Enhancing CHO cell productivity through a dual selection system using Aspg and Gs in glutamine free medium

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Abstract

The dominant method for generating Chinese hamster ovary (CHO) cell lines that produce high titers of biotherapeutic proteins utilizes selectable markers such as dihydrofolate reductase (Dhfr) or glutamine synthetase (Gs), alongside inhibitory compounds like methotrexate (MTX) or methionine sulfoximine (MSX), respectively. Recent work has shown the importance of asparaginase (Aspg) for growth in media lacking glutamine–the selection medium for Gs-based selection systems. We generated a Gs/Aspg double knockout CHO cell line and evaluated its utility as a novel dual selectable system via co-transfection of Gs-Enbrel and Aspg-Enbrel plasmids. Using the same selection conditions as the standard Gs system, the resulting cells from the Gs/Aspg dual selection showed substantially improved specific productivity and titer compared to the standard Gs selection method, however, with reduced growth rate and viability. Following adaptation in selection medium, the cells improved viability and growth while still achieving ~5-fold higher specific productivity and ~3-fold higher titer than Gs selection alone. We anticipate that with further optimization of culture medium and selection conditions this approach would serve as an effective addition to workflows for the industrial production of recombinant biotherapeutics.

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Abstract

The dominant method for generating Chinese hamster ovary (CHO) cell lines that produce high titers of biotherapeutic proteins utilizes selectable markers such as dihydrofolate reductase (Dhfr) or glutamine synthetase (Gs), alongside inhibitory compounds like methotrexate (MTX) or methionine sulfoximine (MSX), respectively. Recent work has shown the importance of asparaginase (Aspg) for growth in media lacking glutamine–the selection medium for Gs-based selection systems. We generated a Gs/Aspg double knockout CHO cell line and evaluated its utility as a novel dual selectable system via co-transfection of Gs-Enbrel and Aspg-Enbrel plasmids. Using the same selection conditions as the standard Gs system, the resulting cells from the Gs/Aspg dual selection showed substantially improved specific productivity and titer compared to the standard Gs selection method, however, with reduced growth rate and viability. Following adaptation in selection medium, the cells improved viability and growth while still achieving ~5-fold higher specific productivity and ~3-fold higher titer than Gs selection alone. We anticipate that with further optimization of culture medium and selection conditions this approach would serve as an effective addition to workflows for the industrial production of recombinant biotherapeutics.

Keywords

Selection system, asparaginase, glutamine synthetase, Chinese hamster ovary cells, biotherapeutic production, mammalian cell line

The integration of exogenous DNA into cultured cells is often facilitated by co-introduction of the desired DNA alongside a selectable element and growth under the corresponding selective pressure (e.g., transfection of the desired gene + a gene imparting antibiotic resistance combined with growth in medium containing the antibiotic). In the biopharmaceutical industry, metabolic selection systems that restore nutritional prototrophies are routinely used to generate mammalian cell lines producing high quantities of life-saving biotherapeutic protein drugs. Dihydrofolate reductase (Dhfr) or glutamine synthetase (Gs) are the most commonly leveraged metabolic selectable markers (Cockett et al., 1990; Kaufman & Sharp, 1982). However, the cell line generation process using these is time-consuming and laborious, often requiring one (Gs) or several (Dhfr) rounds of gene amplification driven by the addition of inhibitory compounds (e.g., methotrexate and methionine sulfoximine) during selection as well as the screening of 100s to 1000s of clones to identify clones with the desired production and quality profiles. It has been shown that utilizing these two systems simultaneously increases the probability that a highly productive cell will be generated–as well as improving the maximum product titer (Li et al., 2010). Recent work has demonstrated the feasibility of additional metabolic selection systems in CHO (Budge et al., 2021; Capella Roca et al., 2019; Pourcel et al., 2020; Sun et al., 2020; Zhang et al., 2020) with one study (Zhang et al., 2022) showing that using 8 selectable markers simultaneously can significantly increase the productivity of the resulting cell lines (although the cells grew very slowly). The orthogonality of these new selection systems (e.g., each requiring the dropout of a different medium component for selective pressure) and/or need for multiple genetic edits led us to explore whether it was possible to increase the selective stringency of glutamine deprivation in a simpler manner, hereby enhancing the selective pressure of one of the most established tools in the clinical cell line generation workflow, without requiring changes from established selection conditions.

Using a CRISPR-Cas9 knockout screen targeting metabolic genes, we identified asparaginase (Aspg) as putatively essential for CHO cell growth in medium lacking glutamine (Karottki et al., 2021). As Gs was

also identified as essential in that condition, we hypothesized that a dual selection system based on Aspg together with Gs could enhance selection without requiring alteration of the selective pressure.

To confirm the importance of Aspg for growth in glutamine-free media and assess its viability for use as an additional selectable marker simultaneously with Gs, we generated three clonal knockout (KO) cell lines using CRISPR/Cas9: Gs-KO, Aspg-KO, and Gs/Aspg-KO. Knockouts had verified frameshift insertions or deletions in all alleles and also showed decreased mRNA expression (Supplementary Figure 1). Both clones lacking Aspg showed decreased maximum viable cell density when grown in a glutamine-containing medium (Supplementary Figure 2A) but with comparable growth rates to the Gs KO cell line (Supplementary Table 1). When grown without glutamine, Aspg knockout cells showed negligible growth, but remained viable. Gs/Aspg-KO cells, on the other hand, showed a dramatic decrease in cell viability–even more quickly than Gs-KO cells (Supplementary Figure 2B). This suggested that a double selection system using Gs and Aspg simultaneously would be more stringent than Gs alone–while still using only glutamine deprivation as the sole selective pressure.

We generated Enbrel-producing cells from the different knockout cell lines via 5 different transfections: 1) GS-KO cells with a Gs+Enbrel plasmid, 2) GS-KO cells with both Gs+Enbrel and Aspg+Enbrel plasmids, 3) Aspg-KO cells with Aspg+Enbrel plasmid, 4) Aspg-KO cells with both Gs+Enbrel and Aspg+Enbrel plasmids, and 5) double Gs/Aspg-KO cells with both Gs+Enbrel and Aspg+Enbrel plasmids. We tested selection in both static minipools (192/transfection) and bulk suspension format (duplicates in 6 well suspension, permitting quantification of recovery profiles). Following recovery, surviving minipools were split 1:2 and evaluated for terminal cell count and titer. After 5 days of culture, minipools derived from clones with Aspg knockouts showed lower cell density and product titer, however, minipools derived from the Gs/Aspg double knockout transfected with both plasmids showed ˜3-4-fold higher median cell-normalized product titer than Gs knockout derived minipools (Figure 1, Supplementary Table 2). We observed no change in recovery timelines in the bulk suspension format and, after characterization of recovered pools in batch culture, the Gs/Aspg double knockout cells transfected with both plasmids again showed decreased growth, but significantly (˜16-fold) higher titer and specific productivity (Supplementary Figure 3B and Supplementary Table 3).

We then tested if minipools could obtain improved performance after being transitioned to suspension culture. Top minipools from all transfections were expanded and characterized in 6 well suspension culture. The trend of higher titer and specific productivity in Gs/Aspg double knockout derived minipools was maintained, but minipools derived from cells with Aspg knocked out showed low VCD (Supplementary Figure 4). We thus continued expanding the top 3 Gs/Aspg knockout derived and Gs knockout derived minipools (based on titer) to test if prolonged time in suspension culture would improve the performance of the former.

After expansion in shake flask culture, the growth and viability of Gs/Aspg double knockout derived minipools were still decreased compared to minipools derived from Gs knockout cells (Figure 2A), but both were improved compared to their performance in 6-well plates (Supplementary Figure 4). We again saw significantly higher production of Enbrel, both in titer (2-4-fold higher in the best performing Gs/Aspg-KO derived minipool) and specific productivity (10-15-fold improvement in Gs/Aspg-KO derived minipools) (Figure 2B and Supplementary Table 4).

The improvement in growth and viability from 6-well to shake flask led us to explore whether further adaptation of minipools derived from Gs/Aspg knockout cells would improve performance in selection conditions designed for Gs knockout derived cells. Following ˜1 month of adaptation (Supplementary Figure 5), we evaluated the top minipool from each transfection: The Gs/Aspg KO derived minipool showed significant improvements in growth and viability while still outperforming the Gs knockout derivatives in titer and specific productivity (Figure 3 and Supplementary Table 5). We further assessed the long-term stability of the dual selection strategy and found that after an additional month of passaging, minipool performance remained stable (Figure 4 and Supplementary Table 6).

Finally, we explored the cause of poor growth in the Gs/Aspg-KO derived pools. Cell growth in this selection

system depends on the rescue of both knocked out enzymes (Gs and Aspg) through the uptake and integration of both transfected plasmids. It is possible that the observed low cell growth results from low expression of either or both plasmids after selection. However, both Gs and Aspg expression levels in the Gs/Aspg-KO derived pools were at least as high as that of Gs-KO derived pools following selection and recovery, prior to adaptation (Supplementary Figure 6); thus, the expression should be sufficient for robust growth. As adaptation partially recovered growth (and considerably improved viability) we anticipate that additional media and/or platform optimization (e.g., altering the plasmid ratio) could further improve the performance of this system.

The dual Gs/Aspg selection system thus is an intriguing option to generate more highly productive cell lines. As such it only requires a single additional genetic edit to the starting cell line and does not require changes to the traditional Gs-based selection workflow. Furthermore, it has the potential to be used as an alternative system for not only the production of proteins but also the expression of several genes of interest without the use of antibiotics (as seen with Dhfr/Mtx co-selection (Lee et al., 2018)). Continued work with cells generated by this approach, e.g., single-cell cloning, expansion, and characterization in fed-batch bioreactors, will further demonstrate the value of this system for cell line generation for biotherapeutic protein production.

Materials and Methods

Plasmid Construction. The Gs-Enbrel plasmid and Aspg-Enbrel plasmid were constructed by uracil-specific excision reagent (USER) cloning using flexible assembly sequence tags as previously described (Lund et al., 2014; Pristovšek et al., 2018). Each plasmid was generated using 4 input PCR products: 3 common inputs–a backbone, the Enbrel gene, and an early SV40 promoter–in addition to the selectable marker (Gs or Aspg). All inputs were generated via PCR amplification of DNA fragments (Supplementary Table 7) with Phusion U Hot Start DNA polymerase (cat. no. F533S, Thermo Fisher Scientific) and uracil-containing primers (Integrated DNA Technologies, inc.) shown in Supplementary Table 8. The PCR settings used for the amplification are described in Supplementary Table 9. The plasmids were constructed by assembling the DNA bricks with USER Enzyme (cat. no. M5505S, New England Biolabs, Ipswich, MA) and CutSmart R Buffer (cat. no. M5505L, New England Biolabs) according to the manufacturer's protocol. Constructed plasmids were transformed into E. coli Mach1 competent cells (Thermo Fisher Scientific). All the constructs were verified through Sanger sequencing by Eurofins Genomics (Eurofins Scientific, Luxembourg). Two primers were used for each construct to verify the Enbrel sequence (5'-CGAAATCGGCAAAATCCC-3') and the selection gene (Aspg or Gs) (5'- TTTTATTTATGCAGAGGCCGAG-3'). Confirmed constructs were purified using NucleoBond Xtra Midi EF kit (Macherey-Nagel) according to the manufacturer's instructions.

Cell line generation and culture maintenance. CHO-S (Life Technologies, Carlsbad, CA) cell lines were established using CRISPR/Cas9 as previously described (Grav et al., 2015) to knockout Aspg and/or Gs. The Gs/Aspg-KO was established by knocking out Aspg in the Gs-KO cell line. All primers for gRNA plasmid construction and sequence verification are listed in Supplementary Table 10. All cell lines were cultured in CD-CHO Medium (cat. No 10743029, Thermo Fisher Scientific, Waltham, MA) with 0.2 % Anti-Clumping Agent (cat. no. 0010057AE, Gibco, Waltham, MA) and 8 mM glutamine unless otherwise specified. Cells were passaged every 2-3 days in 30 ml of medium in 125-ml shake flasks (Corning, Corning, NY). Viable cell densities and viabilities were measured using NucleoCounter® NC-200 or NucleoCounter(r) NC-250 (ChemoMetec, Allerod, Denmark). All cultures were incubated at 37oC, 80% humidity, and 5% CO_2; suspension cultures with working volumes >500 µl were shaken at 120 rpm.

Batch culture experiments and cell line characterization. Cells were seeded at an initial cell density of 3 x 10^5cells/mL in 125 mL shake flasks (Corning) containing 30 mL medium with or without 8 mM glutamine.

Quantitative real-time polymerase chain reaction (qRT-PCR). The expression level of Gs and Aspg genes was evaluated by qRT-PCR as described previously (Kallehauge et al., 2017). Oligos for qRT-PCR are listed in supplementary table 11. Gapdh was used as a housekeeping gene in all the calculations.

96 well-based minipool generation. Cells were passed into medium without Anti-Clumping Agent two days before transfection. Cells were seeded at an initial cell density of 1 x 10^6cells/mL in 6-well plates. Afterward, transfection was performed with FreeStyleTM MAX Reagent (cat. no. 16447100, Gibco) according to the manufacturer's protocol. After 24h, VCD and viability of transfected cells were analyzed using NucleoCounter® NC-200. Two 96-well plates for each transfection condition were seeded with an initial cell density of 5000 cells/well. Transfected cells were seeded in cloning media (20% of CD CHO Medium + 80% EX CELL(r) CHO Cloning (Sigma-Aldrich, St. Louis, MO) with 0.2% Anti-Clumping Agent) after a previous wash step to remove all the media with glutamine.

Bulk pool generation. 24hr after transfection, cells were seeded in 6 well plates into selection medium (without glutamine) at a cell density of 0.3 x 10^6 cells/mL. Every 2-3 days cells were passed or spun and resuspended into fresh selection medium until viability exceeded 90 %.

Adaptation. Cells were inoculated at a concentration of 0.5 x 10^6 cells/mL in 125 mL flasks with 30 mL of culture medium without glutamine. Every 2 – 3 days, cells were passed into fresh culture medium without glutamine until the cell viability reached over 90%.

Stability testing. Cells were passed every 2 – 3 days at a concentration of 0.3 x 10^6 cells/mL in 125 mL Erlenmeyer flasks with 30 mL of culture medium without glutamine for a month. Batch culture was performed simultaneously for cells with and without one-month passaging time.

Titer measurement. The mAb concentration was measured using an Octet RED96 (Pall, Menlo Park, CA, USA), as described previously (Kallehauge et al., 2017).

Statistical analysis. Values are expressed as mean +- standard deviation unless otherwise noted. The data were analyzed with a two-tailed Student's t -test and differences were considered statistically significant at $p < 0.05$.

Acknowledgments

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Figures and tables

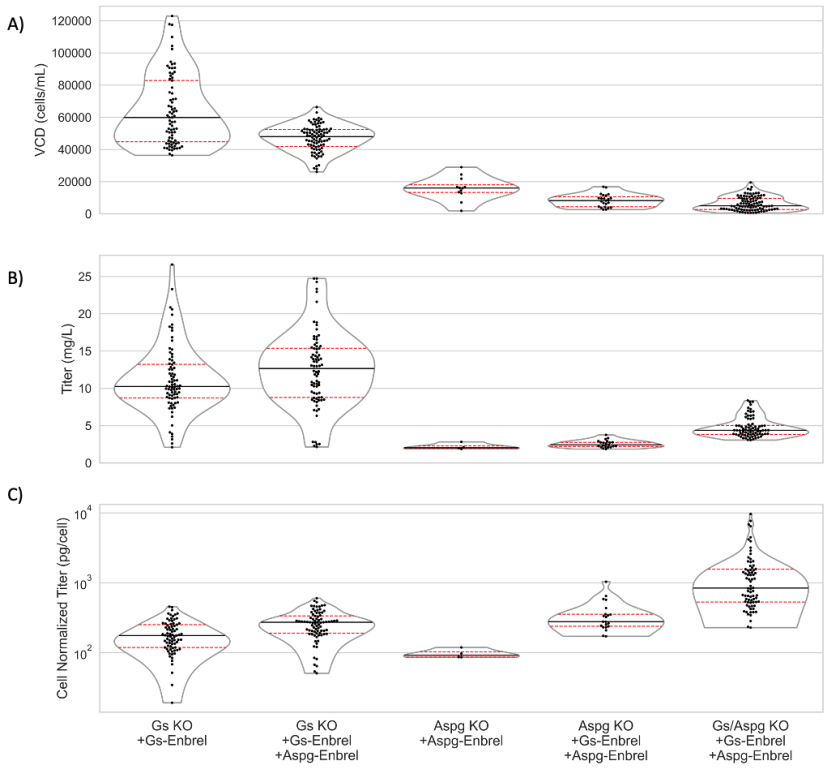

Figure 1 - Minipool culture in 96 well plates after 5 days. Viable cell density (A), titer (B) and cell normalized titer (C, titer divided by cell density). Red dashed lines indicate 1st and 3rd quartiles and black solid lines indicate medians. While the viable cell density of the Gs/Aspg double KO was much initially lower, the amount of protein produced per cell in minipools was substantially higher for the mutant.

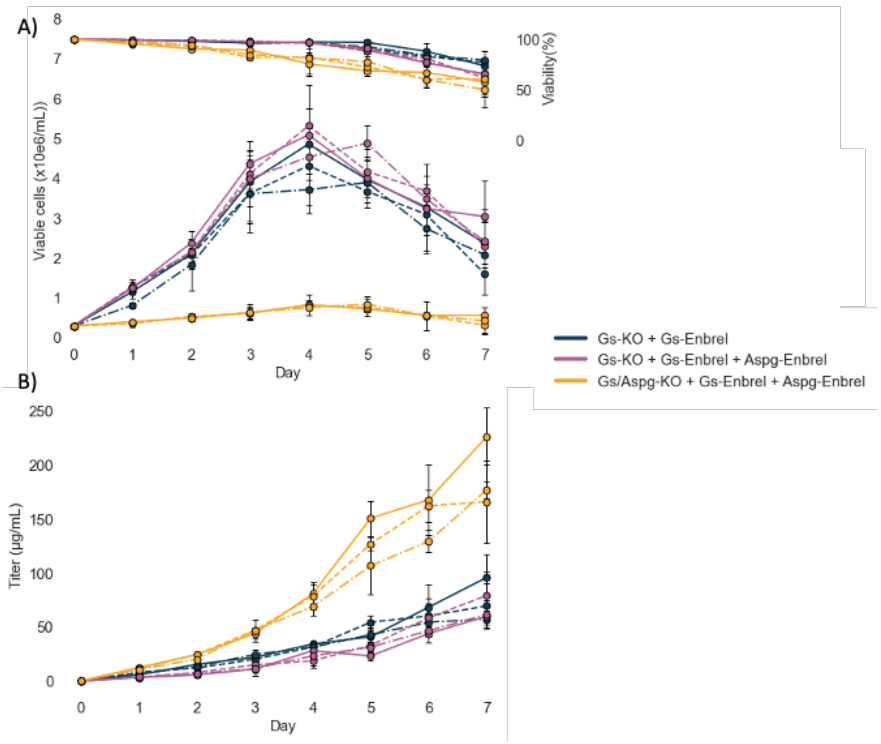

Figure 2 - Shake flask batch culture of top-producing minipools. Viable cell density and viability (A) and titer (B). All cell line conditions are grown in three replicates. Error bars represent standard deviation. Despite its lower viable cell density in shake flasks, the minipools from Gs/Aspg selection showed many-fold increased titers.

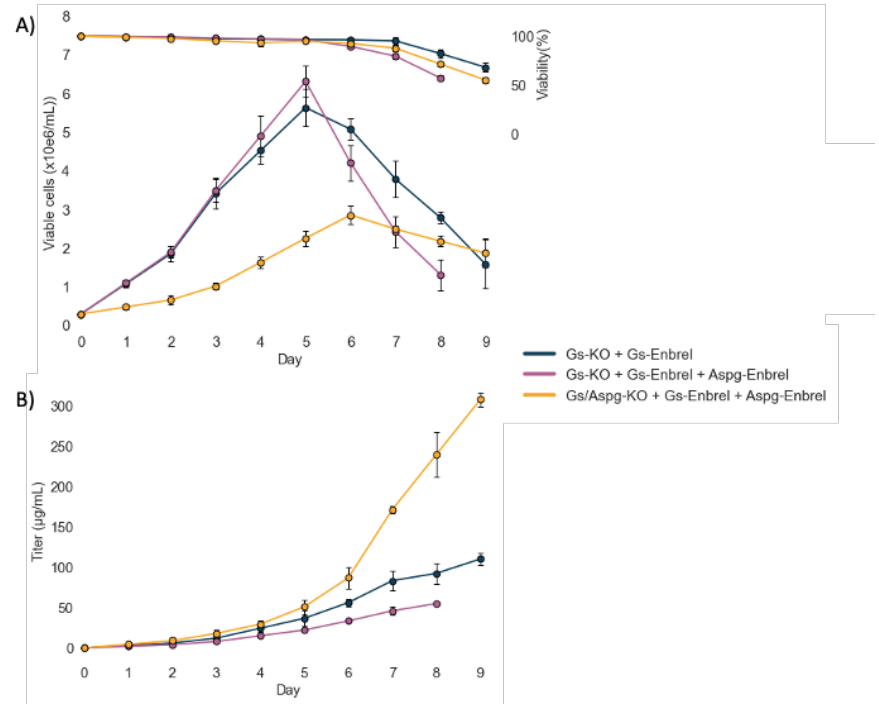

Figure 3 - Shake flask batch culture of the top performing minipool after adaptation showed improved viable cell density and titer. Viable cell density and viability (A) and titer (B). All cell lines were grown in three replicates. Error bars represent standard deviation.

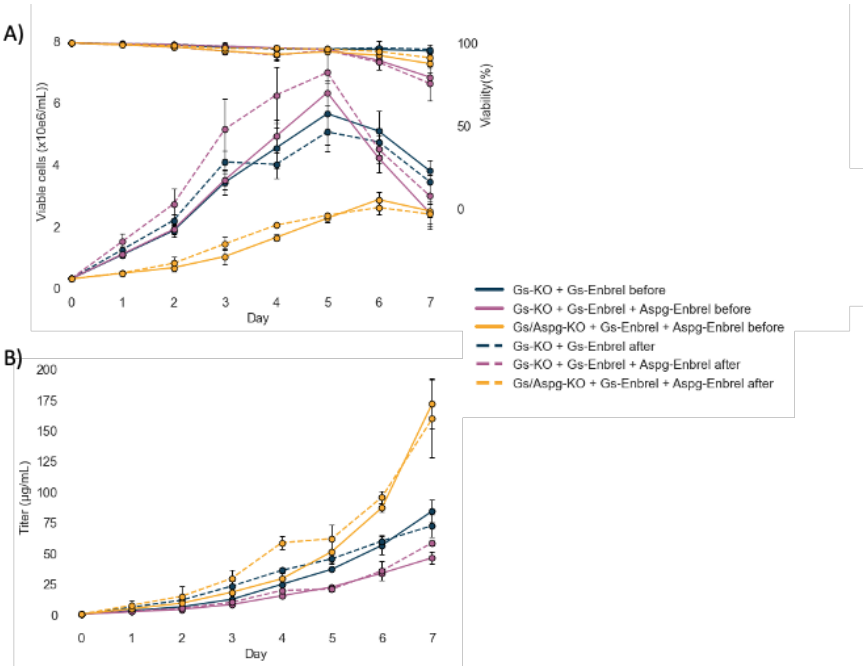

Figure 4 - Stability of adapted cell lines. A batch culture was performed before and after 4 weeks of culture to test the long-term stability of viable cell density and viability (A) and titer (B). All cell line conditions are grown in three replicates. Error bars represent standard deviation.

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