Original Article

Development of Genetically Modified Chinese Hamster Ovary Host Cells for the Enhancement of Recombinant Tissue Plasminogen Activator Expression

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Abstract

Background: **Chinese hamster ovary (CHO) cells are the most commonly used host system for the expression of high quality recombinant proteins. However, the development of stable, high-yielding CHO cell lines is a major bottleneck in the industrial manufacturing of therapeutic proteins. Therefore, different strategies such as the generation of more efficient expression vectors and establishment of genetically engineered host cells have been employed to increase the efficiency of cell line development. In order to examine the possibility of generating improved CHO host cells, cell line engineering approaches were developed based on ceramide transfer protein (CERT), and X-box binding protein 1s (XBP1s).**

Methods: **CHO cells were transfected with CERT S132A, a mutant variant of CERT which is resistant to phosphorylation, or XBP1s expression plasmids, and then stable cell pools were generated. Transient expression of t-PA was examined in engineered cell pools in comparison to un-modified CHO host cells.**

Results: **Overexpression of CERT S132A led to the enhancement of recombinant tissue plasminogen activator (t-PA) expression in transient expression by 50%. On the other hand, it was observed that the ectopic expression of the XBP1s, did not improve the t-PA expression level.**

Conclusion: **The results obtained in this study indicate successful development of the improved CHO host cells through CERT S132A overexpression.**

Keywords: *cell engineering, CHO cells, tissue plasminogen activator*

Introduction

Mammalian cells are the most commonly used host system for the production of recombinant therapeutics due to their ability to produce correctly folded and biologically active proteins (1,2). Among available mammalian cell lines, Chinese hamster ovary (CHO) cells are of particular interest for the industrial manufacturing of biopharmaceuticals (3). However, the development of high protein producing CHO cell clones has proven to be a time-consuming and expensive process. Therefore, different strategies have been investigated to reduce process development time and production costs including development of genetically engineered host cells, optimisation of the expression vectors and adjustment of the culture conditions (4–6).

Protein expression in mammalian cells is regulated at different levels from transcription, post-transcription, translation, and post-transcription to protein processing and secretion (7). Although cell line engineering strategies can be potentially applied to each of these steps,

identification of the proper target genes in each pathway is critical for the successful application of this approach (8).

It is well known that protein secretion is a potential bottleneck in mammalian cells (9). In fact, different studies have shown that after a certain point, the amount of secreted product does not proportionally correlate with the intracellular protein concentration; indicating that cells might not be able to process extra amount of the recombinant proteins (10). Therefore, different modulators of the protein secretory pathway were examined for their potential to improve protein productivity in mammalian cells (8,11,12). Although protein secretion in mammalian cells is a complicated process, the genetic engineering strategies based on the overexpression of individual components of the secretory pathways might not always improve product secretion. Consequently, targeting the key mediators of the secretory pathway could be a more effective strategy (13,14).

Ceramide transfer protein (CERT) mediates non-vesicular transfer of ceramide from the endoplamic reticulum (ER) to the trans-Golgi network (TGN), where it is converted to sphingomyelin (SM), and diacylglycerol (DAG) (15). Accumulation of the DAG at TGN affects the membrane structure and also recruits protein kinase D (PKD). PKD functions as a major regulator of the secretory pathway at the TGN by phosphorylating proteins that are involved in vesicle formation. PKD also regulates the activity of CERT by its phosphorylation at the serine 132 residue (16,17).

X-box binding protein 1s (XBP1s) is an alternatively spliced transcription factor which plays an essential role in the plasma cell differentiation, immunoglobulin secretion, and the unfolded protein response (UPR) (18). UPR has been known as a stress response pathway which is activated through accumulation of the unfolded proteins in the endoplasmic reticulum lumen (19). During UPR, XBP1s is generated by removal of a 26 nucleotide intron from the XBP1 transcript. The resulting variant is a potent transcription factor which mediates expansion of the ER and expression of chaperones and foldases (9, 20).

Cell line engineering approaches based on CERT and XBP1s genes have been used to enhance productivity of recombinant protein expressing CHO cells. For example, Florin et al. (21), have shown that the heterologous expression of CERT and its mutant variant CERT S132A resulted in enhanced secretion of the monoclonal antibody and human serum albumin from recombinant CHO cells by 60 and 26%, respectively. The ectopic expression of XBP1s has been reported to enhance the production of monoclonal antibodies, human placental secreted alkaline phosphatase (SEAP), *Bacillus stearothermophilus* derived α-amylase (SAMY), and human vascular endothelial growth factor 121 (VEGF 121) in CHO cells but not in HEK 293 or HT1080 cell lines (22).

The aim of the current study was to develop a new CHO cell line with improved capacity in expression of the recombinant proteins. None of the previous works have compared the potential of CERT S132A and XBP1s genes for development of an engineered host system. Therefore, CHO-K1 stable cell pools were generated for each of these genes. The protein expression capacity of each cell line was evaluated in transient expression assay using the human tissue plasminogen activator (t-PA) as a model protein.

Materials and Methods

Plasmid construction

To construct the pcDNA3.1-CERT S132A vector, the CERT S132A coding sequence was amplified from the pcDNA3-CERT S132A plasmid (provided by Dr. Monilola Olayioye, University of Stuttgart, Germany) and cloned into the pGEM-T Easy intermediate vector (Promega, USA). A FLAG-tag coding sequence was included in the reverse primer for detection of the CERT S132A protein in Western blotting. After sequencing, the fragment was excised from the intermediate plasmid with *XbaI* and *HindIII* enzymes and cloned into the same sites of the pcDNA3.1/Hygro/LacZ vector (Invitrogen, USA). The human tissue plasminogen activator coding sequence was sub-cloned from the pTZ57R-t-PA vector into *Xho I* and *Not I* sites of the pEGFP-puro plasmid. The EGFP coding region was replaced to create pTPA expression vector. 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

Adherent CHO-K1 cells (ATCC CRL-9661) were routinely cultivated in DMEM/F12 medium 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. Trypan blue exclusion method was used for determination of the cell concentration

and viability. Transfection was performed using lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol in 12 well plates with the cell density of 0.35 × 10^6 cells/mL.

Transient Expression

To optimise plasmid DNA concentration for transient expression of t-PA, different concentrations of the circular pTPA expression plasmid (0.5, 1, 2 and 3 µg) were transfected to CHO-K1 cells and expression analysis was performed 48 h after transfection. The optimized plasmid concentration was then used in transient expression assays throughout the study. For each experiment, transfections were performed in duplicates and repeated three times.

Development of stable cell pools

Stable CERT S132A and XBP1s cell pools were generated by transfection of CHO-K1 cells with BglII linearised pcDNA3.1-CERT S132A/hygro or pcDNA3 XBP1s/neo expression vectors in duplicates. Subsequently, 48 h post-transfection, the double transfectants of each gene were mixed together and then diluted in 1:6 ratio and cultured in selective medium containing 200 µg/mL hygromycin or 400 µg/mL G418 (Invitrogen, USA) for 4 weeks. When the confluency of the transfectants reached above 90%, cells were diluted again and grown in higher concentration of the antibiotic (twice as the initial concentration) for another 3–4 weeks. The resulting cell pools were cultured in antibiotic free medium for at least 15 passages. To analyse the stable integration of heterologous genes into the cell genome, genomic DNA was isolated from stable cell pools as well as from parental CHO-K1 cells using a DNA extraction kit (Roche, Germany) after which polymerase chain reaction (PCR) was performed with gene specific primers.

Real time RT-PCR analysis

Total RNA was extracted from 2–3 × 10^6 cells using RNX plus reagent (Cinnagen, Iran). To ensure the purity of the isolated RNA, DNase treatment was performed with RNase free DNase enzyme (Fermentas, Lithuania). cDNA was prepared using Revert Aid first-strand cDNA synthesis kit (Fermentas, Lithuania). Quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR) was performed in an ABI 7500 system (Applied Biosystems, USA) using SYBR green PCR master mix. However, glyceraldehyde-3-phosphate dehydrogenase GAPDH housekeeping gene was used for normalisation. For detection of the spliced variant of the XBP1 transcript, a reverse primer was designed to overlap the unique exon junction of XBP1s. For the first denaturation step, the amplification program was set at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. A melting curve was set for one cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Relative quantification analysis was performed using the comparative CT (ΔΔCT) method. The primer pairs used in this study are shown in Table 1.

Western blotting

For this experiment, 5 × 106 cells were harvested from a 3 days cell culture, washed with PBS, and suspended in the cell lysis buffer containing protease inhibitor cocktail (Roche, Germany). 50 µg of the total cell lysate was loaded on 12% SDS-PAGE and transferred to a nitrocellulose membrane. Rabbit anti-FLAG polyclonal antibody (Cell Signalling Technologies, USA) was used as the primary antibody in a 1/1000 dilution, while goat anti-rabbit HRP antibody (Santa Cruz Biotechnology, USA) was applied as the secondary antibody in a 1/1500 dilution. For detection, ECL plus reagent (Amersham Biosciences, Germany) was employed.

Analysis of the t-PA expression

To determine t-PA units, Trinilized t-PA activity assay kit (Trinity Biotech, Ireland) was used. Briefly, 100 µl of the tPA standards (0, 0.5, 1.0, 1.5, 2.0 IU/mL) and 100 µL of the diluted supernatant were applied to the microtest strip wells. Samples were incubated for 20 min on a plate shaker at room temperature. After three washing steps, plasminogen and chromogenic substrate were added to each well and incubated for 75 min. Then, the reaction was stopped and samples were read at 405 nm. Units of t-PA were determined using the standard t-PA provided by the kit. Specific productivity (Qp) was calculated

Table 1: Sequence of the primer pairs used in quantitative RT-PCR assay

Primer	Sequence (5′ to 3′)
GAPDH-F	CACTCTTCCACCTTTGATGCTG
GAPDH-R	GTCCACCACTCTGTTGCTGTAGC
CERT-F	AGATTTGTGCAAAAGGTTGAAGAG
CERT-R	ACTCCTTTAACTGCATGGGTAGC
XBP1s-F	GGAGAGAAAACTCATGGCCTTG
XBP1s-R	CTGCACCTGCTGCGGACTC

using the following formulas where P is t-PA concentration, X is viable cell density, t is culture time in days and IVCD is integral viable cell density (23):

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

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

Statistical analysis

Statistical analysis was performed with SPSS 18 software. Un-paired t-test was used to compare means between two groups and one-way ANOVA was used to compare means among multiple groups. The differences between means were considered significant at $P < 0.05$.

Results

Development of CERT S132A and XBP1s stable cell pools

Stable CHO-K1 cell pools for CERT S132A or XBP1s genes were generated in an antibiotic containing medium. After several passages in antibiotic free medium, PCR analysis on genomic DNA of stable cell pools confirmed the integration of each gene cassette in the CHO-K1 genome. The genomic DNA of un-transfected CHO-K1 cells was used as a negative control. The results of PCR analysis are shown in Figure 1. The appearance of the specific bands at 1797 bp for CERT S132A (Figure 1a), and 1131 bp for XBP1s (Figure 1b), confirmed the integration of each gene in the host cell genome. The resulting stable cell pools were named as CHO CERT and CHO XBP1s.

Quantitative real time RT-PCR analysis

Quantitative real time RT-PCR analysis was performed to detect the expression of each gene at the mRNA level in genetically modified cells. Levels of CERT and XBP1s mRNA in stable cell pools were quantified in comparison to parental CHO-K1 cells. The GAPDH housekeeping gene was used as the internal control, to normalise possible variation in the amount and quality of RNA between samples. Real time RT-PCR revealed significant increase in CERT and XBP1s mRNA levels in stable cell pools. As shown in Figure 2, up to 4.3 fold increase was observed in XBP1s mRNA in CHO XBP1s cells and 5.8 fold increase in CERT mRNA in CHO CERT cells.

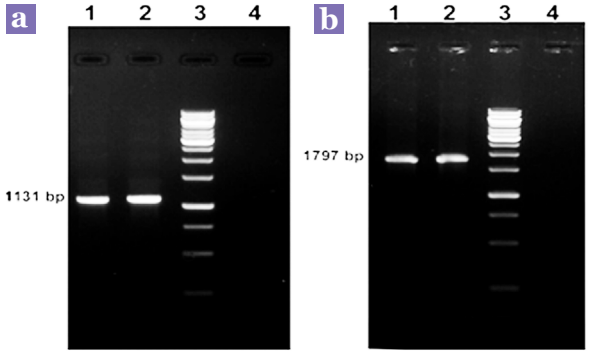

Figure 1: Agarose gel electrophoresis. XBP1s and CERT S132A genes were amplified from genomic DNA of stable cell pools and parental CHO-K1 cells. (a) lanes 1 and 2 XBP1s stable cell pools, lane 3 size marker, and lane 4 un-transfected CHO-K1 cells. (b) lanes 1 and 2 CERT S132A stable cell pools, lane 3 size marker and lane 4 un-transfected CHO-K1 cells.

Western blot analysis

In order to detect expression of the CERT S132A and XBP1s proteins in CHO-K1 cells, lysates from each stable cell pool were examined by Western blot analysis. The CERT S132A and XBP1s genes were expressed as FLAG-tagged proteins; therefore, anti-FLAG primary antibody was used for detection of the related proteins. The results of Western blot analysis are shown in Figure 3. In this test, clear bands corresponded to FLAG-tagged XBP1s at 50 KDa in CHO XBP1s cells (Figure 1a), whereas the FLAG-tagged CERT S132A was observed at 69 KDa in CHO CERT cells (Fig.3B), though no bands were observed in the CHO-K1cells. These results confirmed the successful expression of each protein in stable cell pools.

Transient transfection of tPA in CHO-K1 and stable cell pools

To identify the optimal concentration of plasmid DNA for transfection of CHO-K1 cells, different concentrations of the t-PA expression plasmid were used for transfection of CHO-K1 cells. The cell culture supernatants were analysed for the active t-PA expression level after 48 h. The results were compared to the supernatant from the un-transfected CHO cells as the reference group. However, t-PA expression was found to be enhanced in the cells with increase in plasmid concentration to 1.5 µg, but after this point no

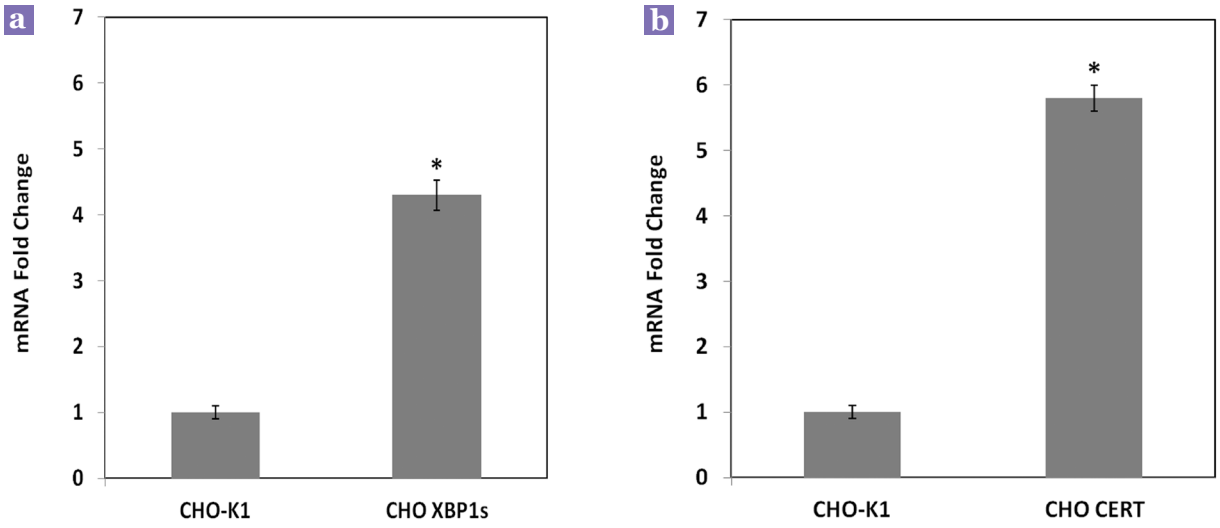

Figure 2: Real time RT-PCR assay for XBP1s and CERT mRNA. (a) Analysis of the XBP1s mRNA level in XBP1s stable cell pools in comparison to parental CHO-K1 cells. (b) Analysis of the CERT mRNA level in CERT stable cell pools in comparison to parental CHO-K1 cells. Error bars represent standard deviation of three independent analyses. Statistical analysis was performed using student t-test and differences in comparison to control CHO-K1 cells were considered significant at $P < 0.05$ as shown by asterisk above each bar.

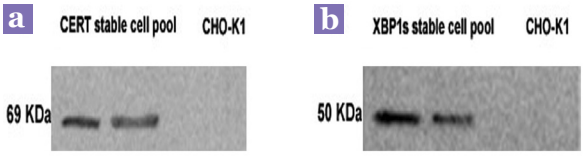

Figure 3: Western blot analysis for detection for CERT S132A, and XBP1s genes. (a) Analysis of CERT S132A expression in CHO CERT stable cells. (b) Analysis of XBP1s expression in CHO XBP1s stable cells. The lysate of parental CHO-K1 cells was used as negative control.

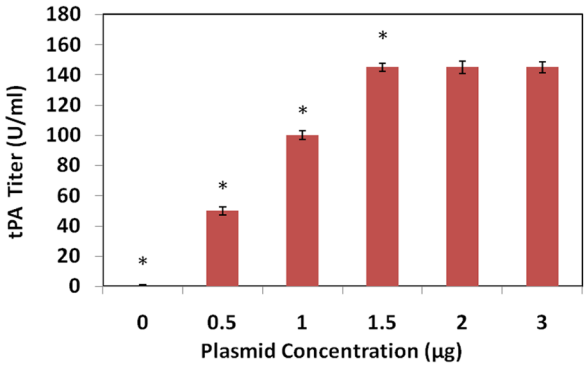

Figure 4: Optimisation of the plasmid concentration for transfection of CHO-K1 cells. Cells were transfected with increasing concentrations of the t-PA expression plasmid and supernatants were analysed for t-PA expression level after 48 hours. Error bars represent standard deviation of two independent analysis. Results were analysed using one-way ANOVA and the differences between means were considered significant at $P < 0.05$ as shown by asterisk above the bars.

improvement in productivity was observed (Figure 4). According to this result, 1.5 µg of plasmid DNA was used for transfection of CHO-K1 cells in the next step.

To examine the expression capabilities of the modified CHO-K1 cells, transient expression of t-PA was assessed in each cell pool during 48 h of culture. Figure 5 shows t-PA titer as well as specific productivity for each cell pool and parental cells. The parental cells were used as the reference group. Interestingly, while t-PA expression level was enhanced up to 50% in CHO

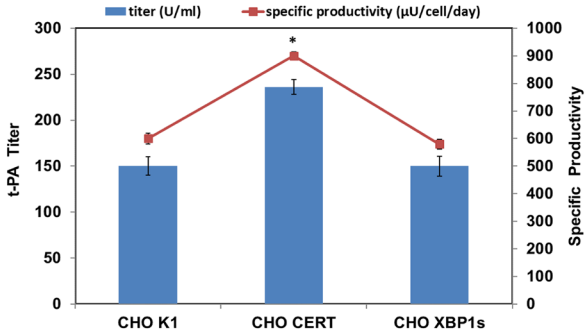

Figure 5: Analysis of t-PA productivity of CHO-K1, CHO CERT and CHO XBP1s cells in transient expression. Supernatants were collected after 48 hours. The presented values are the mean of three independent experiments and error bars indicate standard deviation. Comparison of the means between the three groups was performed using one-way ANOVA and asterisk shows a statistically significant difference with $P < 0.05$.

CERT cells, the productivity of CHO XBP1s cell pool did not show any difference when compared with CHO-K1 cells. This result indicated that only CERT S132A was able to increase t-PA expression in the transient expression set up of this study.

Discussion

The aim of this study was to improve the recombinant protein productivity of CHO cells. Genetic modification of the host cells has proven to be a promising strategy to eliminate or reduce the bottlenecks in recombinant protein expression (24). In this regard, recombinant protein folding and secretion has been an attractive target for cell line engineering. Since recombinant protein secretion is a complex process, it is critical to target the main regulatory molecules which can control the overall activity of the entire pathway (13). This study focused on CERT and XBP1s genes as key mediators of secretory vesicle formation and unfolded protein response, respectively.

Fugmann and co-workers (17) have previously examined the effects of CERT and its phosphorylation mutant in transient expression through co-transfection of this gene with the HRP (horseradish peroxidase) reporter gene in HEK 293 cells. The authors also showed that the CERT S132A, a mutant variant of CERT that is resistant to phosphorylation, was more effective in the enhancement of HRP expression in HEK 293 cells (17). Moreover, ectopic expression of the CERT and its mutant variant has been shown to increase productivity of recombinant protein producing CHO cells (21). In the current study, the potential of this gene to develop improved mammalian host cells was examined. In other studies, XBP1s was the target of cell engineering approaches; however, so far the cell line engineering of XBP1s has resulted in mixed outcomes. XBP1s, as a part of the mammalian unfolded protein response, is activated in response to different types of stress signals including accumulation of recombinant proteins in the endoplasmic reticulum (25). Tigges and co-workers (22) have reported the successful development of modified CHO cells through overexpression of XBP1s. Conversely, Ku et al. (13) claimed that expression of XBP1s can only increase productivity of the high producer clones. Recently, Cain et al. have developed a new CHO host cell line for transient gene expression by simultaneous expression of XBP1s and ERO1-Lα (endoplasmic reticulum oxidoreductase) in CHO-S cells. In this study, monoclonal antibody expression was improved up to 6 folds in the new host cell line (26).

CERT and XBP1s are involved in different levels of the protein secretion pathway. Therefore, comparing the effects of these genes in one experimental set-up can reveal more details about their potential as candidates for cell line engineering.

Since the expression level of the CERT S132A and XBP1s genes might be important for their activity. It was necessary to first confirm that they have been expressed in detectable amounts in CHO cells. The quantitative RT-PCR analysis was performed to evaluate the expression of each gene at the mRNA level. The PCR primers were designed for the native CERT and XBP1s genes; therefore, it was possible to determine the enhancement of mRNA level for each gene in comparison to the parental cells. The quantitative RT-PCR results of the present study showed enhanced mRNA levels of each gene in stable cell pools. Interestingly, the XBP1s mRNA was also detected in parental CHO-K1 cells. The XBP1s, as a UPR mediator, is expected to be expressed under stress conditions. However, the basal level of XBP1s expression in parental cells has been indicated by other groups as well. For example, Ku et al. (13) detected XBP1s mRNA in parental CHO-K1 and DG44 cells. These observations suggest the presence of basal IRE1 endoribonuclease activity in CHO cells. In the present study, Western blot analysis was also

performed on lysates from stable cell pools, to assess the expression of each gene at the protein level. The obtained results showed clear bands for each protein suggesting that they have been successfully expressed in cell pools.

In order to evaluate the efficiency of genetically modified CHO cells as improved hosts, the human tissue plasminogen activator (t-PA) was chosen as a model protein. The molecular nature of the t-PA as a 67 KDa glycoprotein which consists of 17 intra-molecular disulfide bonds and 4 N-linked glycosylation sites, makes it a suitable model protein for our study. The capability of each cell pool to express recombinant proteins was assessed in transient expression.

In the transient expression analysis of this study, no improvement was observed in the t-PA expression level in CHO XBP1s cell pools; however, t-PA titer and specific productivity of CHO CERT cells were enhanced up to 50% when compared with that of the parental cells. The findings of this study regarding CHO CERT cells are in agreement with the previous works in HEK 293 and CHO cells (17,21).

The results obtained from this study regarding CHO XBP1s cells are in contrast to those obtained by Tigges et al. (22). They reported that transient 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) can be enhanced by 3-5 fold in XBP1s engineered cells. The authors also showed that the increase in recombinant protein production caused by overexpression of XBP1s was independent of the expression levels of the proteins.

Differences observed in other studies and in the present study may be because of protein specific effects of the XBP1s gene. The production of different proteins may impose different metabolic burdens on the cells, therefore, the effects of a target gene on productivity could be specific to the particular recombinant proteins or their production level.

Conclusion

The results of this study indicated that although secretion engineering can be an efficient method to enhance recombinant protein productivity of CHO cells, identification of the proper target gene is a major challenge for the successful application of this strategy.

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Conflict of Interest

None.

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Authors' Contribution

Conception and design, analysis and interpretation of the data, drafting of the article: AR, FM
Final approval of the article, obtaining of funding: FM
Provision of study materials or patients: FB, AA
Administrative, technical, or logistic support: AR, RA, FB, AA
Collection and assembly of data: AR, RA

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