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Research Article

Time and Cost-Effective Genome Editing Protocol for Simultaneous Caspase 8 Associated Protein 2 Gene Knock in/out in Chinese Hamster Ovary Cells Using CRISPR-Cas9 System

Soofia Sorourian[1], Abbas Behzad Behbahani[2*], Mohsen Forouzanfar[1], Mojtaba Jafarinia[1], Fatemeh Safari[2]

[1]Department of Biology, Marvdasht Branch, Islamic Azad University, Marvdasht, Iran
[2]Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran

[*]*Corresponding author*: Abbas Behzad Behbahani, Diagnostic Laboratory Sciences and Technology Research Center, School of Paramedical Sciences, Shiraz University of Medical Sciences, Meshkinfam Ave, Shiraz, Iran. Tel/ Fax: +98-7132270301, E-mail: behzadba@sums.ac.ir

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Background: CHO cells are preferred for producing biopharmaceuticals, and genome editing technologies offer opportunities to enhance recombinant protein production. Targeting apoptosis-related genes, such as Caspases 8-Associated Protein 2 (CASP8AP2), improves CHO cell viability and productivity. Integrating robust strategies with the CRISPR-Cas9 system enables its application in CHO cell engineering.

Objectives: This study was performed to develop a cost-effective protocol using the CRISPR-Cas9 system combined with the HITI strategy for simultaneous CASP8AP2 gene deletion/insertion in CHO cells and to assess its impact on cell viability and protein expression.

Materials and Methods: We developed an efficient protocol for CHO cell engineering by combining CRISPR/Cas9 with the HITI strategy. Two distinct sgRNA sequences were designed to target the 3' UTR region of the CASP8AP2 gene using CHOPCHOP software. The gRNAs were cloned into PX459 and PX460-1 vectors and transfected into CHO cells using the cost-effective PEI reagent. A manual selection system was employed to streamline the process of single-cell cloning. MTT assays assessed gene silencing and cell viability at 24, 48, and 72 hours. Flow cytometry evaluated protein expression in CASP8AP2-silenced CHO cells.

Results: The study confirmed the robustness of combining CRISPR-Cas9 with the HITI strategy, achieving a high 60% efficiency in generating knockout clones. PEI transfection successfully delivered the constructs to nearly 65% of the clones, with the majority being homozygous. The protocol proved feasible for resource-limited labs, requiring only an inverted fluorescent microscope. CASP8AP2 knockout (CHO-KO) cells exhibited significantly extended cell viability compared to CHO-K1 cells when treated with NaBu, with IC50 values of 7.28 mM and 14.25 mM at 48 hours, respectively (P-value: 24 hours $\leq$ 0.0001, 48 hours $\leq$ 0.0001, P-value: 72 hours = 0.0007). CHO CASP8AP2-silenced cells showed a 1.3-fold increase in JRed expression compared to native cells.

Conclusions: CRISPR-Cas9 and HITI strategy was used to efficiently engineer CHO cells for simultaneous CASP8AP2 gene deletion/insertion, which improved cell viability and protein expression.

Keywords: Caspases, Cell engineering, CHO cells, CRISPR-Cas9 system, Transfection

1. Background

The biopharmaceutical industry is witnessing a significant rise in the production of recombinant therapeutic proteins (RTPs), with over 25 unique RTPs approved by the FDA in recent years. Global market projections indicate that the biopharmaceutical industry is expected to reach approximately $389 billion by 2024 (1). The CHO cell line hosts six out of the top ten biopharmaceuticals, offers high productivity (0.1-1 $g.L^{-1}$ in batch cultures and 1-10 $g.L^{-1}$ in fed-batch cultures). It is long-lived and adaptable to large-scale industrial environments (2).

Caspases involved in apoptosis induction serve as key targets for enhancing protein production in CHO cells. The objective is to increase the culture lifespan and maximize productivity for industrial applications (3).

The caspase 8 associated protein 2 (CASP8AP2) plays an important role in the apoptotic and pro-apoptotic processes. FLICE-associated large protein-encoding CASP8AP2 (FLASH) system regulates a wide range of biological processes including apoptosis and histone gene expression. Upon activation of the Fas-FasL signalling pathway, FLASH initiates the death-inducing signalling complex (DISC), which is a component of Fas-mediated apoptosis. The proteolytic caspase produced by the DISC complex activates procaspase 8 to cause extrinsic apoptosis (4). This approach enables us to harness the regulatory role of CASP8AP2 in apoptosis to optimize cell viability and enhance protein expression levels in CHO cells within the biopharmaceutical industry.

Traditional rCHO cell line development involves random gene integration, leading to unstable cell lines with reduced production and phenotypic heterogeneity (5). Targeted integration using site-specific nucleases, such as CRISPR-associated (Cas) RNA-guided nucleases, provides a solution (6). This technology enables precise and targeted indel mutations or site-specific sequence integration in CHO cells (7, 8).

Easi-CRISPR is a site-specific integration technique that utilizes long single-stranded DNA (ssDNA) donors. It demonstrates the efficient generation of mice with conditional alleles (9). Easi-CRISPR uses expensive ssDNA donors and has a size limitation for insertion (<1 kb) (10). Tild-CRISPR, which utilizes linearized dsDNA donors, shows effective targeted integration in mouse and human embryos in vivo (11). It allows for larger DNA fragment insertions (up to 200 kb), but optimizing insertion efficiency remains a complex challenge (12).

Site-specific integration in CHO cells is limited, particularly in resource-limited laboratories. A user-friendly protocol is needed to address this limitation. Homology-Independent Targeted Integration (HITI) combined with CRISPR enhances transgene integration rates in dividing and non-dividing CHO cells (13). HITI simplifies targeting vectors by requiring short sequences for gRNA targets identical to the target locus, which can be added via PCR or synthetic oligonucleotide insertion (14).

2. Objectives

The objective of the study was to streamline the knockout/in procedure in CHO cells using CRISPR-Cas9 and HITI strategy. Specific locations in the CASP8AP2 gene's 3' untranslated region (3'UTR) were targeted using two donor vectors. A GFP expression cassette was utilized as the reporter gene. Manual single-cell cloning, guided by a fluorescent microscope, replaced the need for cell sorting and expensive flow cytometry equipment. This cost-effective protocol for targeted integration in CHO cells, suitable for biopharmaceutical manufacturing, eliminated the requirement for homology arms or a cell sorter. The impact of CASP8AP2 silencing on cell viability was assessed through an MTT assay after exposure to Sodium butyrate (NaBu) for 24, 48, and 72 hours. Flow cytometric analysis was employed to evaluate the effects of CASP8AP2 silencing on protein production.

3. Materials and Methods

3.1. Chemicals and Reagents

RPMI-1640 and FBS were obtained from Gibco (Gibco, NY, USA (Cat #11995-040; Gibco); with 10% fetal bovine serum (FBS) (Cat #S11150H; Atlanta Biologicals), Favor Prep TM Plasmid Extraction Mini Kit (FAVORGEN® recommendations, National Biotechnology Park, Taiwan); streptomycin, and penicillin (Sigma–Aldrich, USA); 10%, glutamine (4.05 mM), streptomycin (100 $\mu g.mL^{-1}$) (Cat #15140-122; Gibco), T4 DNA Ligase (Thermo Fisher Scientific, USA (Cat #el0016), MTT (Cat #M8180; Solarbio).

3.2. Cell Line and Cell Culture

The Chinese hamster ovary adherent cells (CHO-K1

cell line) were obtained from the Pasteur Institute in Tehran, Iran. The cells were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals), glutamine (4.05 mM, Gibco, USA), penicillin (100 U. mL^{-1}, Sigma–Aldrich, USA), and streptomycin (100 µg. mL^{-1}, Gibco, USA). The cultures were maintained in a humidified incubator at 37 °C with 5% CO_2.

3.3. Cloning of gRNAs in CRISPR and Donor Vectors

The Homo sapiens CASP8AP2 gene sequence was found in the GenBank sequence collection of the National Center for Biotechnology Information (National Biosciences, Inc., Plymouth, MN). The CASP8AP2 gene and its promoter are 44,537 bp in length. Normal gRNAs and PAM (protospacer adjacent motif) plus gRNAs were designed using the http://chopchop.cbu.uib.no platform and ordered as oligonucleotides targeting the 3'UTR sequence of the CASP8AP2 gene of CHO cells. To identify any possible off-target sites, determine the number of mismatches, and evaluate the size of these mismatches, Cas-offinder software (http://www.rgenome.net/cas-offinder/) was used. The website was utilized to analyse each designed sgRNA and confirm the cleavage efficiency of the two gRNAs in the donor vector. The primary vector utilized in this study was the pSpcas9 (BB) 2A-Puro V2.0 vector (Addgene), commonly referred to as the pSpcas9 vector (PX459).

Two sgRNAs, namely sgRNA-a (sequence: CACCG GACTAGATGATTATCGTGCT) and sgRNA-b (sequence: AAACAGCACGATAATCATCTAGTCC), were employed in the study. Normal gRNAs were cloned into the PX459 plasmid (Plasmid #118632), kindly provided by the Royan Institute in Tehran, Iran. PAM plus gRNAs were cloned in PX460-1, which contains a CAG promoter- Enhanced green fluorescent protein (EGFP)-bovine growth hormone polyadenylation signal and a U6 promoter-sgRNA insertion site-sgRNA scaffold. This cloning process was carried out using the digestion/ligation method. (4).

3.4. Amplification of the Vector

Plasmids containing gRNAs were transformed into DH5α-competent *E. coli*, and plasmid DNA was harvested using the Miniprep Kit (Favorgene, Taiwan). Cloning of gRNAs was confirmed by using PCR and characterized by a NanoDrop spectrophotometer (Thermo Fisher Scientific). For quality assessment, the absorbance ratio of 260 nm to 280 nm was measured using the methodology outlined in the work of Sambrook and Russell (2001) (15).

All sequences of gRNAs are listed in **Table 1**.

3.5. CHO Cell Transfection and Single-Cell Cloning

Polyethyleneimine (PEI) was used as a transfection reagent according to the manufacturer's instructions.

Table 1. The details of oligonucleotide primers utilized for PCR

Name of primers	Sequences	Annealing Temperature (°C)
gRNA-a	5′CACCGGACTAGATGATTATCGTGCT3′	66
gRNA-a plus pam	5′CACCGGACTAGATGATTATCGTGCTtgg3′	70
gRNA-b	5′ AAACAGCACGATAATCATCTAGTCC3′	63
gRNA-b plus pam	5′AAACccaAGCACGATAATCATCTAGTCC3′	67
gRNA-c	5′CACCGGATTCCTCATGTTTTAAATG3′	63
gRNA-c plus pam	5′CACCGGATTCCTCATGTTTTAAATGagg3′	67
gRNA-d	5′AAACCATTTAAAACATGAGGAATCC3′	59
gRNA-d plus pam	5′AAACcctCATTTAAAACATGAGGAATCC3′	64
Genomic primers FW	5′ GGATTTTACGGCGTTTCATTTA3′	57
Genomic primers RW	5′TCCCTGTTGCTTTTATTCTTTCA3′	58
Cloning primer FW	5′ GCCTTTTGCTGGCCTTTTGCTC3′	64
Cloning primer RW	5′CGGGCCATTTACGTAAGTTATGTAACG 3′	68

CHO cells were co-transfected with the PX459 and PX460-1 plasmids. Upon successful transfection and completion of multiple rounds of cell passaging to confirm the consistent expression of EGFP in transfected CHO cells, the cells were subsequently seeded at a concentration of 5×10^4 cells in an 8cm cell culture plate. After one week of growth, individual cells underwent division and formed clones. The manual selection system was preferred for isolating single-cell cloning instead of utilizing Cell sorting by FACSC (Fluorescence-Activated Cell Sorting) due to its cost-effectiveness and time efficiency. Using a fluorescent microscope as a guide, we harvested various clones and transferred them to a 96-well plate. These clones were subsequently transferred to a 24-well plate for further analysis. After expanding single clones, Genomic DNA was isolated from each clone, and PCR was performed on all of them to identify the knockout cells that exhibited stable EGFP expression.

3.6. Genomic DNA Extraction

In this study, approximately 1×10^6 cells were collected through centrifugation. Subsequently, the DNG Extraction-PlusTM reagent, following the recommended procedures by the manufacturer (Sinaclon, Iran), was utilized to extract genomic DNA.

3.7. Validation of CASP8AP2 Silencing

To confirm the knockout of CASP8AP2, we performed PCR and sequencing. Genomic primers were utilized in PCR to validate the integration of the PX460-1 vector at the targeted site. It was predicted that the integration of the vector at the targeted site in the CHO genome would result in no PCR band due to the inability of the Taq DNA polymerase to amplify such a large sequence of more than 5000 base pairs. Therefore, using the genomic primer as the forward and the vector primer as the reverse primer confirmed the integration of the donor vector at the targeted site in CASP8AP2. Upon confirming integration, the clones that exhibited successful integration were selected for sequencing and other downstream experiments. PCR amplification was performed on the CASP8AP2 gene using a 20 µL reaction mixture. The mixture consisted of 1 µL of dNTP mixture (0.2 µM concentration), 2 µL of MgCl2 (2 mM), 2 µL of 1X PCR buffer, 0.25 µL of Taq DNA polymerase (Thermo Fisher Scientific, USA), 1 µL of Forward and 1 µL of Reverse primers (1 µM concentration), and 1 µL of DNA (50 ng concentration). The amplification process was carried out utilizing a Biometra thermal cycler (Applied Biosystems, USA) with the following parameters: initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 58 °C for 30 seconds, elongation at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes. Subsequently, all samples were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

3.8. Cell Viability (MTT) Assay

Parental CHO-K1 cells and CASP8AP2-KO single-cell clones were seeded in 96-well plates at a concentration of 5×10^3 per well to evaluate cell viability. The plates were cultured with normal growth media without drugs for 24 hours and kept in an incubator at 37 °C. After 24 hours, the cells adhered to the surface, and then they were treated with different concentrations of NaBu (2.5, 5, 10, 25, 50, and 100 mM). The plates were placed back in the incubator and kept at 37 °C for 24, 48, and 72 hours. After each day of incubation, the media from each well were completely withdrawn and replaced with 100 µL of culture medium without FBS containing 10 µL of a 12 mM Solarbio MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] stock solution. After 4 hours of MTT treatment, the media from the surface cells were collected completely. Then, the medium was replaced with 100 µL of DMSO and thoroughly mixed using an orbital plate shaker. The plates were incubated for another 20 minutes, and the absorbance optical density (OD) was examined at reference wavelengths of 545 and 630 nm using an ELISA reader to detect MTT metabolism and purple formazan production. The relative viability (IC50) of each clone was analysed based on the absorbance of sodium butyrate compared to the CHO-K1 control. Statistical analysis of the data was conducted using GraphPad Prism 8.0. This experiment was repeated three times with six replicates for each repetition to ensure the accuracy and reliability of the results (16).

3.9. Protein Expression Assay

The pLEX-JRed vector was obtained from the Royan Institute in Tehran. Approximately 6×10^4 cells were seeded in a 24-well plate for each knockout CHO cell type and native CHO cell type. The cells were then incubated at 37 °C for 24 hours. PEI was chosen as

the transfection reagent. Both CHO-K1 and CHO-KO cells were transfected with the pLEX-JRed vector in triplicate. Each cell was transfected with 100 ng of the pLEX-JRed vector. The expression of JRed was assessed using flowcytometry (Beckman Coulter FC500; Beckman Coulter) at 72 hours after transfection. FlowJo (version 7.6) software was used to examine the data.

3.10. Statistical Analysis

All assays were performed in triplicates and repeated. The mean difference between the groups was estimated using the T-test or analysis of variance (one-way ANOVA or two-way ANOVA). GraphPad Prism version 8.0 for Windows (GraphPad Software, USA) was used, and the data were represented as mean ± S.E.

4. Results

4.1. Plasmid Construction and Cloning of gRNAs

The cloning of normal gRNAs in PX459 resulted in a PCR product with a length of 300 bp. Additionally, the cloning of gRNAs containing the PAM sequence in PX460-1 produced PCR products with a length of 300 bp (**Fig. 1**). This experiment successfully validated the presence of the required gRNA constructs in the respective vectors.

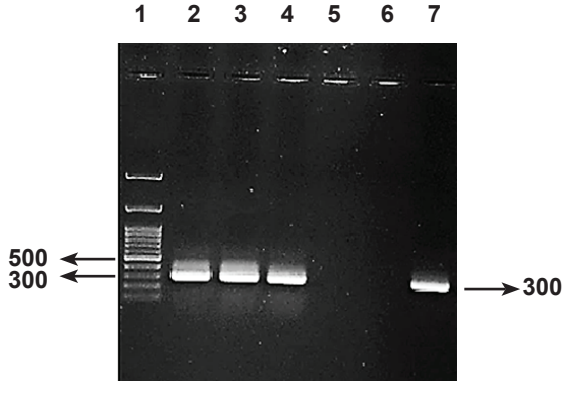

Figure 1. Validation of gRNA cloning in PX459 and PX460-1 plasmids by PCR. Lane 1; 100 bp DNA ladder, Lane 2; using the forward primer and gRNA-b as the reverse primer, Lane 3; using the forward primer and gRNA-d as the reverse primer, Lane 4; using the forward primer and gRNA-b plus PAM as the reverse primer, Lanes 5 and 6; Empty lanes, Lane 7; using the forward primer and gRNA-d plus PAM as the reverse primer.

4.2. Transfection of CHO-K1 Cells, Selection of Single Clones, and Knockout of CASP8AP2 Detection

CHO cell transfection was performed using Polyethyleneimine, using two PX459 vectors containing normal gRNAs targeting two sites in the 3'UTR of CASP8AP2, along with two PX640-1 vectors containing PAM plus gRNAs. By utilizing an inverted microscope and fluorescent light, the count of GFP-transfected cells was determined. The findings revealed that around 65% of the cells demonstrated stable GFP expression (**Fig. 2**).

4.3. Single-Cell Cloning and Confirmation of CASP8AP2 Silencing

After the first round of transfection, single cells were isolated by manual cell sorting, and a colony of cells stably expressing EGFP was expanded. **Figure 3** depicted a clone that demonstrated persistent expression of GFP. After DNA extraction, Forward and Reverse Genomic DNA primers were utilized to confirm the silencing of CASP8AP2 clones. The knockout of the CASP8AP2 gene in CHO cells was subsequently verified through PCR and sequencing analysis. HITI-mediated CASP8AP2 gene silencing resulted in the absence of a PCR band due to the integration of PX460-1 at the targeted site of the gene when using genomic primers. However, when a genomic forward primer and a primer flanked on the vector were used as the reverse primer, a 270 bp band was successfully amplified.

To enhance the confirmation process, an additional primer was utilized as a control, which produced a 400 bp band in PCR analysis. It was observed that some of these clones were heterozygotes, indicating the presence of one knockout allele and another allele that still represented a band at the targeted gene locus (results not represented).

Furthermore, it should be noted that some clones were not modified; that is, no gene knockout occurred. Additionally, it was observed that most of the clones were homozygotes. Homozygous clones did not yield a PCR product due to the integrated DNA fragment being over 5000 bp in length, which is beyond the amplification capacity of regular Taq polymerase. These findings validated the effective silencing of the CASP8AP2 gene in 60% of the clones, further confirmed through sequencing (**Fig. 4**).

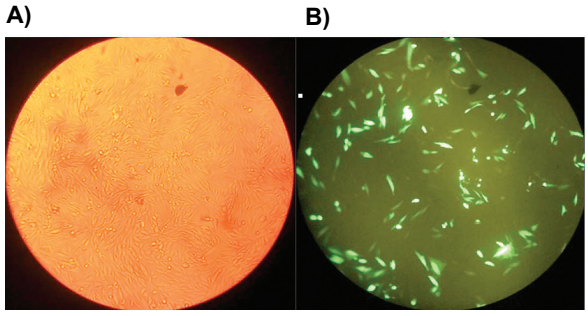

Figure 2. The transfection of CHO-K1 cells. **A**) Transfected CHO cells observed under visible light using an inverted microscope. **B)** Transfected CHO cells observed under fluorescent light.

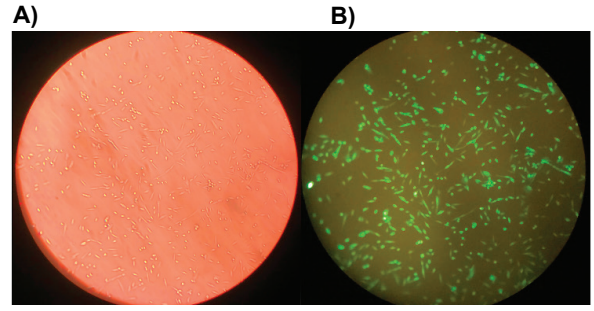

Figure 3. The clonal selection of EGFP expressing CHO-K1 cells. A) The picture of CASP8AP2 knockout CHO cells expressing permanent EGFP captured under visible light using an inverted microscope. **B)** The picture of a single-cell clone of CASP8AP2 knockout CHO cells captured under fluorescent light using an inverted microscope.

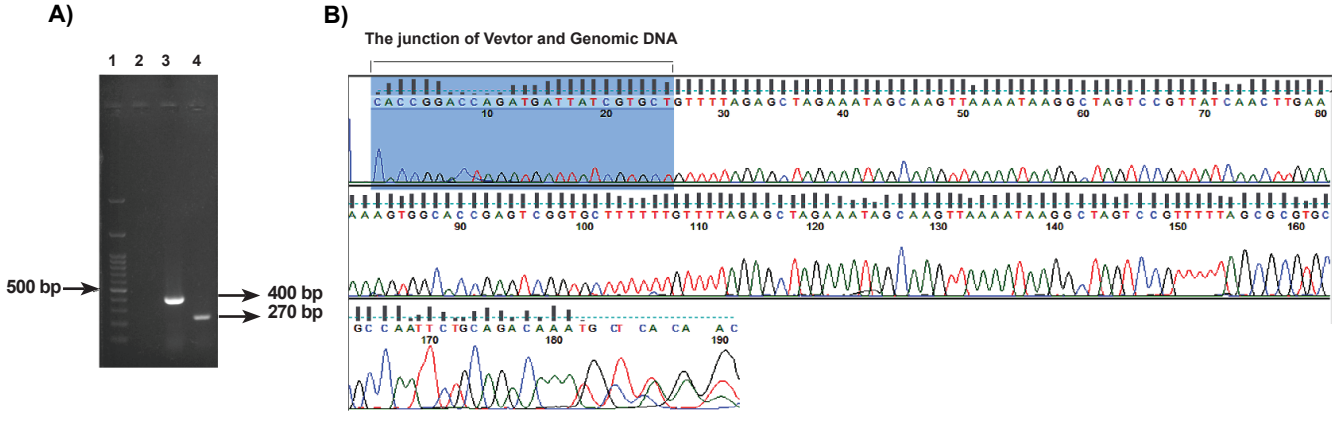

Figure 4. Knockout of the CASP8AP2 gene. A) Agarose gel electrophoresis of polymerase chain reaction (PCR) amplified CASP8AP2. Lane 1; 100 bp DNA ladder, Lane 2; This electrophoresis band shows PCR products obtained using forward and reverse genomic primers, Integration of a vector with more than 5000 bp, containing an EGFP expression cassette in targeted sites, resulted in no PCR band due to the low amplification efficiency of Taq DNA polymerase for 5000bp PCR products, Lane 3; This electrophoresis band represents a product of PCR (400bp) using forward and reverse primers of the Caspase 7 gene as a positive control, Lane 4; This electrophoresis band represents the product of PCR using a genomic primer as the forward primer and a cloning primer as the reverse primer (270bp), confirming the integration of the PX460-1 vector at the targeted site of the CASP8AP2 gene. **B**) The picture depicts the sequencing of a 270 bp fragment that includes a portion of the PX460-1 vector, confirming the integration of EGFP in the target site of CASP8AP2.

4.4. Effect of CASP8AP2 Knockout on Cell Viability

In this study, NaBu was employed to investigate the growth inhibitory effects of CASP8AP2 gene silencing clones. The viability of these cells was assessed using the MTT method for 24, 48, and 72 hours at various NaBu concentrations (2.5, 5, 10, 25, 50, and 100 mM). The results indicated that CASP8AP2 gene silencing increased the vitality of CHO-KO cells compared to CHO-K1 cells over the course of three days, as determined through statistical analysis using

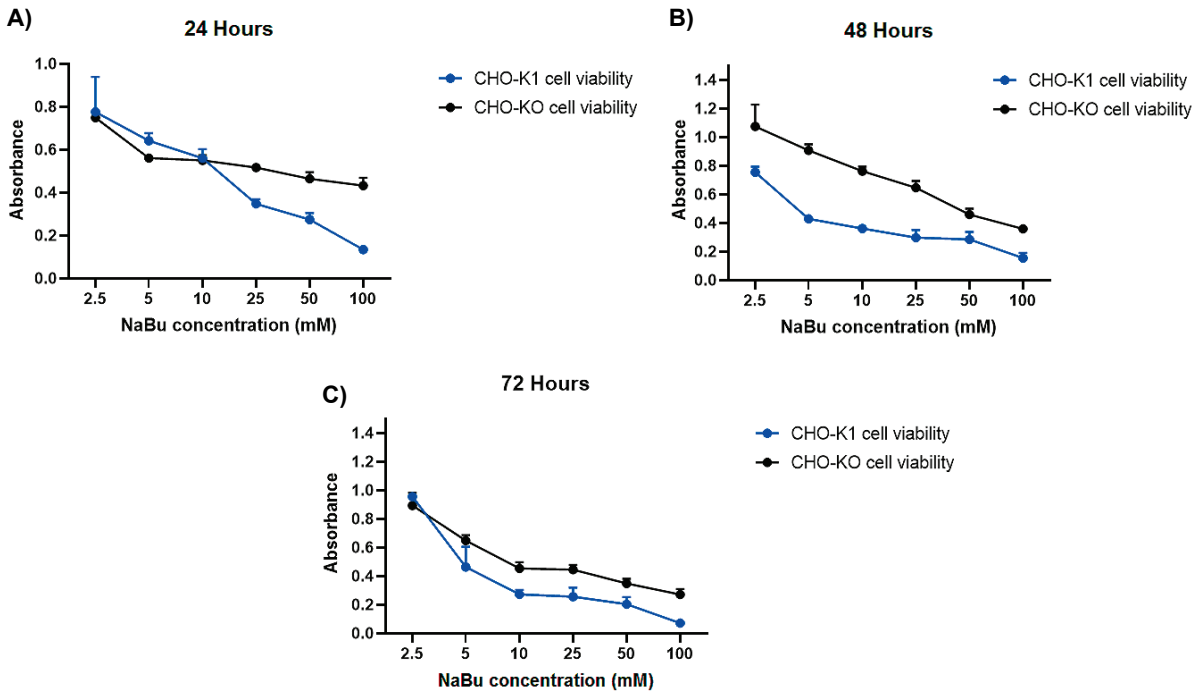

Figure 5. Cell viability assay. The CHO-K1 cell line and clone of CHO-CASP8AP2 manipulated cells were assessed for viability using the MTT method (sodium butyrate was utilized at concentrations of 2.5, 5, 10, 25, 50, and 100 mM). **A**) Viability assay with MTT after exposure to NaBu after 24 hours. **B**) MTT test after exposure to NaBu after 48 hours. **C**) MTT test after exposure to NaBu after 72 hours. The results demonstrated that CHO-KO cells exhibited higher viability compared to CHO-K1 cells due to CASP8AP2 knockout when they were exposed to NaBu.

the ANOVA test (**Fig. 5**).

The CASP8AP2 gene knockout (CHO-KO) cells exhibited a higher IC50 compared to the CHO-K1 control group for 48 and 72 hours. Specifically, at 48 hours, the IC50 for CHO-K1 was measured as 7.28 mM; also, for CASP8AP2 gene knockout, it was increased to 14.25 mM. at 72 hours, the IC50 for CHO-K1 was measured as 5.56 mM, and for CHO-KO was increased to 7.80 mM. This finding was substantiated by statistically significant P-values at different time points, (P-value: 24 hours ≤ 0.0001, P-value: 48 hours ≤ 0.0001, P-value: 72 hours = 0.0007).

4.5. CASP8AP2 Silencing Enhances the Expression of JRed in Knockout CHO Cells

The JRed plasmid was delivered to CHO-K1 and CHO-KO cells using PEI. Subsequently, after a 72-hour incubation period, the cells were observed under a fluorescent microscope with red light to assess the level of successful transfection. The effect of CASP8AP2 silencing on the constitutive expression of JRed was investigated using flow cytometry, showing that the expression of JRed increased by up to 1.3 folds in knockout CHO cells in comparison with CHO-K1 cells (**Fig. 6**).

5. Discussion

The CHO cell line is preferred for biopharmaceutical production due to its superior protein modification capabilities (17). Genome editing using CRISPR in CHO cells is effective in enhancing protein production and glycosylation efficiency (2, 18). HITI, Easi-CRISPR, and Tild-CRISPR are available for integrating transgenes in CHO cells, each with distinct advantages and limitations (10). Easi-CRISPR is a precise and efficient gene-editing technique that uses the CRISPR-Cas9 system (19). It uses a single-stranded DNA fragment as a donor template with homology to the target site and a matching PAM sequence. Cas9, guided by a specific gRNA, cuts the target site, and

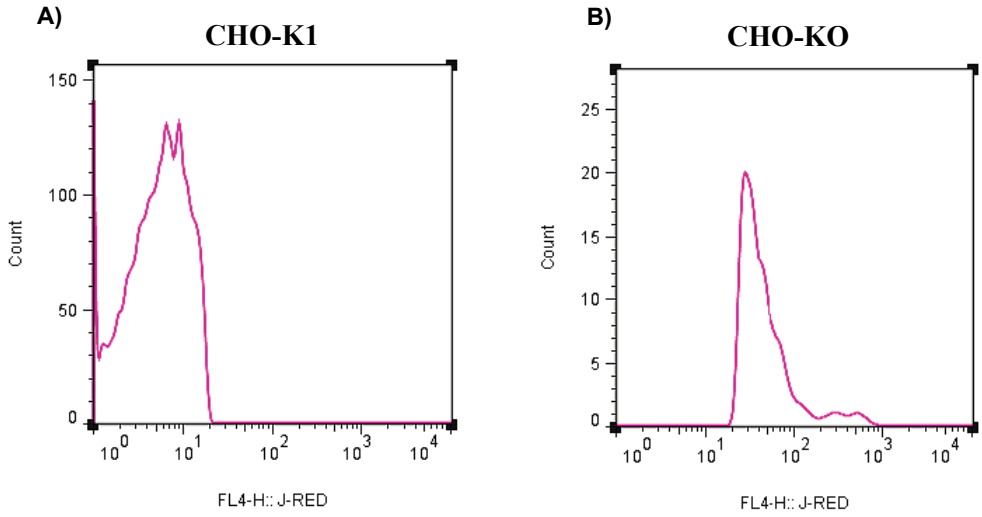

Figure 6. JRed expression assay. **A**) The histogram of JRed expression in native CHO cells after 72 hours of transfection. **B**) The histogram of JRed expression in CASP8AP2 knockout CHO cells after 72 hours of transfection.

the ssDNA donor template facilitates DNA repair. Easi-CRISPR enables the accurate insertion of large DNA fragments at specific genomic locations, making it valuable for incorporating coding sequences or regulatory elements (9). Tild-CRISPR is a promising technique for safer and more effective CRISPR-Cas9 system gene editing (20). It employs the homology-mediated end-joining (HMEJ) strategy and efficiently integrates double-strand linearized DNA. Tild-CRISPR demonstrates high knock-in efficiencies in mouse and human embryos using template donor DNA with 800 bp homology arms, which are prepared through PCR amplification or enzymatic cutting of the transgene donor (11).

Easi-CRISPR and Tild-CRISPR have the limitations of lower efficiency and complex donor libraries for Easi-CRISPR, random integrations, and challenging PCR amplification for Tild-CRISPR (21). To overcome these challenges, we introduced a simple and cost-effective protocol based on the HITI-joined CRISPR strategy for genome editing of the CHO cell line. This cell line is a significant cell factory for biopharmaceuticals, particularly in developing countries (14).

HITI is a strategy for targeted gene insertion using non-homologous end joining (NHEJ) (7). It allows for the insertion of larger gene sequences by introducing a donor DNA template flanked by a CRISPR-Cas9 target site. Cas9 cleaves both the target site and the donor vector, integrating the DNA template through NHEJ. HITI overcomes limitations of traditional CRISPR-Cas9 methods, such as the need for homology-directed repair templates and off-target effects, while accommodating larger gene sequences (22).

The HITI protocol enables site-specific gene knockout and EGFP knock-in without requiring homology arms. It involves two sets of gRNAs, transfection, manual single-cell cloning, and PCR validation to confirm efficiency. Manual clonal selection of EGFP-expressing cells simplifies the process, eliminating the need for cell sorting or antibiotic selection (7). PCR analysis showed 60% positive single-cell clones and sequencing confirmed homozygous CASP8AP2 gene silencing.

HITI-based genome editing is proving successful both *in vitro* and *in vivo*. It allows site-specific transgene integration using rAAV6 and sgRNA/Cas9 RNP complexes in human $CD34^+$ cells (23). HITI has also been employed for *in vivo* imaging and knock-in of large DNA donor constructs (13). Based on HITI, the ORANGE platform facilitates precise tagging of neuronal proteins at nanoscale resolution (24). HITI provides a fast and cost-effective approach to genome editing across diverse applications.

One drawback of combining the CRISPR-Cas9 system with the HITI strategy is the potential for off-target

effects, which are unintended genetic alterations in non-target regions. These effects can arise due to the non-specific nature of the system and HITI, leading to genomic instability or undesired mutations. To alleviate this concern, careful design and validation of guide RNAs (gRNAs) is crucial to ensure specificity and minimize off-target effects. A study illuminated that truncating the 5' end of the sgRNA reduces its affinity to potential off-target sites, mitigating the risk of off-target effects (25).

We utilized the Cas-OFFinder software (http://www.rgenome.net/cas-offinder) to validate the cleavage efficiency of the gRNAs in the donor vector. This software, Cas-OFFinder, identifies potential off-target sites in the genome during CRISPR-Cas9 gene editing. It predicts binding sites across the entire genome by comparing the gRNA sequence with the target genome, helping detect regions where genetic alterations may occur (26). After evaluating our two gRNAs utilized in this study through the Cas-OFFinder website, it was determined that both gRNA sequences employed in this study predicted the lowest number of off-target sites, and there were zero mismatches.

Furthermore, Rahimi *et al*. (27), discovered that disrupting the repression of the BAX gene had a similar effect on enhancing cell survival in CHO cells. By utilizing the CRISPR-Cas9 system to disrupt the BAX gene, the researchers observed increased cell survival in the engineered cell lines compared to the normal CHO-K1 cell line. This finding was validated using the MTT method. Our study also aligns with Rahimi *et al*.'s findings, as both studies utilized the CRISPR-Cas9 system to knockout or disrupt genes involved in apoptosis and pro-apoptotic pathways in the CHO-K1 cell line, resulting in increased cell viability.

Abaandou *et al*. (28), discovered that suppressing the CASP8AP2 gene in HEK293 cells resulted in G0/G1 cell cycle arrest. Interestingly, this suppression did not significantly affect cell viability but led to increased expression of recombinant proteins. Our study was in line with Abaandou *et al*.'s work in that both studies indicated protein expression. We clarified that with knockout of CASP8AP2 on the CHO cell line led to an increase in protein expression. Moreover, Abaandou *et al*.'s study confirmed the role of CASP8AP2 in regulating protein expression in HEK293 cells. Targeting CASP8AP2 enhanced protein production, highlighting its potential as a target for optimizing recombinant protein expression in various cell types. These findings underscore the significance of CASP8AP2 in biotechnological applications.

Our findings confirmed the effectiveness of the combined use of CRISPR-Cas9 and the HITI strategy, resulting in the development of a straightforward technique for genetically engineering CHO cells to enhance productivity. The manual selection approach allows us to select the desired clones more quickly and accurately than FACS methods. Additionally, through the knockout of CASP8AP2 genes, we successfully achieved clones with enhanced viability, improved stability, and elevated protein expression levels. CASP8AP2 is important due to its involvement in crucial cellular processes, association with diseases, and potential as a therapeutic target.

6. Conclusion

This study represents the first investigation of Simultaneous Caspase 8 Associated Protein 2 gene knock-in/out in Chinese Hamster Ovary Cells Using the CRISPR-Cas9 system. Eliminating the CASP8AP2 gene in the CHO cell line using the CRISPR-Cas9 system increased cell survival and elevated protein expression. Further research on CASP8AP2 can contribute to a better understanding of its biological functions and implications for human health and disease.

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