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Enhanced transient recombinant protein production in CHO cells through the co-transfection of the product gene with *Bcl-x_L*

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Abstract

Transient gene expression is gaining popularity as a method to rapidly produce recombinant proteins in mammalian cells. Although significant improvements have been made, in terms of expression, more improvements are needed to compete with the yields achievable in stable gene expression. Much progress has come from optimization of transfection media and parameters, as well as altering culturing conditions to enhance productivity. Recent studies have included using cell lines engineered for apoptosis resistance through the constitutive expression of an anti-apoptotic protein, Bcl-x_L. In this study we examine an alternative method of using the benefits of anti-apoptotic gene expression to enhance the transient expression of biotherapeutics, namely, through the co-transfection of *bcl-x_L* and the product-coding gene. CHO-S cells were co-transfected with the product-coding gene and a vector containing *Bcl-x_L* using polyethylenimine. Cells co-transfected with *Bcl-x_L* showed reduced levels of apoptosis, increased specific productivity, and an overall increase in product yield of approximately 100%. Similar results were produced by employing another anti-apoptotic protein, Bcl-2 delta in CHO cells, or through the co-transfection with *bcl-x_L* using HEK-293E cells. This work provides an alternative method for increasing yields of therapeutic proteins in TGE applications without generating a prior stable cell line and subsequent screening which are both time and resource consuming.

Keywords

transient gene expression; anti-apoptosis; CHO; Polyethyleneimine (PEI)

1 Introduction

The demand for recombinant proteins continues to grow both for use in basic research and for biotechnological applications. There are hundreds of molecules in the pipeline to be clinically evaluated and as patents on existing biologics expire there will be opportunity to produce and test biosimilar or biosuperior versions of these proteins as well [1]. Most of

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these proteins require the post-translational modifications performed by mammalian cells for proper activity [2, 3], as such, mammalian cells are the preferred production system for these recombinant proteins. The traditional method of generating these proteins involves a considerable investment in time and resources for stable cell line selection and screening. With many of the candidate proteins needed initially for research purposes only, it may be expensive to make this investment without assurance that the protein will be approved for clinical applications. As a result, transient gene expression (TGE) has developed as a means to address the need for protein for research purposes. Indeed, advances in TGE have recently shown that g/L quantities of antibody are possible without a significant time investment [4], thus becoming an attractive option to meet the need for rapid delivery of high-quality recombinant proteins.

There are several methods of transient gene delivery and expression but only a few are reasonably acceptable for use at large scale [5]. Due to its low cost and relatively low cytotoxicity, polyethylenimine (PEI) is one of the favored reagents to facilitate DNA delivery into mammalian cells. It has been routinely used to produce mg/L quantities in of recombinant protein in a matter of days [5-11]. While both Human Embryonic Kidney (HEK) 293 and Chinese Hamster Ovary (CHO) cells are widely used host cell lines for TGE, yields from HEK-293 cells tend to be higher, now sometimes exceeding 1 g/L [4] compared to the maximum levels in CHO of 300 mg/L [12]. However, since CHO cells are most commonly used for commercial production of biotherapeutics, it is important to develop methods to improve TGE in these cells to ensure comparable product quality. In addition, advances of TGE in CHO cells will ensure a more seamless transition from preclinical to clinical development and finally approved commercial manufacturing [5]

Several strategies have worked well to improve overall yields using CHO cells for TGE including, transfection media optimization, high density transfections, mild hypothermia, and improved vector design [12-19]. On the other hand, host cell engineering has also been helpful in increasing transient gene expression by engineering the cells to constitutively overexpress the anti-apoptotic protein Bcl-x_L [8]. In the work by Majors et al similar increases in transient product expression were seen after anti-apoptotic engineering as compared to stable gene expression in cell lines stably expressing Bcl-x_L where increases are mainly due to extended culture viability and potential increases in specific productivity [20-24]. While this method of improving TGE showed promise, one still must take the time to develop a robust clone stably expressing Bcl-x_L. In addition this approach requires modifications of the host cells, which may not be desirable if the final product will be produced in the original host cell. Therefore, we considered an alternative approach to take advantage of the positive effects of the anti-apoptotic gene by transiently transfecting the original cell line without stable cell engineering. Here we demonstrate that improvement in product titer can be achieved through the co-transfection of the host cell with the product gene and Bcl-x_L without having to undergo the lengthy process of clonal isolation and screening.

2 Materials and Methods

2.1 Cell lines/maintenance

Cell lines tested for TGE included CHO-S (Invitrogen), a CHO-S cell line stably expressing Bcl-x_L created using the same vector used for the transient expression of Bcl-x_L as described below, and the HEK-293E (ATCC) cell line. The HEK-293E cell line is a suspension adapted HEK293 cell line stably expressing the Epstein–Barr virus nuclear antigen (EBNA-1) allowing for episomal replication of ori-P containing plasmids, and has been shown to increase transgene expression [25]. CHO cells were maintained in SFM4CHO (Hyclone) media supplemented with 8 mmol L-glutamine and 10 ml/L HT supplement while HEK-293E cells were maintained in a 50/50 mixture of SFM4HEK 293 (Hyclone) and FreeStyle 293 (Gibco). These media are here to after referred to as “maintenance medium”. All cultures were grown in a 37°C incubator with 5% CO₂ and shaken at 125 rpm either in 125 mL shake flasks or a six-well plate and passaged at a seeding density of 2×10^5 cells/mL every 3–4 days. Viable cell counts were assessed using the Nova Bioprofile flex (Nova Biomedical) or the Guava EasyCyte plus system (Millipore) with the Nexin or viacount assay per the manufacturer's instructions.

2.2 Plasmids for expression of product and anti-apoptotic proteins

The product expression plasmid was constructed in the Biopharmaceutical Development Program of the SAIC-Frederick Inc. and Frederick National Laboratory for Cancer Research. The plasmid contains the product-encoding sequence to express the model product, a fusion protein, cytokine IL-2 fused with the Fc fragment of Immunoglobulin G 1 (IL-2/Fc) that originally was from Dr. Terry Strom at Beth Israel Deaconess Medical Center (BIDMC), Harvard Medical School. The fusion protein expression is driven by a CMV promoter. To determine transfection efficiency a yellow fluorescent protein (YFP) containing vector, peYFP-c1 (Clontech) was used. For TGE, *Bcl-x_L* and *Bcl-2* delta were cloned into pcDNA3.1 +/zeo (Invitrogen). The human *Bcl-x_L* wt gene was cloned between the XhoI-Xba as described previously [22]. The *Bcl-2* delta gene lacking the coding sequence for amino acids 32–80 of the human wild type gene was obtained from Craig Thompson (University of Chicago) and cloned between the Xba restriction site using PCR primers 5'-GGC GGC tctaga ATG GCG CAC GCT GGG AGA -3' and 5'-GGC GGC tctaga TCA CTT GTG GCC CAG ATA GGC-3' as the 5' and 3' primers respectively. The construct was then sequenced to ensure proper insertion direction. The pcDNA 3.1+/zeo vector was used as a null control. All plasmids were prepared using an Endo-free Maxi-prep kit (Qiagen) following the manufacturer's instructions and stored at a concentration of 0.5–1 mg/mL in endotoxin-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4).

2.3 Transfection

A generic procedure was used for transfection of both cell lines with variations specified below. For both HEK-293E and CHO-S cells the maintenance medium was used for growth, however, was not suitable for transfection. Freestyle 293 and CHO-SSFMII was used for transfecting HEK-293E and CHO-S cells respectively. Linear PEI with molecular weight (MW) of 25,000 (Polysciences) was used as a transfection reagent. A stock solution of 1 mg/mL pH 7 was prepared in Milli-Q water and sterile filtered. Exponentially growing cells

were passed to 1×10^6 cells/mL in maintenance medium on the day prior to transfection. On the day of transfection, cells were spun down ($100 \times g$, 5mins) and re-suspended in transfection medium at the indicated cell density in 90% of the final culture volume at transfection (i.e. if the final cell concentration is 1×10^6 cells/mL and the final volume was to be 2 mL then 2×10^6 cells were resuspended in 1.8 mL of transfection medium). DNA and PEI solutions were prepared separately in 5% of the final culture volume in 150 mM NaCl so that the final concentration of the IL-2/Fc vector or PEI during transfection was as indicated. For dual transfections both DNA vectors were added to the same tube without increasing the final volume. The amount of non-product-containing DNA used during co-transfection is indicated as a weight percentage of the IL-2/Fc vector DNA. In most cases, 3.5 $\mu\text{g/mL}$ of product-coding-containing DNA was used therefore a concentration of 0.35 $\mu\text{g/mL}$ of non-product-coding-containing DNA would be represented as 10%. The PEI solution was added to the DNA solution and they were briefly vortexed and allowed to form complexes for 10 min. The complexes were then added to the cultures to bring the volume to the level indicated. Four hours after transfection the cultures were fed with 2 volumes of growth medium. Cultures were sampled daily until harvest, normally 6 days after transfection. For the purposes of this manuscript both 24 and 6-well plates were used however the technique has also been used in our lab at the 500 mL shake flask scale to generate larger amounts of recombinant protein.

2.4 Optimization of transfection efficiency

For determining the optimal conditions to maximize transfection efficiency, cells were plated in 24-well plates at $1-3 \times 10^6$ cells/mL with a transfection volume of 0.5 mL. The cells were transfected with the peYFP vector at concentrations ranging from 2-4 $\mu\text{g/mL}$ with DNA to PEI ratios from 1:1 to 1:3 (w/w). Samples were drawn 24 and 48 hours after transfection and analyzed for transfection efficiency using the Guava EasyCyte plus flow cytometer (Guava) with the express-plus assay per the manufacturer's instructions. All conditions were tested in triplicate.

2.5 Apoptosis analysis

The assessment of apoptosis was conducted using the Guava Nexin assay run on the Guava following the manufacturer's instructions. Cells were gated according to viable, early apoptotic (Annexin-V only) and dead (Annexin-V and 7-AAD positive) cell populations. Unless otherwise noted the error bars represent the standard error of the mean (SEM) in three or more independent experiments.

2.6 Determination of product expression

On the day of harvest, the cultures were spun down at $100 \times g$ for 5 minutes; the supernatant was then 0.2 μm filtered. The supernatants were purified by Protein A affinity chromatography. 100 μL of Mabselect SuRe protein A beads (GE) were added to ~ 5 mL of the filtered supernatants for overnight binding at 4°C . Resin suspensions were added to poly-Prep columns (Biorad) and allowed to drain by gravity flow. The resin was then rinsed with 2×1 mL of wash buffer (10 mM Citrate, 0.1 M NaCl pH 6.0) and then eluted with 2×350 μL Elution buffer (20 mM Citrate, 0.1 M NaCl pH 3.0) into 50 μL 1 M Tris-HCl pH 9.0. Protein Aeluates were then subject to SDS-PAGE under reduced conditions using NuPage

4-12% bis-tris gels (Invitrogen). On each gel, three product standards were included at 15.0, 7.5 and 3.75 µg/mL to create a standard curve. Gels were stained with Simply Bluesafestain (Invitrogen) and images were taken using the FlourChem E imager (Cell Biosciences, Inc.). Protein Aeluate concentrations were then determined by densitometry using Alpha view SA software (Cell Biosciences, Inc.). The total amount of product produced in each culture was then calculated and normalized in concentration to the starting culture volume. Specific productivity, q_p , was calculated as an average q_p over the length of the batch culture assuming all cells in culture were producing at a constant rate using the formula

$$q_p = \frac{P}{IVCD}$$

Where P is final product concentration and the integral viable cell density (IVCD) is given by

$$IVCD_n = \left\{ \frac{(x_{n-1} + x_n)}{2} \times [n - (n - 1)] \right\} + IVCD_{n-1}$$

Where x is the cell concentration on day n. Unless otherwise noted the error bars represent the SEM in three or more independent experiments.

For determining the theoretical quantity of yfp expression the average intensity of expressing cells on day one was multiplied by the concentration of expressing cells on day one representing the total amount of yfp produced day one. This product was then added to the product of the average intensity and concentration of expressing cells on day 2. This sum was intern added to the product of the average intensity and concentration of expressing cells on day 3 and so forth until day 6 where this cumulative sum of products gave a theoretical level of recombinant protein produced over the 6 day period. This calculation assumes the yfp expressed on day 1 was theoretically excreted and remained in culture through day 6.

2.7 Analysis by Western blot

24 hours after transfection cells co-transfected with *Bcl-x_L* at 5-75% by weight of the product vector were harvested and lysed in 6 M urea. Supernatants were run on SDS-PAGE under reduced conditions using NuPage 4-12% bis-tris gels (Invitrogen). The load was normalized for total protein as measured using a BCA assay (Pierce). The proteins were transferred to a PVDF membrane using 20% methanol Nupage transfer buffer. Bcl-x_L was detected using Bcl-xs/l (L-19) Rabbit polyclonal antibody (Santa Cruz Biotechnology).

3 Results

3.1 Parameter optimization

Before examining the potential benefits of *Bcl-x_L* co-transfection on TGE, we determined the optimal conditions for using PEI to transfect our CHO-S cell line using a YFP

expression vector. Optimal transfection conditions will differ among cell lines and cell types, therefore, we evaluated DNA concentrations of 2.0-4.0 $\mu\text{g/mL}$, cell concentrations $1-3 \times 10^6$ cells/mL and DNA to PEI ratios of 1:1-1:3 (w:w) to find the optimal for our system. In acknowledgement that this optimization is strictly applicable to our cell line, we have chosen to show the results of this study in a single 3-D plot, Figure 1. From our experiments, transfection efficiency was found as a function of starting cell concentration, DNA:PEI ratio and DNA concentration. Two local maximums in the percentage of cells expressing YFP were found in the range of parameters tested both occurring when the cells were transfected at a density of 1×10^6 cells/mL. We observed a sharp decrease in efficiency with increased cell concentrations for the 1:1 ratio and a slower decline in efficiencies with higher ratios. At a cell concentration of 1×10^6 cells/mL, over 65% of the cells were found to express YFP 48 hours after transfection (using 3.5 $\mu\text{g/mL}$ DNA and a DNA:PEI ratio of 1:1) whereas a transfection efficiency around 30% was seen at cell concentrations of 2.0×10^6 cells/mL or greater with the other parameters held constant. In contrast to the negative correlation of decreased efficiencies with increased cell densities, we observed an increase in transfection efficiency with increasing DNA concentration up to the optimal concentration of 3.5 $\mu\text{g/mL}$ for both the 1:1 and 1:2 ratio. For the 1:3 ratio the highest efficiency was found at lower DNA concentrations as the increased PEI concentration at the higher DNA concentrations proved toxic to the cells. Since both the 1:1 and 1:2 ratios achieved similar maximum transfection efficiencies at 3.5 $\mu\text{g/mL}$ and 1×10^6 cells/mL we chose a more subtle difference to determine the optimal condition for our system, namely the sustained higher transfection efficiencies for the 1:2 ratio with increased cell concentration. For example, the transfection efficiency at the 1:2 ratio dipped to 51% with 2×10^6 cells/mL but dropped to 33% with the 1:1 ratio at the same cell concentration. Consequently, we chose the 1:2 DNA/PEI ratio to be optimal for our system. Additionally, although lower cell concentrations could have produced even higher transfection efficiencies, when considering recombinant protein production, it is ultimately the number of cells that become transfected that determine the final titer in batch culture. If 65% of 1×10^6 cells become transfected there will be 0.65×10^6 cells producing the protein of interest, on the other hand, if 51% of 2×10^6 cells become transfected there would be 1.0×10^6 cells producing the product. That is roughly 50% more cells expressing product. Error bars were eliminated from the 3-D figure for clarity but the average standard deviation for all points tested in triplicate independent runs was approximately 7.4%. Ultimately, for maximal transfection efficiency in subsequent studies we chose to transfect 1×10^6 cells/mL with a DNA/PEI ratio of 1:2 and a DNA concentration of 3.5 $\mu\text{g/mL}$.

3.2 Co-transfection with *Bcl-x_L*: Determination of optimal percentage of *Bcl-x_L*

Using the above-stated optimal transfection conditions, initial experiments were conducted in singlet in order to examine the effect on productivity of co-transfecting different amounts of *Bcl-x_L*. We examined *Bcl-x_L* DNA concentrations that varied from 5 to 75% by weight of the vector containing the product gene. The product is a fusion protein containing cytokine, IL-2 and the Fc region of IgG as described in Materials and Methods section. To confirm *Bcl-x_L* expression, samples were drawn 24 hours after transfection and analyzed by Western blot (see online data). An increase in *Bcl-x_L* expression level was observed with an increase in *Bcl-x_L* plasmid concentration. Samples were also taken 6 days after transfection and

examined for changes in product expression. With *Bcl-x_L* vector concentrations as low as 10% of the product-coding plasmid, a considerable increase in titer was found. Levels greater than 25% led to diminishing increases in titer. Therefore, in order to keep the non-product-coding-containing DNA to a minimum, a *Bcl-x_L* concentration of 10% of the product plasmid was used for the remainder of the studies.

3.3 Co-transfection with *Bcl-x_L*: Effect on Apoptosis

To determine whether co-transfection with *Bcl-x_L* altered the onset of apoptosis, we compared the percentage of cells undergoing apoptosis after transfection with the product containing vector only (i.e. the standard condition) to the percentage of cells undergoing apoptosis after co-transfection with the product-containing vector and either a null vector or one containing *Bcl-x_L*. Samples were drawn from 24 to 72 hours after transfection and analyzed for the presence of apoptosis by an Annex in-V binding assay using a flow cytometer as described in the Materials and Methods section. Additionally, we compared these levels of apoptosis to those present after transfecting the target gene into a cell line engineered to stably express *Bcl-x_L*. As shown in Figure 2A, co-transfection with the null vector provided no significant difference in the percentage of cells showing early signs of apoptosis compared to the standard transfection condition up to 72 hours post transfection. Both groups had between 8 and 10 percent of the total population showing early signs of apoptosis. It should be noted that cells may be at other stages of apoptosis not detectable by this assay [20]. In contrast, cells co-transfected with *Bcl-x_L*, showed a significant reduction in the onset of apoptosis, where only 2 percent of the population receiving the *Bcl-x_L* plasmid was undergoing apoptosis (Figure 2A). This was similar to the cell line stably expressing *Bcl-x_L*, in which, at 24 hours, only 2 percent of the population exhibited signs of apoptosis (Figure 2A). This improvement was sustained for 48 hours whereas differences were not as great by 72 hours once the dying cells were committed to late stage apoptosis. The differences in the population undergoing late stage apoptosis were also examined and the results can be seen in Figure 2B. Understandably, there was little difference between conditions until 48 hours after transfection when the cells committed to death had begun to show signs of late stage apoptosis. As seen in figure 2B there is a significant increase in the percentage of cells in late stage apoptosis for the transfectants of the standard condition or null vector, while there was a decrease in population for cells expressing *Bcl-x_L* since fewer cells were committed to death following the insult of transfection.

3.4 Co-transfection with *yfp* and *Bcl-x_L*

To determine if the co-transfection with *Bcl-x_L* had an effect on transfection efficiency of the product gene, cells were co-transfected with target gene, *YFP*, and *Bcl-x_L* or a null vector. The percentage of cells expressing YFP on the days following transfection was then compared. We found that co-transfection with the *Bcl-x_L* vector had no significant impact on the transfection efficiency compared to cells receiving the peYFP vector only or co-transfected with the null vector and peYFP (Figure 3A). It must be noted that overall transfection efficiencies were lower in this experiment than reported for the optimization experiments (Figure 1) and this is believed to be due to differences in scale for the two experiments. The optimization was done at the 24-well plate scale while the effect of *bcl-x_L* experiment was conducted at the six-well plate scale. While no differences were seen in

transfection efficiency, between the types of transfection, we also compared the fluorescence intensity of expressing cells and observed a consistent approximate 20% increase in the average fluorescence intensity of cells co-transfected with *Bcl-x_L*, suggesting an increase in specific productivity (Figure 3B). In addition, the fluorescence intensity of cells not co-transfected with *Bcl-x_L* continued to decline toward the end of batch culture but cells expressing *Bcl-x_L* maintained a statistically significant ($p = 0.05$) higher level of fluorescence intensity from days 4 to 6.

Through resistance to the onset of apoptosis after the initial shock of transfection and an increase in specific expression demonstrated by the peYFP construct, overall improvements in product titer were expected. A cumulative total (Figure 3D) of the average YFP intensity per cell (Figure 3B) multiplied by the concentration of producing cells (Figure 3C) indicates that total YFP expression may have doubled when using co-transfection with *Bcl-x_L* compared with the standard condition. Since YFP is not secreted, this sum may not represent total production but nonetheless was taken as a positive indication that *Bcl-x_L* co-transfection could improve production of recombinant proteins in CHO cells through increased specific productivity.

3.5 Co-transfection of *Bcl-x_L* and product gene: Effect on growth and productivity

To examine the effect of *Bcl-x_L* co-transfection on a biotherapeutic protein titer, CHO-S cells were transfected with the IL-2/Fc-coding-containing vector only (the standard condition), the product-coding vector together with 10% w/w null vector or 10% w/w *Bcl-x_L*-containing vector. As shown in Figure 4A the titers for the single transfection and the null vector co-transfection were similar. In contrast, product titers were observed to increase ~100% when CHO cells were co-transfected with the *Bcl-x_L*-containing vector to approximately 860 µg/L. Surprisingly there was little difference in growth observed after transfection between the three conditions as shown in Figure 4B. The growth rate and maximum cell density of the *Bcl-x_L* transfected cells was slightly lower; although the cells reached similar final viable cell densities with statistically insignificant differences in final integral viable cell density (IVCD), (student's t-test, $p = 0.41$). Similarly, the viabilities of the cultures were closely matched with the *Bcl-x_L* expressing culture achieving only a marginal albeit, statistically significant (student's t-test $p = 0.001$) extension of higher viabilities at the end of the run. However, when considering specific productivity, assuming similar percentage of cells in culture were producing the product, there was no difference between the standard condition and that of transfections with the null vector, whereas a 140% increase in specific productivity was observed for cultures co-transfected with *Bcl-x_L*, (Figure 4C).

3.6 Co-transfection of *Bcl-2* delta and product gene: Effect on growth and productivity

To test whether other anti-apoptotic Bcl-2 family members could enhance product expression in a transient expression system, CHO-S cells were co-transfected with a mutant form of *Bcl-2*, called *Bcl-2* delta together with the product coding vector. Previous studies in our laboratory have shown that stable cell lines expressing *Bcl-2* delta have increased resistance to apoptotic insults[26], although we have not tested them in transient expression studies. In a separate experiment, the prevalence of early signs of apoptosis after transfection

with the product coding vector only, or the product coding vector together with either a null vector or one containing either *Bcl-x_L* or *Bcl-2* was compared (Figure 5A). As in the earlier comparison, cultures co-transfected with the anti-apoptotic gene showed significantly lower levels of apoptosis 24 and 48 hours after transfection compared to controls. While 24% of the null vector co-transfected cultures were undergoing apoptosis 24 hours post transfection, only 7% of the *Bcl-2* delta cultures were experiencing the onset of apoptosis at that time. The growth and viability of all cultures was similar through the 6-day culture (data not shown), however, the final product titers differed significantly (Figure 5B). More than a 2-fold increase in product titer, from 360 to 800 µg/L, was observed using *Bcl-2* delta co-transfection compared to the standard condition. This was similar to that seen with the *Bcl-x_L* vector co-transfection (Figure 4).

3.7 HEK-293E cells as a host for TGE

To further investigate the application of *Bcl-x_L* expression during transient gene expression, HEK-293E cells were co-transfected with the product (IL-2/Fc) containing vector and 10% *Bcl-x_L*. Growth and viability were similar between the anti-apoptosis and control cultures (data not shown). Nonetheless, a significant, 64% ($p = 0.038$) increase in titer was also observed for HEK293E cells co-transfected with 10% *Bcl-x_L* (Figure 5C).

4 Discussion

Recent advances in TGE have enabled this method to emerge as a feasible alternative to stable gene expression for rapid recombinant protein production[3]. Improvements are needed to enhance product yield especially for the more commercially relevant CHO cell line. Following improvements achieved from processing modification including nutrient feeding, high density cultivation, and temperature modulation [11, 16, 17, 19, 27-30], more recently, cellular engineering techniques have led to improved cell survival, cell growth, and product expression[8, 31-33]. The improved cell survival is especially relevant for TGE due to the high stresses imposed on the cells during transfection. Here we demonstrate in batch culture that, in the popular TGE cell hosts, CHO and HEK293E, significant improvements in product yield are possible without having to go through the extended process of host cell engineering. This was shown using co-transfection of a vector containing the anti-apoptotic *Bcl* gene together with a vector for the product gene.

The optimal parameters for transfection efficiency found for our cell line were a DNA concentration of 3.5 µg/mL a DNA:PEI ratio of 1:2 and a cell concentration of 1×10^6 cells/mL, which is consistent with some previous studies [5, 8]. Transfection efficiencies approaching 70% were seen under these conditions in a 24-well plate. When determining the optimal amount of *Bcl-x_L* DNA to introduce during co-transfection it was important to consider the extra burden associated with co-transfection of a second plasmid. Thus we found that a beneficial balance between apoptosis inhibition and increased metabolic load on the cell was reached by using a *Bcl-x_L* DNA concentration of only 10% by weight compared to the IL-2/Fc vector. The benefits however were seen to diminish when greater than 25% by weight *Bcl-x_L* was used. Previous studies with stable cell lines have also indicated there is an optimal anti-apoptosis level with diminishing returns at higher expression levels [20]. In the future it may be advisable to design a single vector to express *Bcl-x_L* under a weaker

promoter together with the product gene under a stronger promoter or under one promoter with insertion of an internal ribosome entry site in between. This combined vector would eliminate the need for co-transfection entirely.

PEI is thought to have relatively low toxicity at the concentrations used for transfection [15]. Nonetheless, approximately 10% of the total cell population initiated the apoptosis cascade after transfection as was indicated by the positive staining with Annex in-V. This is in agreement with a study done showing that PEI induces both necrotic and apoptotic cell death during gene delivery [34]. In contrast, cultures co-transfected with *Bcl-x_L* exhibited only a background level of apoptosis similar to untransfected cells and similar to cells stably expressing the protein. Subsequent studies involving *Bcl-2* co-transfection showed even higher levels of apoptosis for the control group, almost 25% whereas those receiving the anti-apoptotic gene had much lower levels of the onset of apoptosis. This overall higher incidence of apoptosis may have been due to the health of the starting culture or other variables not taken into account. On average, however, (in more than 16 trials) the control cultures had approximately 10% of the culture undergoing apoptosis 24 hours after transfection. While only 10% of the total population seems low, it could account for as much as 25% of the cells that would be expressing the product if only 40% of the total population were transfected. Even though the population of translocated phosphatidylserine (PS) positive cells decreases to baseline levels 3 days after transfection, it is those first few days after transfection where the product expression level per cell is the highest, as indicated in the YFP expression studies (Figure 3). Therefore the ability of the cells receiving *Bcl-x_L* to achieve higher specific productivity initially, added in their ability to achieve higher final volumetric yields. While the specific productivity of the cells diminishes over time this may be an advantage of transiently transfecting *Bcl-x_L* over stable expression of *Bcl-x_L*, since there will be a higher level of specific *Bcl-x_L* expression immediately following transfection. This gives the cell added protection during the recovery from the insult of transfection while later diminishing expression of *Bcl-x_L* allows more cellular resources to be devoted to product expression.

The overall trend in the percent increase in productivity using the anti-apoptotic engineering co-transfection method compared to the standard method remained relatively constant with a roughly two fold increase in product titer observed in 6 sets of 3 independent experiments each (Figure 4A). There was no significant difference in titers observed for the null vector dual transfectants over the standard condition indicating the extra DNA alone played no part in increased titers. Surprisingly, when comparing the benefit of *Bcl-x_L* co-transfection on product expression and cell growth to the co-transfection with a null vector or the standard condition, there was a slight decrease in viable cell density observed for the dual transfectants. Thus, even though *Bcl-x_L* inhibits apoptosis, it appears to also slow cell growth which has also been observed previously in our lab and others [26]. The slower growth may be caused by the extra metabolic burden associated with processing of the *Bcl-x_L* plasmid or some change in cellular metabolism manifested by *Bcl-x_L* expression [35].

The higher titers achieved together with the lower cell numbers for the *Bcl-x_L* transfected cells resulted in a dramatic increase in specific productivity. In this application we did not have the added complexity of discerning clonal isolation effects when determining whether

the increase in specific productivity was directly linked to Bcl-x_L expression as exists in clonal studies. It is likely that, in the days immediately following transfection, the presence of Bcl-x_L allowed for the cells to maintain a healthy status thereby producing more product during that time giving an instantaneous, and therefore an overall, increase in specific productivity. It is also possible that Bcl-x_L expression provides benefits to those cells not apoptotic if it increases the overall health and productive capabilities of all transfected cells. However, the increase might not only be due to the anti-apoptotic effects of Bcl-x_L. Indeed the involvement of Bcl-x_L in maintaining mitochondrial membrane integrity and calcium flux may lead to higher specific productivities in ways independent of its known anti-apoptotic effects [36-39].

We further demonstrated that other Bcl-2 family member proteins could be used to achieve increased productivities similar to Bcl-x_L although a complete characterization was not done to see which protein proved more advantageous. It would be interesting to see whether other anti-apoptotic proteins such as the caspase inhibitor XIAP can also provide improved survival and increased protein yield for transient expression [40]. While advances in TGE for CHO cells using *Bcl-x_L* were the focus of this study, we also demonstrated that this method of co-transfection could enhance productivities in HEK-293E cells. This represents, the first time anti-apoptotic genes have been expressed and shown to be effective for HEK-293E cells in TGE. As HEK-293 cells are widely used for TGE, this approach could be applied in many laboratories. More importantly, these findings demonstrate that the stress on mammalian cells from TGE is likely felt across different cell types and anti-apoptosis proteins can be used in the different cell types to improve the cell health.

It should be noted that co-expression of anti-apoptosis proteins is not the only approach to improve yields from TGE. Certainly one should consider vector optimization as well as media screening and controlled processing conditions as ways to further enhance productivities. As with stable gene expression, the use of anti-apoptotic engineering should be compatible with other orthogonal methods to enhance productivities such as, temperature shifts, hyperosmolality, or inducible cell cycle arrest. Anti-apoptotic engineering methods may even offer synergistic enhancement when combined with these as some of these treatments may specifically induce apoptosis [24, 41-43].

Stable expression of anti-apoptotic proteins has been widely demonstrated as helping increase the survival and yields of recombinant proteins in both stable and transient expression. In the current study we eliminated the need to create and screen for apoptosis resistant clones prior to TGE by co-transfecting the anti-apoptotic gene together with the product gene at levels in the range of 10% by weight of the product vector. By doing so we have demonstrated a simple and effective means to help meet the growing need for advances in TGE for CHO cells. Anti-apoptosis proteins were found to reduce apoptosis in transfected CHO cells leading to higher productivities in both CHO-S and HEK-293E cells. This method, in combination with other process engineering improvements may soon make TGE in CHO cells a go-to platform for the rapid production of recombinant therapeutic proteins in the coming decades.

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Abbreviations

PEI	Polyetheleneamine
HEK	Human Embryonic Kidney
CHO	Chinese Hamster Ovary
TGE	Transient gene expression
YFP	Yellow fluorescent protein

Figure 1. Optimization of transfection efficiency in CHO-S cells

3D plot showing the transfection efficiencies achieved using CHO-S cells transiently transfected with the peYFP vector at various DNA concentrations, DNA:PEI ratios, and cell concentrations. Culture samples were analyzed 48 hours after transfection for YFP expression.

Figure 2. Apoptosis analysis after transfection

Percentage of CHO-S cells undergoing early stage (A) and late stage (B) apoptosis 24-72 hours after transfection of the product containing vector alone or after the co-transfection of the product containing vector together with a null vector or one containing *Bcl-x_L*. The apoptosis levels were also compared to those present after transfection of the product containing vector only in a CHO-S cell line stably expressing Bcl-x_L. Error bars represent SEM for n independent experiments where n is as indicated.

Figure 3. Transfection efficiency and productivity comparison after the co-transfection of YFP and *Bcl-x_L*

Transfection efficiency 48 hours after transfection for cells receiving the peYFP vector only (standard condition), co-transfected with a null vector or one containing *Bcl-x_L* (A), Effect of *Bcl-x_L* co-transfection on the average intensity of transfected cells from 24 to 144 hours after transfection (B), Concentration of cells expressing YFP (C) and cumulative quantification of YFP production calculated as the cumulative sum of the concentration of cells producing multiplied by the average intensity of expressing cells (D). Error bars represent SEM for n independent experiments where n is as indicated. The average fluorescence intensity of the transfected cells at each time point was divided or called normalized by the average intensity of the Bcl-xL co-transfected cells 24 hrs after transfection and then multiplied by 100.

Figure 4. Growth and Productivity comparison with *Bcl-xL* co-transfection

Comparison of product titer at the end of 6 day batch culture with or without the co-transfection with *Bcl-xL* (A), Viable cell density profiles after transfection with the product containing vector only, or co-transfected with a null vector or one containing *Bcl-xL* (B), percent increase in average specific productivity and titer over the standard condition when using co-transfection with *Bcl-xL* (C). Error bars represent SEM for n independent experiments where n is as indicated or 3.

Figure 5. Apoptosis comparison with *Bcl-2* delta in CHO-s cells and productivity comparison in CHO and HEK-293E cells after *Bcl-2* or *Bcl-xL* co-transfection

CHO cells were transfected with the product containing vector only (standard condition) or co-transfected with the product containing vector and a null vector or vector containing *Bcl-xL*, or *Bcl-2* delta at 10% by weight of the product containing vector and were monitored throughout a six day batch culture for late stage apoptosis (A) and final product titer (B). Titer comparison after a six day batch culture of HEK-293E cells transfected with the product containing vector only (standard condition) or co-transfected with the product containing vector and a null vector or one containing *Bcl-xL* at 10% by weight of the product containing vector (C). Error bars represent SEM for 3 independent experiments.