Pharmaceutical BIOPROCESSING

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Optimizing Chinese hamster ovary cell line development via targeted control of N-glycosylation

The glycosylation profile of a protein is vital to its success as a biotherapeutic as it affects its efficacy and function and, thus, has been extensively studied for proteins produced in Chinese hamster ovary cells. Improvements in the sensitivity and throughput of various analytical assays have allowed for more accurate glycosylation information earlier in the cell line development process. Bioprocess parameters and media additives have been used to modulate the glycosylation of proteins. Genome editing techniques have enabled the regulation of glycosylation genes to achieve a targeted glycan profile. This review highlights advances made in engineering strategies and improvements in high-throughput N-glycan assessment that allow for generation of high-productivity clones with a specific product quality profile.

Keywords: bioprocess optimization • CHO cells • genetic engineering • host diversity • HTP analytical methods • N-glycosylation

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The generation of high-producing mammalian cell lines with targeted product quality attributes is still considered a challenge in the development of biotherapeutics. Chinese hamster ovary (CHO) cells are widely used for the manufacture of biotherapeutics, in part because of their ability to produce proteins with desirable properties, including 'human-like' glycosylation profiles. Glycosylation plays a critical role in protein function through its effects on half-life and efficacy of the molecule and has the potential to adversely affect the safety profile of a glycoprotein [1–3]. Historically, the target glycoprotein therapeutic was not necessarily designed with product quality in mind as developing a cell line that generates enough material for clinical and commercial demand was its own challenge. With the advent of biosimilars and the wide breadth of knowledge on how glycosylation and other product quality attributes can affect a therapeutics efficacy, scientists are using many different avenues to control the glycosylation profiles of their therapeutic molecule. Several approaches to achieve this result have been utilized including modification of process conditions [4,5], supplementation of various media additives [6], use of different host cell lines and genetic modification of the host cell line. Each of these approaches has had various degrees of success in achieving the goal of modulating glycosylation of the glycoprotein product. Additionally, to facilitate screening of these attributes, several new technologies that include miniaturization, use of robotics and increase in throughput have been employed in cell line screening assays to generate quality clone data in an easily automated, high-throughput fashion. The various methods that have been developed each have their advantages with regard to speed and throughput allowing for their implementation at various points in cell line development. This review focuses on how deliberate strategies to affect protein glycosylation in CHO cells, specifically via genetic engineering, can be coupled with high-throughput analytical techniques to select a final cell line that gives high product yields with consistent and desirable product quality.

Glycoprotein product quality generally refers to various post-translational modifi-

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Key terms

ADCC: Antibody-dependent cellular cytotoxicity (ADCC) is a mechanism of cell-mediated immune response whereby an effector cell uses antibodies to bind to cell surface antigens on a target cell and lyses it.

Macro- and micro-heterogeneity: Macroheterogeneity refers to the variability in the location and number of oligosaccharides attached to the glycoprotein. Microheterogeneity is the variability in the oligosaccharide structures at specific glycosylation sites.

Glycosylation mutants: CHO mutant cells with altered glycosylation that have been isolated by selection for resistance to the cytotoxicity of plant lectins. They have been used to characterize glycosylation pathways and identify glycosylation genes.

ZFN: Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate genome editing by creating a double-stranded break in DNA at a user-specified location.

miRNA: microRNA is a small noncoding RNA molecule (~22 nucleotides) that functions in RNA silencing and post-transcriptional regulation of gene expression.

CRISPR-Cas9: A genome-editing technology that permits targeted gene cleavage and editing via specific endonuclease cleavage that is guided by RNA sequences.

cations, most notably protein N-glycosylation which plays an essential role on the efficacy and immunogenicity of proteins. The protein function is affected by glycosylation via a variety of mechanisms including the binding, solubility, stability and folding of the protein therapeutic [7,8]. The pathways by which mammalian cells process glycans are complex and have been extensively reviewed [4–5,9–11]. Briefly, formation of N-linked glycans occurs via a precise series of steps in the endoplasmic reticulum (ER) and Golgi apparatus that involve removal of monosaccharides and addition of moieties by specific enzymes. N-linked glycosylation begins in the ER with the synthesis of a lipid-linked oligosaccharide moiety consisting of dolichol phosphate bound to 14 sugars, which is added to a specific asparagine residue on the nascent polypeptide by cotranslational *en bloc* transfer [12]. The subsequent modifications via trimming and addition of monosaccharides to the common core sugar sequence can result in glycan heterogeneity due to the fact that not all enzymatic reactions proceed to completion. This heterogeneity can result in a variety of glycoforms that include high mannose, complex and/or hybrid glycan structures (Figure 1). High mannose structures consist of only mannose residues attached to the protein core, whereas complex glycans contain 'antennae' initiated by N-acetylglucosaminyl transferases. Hybrid glycan structures contain mannose on one arm and one or two antennae are on the other arm. Bi-antennary, complex N-glycans have two antennae or branches initiated by the addition of two terminal N-acetylglucosamine residues. Additional branches can be initiated on the core mannose residues to yield tri- and tetra-antennary N-glycans [13]. The presence of high mannose glycoforms has been linked to faster clearance rates of the protein *in vivo* via liver mediated clearance and increased rate of glycan cleavage [14]. IgGs containing high mannose glycans also have higher binding affinity to FcγRIIIa and hence higher antibody dependent cellular cytotoxicity (ADCC) activity [14].

An additional structure of biological significance is mannose-6-phosphate (Man-6-P) which is recognized by P-type lectins for trafficking to lysosomes (Figure 1) [13]. Specifically, hydrolase enzymes are phosphorylated in the Golgi, followed by a glycosidase step that generates the Man-6-P residue. The P-type lectin receptors recognize the Man-6-P domain on the lysosomal hydrolase and target it to the lysosomes where it can degrade proteins. Additionally, protein glycosylation has been shown to have pathological significance, specifically altered branching of N-glycans plays a role in biology of cancer [15]. These considerations highlight the importance of monitoring and manipulating glycosylation to ensure that the final therapeutic protein has the desired product quality profile.

When analyzing glycans it is important to assess the macroheterogeneity (i.e., variable site occupancy), which refers to the variability in the location and number of oligosaccharides attached to the glycoprotein. In addition, microheterogeneity, the variability in the oligosaccharide structures at specific glycosylation sites, also affects glycoprotein function. Both macro- and micro-heterogeneity affect the efficacy and immunogenicity of glycoproteins and hence can result in functional diversity [16].

Elongation of N-linked glycans can result in a variety of core structures which can be further modified via fucosylation, sialylation, acetylation, methylation or sulfation. The wide variety of N-linked glycans that are produced gives rise to substantial diversity in product quality, some of which is desirable, while some are immunogenic or may compromise efficacy of the product. Most notable are the terminal glycan processing steps of either sialyation or galactosylation, which have been shown to directly affect half-life of proteins in the circulation.

Sialylation represents the terminal step for many glycans with sialic acid playing an crucial role in biological function often prolonging circulatory half-live *in vivo* and improving efficacy of the glycoprotein therapeutic by shielding the galactose residues from recognition by asialoglycoprotein receptors on hepatocytes [1,2]. Sialic acid refers to a class of acidic sugars

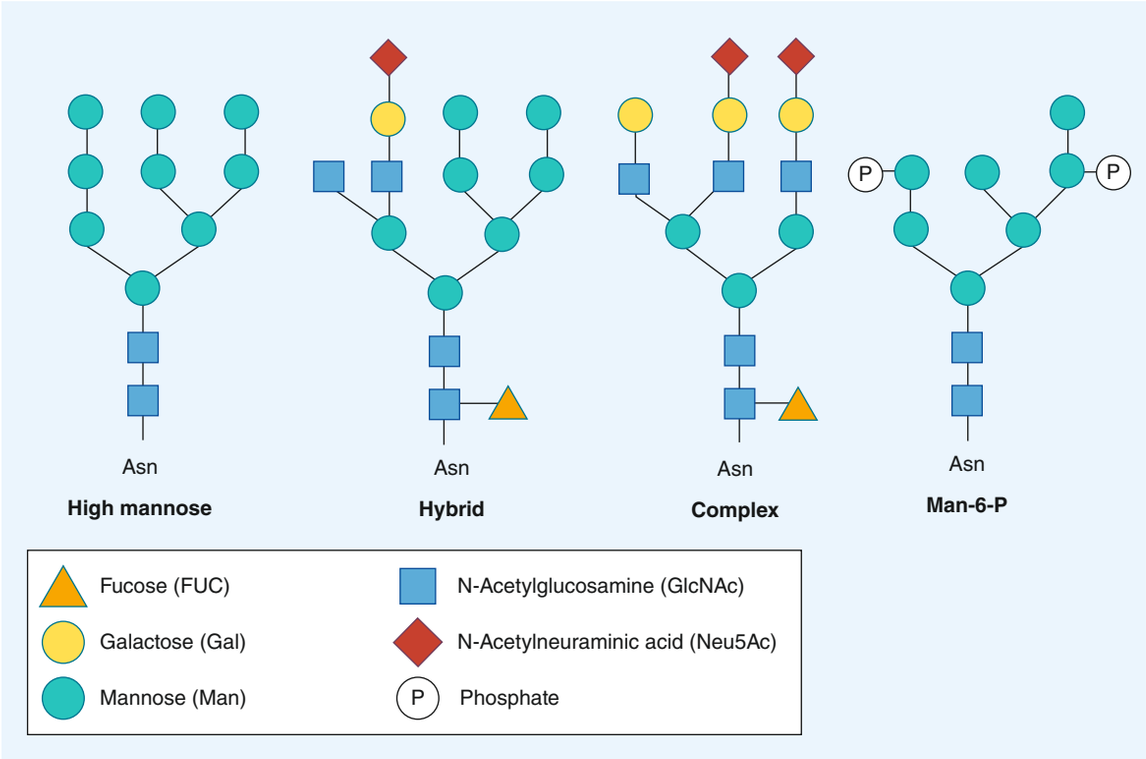

Figure 1. Types of N-linked glycans. N-glycans are attached to proteins at asparagine residues that have an Asn-X-Ser/Thr sequence. There are several potential types of N-glycan mature glycoproteins which include high mannose, complex, hybrid and Man-6-P types, all of which contain a common core of three mannose and two GlcNAc residues attached to the asparagine.
Adapted from [13].

consisting of a nine carbon backbone that are typically found at the terminal end of N-glycans [13]. Sialylation of N-glycans is accomplished by sialyltransferase enzymes which transfer the sialic acid from CMP-sialic acid onto the terminal galactose of the protein. CHO-derived glycoproteins differ significantly from human-derived glycoproteins with regard to the type of sialic acid linkage. Natural human glycoproteins usually contain sialic acid with α2,3-, α2,6- and α2,8-linkages, whereas CHO-derived glycoproteins have only α2,3-linked sialic acid [17]. Another significant difference between human and CHO glycoproteins is the presence of N-glycolylneuraminic acid (Neu5Gc or NGNA), which does not exist in humans due to a missing exon in the CMP-N-acetylneuraminic acid hydroxylase gene [18]. As a result of the lack of Neu5Gc residues on human proteins, recent findings indicate that humans can sometimes have high levels of circulating anti-Neu5Gc antibodies [19]. Because of this, the presence of Neu5Gc on glycoproteins can elicit an immune response, making them an undesirable product quality attribute.

Galactosylation of glycoproteins is catalyzed by glycosyltransfereases that transfer galactose residues onto the growing glycan structure. CHO cells synthesize the α-1,3-galactose (α-Gal) epitope on N-glycans via activity of the N-acetyllactosaminide 3-α-galactosyltransferase-1 [20]. Humans are genetically deficient in this enzyme and spontaneously express anti α-Gal antibodies [21]. Glycoengineering strategies are required to lower the levels of α-Gal immunogenic residues in recombinant proteins to lower complement-dependent cytotoxicity [22]. Another important product quality attribute that has been a popular target for modulation is fucose. Removal of the core fucose on glycosylation sites of monoclonal antibodies is known to enhance activity by improving FcγRIIIa binding, leading to increased ADCC which is triggered upon binding of lymphocyte receptors (FcγRs) to the constant region (Fc) of the antibodies [23]. Other factors that can affect ADCC activity are sialic acid content and galactose levels, as well as the presence of bisecting N-Acetylglucosamine (GlcNAc) on antibodies making all of these potential targets for modification [24–26].

This review introduces the various analytical techniques required to characterize N-glycosylation of proteins. Methods of affecting the glycan profile via bioprocess tools such as optimization of process conditions and use of media supplements during the process are discussed. The ability to utilize the natural

diversity that exists in the multitude of CHO cell lineages available for production of biotherapeutics is also considered. Lastly, utilization of genetic engineering tools to either overexpress, reduce or remove various glycans is reviewed.

Analytical technologies for glycosylation analysis

Owing to the broad array of potential protein glycosylation targets it is vital to have adequate methods for detection of these modifications. To support the cell culture and cell engineering work outlined herein, simple, robust, accurate and reproducible analytical methods need to be developed. There is no universal method for the rapid and reliable identification of glycan structures, but recently more rapid methods with low sample volume requirements have been developed to facilitate the larger number of samples required for optimization of protein glycosylation profiles [27].

Various analytical methods have been developed to analyze not only the macro- and micro-heterogeneity of glycoproteins, but also the intracellular nucleotide sugar content, glycosyltransferase activity and glycosidase activity (Figure 2). These analytical methods are critical for various purposes in cell line development, such as confirming the removal or insertion of specific glycosyltransferases and glycosidases, screening hundreds of clones that exhibit heterogeneity in their glycoforms and detailed characterization of glycan structures. Work continues to focus on higher throughput and small sample requirements to enable more rapid analysis of protein glycosylation.

In macroheterogeneity analysis, CE-based platforms, such as LabChip GXII (Perkin Elmer) and Peggy Sue (Protein Simple) can be used for high-throughput quantification of relative percentage of glycan occupancy. The LabChip GXII with the Protein Express Reagent kit and chip can analyze a full 96-well plate within approximately 2 h with approximately 8 h of sample preparation, and Peggy Sue with the Size Separation Assay within approximately 24 h with approximately 3 h of sample preparation. The LabChip GXII requires purified sample for analysis, while Peggy Sue can analyze unpurified samples. Both of these platforms are suitable for high-throughput screening of clones based on glycan occupancy. Recently, a hydrophilic interaction liquid chromatography (HILIC) method has been developed to resolve unglycosylated, partially glycosylated and fully glycosylated proteins [28]. This LC method takes only approximately 15 min to analyze each purified sample and offers the optimum balance between throughput and resolution for screening a large number of samples. For detailed analysis, liquid chromatography and tandem mass spectrometry (LC-MS/MS) with Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD) fragmentation methods can be used to analyze the occupancy of specific glycosylation sites on the polypeptide backbone [27,29–32].

Glycosylation microheterogeneity analysis can be performed by a variety of techniques, including monosaccharide compositional analysis and structural analysis of N-linked glycans. Monosaccharide compositional analysis of all the glycans can be carried out by acid hydrolysis of the monosaccharides, followed by 2-aminobenzamide (2-AB), 2-anthrallic acid (2-AA) or 1,2-diamino-4,5-methylenedioxybenzene labeling and reversed-phase liquid chromatography (RP-LC) [33,34]. In addition, high-throughput screening methods for quantifying sialylation have also been developed using chemical and enzymatic reactions in a 96-well plate format [35,36], and affinity binding in micro-engraved arrays containing 2025 wells per array, in which single cells were cultured and secreted proteins are sandwiched by antibody and fluorescently labeled lectin [37]. The former can analyze a full 96-well plate within 2 h including sample preparation, while the latter can analyze several microarrays in 1 to 2 days with approximately 5 h of sample preparation.

Structural analysis of N-linked glycans typically involves release of intact N-linked glycans by PNGase-F, labeling of the released glycans, clean-up steps of the labeled glycans and separation of the different glycan species by LC, CE or MS. LabChip® GXII (Perkin Elmer) with the Glycan Screening Reagent kit and chip can be used for high-throughput analysis of neutral glycans mostly found in monoclonal antibodies. This platform can analyze a full 96-well plate within 2 h. If the glycoproteins contain charged glycans, other methods such as capillary IEF (cIEF) immunoassay on Peggy Sue™ (Protein Simple) and HILIC are required. The cIEF Peggy Sue platform can analyze a full 96-well plate within approximately 14 h and provide a fingerprint of protein sialylation from unpurified cell culture samples. The HILIC method can provide glycan structure information and takes approximately 20 to 80 min to analyze each sample depending on the method gradient. A typical short and steep gradient method takes approximately 20 min and would be suitable for screening a large number of samples, while a long 80-min gradient can be used for more detailed characterization of a smaller subset of samples. Other methods, such as RP-LC and anion exchange chromatography are also available for N-linked glycan profiling, but HILIC has been more widely used due to its high resolution [27,38–39]. The short HILIC method combined with high-throughput N-glycan sample preparation kits, such as GlykoPrep®

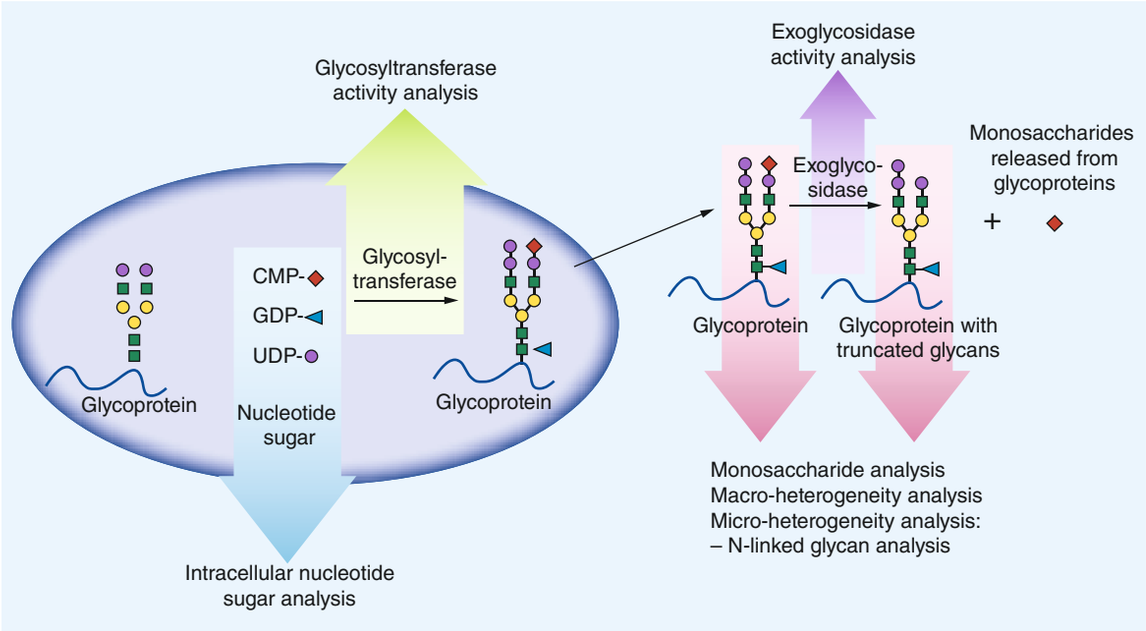

Figure 2. Simplified diagram of protein glycosylation and analytical methods to analyze protein glycosylation. Protein glycosylation involves a complicated network of enzymatic reactions that transfer carbohydrates from nucleotide sugar to protein to produce glycoproteins. Upon secretion of the glycoproteins by the cell, the glycoproteins are subject to glycosidases that can cleave monosaccharides, producing glycoproteins with truncated glycans and free monosaccharides. This diagram provides a simplified overview of the glycosylation process and the analytical methods that can be used to analyze each part of this process. For instance, intracellular nucleotide sugar analysis can be used to analyze the level of intracellular nucleotide sugars that are available for the glycosyltransferase reactions with glycoproteins. In addition, glycosyltransferase activity analysis can be used to analyze the expression level of glycosyltransferases inside the cells. The glycan structures of secreted glycoproteins can be analyzed by various methods such as monosaccharide, macroheterogeneity and N-linked analysis. Furthermore, exoglycosidase activity analysis can be used to analyze the level of exoglycosidase present in the cell culture media.

(Prozyme) [40] fully automated on Bravo™ (Agilent) or RapiFluor-MS (Waters) [28] enables N-glycan structure analysis of many samples as would be seen during early cell line development. If detailed characterization is not required, a weak anion-exchange chromatography using GlycoSep C column can be used to obtain a simplified glycan profile, in which 2-AB labeled glycans are separated into groups of neutral glycan, monosialylated glycan, di-sialylated glycan, tri-sialylated glycan and so forth [41]. Alternatively, lectin microarray may also be used for high-throughput analysis of N-linked glycans. The types of glycan structures that can be analyzed by this method depend on the availability of the lectins. In addition, multiple lectins are required to quantify a specific glycan structure as lectins are specific to certain glycosidic linkages, not the whole glycan structures [42,43]. For more detailed characterization, such as linkage analysis and characterization of unknown glycan species, elaborate methods using LC and MS are also available [27,28].

In addition to analysis of protein glycosylation, analysis of intracellular metabolites and enzymes involved in protein glycosylation are important for development of cell lines with engineered glycosylation machinery. For instance, an α-galactosyltransferase (α-galT) activity assay has been used to confirm that CHO cells express α-galT and are capable of producing the immunogenic α-galactosylated proteins [20]. The same method will be critical in developing an α-galT knock out cell line. Moreover, ion pair [44–46] and anion exchange [47] LC methods for analysis of nucleotide sugar content would provide insight into which pathways to engineer in order to modify the structures of glycoproteins produced by the cells. Last but not least, sialidase activity assays have been used to develop sialidase knock out cell line in order to minimize protein desialylation during cell culture [48]. Overall, all of these analytical methods (Table 1) provide a comprehensive set of tools that will enable cell line engineering to produce glycoproteins with the appropriate glycoforms.

Glycosylation control via process optimization

There have been a large number of studies that focus on studying process conditions affect glycosylation [4–6,51]. Parameters that have been optimized include dissolved

Table 1. Analytical methods for glycan analysis.

Analytical method	Readout	Analytical platforms	Ref.
Macro-heterogeneity analysis	Glycan occupancy (a-gly, mono-gly, di-gly, etc.)	LabChip GXII, Peggy Sue, HILIC, LC-MS/MS	[27–32]
Monosaccharide compositional analysis	Sialic acid, fucose, galactose, mannose, GlcNAc, etc.	RP-LC, high-throughput (HTP) chemoenzymatic assay, HTP microengraved assays	[33–35,37,49]
N-Glycan structure analysis	High-mannose, α-Gal, afucosylation, sialylation, etc.	LabChip GXII, cIEF, HILIC, RP-LC, anion exchange LC, LC-MS, lectin microarray	[27–28,38–43]
Intracellular nucleotide sugar analysis	GDP-Fucose, CMP-NANA, UDP-Galactose, etc.	Ion pair and anion exchange LC	[45–47,50]
Glycosyltransferase activity analysis	α-GalT activity, sialylT activity, etc.	Reaction with labeled substrate followed by LC or LC-MS	[20]
Exoglycosidase activity analysis	Sialidase activity	Reaction with labeled substrate followed by fluorescence spectrometry	[48]

oxygen (DO), pH, shear, temperature and culture duration, among others. The ability to affect the glycosylation profile of a protein by varying process conditions is an attractive option because it can be accomplished as part of the bioreactor control strategy. Although many studies have been published that demonstrate an effect of each parameter on glycosylation, the results have been mixed and may be dependent on the cell line and protein being expressed. This variability in the effect on glycan profile requires careful characterization of the process for a given molecule and CHO host and may not be uniform across products. The effects of manipulating temperature, DO, pH, ammonium concentration, shear stress and culture duration on glycosylation are discussed.

Shifting culture temperature during a process has been used to extend the culture duration and maintain cell viability while also increasing specific productivity [52,53]. The shift to lower culture temperatures in CHO processes also can affect glycosylation in a variety of ways. Studies using CHO cells expressing human tissue-type plasminogen activator (t-PA) correlated to an increase in glycan site occupancy with reduction in temperature [54,55]. Other work focused on the highly glycosylated fusion protein Epo-Fc determined that reduced culture temperature decreased product sialylation [56]. Similar work on DUXB11 cells expressing human EPO found that temperatures below 32°C lead to reduced tetra-sialylated N-glycans, acidic isoforms and tetra-antennary structures [53]. Temperature has also been used to control levels of the N-glycolylneuraminic acid (NGNA or NeuGc) form of sialic acid, specifically reducing levels of NGNA when temperature is shifted down later in culture [57].

DO is another parameter that has been used to affect glycosylation of therapeutic proteins. Chotigeat and colleagues found that increased DO concentrations increased the specific productivity of CHO cells expressing human follicle stimulating hormone (hFSH) as well as the sialic acid content of the protein via an increase in the sialyltransferase activity [58]. In contrast, studies on the Epo-Fc fusion protein [56] and rEPO [59] found no effect of DO on sialylation. Further studies on CHOK1 cells expressing EPO showed no effect of DO on the degree of antennarity, but there was an optimal DO range for maximizing core fucosylation. With respect to terminal galactosylation of an IgG1, a shift toward reduced galactosylation of glycan chains was observed as DO concentration decreased [60].

Some studies have focused on the effect of culture pH on various aspects of glycosylation. Sialylation levels of an Epo-Fc fusion protein were unaffected by culture pH [56], but work with a recombinant enzyme showed a decrease in sialic acid content as culture pH increases [55]. Additional work with this same enzyme showed that glycan site occupancy is reduced by an increase in the pH of the process. Decreases in the extent of glycosylation of CHO expressed recombinant protein mouse placental lactogen-I (mPL-I) were observed at low (below 6.9) and high (above 8.2) extracellular pH [61]. This effect at high pH could be recreated by adding ammonium chloride to the cultures which caused a reduction in the most heavily glycosylated forms of mPL-I [62]. Many studies focused on the effect of ammonium concentration, which has a significant impact on the pH of a culture, have been performed. Yang and Butler have done several studies aimed at determining the effect of ammonium on the molecular heterogeneity of human EPO expressed in CHOK1, initially showing that increased ammonium results in decreased terminal sialyation [63]. In a subsequent study [64] to elucidate the mechanism behind the effects, the proportion of tetrasialylated and tetra-

antennary glycan structures were reduced in the presence of ammonium, which was linked to a significant increase in the intracellular UDP-N-acetylhexosamine (UDP-GNAc) pool. A decrease in sialyation with increasing ammonium concentrations has been seen in the expression of granulocyte CSF [65] and recombinant tumor necrosis factor-IgG (TNFR-IgG) [66]. The high ammonium also reduced the levels of terminal galactosylation of TNFR-IgG, likely through a pH mediated effect on the glycosyl transferase activity.

There are also other culture parameters that have been shown to play a role in affecting the glycoprotein quality. Senger and Karim studied the effect of shear stress on glycosylation of r-tPA, finding that increased shear stress resulted in decreased glycan site occupancy [67]. In this work, a model of glycan addition using plug flow reactor kinetics was used to propose that the shear protective function of the cells results in decreased residence time of the protein in the ER as a result of increased protein synthesis. This limits the contact duration of the glycosylation site with the oligosaccharyltransferases resulting in decreased glycan site occupancy. The same group also studied the effect of culture duration and feeding strategy on tPA glycoform inactivation (via the absence of a glycan at Asn-184) to identify an optimal set of conditions for maximizing the active form of the molecule [68]. Even with the wide breadth of literature on optimization of process conditions to modulate glycosylation, the results are varied depending on the molecule being expressed and may be affected by other process parameters in a given system.

Supplementation of media components

In addition to optimizing cell culture process conditions, media supplementation has been a prominent area of focus for modulating protein glycosylation. The components that have been studied vary from basic sugars such as glucose and glutamine to metals and nucleotide precursors. Glucose limitation has been shown to have a profound effect on the degree of glycosylation in CHO cultures. In studies using IFN-γ, reduced glucose lowered the level of sialylation and increased the proportion of hybrid and high mannose glycoforms [69]. Similarly Hayter *et al.* found that glucose limited cultures resulted in an increase in nonglycosylated γ-IFN [70]. The glycosylation pattern of a chimeric heavy chain antibody (EG2) produced from CHO cells was affected by the glucose depravation in culture with the proportion of nonglycosylated mAb increasing with exposure time of cells to media depleted of glucose [71]. Glutamine limitation also influenced glycosylation of IFN-γ by reducing amino sugar formation and hence UDP-GNAc concentration [72]. Work with human chorionic gonadotrophin (HCG) found that decreased glutamine reduced sialyation, fucosylation and degree of antennarity, while increasing neutral structures via decreased intracellular UDP-GlcNAc pools [73]. Recent work with alternative sugar sources showed that both tagatose and sucrose increase the percentage of high mannose N-glycan species and to a lesser degree reduce the levels of fucosylation in multiple CHO cell lines [74].

The supplementation of cultures with nucleotide sugars and their precursors is a logical approach to modulating glycoform profiles as it provides the cells with the building blocks for glycosylation. Addition of glucosamine to cells expressing human tissue inhibitor of metalloproteinases 1 (TIMP1) resulted in decreased sialylation and increased levels of intracellular UDP-N-acetylhexosamine which was associated with an increase in the antennarity of the glycans [75]. In the same study, supplementation with N-acetylmannosamine (ManNAc) had no effect on sialyation but did increase intracellular levels of CMP-sialic acid. Yang and Butler also found that glucosamine decreased the tetra-sialyated glycan content of EPO but they observed a decrease in the amount of tetra-antennary glycan [64]. However, Gawlitzek *et al.* had found no effect on the degree of N-glycan branching of TNFR-IgG upon glucosamine feeding [66]. In contrast to the decreases in sialylation seen in these studies, others have found that increasing the glucosamine concentration increased the degree of sialylated IFN-γ. Similarly, in this same work, galactose and ManNAc supplementation also increased IFN-γ sialic acid content which was attributed to increases in intracellular pools of CMP-sialic acid and UDP-HexNAc [76]. Galactose supplementation alone has also shown up to a 44% increase in total sialic acid content and 20.3% for sialylated glycans for an Fc-fusion protein expressed in a CHO-GS host [77]. In contrast, galactose feeding of CHO cells producing a recombinant IL-4/13 cytokine trap fusion showed no effect on sialic acid content [78]. Other nucleoside precursors, such as uridine, guanosine and mannose have been shown to have either no effect or cause only a slight reduction in glycan site occupancy of tPA [55]. In the same study, the addition of manganese increased the fraction of fully occupied glycan. In more recent work, manganese has been shown to increase M5 high mannose glycans when supplemented in glucose limited cultures [79]. In CHOK1SV cells expressing IgG4 antibodies or fusion proteins changes in asparagine concentration resulted in a corresponding change in distribution of galactosylated glycoforms with zero (G0F), one (G1F) or two (G2F) galactose sugars [80].

Sodium butyrate inhibits histone deacetylase activ-

ity and as such plays a role in chromatin structure and function [81]. It has also been used extensively to affect the glycosylation of proteins in culture. Supplementation of CHO cultures expressing tPA with sodium butyrate has resulted in increased glycan site occupancy [54]. Sodium butyrate has also been shown to increase the specific productivity of cells expressing hFSH and the sialic acid content of the protein via increased sialyltransferase activity [58].

Several other studies have shown effects on glycosylation via addition of serum or hydrolysates [82,83], but the industry shift toward chemically defined media makes these additives an undesirable option. The variability in the effect of various media supplements discussed here highlights the need to test each component on the particular cell line being used and glycoprotein being expressed. Additionally, many of these additives, especially the nucleoside precursors are costly at industrial scales, suggesting that there may be better alternatives for modulating protein quality via either use of alternative hosts or host engineering.

Leveraging host diversity to modulate glycan profile

A complementary approach to manipulating the process or supplementing the culture with additives is to harness the diversity that exists in the various CHO cell lineages that are available for biotherapeutic production. The original CHO cell line isolated by Puck in 1957 has undergone extensive subcloning and mutagenesis to establish several different lineages that are commonly used across the industry (Figure 3) [84–86]. The inherent genetic diversity and the high rate of genetic change of CHO cells has resulted in the concept of CHO 'quasispecies' suggesting that even within a population, there exists constant remodeling of genomic structure [87]. The CHOK1 cell line was derived from a subclone of the original culture established by Puck [84]. The Chasin lab then subjected this line to extensive mutagenesis to generate dihydrofolate reductase (DHFR) deficient host, known as DUXB11, which contains a missense mutation in one allele of DHFR and deletion of the other DHFR allele. Similarly, the group took another subclone and utilized mutagenesis to obtain DG44 which lacks both DHFR alleles [85,86]. Because of the deficiency in DHFR these hosts are dependent upon an exogenous source of nucleotide precursors for growth. Lonza Biologics has taken the wild-type CHOK1 to create CHOK1SV which uses the glutamine synthetase (GS) selection system [88]. Life Technologies has utilized the CHO-S subclone to create several systems for expression of recombinant proteins. Studies where genetic diversity was incorporated via the use of multiple CHO derivatives have shown varying potential for increasing expression of problematic molecules [89,90].

Characterization of clonal hosts derived from the same original CHOK1SV host pool showed that not only is there diversity in the productivity among the clones but there is variation among the hosts with respect to mAb N-glycan microheterogeneity and macroheterogenity of an Fc fusion protein [91]. Additionally, work has been presented that shows that different CHO hosts yield vastly different ranges of monomer, total sialic acid and NGNA when transfected with the same Fc-fusion protein [92]. These studies highlight the benefit of leveraging genetic diversity that exists in the available panel of CHO hosts to generate molecules with targeted product quality profiles. Although the concept of a host cell toolbox is attractive for accessing a wider product quality space in a targeted manner, characterization of the various host clones or lineages does require a large upfront effort. In addition to the creation and screening of these hosts, substantial effort may be required to adapt these cells to media and processes suitable for bioprocessing applications.

Host cell engineering of glycosylation

Despite the large body of work on controlling process conditions, addition of media supplements and utilizing the diversity of various CHO host lineages, the most direct way to modulate the protein glycosylation profile of a molecule is via genetic manipulation of specific target genes. This can be achieved by removal or insertion of specific enzymes in the glycosylation pathway or by targeted decrease or increase of enzyme expression.

Glycosylation mutants have provided a method to understand the pathways of glycan synthesis and degradation and to identify the genes involved in the enzymatic reactions of glycosylation. Usually these mutants have loss-of-function mutations that depress the activity of an enzyme in a pathway; but there are also gain-of-function mutations that activate a silent glycosylation gene, elevate the expression of an existing pathway or inactivate a negative regulatory factor [13]. There are dozens of CHO glycosylation mutants that have been identified and reviewed in the literature that affect a wide variety of product quality attributes from N-glycan synthesis pathways to the synthesis of glycolipids, glycosaminoglycans or glycosylphosphatidylinositol membrane anchors [93]. These mutants provide valuable information about glycosylation pathways and a way to generate glycoproteins with specific product quality attributes, but they have not been widely used in biotherapeutic production as they are not optimal for high levels of protein expression or the high cell densities and viabilities necessary for glycoprotein manufacture. To address the

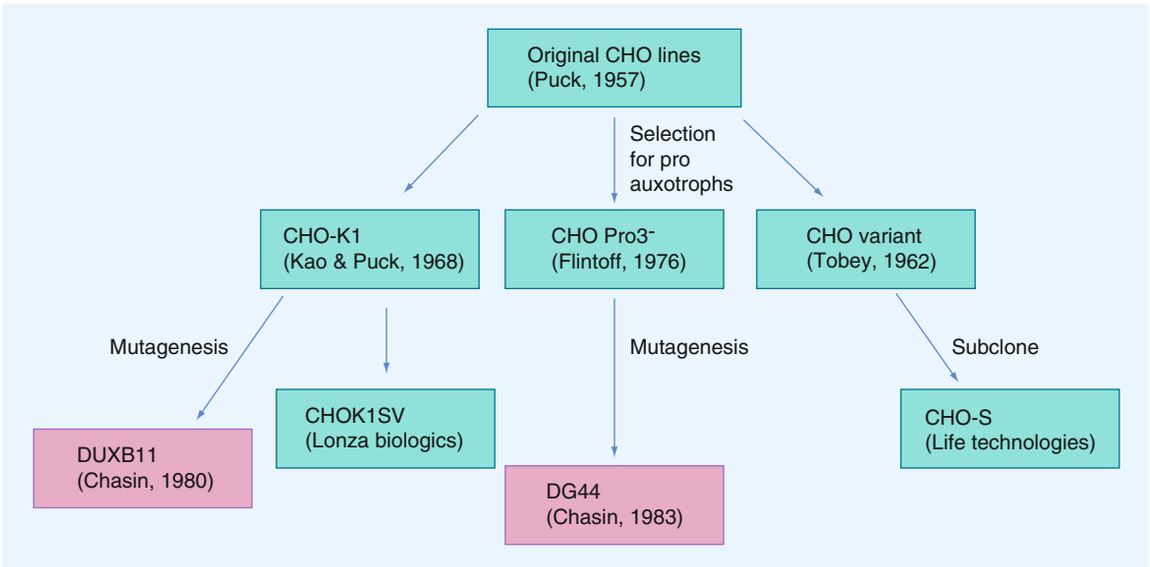

Figure 3. History and lineage of Chinese hamster ovary cell lines. Cell lines derived by mutagenesis to be DHFR deficient are highlighted in pink.
DHFR: Dihydrofolate reductase.

need for CHO cell lines with specific glycan patterns, genetic engineering methods have been successfully employed to overexpress, knock down or knock out various glycosylation genes. Methods discussed here include: overexpression of enzymes in the glycosylation pathway, RNA interference, microRNAs, zinc finger nucleases (ZFNs) and more recently developed genome editing tools.

Engineering of gene targets

Modulating glycosylation of proteins via genetic manipulation of genes that encode glycosylation enzymes is a widely used technique for CHO cell cultures (Table 2). Approaches have included up- and down-regulating endogenous enzyme expression or introduction of enzymes from other organisms. DNA for the gene of interest is cloned into a mammalian expression vector, usually a plasmid, and then transfected either by electroporation or lipofection into the target cells. The gene can be stably integrated into the genome of the cells via the use of selective pressure. A consideration when using plasmids for introduction of genes is that they integrate randomly, do not target specific integration sites within the genome, and can potentially destroy existing reading frames.

The enzymes that affect the degree of protein fucosylation have been extensively studied and used as targets in CHO cells to affect the ADCC activity of antibodies and glycoproteins. A DG44 cell line for antibody production with optimized ADCC activity was achieved by disrupting both *FUT8* alleles using sequential homologous recombination [94]. The resulting cell line produced completely defucosylated ant-CD20 IgG1 with 100-fold higher ADCC activity than the control. Defucosylated antibodies were also produced in DG44 cells engineered to overexpress the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD) within the cytosol which efficiently deflects the fucose *de novo* pathway [103].

Sialic acid content is another target that has been modified via cell line engineering. The fact that CHO cells do not express α2,6 sialyltransferase (ST6GalI) has led to its expression in a large number of studies. Early work by Zhang *et al.* resulted in a CHO host with stably expressed ST6GalI [104]. The modified host was then transfected with human EPO and shown to contain sialic acid in α2,6 linkages, but no effect was seen on the bioactivity of the modified EPO in mice *in vivo* compared with the control. More recently, ST6GalI was overexpressed to generate clones that were then transfected with a human IgG that not only contained α2,6 linkages, but also increased total sialic acid content [105]. Others have overexpressed both ST6GalI and the α2,3 sialyltransferase (ST3Gal IV) in CHO to achieve clones with increased sialylation of human IFN-γ [106]. Co-expression of ST3Gal IV with β 1,4-galactosyltransfersase (β1,4-GT) synergistically enhanced the sialic acid content and amount of tri-sialylated glycans of EPO to a greater extent than just expression of ST3Gal IV alone [107]. Transfection of the CMP-sialic acid transporter (CMP-SAT) in a CHO cell line expressing human IFN-γ has also been shown to increase the expression of CMP-SAT up to 2.8 fold

Table 2. Summary of engineering methods used to modulate glycosylation.

	Cellular target	Engineering method	Outcome	Ref.
Fucosylation	*FUT8*	Homologous recombination	Complete defucosylation, increased ADCC	[94]
	FUT8	siRNA	Complete defucosylation, increased ADCC	[95]
	FUT8	shRNA	Reduced fucosylation	[96]
	FUT8	ZFN Knockout	Complete defucosylation	[97,98]
	GDP-Fucose Transporter (GFT)	siRNA	Increased defucosylation	[99]
	GMD	siRNA	Defucosylation	[100]
	GMD and *FUT8*	siRNA	Complete defucosylation, increased ADCC	[101,102]
	RMD	Overexpression	Defucosylation	[103]
Sialylation	ST6GalI	Overexpression	Presence of α2,6-linkages, increased TSA	[104,105]
	ST6GalI and ST3Gal IV	Overexpression	Increased sialylation	[106]
	ST3Gal IV and β1,4-GT	Overexpression	Increased sialylation and trisialylated glycans	[107]
	ST3Gal III, ST3Gal IV and ST3Gal VI	siRNA	Reduced sialylation	[108]
	CMP-SAT	Overexpression	Increased sialylation, increased CMP-SAT	[109]
	GNE/MNK	Overexpression	Increased sialylation	[110]
	GNE/MNK, CMP-SAT and ST3Gal IV	Overexpression	Increased sialylation, increased tetra-sialylated glycan	[110]
	30Kc19	Overexpression	Increased sialylation	[111]
	Sialidase A	Overexpression	De-sialylation	[26]
	Sialidase antisense RNA	Constitutive expression	Increased sialylation	[112]
	Sialidase	siRNA	Increased sialylation	[48]
	Neu1 and *Neu3*	siRNA/shRNA	Increased sialylation	[113]
Glycosyltransferases	GnT-III	Overexpression	Bisected glycoforms, Increased afucosylation, increased hybrid oligosaccharides increased ADCC	[24,114–115]
	Chimeric GnT-III	Overexpression	Increased afucosylation, increased hybrid oligosaccharides, increased ADCC	[116]
	GnT-IV and/or GnT-V	Overexpression	Increased triantennary and tetraantennary sugar chains	[117]
	Mgat1 (GnTI)	ZFN Knockout	Predominantly Man5	[118]

ADCC: Antibody-dependent cellular cytotoxicity; TSA: Tumor-specific antigen; ZFN: Zinc finger nuclease.

and result in a 4–16% increase in sialylation of the secreted IFN-γ [119].

The enzyme uridine diphosphate-N-acetyl glucosamine 2-epimerase/N-acetyl mannosamine kinase (*GNE/MNK*) plays an important role in the biosynthesis of sialic acid and is regulated by CMP-sialic acid through a feedback mechanism [13]. Patients with the sialuria mutation have defective feedback regulation of the biosynthesis pathway and hence make proteins with high sialic acid content. Use of a sialuria mutated *GNE/MNK* leads to increased sialic acid content of CHO expressed human EPO [110]. When this mutated *GNE/MNK* was co-expressed with both the CMP-SAT and ST3Gal IV genes in cells expressing human EPO, the sialic acid content was increased 43% over the control and the ratio of tetra-sialylated glycans was also increased 32% [120]. Other less obvious genes, such as the *30Kc19* from the silkworm hemolymph, which promote the transfer of sialic acids onto glycoproteins in insect cells

have been used to affect sialylation. Overexpression of *30Kc19* in CHO cells producing EPO resulted in an 87% increase in sialylation via increased activity of α2,3 sialyltransferase [111].

Work has also been done to limit the degree of sialylation of recombinantly expressed antibodies in order to produce antibodies with increased effector function via increased ADCC activity. To this end, cells were engineered to secrete sialidase A, which is capable of removing sialic acid from antibodies co-expressed in the same CHOK1 cells [26]. In a very different approach, CHO cells were engineered to constitutively express sialidase antisense RNA to reduce the level of sialidase and prevent desialylation of the glycoprotein, which resulted in a 20–37% increase in sialic acid content of the protein [112].

The glycosylation patterns of recombinant therapeutic glycoproteins can be engineered by overexpression of glycosyltransferases in the host cells used for glycoprotein production. α-1,4-N-acetylglucosaminyltransferase (GnT-III) creates bisecting oligosaccharides on N-glycans by catalyzing the transfer of GlcNAc from UDP-GlcNAc to the α-mannose of asparagine-linked carbohydrates [121]. GnT-III is typically not expressed at significant levels in CHO cells but a cell line capable of producing IFN-α bisected oligosaccharides on glycoproteins was created by overexpression of rat GnT-III [114]. In another study, rat GnT-III expressed in a CHO cell line producing a chimeric mouse/human anti-CD20 IgG1 antibody was able to add bisecting GlcNAc residues in 48% to 71% of the N-linked oligosaccharides [24]. In an ADCC assay, the engineered antibody was able to promote killing of CD20-positive target cells at concentrations 10-to 20-fold lower than the control.

GnT-III overexpression has been shown to increase non-fucosylated and hybrid oligosaccharides by blocking core-fucosylation and conversion of hybrid to complex glycans, which resulted in increased ADCC activity of an IgG1 antibody [115]. The same group then created chimeric GnT-III by fusing the catalytic domain of GnT-III to the localization domain of other Golgi-resident enzymes [116]. They found that chimeric GnT-III can compete even more efficiently with α1,6 fucosyltransferase and α-mannosidase II, yielding a higher proportion of bisected non-fucosylated hybrid glycans with reduced complement-dependent cytotoxicity activity. The co-expression of GnT-III and ManII led to a similar degree of non-fucosylation, but with mostly complex glycan linkages which significantly increased ADCC activity compared with the unmodified antibody.

To produce IFN-γ with branched glycoforms, Fukuta and colleagues introduced the genes for GnT-IV (β-1,4-N-acetylglucosaminyltransferase) and/or GnT-V (β-1,6-N-acetylglucosaminyltransferase) into IFN-γ expressing CHO cells. Either GnT-IV or GnT-V alone were able to increase the proportion of tri-antennary sugar chains up to 66.9 and 55.7%, respectively, while co-expression of both enzymes resulted in a 56% increase in tetra-antennary sugars [117]. The same group showed that GnTIII affects IFN-γ N-glycan processing by competing with GnT-IV, GnT-V and α-mannosidase II and also by other mechanisms that suppress the conversion of high-mannose-type sugar chains to the hybrid type [122].

Short interfering RNA (siRNA)

RNA interference is a popular method for disruption of genes via introducing double stranded RNA into a cell to silence a specific sequence of complementary mRNA. Short interfering RNA (siRNA) function through a multi-step mechanism that starts with the processing of long dsRNA by the ribonuclease enzyme Dicer. Dicer breaks the dsRNA into smaller duplexes of about 21 base pairs that have a 3' overhang of two nucleotides in length. The duplexes are then unwound and incorporated into the RNA-induced silencing complex (RISC). The target mRNA is then recognized by base pairing and silenced via different mechanisms which include translational repression, mRNA cleavage or degradation [123]. siRNA has been used extensively to silence genes related to glycosylation in CHO cells. Mori and colleagues utilized small interfering RNA (siRNA) [95] or short hairpin RNA (shRNA) [96] against α1,6 fucosyltransferase (*FUT8*) to enhance ADCC in antibodies expressed in DG44. This group also investigated another target involved in oligosaccharide fucose modification, GDP-mannose 4, 6-dehydratase (*GMD*), creating *GMD* knockout DG44 cells that were confirmed to be devoid of intracellular GDP-fucose and to produce completely non-fucosylated antibodies [100]. To further improve the ADCC activity of the DG44 expressed IgG1 antibody, they used siRNAs against *FUT8* and *GMD* to achieve double knockout cells with synergistic reduction of fucosylation, generating clones exhibiting fucose content as low as 2% [101]. More recent studies have demonstrated that addition of *FUT8* and *GMD* siRNA directly to a bioreactor resulted in antibodies with increased levels of afucoslyation (63%) with no negative effect on cell growth, product titer or specific productivity [102]. Another fucosylation target for siRNA is the GDP-fucose transporter (GFT), whose knockdown with siRNA resulted in up to a 40% increase in defucosylated antithrombin III [99].

Sialylation has also been targeted by siRNA in CHO cells mostly by prevention of desialyation of

the glycoprotein by sialidase, a glycosidase responsible for cleaving terminal sialic acids. siRNA was utilized to silence the activity of sialidase on IFN-γ produced by CHO cells, resulting in stable clones with a 60% reduction in sialidase activity and allowing for retention of full sialic acid content of the recombinant IFN-γ [48]. In a separate study using CHO cells expressing human IFN-γ, siRNA and shRNA were used to reduce expression of *Neu1* and *Neu3* [113]. Knocking out *Neu3* resulted in a 98% reduction in the sialidase function in these cells and up to a 33% increase in the sialic acid content of the IFN-γ.

siRNA was used to knockdown various sialyltransferases (ST3Gal III, ST3Gal IV and ST3Gal VI) in CHO cells expressing EPO to elucidate which genes were involved in sialyation of N-glycans [108]. In this work, siRNA transfection of all three targets exhibited the largest reduction in α2,3-linked sialylation, but of the three sialyltransferases, ST3Gal IV showed the most pronounced reduction when transfected alone, suggesting that it may play the most critical role in glycoprotein sialylation.

microRNAs

microRNAs (miRNAs) are short RNA molecules of about 22 nucleotides in size that are capable of modulating target gene expression via destabilizing mRNA in a mechanism similar to siRNAs or by inhibition of translation. There is a large body of literature investigating the effects of miRNA on CHO cell productivity, energy metabolism, cell growth and apoptosis [124,125], but there have been no studies to date that investigate the role of miRNA target sites on enzymes involved in N- and O-glycosylation in CHO cells. There have been several studies that indicate that miRNAs may play a role in the control of product quality attributes in other cell types. It was recently found that a specific miRNA, miR-148b, modulates the expression of β1,3-galactosyltransferase-1 (C1GALT1), an important enzyme in the synthesis of O-glycosylation [126]. Other work on the regulation of glycosylation in cancer metastasis, demonstrated that N-acetylgalactosamine transferases (GALNTs) which initiate O-linked glycosylation were suppressed by the up-regulation of microRNA clusters [127]. Specifically, miR-30b/30d expression was shown to silence *GALNT7*, resulting in defective glycosylation. Bernardi *et al.* found that forced expression of miR-122 and miR-34a induces a decrease of *FUT8* levels and also affected core fucosylation of secreted proteins by specifically interacting with and regulating the 3'UTR of *FUT8* in human hepatocarcinoma cells [128]. These reports provide evidence that microRNAs are involved in these processes and are a potential mechanism for control of CHO glycosylation genes.

Zinc finger nucleases (ZFNs)

Advances in genome editing techniques have resulted in several technologies that allow for precise and efficient introduction of genetic alteration in mammalian cells. ZFNs are composed of an engineered zinc-finger domain, typically an array of 4–6 synthetic Cys_2–His_2 zinc finger motifs, fused through a short linker to a modified FokI endonuclease domain. ZFNs catalyze a double strand break in the target DNA by the action of two units that assemble in a tail-to-tail orientation on opposite DNA strands resulting in dimerization of the FokI domains [129]. Once the double strand break has occurred, the cell can repair the damage by two different mechanisms, homologous recombination (for gene insertion) or nonhomologous end joining (for gene silencing) [130].

This technique has been used to knockout the *FUT8* gene in CHO cells both alone and in combination with the GS and DHFR genes [98]. As the cells were not expressing a therapeutic protein, the knockout functionality was assessed by a FACS-based *Lens culinaris* agglutin (F-LCA) binding assay and it was shown that cell surface proteins lacked core fucosylated oligosaccharides. This same method was used to disrupt *FUT8* in CHO cell lines stably producing an antibody, which was shown to completely lack core fucosylation [97].

ZFNs have also been used to create cell lines deficient in mannosyl (α1,3)glycoprotein β-1,2-N-acetylglucoseaminyltransferase (Mgat1 or GnT1) which produce recombinant proteins with Man5 as the predominant N-linked glycan, which could be useful for mannose receptor-targeted therapeutics [118]. Although the use of ZFNs has many advantages, there are several considerations when designing and using them for modulation of glycosylation genes. Like most genome editing tools, information about the genetic sequence to be targeted is essential for proper design of the ZFN-binding motifs. Additionally, construction of ZFNs by modular assembly has been shown to have a high failure rate for engineering functional zinc finger arrays [131].

Future directions in genome editing for glycosylation engineering

Recently a class of proteins called transcription activator-like effectors (TALEs) has led to the identification of a novel DNA-binding domain known as TALE repeats, which consist of tandem arrays with up to 30 repeats that recognize and bind to extended DNA sequences. By fusing TALE repeats to the FokI endonuclease, a new genome editing tool, TAL effector nucleases (TALENs), have been created. TALENs are similar in mechanism to

ZFNs in that dimerization of the FokI domains results in a double strand break in the target DNA sequence, which allows for knock out or knock in of genes. TALENs may have some advantages over ZFNs in that they are easier to design, can bind longer bp sequences, have fewer constraints on site selection and can bind to desired DNA sequences with high affinity [132]. Some potential disadvantages include off-target effects, which are also a consideration with ZFNs and the significantly larger size than ZFNs. Because the typical size of TALENs cDNA (~3 kb) is larger than ZFNs (~1 kb) it is more difficult to deliver and express the TALEN pairs and package them in certain viral vectors.

Another class of genome engineering technology are RNA-guided nucleases (RGNs) built on components from clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated Cas systems that evolved in bacteria as an immune mechanism against foreign nucleic acids. The most common RGNs use the CRISPR-Cas9 nuclease system from *Streptococcus pyogenes* which utilizes a combination of short RNAs and proteins to target specific DNA sequences. The Cas9 protein contains two nuclease domains and is fused to guide RNA which recognizes and hybridizes to a 20 bp protospacer in the genome, allowing the nucleases to catalyze a double strand break in the DNA [129,133]. Because the target sequence specificity of CRISPR-Cas9 is governed by RNA–DNA hybridization as opposed to protein-DNA interactions in ZFNs and TALENs, the system is conducive to multiplexing via using multiple guide RNAs in parallel. The RNA dependent binding is also advantageous over ZFNs and TALENs because it is relatively easy to target various areas of the genome via changing the 20 bp protospacer of the guide RNA, as opposed to using large DNA segments to code for protein binding domains. The ease of using CRISPR-Cas9 for new targets lends itself to generation of genome-wide libraries or large vector sets to target multiple sites. A disadvantage of this system is the size of the Cas9 protein; the cDNA encoding the protein is 4.2 kb, larger than TALEN cDNA, making it a challenge to deliver using viral vectors. Another issue with CRISPR-Cas9 is the off-target effects due to the promiscuous nature of the 20 bp protospacer which tolerates single mismatches well and can even tolerate multiple mismatches depending on their location in the sequence [134]. These off-target affects are specific to the guide RNA and work is ongoing to improve the specificity of the system [133].

Conclusion

Improvements in the sensitivity and throughput of various analytical assays have allowed for more accurate and rapid glycosylation profiling of proteins. These improvements enable more extensive study of how N-glycosylation can be affected via process changes or engineering strategies. The use of bioprocess parameters and media additives to modulate the glycosylation of proteins has yielded mixed results and may be dependent on the molecule being expressed and/or affected by other process conditions or media components. Engineering of the CHO host maybe the most straightforward way to affect N-glycosylation. The ability of the new genome editing techniques to modulate specific genes makes these approaches extremely attractive option for glycosylation engineering.

Future perspective

Despite the availability of a wide range of potential hosts including *Escherichia coli*, yeast and insect cells for biotherapeutic production, CHO remains the most prevalent host cell of choice for current and emerging therapeutics. More recently human cell lines have been used to express glycoproteins that require post-translational modifications that are consistent with those seen on endogenous human proteins. The potential for CHO cells to produce proteins with α-gal, NGNA and other immunogenic sugars makes using a human cell line an attractive option, but to date human hosts have yet to reach the productivities seen in CHO. Recent advances in synthetic biology are leading to the development of synthetic hosts that could have completely custom product quality profiles, but these systems are early in development. Genome editing tools have the potential to specifically engineer the glycosylation pathways of alternative hosts such as yeast or fungi which have capacity for higher specific productivities than CHO cells. Despite all these factors, the industry's extensive experience with CHO and the ability to customize the CHO host with little to no effect on its growth and productivity characteristics will likely enable it to continue to dominate the glycoprotein production landscape in the near term.

The advent of novel genome editing tools has opened up the field of cell line engineering and offers many options for engineering the glycosylation of therapeutics produced in CHO cells. Compared with the use of glycosylation mutants or standard overexpression of genes, the new technologies are more efficient and less labor intensive. The common prerequisite for use of these techniques is accurate genome information to design the binding domains, guide RNA, siRNA or miRNA to get specific and effective targeting. Luckily, the CHO genome has improved substantially in recent years and continues to be refined and annotated, making the implementation of these genome editing tools in CHO a reality. The ability to leverage the information available in the ever evolving CHO genome and corre-

late this knowledge with metabolomics and proteomics will unveil new targets for genetic engineering that can be used to hone in on specific glycosylation profiles. The ability to multiplex genes via the Crispr-Cas9 system will further refine our ability to develop clones that produce a targeted glycan profile. The key to implementation of these techniques in cell line development is to not compromise the productivity of the resulting clones. As continual improvements are made in the analytical methods used to assess the glycan profile of these molecules, our ability to quickly and accurately isolate clones with both high specific productivity and desired glycoforms will improve. By coupling these analytical methods to the cell line engineering work aimed at creating designer glycoproteins, cell line development can be optimized generate clones with a targeted glycosylation profile.

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The authors have no 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. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Executive summary

Background

- Generating biotherapeutics with targeted glycosylation profiles in Chinese hamster ovary (CHO) cells is important for the function of the molecule.
- Protein glycosylation encompasses N- and O-linked glycans and their micro- and macro-heterogeneity.

Analytical technologies for glycosylation analysis

- Many methods for analyzing protein glycosylation have been developed with increased throughput and accuracy providing many options depending on the application.

Glycosylation control via process optimization & media supplementation

- Parameters such as pH, dissolved oygen, temperature and others have been used to modulate glycosylation with varying effects depending on the cell line and molecule.
- Media supplementation via nucleotide sugars and their precursors can be used to modify glycan profiles in the cell culture process.

Host engineering of specific targets

- Overexpression of glycosylation enzymes is a direct method to vary the degree to which proteins are glycosylated.
- siRNA knockdown has been used to target product quality attributes, most notably fucosylation.
- Zinc finger nucleases also provide a targeted way to edit the genes involved in glycosylation.

Future perspective

- New classes of genome editing techniques including transcription activator-like effector nucleases and Crispr-Cas9 offer a way to utilize information in the CHO genome to target DNA or RNA involved in glycosylation cell machinery.

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