Methionine Sulfoximine Supplementation Enhances Productivity in GS-CHOK1SV Cell Lines through Glutathione Biosynthesis

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Abbreviations: MSX: L-methionine sulfoximine, mAb: monoclonal antibody, CHO: Chinese hamster ovary, GCL: glutamate cysteine ligase, GS: glutamine synthetase, DHFR: dihydrofolate reductase, MTX: methotrexate, GCLc: glutamate cysteine ligase catalytic subunit, GCLm: glutamate cysteine ligase modifier subunit, Ero1: endoplasmic reticulum oxido-reductase 1, ESX: α-ethylmethionine sulfoximine, UPR: unfolded protein response, tPA: tissue plasminogen activator, BSO: L-buthionine sulfoximine.

Abstract

In Lonza Biologics' GS Gene Expression System™, recombinant protein-producing GS-CHOK1SV cell lines are generated by transfection with an expression vector encoding both GS and the protein product genes followed by selection in MSX and glutamine-free medium. MSX

is required to inhibit endogenous CHOK1SV GS, and in effect create a glutamine auxotrophy in the host that can be complemented by the expression vector encoded GS in selected cell lines. However, MSX is not a specific inhibitor of GS as it also inhibits the activity of GCL (a key enzyme in the glutathione biosynthesis pathway) to a similar extent. Glutathione species (GSH and GSSG) have been shown to provide both oxidizing and reducing equivalents to ER-resident oxido-reductases, raising the possibility that selection for transfectants with increased GCL expression could result in the isolation of GS-CHOKISV cell lines with improved capacity for recombinant protein production. In this study we have begun to address the relationship between MSX supplementation, the amount of intracellular GCL subunit in and mAb production from a panel of GS-CHOK1SV cell lines. We then evaluated the influence of reduced GCL activity on batch culture of an industrially relevant mAb-producing GS-CHOK1SV cell line. To the best of our knowledge, this paper describes for the first time the change in expression of GCL subunits and recombinant mAb production in these cell lines with the degree of MSX supplementation in routine subculture. Our data also shows that partial inhibition of GCL activity in medium containing 75 μM MSX increases mAb productivity, and its more specific inhibitor BSO used at concentration of 80 μM in medium increases the specific rate of mAb production 8-fold and the concentration in harvest medium by 2-fold. These findings support a link between the inhibition of glutathione biosynthesis and recombinant protein production in industrially relevant systems and provide a process-driven method for increasing mAb productivity from GS-CHOK1SV cell lines.

1.0 Introduction

CHO cells are the most widely utilized mammalian cell type for the commercial production of recombinant proteins[1]. Currently, there are two major CHO cell-based expression systems used in the pharmaceutical industry: DHFR (dihydrofolate reductase)[2-3] and GS (glutamine synthetase)[4]. When using the DHFR gene as a selectable marker, selection occurs in the absence of the metabolites hypoxanthine and thymidine. Gene amplification is accomplished

by exposing the cells to increased concentrations of MTX, which inhibits DHFR enzyme activity[5]. Alternatively, when using the GS gene a selectable marker, stable cell lines are selected through the ability of the product of an exogenous GS gene to complement a glutamine auxotrophy (brought about by the inhibition of endogenous GS during selection in glutamine-free medium and MSX[4]). Transfectants containing an integrated exogenous copy of the GS gene in an transcriptionally active locus are able to synthesize glutamine from glutamic acid, ammonia and ATP[1] and thus survive selection. In the GS Gene Expression System™, a tight genetic linkage between the transcription cassettes of GS selectable marker and genes of interest favors those recombinant cells where a co-integration event at an active locus has occurred. When using MSX as a positive selection pressure in CHO cells, 3 µM inhibits endogenous enzyme activity[6], 25-50 µM improves selection stringency[7] and 200 µM selects for gene amplification[8]. Whilst bi-allelic GS knockout host cell lines have been constructed lacking endogenous glutamine synthetase (e.g. Lonza's GS Xceed™ System), 25-50 µM MSX is still used during the initial stages of selection in order to maintain stringency[9].

In addition to GS, MSX inhibits the activity of the enzyme GCL, which catalyzes the synthesis of reduced glutathione (GSH) from its precursor amino acids: glutamate, cysteine and glycine[10]. In mammalian cells, the rate of *de novo* GSH synthesis is determined by the availability of cysteine and expression of two distinct gene products encoding the subunits of the heterodimeric GCL complex[11-12]. The larger GCLc subunit (~75 kDa) contains the active site whilst the smaller GCLm subunit (~30 kDa) acts to increase the catalytic efficiency of the enzyme through its interaction with GCLc[13]. Direct and indirect functional measurements of the amounts of intracellular GCL subunits demonstrate that GCLm limits GCL holoenzyme complex formation in most cell types and tissues[14]. Thus, expression of GCLc and GCLm are strong determinants of GCL activity which directly determines the rate of *de novo* GSH synthesis.

GSH is synthesized in a reduced state as a linear tri-peptide, and as electrons are lost, two GSH molecules become linked by a disulfide bond to form oxidized glutathione (GSSG)[15]. When Hwang *et al.* determined the concentration of GSH and GSSG within hybridoma CRL-1606 cells[16], the ratio of reduced GSH to GSSG within the secretory pathway ranged from 1:3 to 3:1, whereas the overall cellular ratio ranged from 30:1 to 100:1, suggesting that the secretory pathway is more oxidative than that of the cytosol. These findings, and the observation that GSSG concentrations in freshly prepared ER-derived microsomal vesicles are similar to those required for *in vitro* folding[17], have supported the hypothesis that the cellular balance of GSH to GSSG influences, and is important in maintaining the correct cellular environment for protein folding.

The mechanism by which glutathione interacts with the folding machinery remains poorly defined. GSSG has been shown to oxidize PDI maintaining the protein in a more active state[18], however this oxidation can occur independently of GSSG through Ero1p[19-21]. It has also been suggested that GSH might have a role for maintaining PDI and other oxido-reductases (e.g. Erp57) in a reduced state so that they can catalyze the reduction part of the isomerization reactions necessary to correct miss-paired disulfide bonds[22-23]. Recent data suggests that GSH serves as a reductant in the ER to off-set Ero1p oxidation, thus protecting the cell from ER-generated ROS[24-25]. The oxidation of PDI by Ero1 is thought to occur through disulfide exchange that results in the formation of reduced Ero1[26]. Ero1p can be oxidized rapidly in the presence of flavin adenine dinucleotide (FAD) and oxygen, indicating that oxygen is the ultimate electron acceptor[27]. The reactivation of Ero1p by molecular oxygen generates reactive oxygen species (ROS) in the ER which in recent models are buffered by GSH[28].

With regard to recombinant protein production in mammalian cell lines, any alteration in the amount of glutathione (either GSH or GSGG forms) could have dramatic consequences on folding of recombinant protein. For example, it is known that larger pools of GSH have been

found in highly productive antibody producing DHFR-CHO cells[29]. Additionally, it was found that lowering the amount of intracellular glutathione in tPA-producing CHO cell lines accelerates disulfide bond formation but does not lead to correct protein folding as the reduction of incorrectly paired disulfide bonds were compromised, preventing disulfide bond isomerization occurring[30]. Here we investigate the intriguing possibility that MSX supplementation in GS-CHO cell line construction alters recombinant protein production capacity, through GCL activity, as this is of direct relevance to users of GS selection systems[9]. We initially used expression analysis to identify relationships between MSX supplementation, GCL subunit expression and mAb production in a GS-CHOK1SV cell line. We then describe an approach to evaluate the influence of inhibition of GCL activity on batch culture of a mAb-producing GS-CHOK1SV cell line derived from the industrially relevant CHOK1SV host.

2 Materials and methods

2.1 Reagents

Chemicals were purchased from Sigma-Aldrich (Dorset, UK) and cell culture reagents were sourced from Gibco/ThermoFisher (Paisley, UK) unless otherwise stated. DNA size markers, enzymes and PCR reagents were purchased from Promega (Hampshire, UK). DNA and RNA extraction kits were purchased from Qiagen (West Sussex, UK). Oligonucleotide primers were commercially synthesized by MWG-Biotech (Hertfordshire, UK).

2.2 Cell Lines and Cell Culture

GS-CHOK1SV cell lines expressing a model mouse-human chimeric IgG4 mAb, cB72.3, were supplied by Lonza Biologics Plc (Slough, UK). The panel of twelve cell lines selected for this study exhibit a range of cell specific production rates (qmAb) and the time integral of viable cell concentrations (IVC) in fed batch suspension culture (Supplementary Table 1). All GS-CHOK1SV cell lines were constructed as described in Porter *et al*[31]. The generation of these cell lines is described in Porter *et al.*[31] with the exception of LB01, Null 8, Z1 and Z14 which

were constructed during an internal study (unpublished). Null 8 is a "null" cell line which has been constructed using an empty GS vector. GS-CHOK1SV cell lines were grown in CD-CHO medium in 100 mL shake flask suspension cultures at 37°C in 5% CO_2 (v/v in air) using a humidified shaking incubator rotating at 140 rpm. Cell counting was performed daily with a ViCell instrument (Beckman Coulter) using settings designed for CHO cells supplied by the vendor. IVC and qmAb were estimated as described in Porter *et al.*[31]. Pearson's correlation coefficients (r_{xy}) and two-tailed paired T-test statistical significance were calculated in Microsoft Excel 2010.

2.3 Gel migration assay

10^6 cells were pelleted from a suspension culture and were re-suspended in 1 mL PBS. Reduced and oxidized controls were generated by treating with either a final concentration of 10 mM DTT or a final concentration of 1 mM dipyridyl sulfide, respectively. The redox status of all samples was then trapped by alkylation of free thiols in a final concentration of 25 mM NEM followed by lysing the cells in 50 mM Tris-HCl containing 150 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Triton X-100. The lysate was denatured by boiling for 2 min in the presence of 1% (w/v) SDS. Tris[2-carboxyethyl]phosphine (TCEP) was added to a final concentration of 10 mM in order to reduce existing disulfide bonds, which were then alkylated by the addition of 30 mM AMS (Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature. The lysates were separated by Bis Tris SDS-PAGE and subjected to western blot analysis in order to detect various oxido-reductases (section 2.4).

2.4 Gel Electrophoresis and Western Blot Analysis

Total protein was extracted from 10^7 viable cells by the addition of RIPA buffer[32]. Cells were washed twice with PBS and once with 0.35 M sucrose before the addition of the RIPA buffer. SDS-PAGE and western blot analysis was undertaken as previously described[33] using the following antibodies and dilutions: GCLm (AB40929, Abcam), GCLc (AB17926 Abcam,

Cambridgeshire, UK), GS (G2781, Sigma, Dorset, UK), ERo1-Lα (PA146120 Affinity Biosystems, UK) 1:1000 and β-actin (A5441, Sigma, Dorset, UK) 1:10,000. Western blots were developed using an enhanced chemiluminescence system (ECL, GE Healthcare) exposed to hyperfilm (GE Healthcare) and scanned using a GE-800 image scanner (GE Healthcare).

2.5 Quantitative Real Time PCR Analysis of mRNA Levels

Quantitative real-time PCR (qRT-PCR) was performed using an IQ5 instrument (Bio-Rad, UK) and the iScript One-Step RT-PCR Kit (Bio-Rad, UK) as described by Marchant *et al.,*[34] using the following primers; GCLm Forward 5'-gtcagggagtttccagat-3', Reverse 5'-gtcagggagtttccagat-3'; GCLc Forward 5'-ccaattgttatggctttgagtgc-3', Reverse 5'-ccaattgttatggctttgagtgc-3' and values were normalized against TAF-7 (Forward 5'-cgactccctagaacatgatgaa-3, Reverse 5'-gatcttctcggtgtcga-3'), β-actin and YWHAZ (primers supplied by Primer Design, UK). These three genes had been selected from a panel of the ten housekeeping genes using geNorm^{TM} selection software (Primer Design, UK)[35] and were deemed to exhibit the most stable expression in the GS-CHO cell lines analyzed in the study.

2.6 Determination of Recombinant Chimeric IgG4 mAb Concentration

The concentration of mAb in cell culture supernatant was determined by quantitative Protein A HPLC[31]. Clarified supernatants were loaded onto a Poros Protein A immunodetection column, connected to an Agilent 1100 HPLC. The column was washed and bound IgG was eluted, by lowering the pH of the solvent. Eluted IgG was quantified using ChemStation software against a generic IgG standard.

3.0 Results

MSX lacks a high degree of specificity for GS; *in vitro* experiments have demonstrated as little as 50 μM is sufficient to inhibit purified sheep brain GS activity by 62% and purified rat kidney GCL activity by 85%[36] .In this study we initially investigated the influence of MSX-based

supplementation (in selection and routine subculture) on the expression of endogenous GCL and mAb production in GS-CHOK1SV cell lines (section 3.1). Subsequently, the effect of specific inhibition of GCL on the performance of a mAb-producing GS-CHOK1SV cell line was evaluated using the MSX analog BSO which lacks specificity for GS[37-38] (section 3.2).

3.1 GCL expression in GS-CHOK1SV cell lines

In the GS expression System™, selection of transfectants derived from the CHOK1SV pool containing stably integrated copies of the GS selection marker linked to product-encoding genes is achieved through supplementation of glutamine-free medium with 25 μM MSX[9]. As MSX also inhibits GCL activity, we initially hypothesized that GS-CHOK1SV cell lines selected in the presence of 50 μM MSX have also been selected with increased expression of endogenous GCL relative to the CHOK1SV host.

Firstly, we compared GS and GCL expression (both mRNA and protein) to the amount of endogenous glutathione in CHOK1SV host cells (cultured in the absence of MSX) and seven mAb-producing GS-CHOK1SV cell lines[9] (see Supplementary Table 1) selected in 50 μM MSX and cultured in glutamine-free medium supplemented with 25 μM MSX (Supplementary Figure 1). Whilst GCL expression fluctuated between GS-CHOK1SV cell lines, the difference in the amount of GCL or glutathione between the CHOK1SV host and the GS-CHOK1SV cell lines was not statistically significant using the two-tailed paired T-test (Table 1). In a subset of these seven cell lines (54, 114, 53, 142 and 38) we also measured endogenous glutathione concentrations, which were shown similar to that detected in the CHOK1SV host (Supplementary Figure 2). This disproved our initial hypothesis, implying that these data do not support the theory that selection of GS-CHOK1SV cell lines in glutamine-free medium supplemented with 25 μM MSX influences the intracellular content of GCL and glutathione across these cell lines. However, there is an inherent limitation in the comparison: The host cell lines require medium supplement with 6 mM glutamine (to prevent the selection of host

cells with higher endogenous GS activity), whereas the mAb-producing cell lines are selected and cultured in glutamine-free medium (usually with MSX supplementation). If glutamine was added to the medium used to select and culture the mAb-producing GS-CHOK1SV cell lines in order to make the medium similar to that used for the host, it would likely reduce selection pressure for the integration of exogenous GS and could result in cell line instability. Comparisons of a wider panel of CHO expression systems (e.g. DHFR, puromycin N-acetyl-transferase and neomycin phosphotransferase selection markers) would perhaps be a better route to examine the influence of different selection conditions on GCL subunit expression. However this would not be relevant to the CHOK1SV cell line.

GS-CHOK1SV cell lines are routinely cultured in 25-50 μM MSX to maintain stable integration of the expression vector and/or stable expression of genes encoded by the vector[9]. As previously discussed, these MSX concentrations are sufficient to inhibit purified rat kidney GCL activity[36]. Therefore, we next investigated the relationship between MSX supplementation and endogenous GCL expression in routine subculture. If indeed MSX supplementation results in inhibition of GCL activity, we expected expression of GCL subunits to be increased in order to compensate for the resulting glutathione depletion[12, 39]. A rolling culture (i.e., defined as a regime of serial 96 h sub-cultures over a set number of cell generations) was completed for the mAb-producing GS-CHOK1SV cell line LB01 in duplicate 100 mL cultures supplemented with either 0, 25, 50 or 75 μM MSX. Following 1344 h of subculture, the quantity of both GCL subunit mRNA and protein were determined by qRT-PCR and western blot analysis, respectively. No correlation was evident between the concentration of MSX in the medium and the intracellular amount of GCLc mRNA (Figure 1A: r_{xy} = -0.165). However, a strong correlation between the concentration of MSX in the medium and the amount of intracellular GCLc protein was observed (Figure 1F: r_{xy}= +0.889). In contrast, the relationship between the GCLm subunit and the correlation of MSX concentration in the culture medium was stronger for mRNA (Figure 1B: r_{xy}= +0.905) than for protein (Figure 1G: r_{xy}= +0.359). The magnitude

of change in GCL subunit expression (1.4 to 4-fold) was greater at ≥ 25 μM MSX. To the best of our knowledge, we believe that this is the first evidence that routine subculture of a mAb-producing GS-CHOK1SV cell line in medium containing different concentrations of MSX influences the expression of endogenous GCL subunits.

Following this, we asked the question: Are changes in the amount of GCL subunits with increasing MSX supplementation correlated with the specific production rate of the mAb (qmAb) and other cell culture parameters? To do this, fed-batch cultures of each mAb-producing cell line were undertaken and subsequently the concentration of mAb in the harvest medium was determined by Protein A HPLC. Data for GS-CHOK1SV cell lines Z1 and Z14 (Supplementary Table 1), generated using in the same experiment, are presented in Supplementary Figure 3. In the GS-CHOK1SV cell line LB01 the concentration of MSX supplementation correlated with IVC (Figure 2A: r_{xy} = +0.681), qmAb (Figure 2B: r_{xy} = +0.795) and the concentration of mAb in the harvest medium (Figure 2C: r_{xy} = +0.988). The expression of GCL mRNA and protein (Figure 1) was analyzed with respect to IVC, qmAb and mAb concentration in the harvest medium. Only a weak correlation was observed between the amount of GCLc mRNA and either IVC (Figure 2D: r_{xy} = +0.441), qmAb (Figure 2E: r_{xy} = +0.567) or the mAb concentration in harvest medium (Figure 2F: r_{xy} = +0.211). In contrast, the amount of intracellular GCLc protein was more strongly correlated with the concentration of mAb in harvest medium (Figure 2I: r_{xy} = +0.829) and less strongly correlated to IVC (Figure 2G: r_{xy} = +0.617) and qmAb (Figure 2H: r_{xy} = +0.589). For GCLm, there was only a strong correlation between the mRNA and the concentration of mAb in the harvest medium (Figure 2L: $r_{xy:}$ = +0.903) and less so for protein species (Figure 2O: r_{xy} = +0.359). This inconsistency in the correlations of mRNA or protein for individual subunits with indicators of cell culture performance is probably the result of different regulation mechanisms. Whilst a strong correlation was observed between the amount of GCLm mRNA or protein with qmAb (Figure 2K: r_{xy} = +0.844, Figure N: +0.708, respectively), a weak correlation was observed for IVC

(Figure 2J: r_{xy} = +0.452, Figure 2M: r_{xy} = +0.330, respectively). Aside from this point, these data support a link between routine subculture in medium containing different concentrations of MSX and the ability of the GS-CHOK1SV cell lines to produce mAb.

3.2 Effects of GCL inhibition on cB72.3 Production

We next evaluated the effect of GCL inhibition on growth and productivity of a mAb-producing GS-CHOK1SV cell line. As MSX inhibits both GS and GCL activity it is difficult to uncouple the influence of inhibition of each enzyme. BSO is a synthetic analog that has specificity for GCL without any detectable inhibition of GS activity[37]. Therefore, in contrast to our data obtained with MSX (section 3.1), any productivity change in response to BSO supplementation can be directly attributed to GCL activity. The inhibition of GCL using BSO has been well characterized in CHO-K1 by Clark *et al.*[38]. and, therefore, their conditions were used as a starting point for our study. Furthermore, to ensure that only endogenously derived GSH from the GCL pathway was available to the cell, the GS-CHOK1SV cell line used for this work (cell line 42, Supplementary Table 1) was adapted to and cultured in a medium lacking GSH and MSX. 100 mL cultures were supplemented with BSO at concentrations selected to give partial inhibition (i.e., 20 to 100 μM) together with enzyme-inhibitor saturation concentrations (i.e., 250 μM to 5 mM) based on the data reported by Clark *et al.*[38]. Cultures were incubated for 288 h and viable cell concentration, viability and cell size were monitored. Supernatant samples were removed at harvest (i.e., 288 h of culture time) for measurement of the concentration of mAb in the medium.

Viable cell concentrations in cultures supplemented with BSO showed three different but distinct growth profiles throughout the 288 h batch suspension culture (Figure 3). The 0 μM BSO culture (i.e., negative control) reached a maximal viable cell concentration of 6 x 10^6 cells/mL at 144 h culture time and a plateau for the remaining 144h. Cultures supplemented with 20 to 100 μM BSO (i.e., partial inhibition) showed a similar growth profile, however the

maximum viable cell concentration was approximately 70% lower than the control culture (Figure 3). Cultures treated with ≥ 250 μM BSO (i.e., enzyme-inhibitor saturation) did not enter exponential growth phase, and the majority of cultures had maximum viable cell concentration 85% lower than the control culture. A sample of the culture treated 250 μM BSO was stained with DAPI and analyzed by immunofluorescence microscopy. The results (data not shown) suggested that these cells were apoptotic, as determined from the observed chromatin condensation and cytoplasmic membrane blebbing as previously described by Arden and Betenbaugh[40]. Figure 4 summarizes the effect of BSO supplementation on growth (A: mean cell diameter and B: IVC) and productivity (C: secreted cB72.3 concentration and D: qmAb). IVC declined with increased BSO supplementation (Figure 4B). At conditions proposed to give partial inhibition (*pa*, Figure 4) of GCL (i.e., 20 to 100 μM BSO) a 2-fold increase in the concentration of mAb in the harvest medium was evident (Figure 4C) and this change correlated with either increased cell size (Figure 4A) (r_{xy}: +0.721), increased qmAb (Figure 4D) (r_{xy}: +0.865) or decreased IVC (Figure 4B) (r_{xy}: -0.919). These data suggest that at conditions proposed to give partial inhibition of GCL increased secreted concentrations of cB72.3 are achieved as a result of increased qmAb perhaps as the results of a cell cycle pause and at the expense of IVC.

As the glutathione balance (GSH:GSSG) in the ER lumen is hypothesized to influence oxidative folding through a modulation oxido-reductases such as Ero1-L, we next determined the redox status of Ero1-Lα using a 1D-SDS-PAGE gel migration assay (see Methods) in cell samples derived from the batch cultures of cell line 42, which showed increased mAb production (Figure 5: 20 and 100 μM BSO). When cells were not treated with BSO, the Ero1-Lα protein migrated with the mobility of a reduced protein (Figure 5, DTT treated), demonstrating that Ero1-Lα is predominantly in a reduced form in the steady-state. Treatment with BSO to reduce GCL activity resulted in the Ero1-Lα protein progressively moving towards a more oxidized state (Figure 5), as shown by the progressive decrease in gel migration distance of the Ero1-Lα band

with BSO concentration. At the highest BSO concentration tested, the migration distance of Ero-1α tended to that of the oxidized DPS control. However, a diffuse banding pattern was observed at ≥ 100μM, which is thought to represent heterogeneity due to a mixture of partial and fully oxidized forms of Ero1-Lα co-existing at the same time. This is consistent with diffuse bands previously reported for PDI, that were thought to represent two semi-oxidized forms in which either the first or second active site are oxidized[41]. A shift of the steady-state from reduced to oxidized Ero1-Lα is aligned with increased mAb production in medium containing increasing concentrations (20-100 μM) of BSO. We were unable to determine if this was the result of an indirect effect of BSO, or if a more oxidized Ero1-Lα accounts for the 2-3 fold increase in mAb production.

4.0 Discussion

We postulated that CHOK1SV-derived GS cell lines constructed under MSX selection in the GS Expression System™ are more competent for recombinant protein expression, owing to selection for increased GCL expression maintaining ER localized glutathione pools[9].

GCLm mRNA and GCLc protein amounts are increased with MSX supplementation

A positive relationship was observed between MSX supplementation concentrations and the amount of intracellular GCLm mRNA (Figure 1B). A similar dose-dependent increase in GCLm has been demonstrated in HepG2/C3A cells with exposure to 50 μM to 0.3 mM MSX[14]. No correlation was evident between the concentration of MSX in medium and GCLc mRNA (Figure 1A). Experiments performed in HepG2 and MCF7 mouse cell lines have demonstrated that GCLc mRNA is rapidly turned-over[42] and perhaps suggest a tight control of expression in mammalian cell lines. A strong correlation was observed between the concentration of MSX in the medium and the amount of GCLc protein (Figure 1F) which advocates a post transcriptional up-regulation GCLc protein expression in GS-CHOK1SV cell lines cultured in higher concentrations of MSX. No such increase in GCLm protein was detected; however it is

plausible that observed increased amounts of mRNA (Figure 1B) are required to maintain steady-state concentrations of the GCLm subunit.

The mechanism by which GCL expression is increased with MSX supplementation is likely to be indirect. MSX binds to the glutamate active site of the GCL enzyme and in the presence of ATP and metal ions (Mg^{2+} and Mn^{2+}) and is converted to methionine sulfoximine phosphate which reversibly inhibits the enzyme[10]. We did not measure cellular glutathione and ROS concentrations in this experiment, however as *de novo* synthesis of glutathione is limited by GCL activity, we would expected that any inhibition will result in a lowering of GSH pools and result in an increased amount of oxidative species. Both the intracellular concentrations of GCLc and GCLm, are positively regulated by transcription, mRNA turnover and post-translation in response to oxidative stress[12] which accounts for the increase in GCL mRNA and protein observed in GS-CHOK1SV cultures supplemented with MSX. These experiments confirm that MSX-based selection interacts with systems aside from glutamine bio-synthesis.

Inhibition of GCL activity increases mAb specific production rate and mAb concentration in harvest medium

Partial inhibition of GCL activity in medium containing 75 μM MSX increases mAb productivity (Figure 2C), and its more specific inhibitor BSO used at concentration of 80 μM in medium increases the specific rate of mAb production 8-fold (Figure 4D) and the concentration in harvest medium by 2-fold (Figure 4C). A similar increase in productivity from fed batch cultures has been reported when MSX was added to GS-knockout CHO IgG, producing clones generated in a MSX free process[43] and this report did not identify any significant differences in gene copy number (GS and IgG) in the presence and absence of MSX treatment, suggesting that this increase is not the result of transgene amplification. The treatment of tPA expressing CHO cells with 500 μM BSO for 16 hours has been reported to accelerate disulfide bond formation at the expense of non-native bond isomerization by using pulse chase experiments[29]. Treatment of

cell-free extracts with BSO is standard practice and has been shown to increase the yield of an active model protein containing 9 disulfide bonds by 50%[44]. In this study, at lower concentrations of BSO than that used by Oh *et al*[44] (20 to 100 μM BSO), we observe an increase in secreted assembled mAb (measured by affinity for Protein A). Similar reductions in cell growth leading to an increase in recombinant protein concentration in harvest medium have been reported in CHO when using a range of chemical compounds[45]. Our data suggests that with lower concentrations of BSO (80 μM) or MSX (75 μM) benefits in terms of mAb production can be obtained but concentrations any higher are toxic, at least in the case of BSO. The findings of this and other internal unpublished studies performed in house have led us to investigate selection and maintenance of recombinant protein producing GS-CHOK1SV cell lines in glutamine free medium containing ≥50μM MSX .

Increased mAb production is co-correlated with increased GCL expression, increased cell diameter and a shift from steady-state reduced to oxidized Ero1-Lα

Increased mAb concentrations correlated with GCLc protein (Figure 2I) and GCLm mRNA (Figure 2L). As previously discussed we predict that MSX supplementation will reduce the activity of GCL, deplete GSH pools, reducing the capacity for ROS buffering. The presence of ER-localized ROS in mammalian cells generates an integrated stress response termed the UPR[46], resulting in elevated glutathione synthesis[47] and ER expansion[46] which could explain the observed co-correlation between GCL subunits, mAb production and MSX supplementation.

A strong positive correlation was observed between cell diameter and secreted cB72.3 concentration (Figure 4A and 4C). Depletion of glutathione with BSO has been shown to arrest cell division, suggesting that de novo glutathione synthesis is required for progression through the cell cycle and results in increased cell size[48]. It has been reported that in CHO-K1-15_{500} permanently producing tPA, cell size is a major determinant of productivity[49-50]. However, this observation was not replicated in clones derived from a CHOK1SV pool transiently expressing

cB72.3[51]. Our data provides evidence that increased cell size, as a result of inhibition of glutathione synthesis, relates to increased cB72.3 production. This further adds to the case for partial inhibition of glutathione pathways with BSO as a possible route to enrich populations with larger super producer clones and/or to the initial selection protocol.

A shift of the Ero1-Lα from reduced to oxidized steady-state aligned with increased mAb production in medium containing increasing concentrations of BSO (20-100 μM). As previously discussed Ero1Lα is thought to oxidize PDI, which in turn transfers oxidative equivalents to newly synthesized cargo proteins which directly contributes to disulfide bond formation[23]. Therefore it is possible that a more oxidized Ero1 maintains PDI in a more active oxidative state which in turn could account for the 2-fold increase in mAb concentration we observed in the harvest medium.

The report reaffirms the importance of glutathione biosynthesis as a potential route for enhancing recombinant CHO cell expression systems and highlights previously undefined ways that the GS system takes advantage of the promiscuity of MSX. We initially set out to demonstrate that CHOK1SV-derived GS cell lines constructed under MSX selection in the GS Expression System™ are more competent for recombinant protein expression. Whilst investigating this hypothesis we successfully demonstrated that specific inhibition of the GCL enzyme during subculture of GS-CHOK1SV cell line can have significant positive effects on mAb production.

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Conflict of interest

M Feary, A Racher and R Young are employed by Lonza Biologics, who developed and license the GS expression system. The authors declare no other financial or commercial conflict of interest.

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Table I: Statistical analysis of normalized GCL mRNA and protein comparisons in CHO, N8 and cB72.3 expressing GS-CHOK1SV cell lines. CHO refers to the non-transfected CHOK1SV host and Null8 is a "null" cell line constructed using an empty GS vector (see section 2.2). mAb refers to cB72.3-producing GS-CHOK1SV cell lines (cell lines 41, 54, 114, 53, 142, 147 and 38)

Comparison		GCLc		GCLm		GS	
		T-test (p)[(a)]	Fold change[(b)]	T-test (p)[(a)]	Fold change[(b)]	T-test (p)[(a)]	Fold change[(b)]
CHO vs Null 8	mRNA	N/A	1.41	N/A	1.51	N/A	N/A
	Protein	N/A	0.91	N/A	1.10	N/A	2.15
CHO vs mAb	mRNA	0.44	0.92	0.21	0.86	N/A	N/A
	Protein	0.15	0.83	0.14	1.37	0.01	1.79
CHO vs GS-CHO	mRNA	0.92	0.98	0.75	0.94	N/A	N/A
	Protein	0.14	0.85	0.16	1.33	0.01	1.85

[(a)] two tailed paired T-test, [(b)]relative to CHO (mean of A and B)

Supplementary Table I: Characteristics of CHOK1SV and GS-CHOK1SV cell lines in fed-batch suspension culture. The data presented was generated from cell lines revived from the same frozen stocks as analyzed in this study. CHO refers to the non-transfected CHOK1SV host and Null8 is a "null" cell line constructed using an empty GS vector (see section 2.2). All cell lines were cultured in 10 L airlift reactors, with the exception of Z1 and Z14 which were cultured in 2 L shake flasks. Airlift reactors were operated as a direct scale down of industrial manufacturing conditions. Data supplied by Tracy Root and Alison Porter (Lonza Biologics, Slough). The GS-CHOK1SV cell lines expressing cB72.3 are arranged by qmAb rank order.

Cell Line	Max Xv (10^6 cells /mL)	IVC (10^6/mL.h)	[Product] at harvest (µg/mL)	qmAb (pg/cell.h)
CHO	6.50	1408	N/A	N/A
Null 8	9.50	1790	N/A	N/A
Z1	12.00	2254	162	0.100
41	12.63	2533	364	0.154
54	8.05	1784	480	0.300
Z14	11.10	2295	870	0.400
53	18.69	3869	1464	0.450
142	18.25	3928	1629	0.480
114	12.44	2275	1312	0.640
38	15.90	3145	2221	0.770
147	11.25	2582	1964	0.801
42	14.57	2835	3220	1.290

Figure legends

Figure 1: GS-CHOK1SV cell line LB01 grown in varying amounts of MSX. Duplicate batch suspension cultures supplemented with 0, 25, 50 and 75 μM MSX were sub-cultured every 96 h. Following 1344 h total GCL expression was determined by qPCR and western blot. MSX concentration is plotted against the normalised amounts of GCLc (**A**) and GCLm (**B**) mRNA determined by qPCR (Mean ± SD). Protein extracts were separated by 1D-PAGE and blotted with antibodies for GCLc, GCLm, and β-Actin (**C, D** and **E**). Normalised amounts of GCLc and GCLm protein were determined by densitometric analysis of the bands (F-G respectively).

Figure 2: The effect of MSX on growth and productivity of cell line LB01 in fed batch suspension culture. Fed batch suspension culture of lineages supplemented with 0, 25, 50 and 75 μM MSX for 1344 h total culture time (Figure 1) were completed. L-MSX concentration is plotted against IVC (**A**), qmAb (**B**), secreted mAb concentration (**C**) from fed batch cultures. GCLc and GCLm normalized mRNA and protein amounts plotted against IVC (**D**, **G**, **J** and **M**), qmAb (**E**, **H**, **K** and **N**) and secreted concentrations of cB72.3 (**F**, **I**, **L** and **O**) respectively for individual fed-batch suspension cultures performed from cryopreserved cells following MSX supplementation.

Figure 3: Inhibition of GCL in batch suspension culture. GS-CHOK1SV cell line 42 was cultured in 100 mL cultures and supplemented with 0 to 5 mM BSO. To ensure that only endogenously derived GSH from the GCL pathway was available to the cell, the GS-CHO cell line used for this work (cell line: 42) was adapted to and cultured in a medium formulation lacking GSH and MSX. 100mL cultures were supplemented with BSO at concentrations selected to give partial inhibition (20 to 100 μM) together with enzyme-inhibitor saturation concentrations (250 μM to 5 mM) based on the data reported by Clark et al. (1984).[36] Viable cell concentration (black full lines) and culture viability (grey dashed lines) were monitored over 288 h. Supernatant samples were removed at 288 h for quantification of the amount of secreted cB72.3 by Protein A HPLC.

Figure 4: The effect of BSO on growth and productivity of cell line 42 in batch suspension culture. 30 mL cultures in GSH free medium were supplemented with 0 to 5000 μM BSO and grown in batch suspension mode over 288 h. Conditions predicted to give partial (*pa*) and full *(fu)* inactivation of GCL activity are shown based on Clarke *et al.*[36]). Viable cell concentration (Figure 3) and Mean cell diameter (**A**) were monitored using a Vi-Cell (Beckman Coulter, UK). The integral viable cell concentration (IVC) was determined for each culture (**B**). Supernatant was removed at harvest (288 h) and subsequently analysed for the presence of cB72.3 (**C**) by Protein A HPLC. qmAb was then determined for each culture (**D**).

Figure 5: Ero1-Lα redox shift assay. GS-CHOK1SV cells in suspension were untreated (0 μM BSO) or treated with 20, 100 and 250 μM BSO. In addition reduced (10 mM DTT) and oxidized (10 mM DPS) controls were included. All cell samples were treated with a membrane permeable alkylating agent to freeze redox status following treatment with AMS to determine any change in migration relative to redox controls following SDS and western blot analysis with an antibody for Ero1-Lα.

Supplementary Figure 1: Comparison of the amounts of GCL mRNA and protein in GS-CHOK1SV cells lines and the CHOK1SV host. Samples were removed from batch suspension cultures after 96 h of culture time. CHO A and CHO B are replicate cultures of CHOK1SV host. **(A)** GCLc and **(B)** GCLm subunit mRNA amounts as determined by qRT-PCR, for 7 GS-CHOK1SV cell lines expressing cB72.3, CHOK1SV host and Null 8. Data shown are the mean of experimental replicates ± SD (n=3). **(C)** Western blot analysis of GCL subunits and GS in the CHOK1SV host, Null 8 and 5 GS-CHOK1SV cell lines expressing cB72.3. Protein extracts were separated by 1D-PAGE and western blotted, the blots were probed with antibodies for GCLc, GCLm, GS and β-Actin. **(D-F)** Amounts of GCLc, GCLm and GS proteins as determined by densitometric analysis of the relevant bands observed on the western blot (Figure 1C). All numercial data presented in Figures **A**, **B** and 1**D-F** have been normalized to CHOK1SV replicate A (CHO-A).

Supplementary Figure 2: Mean GSH concentrations in CHOK1SV host and GS-CHOK1SV cell lines. Samples were removed from batch suspension cultures of CHOK1SV host (CHO) and GS-CHOK1SV cell lines 54, 114, 53, 142 and 38 after 96 h of culture time. GSH concentration was subsequently determined using the GSH-Glo kit (Promega, UK) according to manufacturer's instructions. Data are the mean of two independent samples from the same culture ± SD.

Supplementary Figure 3: The effect of MSX on growth and productivity of cell lines Z1 and Z14 in fed batch suspension culture. Fed batch suspension culture of lineages supplemented with 0, 25, 50 and 75 μM MSX for 1344 h total culture time were completed. L-MSX concentration is plotted against IVC (**A** (Z1), **D** (Z14)), qmAb (**B** (Z1), **E** (Z14)), secreted mAb concentration (**C** (Z1), **F** (Z14)) from fed batch cultures for cells lines Z1 and Z14.

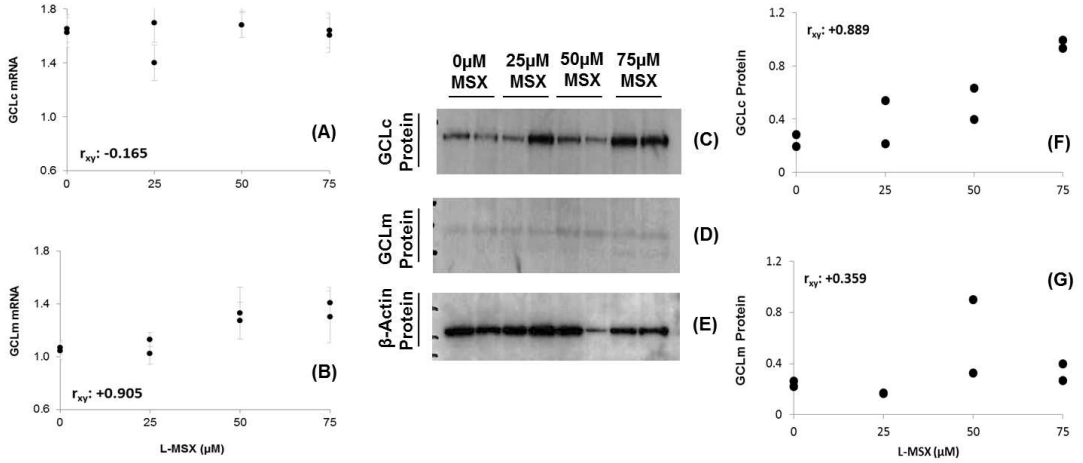


Figure 1

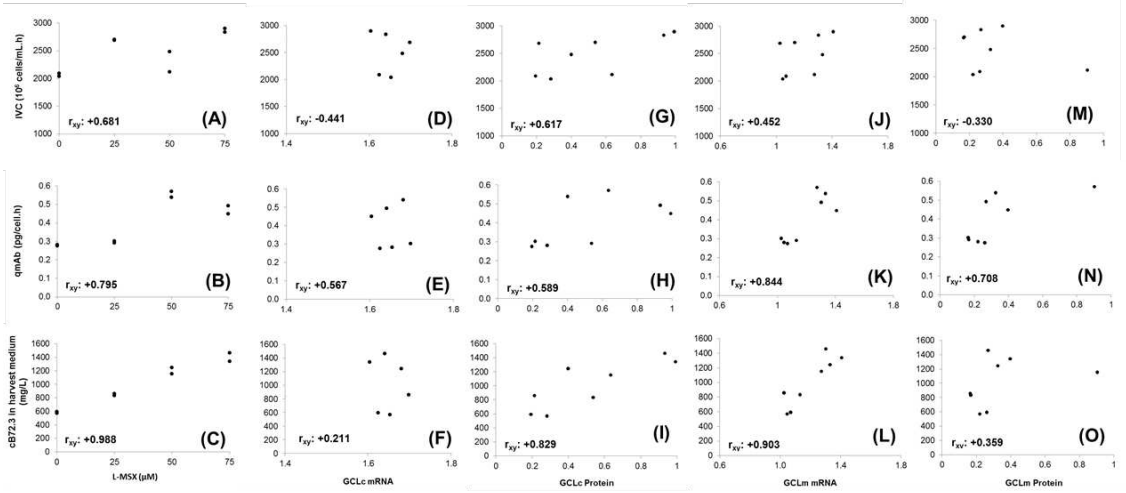


Figure 2

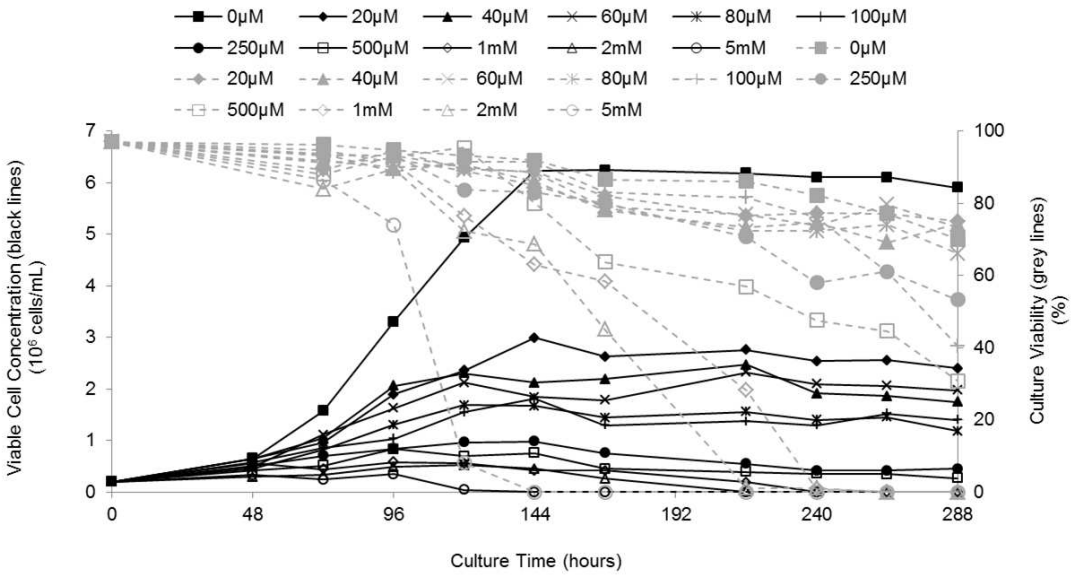


Figure 3

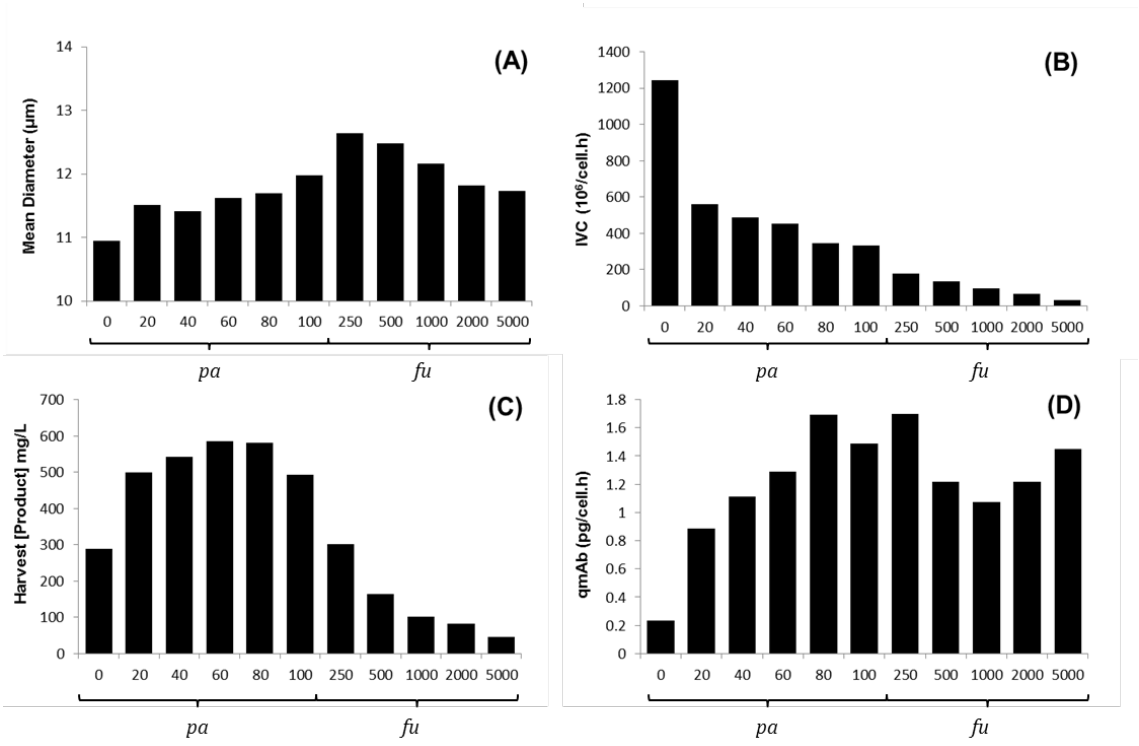


Figure 4

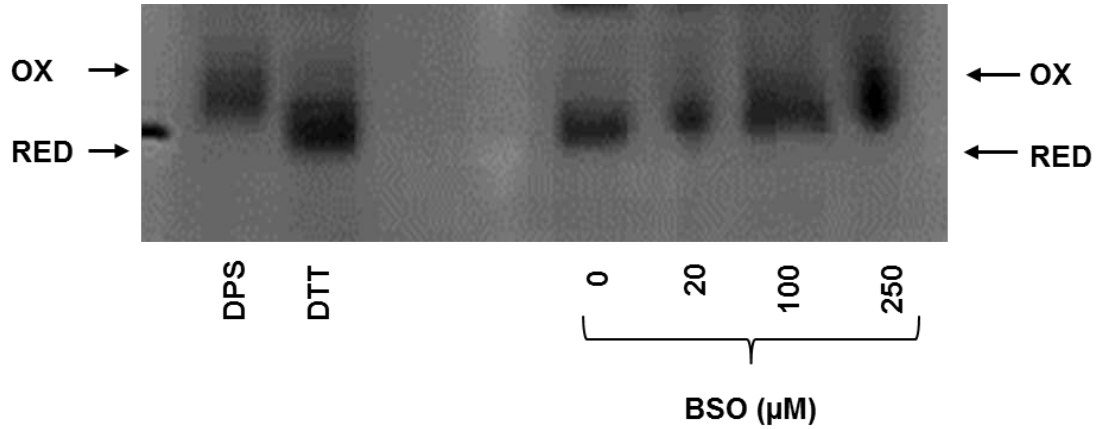
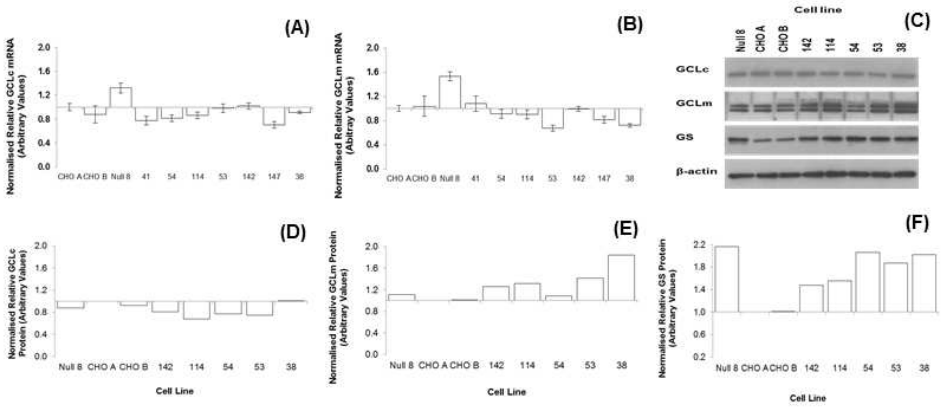
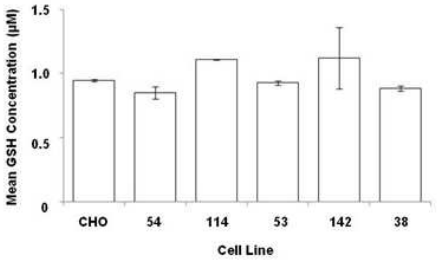


Figure 5

Supplementary I



Supplementary II



Supplementary III