Pharmaceutical BIOPROCESSING

Chinese hamster ovary mutants for glycosylation engineering of biopharmaceuticals

"...manipulation of glycosylation to enhance the properties of glycoproteins can be performed at any stage of the development of a production cell line using CRISPR/Cas9 technology."

Keywords: CHO glycosylation mutants • functional glycans • glycoproteins • N-glycans • O-glycans • targeted mutation

Pamela Stanley
Department of Cell Biology, Albert Einstein College of Medicine, New York, NY 10461, USA
pamela.stanley@einstein.yu.edu

The Chinese hamster ovary (CHO) cell has long been the cell of choice for the biopharmaceutical industry. The reasons for this are partly historical and partly because CHO cells produce glycoproteins with a glycan complement similar to that synthesized by human cells. The CHO-DUKX-B11 line was established early in the development of the biotechnology industry and has the advantage of allowing selection for high expression of transgenes. In addition, CHO cells glycosylate glycoproteins with a subset of glycans typical of human cells and essentially lacking antigenic sugar residues like α(1,3)Gal, β(1,2)xylose or N-glycolylneuraminic acid, typical of rodent or plant cells. In addition, CHO cells have a pseudo-haploid genome allowing stable mutants with desirable properties to be readily isolated. It is becoming increasingly evident that the manipulation of glycosylation pathways to produce secreted glycoprotein therapeutics with glycans appropriate for optimal half-life, cellular targeting and functional activity, is very important. This editorial will discuss CHO glycosylation mutants that are currently available, and the potential for glycosylation engineering to produce optimal biopharmaceuticals in the future.

CHO cell mutants with altered glycosylation were initially isolated by selection for resistance to the cytotoxicity of plant lectins [1]. Cells selected for resistance to one lectin, were often resistant to others with related glycan binding properties, and also hypersensitive to lectins recognizing sugars that became newly terminal due to the nature of the glycosylation defect. By comparing resistance to a panel of plant lectins, lectin-resistant mutants could be grouped into lectin resistance phenotypes. In addition, somatic cell hybridization was used to define genetic complementation groups. Once genes encoding glycosylation activities were cloned, mutations giving rise to each complementation group were identified and numerous allelic series defined. There are now a large number of CHO glycosylation mutants with known genetic and biochemical defects that may be used to engineer glycoproteins in order to optimize their properties. These include mutants that generate glycoproteins with modified N-glycans and/or O-glycans [2], glycosaminoglycans [3] or glycophosphotidylinositol-linked glycans [4]. However, for these mutants to be useful in the industrial level production of glycoprotein therapeutics, a specific glycosylation mutant must be employed from the very beginning of engineering a cell line for high productivity. While it is often stated that existing CHO mutant lines are not high producers, this may

FUTURE SCIENCE part of fsg

simply reflect the fact that few, if any, have been subjected to strong selection or screening for high levels of secretion due to the tedious process required to obtain cell lines that produce grams/liter of glycoproteins. Fortunately, there is now gene editing that can be performed to manipulate glycosylation genes at any stage in the isolation of the final production cell line [5,6].

> "Although it is now comparatively easy to engineer the glycosylation of a glycoprotein after a high-production cell line has been developed, it would be preferable to include glycosylation engineering in the production strategy from the beginning."

Which CHO glycosylation mutants might be useful for facilitating the large-scale production of glycoproteins with optimized properties? One example is the CHO mutant Lec1 that could be used for the production of glucocerebrosidase used to treat Type 1 and Type 3 Gaucher's disease. Commercial glucocerebrosidase (imiglucerase, Cerezyme® [Genzyme, MA, USA]) is produced from wild-type CHO cells and subsequently treated with three glycosidases to convert complex N-glycans to N-glycans terminating in mannose which targets the enzyme to reticulendothelial cells where it is most effective [7]. Lec1 CHO cells lack the glycosyltransferase MGAT1 and consequently do not make complex N-glycans [8]. The oligomannosyl N-glycans made in their place are ideal for targeting a glycoprotein to reticuloendothelial cells. A production line engineered from Lec1 CHO cells would produce glucocerebrosidase immediately ready for use. More recently, production from human cells treated with kifunensine, an inhibitor of N-glycan processing, has been used to generate glucocerebrosidase carrying immature oligomannosyl N-glycans with up to nine mannose residues per N-glycan [9]. Inactivation of the *MGAT1* gene from this cell line by gene editing would preclude the necessity of culturing in the presence of the mannosidase inhibitor. In fact, gene editing using clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology in CHO cells to introduce beneficial glycosylation gene mutations has recently been reported [10].

Although it is now comparatively easy to engineer the glycosylation of a glycoprotein after a high-production cell line has been developed, it would be preferable to include glycosylation engineering in the production strategy from the beginning. Thus, recently described *Mgat1* mutants in the CHO-DUKX line that allows rapid amplification of introduced genes, should be useful [11]. Removing a glycosylation activity from a production cell line has also proved effective. For example, zinc-finger nucleases (ZFN) designed to delete a portion of the catalytic domain of FUT8 were used to obtain *Fut8*$^{[-/-]}$ CHO cells at high frequency without selection from an antibody-secreting production line [12]. Detailed analysis showed that antibody from the ZFN-generated *Fut8*$^{[-/-]}$ cells lacked fucose in the complex N-glycan of the Fc domain as expected, was secreted at similar levels, and had properties equivalent to those of the same antibody secreted from *Fut8*$^{[-/-]}$ cells prepared by homologous recombination. However, the time frame for ZFN-generated knockout was rapid (~3 weeks). CRISPR/Cas9 methods should be similarly rapid and have been used to generate *Fut8*$^{[-/-]}$ CHO cells specific for generating complex N-glycans lacking a core fucose, as well as COSMC$^{-/-}$ CHO cells, specific for generating core 1 and core 2 O-glycans that cannot be extended beyond the addition of GalNAc [10]. The paper by Ronda *et al.* also describes a new database termed CRISPy that identifies single guide RNA sequences designed to target each CHO coding exon. The database contains approximately 2 million CRISPR targets in approximately 27,500 genes and identifies off-target sequences in the CHO genome. This tool is available on the CRISPy database [13].

At this time by far the greatest number of recombinant biologicals in production from cultured mammalian cells are monoclonal antibodies. Since the complex N-glycan of IgG determines interactions with Fc receptors, and thereby the efficacy of IgG in antigen-dependent cellular cytotoxicity, modifying the N-glycan to improve antigen-dependent cellular cytotoxicity has been, and will continue to be, a major focus. To date the most effective modification has been to prevent the addition of fucose to the core of the complex N-glycan by inactivating the *Fut8* gene. Alternative approaches include knocking out an enzyme required to make GDP-Fuc, the substrate of FUT8, or the major GDP-Fuc transporter SLC35C1. The advantage of the latter methods is that they target all fucosyltransferases, not just FUT8. While the literature describes numerous strategies to achieve knockout of an activity, the time is past for antisense cDNA, shRNA or transgenic overexpression strategies to remove or reduce fucosylation because CRISPR/Cas9 or ZFN technologies are much more precise, efficient and complete. However, in cases for which increasing glycosylation of a recombinant therapeutic is desirable, overexpression strategies are necessary. For example, fully sialylated erythropoietin has a longer half-life in the circulation, and IgG used for passive immunotherapy that is highly sialylated on the complex N-glycan induces an anti-inflammatory cascade that dampens the autoimmune response. However, transgenes are

often silenced during culture in mammalian cells. To avoid the problem of transgene silencing, a robust integration locus, that is resistant to epigenetic effects and surrounding chromatin, should be used. Success has been reported for obtaining stable, long-term expression using transgenes introduced into CHO cells on bacterial artificial chromosome vectors [14]. Enhancing overall synthesis of complex N-glycans may also lead to enhanced sialylation.

In summary, many CHO glycosylation mutants obtained by somatic cell genetic selection strategies, or more recently by gene editing strategies, have been developed to optimize glycans for the production of therapeutic recombinant glycoproteins. In future, manipulation of glycosylation to enhance the properties of glycoproteins can be performed at any stage of the development of a production cell line using CRISPR/Cas9 technology. This should facilitate research into understanding which glycans will promote, and which might inhibit, the production of an optimal recombinant glycoprotein therapeutic.

Financial & competing interests disclosure

The author has 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.

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

References

1 Stanley P. Glycosylation mutants of animal cells. *Annu. Rev. Genet.* 18, 525–552 (1984).

2 Patnaik SK, Stanley P. Lectin-resistant CHO glycosylation mutants. *Methods Enzymol.* 416, 159–182 (2006).

3 Zhang L, Lawrence R, Frazier BA, Esko JD. CHO glycosylation mutants: proteoglycans. *Methods Enzymol.* 416, 205–221 (2006).

4 Maeda Y, Ashida H, Kinoshita T. CHO glycosylation mutants: GPI anchor. *Methods Enzymol.* 416, 182–205 (2006).

5 Zhang P, Chan KF, Haryadi R, Bardor M, Song Z. CHO glycosylation mutants as potential host cells to produce therapeutic proteins with enhanced efficacy. *Adv. Biochem. Eng. Biotechnol.* 131, 63–87 (2013).

6 Steentoft C, Bennett EP, Schjoldager KT, Vakhrushev SY, Wandall HH, Clausen H. Precision genome editing – a small revolution for glycobiology. *Glycobiology* 24(8), 663–680 (2014).

7 Tekoah Y, Tzaban S, Kizhner T *et al.* Glycosylation and functionality of recombinant β-glucocerebrosidase from various production systems. *Biosci. Rep.* 33(5), pii: e00071 (2013).

8 Chen W, Stanley P. Five Lec1 CHO cell mutants have distinct *Mgat1* gene mutations that encode truncated N-acetylglucosaminyltransferase I. *Glycobiology* 13(1), 43–50 (2003).

9 Brumshtein B, Salinas P, Peterson B *et al.* Characterization of gene-activated human acid-beta-glucosidase: crystal structure, glycan composition, and internalization into macrophages. *Glycobiology* 20(1), 24–32 (2010).

10 Ronda C, Pedersen LE, Hansen HG *et al.* Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool. *Biotechnol. Bioeng.* 111(8), 1604–1616 (2014).

11 Zhong X, Cooley C, Seth N *et al.* Engineering novel Lec1 glycosylation mutants in CHO-DUKX cells: molecular insights and effector modulation of N-acetylglucosaminyltransferase I. *Biotechnol. Bioeng.* 109(7), 1723–1734 (2012).

12 Malphettes L, Freyvert Y, Chang J *et al.* Highly efficient deletion of FUT8 in CHO cell lines using zinc-finger nucleases yields cells that produce completely nonfucosylated antibodies. *Biotechnol. Bioeng.* 106(5), 774–783 (2010).

13 CRISPy Database. http://staff.biosustain.dtu.dk/laeb/crispy/

14 Kunert R, Casanova E. Recent advances in recombinant protein production: BAC-based expression vectors, the bigger the better. *Bioengineered* 4(4), 258–261 (2013).