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# Engineering the Cellular Protein Secretory Pathway for Enhancement of Recombinant Tissue Plasminogen Activator Expression in Chinese Hamster Ovary Cells: Effects of CERT and XBP1s Genes

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Cell line development is the most critical and also the most time-consuming step in the production of recombinant therapeutic proteins. In this regard, a variety of vector and cell engineering strategies have been developed for generating high-producing mammalian cells; however, the cell line engineering approach seems to show various results on different recombinant protein producer cells. In order to improve the secretory capacity of a recombinant tissue plasminogen activator (t-PA)-producing Chinese hamster ovary (CHO) cell line, we developed cell line engineering approaches based on the ceramide transfer protein (CERT) and X-box binding protein 1 (XBP1) genes. For this purpose, CERT S132A, a mutant form of CERT that is resistant to phosphorylation, and XBP1s were overexpressed in a recombinant t-PA-producing CHO cell line. Overexpression of CERT S132A increased the specific productivity of t-PA-producing CHO cells up to 35%. In contrast, the heterologous expression of XBP1s did not affect the t-PA expression rate. Our results suggest that CERT-S132A-based secretion engineering could be an effective strategy for enhancing recombinant t-PA production in CHO cells.

**Keywords:** Chinese hamster ovary cells, cell line engineering, X-box binding protein 1, ceramide transfer protein, tissue plasminogen activator

## Introduction

Recombinant proteins appear to be the most promising category of pharmaceuticals, because they provide novel preventive and therapeutic opportunities for a large number of human disorders. Today, about 200 recombinant therapeutic proteins have been approved and much more are in clinical trials. Therefore, the development of innovative methods for the rapid production of recombinant proteins is essential [35].

Although a variety of expression systems have been used for the expression of heterologous genes, mammalian cells are considered the most suitable host system for the expression of fully active recombinant proteins with correct posttranslational modifications. Currently, about 60% of recombinant therapeutic proteins are produced in this expression system [4, 34]. Among the available mammalian cell lines, Chinese hamster ovary (CHO) cells are of particular interest for the industrial manufacturing of biopharmaceuticals. The main advantages of CHO cells are the feasibility of gene manipulation, the ability to create human-like glycosylation patterns, and the capacity for growth in high cell densities in serum-free suspension culture. The major disadvantage of CHO cells is their lower protein expression rate in comparison with bacterial and lower eukaryotic expression systems [13, 16].

Different strategies, such as optimization of the cell culture process, adjustment of medium components, generation of more efficient expression vectors, and development of genetically engineered host cells, have been used to improve recombinant protein expression in CHO cells [18, 34]. Cell line engineering approaches have been utilized for the

modulation of the cell proliferation rate [9, 22], the inhibition of apoptosis [6, 31, 36], the enhancement of protein secretion [3, 14], the establishment of new host cells for proper posttranslational protein modifications [25, 33], and decreasing the rate of metabolic waste-products formation [17, 26].

Protein secretion is known as one of the major bottlenecks in the productivity of mammalian cells [2]. Several genes have been used to improve protein secretion from CHO cells, including molecular chaperones and mediators of secretory vesicle formation along the secretory pathway. The influence of genetic modification of the secretory pathway on the productivity of recombinant proteins, however, appears to vary among mammalian cell types, effector genes, and target proteins [7, 15, 27].

Ceramide transfer protein (CERT) has been found to have a critical role in the trafficking of ceramide from the endoplasmic reticulum (ER) to the Golgi, where it is used as a precursor for the synthesis of diacylglycerol (DAG) and sphingomyelin. Accumulation of DAG at the trans-Golgi network (TGN) leads to the recruitment of protein kinase D (PKD). Upon activation at the TGN, PKD plays a central role in protein secretion through activation of the proteins involved in secretory vesicle formation [1, 11]. In addition, CERT is a PKD substrate, and the phosphorylation of CERT at serine 132 reduces its lipid transfer function and its affinity for Golgi [8]. In a report by Florin *et al*. [7], ectopic expression of CERT and its mutant variant CERT S132A resulted in enhanced secretion of a monoclonal antibody and human serum albumin from recombinant CHO cells.

X-box binding protein 1 (XBP1) is a basic leucine zipper (bZIP) transcription factor that is involved in the mammalian unfolded protein response (UPR). The UPR is a stress response pathway, which is activated by accumulation of misfolded or unfolded proteins in the lumen of the ER. Two major isoforms of XBP1 are known: XBP1u, the unspliced variant that is inactive; and XBP1s, the spliced variant that is a potent transcription factor. Upon UPR activation, XBP1s is generated by removal of a 26 nucleotide intron from the XBP1 transcript. The spliced isoform then stimulates upregulation of the endoplasmic reticulum chaperones and foldases. This protein also induces enlargement in the ER, Golgi, and cell size. Therefore, the result of XBP1s expression would be an improvement in the secretory capacity of the cells [21, 29]. Heterologous expression of XBP1s has been shown to enhance the production of monoclonal antibodies [2], human placental secreted alkaline phosphatase (SEAP), *Bacillus stearothermophilus* derived α-amylase (SAMY), and human vascular endothelial growth factor 121 (VEGF 121) in CHO cells [32].

The human tissue plasminogen activator (t-PA) is a 68 kDa serine protease known as the key component of the fibrinolytic system. t-PA converts the proenzyme plasminogen to plasmin, which in turn mediates the degradation of fibrin. Recombinant human t-PA has been extensively used as a therapeutic agent for the treatment of thrombotic diseases owing to its greater safety and efficiency compared with other plasminogen activators like urokinase and streptokinase. At present, CHO cells are the main source for the commercial production of full-length recombinant human t-PA [28].

In the study presented here, we investigated the effects of CERT and XBP1s, two major proteins involved in mammalian cell protein secretion, on the t-PA expression level in CHO cells. Our results here provide further details about the successful application of the cell line engineering approach for enhancement of the recombinant protein expression.

## Materials and Methods

### Vector Construction

The cDNA for human CERT S132A was amplified from the pcDNA3-CERT S132A vector (kindly provided by Dr. Monilola Olayioye, University of Stuttgart, Germany), cloned into the pGEM-T Easy vector (Promega, USA), sequenced, and then cloned into the *Hin*dIII and *Xba*I sites of the pcDNA3.1/hygro (+) expression vector (Invitrogen, USA). For detection of the CERT S132A protein, a sequence encoding the FLAG epitope was incorporated in the reverse primer. The pcDNA3-FLAG-XBP1s vector, containing the spliced variant of human XBP1s, was a generous gift from Dr. Ling Qi (Cornell University, USA).

### Cell Culture

CHO 1-15(500) t-PA-producing cells (ATCC CRL-9606) were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco, USA) at 37°C in a humidified incubator containing 5% $CO_2$. Cultures were passaged every 2–3 days at the cell density of 0.2–0.3 × $10^6$ cells/ml. To determine the cell concentration and viability, the trypan blue exclusion method was used.

### Transfection and Development of Stable Cell Lines

Transfection was performed using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. To develop stable CHO 1-5(500) cells overexpressing CERT S132A or XBP1s, cells were transfected with *Bgl*II linearized pcDNA3.1-CERT S132A/hygro or pcDNA3-XBP1s/neo expression vectors, in triplicate. After 48 h, transfectants were diluted in 1:6 ratio and stable cell pools were selected in medium containing 200 μg/ml hygromycin or 400 μg/ml G418 (Invitrogen, USA) for 4 weeks. To confirm the

integration of the CERT S132A and XBP1s genes into the CHO cell genome, genomic DNA was isolated from stable cell pools as well as parental CHO cells using a DNA extraction kit (Roche, Germany), and a PCR was performed with gene-specific primers.

**Quantitative RT-PCR**

Total RNA was isolated from cells by RNX plus reagent (Cinnagen, Iran) after 72 h of cultivation. First-strand cDNA was synthesized using 2 µg of DNase-treated RNA with the RevertAid first-strand cDNA synthesis kit (Fermentas, Lithuania). Quantitative real-time PCR was performed in an ABI 7500 system using a SYBR green PCR master mix (Applied Biosystems, USA). the GAPDH housekeeping gene was used for normalization. Primers used for the real-time RT-PCR were 5'-CACTCTTCCACCTTTGATGCTG-3' and 5'-GTCCACCACTCTGTTGCTGTAGC-3' for GAPDH; 5'-CTG GGAAGTGCTGTGAAATAGATACC-3' and 5'-GCTGTTCCAGTT GGTGCACTC-3' for t-PA; 5'-AGATTTGTGCAAAAGGTTGAA GAG-3' and 5'-ACTCCTTTAACTGCATGGGTAGC-3' for CERT; and 5'-GGAGAGAAAACTCATGGCCTTG-3' and 5'-CTGCAC CTGCTGCGGACTC-3' for XBP1s. For selective amplification of the spliced variant of the XBP1 transcript, a reverse primer was designed to overlap the unique exon junction of XBP1s. The amplification program was set as 10 min at 95°C for the first denaturation step, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Relative quantification analysis was performed using the comparative CT (ΔΔCT) method.

**Western Blotting**

Western blot analysis was performed to detect the intracellular expression of CERT S132A and XBP1s proteins in CHO cells. For preparation of total cell lysates, $5 \times 10^6$ cells were harvested after 72 h of cultivation, washed with PBS, and lysed in cell lysis buffer (20 mM Tris HCl, pH 7.5, 0.15 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Germany). Then, 50 µg of the cell lysates was electrophoresed on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. After 1 h of blocking in 5% skimmed milk, rabbit anti-FLAG polyclonal antibody (Cell Signaling Technologies, USA) was used as the primary antibody in a 1/1,000 dilution and goat anti-rabbit HRP antibody (Santa Cruz Biotechnology, USA) was applied as the secondary antibody in a 1/1,500 dilution. For detection, ECL plus reagent (Amersham Biosciences, Germany) was employed.

**Quantification of t-PA in Cell Culture Supernatant**

To determine the t-PA units, cells were seeded in 6-well plates at the concentration of $0.2 \times 10^6$ cells/ml. Culture supernatants were collected after 72 h, and t-PA titers were measured using a trinilized t-PA activity assay kit (Trinity Biotech, Ireland) according to the manufacturer's guidelines. Briefly, 100 µl of the diluted culture supernatant was applied to the microtest wells containing anti-t-PA antibody for 20 min at room temperature. After three washing steps, plasminogen and chromogenic substrate were added to each well and incubated for 75 min. Samples were read at 405 nm and units of t-PA were determined using the standard t-PA provided in the kit. The integral viable cell density (IVCD) and specific productivity (Qp) were calculated using the following formulas where P is the t-PA concentration, X is the viable cell density, and t is the culture time in days [10].

$$IVCD = \frac{X_2 - X_1}{2} \times (t_2 - t_1)$$

$$Qp = \frac{P_2 - P_1}{IVCD}$$

## Results

**Stable Expression of CERT S132A and XBP1s Genes in CHO 1-15(500) Cells**

To evaluate the effects of CERT S132A and XBP1s overexpression on the t-PA expression level, we used CHO 1-15(500), a stable t-PA-producing CHO cell line. CHO 1-15(500) cells were transfected with CERT S132A or XBP1s expression plasmids, and three stable cell pools were generated for each gene. After several passages, integration of the CERT S132A or XBP1s expression plasmids into the CHO cell genome was examined through PCR analysis on genomic DNA of parental CHO cells as well as CERT S132A or XBP1s transfected cell pools using gene-specific primers. The appearance of bands with the expected size of 1,131 bp for XBP1s (Fig. 1A) and 1,797 bp for CERT S132A (Fig. 1B) verified the presence of these genes in the genome of genetically modified cells.

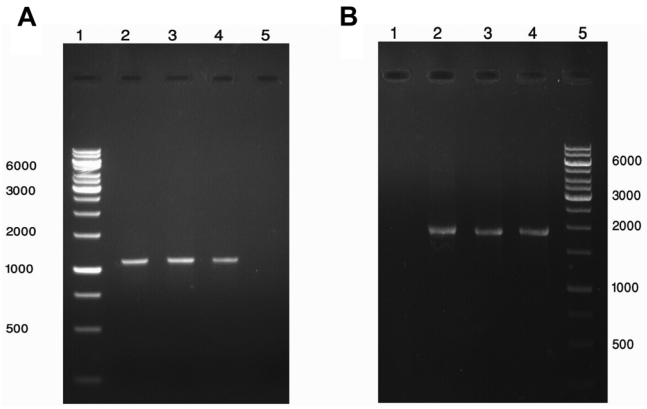


**Fig. 1.** Agarose gel electrophoresis of amplified CERT S132A and XBP1s genes from genomic DNA of CHO 1-15(500) stable cell pools and parental cells.

(**A**) XBP1s stable cells: lane 1, size marker; lanes 2–4, three XBP1s stable cell pools; and lane 5, un-transfected CHO 1-15(500) cells. (**B**) CERT S132A stable cells: lane 1, un-transfected CHO 1-15(500) cells; lanes 2–4, three CERT S132A stable cell pools; and lane 5, size marker.

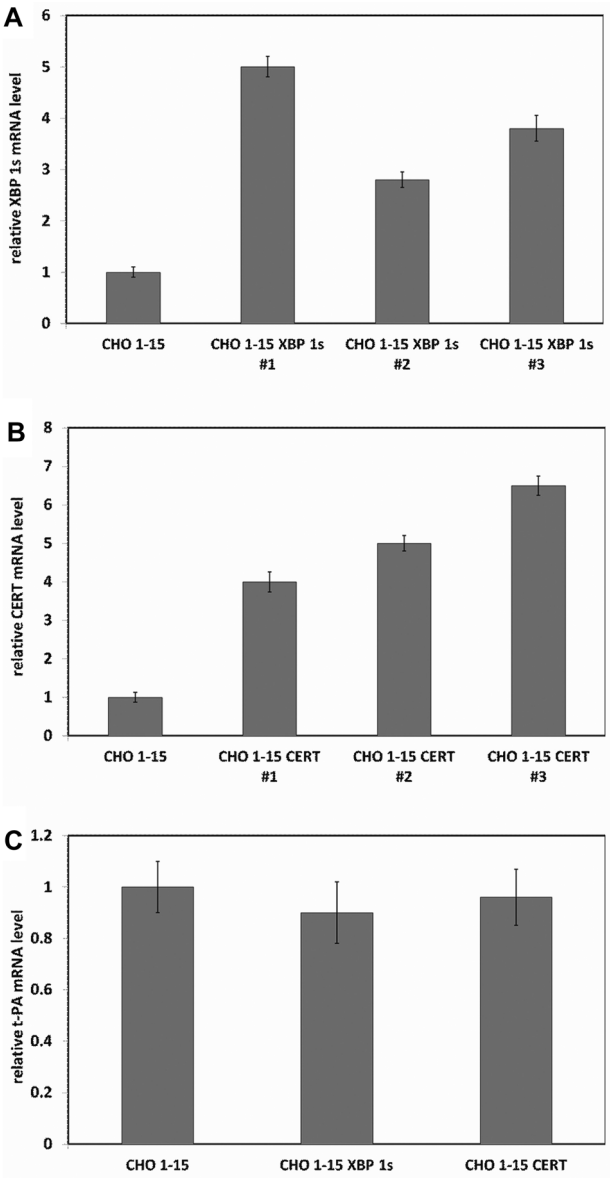


**Fig. 2.** Real-time RT-PCR analysis of XBP1s (**A**), CERT (**B**), and t-PA (**C**) mRNA levels in parental CHO 1-15(500) cells and XBP1s or CERT stable cell populations (indicated as CHO 1-15 XBP1s #1, #2, #3, and CHO 1-15 CERT #1, #2, #3).

The bars represent the mean values of three independent measurements for each cell pool in **A** and **B**, and mean values obtained from three stable cell pools per genotype (CHO 1-15 XBP1s and CHO 1-15 CERT) in **C**. Error bars represent the standard deviation.

### Determination of CERT, XBP1s, and t-PA mRNA Levels

In order to further characterize XBP1s and CERT S132A expressing cells, the CERT, XBP1s, and t-PA transcript levels were quantified in CHO 1-15(500) and stable cell pools. As indicated in Figs. 2A and 2B, the mRNA levels of XBP1s and CERT were significantly increased in the stable

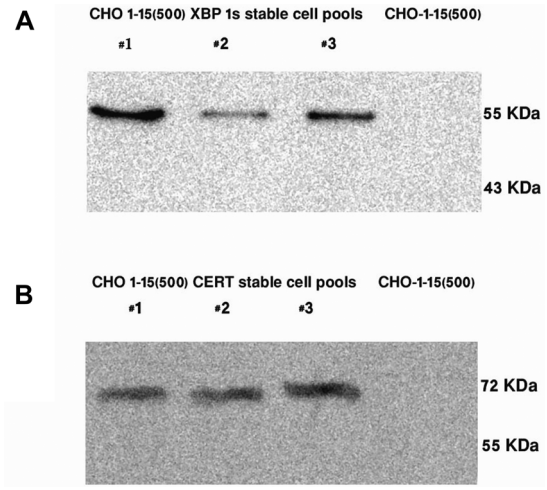


**Fig. 3.** Western blot analysis for detection of FLAG-tagged XBP1s (**A**) and CERT S132A (**B**) in CHO 1-15(500) cells engineered for constitutive expression of XBP1s or CERT S132A genes.

Three stable cell pools were analyzed for each gene, and the lysate from parental CHO 1-15(500) cells was used as the negative control. Equal amounts of cell lysate (50 μg) were loaded in each well.

cell pools as compared with the parental cells. On the other hand, the quantitative real-time PCR did not reveal any difference in t-PA mRNA levels between the parental CHO 1-15(500) and XBP1s or CERT S132A genetically modified cell pools (Fig. 2C). This result confirms that genetic manipulation of CHO cells has not affected the t-PA transcription level.

### Western Blot Analysis

Each set of stable cell pools was analyzed for the expression of heterologous CERT S132A or XBP1s proteins using western blot analysis. Since these genes were designed to be expressed as FLAG-tagged proteins, anti-FLAG antibody was used for detection. As can be seen in Fig. 3, corresponding bands to recombinant XBP1s or CERT S132A proteins appeared in all three transgenic cell lines for each gene.

### Effects of CERT S132A and XBP1s Expression on t-PA Productivity in CHO 1-15(500) Cells

XBP1s and CERT S132A stable cell pools as well as parental CHO cells were cultured at the initial cell density of $0.2 \times 10^6$ cells/ml. After 72 h of cultivation, the specific productivity was calculated based on estimated t-PA titers and IVCD values. As indicated in Fig. 4A, no improvement in t-PA expression was observed in the XBP1s-transfected

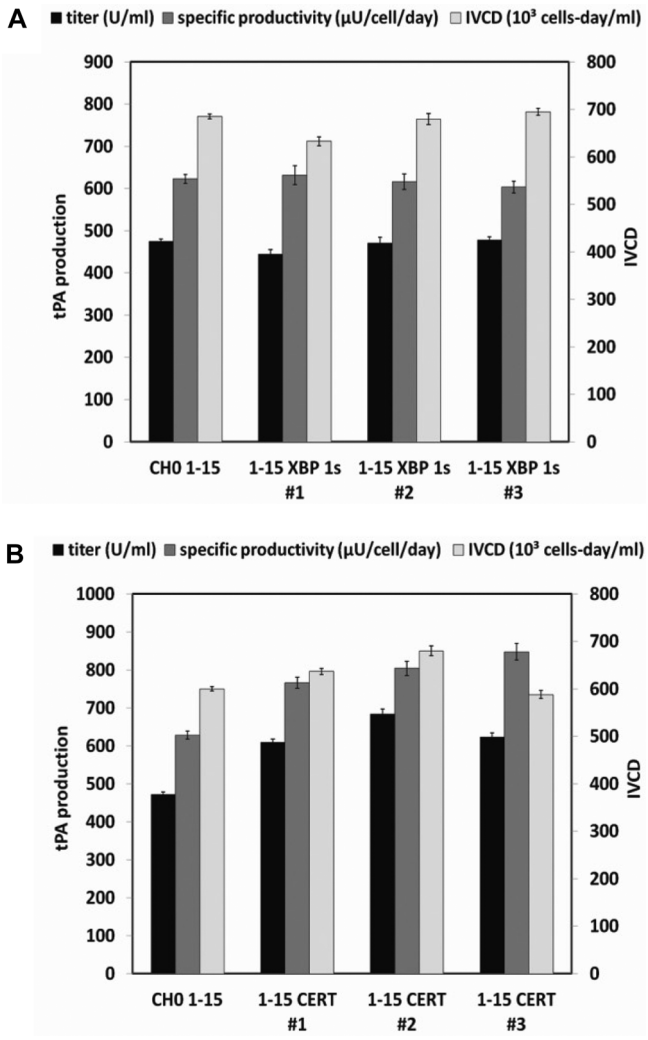


**Fig. 4.** Effects of XBP1s (**A**) and CERT S132A (**B**) expression on the productivity of t-PA-producing CHO 1-15(500) cells.
t-PA units and viable cell densities were measured for stable cell pools of XBP1s (CHO 1-15 XBP1s #1, #2, #3), CERT S132A (CHO 1-15 CERT #1, #2, #3), and parental CHO 1-15(500) cells after 72 h of culture. t-PA titer, specific productivity, and IVCD are indicated for each sample. Error bars represent the standard deviation of measurements of three independent experiments.

cells as compared with CHO 1-15(500) cells. However, ectopic expression of CERT S132A increased the t-PA titer and specific productivity of CHO 1-15(500) cells by 29%–45% and 22%–35%, respectively (Fig. 4B).

## Discussion

During the last two decades, significant efforts have been made to increase recombinant protein productivity in mammalian cells. In fact, today, more than 100-fold enhancements in product titer have been achieved through the optimization of different aspects of protein production [4, 27].

This progress has mostly been acquired through the optimization of the culture medium and bioprocess conditions, but improvements in cell line development have also been beneficial. The development of more efficient expression vectors in conjugation with new screening technologies has greatly enhanced the efficiency of obtaining high producer cells [5]. Moreover, cell line engineering has been considered as a promising strategy for the enhancement of protein productivity of mammalian cells. For this purpose, several regulatory genes like those involved in transcription, translation, protein processing and secretion, and cell survival have been used [27]. Since CHO-cell-derived recombinant proteins are usually secreted into the culture medium, manipulation of the protein secretion pathway has been of particular interest in the development of high producer cells.

To study the influence of XBP1s and CERT genes on t-PA expression, these genes were overexpressed in CHO 1-15(500), a monoclonal population of t-PA-producing CHO cells. CERT S132A, the phosphorylation mutant of CERT, was used based on previous studies in HEK 293 and CHO cells, which have shown enhanced activity of this variant in comparison with wild-type CERT [8].

Overexpression of CERT and XBP1s at the mRNA and protein levels was indicated by real-time PCR and western blot analysis, respectively. The appearance of clear bands for the CERT and XBP1s proteins in lysates of transfected cells confirmed the successful expression of each gene in the stable cell pools.

Analysis of the t-PA concentration in the cell culture supernatant revealed a 22%–35% enhancement in the specific productivity of CERT-S132A-expressing cells compared with parental cells. As shown by qRT-PCR, the t-PA mRNA level remained constant in parental and CERT S132A stable CHO 1-15(500) cells, suggesting that the enhanced expression of t-PA titer is mediated by a posttranscriptional mechanism. The observed improvement in t-PA expression level is in agreement with the previous findings of Florin *et al*. [7] that showed CERT and its mutant form CERT S132A can enhance protein secretion of CHO cells producing human serum albumin and monoclonal antibodies by 60% and 26%, respectively. The authors also reported a correlation between the CERT S132A expression level and increased antibody productivity [7]. In our work, we detected different rates of t-PA enhancement in three CERT-S132A-expressing

CHO cell pools. Although we did not compare the CERT S132A protein level among these cell populations, this observation could also be a reflection of various levels of CERT S132A expression in the three stable cell pools.

Conversely, XBP1s overexpression did not improve the t-PA expression in our experiment. So far, several groups have investigated the influence of XBP1s on CHO cell productivity in transient and stable expression set-ups, but the outcomes of this approach seem to be inconsistent. While Tigges and Fussenegger [32] showed that XBP1s can enhance the expression of the human placental secreted alkaline phosphatase (SEAP), *Bacillus stearothermophilus* derived secreted α-amylase (SAMY), and human vascular endothelial growth factor 121 (VEGF 121) by 3–5-fold, Ku *et al*. [19] claimed that the influence of XBP1s on heterologous protein expression is dependent on the protein expression level, and this gene can only be effective when the secretory capacity of cells is saturated. In another experiment, Ohya *et al*. [24] explained the fact that during cell line development, expression of the XBP1s gene might be activated in some producer cell clones, and therefore, ectopic expression of this gene cannot improve the productivity of these clones any further.

Our qRT-PCR result indicated basal levels of XBP1s expression in parental CHO 1-15(500) cells. In fact, inherent expression of the spliced variant of XBP1 in non-protein-producing CHO-K1 and DUXB11 cells has been reported by other groups [20, 24]. Although, in our work, it remained unclear whether XBP1s expression was induced as a result of UPR activation during the development of t-PA-producing CHO 1-15(500) cells or if it was activated in the original CHO cells, we can assume that this basal level of XBP1s expression has abolished the need for the heterologous expression of this gene.

Recent advances in molecular biology and high-throughput "omics" technologies have increased our knowledge of the identification of key regulatory molecules in different levels of gene expression that can be used as potential targets for cell line engineering [12, 30]. However, protein production in mammalian cells is a complicated process, and for this approach to be successful, several considerations should be taken into account. In this regard, the nature of the heterologous protein seems to be an important factor, as different proteins may cause different burdens on producer cells. It is also well-proved that mammalian cells are genetically heterogeneous, and during cell line development, it is possible for a subpopulation of cells to become more competent for the extra metabolic load caused by the recombinant protein [23]. Therefore, cell line engineering can sometimes lead to unexpected or different outcomes for different cell populations.

In conclusion, our results emphasize that although engineering of the secretory pathway can be an efficient method to enhance the recombinant protein productivity of CHO cells, the identification of an appropriate target gene is a major challenge for the successful application of this approach in a given recombinant protein producer mammalian cell line.

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## References

1. Bard F, Malhotra V. 2006. The formation of TGN-to-plasma-membrane transport carriers. *Annu. Rev. Cell Dev. Biol.* **22:** 439-455.
2. Becker E, Florin L, Pfizenmaier K, Kaufmann H. 2008. An XBP-1 dependent bottle-neck in production of IgG subtype antibodies in chemically defined serum-free Chinese hamster ovary (CHO) fed-batch processes. *J. Biotechnol.* **135:** 217-223.
3. Borth N, Mattanovich D, Kunert R, Katinger H. 2005. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. *Biotechnol. Prog.* **21:** 106-111.
4. Butler M. 2005. Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. *Appl. Microbiol. Biotechnol.* **68:** 283-291.
5. Cacciatore JJ, Chasin LA, Leonard EF. 2010. Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production using the Dhfr-based CHO cell selection system. *Biotechnol. Adv.* **28:** 673-681.
6. Figueroa Jr B, Chen S, Oyler GA, Hardwick JM, Betenbaugh MJ. 2004. Aven and Bcl-xL enhance protection against apoptosis for mammalian cells exposed to various culture conditions. *Biotechnol. Bioeng.* **85:** 589-600.
7. Florin L, Pegel A, Becker E, Hausser A, Olayioye MA, Kaufmann H. 2009. Heterologous expression of the lipid transfer protein CERT increases therapeutic protein productivity of mammalian cells. *J. Biotechnol.* **141:** 84-90.
8. Fugmann T, Hausser A, Schoffler P, Schmid S, Pfizenmaier K, Olayioye MA. 2007. Regulation of secretory transport by protein kinase D-mediated phosphorylation of the ceramide transfer protein. *J. Cell Biol.* **178:** 15-22.
9. Fussenegger M, Schlatter S, Datwyler D, Mazur X, Bailey JE. 1998. Controlled proliferation by multigene metabolic

engineering enhances the productivity of Chinese hamster ovary cells. *Nat. Biotechnol.* **16:** 468-472.
10. Geserick C, Bonarius HP, Kongerslev L, Hauser H, Mueller PP. 2000. Enhanced productivity during controlled proliferation of BHK cells in continuously perfused bioreactors. *Biotechnol. Bioeng.* **69:** 266-274.
11. Hanada K, Kumagai K, Yasuda S, Miura Y, Kawano M, Fukasawa M, Nishijima M. 2003. Molecular machinery for non-vesicular trafficking of ceramide. *Nature* **426:** 803-809.
12. Jaluria P, Chu C, Betenbaugh M, Shiloach J. 2008. Cells by design: a mini-review of targeting cell engineering using DNA microarrays. *Mol. Biotechnol.* **39:** 105-111.
13. Jayapal KP, Wlaschin KF, Hu WS, Yap MGS. 2007. Recombinant protein therapeutics from CHO cells – 20 years and counting. *Chem. Eng. Prog.* **103:** 40-47.
14. Josse L, Smales CM, Tuite MF. 2012. Engineering the chaperone network of CHO cells for optimal recombinant protein production and authenticity. *Methods Mol. Biol.* **824:** 595-608.
15. Khan SU, Schroder M. 2008. Engineering of chaperone systems and of the unfolded protein response. *Cytotechnology* **57:** 207-231.
16. Kim JY, Kim YG, Lee GM. 2012. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. *Appl. Microbiol. Biotechnol.* **93:** 917-930.
17. Kim SH, Lee GM. 2007. Functional expression of human pyruvate carboxylase for reduced lactic acid formation of Chinese hamster ovary cells (DG44). *Appl. Microbiol. Biotechnol.* **76:** 659-665.
18. Kramer O, Klausing S, Noll T. 2010. Methods in mammalian cell line engineering: from random mutagenesis to sequence-specific approaches. *Appl. Microbiol. Biotechnol.* **88:** 425-436.
19. Ku SC, Ng DT, Yap MG, Chao SH. 2008. Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells. *Biotechnol. Bioeng.* **99:** 155-164.
20. Ku SC, Toh PC, Lee YY, Chusainow J, Yap MG, Chao SH. 2010. Regulation of XBP-1 signaling during transient and stable recombinant protein production in CHO cells. *Biotechnol. Prog.* **26:** 517-526.
21. Lee AH, Iwakoshi NN, Glimcher LH. 2003. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell Biol.* **23:** 7448-7459.
22. Meents H, Enenkel B, Werner RG, Fussenegger M. 2002. p27Kip1-mediated controlled proliferation technology increases constitutive sICAM production in CHO-DUKX adapted for growth in suspension and serum-free media. *Biotechnol. Bioeng.* **79:** 619-627.
23. O'Callaghan PM, James DC. 2008. Systems biotechnology of mammalian cell factories. *Brief. Funct. Genomic. Proteomic.* **7:** 95-110.
24. Ohya T, Hayashi T, Kiyama E, Nishii H, Miki H, Kobayashi K, *et al*. 2008. Improved production of recombinant human antithrombin III in Chinese hamster ovary cells by ATF4 overexpression. *Biotechnol. Bioeng.* **100:** 317-324.
25. Onitsuka M, Kim WD, Ozaki H, Kawaguchi A, Honda K, Kajiura H, *et al.* 2012. Enhancement of sialylation on humanized IgG-like bispecific antibody by overexpression of alpha2,6-sialyltransferase derived from Chinese hamster ovary cells. *Appl. Microbiol. Biotechnol.* **94:** 69-80.
26. Park H, Kim IH, Kim IY, Kim KH, Kim HJ. 2000. Expression of carbamoyl phosphate synthetase I and ornithine transcarbamoylase genes in Chinese hamster ovary DHFR-cells decreases accumulation of ammonium ion in culture media. *J. Biotechnol.* **81:** 129-140.
27. Peng RW, Fussenegger M. 2009. Molecular engineering of exocytic vesicle traffic enhances the productivity of Chinese hamster ovary cells. *Biotechnol. Bioeng.* **102:** 1170-1181.
28. Rouf SA, Moo-Young M, Chisti Y. 1996. Tissue-type plasminogen activator: characteristics, applications and production technology. *Biotechnol. Adv.* **14:** 239-266.
29. Schroder, M. 2006. The unfolded protein response. *Mol. Biotechnol*. **34:** 279-290.
30. Seth G, Charaniya S, Wlaschin KF, Hu WS. 2007. In pursuit of a super producer – alternative paths to high producing recombinant mammalian cells. *Curr. Opin. Biotechnol.* **18:** 557-564.
31. Tey BT, Singh RP, Piredda L, Piacentini M, Al-Rubeai M. 2000. Bcl-2 mediated suppression of apoptosis in myeloma NS0 cultures. *J. Biotechnol.* **79:** 147-159.
32. Tigges M, Fussenegger M. 2006. Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells. *Metab. Eng.* **8:** 264-272.
33. Wong NS, Yap MG, Wang DI. 2006. Enhancing recombinant glycoprotein sialylation through CMP-sialic acid transporter over expression in Chinese hamster ovary cells. *Biotechnol. Bioeng.* **93:** 1005-1016.
34. Wurm FM. 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. *Nat. Biotechnol.* **22:** 1393-1398.
35. Zhu J. 2012. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol. Adv.* **25:** 1158-1170.
36. Zustiak MP, Dorai H, Betenbaugh MJ, Sauerwald TM. 2012. Controlling apoptosis to optimize yields of proteins from mammalian cells. *Methods Mol. Biol.* **801:** 111-123.