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The use of glutamine synthetase as a selection marker: recent advances in Chinese hamster ovary cell line generation processes

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Central to the needs of the biopharmaceutical industry to support the development of innovative biologics and biosimilars are more effective and efficient manufacturing processes which require highly productive cell lines with desired quality attributes. New designs of molecules such as antibody humanization have greatly reduced immunogenicity concerns, and advances in cell culture technology including media optimization and process control have driven monoclonal antibody productivities in excess of 10 g/l with peak cell densities in bioreactors climbing to over 35 million per ml. However, over this same timeframe, the fundamental processes utilized for cell line generation have not changed significantly, especially in the selection step for top-producing clonal cell lines. Cell line generation continues to be time consuming and labor intensive and has become the timeline limiting step for the majority of the industry. In order to meet the 'Fast-to-Proof-of-Principle' strategy, multiple efforts including host cell and expression plasmid engineering have been pursued in order to improve the effectiveness and efficiency of cell line generation processes. This review will summarize the recent advancements in cell line generation processes in Chinese hamster ovary cells, with a focus on the glutamine synthetase selection system.

Since the first approval of recombinant insulin in the early 1980s, more than 100 new recombinant protein therapeutics have been approved by the US FDA or the European Medicines Agency [1]. The biopharmaceutical industry has been rapidly growing at a pace of 10–20% annual increase in revenue worldwide [2] and with biologics' sales in 2010 exceeding US$100 billion [201,202]. At the same time, with biologic drugs worth more than $80 billion in global sales losing patent protection through 2015, biosimilars have also become an emerging area of interest garnering substantial investment by many biotech and large pharmaceutical companies [3]. Central to the needs of the industry to support the development of innovative biologics and biosimilars are more effective and efficient manufacturing processes which require highly productive cell lines with desired quality attributes.

Choosing an appropriate expression system is one of the critical steps for biopharmaceutical product development. Multiple expression systems including micro-organisms [4,5], mammalian cell lines [6,7], plants [8–10] and animals [11] are currently available for biopharmaceutical production. Though the gap has been narrowed, microbial systems, as exemplified by *Escherichia coli*, have the advantages of low cost and short timeline in establishing a production strain, quick production cycle, easy in-process control, and comparable productivity compared with mammalian expression systems. However, there are various limitations for prokaryotic systems such as *E. coli*. It is quite challenging for microbial systems to express large complex proteins containing multiple subunits, requiring cofactors, disulfide bonds, and post-translational modifications [12], since the post-translational metabolic machinery

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Key Terms

Suitability: A phenotypic term that describes the change in productivity as a function of increasing generation number accumulating through culturing (passaging) of a cell line. It is not necessarily the result of any product-related genetic instability. Cell lines with <20% productivity drop over the manufacturing window are acceptable.

Stability: Reserved for genetic changes to the coding sequence that could have potential for impacting the quality or integrity of the recombinant product.

is only available in eukaryotic cells. Many recombinant proteins including monoclonal antibodies (mAb), the largest class of therapeutic proteins currently under development, require post-translational modifications such as glycosylation for their optimal biological functions and pharmacokinetics [13–15]. Plant and transgenic animal systems have been encouraging for protein therapeutics manufacturing with their eukaryotic protein processing. However, challenges and concerns still remain and have to be addressed with respect to drug safety. These are, however, outside the scope of current review [9,16].

More than half of the therapeutic proteins approved and currently marketed are produced using Chinese hamster ovary (CHO) cells [16–21], mainly due to CHO cells' unique characteristics, such as human-like post-translational modification of the product and it amenability to bioprocess development and large-scale manufacturing [18,22,23]. DHFR-based methotrexate (MTX) selection or glutamine synthetase (GS)-based methionine sulfoximine (MSX) selection are two of the most commonly leveraged CHO expression systems [18]. In the case of the DHFR-based system, CHO-DG44, a CHO derivative lacking DHFR activity, has been widely used via selection, for an exogenously-introduced *DHFR* gene, in glycine, hypoxanthine and thymidine (GHT) deficient medium [6,24–27]. Historically, amplification of the *DHFR* and gene of interest (GOI) was needed to improve productivity, resulting in a longer timeline for cell line generation and introducing potential suitability and genetic stability risk arising from a loss of gene copies following removal of MTX selective pressure. MTX is generally removed from the cell culture process to avoid potential drug product safety concerns (MTX is a highly toxic drug), and to simplify processing by removing the need to demonstrate that the purification process has cleared MTX from the drug product. In contrast, a GS-based system [203] does not typically require an amplification step, thus resulting in fewer copies of the transgene per cell needed to achieve highly productive clonal cell lines [28,29].

Over the past two decades, advances in new designs of molecules such as antibody humanization have greatly reduced immunogenic concerns. Cell culture processes including media, process control and bioreactor design have also changed significantly to drive a five- to ten-fold increase of peak cell densities in mammalian cell cultivated bioreactors and this has reached >10 g/l in mAb and Fc-fusion protein production [6,23,30–32]. However, over this same timeframe, the fundamental processes utilized for cell line generation have not changed significantly, especially in the selection step for top-producing clonal cell lines. Hundreds to thousands of individual clonal cell lines need to be screened to identify the final top producers, which makes cell line generation time consuming and labor intensive, and this has become the timeline limiting step for the majority of the industry. It is only recently that the increased use of new high-throughput screening technologies including fluorescence-activated cell sorting (FACS) [33–36], ClonePix [37], CellXpress [38,39] and mRNA expression levels [40], together with new CHO cell lines, have created opportunities that have substantially shortened cell line development timelines. Numerous outstanding reviews have previously summarized expression systems [7], cell culture process developments [6,23,41], metabolic pathway and host cell engineering [42]. This review will focus on the latest advancements, achieved through host cell and expression plasmid engineering approaches, in cell culture selection systems used in the cell line generation process, as exampled by GS-CHO.

Nature of the GS gene: biological function & the molecular mechanism used as a selection marker

GS (L-glutamate:ammonia ligase, E.C. 6.3.1.2) is a universal housekeeping enzyme that catalyzes the synthesis of glutamine from glutamate and ammonia using the hydrolysis of ATP to ADP and phosphate to drive the reaction (Figure 1A) [43]. It is the only enzyme that can synthesize glutamine *de novo*. The function of GS in human cells is tissue specific [44,45]. In the brain, GS regulates the level of toxic ammonia and converts neurotoxic excess extracellular glutamate to harmless glutamine. The potential links between GS enzyme's malfunctioning and illnesses such as Alzheimer's disease [46,47], schizophrenia, Parkinson's disease [48], and Huntington's chorea [49] have stimulated studies on GS enzyme over the past 50 years [50]. In the liver, GS is one of the enzymes responsible for the removal of ammonia [51]. The complete knockout of the mouse GS gene results in early embryonic lethality [52]. In bacteria and plants, GS is responsible for fixation and re-assimilation of ammonia, allowing glutamine to be used as a nitrogen source for metabolism [53,54].

The CHO GS gene was identified from a CHO cell line over expressing GS [55]. It is comprised of six exons, with exon 5 comprising the sequence critical for GS activity (Figure 1B) [51,56,57]. Mammalian GS is a decameric protein consisting of two pentamers with a total of ten active centers, each located at the interface of two adjacent subunits in the pentameric ring [51]. The amino acid sequences of GS are highly conserved across species

and the differences in the crystal structures between the mammalian and plant enzymes are extremely subtle [51]. The metabolic pathways that regulate GS in bacteria and mammalian cells have been well established [45,50,58].

Glutamine is one of the essential amino acids needed by CHO cells when cultured *in vitro* [59]. The primary functions of glutamine in cell culture are as an energy source, and as a nitrogen donor in the synthesis of amino acids and nucleotides. The GS system™ was designed originally for use in cell lines that do not produce sufficient GS to support growth. Hybridomas (Sp2/0-Ag14) and cell lines, such as mouse myeloma (NS0) lines [60] naturally do not express sufficient GS even though the GS gene (*GLUL*) is present (inferred by the ability to obtain glutamine-independent revertants). For other cell lines (e.g., CHO) that are glutamine prototroph, this trait can be introduced by genome editing [57] or inclusion of an appropriate enzyme inhibitor (MSX) in the medium [61]. The complementation of a glutamine auxotrophy by recombinant GS provides the basis for use of GS as a selectable marker [6,62].

MSX, an analogue of glutamate, has been identified as an inhibitor of GS by binding to the glutamate site of the enzyme [50,63]. Irreversible inhibition of GS results from binding of the phosphorylated form of MSX to the glutamate binding site [64]. More specifically, MSX blocks glutamate entry through stabilization of the flexible loop in the active site [63,65]. In a concentration-dependent manner, MSX, inhibits enzyme activity in cells that possess endogenous GS activity (3 µM [55]), improves selection stringency (25–50 µM [66]); or can be used to select for gene amplification (200 µM [67]).

It is worth mentioning that MSX is not a specific inhibitor of GS. MSX also inhibits γ-glutamylcysteine synthetase, the first enzyme of both glutathione (GSH) synthetic pathways [68]. GSH has a crucial role in maintaining an environment within the endoplasmic reticulum (ER), conducive for protein folding. GSH has been implicated in the formation of native disulfide bonds within the mammalian ER, where it functions to maintain the ER oxidoreductases in the reduced state [69,70]. Mammalian cells respond to the formation of reactive oxygen species resulting from disulfide bond formation in the ER by increasing the synthesis of GSH [71]. Larger pools of GSH have been found in highly productive antibody-producing DHFR-CHO cell lines whilst the benefit of over expressing the enzymes of disulfide bond formation is not clear [70,72]. This raises an intriguing possibility: in cell lines selected for their ability to complement a chemically induced glutamine auxotrophy, have we also inadvertently selected for cell lines with a greater inherent capacity for protein secretion?

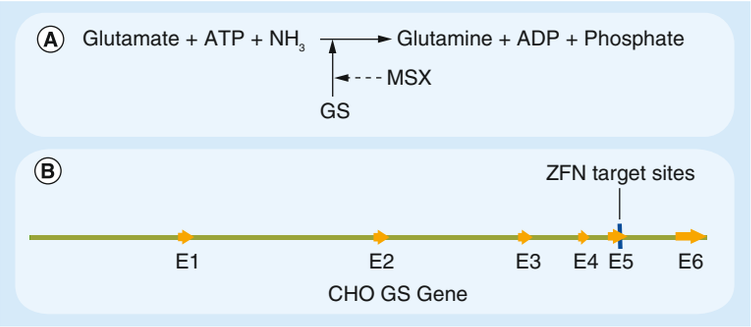

Figure 1. Function and gene structure of mammalian glutamine synthetase. (A) GS plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. Methionine sulphoximine irreversibly inhibit GS activity; **(B)** CHO GS gene structure. ZFN target sites indicated the mutation sites in GS-knockout cells. CHO: Chinese hamster ovary; E: Exon; GS: Glutamine synthetase; MSX: Methionine sulphoximine.

History of the development of the GS selection system in CHO cells for the production of biologics

The GS gene expression system [60,62], uses complementation of a glutamine auxotrophy by a recombinant GS gene to select for high-level expression of proteins from mammalian cells. As the GS System is intended for the commercial manufacture of therapeutic proteins, materials and procedures are developed so that cell lines are selected to fit a commercially relevant platform. To-date (August 2013), 13 licensed therapeutic proteins are manufactured using the GS system: eight antibodies (including Soliris®, Synagis® and Zenapax®) are manufactured using GS-NS0 cell lines, and five non-antibodies are manufactured using GS-CHO cell lines [Lonza, Unpublished Data].

Expression Vectors

GS expression vectors comprise the GS gene plus up to three highly efficient transcription cassettes for the GOIs [66]. Initially, different vectors were used to create GS-NS0 and GS-CHO cell lines (pEE12 and pEE14 respectively, Figure 2). These vectors differed in the format of the GS gene and the structure of promoter used to drive expression of the GOIs. The GS transcription cassette for vector pEE12 uses a GS cDNA, SV40E promoter, and SV40 splicing and polyadenylation signals. The GS transcription cassette in the pEE14 vectors uses GS mini-gene plus the SV40L promoter and two polyA signals. The mini-gene, unlike the GS cDNA, contains intron 6 of the genomic CHO GS gene. In both vectors, the transcription cassette for the GOI includes the hCMV-MIE promoter and 5′-untranslated sequences from the hCMV-MIE gene, including the first intron, to enhance mRNA levels and translatability. In the current version of the GS System™ for use with CHO cell lines,

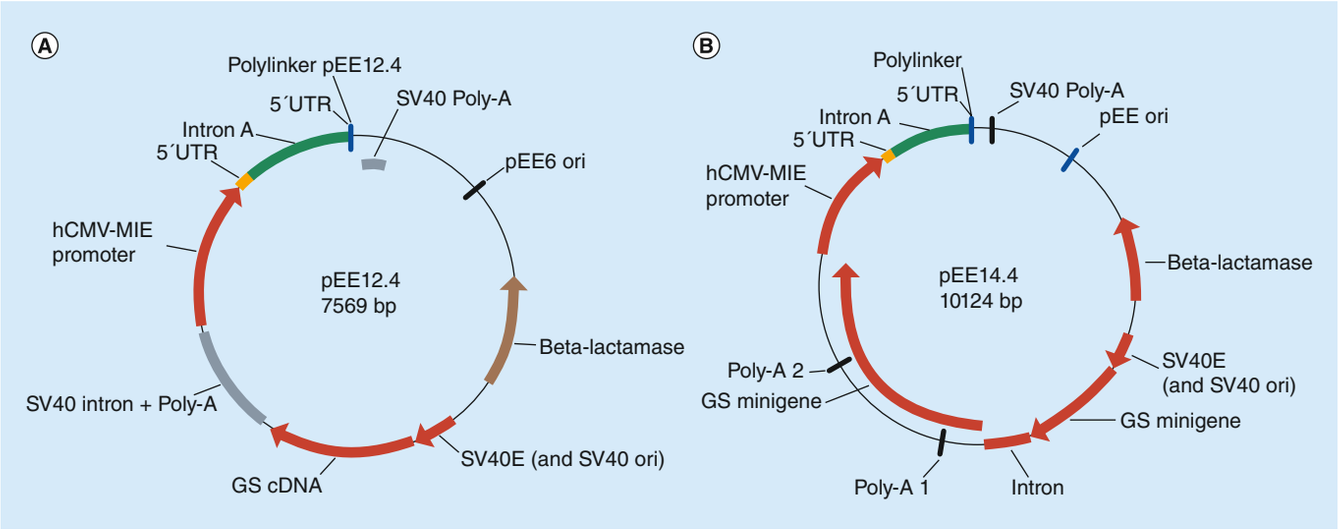

Figure 2. pEE12.4 and pEE14.4 vectors. (A) pEE12.4 uses a GS cDNA, SV40E promoter, and SV40 splicing and polyadenylation signals; **(B)** pEE14.4 uses GS mini-gene plus the SV40L promoter and two polyA signals. In both vectors, the transcription cassette for the gene of interest. includes the hCMV-MIE promoter and 5-untranslated sequences from the hCMV-MIE gene.

GS Xceed™, the mCMV promoter is used as this has advantages in terms of how quickly transfectants appear and their number [204].

The pEE14 vectors were recommended for protein expression in CHO-K1 cells because they produced higher numbers of transfectants compared with the pEE12 vectors. pEE12 vectors were used with the NS0 host cell line. During the development of the CHOK1SV host cell line, it was observed that pEE12 and pEE14 vectors produced similar antibody concentration distributions (0.4 to 290 mg/l, mean 79 mg/l, for pEE12 *cf.* 0.4 to 209 mg/l, mean 63 mg/l, for pEE14 in a 24-well plate model) [Lonza, Unpublished Data]. However, there were substantial differences in numbers of transfectants recovered (120 transfectants from 20 96-well plates for pEE12 *cf.* 85 transfectants from 239 96-well plates for pEE14). This is in contrast to the result observed with CHO-K1. To simplify use of the GS system and exploit the potential advantages of pEE12 in CHOK1SV, the use of pEE12 was standardized across both GS-NS0 and GS-CHO.

Host cell line

In principle, the GS system can be used with a variety of cell lines [60], but in practice, the most commonly used are CHO and NS0. The mouse myeloma cell line NS0 does not grow in glutamine-free medium and has a reversion frequency to glutamine prototrophy of $<10^{-7}$. The reversion frequency is substantially lower than the value for the murine hybridoma Sp2/0-Ag14 and rat myeloma Y0 [60]. The NS0 cell line is derived from the IgG1 secreting myeloma cell line P3-X27, although it does not synthesize light chain (LC) or heavy chain (HC) itself [73]. As such, it made the NS0 host cell line the preferred choice for manufacturing antibodies as it was considered a professional antibody secreting cell line. The NS0 host cell line, along with Sp2/0-Ag14, are no longer the preferred host cell lines since they produce recombinant proteins containing the immunogenic sugar α-1,3-galactose [74,75]. The default growth mode for CHO cells is as adherent cells in serum-containing media. This has several disadvantages for the rapid development of cell lines suitable for the manufacture, in the current regulatory landscape, of proteins for clinical and commercial uses. The current generation of CHO host cell lines has been selected to circumvent these disadvantages.

Originally, the GS System™ used the cell line CHO-K1 grown initially in attachment mode for cell line construction and early cell line selection before adaptation to suspension growth mode for late stage cell line selection. Additionally, early stages typically used serum-containing media and the later stages used serum-free media. The adaptation to growth in serum-free media as a single cell suspension is time consuming, typically taking in the order of 30 weeks [Lonza, Unpublished Data]. In the 1990s, Sinacore and colleagues demonstrated that the CHO cell line DUKX could be pre-adapted to growth in serum-free media and then be used for the generation of stable, recombinant cell lines [76]. This approach provided an opportunity to reduce the time and resources required to develop large-scale, suspension culture-based manufacturing processes with serum-free medium. To shorten the timeline for selection and development of GS-CHO cell lines, a CHO-K1 host cell line was successfully adapted to growth in suspen-

sion mode as single cells using protein-free media by first adapting the cells to suspension culture in serum-containing medium, and switching to serum-free and then protein-free media (Figure 3). The resultant cell line was renamed CHOK1 SV (CHO-K1 Suspension Variant). CHO cell line contain a functional GS gene and can grow in glutamine-free medium, but growth is inhibited by inclusion of MSX at 3 µM [55].

Cell line development & cell culture process

Highly productive cell lines are selected using the GS system through a combination of a weak promoter driving expression of the selectable marker gene together with stringent selection for transfected cells expressing high levels of the gene product, and tight genetic linkage between the transcription cassettes for the selectable marker gene and the GOIs.

The vectors use a SV40 promoter to drive transcription of the GS gene. By using either glutamine-free media or glutamine-free media containing at least 3 µM MSX, transfected NS0 and CHO cells, respectively, can be selected. By using a MSX concentration above that needed to inhibit growth and selecting for cell lines that grow well, cell lines are selected that synthesize sufficient glutamine through having high levels of GS. As a weak promoter is used in the GS expression cassette, it is postulated that cells are selected where the vector is inserted into a transcriptionally active locus within the genome. Since the GOIs and GS gene are on the same vector and genetically tightly linked, for an antibody the vector is approximately 11 kb, selection for integration of the GS gene into a transcriptionally active locus results in cointegration into the same locus. Expression by the strong promoter in the GOI expression cassette is enhanced by the favorable site of integration.

The development of bioreactor process for GS-CHO cell lines reflects the changes in the bioprocessing industry over the last 25 years and has been covered in several reviews [6,23,41]. The history of the GS-CHO process is summarized in Table 1. Initially, the process

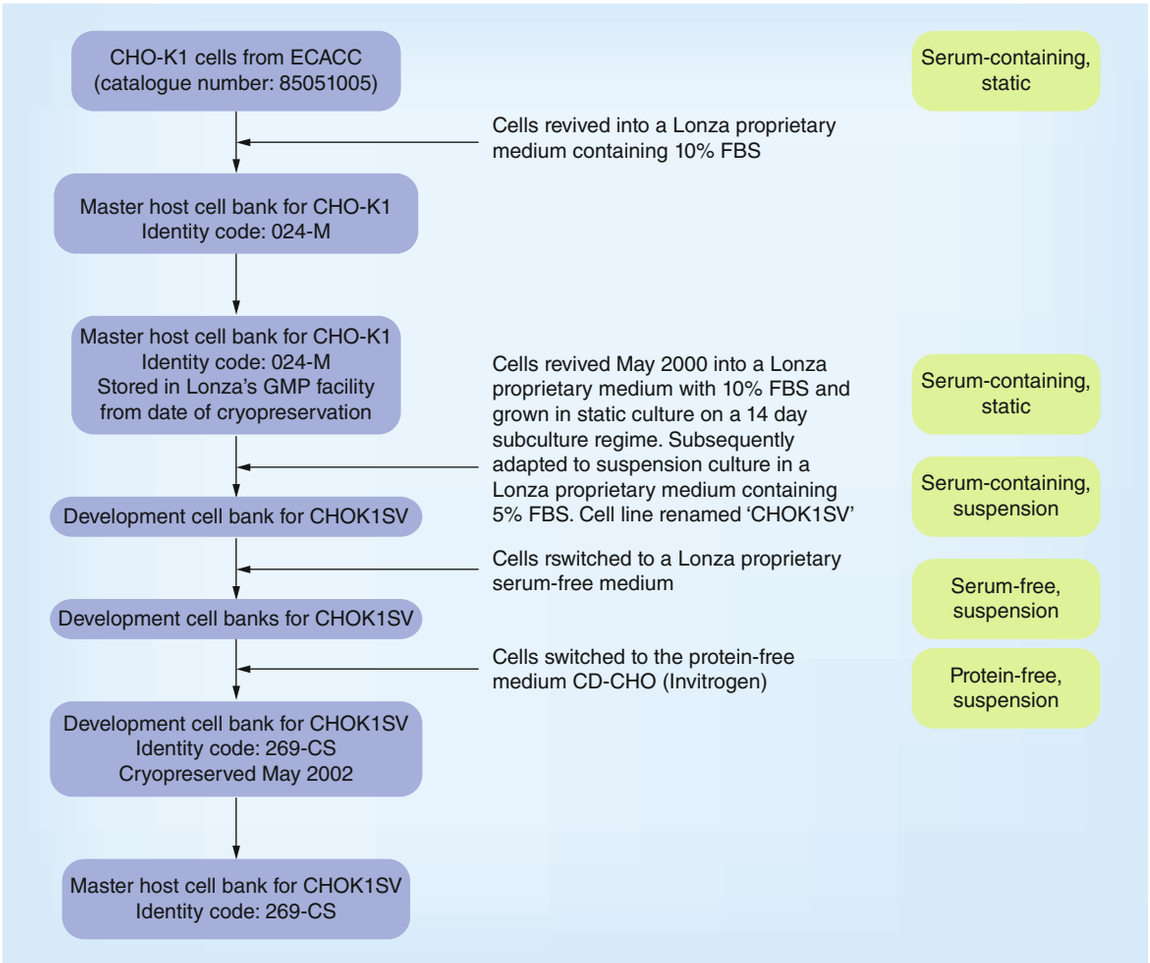

Figure 3. Derivation of cell line CHOK1SV.
CHO: Chinese hamster ovary cells; FBS: Fetal bovine serum.

Table 1. Development of glutamine synthetase-Chinese hamster ovary cells, process from 1990 to 2008. The chimeric antibody cB72.3 was initially expressed using CHO-K1. With launch of CHOK1SV in 2003 was re-expressed in the new host line.

Process version	Maximum viable cell concentration (10^6/ml)	Integral of viable cell concentration (10^6 cells h/ml)	mAb (mg/l)	Specific production rate (pg/[cell h])
22HI1 (CHO-K1)				
Not defined	3.25	328	41	0.13
Not defined	2.29	267	139	0.52
1	4.51	498	334	0.66
2	6.32	1041	585	0.53
LB01 (CHOK1SV)				
2	9.70	2266	1917	0.89
3	14.19	2493	2829	1.17
4	12.40	2254	3560	1.55
5	12.63	3470	4301	1.41
6	15.78	4215	5929	1.52
7	16.30	6048	6851	1.13
8	17.83	5142	8335	1.62

CHO: Chinese hamster ovary cells; mAb: Monoclonal antibody.

was fed-batch using simple feeds with serum-free media. The initial optimization was performed by changing the base medium, from serum-free to a chemically defined, animal component- and protein-free medium, and modifying the feed system based on the approach of spent medium analysis and feed supplementation. This increased the product concentration from 139 to 585 mg/l. The resulting process (version 2) was used as the starting point for developing a process for cell lines developed using the new host cell line CHOK1SV. Switching host cell lines gave a 14-fold increase in mAb concentration. The mAb concentration increased to over 8 g/l by version 8 through a mixture of optimizing process parameters and modification of the feed following spent media analysis.

Key Terms

Selected bulk culture: Describes the enrichment of transfected cells containing copies of external transgenes that stably integrated into its genome through a suspended cell culture condition. The selection processes generally requires the use of a selection marker (auxotrophy, antibiotics) to counter select non-transfectents.

Gene Knockout: This is a genetic term (abbreviation KO) in which one of an organism's genes are disrupted and lost its natural function. It is one of the most popular techniques to study gene that has been sequenced, but which has an unknown or incompletely known function.

Recent advances in the GS selection system: expression host & expression plasmid engineering

Clinical cell line development has a profound impact in biopharmaceutical product development. While the productivity and product quality characteristics of the producing cell line affect the complexity of cell culture and downstream purification processes, the speed of developing a successful cell line can provide significant timeline advantages for product clinical development programs in the current highly-competitive industry [77]. The efficiency of identifying top-producing cell lines largely depends on the selection stringency during cell line generation processes. Considerable efforts have been made in CHO cells to improve the productivity, product quality, cell line stability [78,79], and cell culture process robustness through media optimization, process control, host cell protein dynamics [2,80], and host cell engineering [81–85]. However, improvements in selection systems related to host cells and plasmids were relatively slower due to technology limitations in generating knockout cell lines through plasmid-based homologous recombination.

CHO host cell engineering

It is only most recently, with the advancement in several key technologies including ZFNs [86–88], meganucleases [89], TALE nucleases [90,91], and CRISPR/Cas9 [92,93] that targeted modification of key genes related to the selection process has become practical with successful demonstration of the generation of knockout CHO cell lines to improve selection stringency and product qualities [94,95].

Unlike NS0 cells, CHO cells have constant expression of GS from the endogenous GS genes when grown in glutamine-free media. This 'background' GS expression results in a decrease in selection stringency when the GS selection system is used in CHO cells, as evidenced by the observations that large numbers of non- or low-producing cells exist within the selected

bulk culture population and the recovery of mock-transfected parental CHO cells under standard dosage of MSX (50 µM compared with 5 µM in NS0 cells) (Figure 4A & B). Given the fact that some CHO parental cells can survive in as high as 5 mM MSX selection [55], simply increasing the MSX concentration to improve selection stringency is not feasible since higher MSX will have a negative impact on cell growth. Additionally, transgene amplification could result from increasing MSX levels, which could also cause potential stability concerns on established cell lines. Thus, CHO cells in which the background expression of GS is eliminated are desirable to enable a highly effective and efficient cell line development process in CHO cells.

Recently, attempts to delete or disrupt the endogenous GS gene from CHO cells through gene knockout technology have been very successful [95,96]. Independent development of GS-knockout cell lines has been undertaken and these engineered expression hosts have been implemented in biopharmaceutical processes. Compared to CHOK1SV cells, GS-knockout cells allow for more stringent GS selection conditions to be leveraged in bulk cell cultures after removal of the background expression of the CHO GS gene. Through these stringent selection conditions, significant productivity improvements in bulk cell cultures are achieved, helping meet protein needs for early-stage drug discovery (Figure 5A) [57,97]. It is obvious that the bulk culture productivity improve-

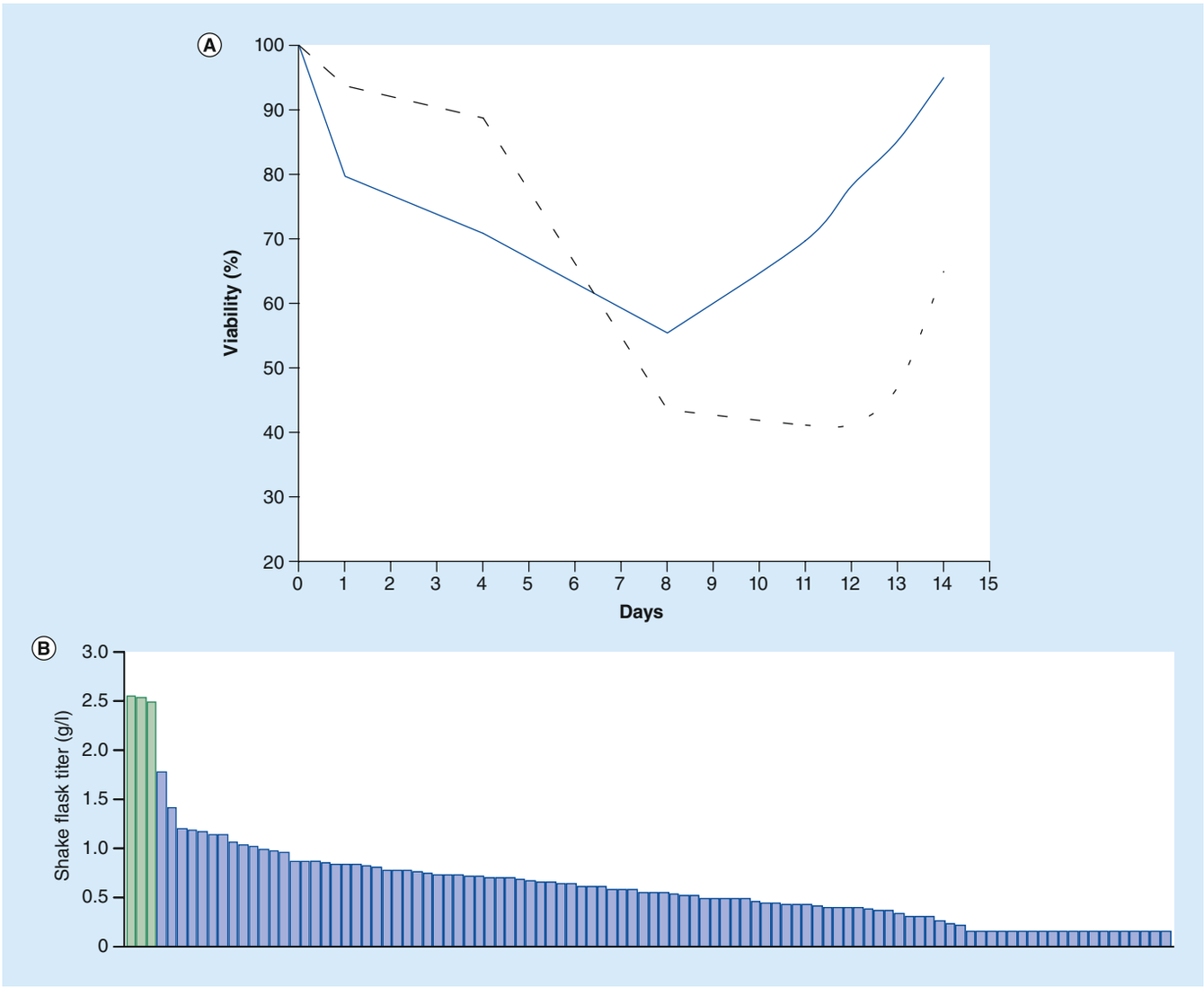

Figure 4. Monoclonal antibody versus bulk cell culture selection in CHOK1SV cells. (A) Viability of selected bulk cell cultures indicating mock transfection (without plasmid DNA, dotted line) recovered at the end of the selection. Solid line represents typical monoclonal anitbody selection. **(B)** Distribution of productivity of clonal cell lines from selected mAb x bulk cell culture in CHOK1SV cells. A total of 114 individual cell lines were randomly picked and scaled up for a 14-day fed batch shake flask study.

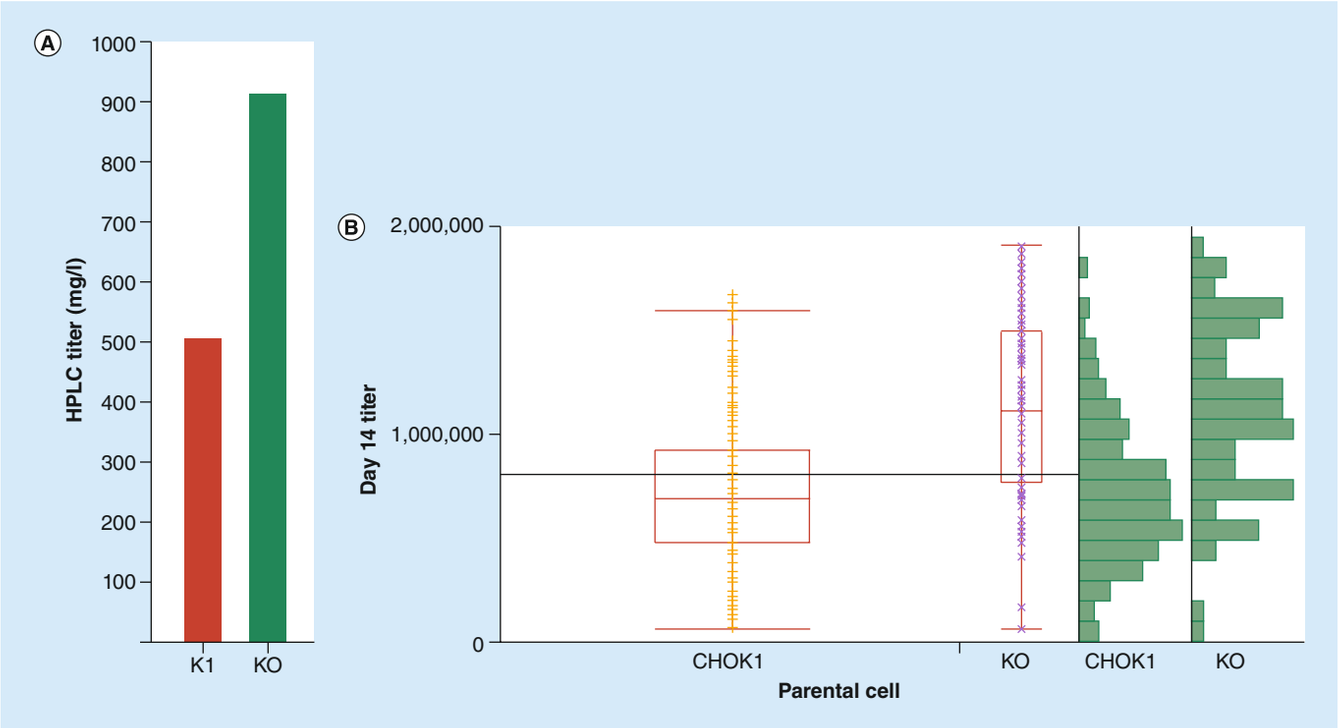

Figure 5. Comparison of selected bulk cultures between CHOK1SV and GS-KO cells. (A) HPLC titers of monoclonal antibody x bulk cultures. KO: GS-KO cells with 25 μM MSX, K1: CHOK1SV cells with 50 μM MSX. **(B)** One-way analysis of day 14 ACES titer data against parental cells used in the experiment. Data was analyzed by JMP 8.0 software. The significance of the mean difference was tested by Tukey–Kramer test. Histograms indicated the titer distribution of individual clones.
CHO: Chinese hamster ovary cells; CHOK1: CHOK1SV parental cells; HPLC: High-performance liquid chromatography; KO: CHOK1SV-derived GS-knockout cells.

ments are largely due to the elimination of non- and low-producing cells within the bulk culture populations (Figure 5B). This shift in selected bulk culture populations to larger percentages of high-producing cells has also impacted the cell line generation screening process resulting in a six- to eight-fold efficiency improvement. In addition, changing the host cell line to a GS-knockout derivative of the CHOK1SV host cell line reduced the cell line development timeline further. With the CHOK1SV host, selected to fit the current GS-CHO inoculum and bioreactor process (version 8; Table 1), a high producing clonal cell line is available about 23 weeks after transfection (Figure 6A). Comparison of Figure 6A & B shows that when using the CHOK1SV GS-knockout host with pXC vectors, the elapsed time to a high producing clonal GS-CHO cell line is 17 weeks. The six-week time reduction results from faster selection and expansion of transfectant pools, faster outgrowth of cell lines after cloning and a faster doubling time for the host.

Furthermore, the 'Fast-to-Proof-of-Principle' strategic requirement of the bioprocessing industry with 'good enough' cell lines making 2–3 g/l of an antibody creates the opportunity to reduce both the number of screening rounds and the number of cell lines screened (Figure 6C). This reduces the timeline by a conservative 2 weeks. Figure 6B & C differ in that there is no intermediate screening stage using 'abbreviated fed-batch cultures,' where feeds are added at a fixed time and volume rather than by, for example, cell concentration. Instead, high ranked cell lines are identified at the clone screening stage and then evaluated in a miniature bioreactor system. Historically, generating a clonal CHO cell line took more than 2 years: with the current generation of cell lines, vectors and equipment, this is now approximately 4 months.

Expression plasmid engineering

While selection stringency could be increased by raising drug (MSX) concentrations in the cell culture medium, this approach is typically not very attractive as the high drug concentration will normally have negative impact on cell growth and lengthen the timeline for cell line generation. Alternative strategies have been employed to modulate the expression level of marker genes through attenuating the selection marker or DNA components that regulate the marker gene expression. Mutation of neomycin phosphotransferase II to reduce its affinity to neomycin has improved the specific mAb productivities from 1.4- to 14.6-fold [98,99]. However, similar strategies have not been used for the GS gene due to the complex-

ity in balancing the potential negative impact on GS' normal metabolic function and the use of GS enzyme as a selection marker.

Modulating the level of transcription of the selection marker gene through the manipulation of DNA components that regulate marker gene expression is an alternative approach to increase selection sensitivity. Limited successes have been achieved with such approaches including the use of dicistronic vector containing an internal ribosome entry site of the encephalomyocarditis virus [100]. Antibody production from an expression vector with HC and LC gene transcription driven by two separate CMV promoters was compared with expression using a single CMV promoter to drive expression of a single HC–LC fusion, where the two polypeptides were linked using a foot-and-mouth disease F2A polypeptide self-cleaving linker sequence [101]. The lower expression of the single polypeptide was attributed to the ER processing/degradation capacity setting a limit on the transcriptional input. AU-rich elements and MODC PEST region as respective mRNA and protein destabilizing elements [102,103] had not been previously tested in the GS-CHO expression system due to the lack of GS knockout cells, because only in GS knockout cells can the needed sensitivity to MSX be achieved. One approach in our lab has been the development of weakened SV40E (ΔSV40E) promoter through a series of nested deletions involving key regulatory elements of the wild type SV40E promoter [104]. When those ΔSV40E promoters, which were 40–60% lower in GS gene transcriptional activity, were used to drive GS expression and were utilized in cell line generation in combination with GS-knockout cells, selection stringency was further improved compared with wild-type SV40E promoter used in combination with the GS-knockout cells. Not only non-producing cells, but a large number of lower-producing cells were eliminated even in cases where no MSX was added to the cell culture medium (Figure 7A). In addition to improvements in screening efficiency, productivities of individual top cell lines also improved dramatically (from 1.5 g/l to 3.5 g/l) when ΔSV40E promoters were used to replace wild-type SV40E promoter (Figure 7B), a significant difference from using GS-knockout cells alone [57]. The removal of MSX from cell culture medium has multiple advantages including eliminating a raw material from cell culture processes, reducing the regulatory concern on trace amounts of MSX in purified drug substance, and providing potential advantages in terms of cell line suitability and stability by maintaining selection pressure in the absence of chemical selection.

Targeted integration

Traditional stable transfection strategies typically result in random integration of transgenes into CHO

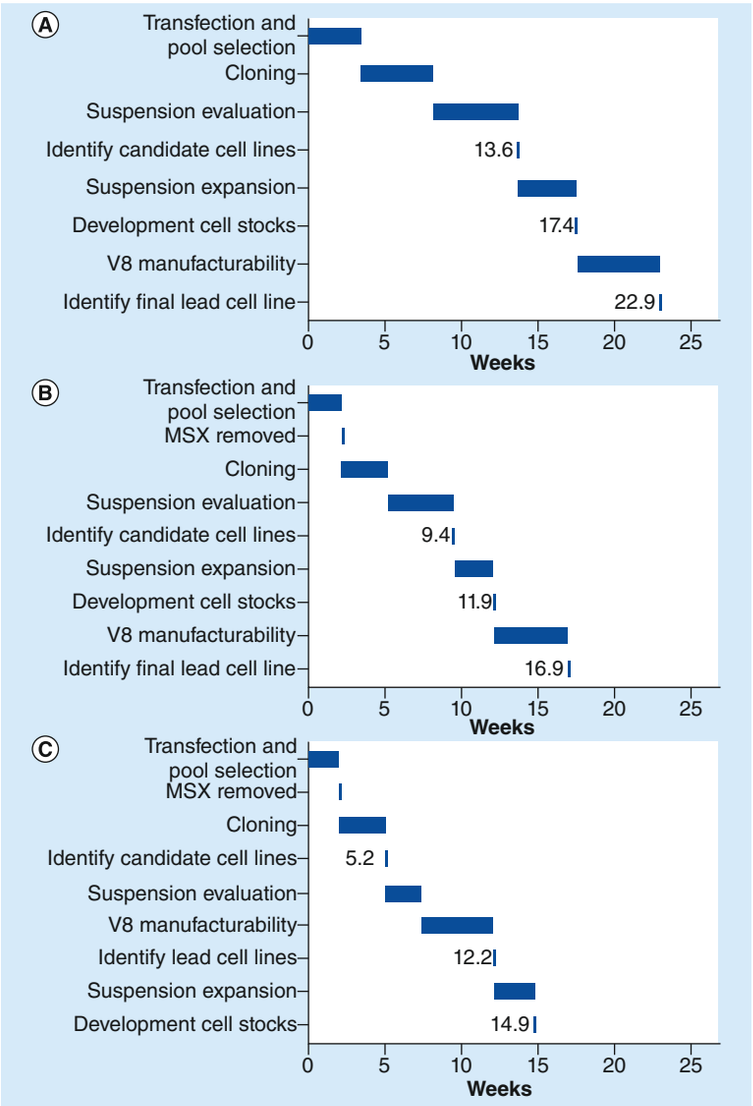

Figure 6. Evolution of cell line generation timeline with GS-CHO system. **(A)** Timeline for cell line selection and development for GS-CHO using CHOK1SV as host, with the objective of selecting a high-producing cell line, >3 g/l. Workflow uses a FACS technology for cloning and then liquid handling robots coupled with use of shaken, deep-well plates for cell line screening. Cell lines are selected to fit the version 8 GS-CHO process. MSX is included in the medium at all development stages. **(B)** Timeline for cell line selection and development for GS-CHO using Lonza's CHOK1SV GS-knockout cells strain as host, with the objective of selecting a high-producing cell line, >3 g/l. Workflow uses a FACS for cloning and then liquid handling robots coupled with use of shaken, deep-well plates for cell line screening. Cell lines are selected to fit the version 8 GS-CHO process. MSX is included in the medium at only at the transfection and pool selection stage, after which it is removed. **(C)** Timeline for cell line selection and development for GS-CHO using Lonza's CHOK1SV GS-knockout cells strain as host, with the objective of selecting a cell line making 2–3 g/l. Workflow uses a FACS for cloning and then liquid handling robots coupled with use of shaken, deep-well plates for cell line screening. Cell lines are selected to fit the version 8 GS-CHO process. MSX is included in the medium at only at the transfection and pool selection stage, after which it is removed. FACS: Fluorescence-activated cell sorting; GS-CHO: GS-Chinese hamster ovary cells; MSX: Methionine sulfoximine.

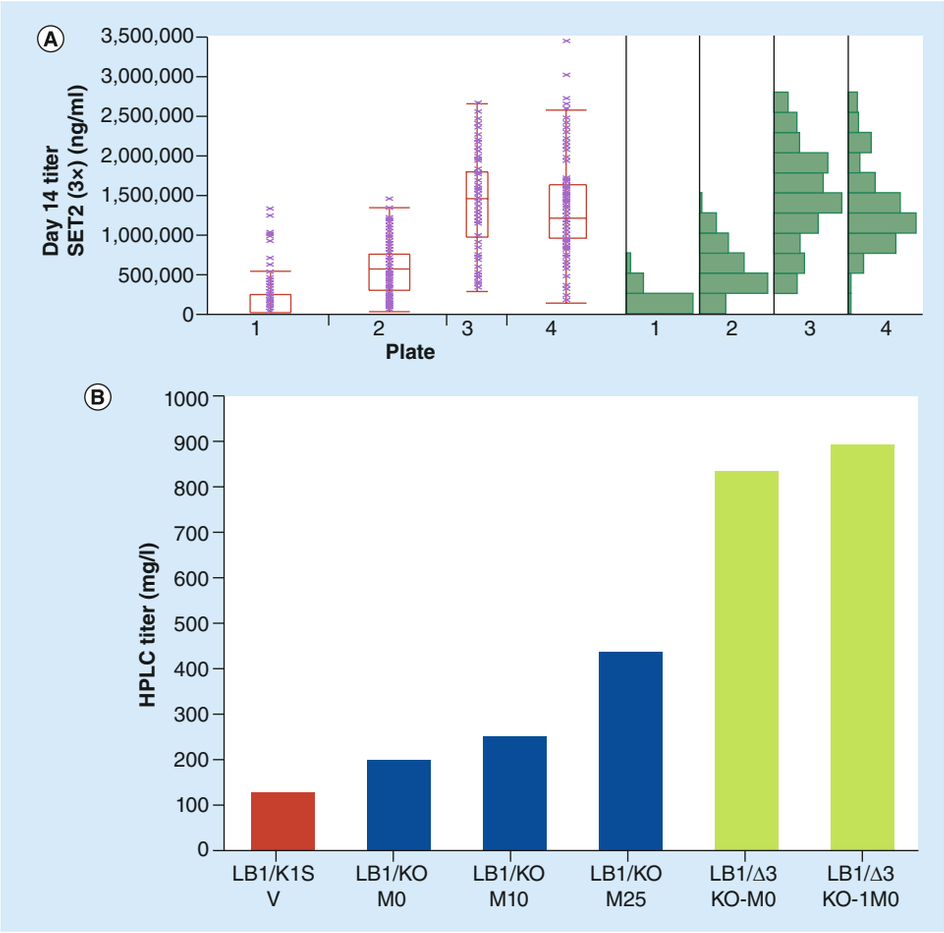

Figure 7. Comparison selected bulk cultures with different SV40E promoters. (A) One-way analysis of day 14 ACES titer data against groups in the experiment. Data were analyzed by JMP 8.0 software. The significance of the mean difference was tested by Tukey–Kramer test. Histograms indicated the titer distribution of individual clones. Group 1: CHOK1SV cells plus wild type SV40E with 50 μM methionine sulfoximine; group 2: GS-knockout cells with wild type SV40E with 25 μM MSX; group 3: GS-KO cells plus D3SV40E with 0 MSX; group 4: GS-KO cells plus D3SV40E with 10 μM MSX; **(B)** Bulk culture productivity. Day 10 protein A titers.
KO: knockout cells; K1SV: CHOK1SV cells; M: MSX: Methionine sulfoximine.

chromosomes. Targeted integration technology offers an alternative strategy to develop highly productive and stable cell lines in a reproducible and predictable manner [105] by targeted integration of transgene into a transcriptionally active genome region(s) – 'hot spots'. Identification of those rare 'hot spots' in the CHO genome and an established technology that can target transgene delivery into the 'hot spots', are the two key components required for the success of targeted integration strategy. Efficient delivery methods have been established with several recombinases including Flp, Cre and PhiC31 and more recently by introducing a site-specific DNA double-strand break on the chromosome that can stimulate homologous recombination efficiency by up to 1000-fold or more in mammalian cells [106–108]. In contrast, screening for and identifying 'hot spots' has been relatively less successful as it depends largely on the selection system for cell line generation, the bioprocess for cell cultures, the understanding of mammalian genome, and the mechanism of gene expression. Phenotypic markers, such as GFP, have been used as tools in screening for transcriptionally active spots, but how representative these reporters are to industrial processes (i.e. expression of secreted therapeutic proteins) is still questionable. As 'hot spots' are extremely rare in the CHO genome, intensive screening of cell lines with single copy integration, high expression levels, and stability is required to identify an ideal target locus. Using FLP/FRT technology for targeted integration in GS-knockout cells, Pfizer has recently achieved four weeks reduction in their cell line generation process [29]. A stringent selection system such as that provided by the combination of ΔSV40E promoters and GS-knockout cells could provide a valuable tool to identify transcriptional 'hot spots' in CHO genome for the development of a targeted integration system, which is deemed as the next generation cell line generation process development [109–111].

Additionally, chromatin-modifying DNA elements that control the chromatin structures on regulation of transgene expression, such as ubiquitous chromatin opening elements (UCOEs) and scaffold or matrix attachment regions (S/MARS) of DNA, could further improve the targeted integration strategy by increasing the stability of transgene expression after the transgenes have been put into the 'hot spots'. UCOEs are methylation-free CpG islands that function as insulator elements against heterochromatin expansion making a DNA region more accessible to transcription machinery [112]. S/MARS of DNA are genomic DNA sequences at which chromatin is anchored to the nuclear matrix during interphase and are linked to histone hyperacetylation. which indirectly recruits DNA

Key Term

Targeted integration: Describes the integration of transgene(s) into specific genomic regions through site specific recombination technologies. It is the counterpart term for 'random integration', which describes the insertion of external transgenes throughout the genome randomly.

demethylase to demethylate DNA in order to make it accessible for transcription [113–115]. Previous studies revealed that both UCOEs and S/MARS augment the expression of a reporter protein in stably transfected mammalian cell lines [116–118].

Future perspective

The effective and efficient generation and identification of highly productive CHO cell lines for biopharmaceutical products continue to be a challenge for the industry. Current cell line generation processes are often time consuming and require improvement to support the growing number of biologics moving into clinical development and the increasing costs for such programs. By utilizing technologies such as FACS/automated colony pickers and automated cell culture evaluation systems allows high-throughput screening, the procedures are often expensive and need constant modification to adapt to different cell lines and protein products. The recent advances involving host cell and expression plasmid engineering and targeted integration have shown promising results in improving the generation and identification of very high-producing cell lines. Further implementation of such strategies has the potential to increase overall efficiency of biopharmaceutical drug development, especially for those non-mAb molecules including non-mAb proteins, peptides, bifunctionals and bispecifics, by reducing the timeline required to supply drug product for clinical studies.

Recent progress in improving GS-CHO selection efficiency and bulk culture productivity has provided a solid foundation for the development of an improved transient CHOK1 expression system. It has been shown that there can be inconsistencies between protein quality from HEK293 and CHO which could impact pharmacokinetic profiles, as well as potentially impact chemical and/or physical characteristics [119]. The detection of aberrant splicing of transgene expression in HEK293 and CHO cells can also be inconsistent and negatively impact the drug development timeline. In order to keep a consistent protein quality source between early discovery research and commercialization, it will be desirable to have a transient CHOK1 expression system to replace current HEK293 cells for early discovery needs. Recent studies with polyethylenimine (PEI) have shown promising progress [119–121] and the combination of the engineered ΔSV40E promoter in combination with the GS-knockout system should provide a valuable tool for this application.

Going forward, the targeted integration strategy is very likely to become the next generation process for cell line development. Leveraging technologies such as ZFNs and TALEN transgenes could be introduced into specific sequence locations in the host genome. One of the central challenges that remains is the identification of genomic sites which are 'hot enough' and 'stable'. One potential option is to utilize reverse-genetic approaches using selection markers to identify clonal cell lines containing 'hot spots' through standard cell line generation process. Even though the timeline for this approach is longer relative to other strategies, the 'hot spots' identified tend to be more representative and robust, more importantly, the new parental host cells could be screened for other characteristics including growth, metabolic profile and host cell proteins to improve cell culture process robustness. In the reverse-genetic approach, use of the metabolic selection marker itself is only necessary for the first round 'hot spots' identification, and may not be needed for subsequent targeted integration efforts. Depending on the design of targeted integration, metabolic selection markers could either be permanently inserted in the new host cell genome, or removed by the replacement plasmid constructs. The combined usage of targeted integration and chromatin regulation elements will possibly generate a homogeneous population of highly productive and stable cells within much shorter time windows, and it will provide great opportunity to cut current timelines from transfection to first human dose study.

Finally, the availability of the CHO genome will likely provide another round of breakthroughs in cell line generation technologies as we continue to further the understanding of the underlying cellular processes of protein production. A good example will be further understanding of the mechanisms causing instability in some CHO cell lines, especially as some authors argue that it "is a consequence of the inherent genetic instability of recombinant CHO cell lines" [122]. Tremendous efforts have been made to illustrate the long -term culture instability issues at a post-mRNA level to metabolic events [78,79]. With further omics data providing more insight into the mechanisms, regulatory events, and linkages underpinning cellular phenotype changes, highly productive and stable GS-CHO cell lines could be generated in more predictive ways.

Financial & competing interests disclosure

A Racher is employed by Lonza Biologics, who developed and license the GS Gene Expression System. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Background

- Central to the needs of the industry to support the development of innovative biologics and biosimilars are more effective and efficient manufacturing processes which require highly productive cell lines with desired quality attributes.
- The fundamental processes utilized for cell line generation have not changed significantly, especially in the selection step for top-producing clonal cell lines over the past two decades.
- Many recent advancements in the field of GS-Chinese hamster ovary (CHO) cell culture selection systems used in the cell line generation process through host cell and expression plasmid engineering approaches.

Nature of the GS gene

- GS catalyses the synthesis of glutamine from glutamate and ammonia and it is the only enzyme that can synthesize glutamine *de novo*.
- Glutamine is one of the essential amino acids needed by CHO cells when cultured *in vitro*. The complementation of a glutamine auxotrophy by recombinant GS provides the basis for use of GS as a selectable marker.
- Methionine sulfoximine, an analogue of glutamate, has been identified as an irreversible inhibitor of GS by binding to the glutamate site of the enzyme.

History of the development of the GS selection system in CHO cells

- The GS gene expression system was developed in 1980s and 13 licensed therapeutic proteins are manufactured using the GS system to date.
- GS expression vectors comprise the GS gene plus up to three highly efficient transcription cassettes for the genes of interest.
- CHO cells are widely used for the manufacture of therapeutic proteins. CHO cells have been adapted to serum-free media and in suspension culture.

Recent advances in the GS-selection system

- Compared to CHOK1SV cells, GS-knockout cells allow for more stringent GS selection conditions resulting in significant productivity improvements in bulk cell cultures, a six- to eight-fold cell line generation efficiency improvement, and reduced the cell line development timeline.
- The combination usage of weakened SV40E (ΔSV40E) promoter and GS-knockout cells further improved selection stringency in GS-CHO system, resulting in additional improvements in screening efficiency, productivities of individual top cell lines and the potential removal of methionine sulfoximine from cell culture medium.
- Targeted integration technology offers an alternative strategy to develop highly productive and stable cell lines in a reproducible and predictable manner by targeted integration of transgene into a transcriptionally active genome region(s).

Future perspective

- The recent advances involving host cell and expression plasmid engineering and targeted integration have shown promising results in improving the generation and identification of very high-producing cell lines .
- Future improvements will focus on transient CHOK1 expression system, stability of clinical cell lines and global metabolic flux.

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