Evaluation of Cellular Mechanisms Involved in Recombinant Antibody Expression in Transiently Transfected Chinese Hamster Ovary Cells

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A Thesis in the Field of Biotechnology

for the Degree of Master of Liberal Arts in Extension Studies

Harvard University

May 2016

Abstract

Transient recombinant antibody production in Chinese hamster ovary (CHO) cells is often used to screen large panels of candidates for potential therapeutic use. However, a significant proportion of antibodies express at a level too low for adequate *in vitro* characterization. This study compares the pathways, including the unfolded protein response (UPR), involved in the transient expression of both low and high expressing antibody clones. Twenty historically low expressing antibodies from two different hybridoma campaigns were re-evaluated for antibody productivity using transient co-transfection of heavy and light chain plasmids in CHO 3E7 cells. Five antibodies had significantly improved antibody production while one antibody had moderately improved antibody production when expressed in CHO 3E7 cells compared to their respective historical data. Seven individual antibody chains were affected at the transcriptional level with minimal or no detectable levels of mRNA. These seven chains were used in different combinations for ten of the antibodies screened. For those ten antibodies, the lack of detectable mRNA, determined by Northern blot analysis, correlated with low levels of intracellular and secreted antibody. The low levels of mRNA could potentially be caused by enhanced degradation due to the physical characteristics of these sequences (Cooper, G. M., 2000). One variant had low levels of light chain mRNA but had secreted titers of antibody similar to its corresponding control and was the only antibody that possessed these features. The remaining four variants had adequate levels of mRNA and intracellular antibody, determined by Western blot analysis, but minimal amounts of

secreted antibody, potentially implicating UPR induction. Antibodies 5L+19H (low expression) and 5L+6H (high expression) along with mock and untransfected cells were analyzed for UPR gene regulation using the Affimetrix CHO Gene array. Several observations were made. First, the transfection process alone had the most impact on differential gene expression affecting 1146 genes out of 29,700 genes assayed. Second, the UPR related gene CHOP was noted to be upregulated for both antibody transfections compared to mock, and HERPUD1 was shown to be upregulated for the low expressing antibody compared to mock. This suggests UPR induction had begun for both experimental transfections, however, the specific pathway or pathways that have been activated cannot be determined with the limited number of genes that were found to be upregulated. Additionally, when the two experimental transfections were compared to each other we found no significant difference in differential gene expression of UPR related genes. This implies that 72 hours post-transfection, the level of UPR induction was similar for an antibody that has low levels of secretion and one that has high levels of secretion. Finally, four key UPR associated genes were found to be downregulated in mock transfected cells compared to untransfected cells, (CHOP, GADD34, ERDJ4 and XBP1).

Acknowledgements

William Brondyk, PhD: Thesis Director, Sanofi Genzyme

Dr. Brondyk is the Vice President of Research Biologics at Sanofi Genzyme and provided guidance and support for this project and critical review of the manuscript.

Stephen Denkin, PhD: Research Advisor, Harvard University

Dr. Denkin is the director of the Biotechnology ALM program, and provided expertise in meeting all program requirements.

Kimberly Bishop

Kimberly is an Associate Scientific Director in Research Biologics at Sanofi Genzyme who provided critical content and editorial review of the manuscript.

Timothy Connors

Timothy is a Senior Scientist in Research Biologics at Sanofi Genzyme who provided archived plasmids and sequence information.

Christine DeMaria & Jin Zhang

Christine is the Associate Scientific Director of the Therapeutic Protein Expression group and Jin is a Staff Scientist I in the same group. Christine and Jin provided protocols, guidance and expertise for Northern blot analysis.

Yuriy Alekseyev, Ph.D.

Dr. Alekseyev is a Research Associate Professor of Pathology and Laboratory Medicine and is the Director of the Microarray and Sequencing Resource Center at Boston University and ran the CHO Array as well as provided support and guidance for array analysis.

Todd Kitterman

Todd is a Senior Principal Systems Engineer at Raytheon who provided critical editorial review of the manuscript.

The 3E7 CHO/EBNA cells and pTT5 expression vector were licensed from the National Research Council Canada (NRC).

The CHO Gene micro-array was performed at the Boston University Microarray facility. All remaining research was performed at Sanofi Genzyme, 49 New York Avenue, Framingham, MA.

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Chapter I

Introduction

The development of humanized monoclonal antibodies for therapeutic use has notably improved over the last few decades, however significant hurdles still remain. Many humanized antibodies are initially evaluated in transient mammalian expression systems which sometimes fail to produce these recombinant proteins. This section will take an in-depth look at the limitations in cellular antibody expression and discuss how further understanding of transient antibody expression may contribute to the development of better cellular production systems.

Classical antibody generation overview

With the development of monoclonal antibodies (mAbs) in 1975, Köhler and Milstein had planted the seed for what is now a multi-billion dollar industry (Ecker *et al*., 2015). Since then we have witnessed the promising evolution of the biologics industry with potential for treating common and rare diseases due to the highly specific nature of mAbs. While the industry is continuing to explore the potential for mAbs to treat cancer and other diseases, the development process to generate viable candidates using the classic hybridoma method remains inherently time consuming.

A typical hybridoma campaign can produce thousands of clones which need to be assayed for desired binding traits (Almagro & Fransson, 2008). Once hybridomas are identified as having positive binding characteristics, they are subject to a humanization process. The concept behind humanization of a murine hybridoma mAb is to reduce the potential for immunogenicity while retaining the binding characteristics of the original murine antibody (Swann *et al*., 2008). One of the first steps to humanization is generating a chimeric antibody derived from the hybridoma mAb. A chimeric antibody will have the murine Fc region replaced with a human Fc region but will retain the murine antigen binding variable domain (Morrison *et al*., 1984). To further decrease the risk of an immune response, additional humanization can be performed by switching flanking regions of the murine complementary determining regions (CDRs) to a human framework (Jones *et al*., 1986).

The number of candidates to screen at the chimeric and CDR grafting stage of humanization can be significant. Generally, only milligram quantities of antibody are needed to perform the desired screening assays and it is standard practice to generate material in a transient system as opposed to a stable system, which can take months to complete (Bandaranayake & Almo, 2013). During this early evaluation, it is common to have multiple chimeric or further humanized antibodies that will fail to transiently express significant amounts of protein. Poorly expressing clones are typically discarded due to the high number of antibodies to be screened but there is the potential that these discarded clones could be relevant therapeutic candidates.

Limitations of cellular expression

Within the cellular expression system there are several stages that could be limiting in regard to transient antibody production. The process could be disrupted at the transcriptional level, at the translational level or post-translational level (including assembly and secretion of the antibody). Some initial efforts have been made to narrow down where the cellular processes are deficient but the results are not consistent. Several studies showed evidence that production in stable cell lines were slowed due to low levels of transcription and corresponding low gene copy number (Jiang *et al*., 2006; Chusainow *et al*., 2008; Mason *et al*., 2012), however, Reisinger *et al*. (2008) found the rate limiting steps to be at the translational and post-translational stage of their stable cell lines. Interestingly, Mason *et al*. (2012) showed that when their antibody was expressed in a transient system, production became limited at the translational and post-translational stages.

UPR regulation

In regard to limitations at the post-translational stages, several studies have focused on effects of the unfolded protein response (UPR). The UPR is triggered when the endoplasmic reticulum (ER) of the cell experiences stress, potentially from accumulation of excess naïve antibody to process (Schröder & Kaufman, 2005; Hussain *et al*., 2014). The activation of the UPR pathway will either direct the cell back to homeostasis by processing or removing the naïve protein or drive the cell toward apoptosis (Schröder & Kaufman, 2005; Hussain *et al*., 2014). Recently, Prashad and Mehra (2015) evaluated induction of the unfolded protein response (UPR) in stable high

and low producing cell lines. In these studies a large array of genes involved in homeostasis maintenance of the endoplasmic reticulum (ER) were examined for expression in a batch culture. It was noted that for both stable cell lines there was some level of induction for all UPR genes assayed, however, several genes appeared to be induced transiently, reaching peak levels between days 3 and 4. The importance of effective transient gene expression for the antibody discovery process supports the need to understand which cellular mechanisms are responsible for low antibody expression with certain antibody clones.

Predictions of limitations in a transient system

We hypothesize that the underlying problem of low yielding humanized antibody clones in a transient system are multi-factorial with issues arising at both the transcriptional and post-transcriptional levels. We further hypothesize that a significant percentage of these poorly expressing antibodies will be limited at the post-translational level and there will be induction of the UPR in this subset. Given the time required to develop therapeutic mAbs, it would be justified to obtain a better understanding of the underlying reasons why some of these early mAb candidates lose their ability to express in a transient system once they have undergone humanization.

Limitations of cellular expression system in a transient environment

In order to further investigate the potential underlying problems in transient systems, we propose to evaluate early stage humanized antibodies that have previously

been discarded due to their poor expression levels. Reduction of expression could occur at the transcriptional level (e.g. inefficient initiation of transcription resulting in low levels of mRNA), at the translational level, resulting in low levels or partially generated nascent amino acid chains, or at further post-translational processes such as protein folding and assembly in the ER and secretion (Watson *et al*. 2008). By evaluating a panel of antibodies directed against different target antigens we seek to determine whether there is a commonality behind those that have poor expression and potentially at which cellular level these antibodies are failing to express.

Elevated stressors at the post-translational level could activate cellular control mechanisms such as the UPR, further diminishing antibody secretion (Prashad and Mehra, 2015; Chakrabarti *et al*., 2011). When the ER is placed under stress for reasons such as too much naïve protein to process, or improper ratios of chains to fold and assemble, the UPR can be induced to either return the cell back to homeostasis or initiate apoptosis. Based on the works of Prashad and Mehra (2015) and Mason *et al*. (2012), antibodies which are identified as having poor expression at a post-translational stage will be evaluated for induction of key UPR genes.

Implications of understanding limitations in a transient system

The results obtained from this study will help us develop a better understanding of limitations of the cell when generating transiently expressed antibodies. Significant efforts have been put into developing stable cell lines that exploit genes of the UPR pathway to increase antibody production but these results have been variable (Prashad

and Mehra, 2015; Pybus *et al*., 2014; Nishimiya *et al*., 2012; Cain *et al*., 2013). However, these studies have a significant impact since they suggest that manipulation of the UPR pathway could improve expression of certain recombinant antibodies. By investigating UPR in a transient system, we have the potential to identify unique gene regulation that might be overlooked in a stable system. This study could also identify antibody features that prevent expression and would aid in future manufacturability and design of antibodies. By evaluating the percentage of antibodies that fail production at a post-translational stage and upregulate the UPR, we could determine the value of generating a new cellular expression system that could exploit key genes involved in regulating UPR. This study could therefore open new avenues to generating higher producing recombinant expression systems, increasing productivity and potentially capturing early stage antibody candidates that are not currently characterized.

Chapter II

Materials and Methods

Multiple methods were employed to investigate the various cellular stages at which protein production may be limited during transient antibody expression. Antibodies with varying historic expression levels were selected for evaluation by transient transfection in an optimized expression system with monitoring of mRNA levels, soluble intracellular protein levels and secreted antibody levels in the conditioned medium. A subset of the antibodies was chosen for further evaluation of gene regulation using microarray analysis. The following chapter will further elaborate on the methods used to perform these experiments.

Expression vector

The pTT5 vector was utilized for all transient transfections and was obtained from the National Research Council Canada. In the pTT5 vector, recombinant gene expression is regulated by the CMV enhancer/promoter. It also contains the Epstein-Barr virus (EBV) origin of replication which has been shown to enhance protein expression by enabling episomal replication of the plasmid (Pham *et al*., 2006). Heavy and light chain sequences of interest were independently transferred into the pTT5 plasmid using Invitrogen's Gateway Cloning system. Additionally, green fluorescent protein sequence

(GFP) was also Gateway cloned into the pTT5 vector. The pTT5/GFP vector was used to determine transfection efficiency. All sub-cloned pTT5 plasmids were verified for the presence of the insert by enzymatic digest. Large-scale plasmid preparations were generated using the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions. All antibody co-transfections were performed using a 1:1 ratio of pTT5/HC to pTT5/LC.

Cell line and cell culture

All transient transfections utilized the CHO 3E7cell line which was licensed through the National Research Council Canada. The CHO 3E7 is a stable suspension CHO clone that expresses a functionally truncated form of EBNA1. This cell line has been shown to increase transient expression levels 3-4 fold when used with a plasmid containing the EBV origin of replication (Pham *et al*., 2006). Cells were cultivated in an orbital shaker (INFORS HT, Bottmingen, Switzerland) maintained at 37^{o}C, 5% CO_2, and 110 rpm using culture growth media consisting of Freestyle F17 (Life Technologies) supplemented with 6mM L-glutamine (Life Technologies) and 0.1% Pluronic F-68 (Life Technologies).

Transient expression

All transfections were performed using 50 ml culture volumes seeded with 1 x 10^6 viable cells/ml in 250 ml Erlenmeyer flasks 24 hours prior to transfection. All antibody transfections were performed in duplicate. The day of transfection, CHO 3E7 cells were

adjusted to 2 x 10^6 viable cells/ml. Cells were co-transfected using 25µg pTT5/HC plasmid, 25µg pTT5/LC plasmid and 250µg Polyethylenimine (PEI) "Max" (Mw 40,000 nominal linear PEI, Polysciences Inc.), or 50µg pTT5/GFP and 250µg PEI "Max". DNA and PEI "Max" were diluted into 1.25 ml growth media and allowed to incubate for 15 minutes at room temperature. Complexes were added directly to culture flasks and allowed to incubate overnight. The following day, cells were fed a supplement, anti-clumping agent (Life Technologies) and the temperature was shifted to 33°C. Cell concentration and viability were determined using the Vi-Cell™ analyzer (Beckman Coulter). Transfection efficiency was determined as the percent GFP positive cells at 72 hours post transfection (Countess II FL, Life Technologies).

Quantitation of secreted antibody

Quantitation of secreted antibody from transfected cultures was performed using the Octet Red 96 (PALL Fortébio) and Protein A Dip and Read biosensor tips for quantitation (PALL Fortébio) with regeneration using 10mM glycine-HCl (GE Healthcare) and primed using cell culture growth media. Samples were assessed at 48 and 72 hours and day 7 post transfection. Culture samples were spun at 10,000 RPM for 5 minutes and 200µl of supernatant was transferred into a black 96-well plate. A standard curve was generated using an in-house purified monoclonal antibody diluted in cell culture growth medium. Samples were all run using the same standard curve. PALL Fortébio's software Data Analysis for Acquisition 9.0 was used to quantitate antibody concentrations in the supernatant.

Western blot analysis

Cell pellets were harvested 72 hours post transfection and normalized to 10 x 10^6 viable cells. Pellets were resuspended in 0.5ml ice cold RIPA buffer with EDTA and EGTA (Boston Bioproducts) containing a protease inhibitor cocktail tablet (Roche). The lysed pellets were spun at 100,000 x g for 30 minutes using the Optima^{TM} MAX-TL Ultracentrifuge (Beckman Coulter). Soluble cell lysates were retained and DNase treated with 5µl of RNase free DNase (QIAGEN) for 30 minutes at 37^{o}C. Cell lysates were resolved under reducing conditions using NuPAGE 4-12% Bis-Tris 1mm pre-cast gels (Life Technologies) in 1x MES SDS running buffer (Life Technologies) for 35 minutes at 200V. Protein samples were transferred onto a nitrocellulose membrane by semi-dry blotting (iBlot system, Life Technologies) according to the manufacturer's protocol and probed using peroxidase-conjugated AffiniPure goat anti-human IgG (H+L) (Jackson ImmunoResearch). Peroxidase signal was detected using Super Signal ELISA Pico Chemiluminescent Substrate (Thermo Scientific) and visualized on film through the X-OMAT 2000 processor (Kodak). Relative quantity of protein was determined by gel densitometry analysis using GeneTools 4.02 Software (SynGene, Synoptics Ltd.). Fold change was determined by comparison of a variant's heavy and light chains to the positive control.

Northern blot analysis

Total RNA was prepared from transiently transfected cell lysates and used in Northern blot analysis to quantitate mRNA levels of heavy and light chain.

RNA isolation

All work surfaces were treated with RNaseZAP prior to RNA isolation. Total RNA was isolated from 10 x 10^6 viable cells using the RNeasy Mini Kit (QIAGEN). Cell pellets were resuspended in 600µl ice cold lysis buffer containing β-mercaptoethanol (Sigma). The cell lysates were passed through a Qiashredder column to homogenize them. The flow through was processed using the RNeasy Mini kit per the manufacturers protocol using the optional on-column DNase digestion. Purified RNA was eluted with 50µl RNase-free water and quantitated in triplicate using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). Samples were normalized to 0.5µg RNA using RNase/DNase free water (Ambion). The normalized RNA samples and RNA digoxigenin-labeled molecular weight marker (RNA Molecular Weight Marker I, digoxigenin-labeled 0.3-6.9 kb, Roche) were prepared using glyoxal loading dye (Ambion) and were denatured at 55^{o}C for 30 minutes.

Gel electrophoresis

Samples were resolved on a 1% agarose gel (UltraPure™ Agarose, Life Technologies) prepared using 1x gel prep buffer (NorthernMax-Gly 10x Gel Prep buffer, Ambion) containing ethidium bromide. A total of 0.5µg RNA was loaded per lane and run in 1x running buffer (NorthernMax-Gly 10x Gel Prep buffer, Ambion) for 2.5 hours at 135V. RNA was visualized briefly using 302nM short wave UV light (Alpha Innotech) to ensure the integrity of samples. Additionally, 18S and 28S ribosomal RNA was assessed using UV light as an internal positive control (Alpha Innotech).

RNA transfer

RNA samples were transferred for 4 hours or overnight onto positively charged nylon membrane (BrightStar-Plus positively charged nylon membrane, Ambion) using NorthernMax transfer buffer (Ambion) and the VacuGene transfer unit (GE Healthcare) set at 35 psi. The RNA was fixed to the membrane by UV crosslinking (UV Crosslinker set to Autocrosslink 1200, VWR).

DNA probe generation

Probes and primer templates are in-house proprietary sequences. Primer sets for heavy and light chain were generated through Integrated DNA Technologies. Test amplifications were performed for all primer pairs using high fidelity PCR cloning mix (CloneAmp HiFi PCR Premix, Clontech) on a Petlier Thermal Cycler-200 (MJ Research). PCR fragments were generated with an initial 2 minute denaturation at 95^{o}C followed by 30 cycles of 30 seconds denaturation at 95^{o}C, 30 seconds annealing at 55^{o}C, and 40 seconds elongation at 72^{o}C. A final elongation step of 7 minutes at 72^{o}C was performed at the end of the 30 cycles. PCR amplified fragments were resolved on a 1.2% agarose gel using the Lonza FlashGel System and visualized using 302nM short wave UV light (UV and Chemiluminescent Imager, Alpha Innotech). Probes for HC and LC were generated using digoxigenin (DIG) labeling following the manufacturer's protocol (PRC DIG Probe Synthesis Kit, Roche) and the previously described PCR amplification scheme. Incorporation of DIG label into the probes was confirmed by comparison to unlabeled PCR product via resolution on a 1.2% agarose gel as previously described. All

DIG labeled probes were cleaned per the manufacturer's protocol prior to use (QIAquick PCR Purification Kit, Qiagen).

Hybridization

All work surfaces were cleaned with RNase ZAP prior to hybridization. DIG Easy Hyb (Roche) was pre-warmed for one hour in a hybridization oven set to 50^{o}C. The blots were placed into hybridization bottles containing the DIG Easy Hyb solution and the membranes were allowed to pre-hybridize for 15 minutes at 50^{o}C. Hybridization solution was prepared by diluting 2µl of probe per 1ml of DIG Easy Hyb buffer, pre-warmed to 50^{o}C. The pre-hybridization solution was removed and 3.5ml of hybridization solution was added. The membrane in hybridization solution was allowed to hybridize at 50^{o}C overnight. The following day, blots were rinsed two times with Wash Buffer I (0.1% sodium dodecyl sulfate, (10x SDS, Life Technologies) + 2x sodium chloride, sodium citrate (10x SSC buffer, Life Technologies)) at room temperature. The membranes were washed twice with pre-warmed Wash Buffer III at 68^{o}C. The remaining procedures were performed at room temperature unless otherwise noted. The membranes were washed briefly in Wash Buffer I then blocked for 30 minutes (DIG Wash and Block Buffer Set, Roche). After blocking, the membranes were incubated with the detection antibody (DIG Nucleic Acid Detection Kit, Roche), washed twice (DIG Wash and Block Buffer Set, Roche), then equilibrated with detection buffer for 5 minutes (DIG Wash and Block Buffer Set, Roche). The membranes were transferred to transparency sheets and CSPD Ready to Use detection reagent (Roche) was added and sealed with an additional transparency sheet. The membranes were incubated at 37^{o}C for 15 minutes then exposed

to film in a development cassette for 1hour up to overnight. The film was developed using the X-OMAT 2000 processor (Kodak). Relative quantity of RNA was determined by gel densitometry analysis using GeneTools 4.02 Software (SynGene, Synoptics Ltd.). Fold change was determined by comparison of a variant's heavy and light chains to the positive control.

Cricetulus griseus (Chinese hamster) gene array

The Chinese hamster gene array was performed to evaluate upregulation of UPR - related genes from transiently expressed antibodies that had low and high yields and was performed at the Microarray and Sequencing Resource Facility at Boston University.

Microarray experimental design

The experiment was comprised of 12 Chinese Hamster Ovary (CHO) 2.0ST arrays, comparing CHO cells that were untransfected ("UnTXN"), mock-transfected with an empty plasmid, or co-transfected with light and heavy chain antibodies corresponding to low (5L+19H) and high (5L+6H) antibody secretion (n=3 per experimental group). Transient transfections were set up as previously outlined and RNA was isolated as previously described. Untransfected cells were subject to temperature shift as were all other samples, however, no other manipulations were performed on the untransfected control.

Microarray

All procedures and statistical analysis were performed at Boston University Microarray Resource Facility as described in GeneChip® WT Plus Kit Reagent Manual (current version available at: http://media.affymetrix.com/support/downloads/manuals/wtplus_reagentkit_assay_manual.pdf, Affymetrix, Santa Clara, CA). Boston University Microarray Resource Facility has kindly provided the following methods and statistical analysis.

RNA integrity was verified using RNA 6000 Pico Assay RNA chips run in Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was reverse-transcribed using GeneChip® WT Plus Kit Reagent Kit (Affymetrix, Santa Clara, CA) and the cDNA product was utilized for *in vitro* transcription yielding antisense cRNA which was purified using Purification Beads (Affymetrix, Santa Clara, CA) and used as a template for reverse transcription to produce single-stranded DNA in the sense orientation. During this step dUTP was incorporated. The DNA was then fragmented using uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) and labeled with DNA Labeling Reagent that is covalently linked to biotin using terminal deoxynucleotidyl transferase (TdT, WTPlus Reagent Kit, Affymetrix, Santa Clara, CA). IVT and cDNA fragmentation quality controls were carried out by running an mRNA Pico assay in the Agilent 2100 Bioanalyzer.

The labeled fragmented DNA was hybridized to the CHO Gene Arrays 2.0ST for 18 hours in GeneChip Hybridization oven 640 at 45^{o}C with rotation (60 rpm). The hybridized samples were washed and stained using Affymetrix fluidics station 450. The first stain with streptavidin-R-phycoerythrin (SAPE) was followed by signal

amplification using a biotinilated goat anti-streptavidin antibody and another SAPE staining (Hybridization, Washing and Staining Kit, Affymetrix, Santa Clara, CA). Microarrays were immediately scanned using Affymetrix GeneArray Scanner 3000 7G Plus (Affymetrix, Santa Clara, CA).

The resulting CEL files were summarized using Affymetrix Expression Console (current version 1.1). Robust Multi-Array Analysis (RMA) algorithm (Irizarray *et al*., 2003) was used to generate gene-level data.

Normalization and quality assessment

All arrays were normalized together using Affymetrix Expression Console and Robust Multiarray Average (RMA) normalization. The expression values are log2-transformed by default. All samples had similar quality metrics, including mean Relative Log Expression (RLE), and Area Under the [Receiver Operating Characteristics] Curve (AUC) values > 0.8.

Student's t test

Student's two-sample *t* test was performed for each probeset between the following pairs of experimental groups to obtain a *t* statistic and *p* value for each gene:

5L+6H versus mock

5L+19H versus mock

Mock versus un-transfected (UnTXN)

Microarray analysis

Affymetrix Expression Console was used to normalize CHO 2.0ST CEL files with the Robust Multiarray Average (RMA) (Irizzary *et al*., 2003) to produce gene-level expression values and to generate quality metrics. Principal Component Analysis (PCA) was performed using the *prcomp* R function with expression values that had been normalized across all samples to a mean of zero and a standard deviation of one. Analyses of variance were performed using the *f.pvalue* function in the *sva* package (version 3.4.0). Pairwise differential expression was assessed by performing Student's t test on the coefficients of linear models created using the lmFit function in the limma package (version 3.14.4). Correction for multiple hypothesis testing was accomplished using the Benjamini-Hochberg false discovery rate (FDR) (Benjamini *et al*., 1995). All microarray analyses were performed using the R environment for statistical computing (version 2.15.1).

Chapter III

Results

The methods previously outlined were utilized to investigate the underlying problems specific to transient transfection systems and antibody production. These experiments were designed to allow binning of the antibodies and identification of those appropriate for further investigation of UPR induction. The following section will report on the detailed findings associated with each of the experiments performed.

Determination of target antibody panel

Several recent antibody campaigns were reviewed to identify candidates that had historically low levels of secreted antibody expression in a transient system (<15µg/ml). Humanized variants of two campaigns were previously expressed in several different transient systems (FreeStyle™-293F, Expi293F™ and CHO 3E7) with differences in transfection methods, transfection reagents, supplemental feeds and cell line growth parameters. To normalize methods for this study, the CHO 3E7 system was chosen as it has shown consistency in transfection reproducibility over time. Additionally, downstream processes of target candidate development occur in CHO-derived cell lines thus allowing a higher likelihood of predicting antibody expression characteristics during

the antibody development process. The objective of this initial study was to assess the reproducibility of the historical expression data in the CHO 3E7 cell line.

Data was collated from two distinct antibody target campaign programs. Included in each panel was a variant which had historically high levels of secreted antibody (>15ug/ml) and was used as a positive control. Five unique antibody clones were selected amongst the two campaigns and from those a total of 25 humanized variants were re-assessed for their secreted antibody expression levels (Figure 1).

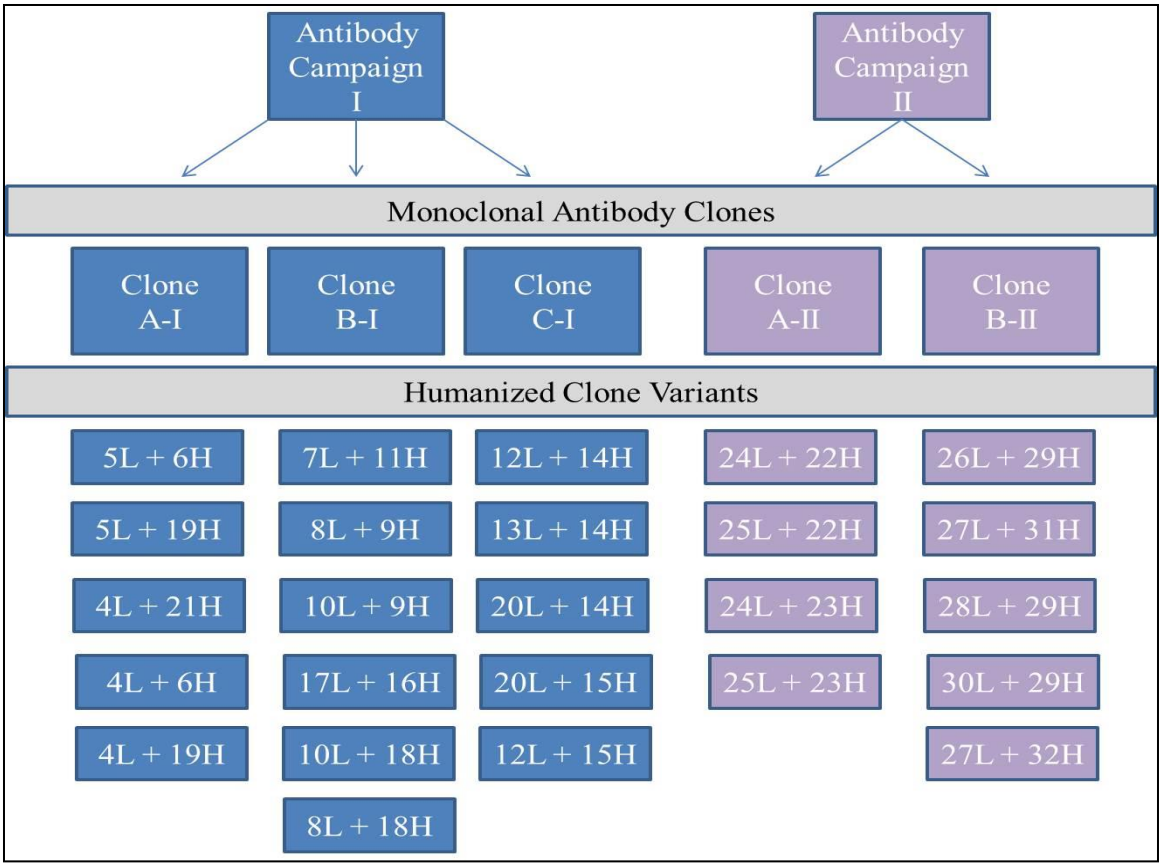


Figure 1. Antibody campaign flow chart. Hierarchical progression of humanized antibody variant panel. The two antibody target campaigns are noted at the top (blue and purple) followed by monoclonal antibodies isolated from each campaign. Finally, the humanized variants are distinguished by their coded light and heavy chains (xL + xH). Note several heavy or light chains were used multiple times in combinatorial pairing during the humanization process.

For expression analysis, transfections of the humanized antibody variants were performed in duplicate (n = 2) and the secreted antibody titers were assessed at 24 hours, 48 hours, and 7 days post-transfection. A single green fluorescent protein (GFP) transfection was performed in parallel to assess transfection efficiency. There was minimal variability in cell density or viability during the course of the transient production and transfected cultures were >70% viable on day 7 (data not shown). For all sets of transfections, the percentage of GFP-positive cells was highest at 72 hours. The

expression profiles trended similarly with historical data. Six variants had cumulative expression higher than 15µg/ml on day 7. These variants were used as additional positive controls for subsequent experiments (Figure 2).

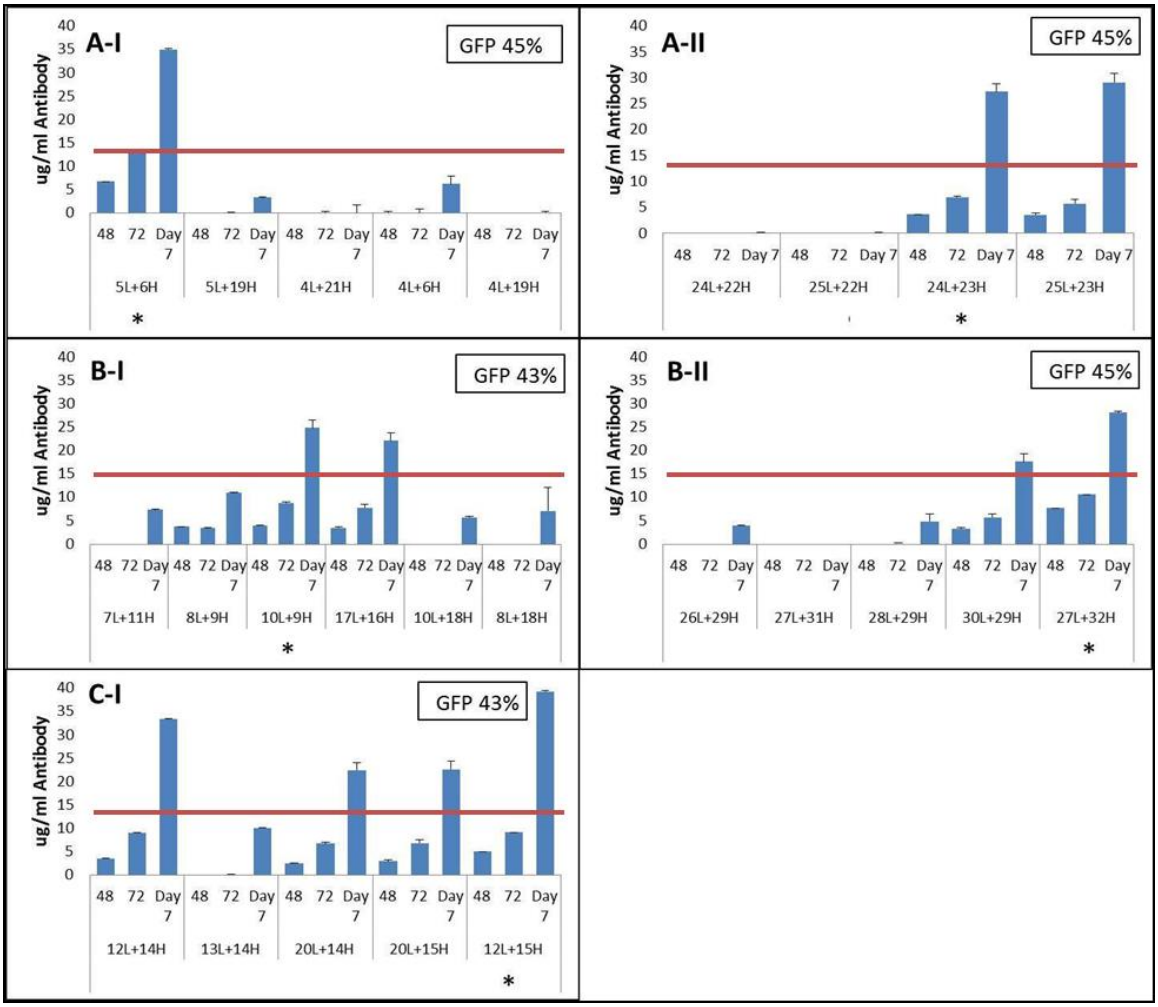


Figure 2. Transient antibody expression titers. Transient expression of secreted antibody was determined at 48 hours, 72 hours and 7 days post transfection. Samples were collected from two independent transfections (n=2). Secreted antibody levels were analyzed from cell supernatants via protein A binding utilizing the Octet Red system and duplicate samples were averaged and graphed. Positive controls are marked with (*) on the x-axis. The red line denotes the 15µg/ml limit for low expression. Transfection efficiency was calculated as the percent GFP positive cells at 72 hours post transfection.

Variability of secreted antibody expression for the duplicate samples was minimal. Additionally the transfection efficiency was highly reproducible between transfection sets (standard deviation of 1.095) with an average of 44.2% GFP positive cells. All A-I clone test variants had less than 15µg/ml secreted antibody on day 7. All B-I clone test variants had less than 15µg/ml secreted antibody on day 7 except 17L+16H, which historically had moderate to low expression levels. Clone C-I had one variant, 12L+14H, with higher than historical levels of secretion. Both C-I variants 20L+14H and 20L+15H had historically moderate levels of antibody expression and had greater than 15µg/ml on day 7 when expressed in the CHO 3E7 cells. A total of 14 antibody variants qualified as having low secreted antibody expression levels (Figure 3).

A-I	B-I	C-I	A-II	B-II
Controls: Historically High Expression				
5L+6H	10L+9H	12L+15H	24L+23H	27L+32H
Low Expression <15ug/ml				
5L+19H	7L+11H	13L+14H	24L+22H	26L+29H
4L+21H	8L+9H		25L+22H	27L+31H
4L+6H	10L+18H			28L+29H
4L+19H	8L+18H			
High Expression >15ug/ml				
	17L+16H	12L+14H	25L+23H	30L+29H
		20L+14H		
		20L+15H		

Figure 3. Organizational chart of antibody expression titers. Figure summarizing positive controls, low expressing antibodies and high expressing antibodies and their corresponding parental antibody clone.

Inhibition at the RNA level

The first step in protein production occurs at the transcriptional level with processing of the gene of interest into messenger RNA (mRNA) for downstream translation. In order to assess the ability of the cell to generate the heavy and light chain mRNA necessary for downstream protein synthesis, Northern blots were performed on total RNA. The intensities of the resulting bands were quantified by densitometry and the fold change compared to the positive control was graphed (see Appendix 1, Figure 4). For this study, a low level of RNA was considered to be at least a 50% fold decrease of mRNA compared to the corresponding control.

Overall, there was significant variability in the quantity of mRNA among the different variants. Several variant chains had no detectable signal by Northern blot analysis. Clone A-I variants that shared the 4L light chain had no detectable light chain signal. This correlated with the low levels of quantitated antibody on day 7. Interestingly, variant 5L+19H, which shares the same light chain as the control, had 19.2% and 16.7% lower relative intensities of heavy and light chain mRNA compared to the control, respectively, however the yield on day 7 was only 3.2µg/ml compared to 35µg/ml from the positive control.

Clone B-I variants had some level of expression for all heavy and light chains. Two variants, 10L+18H and 8L+18H, had 62% and 41% lower relative intensities of heavy chain compared to the control, respectively. The low quantities of heavy chain mRNA corresponded to low levels of secreted antibody. Interestingly, variant 17L+16H had one of the lowest relative quantities of heavy chain mRNA in this subset (92.5% less than control), but had secreted antibody levels equivalent to the control. Variants 7L+11H

and 8L+9H had mRNA levels of both chains slightly lower than the control but their secreted antibody titers were 50% less than the control.

Clone C-I variants all had similar or slightly higher levels of heavy chain mRNA compared to the control. The 12L light chain had similar levels of mRNA compared to control. Variants 20L+14H and 20L+15H had 237% and 139% more relative light chain compared to the control respectively. For all the variants except 13L+14H, the secreted antibody titers were equivalent to the control. The undetectable level of light chain mRNA from variant 13L+14H correlate with 75% lower levels of secreted antibody titer compared to the control.

Two clone A-II variants, 24L+22H and 25L+22H, had no detectable mRNA levels for the heavy chain which correlated to the undetectable levels of quantitated antibody titers. Variant 25L+23H had111% more relative heavy chain mRNA compared to control which corresponded to secreted antibody titers slightly higher than the control. All light chain mRNA levels were nearly equivalent to or higher than the control.

Clone B-II variant 26L+29H and 28L+29H had undetectable levels of light chain mRNA and both variants had above normal levels of heavy chain. The absence of detectable light chain for 26L+29H and 28L+29H correlated with low levels of secreted antibody on day 7 with 3.9 and 4.9µg/ml, respectively. Variant 27L+31H had nearly three times higher levels of relative mRNA for the light chain but had 69.5% less relative heavy chain mRNA compared to the control. This correlated with undetectable levels of secreted antibody titers. Finally, variant 30L+29H had nearly equivalent levels of mRNA for the heavy chain but 72.6% less relative light chain mRNA compared to the control. This correlated to nearly 60% less secreted antibody titer compared to the control.

Intracellular antibody expression

In order to determine whether the cellular machinery was blocked at the post-translational stage, we assessed the ability of the cells to form both heavy and light chains intracellularly. To visualize intracellular composition of the antibody variants, Western blots were performed on cell lysates taken 72 hours post transfection, intensities were quantitated by densitometry, and the fold change compared to the positive control was graphed (see Appendix 2, Figure 5).

As expected, those variants that were noted to have moderate to high levels of secreted antibody expression, including the positive controls, had significant amounts of intracellular heavy chain and light chain.

Clone A-I variants sharing the 4L light chain had no detectable light chain protein by western blot. This correlated with the absence of detectable mRNA and to the low levels of detectable secreted antibody. Interestingly, variant 5L+19H had mRNA levels similar to the control but had 94.4% lower relative quantity of detectable 5L light chain by western blot. This was additionally intriguing as 5L+19H shared the same light chain as the control variant, 5L+6H. Intracellular levels of 5L+19H heavy chain was 36% lower compared to the control.

Clone B-I variants 10L +18H and 8L+18H both had undetectable levels of light chain protein by western blot which correlated to reduced levels of secreted antibody on day 7 with 5.7µg/ml and 7.1µg/ml, respectively. Variants 7L+11H and 8L+9H had 52.9% and 33.8% lower relative intensities of detectable light chain compared to the control, respectively. Additionally, variant 7L+11H had 27.9% lower relative intensity of heavy

chain compared to control. This data correlated with lower levels of secreted antibody expression for both 7L+11H and 8L+9H with 7.38 and 11.0µg/ml secreted antibody on day 7, respectively.

All clone C-I variants except 13L+14H, had equivalent or higher levels of intracellular light and nearly equivalent levels of heavy chain compared to control. This correlated with their high levels of secreted antibody on day 7. Variant 13L+14H had undetectable levels of light chain protein by western blot which correlated with low levels of secreted antibody (10µg/ml).

Clone A-II variants that shared the 22H heavy chain had no detectable signal by western blot. This correlated with the absence of detectable mRNA and the undetectable levels of secreted antibody. Variant 25L+23H had 18.5% higher levels of detectable heavy chain and 5.5% higher levels of light chain compared to the control. This correlated to similar levels of secreted antibody detection on day 7 as the control; 29.1µg/ml for variant 25L+23H and 27.3µg/ml for the control.

Finally, clone B-II variants had the most variability of detectable intracellular protein. Variant 26L+29H had almost no detectable light chain (96.1% lower than the control) but 13.5% higher levels of heavy chain compared to the control. This correlated with low levels of light chain mRNA and low levels of secreted antibody. Variant 27L+31H had no detectable heavy chain which correlated with the undetectable heavy chain mRNA and undetectable levels of secreted antibody. Variant 28L+29H had 87.1% lower levels of light chain but nearly the same quantity of heavy chain compared to the control. This correlated with low levels of detectable mRNA light chain and secreted antibody.

In general, the absence of heavy chain mRNA resulted in undetectable intracellular protein and undetectable secreted antibody titers with the exception of variant 17L+16H which had secreted antibody titers similar to the control. Absence of detectable light chain mRNA typically resulted in reduced levels of intracellular protein and secreted antibody. Conversely, equivalent or higher levels of intracellular heavy chain and light chain correlated with high levels of detectable secreted antibody expression.

An interesting phenomenon was noted for the C-I variants 12L+14H (high producer), 13L+14H (low producer) and the control variant 12L+15H, as a significant band was detected around 38kDa. To a lesser extent, a similar band could be seen for clone B-II variants 26L+29H and 28L+29H (both low producers). Since the 13L+14H variant had no detectable light chain, and the samples were run under reducing conditions, we hypothesized that this was a clipped form of heavy chain. To investigate this matter further, we performed western blot analysis of the corresponding supernatants to see whether the material was being secreted (Figure 6).

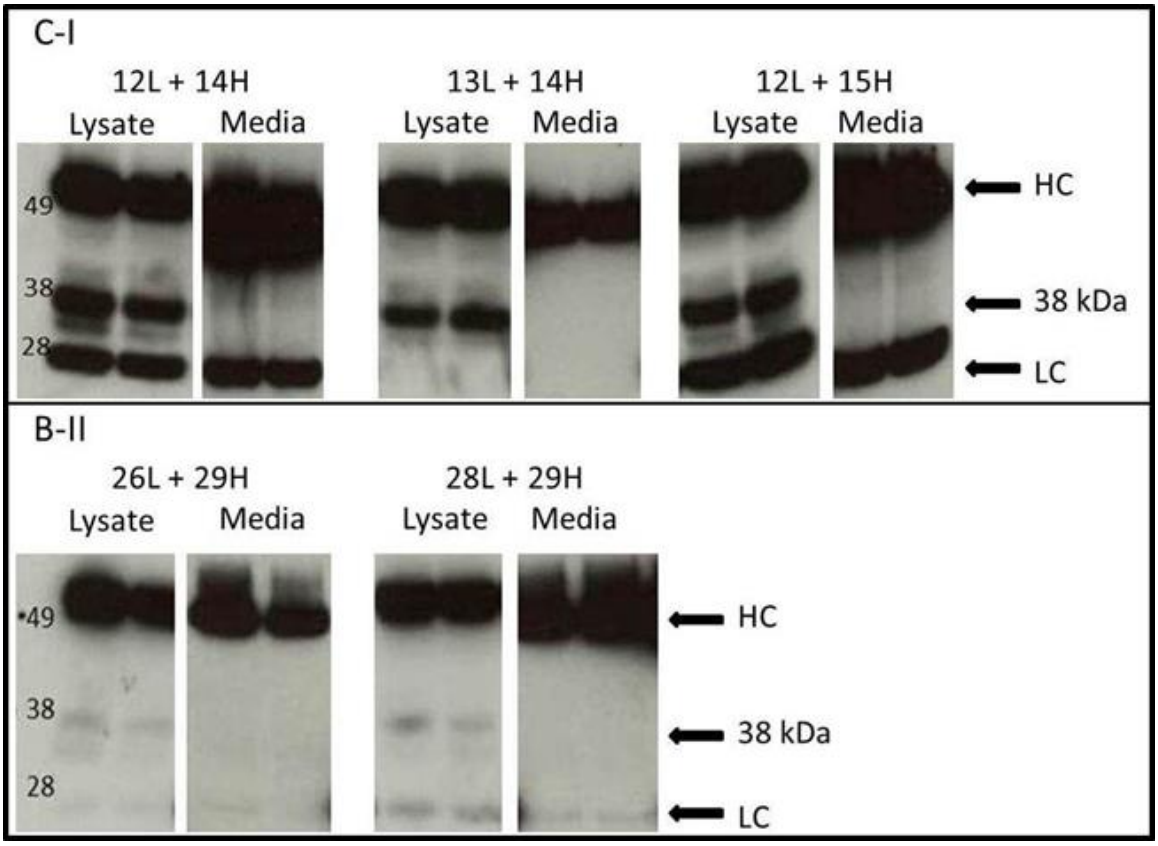
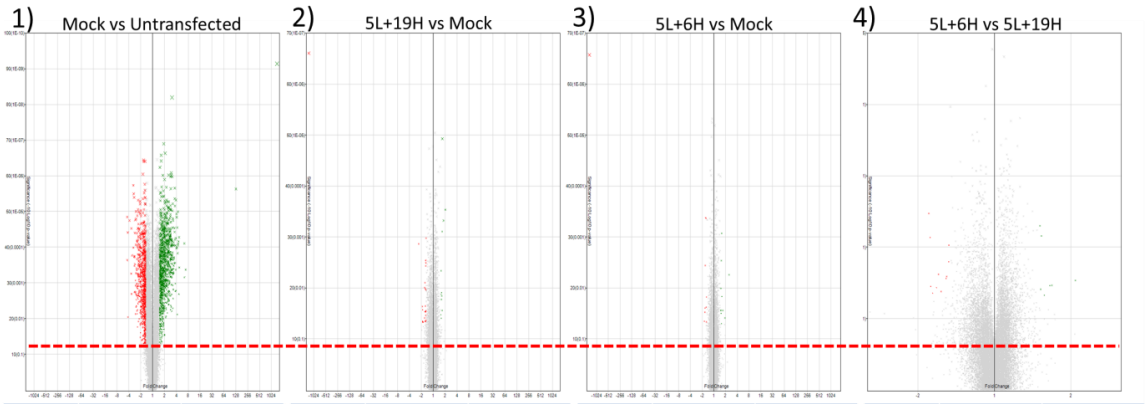


Figure 6. Western blot of conditioned media. Western blot comparison of whole cell lysates and conditioned media supernatants taken 72 hours post transfection. Conditioned media supernatants were run under reducing conditions and probed using peroxidase-conjugated AffiniPure goat α-human IgG (H+L) antibody. Each panel label corresponds to the monoclonal antibody clone (C-I and B-II) and the titles of the wells correspond to the humanized variants for each clone (xL + xH). Cell lysates are shown to the left of their corresponding conditioned media samples. Arrows denote signal from heavy chain (HC), light chain (LC) and the ~38 kDa band.

As shown in the western blots of the media versus cell lysates, the 38kDa band was not being secreted for any of the variants. This extra band does not appear to have a correlation with antibody secretion levels as it was associated with both high and low expressing variants.

Investigation of UPR induction

To gain further insight into the underlying causes of low antibody expression and its correlation with UPR, we chose two variants for in-depth investigation. Clone A-I variants 5L+19H (low yield) and 5L+6H (positive control) were selected for UPR analysis. This pair was of particular interest as both had significant levels of mRNA for the light chain and adequate amounts of heavy chain but variant 5L+19H had significantly lower levels of secreted antibody, significantly lower levels of intracellular light chain (even though it shared the same light chain as the control) and had moderately lower levels of intracellular heavy chain suggesting there may be malfunctions of the cellular protein processing system at the translational or post-translational stage. These are conditions which could potentially result in induction of UPR. In addition to the two test variants, mock transfections using an empty plasmid vector were assayed as well as untransfected cells. The mock transfection was assessed as it would be possible for significant gene level changes to occur due to the supplemental feed, transfection reagents, and plasmid. The untransfected cells were used as a baseline to compare to mock transfections to see which genes, if any, would have differential expression. Analysis was performed using the Transcriptome Analysis Console v3.0 with parameters set to a one way between-subject ANOVA, fold change (linear) <-1.5 or >1.5 and an ANOVA p-value (conditioned pair) <0.05. A total of 29,700 genes, coding and non-coding, were assessed for gene regulation and graphed on volcano plots (Figure 7).



Non-Coding	<-1.5 Fold Change	>1.5 Fold Change	Non-Coding	<-1.5 Fold Change	>1.5 Fold Change	Non-Coding	<-1.5 Fold Change	>1.5 Fold Change	Non-Coding	<-1.5 Fold Change	>1.5 Fold Change
643	392	754	36	5	4	12	4	4	23	3	0

Figure 7. Volcano plots of differential gene expression. Volcano plots for four conditions 1) mock vs. untransfected, 2) 5L+19H (low expression) vs. mock, 3) 5L+6H (high expression) vs. mock, and 4) 5L+19H (low expression) vs. 5L+6H (high expression). Transfections were performed in triplicate and samples harvested at 72 hours. RNA was prepared as previously described and analyzed on the CHO Gene Array 2.0ST. The red dotted line denotes the p-value where p=0.05. Samples that have a p-value less than 0.05 (below the line) are greyed out. The vertical line denotes zero fold change of sample to its counterpart (fold change = 1) with samples between -1.5 and 1.5 greyed out. Genes that are upregulated, compared to their counterpart are noted in green and genes that are downregulated are noted in red. Statistical significance increases as values ascend the vertical axis and move further away from the mid-line. The total number of non-coding, up or down regulated genes with a statistical p-value and fold change <-1.5 or >1.5 are listed below each graph.

In order to understand the effects of plasmid and transfection reagents on the cells, we investigated the differential gene regulation of mock transfected cells to untransfected cells. Using the statistical parameters previously described, a total of 1146 genes were significantly affected by the vector, transfection reagents, temperature shift and supplemental feed alone. Of these genes, 392 were significantly upregulated and 754 were significantly downregulated.

Next we investigated the effect of differential gene expression from the low and high expressing antibody transfections to the mock transfection. When compared to the

mock transfected cells the positive control variant (5L+6H) had only 8 genes differentially expressed with 4 genes significantly downregulated (LOC100760232, FOSB (LOC100755767), LOC100770071, and NSG1 (LOC100772794)) and 4 genes significantly upregulated (CHOP (LOC100763514), AIM2 (LOC100750381), ZNF50 (Nfp647) and LOC100754894). When compared to mock transfected cells, the low expressing variant (5L+19H) had only 9 genes differentially expressed compared to mock with 5 genes significantly downregulated (LOC100765613, RPS26 (LOC100755871), LOC100756262, LOC100757859 and PBLD (LOC100762425)) and 4 genes significantly upregulated (AIM2 (LOC100750381), CHOP (LOC100763514), HERPUD1 and ZNF250 (Zfp647)).

Finally to determine if there would be more significant induction of UPR related genes in an antibody that was difficult to express compared to an antibody that had high levels of expression, the low expressing variant (5L+19H) was compared to the positive control (5L+6H). Only 3 genes were differentially expressed, all were downregulated, and none of those genes have been implicated in UPR (LOC100752635, LOC100765613, and LOC100762498).

Based on the research by Prashad and Mehra (2015), 12 genes involved in UPR were evaluated. GAPDH was used as a house-keeping negative control gene. The UPR regulated genes included endoplasmic reticulum oxidoreductase alpha (ERO1-l), glucose regulated protein 78 (GRP78), X-box binding protein 1 (XBP1), ER-localized DnaJ homolog 4 (ERDJ4), glucose regulated protein 94 (GRP94), growth arrest and DNA damage gene 34 (GADD34), activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein (C/EBP) homologous protein/DNA damage inducible transcript 3

(CHOP/DDIT3), calreticulin (CRT/Calr), Nuclear factor erythroid 2-related factor 2 (NRF2/NFE2L2), and UDP-glucose:glycoprotein glucosyltransferase-variant 1 (UGGT1). Fold induction was graphed for all comparison scenarios as shown in Figure 8.

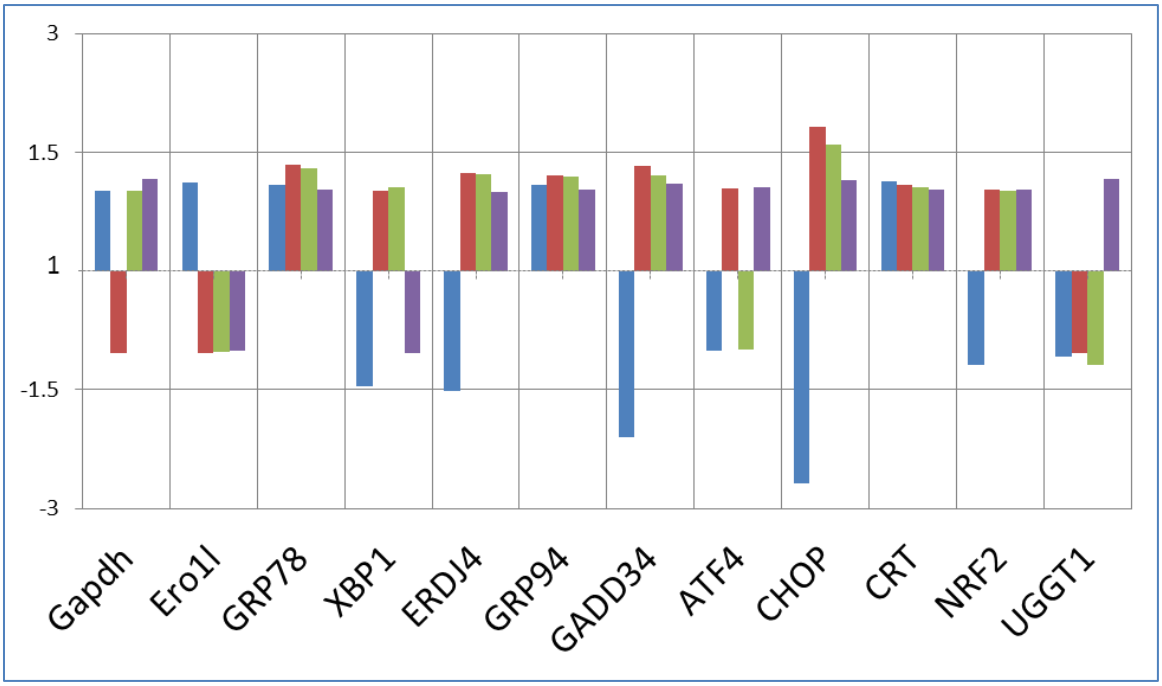


Figure 8. Fold change of UPR specific genes in mock transfected cells versus untransfected cells. Gene regulation of key UPR related genes. The fold change was graphed for each comparison scenario 1) mock vs. untransfected (blue), 2) 5L+19H vs. mock (red), 3) 5L+6H vs. mock (green), 4) 5L+6H vs. 5L+19H (purple). Fold change <-1.5 or >1.5 is considered significant.

As expected, GAPDH had negligible fold change for all scenarios screened. Unexpectedly, three UPR related genes, ERDJ4, GADD34 and CHOP were significantly down-regulated in mock vs. untransfected cells with 6.3%, 13.8% and 15% decreases in expression levels, respectively, and p-values of 0.001747, 0.006842, and 0.002024, respectively. Additionally, XBP1 had a fold change just under the set parameters with

6.27% decrease in expression level compared to untransfected cells and had a p-value of 0.000303. CHOP has been implicated in induction of cell death by direct activation of GADD34 (Marciniak *et al*., 2004) while ERDJ4 is an XBP1 dependent UPR gene which has been shown to stimulate downstream proteins that suppress ER-stress related induction of cell death (Lee *et al*., 2003). Full statistical analysis for mock vs. untransfected cells can be found in the appendix (see Appendix 3, Table 1). There were no statistically significant changes for any of these genes when directly comparing 5L+19H vs. 5L+6H (see Appendix 3, Table 2). However, CHOP, was significantly upregulated in both 5L+19H vs. mock (low expression) and 5L+6H vs. mock (high expression) with 10.7% and 8.2% increases in CHOP expression, respectively, and p-values of 0.000476 and 0.000843, respectively (see Appendix 3, Tables 3 and 4 respectively). All remaining genes were not significantly up or down regulated or did not have statistically significant p-values.

Next, we observed the 17 genes that were significantly up or down regulated for 5L+19H vs. mock and 5L+6H vs. mock (Figure 9).

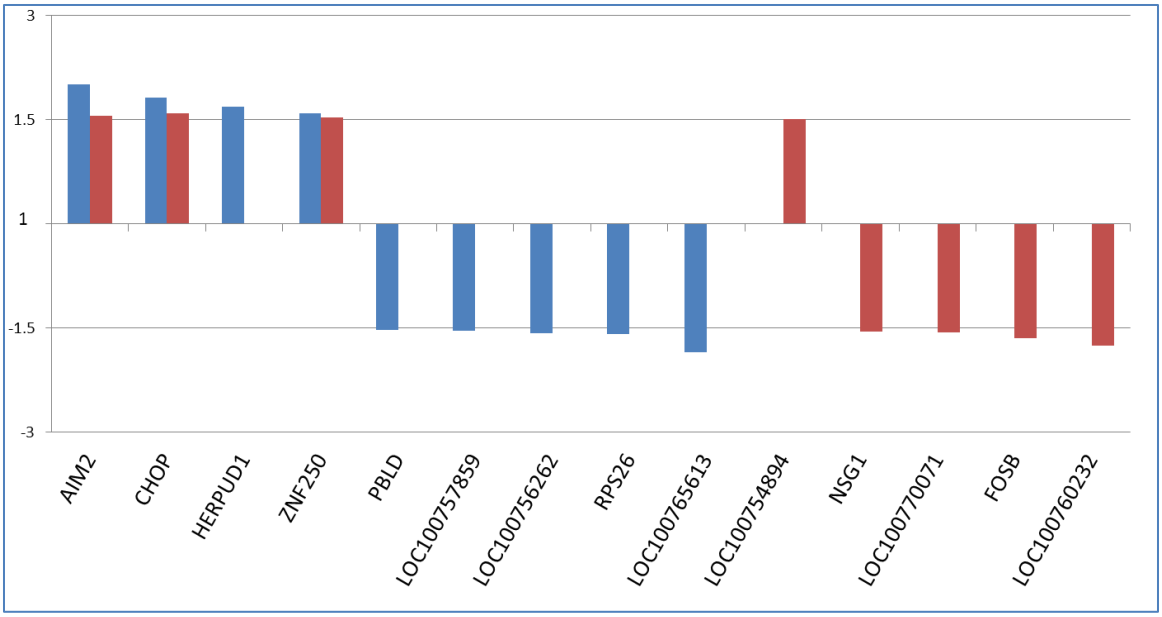


Figure 9. Fold change of differentially expressed genes. Genes that had significant differential gene regulation <-1.5 or >1.5 fold difference. Comparisons of 5L+19H vs. mock are denoted by blue bars and comparisons of 5L+6H vs. mock are denoted by red bars. Genes that have no human homologs are denoted with the preface "LOC".

Only 2 out of the 17 differentially regulated genes, CHOP and HERPUD1, have been noted as playing a role in UPR. While CHOP was previously described to be up-regulated for both comparison sets, HERPUD1 was only upregulated for the 5L+19H vs. mock comparison (low expression) with an 8.1% increase and a p-value of 0.000783. HERPUD1 may play a role in both UPR and ER-associated protein degradation (ERAD) (Ma *et al*., 2004). Full statistical analysis for UPR related genes for 5L+19H vs. 5L+6H can be found in the supplementary data (see Tables

Chapter IV

Discussion

To date, several studies have evaluated the rate limiting steps of antibody production in stable cell lines, but investigation of the steps inhibited in transient expression systems has been minimal. In the present study, we explored several stages that could be rate limiting to transient antibody production and the role UPR regulation may play within a transient system. The following section will further describe these findings and their potential implications.

Experimental review

To date, a large number of studies have evaluated the stages at which recombinant antibody production from stable cell lines becomes limiting; however, these studies have yielded varying results. Collectively, the studies have implicated the rate limiting stages to be at the transcriptional level, post-translational stages, and have also shown UPR induction correlating with productivity levels (Jiang *et al*., 2006; Reisinger *et al*., 2008; Chusainow *et al*., 2008; Mason *et al*., 2012; and Prashad and Mehra, 2015). The differences in these results may be in part due to variances at the site of chromosomal integration as well as the number of copies of the genes integrated. These aspects can affect the accessibility of the gene for transcription as well as the amount of mRNA made

available for translation (Colosimo *et al*., 2000). Additionally, the differences observed could be due, in part, to the unique properties of the antibodies studied. As described by Mason *et al*., (2012), a single amino acid substitution can have significant impact on the expression level of an antibody. Therefore, the differences in the antibodies evaluated in each study could have bottlenecks unique to their specific properties. While a significant amount of time has been spent evaluating the limitations of antibody production in stable cell lines, little has been done to understand the mechanisms involved in a transient system. Understanding the mechanisms that cause decreased antibody production in a transient system could aid in better antibody design as well as developing an improved expression system for future recombinant antibody screening campaigns. In this study, we evaluated transient expression of unique panel of humanized variants from two distinct antibody campaigns to see which stages of antibody production were limiting. Additionally, this was the first study to evaluate the extent of UPR induction in a transient expression system.

We investigated transient recombinant antibody production of 25 humanized monoclonal antibody variants. Five variants yielded historically high levels of secreted antibody and were used as positive controls. The remaining 20 had historically low levels of secreted antibody expression. All variants were re-evaluated for expression in the CHO 3E7 cell line and protein quantitation was determined using Protein A-coated biosensor tips. Of the 20 test variants re-screened, 14 variants were confirmed to have low levels of antibody titer (less than 15µg/ml) with one variant (30L+29H) having a moderately low yield with 17.7µg/ml (63% lower than the positive control). It was interesting to see five of the variants (17L+16H, 12L+14H, 20L+14H, 20L+15H, and

25L+23H) had antibody titers equivalent to their respective controls. Many factors can affect transient cellular protein expression levels including the cell line chosen for expression, the transfection reagents used, the type of vector and any supplemental feeds or temperature reductions during production (Jäger *et al*., 2015, Colosimo *et al*., 2000). Four out of those five antibodies that had increased productivity levels had used alternative expression systems, vectors, supplemental feeds and environmental parameters in the past. It was therefore not too surprising to see some of these variants increase their expression levels using the optimized CHO 3E7 expression system. Although one antibody had previously been expressed in the CHO 3E7 cells in the past and resulted with poor expression, it is worth noting that the storage conditions of the transfection reagents as well as the passage number of the cells can both have a significant impact on protein production. All reagents used in this study were screened for transfection efficiency prior to use and each set of transfections used cells at the same passage number. Additionally, it has also been observed that for transient PEI mediated transfections, an optimized ratio of DNA and PEI is necessary to obtain the highest levels of recombinant protein and transfecting outside of this range can result in significantly lower yields (Rajendra *et al*., 2015). This further illustrates the need to carefully consider the type of expression system for transient antibody production as well as the need to optimize the transfection parameters.

Limitations in transcription

Several investigations of high versus low yielding stable clones have implicated different stages of protein production to be rate limiting. To understand the potential

limitations at the transcriptional level we performed Northern blot analysis on all variants. Samples were taken 72 hours post transfection as mRNA levels typically begin to rise and peak between 12 and 72 hours (Colosimo *et al*., 2000). Four variant chains, 4L, 13L, 22H, and 26L, had no detectable mRNA levels. These were used in co-transfections for seven antibody variants. In addition, three variant chains, 18H, 28L and 31H, had 70%, 75% and nearly 80% lower relative levels of mRNA compared to their controls, respectively. These were used in co-transfections for three antibody variants. Overall, four out of fifteen light chains (27%) and three out of fourteen heavy chains (21%) were blocked at the mRNA level. This finding was interesting as all genes were in the same plasmid backbone and therefore were under the same enhancer and promoter control. This suggests that the issue for those genes may have been specific to their individual sequences. Evidence for the effect of antibody sequence on subsequent antibody production was highlighted in the works of Mason *et al*., (2002) in which a single amino acid substitution resulted in production of a non-secreted heavy chain and therefore significant fold decrease in antibody production. Although performed in stable cell lines, several studies have shown similar observations where the detectable level of mRNA does not necessarily correlate to secreted antibody titers (Jiang *et al*., 2006, Reisinger *et al*., 2008). Since all antibody genes were placed into the same vector and under control of the same promoter it is unlikely that initiation of transcription was the problem, and while identifying the specific issue for these constructs at the transcriptional level was beyond the scope of this study, it is possible that the chains with undetectable or low levels of mRNA could be more susceptible to mRNA degradation due to their nucleic acid sequence properties (Cooper, G. M., 2000). As would be expected, the

absence of detectable mRNA for those seven variants and low levels of detectable mRNA for the additional three variants resulted in poor or undetectable levels of secreted antibody. It should be noted that for those variants that had no detectable heavy chain mRNA, the yield of detectable antibody was zero, however, those variants that had no detectable light chain mRNA typically had low levels of quantitated protein. Protein A specifically binds the Fc portion of antibodies and we were presumably detecting free heavy chain in the cell supernatant. This free heavy chain was most likely released from apoptotic cells as the heavy chain binding protein, BiP, remains associated with heavy chain in the ER until they are able to associate with light chains (Hendershot *et al*., (1987); Wattson *et al.*, (2008)).

One variant, 17L+16H, did stray from the trend of low mRNA levels correlating with low antibody titers. While the detectable light chain was slightly more than the control, the heavy chain had nearly 90% less relative intensity compared to the control, yet the secreted protein titer was nearly equivalent to the control. Although not to the same degree, others have observed mRNA levels do not necessarily correlate with detectable antibody levels in a transient system (Mason *et al.,* (2012)).

Intracellular protein synthesis

In general, intracellular protein quantitation correlated with secreted antibody titers. The variants which had high levels of intracellular heavy chain and light chain, equal to or greater than the controls, all had high levels of secreted antibody and similar findings have been reported by Rajendra *et al*., (2015). Interestingly, several variants had

adequate quantities of mRNA for both heavy and light chain but manifested with low intracellular protein concentrations which in turn correlated to low levels of secreted antibody titers. This would suggest, for those variants, that protein production was being blocked at either translational or post-translational stages and therefore made them potential candidates for UPR induction. These variants included 5L+19H, 7L+11H, 8L+9H, and 8L+18H. Of specific interest was clone 5L+19H as there were abundant levels of mRNA but significantly lower levels of intracellular light chain. This particular clone also shared the same light chain as the control but had ~90% reduction in secreted antibody. Due to these specific characteristics, 5L+19H was chosen for UPR induction evaluation.

UPR regulation in a transient system

Since the transfection process and vector alone would be anticipated to have a significant impact on cellular gene regulation, we first evaluated mock transfected cells compared to untransfected cells (Jäger *et al*., 2015; Pham *et al*., 2006). While there were many genes that were differentially up or down regulated in the mock compared to control transfection, it was of considerable note that four genes that are typically upregulated during UPR induction (XBP1, ERDJ4, GADD34 and CHOP) were downregulated in the mock transfected cells by at least a 1.5 fold difference. As outlined in Figure 10, three of the genes that were noted to be downregulated (XBP1, ERDJ4 and GADD34) are associated with pathways that are pro-survival; however, CHOP is typically associated with pro-apoptotic outcomes. At this point, it is uncertain as to why these specific genes are downregulated in the mock transfections as this has not been

previously observed. While speculative, it is possible that there are compensatory mechanisms in place within cells that are experiencing general stress (such as those caused by the transfection reagents and empty plasmid vector) but not ER-related stress. This could potentially cause UPR specific genes to be down-regulated until homeostasis is regained. It should also be noted that while there is statistical significance to the downregulation of these genes in the mock transfected cells, the fold change was modest compared to other genes which were up or downregulated.

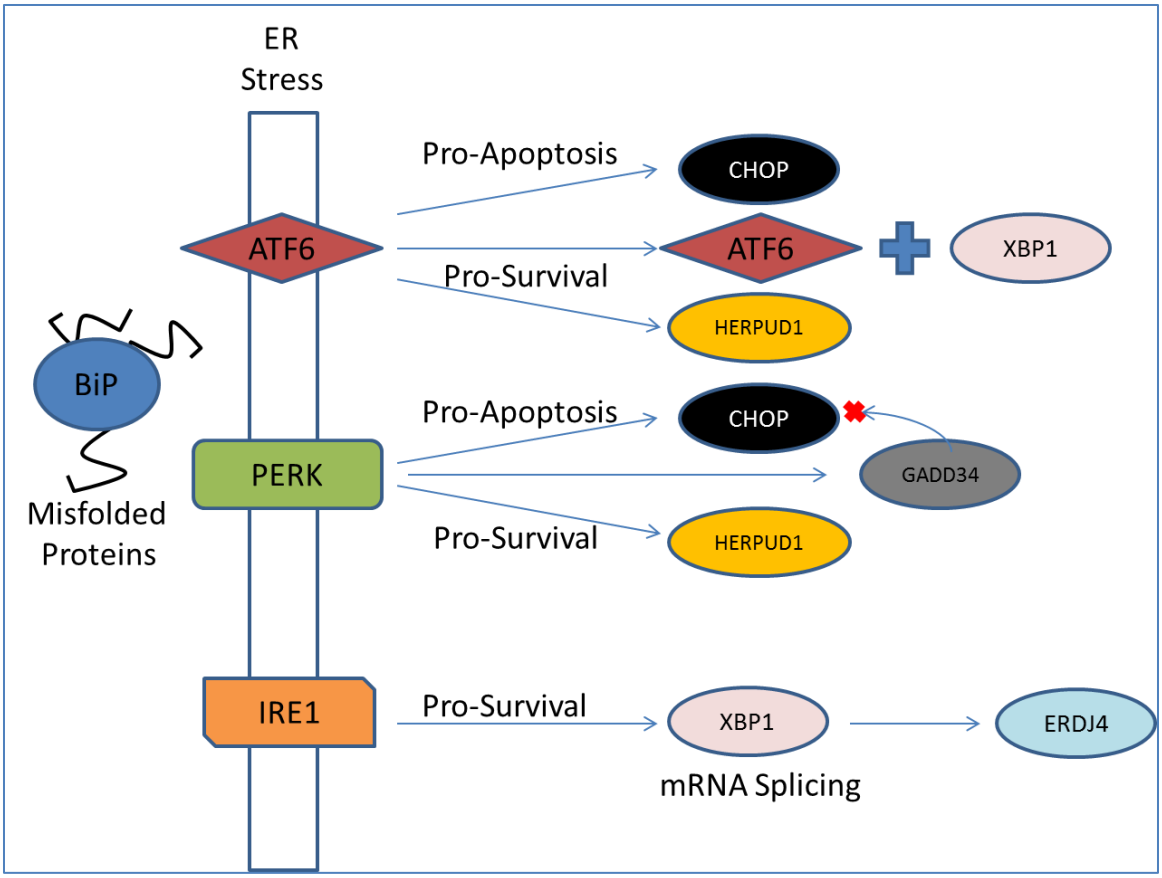


Figure 10. UPR pathway of differentially expressed genes. The three pathways of UPR induction from ER stress. The figure depicts a simplified flow chart of the specific genes which were found to be up or downregulated in our test transfection samples. All three pathways are activated by the dissociation of BiP in the presence of unfolded or misfolded proteins (Chakrabarti *et al*., 2011). The ATF6 pathway has pro-survival characteristics through its downstream upregulation of chaperone proteins HERPUD1 and its interaction with XBP1 which help target degradation of unfolded or misfolded proteins (Schröder & Kaufman, 2005; Yoshida *et al*., 2001; Lee *et al*., 2003). Initially, the ATF6 pathway also causes inhibition of apoptotic activity but can be overrun if homeostasis is not returned and may induce CHOP (Yoshida *et al*., 2001; Lee *et al*., 2003). The PERK pathway can have both pro-survival and pro-apoptotic activities and is associated with ER stress induction and non-ER associated stress induction (Novoa *et al*., 2001). In the initial phases of ER stress PERK causes the activation of several downstream proteins including HERPUD1 which help block translation thereby limiting the amount of new proteins needing to be processed (Novoa *et al*., 2001). However, if homeostasis cannot be achieved, the PERK pathway can activate a pro-apoptotic mechanism which includes the induction of CHOP (Hussain *et al*., 2014). Additionally, a potential negative feedback loop involving GADD34 and its attenuation of CHOP can be initiated to restore the cell to homeostasis (Novoa *et al*., 2001). Finally, the IRE1 pathway plays a pro-survival role through splicing of XBP1 and eventual induction of downstream chaperone proteins including ERDJ4 (Lee *et al*., 2003).

Once it was established which genes were specifically up or down-regulated due to the transfection process alone, we compared our two experimental variables, 5L+19H (low expression) to mock, 5L+6H (high expression) to mock and finally, the differences between 5L+19H to 5L+6H. Somewhat surprisingly, there was very little variability in gene expression between the high and low expressing clones compared to mock and even fewer differences in gene expression between low and high expressing clones when compared to each other. There were a total of 9 genes for 5L+19H and 8 genes for 5L+6H that were either up or downregulated when compared to mock. Of these genes, only two were associated with UPR: CHOP and HERPUD1. For both sets, CHOP was upregulated at least 1.5 fold higher than mock transfected cells with slightly higher induction for the poor expressing variant (5L+19H). The gene HERPUD1 was only upregulated for the poor expressing variant with just over a 1.5 fold increase compared to mock. Although the levels of upregulation of these genes are modest, it suggests that there is some level of UPR induction for both sets of transient transfections. Additionally, it cannot be concluded which UPR pathway or pathways are specifically associated with each transfection set as CHOP and HERPUD1 are both associated with the ATF6 and PERK pathways (Figure 10). A time course study may need to be performed to determine the pathways that are activated. It should also be noted that there was no significant upregulate of UPR related genes between to two test transfections; therefore UPR induction was similar for low expressing and high expressing antibodies at this time point.

Interestingly, previous studies observing the regulation of UPR associated proteins in stable batch cultures over a 7 day time course found that there was significant

induction of several chaperone proteins on day 4. CHOP, GADD34, XPB1 splice variants and induction of downstream chaperone proteins were noted at later time points (Prashad and Mehra (2015)). While we did not witness the same level of induction for our test variants, it was interesting that CHOP, GADD34 and XBP1 were all noted to be downregulated in the mock transfection suggesting that there may be inherent regulatory pathways to limit their synthesis when the cells are not under ER stress. Additionally, several studies of CHO cell lines overexpressing either CHOP, GADD34 or XBP1 (in conjunction with ERO1-Lα) have been shown to increase protein expression levels (Nishimiya *et al*., 2012, Omasa *et al*., 2008 and Cain *et al*., 2013). Further investigation of those genes as well as HERPUD1 may be necessary to determine whether they could also increase antibody titers within a transient system.

Chapter V

Conclusions

This study was designed to obtain a better understanding of the limitations of the CHO cellular protein production system when generating protein in a transient manner using an array of antibody variants. We determined that limitations across the panel of antibodies screened occurred at several different stages of antibody production and encompassed transcriptional and post-translational stages of protein productivity. Of the 20 unique variants screened, seven chains were blocked or limited at the transcriptional level. These chains were associated with ten variants which also had low levels of antibody secretion, and accounted for half of the antibodies evaluated. One chain was limited at the transcriptional level but this did not impair secreted antibody production and was the only antibody to display this particular quality. From the remaining ten variants, five were shown to improve production when placed into an optimized expression system while one antibody had moderately improved production, further underlying the need to thoroughly evaluate cell lines, vectors and optimize transfection processes. The remaining four antibodies had expression blocked at the translational or post-translational stage. For the samples that were analyzed for UPR regulation, we observed modest UPR induction for both high and low expressing antibodies. Additionally, the level of UPR induction was similar for both high and low expressing antibodies. It cannot be determined from this study which UPR pathway or pathways

were activated as CHOP was upregulated for both the low and high expressing variant and HERPUD1 was upregulated for only the low expressing variant, but both genes are associated with the ATF6 and PERK pathways. Overall, the effect of the transfection reagent in conjunction with plasmid had the most significant impact on differential gene regulation. Additionally, four key genes typically upregulated when UPR is induced, were found to be downregulated in mock transfected cells compared to untransfected cells. While speculative, it is possible that cellular stress that is not associated with ER-related stress could cause specific down-regulation of UPR related genes although additional studies would need to be performed to further understand this observation.

Appendix 1

Northern blots

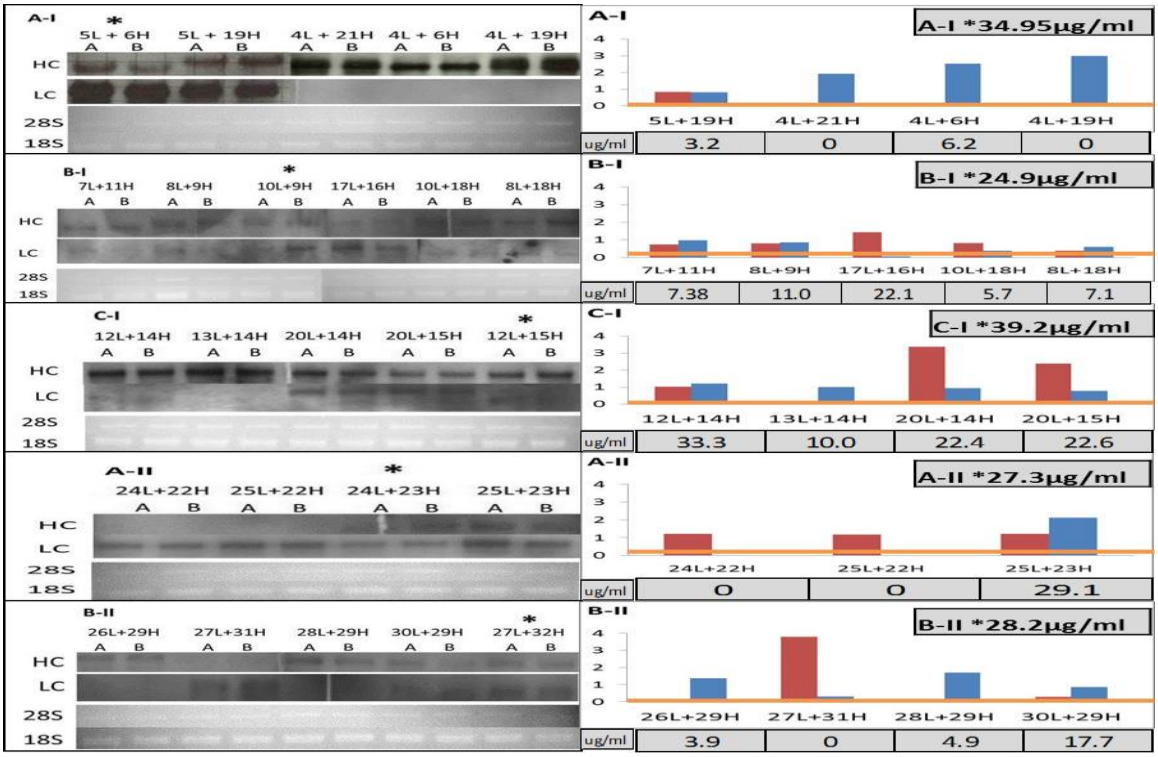


Figure 4. Northern blot and relative quantitated mRNA. Left Panel: Northern blots of 72 hour time point total RNA samples and corresponding 18S and 28S RNA. Isolated total RNA was quantitated by NanoDrop and 0.5µg of RNA was loaded per sample. RNA gels were imaged with UV light for 18S and 28S RNA to insure integrity of the RNA and to confirm normalization of the sample load. Blots were probed with digoxigenin (DIG) labeled heavy and light chain and detected using anti-DIG antibody. Bands were visualized by chemiluminescent excitation on film. Samples are organized by monoclonal antibody (A-I through B-II) and their respective humanized variants (xL + xH). The (*) denotes the positive control for the antibody clone group. A and B denote replicates. Right Panel: Fold change of RNA compared to the positive control and corresponding protein yields. Heavy and light chain bands for the variants were quantitated using densitometry (GeneTools 4.02 software, SynGene, Synoptics Ltd.). Quantity of RNA for light chains (red bars) and heavy chains (blue bars) were calculated relative to the positive control (yellow line). The quantitated antibody titer from day 7 is noted below each variant. The control quantitated antibody titer from day 7 is noted in the upper right corner (*).

Appendix 2

Western blots

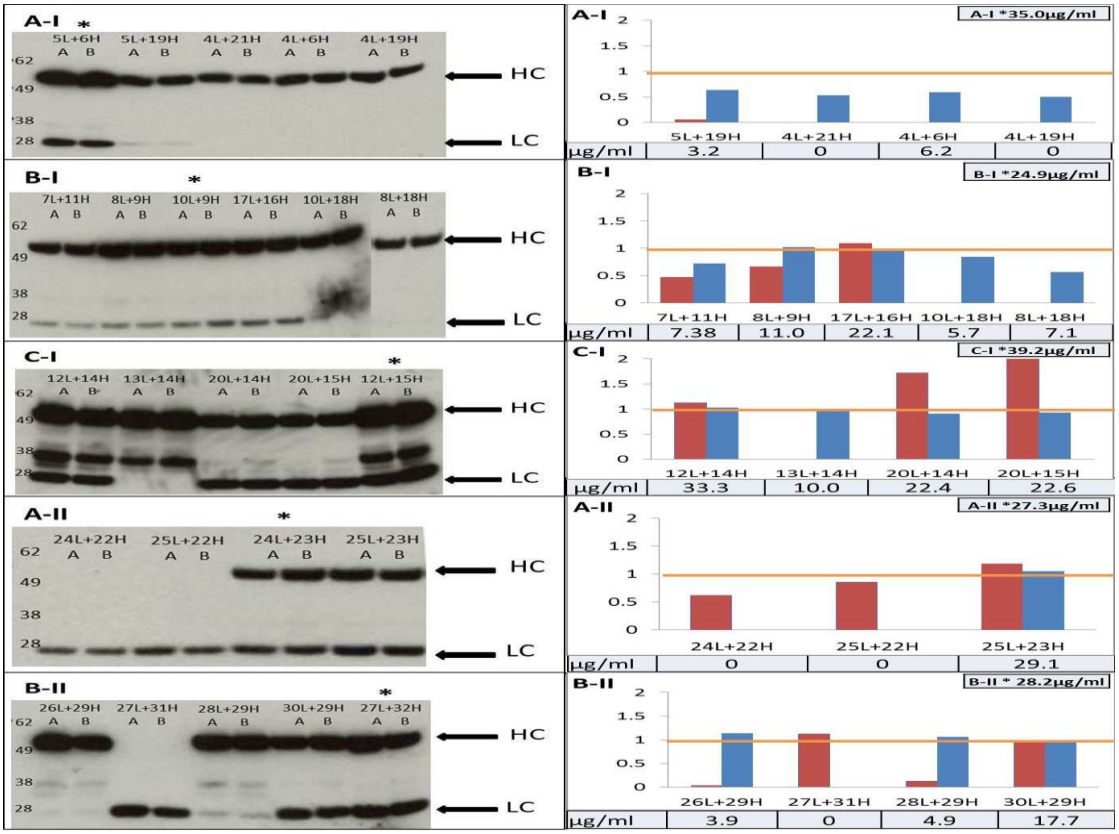


Figure 5. Western blot of intracellular protein. Left Panel: Western blots of whole cell lysates taken 72 hours post transfection. Biological replicate samples of cell lysates (A and B), normalized by cell number, were run under reducing conditions and probed using peroxidase-conjugated AffiniPure goat α-human IgG (H+L) antibody was visualized by chemiluminescent excitation on film. Samples are organized by monoclonal antibody (A-I through B-II) and the humanized variants for each clone (xL + xH). The (*) denotes the positive control for the antibody clone group. Arrows denote signal from heavy chain (HC, ~50 kDA) and light chain (LC ~27 kDa). Right Panel: Fold change compared to the positive control. Heavy and light chains were quantitated by densitometry using GeneTools 4.02 software (SynGene, Synoptics Ltd.). Quantity of protein for the light chains (red bars) and heavy chains (blue bars) were calculated and graphed as the fold change compared relative to their respective positive controls (yellow line). The quantitated antibody titer from day 7 is noted below each variant. The control quantitated antibody titer from day 7 is noted in the upper right corner (*).

Appendix 3

Microarray statistical analysis

Transcript Cluster ID	Mock Bi-weight Avg Signal (log2)	Untransfected Bi-weight Avg Signal (log2)	Mock Standard Deviation	Untransfected Standard Deviation	Fold Change (linear) (Mock vs. Untransfected)	ANOVA p-value (Mock vs. Untransfected)	FDR p-value (Mock vs. Untransfected)	Gene Symbol	Description	Total Probes
18103922	12.64	12.62	0.02	0.03	1.01	0.764737	0.87989	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	26
18118174	8.82	8.65	0.05	0.08	1.12	0.091241	0.25637	Ero1l	ERO1-like (S. cerevisiae)	25
17982311	11.9	11.78	0.03	0.04	1.09	0.021672	0.097066	Hspa5	heat shock protein 5	25
18028468	8.06	8.6	0.08	0.04	-1.45	0.000303	0.008031	Xbp-1	X-box binding protein 1	25
18087796	8.93	9.53	0.12	0.07	-1.52	0.001747	0.019736	LOC100763648	dnaJ homolog subfamily B member 9-like	24
18024760	12.12	12	0.02	0.01	1.09	0.000768	0.012666	Hsp90b1	heat shock protein 90, beta (Grp94), member 1	27
18002683	6.66	7.73	0.2	0.28	-2.1	0.006842	0.045315	LOC100757625	protein phosphatase 1 regulatory subunit 15A-like	25
17964037	11.08	11.09	0.08	0.01	-1.01	0.26618	0.489568	Atf4	activating transcription factor 4	22
18012083	8.05	9.47	0.14	0.32	-2.68	0.002024	0.021545	LOC100763514	DNA damage-inducible transcript 3 protein-like	26
18100054	3.38	3.19	0.1	0.08	1.13	0.088844	0.25183	LOC100760153	calreticulin-like	22
18084963	10.33	10.57	0.05	0.04	-1.18	0.002552	0.024473	LOC100752102	nuclear factor erythroid 2-related factor 2-like	26
17969325	3.84	3.95	0.15	0.06	-1.08	0.599405	0.772839	Ugt1a1; LOC100757172	UDP glucuronosyltransferase 1 family, polypeptide A1; UDP-glucuronosyltransferase 1-7C-like	24

Table 1. Mock transfection versus untransfected cells. Full statistical results of mock vs. untransfected cells for several UPR related genes.

Transcript Cluster ID	Low Expression Bi-weight Avg Signal (log2)	High Expression Bi-weight Avg Signal (log2)	Low Expression Standard Deviation	High Expression Standard Deviation	Fold Change (linear) (Low Expression vs. High Expression)	ANOVA p-value (Low Expression vs. High Expression)	FDR p-value (Low Expression vs. High Expression)	Gene Symbol	Description	Total Probes
17969325	3.81	3.59	0.06	0.2	1.16	0.088851	0.899815	Ugt1a1; LOC100757172	UDP glucuronosyltransferase 1 family, polypeptide A1; UDP-glucuronosyltransferase 1-7C-like	24
18118174	8.77	8.78	0.08	0.01	-1.01	0.616508	0.944219	Ero1l	ERO1-like (S. cerevisiae)	25
17982311	12.32	12.28	0.02	0.04	1.03	0.316343	0.900636	Hspa5	heat shock protein 5	25
18028468	8.08	8.14	0.12	0.02	-1.04	0.789128	0.974343	Xbp-1	X-box binding protein 1	25
18087796	9.22	9.22	0.04	0.02	1	0.381422	0.910264	LOC100763648	dnaJ homolog subfamily B member 9-like	24
18024760	12.4	12.37	0.02	0.03	1.02	0.296563	0.899815	Hsp90b1	heat shock protein 90, beta (Grp94), member 1	27
18002683	7.07	6.93	0.09	0.1	1.1	0.092047	0.899815	LOC100757625	protein phosphatase 1 regulatory subunit 15A-like	25
17964037	11.14	11.08	0.04	0.07	1.05	0.099935	0.899815	Atf4	activating transcription factor 4	22
18012083	8.91	8.71	0.06	0.04	1.15	0.016502	0.899815	LOC100763514	DNA damage-inducible transcript 3 protein-like	26
18055349	12.04	12.01	0.02	0.02	1.02	0.134921	0.899815	Calr	calreticulin	21
18084963	10.38	10.35	0.08	0.03	1.02	0.901582	0.990393	LOC100752102	nuclear factor erythroid 2-related factor 2-like	26
17969325	3.81	3.59	0.06	0.2	1.16	0.088851	0.899815	Ugt1a1; LOC100757172	UDP glucuronosyltransferase 1 family, polypeptide A1; UDP-glucuronosyltransferase 1-7C-like	24

Table 2. Low expression (5L+19H) versus high expression (5L+6H). Full statistical results of low vs. high transfected cells for several UPR related genes.

Transcript Cluster ID	Low Expression Bi-weight Avg Signal (log2)	Mock Bi-weight Avg Signal (log2)	Low Expression Standard Deviation	Mock Standard Deviation	Fold Change (linear) (Low Expression vs. Mock)	ANOVA p-value (Low Expression vs. Mock)	FDR p-value (Low Expression vs. Mock)	Gene Symbol	Description	Total Probes
18103922	12.59	12.64	0.03	0.02	-1.03	0.095161	0.705451	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	26
18118174	8.77	8.82	0.08	0.05	-1.03	0.971028	0.994531	Ero1l	ERO1-like (S. cerevisiae)	25
17982311	12.32	11.9	0.02	0.03	1.34	0.000053	0.196221	Hspa5	heat shock protein 5	25
18028468	8.08	8.06	0.12	0.08	1.01	0.191792	0.72053	Xbp-1	X-box binding protein 1	25
18087796	9.22	8.93	0.04	0.12	1.23	0.013244	0.628635	LOC100763648	dnaJ homolog subfamily B member 9-like	24
18024760	12.4	12.12	0.02	0.02	1.21	0.000031	0.151777	Hsp90b1	heat shock protein 90, beta (Grp94), member 1	27
18002683	7.07	6.66	0.09	0.2	1.32	0.056582	0.69796	LOC100757625	protein phosphatase 1 regulatory subunit 15A-like	25
17964037	11.14	11.08	0.04	0.08	1.04	0.120149	0.705451	Atf4	activating transcription factor 4	22
18012083	8.91	8.05	0.06	0.14	1.82	0.000476	0.43831	LOC100763514	DNA damage-inducible transcript 3 protein-like	26
18055349	12.04	11.92	0.02	0.02	1.09	0.003852	0.534386	Calr	calreticulin	21
18084963	10.38	10.33	0.08	0.05	1.03	0.974898	0.995613	LOC100752102	nuclear factor erythroid 2-related factor 2-like	26
17969325	3.81	3.84	0.06	0.15	-1.03	0.541192	0.864441	Ugt1a1; LOC100757172	UDP glucuronosyltransferase 1 family, polypeptide A1; UDP-glucuronosyltransferase 1-7C-like	24

Table 3. Low expression (5L+19H) versus mock transfected cells. Full statistical results of low vs. mock transfected cells for several UPR related genes.

Transcript Cluster ID	High Expression Bi-weight Avg Signal (log2)	Mock Bi-weight Avg Signal (log2)	High Expression Standard Deviation	Mock Standard Deviation	Fold Change (linear) (High Expression vs. Mock)	ANOVA p-value (High Expression vs. Mock)	FDR p-value (High Expression vs. Mock)	Gene Symbol	Description	Total Probes
18109768	13.07	13.06	0.03	0.01	1.01	0.587732	0.94591	Gapdh	glyceraldehyde-3-phosphate dehydrogenase	25
18118174	8.78	8.82	0.01	0.05	-1.02	0.405535	0.914282	Ero1l	ERO1-like (S. cerevisiae)	25
17982311	12.28	11.9	0.04	0.03	1.29	0.000299	0.436367	Hspa5	heat shock protein 5	25
18028468	8.14	8.06	0.02	0.08	1.05	0.073435	0.895712	Xbp-1	X-box binding protein 1	25
18087796	9.22	8.93	0.02	0.12	1.22	0.015091	0.895712	LOC100763648	dnaJ homolog subfamily B member 9-like	24
18024760	12.37	12.12	0.03	0.02	1.19	0.000126	0.288998	Hsp90b1	heat shock protein 90, beta (Grp94), member 1	27
18002683	6.93	6.66	0.1	0.2	1.21	0.26795	0.89634	LOC100757625	protein phosphatase 1 regulatory subunit 15A-like	25
17964037	11.08	11.08	0.07	0.08	-1	0.919907	0.990621	Atf4	activating transcription factor 4	22
18012083	8.71	8.05	0.04	0.14	1.59	0.000843	0.58084	LOC100763514	DNA damage-inducible transcript 3 protein-like	26
18055349	12.01	11.92	0.02	0.02	1.06	0.018732	0.895712	Calr	calreticulin	21
18084963	10.35	10.33	0.03	0.05	1.01	0.817051	0.977142	LOC100752102	nuclear factor erythroid 2-related factor 2-like	26
17969325	3.59	3.84	0.2	0.15	-1.19	0.085956	0.895712	Ugt1a1; LOC100757172	UDP glucuronosyltransferase 1 family, polypeptide A1; UDP-glucuronosyltransferase 1-7C-like	24

Table 4. High expression (5L+6H) versus mock transfected cells. Full statistical results of high vs. mock transfected cells for several UPR related genes.

Bibliography

Almagro, J. C., & Fransson, J. (2008). Humanization of Antibodies. *Frontiers in Bioscience, 13*, 1619-1633.

Bandaranayake, A. D., & Almo, S. C. (2014). Recent Advances in Mammalian Protein Production. *FEBS Letters, 588* (2), 253-260.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series B, 57* (1), 289-300.

Cain, K., Peters, S., Hailu, H., Sweeney, B., & Stephens, P. (2013). A CHO Cell Line Engineered to Express XPB1 and ERO1-Lα Has Increased Levels of Transient Protein Expression. *Biotechnology Progress, 29* (3), 697-706.

Chakrabarti, A., Chen, A. W., & Varner, J. D. (2011). A Review of the Mammalian Unfolded Protein Response. *Biotechnology and Bioengineering, 108* (12), 2777-2793.

Chusainow, J., Yang, Y. S., Yeo, J. H. M., Toh, P. C., Asvadi, P., Wong, N. S. C., & Yap, M. G. S. (2009). A study of Monoclonal Antibody-Producing CHO Cell Lines: What Makes a Stable High Producer? *Biotechnology and Bioengineering, 102* (4), 1182-1196.

Colosimo, A., Goncz, K. K., Holmes, A. R., Kunzelmann, K., Novelli, G., Malone, R. W., … Gruenert, D. C. (2000). Transfer and Expression of Foreign Genes in Mammalian Cells. *BioTechniques, 29* (2), 314-331.

Cooper, G. M. (2000). *The cell: A molecular approach* (2nd ed.). Sunderland, MA: Sinauer Associates.

Ecker, D. M., Jones, S. D., & Levine, H. L. (2015). The Therapeutic Monoclonal Antibody Market. *mAbs, 7* (1), 9-14.

Hendershot, L., Bole, D. & Kearney, J. F. (1987). Assembly and Secretion of Heavy Chains that Do Not Associate Posttranslationally with Immunoglobulin Heavy Chain-binding Protein. *The Journal of Cell Biology, 104*, 761-767.

Hussain, H., Maldonado-Agurto, R., & Dickson, A. J. (2014). The Endoplasmic Reticulum and Unfolded Protein Response in the Control of Mammalian Recombinant Protein Production. *Biotechnology Letters, 36*, 1581-1593.

Irizarry, R. A., Hobbs, B., Collin, F., Beazer-Barclay, Y. D., Antonellis, K. J., Scherf, U., & Speed, T. P. (2003). Exploration, Normalization, and Summaries of High Density Oligonucleotide Array Probe Level Data. *Biostatistics, 4*, 249-264.

Jäger, V., Büssow, K., & Schirrmann, T. (2005). Transient recombinant protein expression in mammalian cells. In M. Al-Rubeai (Ed.), *Cell engineering: Animal Cell Culture Vol. 9*. (27-64). Cham, Switzerland: Springer International Publishing.

Jiang, Z., Huang, Y., & Sharfstein, S. T. (2006). Regulation of Recombinant Monoclonal Antibody Production in Chinese Hamster Ovary Cells: A Comparative Study of Gene Copy Number, mRNA Level, and Protein Expression. *Biotechnology Progress, 22,* 313-318.

Jones, P. T., Dear, P. H., Foote, J., Neuberger, M. S., & Winter, G. (1986) Replacing the Complementarity-determining Regions in a Human Antibody with those from a Mouse. *Nature, 321* (6069), 522-525.

Köhler, G., & Milstein, C. (1975). Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity. *Nature, 256* (5517), 495-497.

Lee, A., Iwakoshi, N. N., & Glimcher, L. H. (2003). XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response. *Molecular and Cellular Biology*, *23* (21), 7448-7459.

Ma, Y., & Hendershot, L. M. (2004). Herp is Dually Regulated by Both the Endoplasmic Reticulum Stress-specific Branch of the Unfolded Protein Response and a Branch that is shared with Other Cellular Stress Pathways. *The Journal of Biological Chemistry, 279* (1), 13792-13799.

Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., … Ron, D. (2004). CHOP Induces Death by Promoting Protein Synthesis and Oxidation in the Stressed Endoplasmic Reticulum. *Genes & Development, 18*, 3066-3077.

Mason, M., Sweeney, B., Cain, K., Stephens, P., & Sharfstein, S. T. (2012) Identifying Bottlenecks in Transient and Stable Production of Recombinant Monoclonal-Antibody Sequence Variants in Chinese Hamster Ovary Cells. *Biotechnology Progress, 28* (3), 846-855.

Morrison, S. L., Johnson, M. J., Herzenberg, L. A., & Oi, V. T. (1984). Chimeric Human Antibody Molecules: Mouse-Antigen Binding Domains with Human Constant

Region Domains. *Proceedings of the National Academy of Sciences of the United States of America, 81*, 6851-6855.

Nishimiya, D., Mano, T., Miyadai, K., Yoshida, H., & Takahashi, T. (2012). Overexpression of CHOP alone and in Combination with Chaperones is Effective in Improving Antibody Production in Mammalian Cells. *Applied Microbiology and Biotechnology, 97* (6), 2531-2539.

Novoa, I., Zeng, H., Harding, H. P., & Ron, D. (2001). Feedback Inhibition of the Unfolded Protein Response by GADD34-mediated Dephosphorylation of eIF2α. *The Journal of Cell Biology, 153* (5), 1011-1021.

Omasa, T., Takami, T., Ohya, T., Kiyama, E., Hayashi, T. Nishii, H., … Ohtake, H. (2008). Overexpression of GADD34 Enhances Production of Recombinant Human Antithrombin III in Chinese Hamster Ovary Cells. *Journal of Bioscience and Bioengineering, 106* (6), 568-573.

Pham, P. L., Kamen, A., & Durocher, Y. (2006). Large-scale Transfection of Mammalian Cells for the Fast Production of Recombinant Protein. *Molecular Biotechnology, 34* (2), 225-238.

Prashad, K., & Mehra, S. (2015). Dynamics of Unfolded Protein Response in Recombinant CHO Cells. *Cytotechnology, 67* (2), 237-254.

Pybus, L. P., Dean, G., West, N. R., Smith, A., Daramola, O., Field, R., … James, D. C. (2014). Model-directed Engineering of "Difficult-to-express" Monoclonal Antibody Production by Hamster Ovary Cells. *Biotechnology and Bioengineering, 111* (2), 372-385.

Rajendra, Y., Kiseljak, D., Baldi, L., & Wurm, F. M. (2015). Transcriptional and Post-transcriptional Limitations of High-yielding, PEI-mediated Transient Transfection with CHO and HEK-293E Cells. *Biotechnology Progress, 31* (2), 541-549.

Reisinger, H., Steinfellner, W., Stern, B., Katinger, H., & Kunert, R. (2008). The Absence of Effect of Gene Copy Number and mRNA Level on the Amount of mAb Secretion from Mammalian Cells. *Applied Microbiology and Biotechnology, 81* (4), 701-710.

Schröder, M., & Kaufman, R.J. (2005). The Mammalian Unfolded Protein Response. *Annual Review of Biochemistry, 74*, 739-789.

Swann, P. G., Tolnay, M., Muthukkumar, S., Shapiro, M. A., Rellahan, B. L., Clouse, K. A. (2008). Considerations for the Development of Therapeutic Monoclonal Antibodies. *Current Opinion in Immunology, 20* (4), 493-499.

Watson, J. D., Baker, T. A., Bell, S. P., Gann, A., Levine, M., & Losick, R. (2008). *Molecular biology of the gene*. (6th ed.). San Francisco, CA: Pearson/Benjamin Cummings.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., & Mori, K. (2001). XBP1 mRNA is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. *Cell, 107*, 881-891.