder fibroblasts at six concentrations to generate a dose-response curve. Cellular uptake of the ERT was quantified for each ERT dose. The dose response curve was fit to the Michaelis-Menten equation on Graphpad Prism 7. Both CHO and SP2/0 cells have similar $K_{uptake}$, 4.5 and 4.6, respectively (Figure 19). Although $K_{uptake}$ values of SP2/0 and CHO cells are similar, the SP2/0-produced ERT had a higher $V_{max}$ compared to CHO cells. Overall, this experiment demonstrates that cellular uptake of CHO and SP2/0 produced ERTs are comparable.
Figure 18. Western blot of the CHO and SP2/0 produced ERT

0.1 μg ERT sample produced from CHO (CHO-ERT) and SP2/0 (SP2/0-ERT) cell hosts used for protein comparison studies in this experiment were separated by non-reducing SDS-PAGE. The gel was then transferred to a nitrocellulose membrane and probed using a polyclonal antibody specific to the proprietary ERT. The sizes of the ERTs were estimated using the molecular weight markers, labeled in kDa.
Six increasing concentrations of SP2/0-produced ERT (green triangle) and the same ERT produced by CHO cells (blue circle) were administered to lysosomal storage disorder fibroblasts to establish an ERT dose-response curve. Data points were fit to the Michaelis-Menten equation on Graphpad Prism 7.0 to determine $K_{uptake}$ values. Each data point represents the results of two replicates. This experiment was repeated twice. Representative results are shown.

The enzymatic activity decay of ERT uptake into lysosomal storage disease fibroblasts were measured over a 7 day period. The ERT produced in both CHO and SP2/0 cells had half-lives of 36.5 hours and 14.9 hours respectively (Figure 20). When the ERT is internalized into the cell, it is processed from its full length form at 65 kDa into the 25 kDa form (Figure 21). Although enzymatic activity decay of the ERT is similar from both CHO and SP2/0 expression platforms, the ERT processing is different.

The ERT produced by CHO cells was mostly processed from the full-length 65 kDa form

<table>
<thead>
<tr>
<th></th>
<th>CHO</th>
<th>SP2/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>0.008763</td>
<td>0.01444</td>
</tr>
<tr>
<td>$K_m$</td>
<td>4.505</td>
<td>4.607</td>
</tr>
</tbody>
</table>
into the 25 kDa form within 24 hours, with full processing after 48 hours. The ERT produced by SP2/0 cells was not fully processed into its 25 kDa form after 168 hours. The SP2/0-produced ERT are processed slowly, with a continuous increase in expression of the 25 kDa processed form over the 168 hour period. Total protein was stained by Ponceau S, and showed a gradual increase in amounts of protein over time, as a result of fibroblast cell growth (Supplementary Figure 5).

**Figure 20. Determination of ERT half-life from CHO and SP2/0 production hosts**

Lysosomal storage disorder fibroblasts were dosed with 50 nM ERT produced from CHO and SP2/0 expression platforms. After a 4 hour incubation period, the ERT dose was removed and replaced with DMEM media supplemented with FBS. At each time point, the fibroblasts were washed twice and cell lysates were harvested. Cell lysates were assayed using a 6-HMU enzyme activity assay to determine concentration of the
internalized ERT. The curve was fit to a one phase exponential decay curve on GraphPad Prism 7.0 software to calculate the ERT half-life in hours. Each point and error bar represents mean and error of two technical replicates.

Figure 21. Half-life comparison of CHO and SP2/0-produced ERTs

Cell lysates from the half-life uptake experiment were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The western blot on the left shows cell lysates of diseased fibroblasts dosed with 50 nM ERT produced by CHO cells, while the western blot on the right shows cell lysates of diseased fibroblasts treated with 50 nM ERT from SP2/0 cells. Time points are measured in hours after the ERT was removed (4 hours after incubation in the ERT). As a control, diseased fibroblasts were dosed with a diluent, DMEM media, without an ERT. The nitrocellulose membrane was probed with a monoclonal antibody enzyme specific to the proprietary ERT. Full length-ERT is expressed at 65 kDa (indicated by top arrow), and the ERT processed within the lysosome is expressed at 25 kDa (indicated by bottom arrow).

The internalization of an ERT into the cell is mediated by endocytosis after binding to the cation-independent mannose-6-phosphate (CI-M6PR) receptor. To further explore characteristics of the ERTs, M6P binding to the CI-M6PR was measured for seven concentrations of the CHO and SP2/0-produced ERTs, generating an enzyme binding curve (Figure 22). Wells of a 96 well plate were coated with 100 µL of 4 µg/mL
CI-M6PRs. The ERTs produced from CHO and SP2/0 cells were bound to the CI-M6PR during a two hour incubation period at room temperature. ERT bound to the CI-M6PR was detected by measuring enzyme activity of cleaved 4-MU conjugated substrate. Binding affinity ($K_D$) to the CI-M6PR was calculated by linear regression to for both CHO and SP2/0 produced ERTs (Table 1).

**Figure 22. ERT binding affinity to CI-M6PR**

0.4 µg of the CI-M6PR was bound to each well of a 96 well plate. CHO and SP2/0-produced ERT was bound to the CI-M6PR and was measured for fluorescence released from cleaved 4-MU conjugated substrate. Each data point represents variability of two assays, each with two technical replicates.

<table>
<thead>
<tr>
<th>ERT Host</th>
<th>Lineweaver-Burk $K_D$</th>
<th>Hanes-Woolf $K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>12.54</td>
<td>10.23</td>
</tr>
<tr>
<td>SP2/0</td>
<td>10.36</td>
<td>9.21</td>
</tr>
</tbody>
</table>

**Table 1. Binding affinity summary of ERT to CI-M6PR**

Binding affinities of M6P residues on CHO and SP2/0 ERTs to the CI-M6PR was tested. The $K_D$ determined for the binding of the M6P residue on CHO or SP2/0-produced ERT to the CI-M6PR was calculated by linear regression using Lineweaver-Burk and Hanes-Woolf. $K_D$ values are reported in nM.
4.0 DISCUSSION

As research and development groups continue to develop therapeutic proteins, there is increasing demand for efficient expression of a variety of recombinant proteins including biotherapeutics and protein reagents. Some proteins including lysosomal enzymes are especially difficult-to-express, yielding considerably low titers, making the cell line development process timely, costly, or unsuitable for manufacture. Instead of altering multiple molecular pathways to force expression of a difficult protein, another cell line may already possess the cellular machinery required to express the protein of interest. With multiple expression platforms available for recombinant protein production, difficult-to-express proteins can be produced more efficiently, and cell lines can be developed quicker.

To address the lab’s need for additional mammalian recombinant protein expression platforms for production of difficult-to-express proteins, this project explored the use of a SP2/0 expression platform for a difficult-to-express lysosomal enzyme. After establishing a stable cell line, a bioprocess capable of producing milligram quantities of a difficult-to-express recombinant protein was developed. Finally, the same recombinant protein expressed in CHO and SP2/0 were compared to determine whether protein function was affected by the expression host. This project successfully identified a working SP2/0 expression platform capable of producing milligram quantities of a research-grade recombinant protein that is difficult-to-express. The fed-batch process established may be used as an additional expression system for production of difficult-to-express recombinant proteins, for research-grade therapeutics or reagents.
4.1 Assessment of GS-Based Selection System for the SP2/0 Expression Platform

This project first evaluated the GS-based selection method for isolating high-yielding SP2/0 clones. One of the main concerns about using the GS-based selection method for SP2/0 cells was that non-transfected cells would be able to survive due to the low levels of endogenous GS expression. An accepted GS-based selection method for cell lines that endogenously express GS requires the supplementation of small amounts of MSX to the cell culture to inhibit GS (Birch, Mainwaring, & Racher, 2008). To determine whether MSX was required for the selection of transfected SP2/0 cells, the SP2/0 cell line was tested for glutamine dependence. When removing glutamine from the media, SP2/0 cells were not capable of synthesizing enough glutamine for survival, requiring an exogenous source of GS. Thus, the GS-based selection system can successfully eliminate non-transfected SP2/0 cells without the use of MSX. Furthermore, protein expression levels of the top-yielding clone, 2H3, demonstrated long-term maintenance of protein expression (Figure 17). This ensures that the co-expression of GS and the recombinant protein is still favored in glutamine-free conditions even with the expression of endogenous GS. To this end, the implemented clonal selection strategy of combining selection pressure with high resolution imaging can be used to quickly select a high-yielding stable SP2/0 clone within 5-6 weeks. To further improve production yields and long-term recombinant protein expression from SP2/0 cells, a larger number of clones could be screened.

4.2 Bioprocess Analysis

In a fast-paced industry setting, many high-quality recombinant proteins need to
be expressed simultaneously for several different projects at the early research stage. For 
SP2/0 cells to be considered a valuable expression tool for early stage research purposes,
the SP2/0 generated cell lines need to be able to yield high amounts of protein after a few
optimizations. This project focused on three bioprocess optimization strategies and one
selection pressure strategy, each of which could be used to increase protein production
titers.

The first bioprocess optimized for the SP2/0 production platform was the cell
culture media. Cell culture basal media is one of the most critical components of cell
based expression platforms since it is the central supply of nutrients to the cells, directly
influencing cell growth and viability. For this reason, culture media is generally one of
the first few components of the bioprocess that is optimized. In addition to cell health,
media can also influence glycan distribution on recombinant proteins (Wells & Robinson,
2016). Today, culture media with different formulations of sugar content, amino acids,
and serums are commercially available to fit protein production needs for specific cell
lines (Wells & Robinson, 2016). Selecting the optimal media that supports high cell
density, with minimal reduction in viability or cellular phenotype is vital to maintaining
the quality of recombinant proteins. For this project, five commercially available serum-
free media specific for hybridoma or myeloma host cell lines were screened for optimal
cell health and productivity. As seen in Figure 11, different media formulations can
greatly influence cell health and productivity. When comparing Media A to Media C,
basal media was capable of having devastating effects reducing productivity from an
average titer of 19.6 mg/L to 0.33 mg/L, as a result of a dropping in viability from 96.7%
to 3%. Results from this project suggests that Media A boosts productivity and cell
growth, making it an ideal basal media for both production and culture maintenance
(Figure 11). When selecting an optimal media, other parameters for example specific
productivity, the amount of recombinant protein produced per cell, also need to be taken
into consideration. For example, Media A was able to support rapid cell growth rates and
cell density up to 9 million cells per mL, compared to Media D, which maintained cell
density under 1 million cells per mL. Although Media D did not support high cell
density, it supported higher specific productivity, shifting cellular energy was towards
recombinant protein production rather than cell division. This allowed the Media D to
yield an average titer of 15.17 mg/L, comparable to the titers of Media A 19.64 mg/L
despite large differences in cell density. It should be noted that only five types of
myeloma/hybridoma media were screened when there are many other commercially
available media for further optimizations. Perhaps with a larger panel of commercially-
available medium or upon using an optimized in-house media formula for the SP2/0-2H3
clone, higher productivity and extended viability could be achieved. For optimization at
the research stage of therapeutic development, the goal is to identify a few types of media
that can be quickly screened so multiple clones and cell types can be optimized
simultaneously. After leading therapeutic candidates and their cell lines are identified at
the research stage, the production process of these therapeutic candidates will be fully
optimized in later stages of the therapeutic pipeline. Overall, this project demonstrated a
straightforward and quick method for selecting a high-yielding basal media for a SP2/0
production cell line at the research stage of therapeutic development.
The second bioprocess optimized was incubation temperature, a widely-used method to optimize protein production in industrial cell lines. Inducing mild-hypothermia, or temperature shifting, to a lower incubation temperature is known to suppress cell growth, reduce glucose consumption, and extend viability, ultimately enhancing protein productivity (Furukawa & Ohsuye, 1998; Kumar et al., 2008; Moore et al., 1997). Temperature shifting has mainly been studied in CHO cells, and usually supports an increase in productivity. In contrast, the effect of temperature shifting in murine myeloma or hybridoma cells can vary depending on the cell line being used and the protein expressed (Furukawa & Ohsuye, 1998; Mason, Sweeney, Cain, Stephens, & Sharfstein, 2014). Generally, reports of temperature shifting in murine myeloma or hybridoma cell lines decrease productivity or does not affect productivity (Al-Fageeh, Marchant, Carden, & Smales, 2006). Unlike general reports, temperature shifting using our SP2/0 clone slightly benefits protein production when temperature shifted to 32°C on days 0, 1 or 2 of the protein production process (Figure 12). By temperature shifting early in the production run, maximum cell density was not reached until the last day of the production run, ultimately extending viability. This was not the case for cell cultures shifted after day 3 since cultures reached their maximum cell density before the end of the production run. Temperature shifting provided excellent viability throughout the production process, which could contribute to maintaining the quality of recombinant proteins. Protein production in our proof-of-concept model was slightly improved using temperature shifting, but should be optimized per generated SP2/0 cell line as previous reports of temperature shifting on myeloma and hybridoma cell lines can have varied
results. Furthermore, each clone can have varying responses to temperature shifting (Yoon et al., 2004), and temperature shifting may have a different effect depending on the recombinant protein being expressed (Mason et al., 2014). Although reports in the literature have stated that temperature shifting usually does not increase production in murine myeloma cell lines, the results from this project suggests that temperature shifting may be beneficial for clones that require higher viability throughout the protein production process.

To further investigate ways to improve the SP2/0 expression platform, a fed-batch bioprocess was adapted and tested using a panel of serum-free and commercially available nutrient feed. Fed-batch cell culture is commonly used for recombinant protein production since it offers a simple method to boost production by replenishing depleted nutrients, it is reproducible at a large scale, and it gives experimental control over relative nutrient levels (Khattak, Xing, Kenty, Koyrakh, & Li, 2010). For this reason, many therapeutics are manufactured using a fed-batch process with both mouse myeloma and CHO cell hosts, producing therapeutics including but not limited to Synagis (AstraZeneca), Erbitux (Eli Lilly & Company), Humira (AbbVie), Herceptin (Roche), and Rituxan (Genentech and Biogen Inc.). Similar to basal media, nutrient feed had drastic effect on protein production using the SP2/0 clone, either reducing titers from 16.9 mg/L to 0.76 mg/L, or doubling production titers (Figure 14). It was observed that over-feeding the cells by adding feed on day 0, 3 and 5 did not benefit production (Figure 13). From these results, we hypothesize that nutrient overfeeding increased the amount of accumulated toxic waste metabolites in the culture. Feeding strategy was further
investigated by implementing a different feeding strategy, replenishing depleted nutrients by adding ten percent feed on days 3 and 5. The second feed strategy had a beneficial effect resulting in doubling titers, extending viability and cell growth (Figure 13). These results suggest that feeding strategy has a drastic impact on recombinant protein production, and that feed strategy should be optimized to maximize protein titers. Similar to the media optimizations performed in this project, a larger nutrient feed panel of commercially-available feed or an in-house optimized nutrient feed panel could be screened for further optimization. This experiment successfully developed a quick and practical approach to screening a panel of feed for the identification of two types of nutrient feed capable of doubling production titers. Together, nutrient feed and basal media optimizations had the most impact on raising production from the SP2/0 clone.

The SP2/0 cells used in this project expresses low levels of GS, but still require glutamine to survive (Figure 5), similar to what is reported in literature reviews (Birch et al., 2008). Expression systems which utilize the GS expression system can amplify copy number of the gene encoding the recombinant protein by adding MSX, a GS inhibitor, to add selection pressure (Nakamura & Omasa, 2015). The GS expression system is an ideal selection method for SP2/0 cells since they express low levels of endogenous functional GS compared to CHO cells.

The MSX selection pressured SP2/0 clone successfully increased recombinant protein activity using a single round of selection pressure without cloning for all four concentrations of MSX within a three week time frame (Figure 16). Within three weeks of 50 µM MSX selection pressure, titers were able to double. 50 µM MSX seems to be a
reasonable selection pressure concentration for SP2/0 cells according to our results and
previous reports of MSX concentrations for cell lines of varying endogenous GS levels.
For example, NS0 expression systems, which are GS deficient, usually maximizes
recombinant protein expression or gene copy number at MSX concentrations of 10-100
µM. Due to high levels of functional endogenous GS in CHO cells, GS-CHO expression
systems usually maximize gene amplification at 250-500 µM MSX (Barnes, Bentley, &
Dickson, 2000). Although there are limited literature reports reporting the range of MSX
concentrations in SP2/0 cells, the optimal MSX selection pressure concentration is likely
to be between 10-100 µM (GS-NS0 range with little to no endogenous GS) and 250-500
µM (GS-CHO range with higher endogenous GS). By using 50 µM MSX, a slightly
lower MSX concentration predicted for SP2/0 cells, unfavorable effects of MSX
including cellular toxicity and cell line instability are minimized. However, it would be
valuable to explore the effects of higher MSX concentrations on SP2/0 cells. A limitation
to the current gene amplification process by MSX is that adding the best performing
nutrient feed with the 50 uM MSX clone did not have a synergistic effect with nutrient
feed (Supplementary Figure 4). Currently, results from basal media, nutrient feed, and
MSX selection experiments suggest that SP2/0 cells have great potential for titer
improvements after several process optimizations.

4.3 Comparative Analysis of Therapeutic Protein Quality from CHO and SP2/0
Cell Hosts

To consider SP2/0 cell as production hosts for recombinant therapeutics, the
SP2/0-produced protein therapeutic product needed to demonstrate comparable quality to
the same therapeutic known to be research-grade quality. The ERT expressed in CHO
cells was previously tested and is known to be our research-grade ERT for *in vitro* studies. One criteria for recombinant protein production is that protein expression in SP2/0 cells should not interfere with protein functionality. To be considered research-grade, the ERT needs to meet several criteria including similar molecular weight, uptake into a diseased cell, half-life and processing within a diseased cell. The first round of comparative studies verified that the ERT is expressed at the same molecular weight, indicating that the SP2/0 cells are not likely to add or delete any part of the protein (Figure 18). Furthermore, this suggests that any differences identified between SP2/0 and CHO produced ERTs are likely due to host cell post-translational modifications. ERT therapeutic uptake into diseased cells *in vitro* is similar, regardless of the production host. Interestingly, the therapeutic produced in SP2/0 host cells had a higher $V_{\text{Max}}$ which suggests that a higher amount of the SP2/0-produced ERT was able to enter the cells compared to CHO. Alternatively, a higher $V_{\text{Max}}$ could be due to assay variability if more cells were harvested from the fibroblasts treated with SP2/0-produced ERT.

In addition to similar uptake values, SP2/0 and CHO expression platforms produced ERTs with similar binding affinities to the CI-M6PR (Figure 22). This feature is critical to ERT drug efficiency, since the CI-M6PR is the receptor responsible for the binding and internalization of M6P-tagged lysosomal enzymes from the extracellular space into the cell and ultimately to the lysosome (Saftig, 2006). Similar $K_D$ values suggest that binding to the CI-M6PR is not affected and it is unlikely that there are variations in M6P post-translational modifications between the two cell lines. These results seem to correlate to the similar uptake values, suggesting that similar ERT binding
affinities to the CI-M6PR receptor would facilitate similar uptake into the cell.

Although cellular uptake is similar, differences in ERT half-life were identified, showing over two times faster ERT degradation in the SP2/0 produced ERT compared to the CHO produced ERT (Figure 21). Interestingly, fibroblast lysates from an uptake half-life experiment showed differences in intracellular processing of the ERT. Normally, the ERT is known to be processed from its full-length 65 kDa precursor form, into the active 25 kDa form inside the lysosome. The ERT from the CHO cells follows these normal processing steps. In the SP2/0-produced ERT the full-length ERT is maintained in the full-length form and is slowly processed into the 25 kDa form, showing an increase in the 25 kDa expression until the 168 hour time point. These results are inconsistent with the half-life generated by the enzyme activity assay, showing decreasing recombinant enzyme expression over time. We propose two hypotheses explaining the internalized SP2/0-produced ERT western blot results. First, the ERT from the SP2/0 host may not be internalized into the cell and instead, could be immobilized at the cell surface. One reason internalization of the ERT may be affected is due to the semi-pure state of the SP2/0-produced ERT, allowing some molecules to interfere with pathways involving the M6P-mediated internalization. The uptake and processing of the SP2/0-produced ERT could be different after complete purification. Second, the ERT may be internalized into the cell but is not being processed into its active form due to various interactions with other molecules within the cell. Differences in intracellular molecule interactions could be due to variation in SP2/0 post-translational modifications or protein structure. These differences visualized by the western blot need to be further investigated, first by
understanding where the SP2/0-produced ERT localizes into the diseased cells. In addition, comparing protein structure and glycan distribution of the CHO and SP2/0 ERTs could provide better understanding about structural differences which could interfere with the enzyme processing. Understanding these intracellular processes may hold great potential in improving therapeutic efficacy, providing hints to manipulations in the ERT that could extend the half-life.

5.0 CONCLUSION

To address our lab’s need for alternative production platforms for efficient expression of a diverse set of proteins, this project established a working SP2/0 expression platform capable of producing milligram quantities of a difficult-to-express recombinant protein. The cloning process was quick, demonstrating an effective way of establishing a high-yielding SP2/0 clone within 5-6 weeks. Furthermore, this project proposes several optimization strategies for doubling recombinant protein titers in SP2/0 cell lines. Importantly, the therapeutic efficacy of lysosomal enzyme proteins is comparable in both SP2/0 and CHO cells, showing similar cellular uptake and binding affinity to the CI-M6PR. However, there are some differences in intracellular processing of the ERT produced in SP2/0 cells, suggesting some interference with either the internalization of the ERT or the processing of the ERT within the cell. This phenomena could be explored more thoroughly, as extending the half-life could have great potential on improving therapeutic efficacy. From this project, we propose that having SP2/0 cells as an additional mammalian expression platform for recombinant protein production is
valuable since it offers new benefits to our existing expression system including expression of a difficult-to-express protein, quick process optimization to double titers, long-term cell line stability, and demonstrates similar cellular ERT uptake in diseased cells.
**Supplementary Figure 1. Post-transfection media optimization**

24 hours post-transfection, transfected cells were split into three different types of glutamine-free media: SP2/0-BMN (A), SP2/0-BMN-2 (B), and SP2/0-BMN-3 (C). Bright field images were taken 24 hours after incubating in the media. Healthy cells post-transfection are visualized in small cell colonies indicated by the black arrows in figure B. Scale bars represent 400 µm.
Supplementary Figure 2. Media optimization during clone expansion

A cell population of a SP2/0-GS-ERT clone was split into two wells of a 24 well plate, each with different media either SP2/0-BMN-2 (A) or SP2/0-BMN (B). Bright field images were taken 24 hours after media was administered to the cells. During clonal expansion, cells in SP2/0-BMN-2 media adapted to adherent culture conditions, seen by their elongated morphology (A). SP2/0-BMN media kept the cells in a suspension-adapted culture, seen by their round shape. Scale bars represent 400 µm.

Supplementary Figure 3. SP2/0 media optimization summary

Summary of optimal media for growth of suspension-adapted SP2/0 clones during early phases of cell line development.
Supplementary Figure 4. Combined effects of nutrient feed and MSX pressured cells on protein expression and cell health

SP2/0 clone 2H3 was pressured in methionine sulfoximine (MSX) concentrations at either 5 µM, 10 µM, 20 µM, 50 µM, or no MSX for three weeks. On days 3 and 5, 10% volume feed was added to some of the flasks. Shake flasks were seeded at 0.5 million cells per ml and sampled daily to monitor cell health including viability and growth (A, B, D, and E). For days 1-7 of the production run, protein production titers were measured by an enzyme activity assay (C and F).
Supplementary Figure 5. Ponceau S total protein stain of cell lysates dosed with CHO and SP2/0-produced ERT

Cell lysates from the half-life uptake experiment were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane on the left shows cell lysates of diseased fibroblasts dosed with 50 nM ERT produced by CHO cells, while the membrane on the right shows cell lysates of diseased fibroblasts treated with 50 nM ERT from SP2/0 cells. Time points are measured in hours after the ERT was removed (4 hours after incubation in the ERT). As a control, diseased fibroblasts were dosed with a diluent, DMEM media, without an ERT. Protein was stained red with Ponceau S stain.
7.0 REFERENCES


