

## Engineering a Human-Like Glycosylation to Produce Therapeutic Glycoproteins Based on 6-Linked Sialylation in CHO Cells

Nassimal El Maï, Sandrine Donadio-Andréi, Chloé Iss, Valérie Calabro, and Catherine Ronin

### Abstract

When recombinant glycoproteins for therapeutic use are to be produced on an industrial scale, there is a crucial need for technologies that can engineer fast-growing stable cells secreting the protein drug at a high rate and with a defined and safe glycosylation profile. Current cell lines approved for drug production are essentially from rodent origin. Their glycosylation machinery often adds undesired carbohydrate determinants which may alter protein folding, induce immunogenicity, and reduce circulatory life span of the drug. Notably, sialic acid as *N*-acetylneuraminic acid is not efficiently added in most mammalian cells and the 6-linkage is missing in rodent cells. Engineering cells with the various enzymatic activities required for sialic acid transfer has not yet succeeded in providing a human-like pattern of glycoforms to protein drugs. To date, there is a need for engineering animal cells and get highly sialylated products that resemble as closely as possible to human proteins. We have designed ST6Gal minigenes to optimize the ST6GalI sialyltransferase activity and used them to engineer ST6(+)-CHO cells. When stably transfected in cells expressing a protein of interest or not, these constructs have proven to equip cell clones with efficient transfer activity of 6-linked sialic acid. In this chapter, we describe a methodology for generating healthy stable adherent clones with hypersialylation activity and high secretion rate.

**Key words** Sialyltransferases, Glycoengineering, Stable clones, CHO cells, Sialylation

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### 1 Introduction

Most marketed therapeutic proteins, including the wide family of monoclonal antibodies as well as many new candidates under development, are glycoproteins. These glycoproteins are predominantly produced in mammalian (rodent) expression systems such as Chinese hamster ovary (CHO), mouse myeloma NS0, or SP2/0 cell lines because their glycosylation machinery is of the complex type like that of human cells. However terminal glycosylation was found to be significantly different and increase the immunogenicity of the drug (1).

Rodent-type glycans substantially differ from human structures in both N-linked and O-linked carbohydrate chains. Sialic acid is not the preferred terminal sugar in mammals which rather terminate their glycans in  $\alpha$ -linked galactose or poly *N*-acetylglucosamine. Also among sialic acids, the presence of *N*-glycolylneuraminic acid (NeuGc) predominates over *N*-acetylneuraminic acid (NeuAc) in rodents. NeuGc is not found in human glycoproteins because of a mutation in the CMP-neuraminic acid hydroxylase gene (2). Altogether, such terminal glycosylation was found to substantially generate carbohydrate-based immunogenicity and promote clearance. More particularly, antibodies expressed in NS0 or SP2/0 cells were found to generate strong reactivity in patients administered with these proteins (3). Neutralization of the drugs rapidly occurs through anti- $\alpha$ -Gal antibodies since they represent 1% of our circulating immunoglobulins. Anti-NeuGc antibodies have also been found in some patients (4, 5), and are considered as being linked to chronic inflammation (6, 7).

CHO cells have been approved for drug production very early and very luckily, they fail to express the  $\alpha$ 3Gal epitope. They express an  $\alpha$ 2,3-sialyltransferase (ST3) (8) which adds  $\alpha$ 2,3-linked terminal sialic acid (NeuGc/NeuAc) to *N*-glycans. However, during a fermentation process, this enzyme often fails to fully cover all the branches of *N*-glycans, leaving a variable amount of free  $\beta$ -galactose residues detrimental to prolonged circulatory half-life. Indeed, clearance of asialoglycoproteins rapidly occurs through the liver asialoglycoprotein receptors, a galactose lectin receptor capable of removing from serum with a remarkably high capacity (mg/min) (9), as previously described for erythropoietin (10). Various genetic engineering methods have therefore been developed to enhance glycoprotein sialylation in rodent cells (11). These methods have included various approaches such as increasing the intracellular pool of sialic acids, increasing the availability of nucleotide precursor CMP-NeuAc, increasing the number of sialyltransferase acceptor sites, overexpression of sialyltransferases (STs), and decreasing soluble sialidase activities (12–16). All these methods were found to increase the cellular content in sialic acid and in sialylated endogenous components but have limited efficacy on the therapeutic proteins. CHO cells also lack the expression of an  $\alpha$ 2,6-sialyltransferase (ST6) able to transfer sialic acid in the 6-position to N-linked glycans of proteins. Such linkage is the preferred terminal glycosylation of human proteins, especially in blood, and is thus often regarded as the representative human-type glycosylation because yeast, plant, insect, or bird expression systems are not able to carry out 6-linked sialylation. Expressing ST6 activity is thus essential to produce glycoproteins with human-like glycoform pattern.

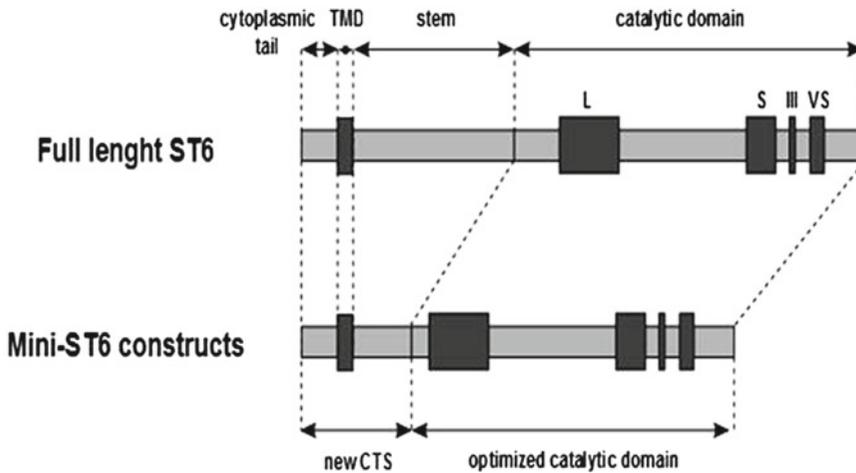
Since the first transfection of the rat ST6GalI gene into CHO cells (8), several groups have attempted to coexpress this enzyme with various proteins of human origin. Recombinant human tPA was expressed in CHO cells engineered with rat ST6GalI (17),

IFN- $\gamma$  in CHO clones containing mouse ST3 and rat ST6 (15) or rat ST6GalI alone (18), and more recently, TSH in CHO cells containing rat ST6cDNA (19). All these studies demonstrated a substantial improvement in glycoengineered cells with a 1.5-fold increase in sialic acid content and an average distribution of 60%  $\alpha$ 2,3 to 40%  $\alpha$ 2,6-linked sialic acid but no substantial increase in the sialylation of the therapeutic protein of interest. Some studies even failed to detect significant difference in overall sialylation following ST6 transfection (20, 21). It was therefore concluded that the wild-type ST6 enzyme was ineffective to compete with the endogenous ST3 enzyme, either because it did not localize in the same subcellular compartments or because it could not be active enough in the cellular context. Some of us could further demonstrate that efficacy on protein acceptors is restricted by a peptide region in the stem domain of the human hST6GalI enzyme (22).

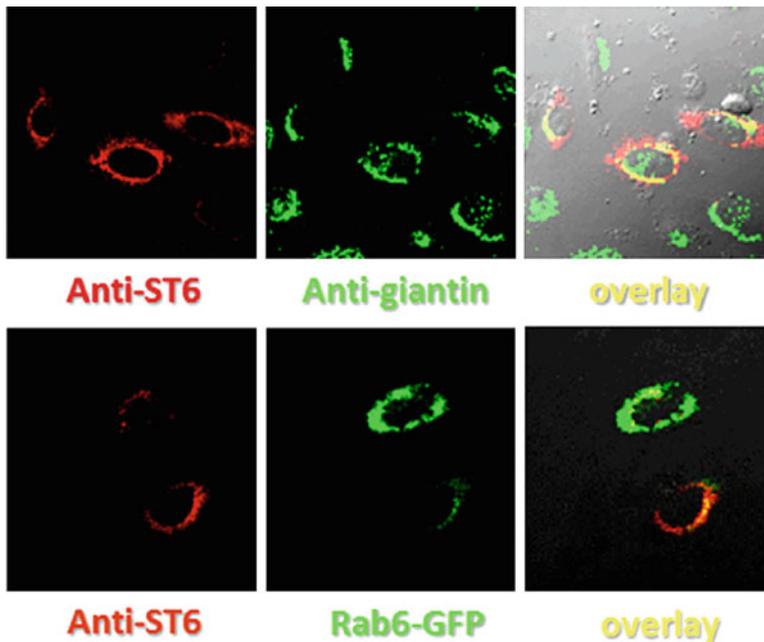
The gene family of human sialyltransferases (STs) is composed of 20 members and distributes in four subfamilies and according to their specificity: ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia. All STs are type II proteins, resident of the *trans*-Golgi/TGN intracellular compartments. They are all constituted of a short cytoplasmic N-terminal tail (1–31 aa), a transmembrane fragment (16–20 aa) followed by a stem region of variable length (20–300 aa), and a C-terminal catalytic domain (CD) facing the Golgi lumen (23). Unlike other glycosyltransferase families, mammalian and human STs display high sequence homology in their catalytic domain, with four consensus sequences designated as sialylmotifs: L (for long) (24, 25), S (for short) (26, 27) VS (for very short) (28), and sialylmotif-3 (third position in the sequence) (29) (Fig. 1). Site-directed mutagenesis demonstrated that the L-sialylmotif contributes to the binding of donor substrate CMP-NeuAc (24), and the motifs III- and VS contribute to the binding of the acceptor substrate (29). The S-sialyl motif participates in the binding of both the donor and acceptor substrates (30). Alternatively, the cytosolic tail, transmembrane region, and a significant portion of the stem, often designed as the CTS region, govern Golgi localization (31). Accordingly, innovative design of the CTS region has been carried out to provide a panel of a nonnatural membrane anchors to the catalytic domain of the human ST6GalI (cf. Footnote). The catalytic domain of the transferase has been optimized to present a 30-fold increase in transfer efficacy and broadly transfer sialic acid to acceptor substrates of different degree of branching compared to the full-length enzyme. Figure 1 describes the design of the minigenes.

All the engineered transferases were found to be located in the Golgi apparatus as shown in Fig. 2.

It could further be observed that the glycoengineered cells are healthy, good producers and possess high hypersialylation activity. We describe below the transfection procedure and selection of stable clones using such minigenes and an adherent CHO cell line.



**Fig. 1** Design of engineered hST6GalII sialyltransferases



**Fig. 2** Localization of engineered sialyltransferases in the Golgi compartments using specific markers and anti-ST6 antibodies. Anti-giantin labeled the *cis* Golgi cisternae in *green*, Rab6 GFP is a marker of *trans* Golgi/TGN (also in *green*), and staining with ST6 antibodies is in *red*. Overlay shows the colocalization in *yellow*. These experiments are from a transient transfection of CHO cells with minigenes

## 2 Materials

### 2.1 Cell Culture

1. CHO cells expressing or not the protein of interest: This cell line was originally derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster. Various CHO cell lines may be used alternatively with similar efficiency.

2. pcDNA3.1(+) vector which encodes a sialyltransferase minigene, ampicillin resistance for selection and maintenance in bacteria, and geneticin (G418) for selection in mammalian cells.
3. Ham's medium with L-glutamine and fetal bovine serum (FBS) Ham's medium is supplemented with 10% FBS with or without 100 U/mL penicillin/100 µg/mL streptomycin. Transfection is performed in the absence of FBS.
4. A commercial transfection reagent.
5. Geneticin selective antibiotic.
6. DPBS (1×) with Ca<sup>2+</sup> and Mg<sup>2+</sup>.
7. Clean forceps with fine tips, autoclaved.
8. Trypsin/EDTA.
9. 6-well culture plates.
10. CO<sub>2</sub> incubator (37°C): It maintains optimal temperature, humidity, and conditions of CO<sub>2</sub> and oxygen content.
11. 2-, 5-, and 10-mL serological pipets.

## **2.2 Selection of Transfected Cells**

1. Optic inverted microscope.
2. Geneticin selective antibiotic liquid 10131-027.
3. 6-well culture plates.
4. DPBS (1×) with Ca<sup>2+</sup> and Mg<sup>2+</sup>.
5. Clean forceps with fine tips, autoclaved.
6. Trypsin/EDTA.
7. 2-, 5-, and 10 mL serological pipets.

## **2.3 Immunostaining**

1. Fluorescence microscope: Filters should be selected to handle double or triple fluorochromes.
2. Fluorescent *Sambucus Nigra* agglutinin and prolong gold DAPI. The SNA lectin is specific for 6-linked sialic acid while DAPI is a specific labeling of the nucleus in cells. The procedure herein describes the use of SNA-FITC but any other fluorophore can be used.
3. DPBS (1×) with Ca<sup>2+</sup> and Mg<sup>2+</sup>.
4. Cover glass and microslides.
5. 24-well plates.
6. PBS-PFA 4%: 4 g of paraformaldehyde are dissolved in 100 mL of DPBS and warmed until a colorless solution is obtained. Do not heat above 60°C. Store at -20°C. Wear a mask when weighing paraformaldehyde.
7. PBS/BSA 1%: Dissolve 1 g bovine serum albumin (BSA) in 100 mL of DPBS (1×) and store at 4°C.

#### **2.4 Selection of Stable Cell Clones**

1. 96-well culture plates.
2. Automatic pipettor and sterile tips.
3. Ham's medium with L-glutamine and FBS Ham's medium is supplemented with 5% FBS with or without 100 U/mL penicillin/100 µg/mL streptomycin.
4. Geneticin.

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### **3 Methods**

#### **3.1 Transfection of CHO Cells**

1. Seed  $5 \times 10^5$  cells/well in a 6-well plate in the presence of Ham's medium + 5% FBS and incubate for 24 h at 37°C under 5% CO<sub>2</sub>.
2. Wash the cells three times with 1 mL of DPBS and then add 2 mL of Ham's medium FBS free.
3. Transfect CHO cells with pcDNA3.1(+) plasmids using the transfectant reagent at various ratios (3:2, 3:8, and 4:5 (µL/µg)) and incubate overnight (see Note 1).
4. Remove the medium, wash once with DPBS, then add 2 mL of new Ham's medium + 5% FBS, and incubate for 24 h to allow cell recovery after transfection.
5. Remove the medium and place in Ham's medium + 5% FBS containing G418 at 100 µg/mL. This step will select cells that have stably incorporated the plasmid into their genomic DNA. It is recommended to treat cells with moderate concentration of antibiotic because it reduces cellular growth. Clones can also be rapidly selected after transfection but the most interesting cells are often the ones which grow slowly at this stage.
6. Following addition of the selection medium, cells still grow for 1–2 days but most cells start to die within a week. There is a lot of debris in the culture at this point. Change the medium regularly over this period.
7. It is recommended to include a negative control prepared with non-transfected cells to estimate cell viability at each concentration of antibiotic (see Note 2).

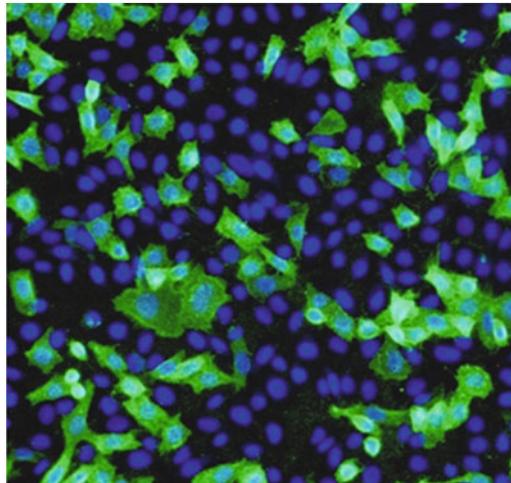
#### **3.2 Selection of Transfected Cells**

1. Observe cells every day with an optic inverted microscope. Once confluence is almost reached, split cells to 1:5 dilution in new selective Ham's medium + 5% SVF + 100 µg/mL G418. Splitting of cells should be done three times a week according to cell growth. It is especially important to well separate cells during splitting. Allow the trypsin to act under gentle agitation to keep the cells separated and avoid aggregates.
2. Repeated splitting allows the removal of non-transfected cells and favor increase of resistant cells.

3. After 3 weeks, increase the G418 concentration of the medium to 150  $\mu\text{g}/\text{mL}$ . After time, a few cells grow in low concentration of antibiotic and the purpose of this step is to prevent their growth. Take care not to pipet directly on the cells; otherwise the concentration of the G418 will be too strong on these cells and not on others. When a massive cell death is achieved, wash off the bottom of the dish, leaving colonies of resistant cells.

### 3.3 Immunostaining

1. Pick  $2.5 \times 10^4$  cells of each well and plate them on sterile cover glass in a 24-well plate.
2. After overnight incubation, the cells are attached to the cover glass and rinsed with DPBS (1 $\times$ ) with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  three times.
3. Each cover glass is treated with 0.5 mL of PBS/PFA 4% for 30 min at RT and then wash three times for 2 min with DPBS (1 $\times$ ) with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ .
4. Add 0.5 mL of PBS/BSA 1% and saturate each cover glass for 30 min at RT. Do not wash the cells after saturation.
5. Prepare the staining solution by adding 5  $\mu\text{L}$  of SNA-FITC to 995  $\mu\text{L}$  of DPBS (1 $\times$ ) with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Cover each cover glass with a 50  $\mu\text{L}$  drop of the staining solution so that cells will be directly in contact with the fluorescent lectin. Stain for 1 h at 4 $^{\circ}\text{C}$  in the dark. Then place cells upward to wash each cover glass three times with DPBS (1 $\times$ ) with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Only cells which show sialyltransferase activity are labeled as shown in Fig. 3.

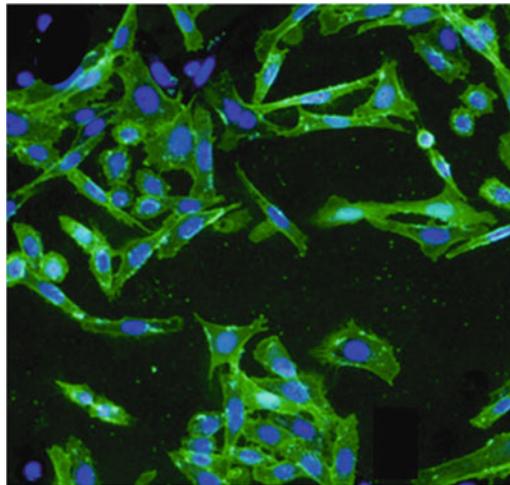


**Fig. 3** Double staining of CHO cells transfected with a sialyltransferase minigene and selected over several weeks with a G418 concentration of 100  $\mu\text{g}/\text{mL}$ . FITC labeling is in *green* and DAPI in *blue*

6. Dry-clean microslides with a paper soaked in alcohol, put down a drop of prolong gold-DAPI (liquid mountant with DAPI nuclear stain) on the microslide and put one drop of prolong gold on cells, and let it dry at RT in the dark. Store at  $-20^{\circ}\text{C}$  in slidebox until use (see Note 3).
7. Count positive stained cells with regard to the total number of cells (based on nuclear staining). Some negative cells still remain at this stage even though cells are under selection. Estimating the number of stained cells allows the identification of wells suitable for further cloning. Save the wells that are best enriched in labeled cells.

### 3.4 Selection of Stable Cell Clones

1. Resistant cells are harvested and counted. Seed a single cell in each well of 96-well plates in the selection medium and incubate overnight at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ . The remaining cells are picked and plated in a new 6-well plate. At this stage, they can be frozen and stored at  $-80^{\circ}\text{C}$  for further use. On the 96-well plate, the cell number may vary among wells. The wells with a single cell are carefully identified. It takes 1–2 weeks for cells to reach suitable density. At this point, the plates should be carefully inspected under microscope (see Note 4).
2. Transfer every single group of cells from 96-well to 24-well plates. Each well is matched with the earlier staining. Use immunostaining to determine the wells which contain only positive cells and then transfer each positive clone to a new well. All cells should be labeled as in Fig. 4.



**Fig. 4** Double staining of a stable clone expressing a 6-sialylation: 1 at this stage, 100% of the CHO cells are stained by FITC (*green*) and DAPI (*blue*) fluorochromes and are resistant to G418 at the final stage of the limit dilution process

3. Stable clones selected after dilution are propagated in selection medium. When cell number reaches almost 80% confluence in T25 flask, aliquots can be frozen at  $-80^{\circ}\text{C}$ . Each selected clone can be propagated at a reduced amount of G418 for maintenance. Keep track of passage numbers to ensure stability of each clone which may vary and even die after a few passages. Periodically check the quality of the clones by immunostaining.

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## 4 Notes

1. The transfection reagent must be adapted to the cell line used. Many different preparations are commercially available and must be tested to optimally allow 10–30% of transfected cells.
2. The wild-type cell line should be initially sensitive to G418 before starting the glycoengineering process. It is recommended to carry out a negative control at each raise in antibiotic concentration.
3. Immunostaining is stable over a week or so. However, it is recommended to analyze the staining as soon as possible to get the best contrast for stained cells.
4. Never mix positive cells from different clones because the sialyltransferase activities may vary from one clone to another. Further selection should determine the best clone to be used in subsequent experiments.

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