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Reconstruction of reverse transsulfuration pathway enables cysteine biosynthesis and enhances resilience to oxidative stress in Chinese Hamster Ovary cells

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ABSTRACT

Cysteine is a critically important amino acid necessary for mammalian cell culture, playing key roles in nutrient supply, disulfide bond formation, and as a precursor to antioxidant molecules controlling cellular redox. Unfortunately, its low stability and solubility in solution make it especially problematic as an essential medium component that must be added to Chinese hamster ovary and other mammalian cell cultures. Therefore, CHO cells have been engineered to include the capacity of endogenously synthesizing cysteine by overexpressing multiple enzymes, including cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CTH) and glycine N-methyltransferase (GNMT) to reconstruct the reverse transsulfuration pathway and overcome a key metabolic bottleneck. Some limited cysteine biosynthesis was obtained by overexpressing CBS and CTH for converting homocysteine to cysteine but robust metabolic synthesis from methionine was only possibly after incorporating GNMT which likely represents a key bottleneck step in the cysteine biosynthesis pathway. CHO cells with the reconstructed pathway exhibit the strong capability to proliferate in cysteine-limited and cysteine-free batch and fed-batch cultures at levels comparable to wildtype cells with ample cysteine supplementation, providing a selectable marker for CHO cell engineering. GNMT overexpression led to the accumulation of sarcosine byproduct, but its accumulation did not affect cell growth. Furthermore, pathway reconstruction enhanced CHO cells' reduced and glutathione levels in cysteine-limited conditions compared to unmodified cells, and greatly enhanced survivability and maintenance of redox homeostasis under oxidative stress induced by addition of menadione in cysteine-deficient conditions. Such engineered CHO cell lines can potentially reduce or even eliminate the need to include cysteine in culture medium, which not only reduces the cost of mammalian media but also promises to transform media design by solving the challenges posed by low stability and solubility of cysteine and cystine in future mammalian biomanufacturing processes.

1. Introduction

To date, the Chinese hamster ovary (CHO) cells are the predominant host organism for recombinant therapeutic proteins such as monoclonal antibodies due to various advantages including their high adaptability to suspension culture conditions and capacity to achieve proper folding and disulfide bond formation of complex proteins as well as desirable post-translational modifications (Baycin-Hizal et al., 2012; Lim et al., 2010; Liu et al., 2015). However, unlike bacteria culture, successful culture of CHO cells requires a much more complex list of nutrients to be present in the culture media, including carbohydrates, amino acids, vitamins, etc. (Eagle, 1959; Ritacco et al., 2018). The very early culture media for CHO cells also required supplementation with serum but serum-free, chemically defined medium was developed later and is predominantly used now to avoid unwanted contamination and to achieve consistent, high productivities (Kim et al., 2020; Ritacco et al., 2018).

Careful design of chemically defined culture medium for CHO and other mammalian cells necessitates the inclusion of up to twenty amino acids given that they are metabolically indispensable and serve as building blocks of biomass and secreted proteins (Chen, Y. et al., 2021; Hosios et al., 2016; Salazar et al., 2016). Amino acids are usually classified as essential or non-essential based on the organisms' capability to synthesize them for sustaining growth and survival and the list of

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essential amino acids can differ when considering organisms or cells having distinct metabolic capabilities (Wu, 2015). Based on the genome-scale metabolic model developed for CHO cells and a corresponding essential nutrient minimization analysis performed by our group, 12 amino acids are identified as essential amino acids for general CHO–S, CHO-DG44 and CHO–K1 cell lines, including arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, valine, and cysteine (Chen, Y. et al., 2019; Hefzi et al., 2016). Although most of the amino acids need to be supplied in the culture media, attempts have been made to metabolically engineer CHO cells to enable a reduction or even removal of certain amino acids from the media by enhancing CHO cells' capability to synthesize them. For example, glutamine synthetase (GS) overexpression allows CHO cells to generate sufficient glutamine from glutamate, allowing cell growth in glutamine-free medium and GS is now widely used as a highly effective selection system for recombinant protein expression (Cockett et al., 1990; Fan et al., 2012; Hefzi et al., 2016). Also, Mulukutla et al. found that CHO cells can proliferate in tyrosine-free culture media after the enzyme phenylalanine hydroxylase was overexpressed which converts phenylalanine to tyrosine (Mulukutla et al., 2019).

Among the identified essential amino acids for CHO cells, cysteine is known to be a key component of the culture media for several reasons. First, apart from being building blocks of biomass and a key residue linking heavy and light chains in monoclonal antibody products, cysteine also plays a key role in mammalian cells as the potential rate-limiting precursor of antioxidant molecules including glutathione (GSH) (Lu, 2009; McBean, 2017; Ripps and Shen, 2012). Indeed, it has been reported that limitations of cysteine in CHO culture media can cause oxidative stress which results in irreversible detrimental effects on cell physiology and growth (Ali et al., 2019; Ghaffari, 2015; Ritacco et al., 2018). Second, due to its low stability and solubility in neutral pH, cysteine present in culture media is oxidized to form the dimer cystine and causes precipitation problems in feed medium, making it a challenge to incorporate in CHO bioprocesses (Rigo et al., 2004). To tackle this issue, several cysteine derivatives and analogs have been used to substitute the L-cysteine present in CHO cell culture media to stabilize culture media (Chevallier et al., 2021; Hecklau et al., 2016). In addition, high concentrations of cysteine have been reported to induce a cytotoxicity effect in mammalian cells (Nakanishi et al., 2005). Thus, extra attention is required when considering the inclusion of cysteine as a nutrient component in the design and development of mammalian cell culture media.

Unlike some other eukaryotic cells such as Sf9 insect cells, CHO cells do not survive and proliferate in cysteine-free medium (Doverskog et al., 1998; Naylor et al., 1976). However, cysteine is known as an organism-wise non-essential amino acid for mammals due to the biosynthetic capability mainly observed in liver (Pajares and Pérez-Sala, 2018; Stipanuk et al., 2006). The active pathway in liver which converts methionine and serine to cysteine is known to be the metabolic pathway responsible to produce cysteine in mammals (Sbodio et al., 2019), and the conversion includes five major steps (see Fig. 1A). While the conversion from methionine to cysteine is sometimes referred to as the reverse transsulfuration pathway, in this article we will use the more general term, "transsulfuration pathway" for overall cysteine biosynthetic pathway. In this study, we introduce the first reconstruction of the transsulfuration pathway in CHO cells enabling the intracellular synthesis of cysteine and allowing cell proliferation in culture media without cysteine or cystine. It is observed that CHO cells lack the expression of two enzymes, cystathionine-beta-synthase (CBS) and cystathionine gamma-lyase (CTH) that convert homocysteine to cysteine, which are known to be expressed in a highly tissue-specific manner, principally in liver (Ali et al., 2020; Werge et al., 2021; Zhu, H. et al., 2018). In this study, we overexpress human CBS and CTH enzymes in CHO cells to complete the transsulfuration pathway, followed by the enhancement of the methylation reaction by overexpressing the human glycine N-methyltranserase (GNMT) enzyme which is identified to be a rate-limiting step of transsulfuration pathway in cancer cells (Weber and Birsoy, 2019; Zhu, J. et al., 2019). Cell lines generated via overexpression of the three enzymes demonstrate strong growth capability in cysteine/cystine-free medium, and the addition of minimal cysteine/cystine can boost their growth to full capacity comparable to unmodified cells growing in ample cysteine/cystine supplement. In addition, the cell lines with cysteine synthetic capability exhibit enhanced growth and resilience under induced oxidative stress conditions in a low cysteine condition. Overall, our work tackles multiple existing challenges in CHO cultures and demonstrates the potential to

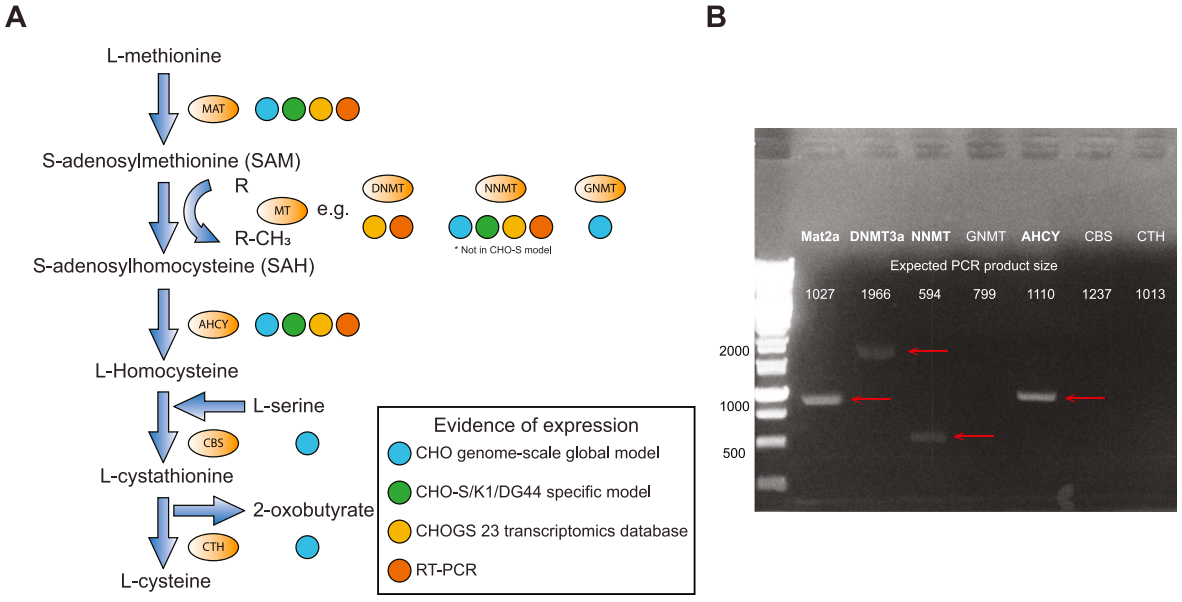

Fig. 1. Investigation of transsulfuration pathway activity in CHO cell. (A) Diagram of the mammalian transsulfuration pathway and evidence of enzyme expression according to multiple sources. MAT: Methionine adenosyl transferase. MT: Methyltransferase. DNMT: DNA N-methyltransferase. NNMT: Nicotinamide N-methyltransferase. GNMT: Glycine N-methyltransferase. AHCY: Adenosylhomocysteinase. CBS: Cystathionine beta-synthase. CTH: Cystathionine gamma-lyase. (B) DNA gel-electrophoresis result of RT-PCR products detecting expression of genes of interest for CHOGS 23 cell line. Genes expressed with detectable PCR products (red arrows) are bolded.

improve mammalian cell-based biopharmaceutical processes via the metabolic reconstruction of a critical essential amino acid biosynthetic pathway.

2. Materials and methods

2.1. Expression plasmid design

The gene sequences of human CBS, CTH and GNMT were identified and DNA fragments were cloned from plasmids acquired from DNASU plasmid repository (HsCD00002251, HsCD00002300 and HsCD00039660) via PCR. DNA fragments coding human CBS and CTH were cloned into the multiple cloning sites of pBudCE4.1 bicistronic mammalian expression vector (Invitrogen) using restriction enzymes and T4 DNA ligase purchased from New England Biolabs (NEB) following corresponding protocols. Specifically, human CBS was cloned into HindIII and SalI sites to be under the regulation of cytomegalovirus (CMV) promoter, and human CTH was cloned into NotI and XhoI under the control of human elongation factor 1 alpha (EF1α) promoter. Human GNMT sequence was cloned into pcDNA3.1$^{(+)}$ (Invitrogen) HindIII and XhoI sites following CMV promoter.

2.2. Stable cell line development

A CHOGS 23 cell line acquired from MilliporeSigma was used as host for overexpression of target enzymes. The CHOGS 23 cell line was maintained in a customized culture medium (referred as medium A) without glutamine. Expression vector containing sequences of human CBS and CTH was introduced into the CHOGS 23 cell line via TransIT-PRO transfection reagent (MirusBio) following vendor's protocol. Stable pool of transfected cells was selected by exposing the cell with 500 μg/mL Zeocin and replacing medium every 3–4 days. After the cells recovered from Zeocin, another selection based on activity of CBS and CTH was performed by culturing the cell pool in another customized medium without cysteine/cystine (referred as medium B) and supplemented with L-homocysteine at 0.5 mM. With medium replacement every 3–4 days, cell proliferation was observed after one to two weeks and the cell pool was stably expressing active CBS and CTH (referred as CHOGS-CC-2 or CC-2). The CHOGS-CC-2 cell pool was then subsequently transfected with the plasmid containing GNMT gene and selected with 500 μg/mL Zeocin together with 350 μg/mL G418. Finally, selection based on the cysteine biosynthesis capability was performed by maintaining the cells in cysteine/cystine-free medium B, and the recovered proliferating cells referred as CHOGS-CCG-3 or CCG-3. Subsequently, single clones for both CHOGS-CC-2 and CHOGS-CCG-3 were isolated via limiting dilution approach and top cell clones were selected based on growth performance in their respective selection condition (homocysteine-based growth and cysteine/cystine-free growth).

2.3. Batch/fed-batch cell culture

Batch and fed-batch cultures were performed by seeding 30 mL of CHO cell culture in cysteine/cystine-free medium B at 0.3×10^6 cell/mL into 125 mL vented Erlenmeyer flasks (Nalgene), supplied with designated amount of L-cysteine or L-homocysteine with prepared stock solution. Flasks were maintained in shaking incubator (Infors HT) operating at 37 °C, 90% relative humidity, 5% CO_2 and shaking at 120 rpm. Every 24 h, 0.35 mL sample was taken for cell density and spent media analysis. Viable cell density and viability were measured with a Cellometer Auto T4 automated cell counter (Nexcelom) with trypan blue staining. Glucose and lactate concentrations were measured using a YSI 2950D biochemical analyzer (Yellow Spring Instrument). Concentrations of methionine, glycine, serine, sarcosine and other amino acids were measured using REBEL bioprocess analyzer (908 Devices). For fed-batch, 1.5 mL of customized feed X (concentrated feed without tyrosine and cysteine/cystine) and 1.5 mL of customized feed Y (tyrosine feed, cysteine supplied separately) were added into the flask every day starting on day 3, and 250 μL of D-glucose stock solution (450 g/L) was added into the culture if measured glucose level drops below 2 g/L before feeding.

2.4. Glutathione assay and menadione test

CHO cell pellets were collected on day 3 or day 5 of culture with medium B supplied with 1 mM, 0.5 mM and 0 mM of L-cysteine and intracellular glutathione (GSH) content was measured with colorimetric glutathione assay kit (G-Biosciences). First, cell pellets were resuspended in deproteination reagent and homogenized by sonication to extract cell lysate. Then, triplicates of oxidized glutathione (GSSG) standard and cell lysate samples were loaded into 96 well plates and incubated with working assay mixture and Ellman's working solution at room temperature following vendor's protocol for total GSH content (GSH + GSSG). For intracellular GSSG content measurement, samples and standards were treated with 4-vinylpyridine before loading. Absorbance at 415 nm was measured 25 min after adding Ellman's reagent to establish standard curve and sample quantification. Reduced GSH content was estimated by subtracting GSSG content from total GSH content.

For menadione-induced oxidative stress test, CHO cells were seeded at 1×10^6 cell/mL into 20 mL cysteine/cystine-free medium B supplied with L-cysteine to achieve an initial concentration of 0.1 mM and maintained in the condition mentioned above. 24 h after seeding, menadione was introduced into the culture at 20 μM, 35 μM and 50 μM by adding stock solution prepared with menadione sodium biphosphate (MSB) and phosphate buffered saline (PBS). Viable cell density and viability were monitored every 12 h afterwards until day 4.

2.5. RT-PCR and Western blot analysis

First, cell pellets were collected by centrifuging day 3 cell culture and discarding supernatant, followed by washing with PBS. For RT-PCR, total RNA was first collected using RNeasy Mini Kit (Qiagen) following vendor's protocol. Reverse transcription (RT) reaction was performed to obtain cDNA using Moloney Murine Leukemia Virus reverse transcriptase protocol together with Oligo$(dt)_{15}$ primer, dNTP mix and RNasin Ribonuclease Inhibitor purchased from Promega. Subsequently, PCR was performed using Phusion DNA polymerase (NEB) with obtained cDNA as template and primers designed for detecting the sequences of genes of interest. Finally, PCR products were visualized via DNA gel electrophoresis approach and expression of genes was evaluated.

For Western blot, cell lysis was performed for the collected CHO cell pellets using M-PER mammalian protein extraction reagent (Thermo Scientific). Bicinchoninic acid (BCA) assay was performed using Pierce BCA protein assay kit (Thermo Scientific) to determine the concentration of protein in the lysate. Collected cell lysate was then denatured by heating at 95 °C for 5 min after addition of beta-mercaptoethanol. Subsequently, SDS-PAGE was performed after loading samples containing 30 μg of total protein per sample, followed by protein transfer onto Immun-Blot PVDF membrane (Biorad). For detection of beta-actin, CBS and CTH protein, anti-beta-actin rabbit polyclonal antibody (Cell Signaling, #4967), anti-CBS rabbit monoclonal antibody (Abcam, ab140600) and anti-CTH rabbit monoclonal antibody (Abcam, ab189916) were used as primary antibody and HRP-conjugated goat anti-rabbit IgG antibody (Abcam, ab205718) was used as secondary antibody. For detection of GNMT, anti-GNMT mouse monoclonal antibody (Novus Biologicals, MAB6526) was used as primary antibody and HRP-conjugated goat anti-mouse IgG antibody (Novus Biologicals, HAF007) was used as secondary antibody. Finally, SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was used to detect the protein signal on the blot.

3. Results

3.1. Examination of transsulfuration pathway in CHO

To investigate possible limitations within the cysteine biosynthesis pathway in CHO cells, expression of enzymes comprising the transsulfuration pathway in CHOGS 23 cell line was investigated by reviewing multiple different sources of evidence (Fig. 1A). Enzymes in the transsulfuration pathway include methionine adenosyl transferase (MAT), methyltransferase (MT), adenosylhomocysteinase (AHCY), cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CTH). The first reaction, MAT, converts L-methionine to S-adenosylmethionine (SAM). For mammals, two homologous catalytic subunits MAT1A and MAT2A are expressed, for which MAT1A is predominantly expressed in liver and MAT2A is widely distributed in extrahepatic tissues (Kotb and Kredich, 1985; Kotb et al., 1997). The second step is regulated by MTs which convert SAM to S-adenosylhomocysteine (SAH), with a methyl group acceptor molecule involved as co-substrate. For MTs, three different N-methyltransferases using common metabolites as co-substrates were selected to be examined, including DNA N-methyltransferase (DNMT), nicotinamide N-methyltransferase (NNMT) and glycine N-methyltransferase (GNMT). The third step, adenosylhomocysteinase (AHCY), converts SAH to L-homocysteine (Hcy). AHCY, known to be one of the most conserved enzymes in many different organisms, is the only enzyme in mammals regulating the conversion from SAH to Hcy and is expressed in most tissues (Chen, N. C. et al., 2010; Vizán et al., 2021). The last two steps of the cysteine synthetic pathway are regulated by CBS and CTH, which convert Hcy and L-serine to form L-cystathionine, which is subsequently broken down to form 2-oxobutyrate and L-cysteine. As mentioned previously, CBS and CTH are both known to be highly tissue specific and are predominantly found in liver, kidney and the pancreas (Ali et al., 2020; Werge et al., 2021; Zhu et al., 2018).

Initially, the transsulfuration pathway was evaluated in global and cell-line specific CHO genome-scale models (Hefzi et al., 2016) and the existence and expression of the relevant genes for these representative CHO cell lines determined. As presented in Fig. 1A, most of the enzymes relevant to transsulfuration pathway are present in the CHO genome as suggested by the CHO genome-scale global model except for DNMT which is not represented as a separate metabolic reaction in the model. However, CHO cell-line specific models which are derived from CHO transcriptomics and proteomics data suggest that GNMT, CBS and CTH do not exhibit sufficient expression for these reactions to be functional (Hefzi et al., 2016; Lewis et al., 2013; Palsson et al., 2011). Next, transcriptomics data was also reviewed specifically for CHOGS 23 cell line, which indicated MAT, DNMT, NNMT and AHCY expression is present in the CHOGS 23 cell line but the expression of GNMT, CBS and CTH enzymes is negligible. In addition, an RT-PCR was performed to further confirm the enzyme expression profile of the CHOGS 23 cell line used in our study (Fig. 1B). This analysis suggests that activity of CBS and CTH is likely to be necessary for CHO cells to achieve robust cysteine biosynthesis, and GNMT may also be a rate-limiting step. Overall, these three enzymes, (GNMT, CBS and CTH) are thus potential targets of metabolic engineering for trassulfuration pathway reconstruction in CHO.

3.2. Overexpression of CBS, CTH and GNMT in CHO cells

Given the absence or lack of specific relevant enzymes within the transsulfuration pathway in CHO and potential target enzymes for overexpression identified, the missing genes, including CBS, CTH, and GNMT were transfected into CHOGS 23 and new CHO cell lines isolated (Fig. 2A). DNA sequences of human CBS and CTH were first cloned into the pBudCE4.1 bicistronic mammalian expression vector, transfected into CHOGS 23, and cell pools surviving after Zeocin selection were further selected for sequential CBS and CTH activities by culturing in cysteine/cystine-free medium B (MilliporeSigma, see Materials and Methods) supplemented with L-homocysteine. The resulting cell pool was designated as the CC-2 pool. Next, the human GNMT sequence was cloned into the pcDNA3.1$^{(+)}$ mammalian expression vector and transfected into the CC-2 cell pool. After G418 selection, the cell pool was maintained in cysteine/cystine-free medium B until proliferation was observed to select for cysteine biosynthetic capability. This CHO cell

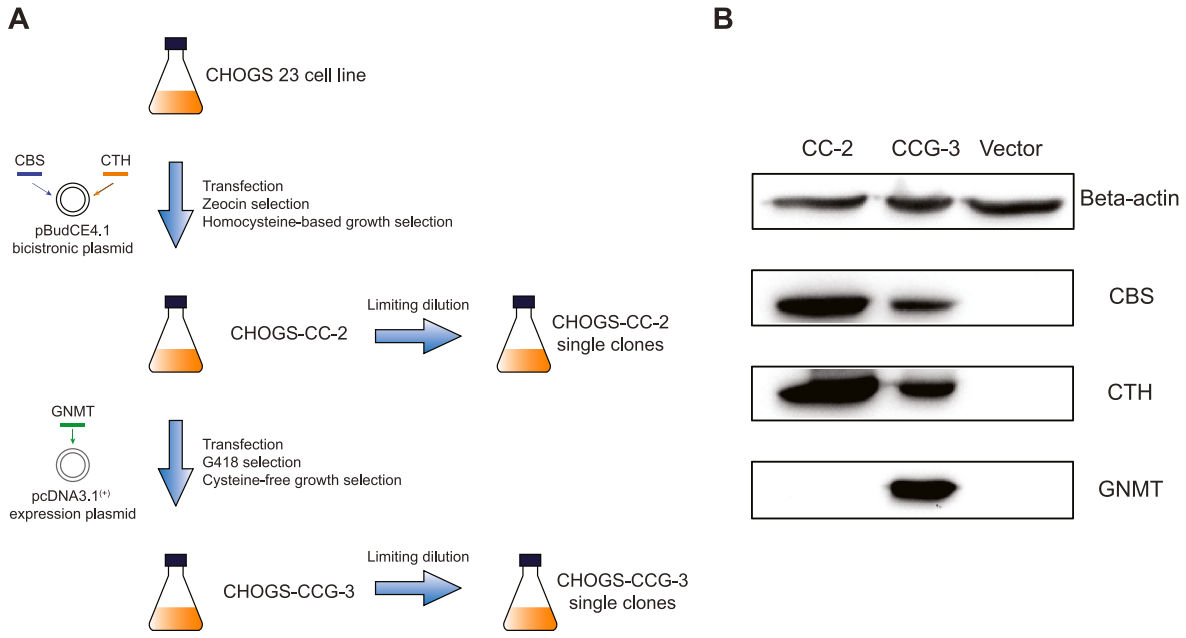

Fig. 2. Development of CHO cell lines with CBS, CTH and GNMT expression and activity. (A) Procedure of generating cell lines via plasmid transfection, antibiotic selection, metabolism-specific selection and single cell cloning. The cell pool/line developed having expression of CBS and CTH is referred as CHOGS-CC-2 (or CC-2), and the cell pool/line with CBS, CTH and GNMT expression is referred as CHOGS-CCG-3 (or CCG-3). (B) Western blot images of developed cell lines for CC-2 and CCG-3 and a negative control cell line transfected with an empty vector. Bands indicate the presence/absence of proteins in cell. Beta-actin was used as a positive control.

pool obtained containing transfected CBS, CTH and GNMT is referred to as the CCG-3 pool. After single cell cloning of CC-2 and CCG-3, Western blot analysis confirmed the expression of human CBS and CTH in the CC-2 and CCG-3 cell lines and human GNMT expression in the CCG-3 cell line (Fig. 2B).

To investigate the effect of the overexpressed CBS and CTH enzymes with or without GNMT on cell metabolism and proliferation in cysteine-depleted environments, batch cultures were conducted for CHOGS-CC-2, CHOGS-CCG-3, a negative control (Vector, transfected with empty vectors) and unmodified CHOGS 23 (WT). Cell clones were exposed to cysteine-limited (0.1 mM L-cysteine [Fig. 3A]), cysteine-depleted medium B with a metabolic precursor (0 mM L-cysteine + 0.5 mM L-homocysteine [Fig. 3B]), and medium B without any cysteine or precursors added (0 mM L-cysteine [Fig. 3C]). When supplied with low levels of cysteine (0.1 mM L-cysteine in Fig. 3A), all the cell lines grew but the unmodified WT cell line only reached a maximum VCD of about 1.4 million cells/mL before experiencing a drop in cell number and viability starting at 4 days. The CC-2 cell line reached a higher VCD of about 1.8 million cells/mL and experienced a slower decline in viability after 4 days of growth. This observation indicates that the introduction of CBS and CTH may enhance, at least moderately, the cysteine generation pathway, indicating endogenous methyltransferases such as DNMT and NNMT present in CHO cells can produce limited amounts of the metabolic precursor SAH. In contrast, the CCG-3 cell line reached a cell density that was nearly 4 times higher than the CC-2 cell line and maintained a viability above 95% throughout the batch culture.

When the media was instead supplemented with 0.5 mM L-homocysteine in complete cysteine-free conditions, CC-2 grew for a longer time period (5 days) and maintained viabilities above 80% over the complete batch culture while the control Vector cells were unable to grow extensively in this medium (Fig. 3B). These results indicate that CBS and CTH were indeed expressed and functionally active in CC-2. Alternatively, the CCG-3 cell line, containing the combination of CBS, CTH and GNMT, exhibited even more robust proliferation capability, reaching a VCD double that of the CC-2 cell lines in homocysteine supplemented media, suggesting that the conversion of SAM to SAH may be a potential bottleneck step in the transsulfuration pathway of CC-2. Finally, only the CCG-3 cells were able to grow significantly in media with 0 mM cysteine (Fig. 3C), reaching 6 million cells/mL with viabilities approaching 100% to indicate that the combination of CBS, CTH and GNMT is able to boost the intracellular cysteine biosynthetic capability and sustain cell growth over the course of the batch experiment.

Extracellular concentrations of L-methionine, the initial reactant in the transsulfuration pathway (reactant of the first reaction, MAT), were measured for CCG-3 batch cultures described above at cysteine-limited conditions (0.1 mM and 0 mM). WT cells cultured in medium B supplied with 1 mM L-cysteine were included as controls representing

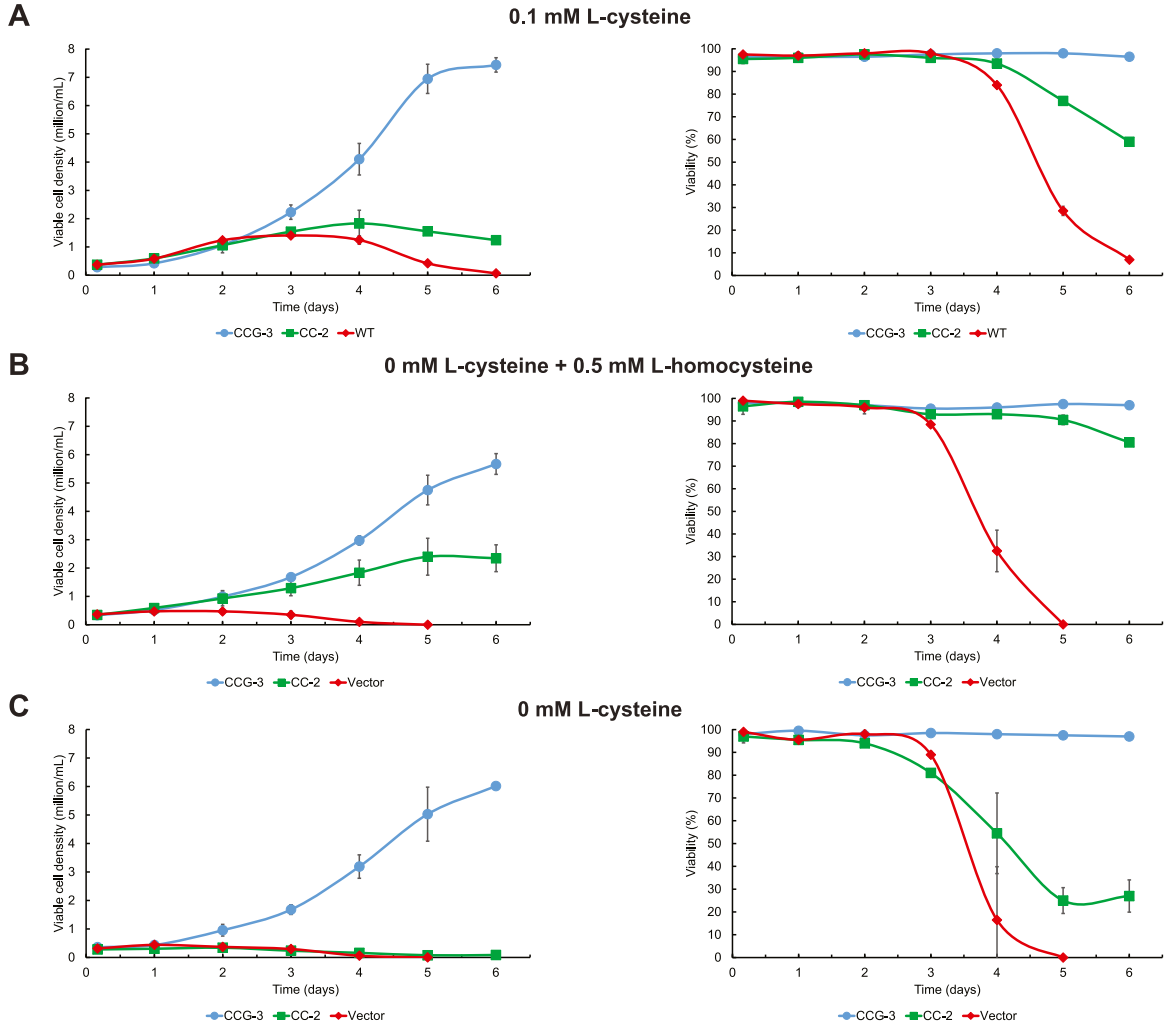

Fig. 3. Viable cell density and viability of CHOGS-CCG-3, -CC-2, negative control (vector) and unmodified CHOGS 23 (WT) cell lines for batch cultures in cysteine/cystine free medium B supplied with different amount of L-cysteine or L-homocysteine. (A) Supplied with 0.1 mM L-cysteine. (B) Supplied with 0 mM L-cysteine and 0.5 mM L-homocysteine. (C) Supplied with 0 mM L-cysteine. Error bars represent standard deviations of the biological duplicates.

typical culture behavior (growth profiles of WT and other cell lines at 1 mM cysteine are shown in Supplementary Fig. S1). As presented in Fig. 4A, methionine levels in the 2 CCG-3 cultures were comparable to WT for the first 3 days but declined at a more rapid rate afterwards. Interestingly, the methionine levels were exhausted first in the CCG-3 cultures supplemented with 0.1 mM cysteine by 5 days and by 6 days in the CCG-3 cultures supplemented with 0 mM cysteine. The enhanced methionine consumption in CCG-3 suggests enhanced MAT activity in CCG-3 cell line compared to WT even though the MAT gene was not overexpressed. As MAT is endogenously expressed in both CCG-3 and WT, it may be that the accumulation of MAT product, SAM, in WT inhibits the activity of MAT in a negative feedback manner. Alternatively, the overexpression of GNMT in CCG-3 reduces excessive SAM which prevents the inhibition and eventually results in elevated methionine consumption Indeed, a previous study suggested that enzymatic activity of MAT is downregulated when SAM levels are high (Quinlan et al., 2017). Also, the increased methionine utilization in the CCG-3 clones with 0.1 mM cysteine at later times is consistent with the enhanced growth observed in the clones with limited cysteine addition as compared to no added cysteine in Fig. 3. Despite higher daily methionine utilization observed for 0.1 mM cysteine case, the cultures with 0 mM cysteine exhibit a slightly higher cell-specific methionine consumption rate, suggesting that more methionine may have been utilized to drive the transsulfuration pathway and generate sufficient cysteine in a cysteine-free environment.

In addition to measuring methionine depletion, levels of extracellular sarcosine were also evaluated. As shown in Fig. 4B, measurable levels of extracellular sarcosine were observed in CCG-3 batch cultures with 0 mM and 0.1 mM cysteine at 2 days and increased over the remainder of the cell culture period. In contrast, no sarcosine was observed in the spent media for the WT cultures supplied with 1 mM cysteine over the duration. Since GNMT converts SAM to SAH and methylates glycine to form sarcosine, CCG-3 with overexpressed GNMT is likely to produce sarcosine intracellularly that is subsequently secreted into the extracellular environment. Interestingly, no significant difference was observed for the depletion or accumulation of other extracellular amino acids and metabolites measured for CCG-3 in comparison to WT, and most amino acids and metabolites remained detectable by the end of the batch culture (data not shown).

To further examine the growth behavior of the cell lines in extended cysteine-deficient culture conditions, CCG-3, CC-2 and WT cell lines were cultured in a fed-batch mode with medium B as basal medium and feed X and Y media supplied in defined volume daily starting at day 3 (see Materials and Methods for details). Basal cysteine/cystine requirements were addressed by supplying L-cysteine at either 0%, 10% or 100% of the amount listed in the original basal and feed media composition. Growth and viability were measured for WT, CC-2, and CCG-3 in 100% cysteine fed-batch (control), the three cell lines cultured in 10% cysteine condition plus CCG-3 in 0% cysteine conditions. As shown in Fig. 5A, viable cell numbers of the WT, CC-2 and CCG-3 exhibited similar growth profiles when supplied with 100% cysteine. The cell lines reached similar cell numbers, although WT reached a peak cell number about 1 or 2 days earlier than the CC-2 and CCG-3 cell lines. The CCG-3 cell line also maintained a higher viability from 9 through 12 days to suggest that the presence of a cysteine biosynthesis pathway may have enhanced the robustness of this cell line. When supplied with 10% cysteine, only the CCG-3 cell line was able to proliferate at a growth performance profile comparable to that in 100% cysteine condition, while growth of CC-2 and WT was limited and viabilities declined earlier due to lack of sufficient cysteine (Fig. 5B). Again, the peak cell number of CC-2 culture was twice as high as the WT culture for the 10% cysteine condition, in agreement with Fig. 3C to indicate that the enzymatic activity of CBS and CTH provides some limited cysteine generation capabilities. Nonetheless, the maximum viable cell number of CCG-3 culture was still around 3 times higher than the CC-2 culture and indicative of the important role that the GNMT enzyme plays in completing the transsulfuration pathway. Furthermore, even when completely deprived of cysteine, CCG-3 (0% cysteine in Fig. 5B) still exhibited strong proliferation capabilities with only a slightly lower peak cell number relative to the 10% cysteine culture.

3.3. *Intracellular biosynthesis of cysteine enhances glutathione content and cell resilience in insufficient cysteine conditions under menadione-induced oxidative stress*

As cysteine is a precursor of glutathione, which is known to be important for maintaining the cell redox balance, intracellular glutathione (GSH) content was also measured for the WT, CC-2 and CCG-3 cell lines in batch culture supplied with 1 mM (approximately 100% of medium B level), 0.5 mM and 0 mM of L-cysteine to investigate the impact of intracellular transsulfuration pathway activity (Fig. 6). In batch cultures supplied with 1 mM cysteine, all three CHO cell lines have comparable intracellular total (reduced and oxidized; Fig. 6A) and reduced GSH (Fig. 6B) content on day 3 suggesting that the cells maintain a healthy redox environment when extracellular cysteine is plentiful. On day 5, the total and reduced GSH levels were slightly reduced across all three cell lines, although the overall levels remained elevated. However, when 0.5 mM cysteine (approximately 50% of medium B level) was initially added to the cultures, total and reduced GSH content in CC-2 and WT cells was significantly reduced by day 5 when compared to the CCG-3 cell line, likely due to a lack of cysteine, which also impacts the growth of WT and CC-2 to a lesser extent

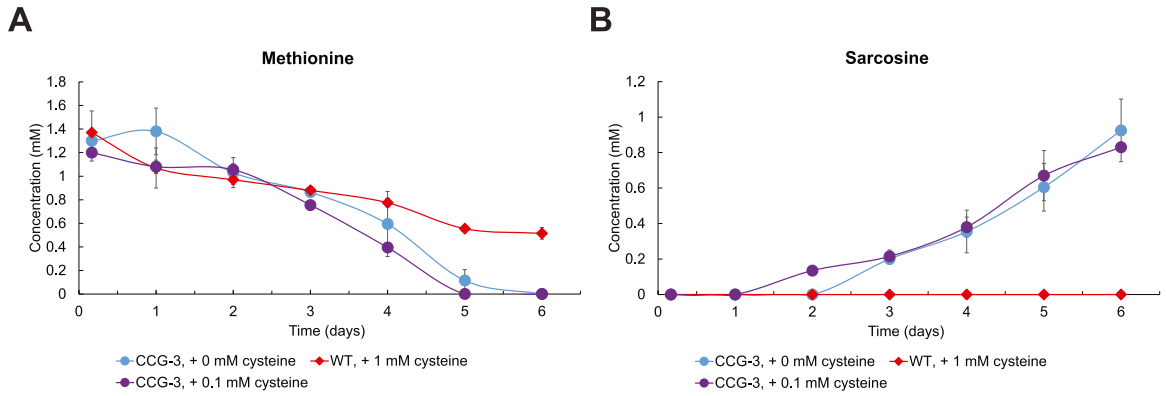

Fig. 4. Concentration profiles of methionine (A) and sarcosine (B) in CCG-3 cultures supplied with 0 mM cysteine (Figs. 3C) and 0.1 mM cysteine (Fig. 3A), compared with concentrations in WT cultures supplied with 1 mM cysteine as control (Supplementary Fig. 1). No sarcosine was detected in WT cultures. Zero value indicates concentration below detection limit. Error bars represent standard deviations of biological duplicates.

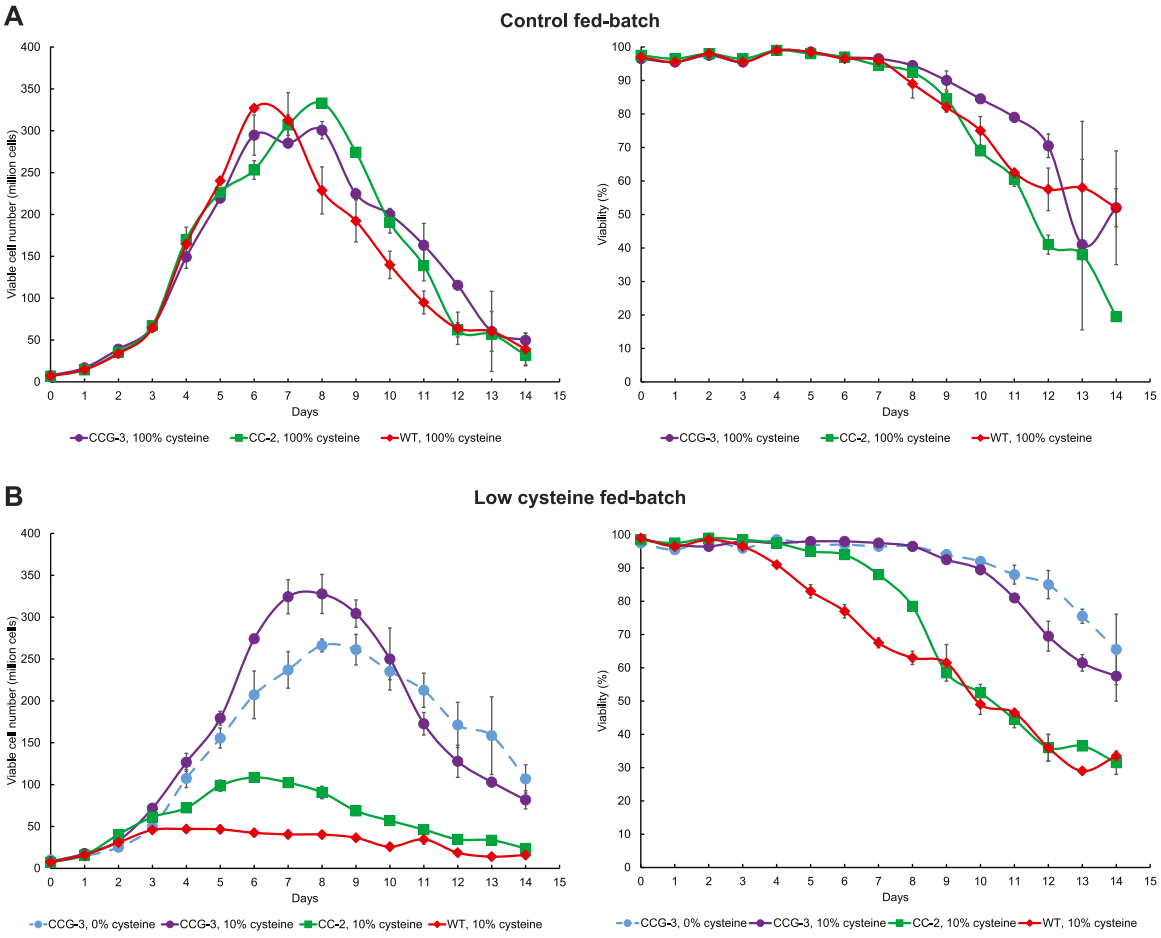

Fig. 5. Measured fed-batch viable cell number and viability profiles of CCG-3, CC-2 and WT. (A) Control fed-batch supplied with 100% L-cysteine quantity in basal and feed media. (B) Low cysteine fed-batch supplied with 10% or 0% (for CCG-3 only) L-cysteine levels in basal and feed media. Error bars represent standard deviations of biological duplicates.

(Supplementary Fig. 3). On the other hand, total and reduced GSH content in CCG-3 was significant at both 0.5 mM and 0 mM cysteine levels, indicating that an active cysteine biosynthetic pathway enables CHO cells to maintain robust total and reduced glutathione levels even in the absence of fed cysteine. In contrast, WT and CC-2 cell lines did not survive to day 5 in cysteine-free condition and thus no glutathione was detected.

To investigate the effects of an active transsulfuration pathway for providing redox protection and cellular homeostasis in a cysteine-deficient medium, the resilience of WT, CC-2 and CCG-3 cells was evaluated in low (0.1 mM) cysteine condition and subjected to menadione, a chemical commonly used to generate excessive reactive oxygen species (ROS) in mammalian cells (Loor et al., 2010). Specifically, the three cell lines were seeded at 1 million cells/mL into medium B with 0.1 mM cysteine and menadione sodium biphosphate (MSB) was introduced into the culture 24 h later at concentrations of 0 μM, 20 μM, 35 μM and 50 μM. As shown in Fig. 7A, the wild type cells grew only marginally to approximately 2.2 million cells/mL and this growth was suppressed at increasing levels of MSB while the viabilities showed increasingly steep declines with increasing MSB concentrations starting on day 2. The CC-2 cell line grew slightly better to reach a final maximum cell density of 2.9 million cells/mL which was progressively impacted at increasing levels of MSB. However, the viability of CC-2 cells was not impacted when exposed to 20 μM and 35 μM MSB but declined at 50 μM MSB to suggest improved resilience over the WT. Finally, CCG-3, with the strongest cysteine producing capability, survived and even proliferated over the culture duration with viable cell densities much higher than that of WT and CC-2 in the absence and presence of MSB. Even when exposed to 50 μM MSB, CCG-3 cells exhibited slow but sustained growth up to 3.8 million cells/mL by day 4 at which point the VCD of the other 2 cultures had fallen below 0.5 million cells/mL. Furthermore, while viability of both WT and CC-2 dropped significantly following treatment with 50 μM MSB, only a very slight viability decline was observed in the CCG-3 culture at the same treatment level. CCG-3 cells were able to maintain overall viabilities above 92% even out to 3 days after treatment with the ROS generating chemical (day 4). Overall, the results presented here demonstrate that cysteine generated intracellularly can enhance cell viability and protection against oxidative stress even in a cysteine-depleted culture environment.

4. Discussion and conclusion

In mammalian cell culture processes, cysteine is often fed in a separate feed medium together with tyrosine apart from the other amino acids due to its low stability and solubility in neutral pH, and this property of cysteine, along with its requirement as an essential amino acid, represents a challenge in optimizing bioprocesses (Salazar et al., 2016). In order to tackle this challenge, attempts have been made to modify the cysteine molecule such as to make cysteine/cystine equivalents and derivatives with improved stability and solubility (Chevallier et al., 2021; Giustarini et al., 2012; Grinberg et al., 2005; Hecklau et al., 2016; Issels et al., 1988). Another angle of solving the problem is to reduce the requirement of cysteine in cell culture through modification to cellular metabolism, as introduced in this study.

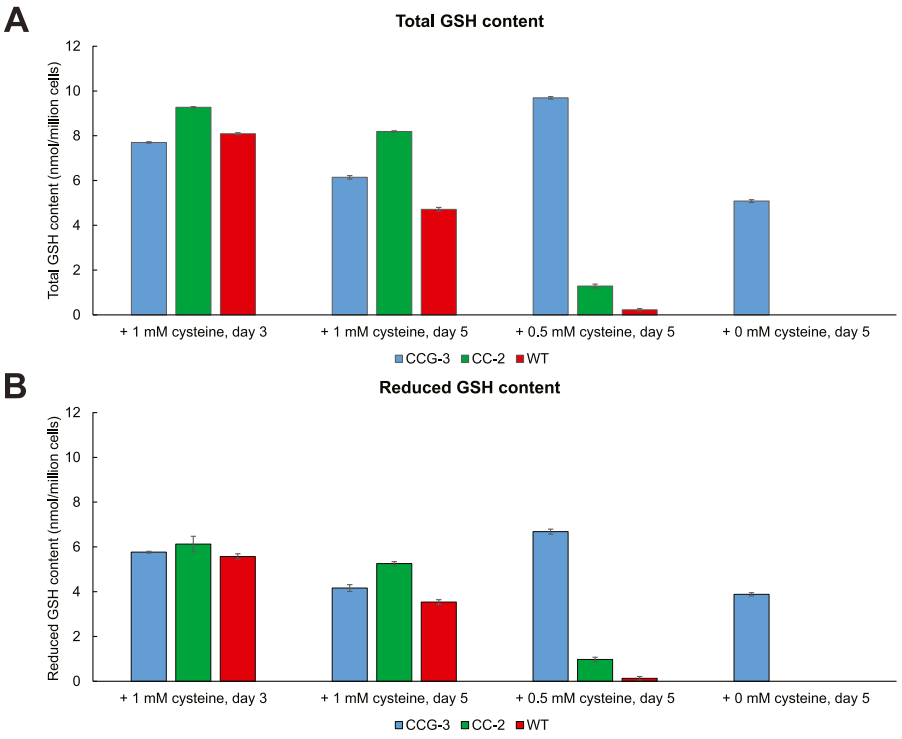

Fig. 6. Intracellular total (A) and reduced (B) glutathione (GSH) content measured for day 3 or 5 CCG-3, CC-2 and WT cells in batch cultures supplied with 1 mM, 0.5 mM and 0 mM L-cysteine at the beginning of culture. CC-2 and WT cells did not survive to day 5 at 0 mM cysteine condition. Error bars represent standard deviation of triplicate measurements.

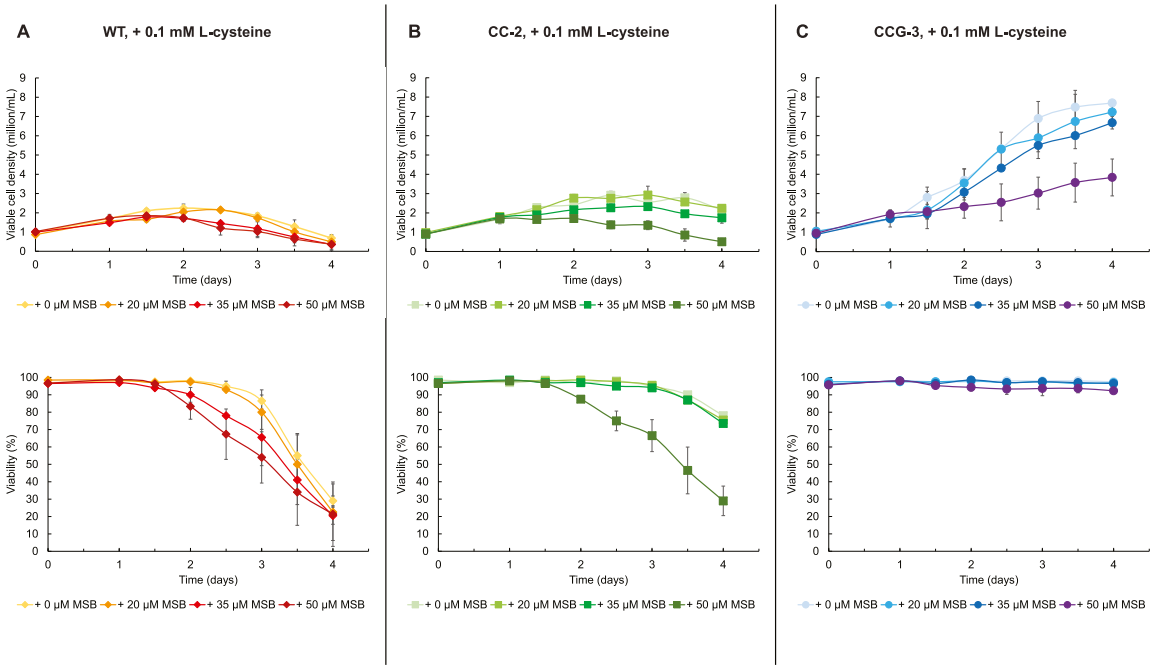

Fig. 7. Viable cell density and viability of WT (A), CC-2 (B) and CCG-3 (C) cells cultured in medium with 0.1 mM L-cysteine supplied menadione sodium biphosphate (MSB) at 0 μM, 20 μM, 35 μM and 50 μM. MSB is added to the cell culture on day 1. Error bars represent standard deviations of biological duplicates.

While the transsulfuration pathway has been well-characterized, few studies have characterized the complete pathway of cysteine biosynthesis in CHO and up to now there is no CHO cell line reported with the capability to generate sufficient cysteine to survive in cysteine-deficient medium. Recently, Ali et al. reported that low or no expression of CBS and CTH was observed in CHO cells according to proteomics and transcriptomics while cystathionine was detected intracellularly, suggesting that a deficiency in the levels of these two enzymes is likely a limiting factor for cysteine production in CHO cells (Ali et al., 2020; Ali et al., 2020). Indeed, transcriptomics- and proteomics-based evidence presented in this study agrees with the hypothesis, and the CC-2 cell line overexpressed with CBS and CTH does demonstrate homocysteine

utilization and limited cysteine generation capabilities not observed in the unmodified CHO cell line. Although presented ubiquitously in mammals, CBS and CTH are known to exhibit highly tissue-specific expression as they are predominantly found in liver, kidney and pancreas (Pajares and Pérez-Sala, 2018; Paul et al., 2014, 2014kovierová et al., 2016; Stipanuk, 2020; Zhu et al., 2018; Zuhra et al., 2020). Besides maintaining cysteine levels, these two enzymes also contribute to mammalian metabolism as a detoxification pathway for homocysteine. Homocysteine is believed to be cytotoxic and the accumulation of which in human and rodents results in a variety of pathologies (Fonseca et al., 1999; Petras et al., 2014, 2014kovierová et al., 2016). Indeed, one study specifically reports significant inhibitory effect on CHO cell growth exerted by homocysteine when the concentration is higher than 0.5 mM (Mulukutla et al., 2019). Thus, overexpression of CBS and CTH in CHO and other mammalian cells may benefit cellular metabolism and growth by preventing the detrimental effect of homocysteine accumulation in certain cases (Krutil, 2001; Werstuck et al., 2001).

Apparently, overexpression of CBS and CTH together in CHO cells still does not produce adequate cysteine to support standalone survival and proliferation. Since cysteine biosynthesis in mammals happens predominantly in the liver, it is rational to review liver metabolism for the reconstruction of transsulfuration pathway (Papet et al., 2019; Stipanuk et al., 2006). Literature evidence suggests that apart from CBS and CTH, GNMT is also mainly a hepatic enzyme, which is not expressed in CHO cells as confirmed in this study (Pajares and Pérez-Sala, 2018). Furthermore, despite the existence of a variety of other hepatic and non-hepatic methyltransferases (MT) such as DNMT, NNMT, phosphatidylethanolamine N-methyltransferase (PEMT) and guanidinoacetate N-methyltransferase (GAMT), GNMT is selected to be the overexpression target due to glycine being its co-substrate. Specifically, the methylation reactions catalyzed by MT requires a methyl acceptor molecule to produce SAH from SAM (R to R–CH_3 as shown in Fig. 1), and glycine is a metabolite constantly produced in CHO cells and present in abundance in culture medium relative to the other potential methyl acceptor substrates of MTs. Indeed, overexpression of GNMT significantly increases the production of cysteine and makes the resulting CCG-3 cell line capable of growing in cysteine-free condition, confirming the conversion of SAM to SAH being the bottleneck in the CC-2 cell line. The entire transsulfuration pathway is activated as a result of the overexpression of CBS, CTH and GNMT, and increased methionine depletion can be observed in the cell culture. Interestingly, despite being the direct carbon source of cysteine generation and co-substrate for GNMT reaction, concentrations of serine and glycine in batch culture do not show significant differences in CCG-3 compared to unmodified cell cultures although the byproduct sarcosine accumulates to measurable levels (Supplementary Figs. 5 and 6). This may be explained by the hypothesis that an increased amount of serine is produced by the cells to support the cysteine biosynthetic pathway as serine can be synthesized from glucose and can be directly converted to glycine. These changes in the metabolism and amino acid utilization patterns emphasize the importance of re-designing basal and feed media compositions to optimize cell culture performance following incorporation of these engineered cell lines.

Sarcosine, also known as N-methylglycine, is an intermediate molecule in mammalian metabolism, which can be synthesized from betaine and glycine and can be degraded to glycine (Umair et al., 2013). Sarcosine is generally known primarily as an intermediate metabolite with few studies found in the literature concerning sarcosine in CHO cells (Fairgrieve et al., 1987). Indeed, sarcosine concentrations are not usually reported for standard CHO cell culture processes and in this study, sarcosine is not detectable in spent media for any CHO cell lines other than CCG-3. However, the reconstructed transsulfuration pathway in CCG-3 cell line significantly increases the sarcosine generation and leads to its accumulation in extracellular environment. However, no negative impact was observed in CCG-3 culture that can be directly related to sarcosine, although its accumulation represents an inefficient utilization of the glycine amino acid. Thus, future efforts should examine methods to limit its accumulation or facilitate its conversion into useful metabolites.

From the perspective of optimizing bioprocesses, activation of the cysteine synthetic pathway in CHO cell facilitates a reduction or elimination of cysteine in the medium, which lowers raw material cost and eases medium design strategies by tackling the challenge of poor stability and solubility of cysteine. Furthermore, cysteine biosynthesis could in the future be used as a selection marker much like glutamine synthesis is used in most current cell line development processes. Besides these advantages, enhancement of cysteine biosynthesis also has the potential to improve culture performance in industrial fed-batch processes by promoting glutathione generation. Several studies have reported a positive relationship between glutathione content and protein productivity in CHO cell, suggesting that glutathione and redox capabilities are potential targets for cell culture optimization (Chevallier et al., 2020; Chong et al., 2012; Orellana et al., 2015, 2017). One possible approach to promote glutathione content in CHO cell is to increase the availability of cysteine to the cells, since cysteine and glutamate are the direct precursors of glutathione, and cysteine is believed to be a limiting factor for glutathione synthesis in mammals (Bornkamm et al., 2006). In this study, the biosynthesis of cysteine introduced in the engineered CCG-3 cell line enabled a retention in the cell's capacity to generate glutathione in conditions lacking enough cysteine. Such characteristics of cells can benefit fed-batch processes by preventing any negative impacts of cysteine underfeeding, including a reduction of growth and productivity and even cell death (Ali et al., 2020; Ali et al., 2020). However, no significant difference was observed between engineered and unmodified cell lines in conditions with ample cysteine. It is possible that when the cysteine level is high, the availability of cysteine does not limit the glutathione generation in the CHO cell lines used in this study. CHOGS 23 cell line used in this study may have developed high glutathione characteristics due to its enhanced capability to generate glutamine, as glutamine-derived glutamate is a precursor for glutathione synthesis. Indeed, many studies suggest that glutamine drives glutathione synthesis and the supplementation of which can potentially elevate glutathione levels in mammalian cells (Amores-Sánchez and Medina, 1999; Lian et al., 2018; Sappington et al., 2016; Yoo et al., 2020).

In conclusion, this study investigated the reverse transsulfuration pathway in CHO cells by identifying rate-limiting reactions followed by pathway reconstruction to enable efficient cysteine biosynthesis. Such cysteine cellular engineering approaches have never been introduced into any mammalian recombinant protein production platforms, and CHO cell lines with a reconstructed transsulfuration pathway exhibit significantly enhanced capability to survive and proliferate in low and deficient cysteine environments and under oxidative stress conditions. This study demonstrates the expanding potential to improve current CHO and other mammalian-based biomanufacturing processes via metabolic engineering approaches.

Author contributions

Yiqun Chen: Conceptualization, Methodology, Investigation, Writing – Original Draft.

Michael J. Betenbaugh: Conceptualization, Supervision, Funding acquisition, Writing – Review & Editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2023.02.010.

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