Biochemical and Biophysical Research Communications 463 (2015) 211–215

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Assessment of the coordinated role of ST3GAL3, ST3GAL4 and ST3GAL6 on the α2,3 sialylation linkage of mammalian glycoproteins

Cheng-Yu Chung, Bojiao Yin, Qiong Wang, Kai-Yun Chuang, Jeffrey H. Chu, Michael J. Betenbaugh*

Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA

ARTICLE INFO

Article history:
Received 27 April 2015
Available online 18 May 2015

Keywords:
CHO cells
Glycosylation
Sialylatransferase
siRNA
Gene knockdown
Sialic acid

ABSTRACT

In this research, we examined which genes are involved in N-linked sialylation in Chinese Hamster Ovary (CHO) cells using siRNA knockdown approaches. Three genes from the sialyltransferase family (ST3GAL3, ST3GAL4 and ST3GAL6) were chosen as knockdown targets with siRNA applied to reduce their expression. Single, double and triple gene knockdowns were investigated, and the reduction levels of sialylation on the total cell lysate were monitored by enzyme-linked lectin absorption assays (ELLA) and sialic acid quantification with high performance liquid chromatography (HPLC). All transfection groups showed effective reduction in 2,3-linked sialylation whereas the trend of reduction levels of triple siRNA transfection outweighed both the dual siRNA groups and single siRNA transfection groups. Next, this transfection approach was applied to CHO cells producing erythropoietin (EPO). Quantification of EPO sialylation showed similar result to total cell lysate except that the ST3GAL4 siRNA transfection exhibited the largest reduction according to the HPLC analysis as compared with other single siRNA transfections. Finally, the N-glycan released from the EPO transfected with ST3GAL4 siRNA showed a prominent reduction in sialyation level among the single siRNA transfections. From these experiments, we concluded that each of these three genes were involved in N-linked sialylation and ST3GAL4 may play the critical role in glycoprotein sialylation of recombinant proteins such as EPO.

1. Introduction

Glycosylation is one of the critical post translational modifications occurring in eukaryotic cells. Glycan structures affect numerous biological properties including development, growth, and survival by serving protective, stabilizing and signaling functions [1]. Sialylation represents the terminal step for many glycans with sialic acid playing an influential role in the biological function due to its size, hydrophilic characteristic and electronegative negative charge [2].

Another feature is that glycoprotein bearing sialic acid can hide the galactose residues from being recognized by asialoglycoprotein receptors (ASPR), a lectin in hepatocytes which captures the non-sialylated glycoproteins from blood circulation [3]. As a result, asialylated proteins will be cleared more rapidly than their sialylated counterparts. Consequently, researchers have observed that glycoprotein therapeutics bearing sialic acid often exhibit prolonged circulatory half-lives in vivo and improved efficacies [4].

On the other hand, limiting sialylation of glycoproteins can have other important in vivo implications for biomedicine and diagnostics. It has been reported that antibodies containing sialic acid exhibit reduced antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [5]. In addition, radiolabeled diagnostic proteins such as radiolabeled EPO represent potential diagnosis candidates in order to evaluate the lung cancer prognosis [6]. However, radioactive diagnostic proteins having long circulatory half-lives may lead to overexposure of patients to radiation and can be detrimental to patient health. Therefore, modulating the sialic acid content on antibodies or radioactive diagnostic proteins may alter the application of these proteins' in biomedical contexts.

Sialylation of N-glycans is accomplished by the sialyltransferases which transfer the sialic acid from CMP-sialic acid onto a glycoprotein terminating in galactose. HEK 293 and NS0, the common protein production vehicles derived from human and murine, contain glycoprotein bearing both α2–3 and α2–6

* Corresponding author.
E-mail address: beten@jhu.edu (M.J. Betenbaugh).

http://dx.doi.org/10.1016/j.bbrc.2015.05.023
0006-291X/

sialylation linkages. However, CHO cells produce glycoproteins having only α2−3 sialylation linkages because CHO cells contain α2−3 but no α2−6 sialyltransferases [7]. Six α2- 3 sialyltransferase family members, ST3GAL1 through ST3GAL6, have been identified and generally differentiated based on each sialyltransferase's substrate [8]. ST3GAL1 and ST3GAL2 have been shown to mask the galactose residues with sialic acids in O-glycans and glycolipids [8]. ST3GAL3 preferentially utilizes type I glycans (Galβ1-3GlcNAc) over the type II glycan (Galβ1-4GlcNAc), whereas ST3GAL4 has a preference of acting on type II glycan from the N-glycan [9,10]. ST3GAL5 is believed to act solely on lactosyceramide substrates [8]. ST3GAL6 has been shown to exhibit weak substrate specificity toward glycoproteins and has acted preferentially on glycolipids with substrates containing Galβ1-4GlcNAc-R [11]. Thus, ST3GAL3, ST3GAL4 and ST3GAL6 are believed to act on glycoproteins.

Previous studies of ST3GAL3, ST3GAL4 and ST3GAL6 have focused primarily on overexpression of these genes to enhance the sialylation and examine the biological consequences such as its effect on glycoproteins' circulatory half-lives and cancer metastasis [4,12,13]. Much less work has been performed to knockdown or knockout these genes individually or together. Mo et al. used siRNA to down-regulate the ST3GAL3 and/or ST3GAL4 expression on the endolyn produced in Madin-Darby canine kidney(MDCK) cells and addressed the effect of reducing sialylation on apical sorting of endolyn [14]. Yang et al. created ST3GAL4 and ST3GAL6 knockout mice and demonstrated that both genes are involved in the generation of functional selectin ligands in vivo [15]. However, previous research has not investigated the relevance of ST3GAL3, ST3GAL4 and ST3GAL6 simultaneously and especially their role on glycosylation of biotherapeutic molecules. Thus, the present study is to transiently knockdown the ST3GAL3, ST3GAL4 and ST3GAL6 genes through siRNA interference and perform a comprehensive discussion of the individual and coordinated roles of ST3GAL3, ST3GAL4 and ST3GAL6 sialylatransferases involved in erythropotein (EPO) expressing CHO cells, a heterologous and highly glycosylated biotherapeutic protein used in the treatment of anemia andradiolabelling imaging studies.

2. Materials & methods

2.1. Cell lines

A CHO-K1 cell line was purchased from Sigma−Aldrich (European Collection of Cell Culture, ECACC), and cultured with Ham's F-12K media with 10% FBS and 2 mM L-glutamine (Life technologies). An EPO-producing cell line was created by transfecting a mammalian cell expression plasmid containing a codon-optimized human EPO cDNA sequence linked with a six-histidine tag at the 3′ end. The CHO-EPO cell line was cultured in the same media as the CHO-K1 cell line except for the addition of 300ug/mL of Zeocin to maintain stable EPO expression.

2.2. siRNA transfection

The siRNAs targeting the ST3GAL genes were designed and purchased from Sigma. A negative control siRNA from Sigma was used as a negative control. The nucleotide sequences for ST3GAL3 siRNA were 5′ GUCACGAAUUGACGACUAUTT 3′ (sense) and 5′ AUAGUCGUCAAUUCGUGACTT 3′ (antisense); for ST3GAL4 siRNA were 5′ CCAUCACUAGCUAUUCUAUTT 3′ (sense) and 5′ AUAGAAUAGCUAGUGAUGGTT 3′ (antisense); and for ST3GAL6 siRNA were 5′ CCUUAAACUUGAUCUAUAATT 3′ (sense) and 5′ UUAUAGAUCAAGUUUAAGGTT 3′ (antisense). The siRNA transfection procedure was adapted from the Lipofectamine RNAiMax transfection protocol following the manufacturer's protocol. Briefly, 5×10^5 cells were seeded to each well in a 6-well plate. 24 h after seeding, dilute 9 ul of lipofectamine RNAiMax transfection reagent with 150 ul Opti-MEM media (Life technologies). Meanwhile, dilute 3 ul of 10 uM siRNA with 150 ul Opti-MEM media. Mix the diluted siRNA solution with the diluted lipofectamine RNAiMax solution and incubate for 20 min. Then the mixture was added to each well in the 6-well plate.

2.3. RT-PCR

RNA was isolated from the siRNA transfected CHO cells using an RNAeasy kit from Qiagen. 2 ug of the isolated RNA were used as the template for the first strand cDNA synthesis. A 15 ul mixture was created containing 2ug of isolated RNA, 1 ul of OligodT, and deionized water. This mixture was incubated at 70 °C for 5 min, and then put on ice immediately. A second mixture, containing 5 μl of 5 × MMLV buffer, 1.25 μl of 10 mM dNTP, 1 μl of RNase inhibitor, and l μl of Moloney murine leukaemia virus (MMLV) reverse transcriptase were added subsequently into the first mixture and incubated at 42 °C for 1 h to synthesize the cDNA.

The gene sequences of the sialyltransferase genes were obtained from CHOgenome.org. Specific PCR primer pairs of sialyltransferase genes were designed by the Primer3 website. The sequences of primers were as follows: ST3GAL3 (forward primer: 5′ CTGCTTGGAAGTTGCACTTG 3′ reverse primer: 5′ GTTTCTACGGAAGCTGGTGA 3′); ST3GAL4 (forward primer: 5′ AATCCCGCTGTAAGTTCCTG 3′ reverse primer: 5′ CACAATGTCTCCCAAGAGGC 3′); ST3GAL6 (forward primer: 5′ TGGCCTTAGTCTTGTGTTCC 3′ reverse primer: 5′ CTACTATGGAAACGCCACCA 3′); The gene fragments were amplified using a Veriti® thermocycler. For each reaction, 12.5 ul of 2x PCR Mastermix (Promega), 1ul each of 10 uM forward and reverse primer, 50 ng of cDNA, and nuclease-free water were added subsequently to make the final volume of 25 ul. Gel electrophoresis was performed to detect the gene expression levels.

2.4. SDS-page and western blotting analysis

CHOK1 cells transfected with siRNAs were lysed using RIPA buffer followed by brief sonication. The total cell lysate was subjected to SDS-PAGE and the protein samples were transferred to a PVDF membrane using Biorad wet blotting system under 100 V for an hour. For western blot, the membrane was blocked with 5% milk in PBST (Phosphate-buffered saline with 0.5% Tween 20) for an hour, followed by another hour of incubation in either rabbit anti-ST3GAL3 or rabbit anti-ST3GAL4 antibody. The membrane was then washed multiple times by 1X PBST and incubated with HRP-conjugated goat-anti-Rabbit IgG antibody for an extra hour. Finally, the membrane was visualized by an ECL detection substrate.

2.5. Maackia amurensis lectin II enzyme-linked lectin absorption assay (MAL II ELLA assay)

The protein concentration of total cell lysate or purified EPO from the siRNA transfected CHO cells was quantified by BCA assay (Pierce Biotechnology). 100 ul of Bicarbonate buffer(pH 9.6) was first added to each well in the 96-well microtiter plate. Add 2 ug of total cell lysate or purified EPO to each well subsequently and allow the protein samples to be coated at 4 °C overnight. Then remove the coating buffer and wash the plate three times with PBST (phosphate buffered saline with 0.5% (v/v) Tween 20). The wells were then blocked by 1X Carbo-Free blocking solution (Vector Lab) at 37 °C for 1 h. Remove the blocking buffer and wash the plate with PBST three times. 100 ul of 2 ug/mL of biotinylated Maackia amurensis lectin II (Vector Lab) in PBST was added to each well,

following 37 °C incubation for 2 h. Remove the lectin solution and wash the plate with PBST four times. Then each well was incubated with 100 ul of 0.5 ug/mL of horseradish peroxidase conjugated Streptavidin (Vector Lab) at 37 °C for 1 h. The color was developed with 100 ul of TMB Liquid Substrate (Sigma) and stopped by 50 ul of 2 M H_2SO_4. The plate was read on a microplate reading at 405 nm.

2.6. Ni-NTA purification of EPO

30 mL of culture supernatant produced by siRNA transfected CHO-EPO cells was collected and filtered through a 0.45 um filter in a conical centrifuge tube. The conditioned media was incubated with 500 ul of Ni-NTA resin with 50% slurry (Qiagen) for 1 h at room temperature. The resin was collected by centrifugation (2000 × g, 5 min) and washed twice using washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0). The protein was eluted with an elution buffer containing 300 mM NaCl, 500 mM imidazole, 50 mM NaH_2PO_4, at pH 8.0. The protein concentration of eluant was quantified using BCA assay, and the samples were stored at −20 °C.

2.7. Sialic acid content analysis

Sialic acid content was quantified according to the method developed by Hara et al. [16]. Briefly, 5 ul of protein sample was added to 200 ul of 25 mM sulfuric acid, and incubated at 80 °C for 1 h to hydrolyze the sample. After cooling the sample to room temperature, the sample was incubated at 60 °C for 2.5 h with 200 ul of 7.0 mM DMB solution to develop fluorescence.

For sialic acid quantification on the N-glycan, the EPO protein was denatured and digested with PNGaseF (New England Biolabs) for 1 h 200 ul of PBS was added to the digested EPO samples, and the N-glycans were separated from the EPO by using a 10 kDa.

MWCO spin column (Millipore), the flow-through which contained the released N-glycans was collected and subjected to sialic acid analysis as described above.

3. Result and discussion

3.1. Confirming the gene knockdown efficiency of single and triple siRNA transfection

To understand the function of alpha 2,3 sialyltransferases' function, RNAi induced gene knockdown is a useful approach for rapidly identifying which gene(s) may play roles in N-linked sialylation. Therefore, three different alpha 2,3 sialyltransferase gene sequences, ST3GAL3, ST3GAL4, and ST3GAL6 were obtained from CHOgenome.org [17] and individual siRNAs were designed by Sigma. A negative control siRNA served as the negative control. CHOK1 cells were transfected with individual or a combination of all three sialyltransferase siRNAs, and then the knockdown efficiencies were evaluated. After three days post transfection, RNA expression levels for both the single and triple siRNA transfections were analyzed by RT-PCR (Fig. 1A).

As demonstrated in Fig. 1A, siRNA transfection resulted in a decline in the gene expression of the representative ST3GAL gene. Furthermore, the co-transfection of all 3 siRNAs yielded a decline in the expression of all the genes. Next, we analyzed the effect of introducing siRNA at the protein level using western blot analysis. As shown in Fig. 1B, CHO cells transfected with ST3GAL3 or ST3GAL4 siRNAs exhibit a decreased level of enzyme expression compared with the negative control. The ST3GAL6 western blot was not performed due to a lack of an appropriate antibody source. Confirming the effect of siRNA on the reduction of mRNA and protein levels, we next evaluated whether the reduced expression of these sialyltransferase altered sialylation in the cells.

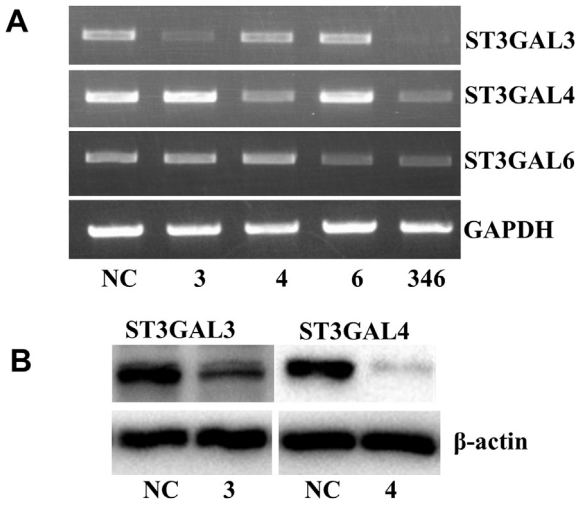

Fig. 1. Evaluate the knockdown efficiencies of ST3GAL siRNAs. (A) Measurement of the mRNA expression levels of the ST3GAL siRNA transfected CHOK1 cells. CHOK1 cells were transfected with siRNAs targeting ST3GAL3, ST3GAL4 and ST3GAL6 genes separately or with all three siRNAs. The RNAs isolated from the cells were reverse transcribed into cDNAs. The gene knockdown levels were then examined by PCR amplification. The genes on the right side of the figure represent which gene was amplified during the PCR. NC: transfected with negative control siRNA; 3: transfection with ST3GAL3 siRNA; 4: transfected with ST3GAL4 siRNA; 6: transfected with ST3GAL6; 346: transfected with all three siRNAs mentioned above. (B) Western blot analysis of siRNA transfected CHOK1 cells showed reduced expression of ST3GAL3 and ST3GAL4 sialylatransferases. Left panels: comparisons of the total cell lysate between the negative control siRNA transfected CHO cell and the ST3GAL3 siRNA transfected CHO cell. Right panels: comparison of the total cell lysate between the negative control siRNA transfected CHO cell and the ST3GAL4 siRNA transfected CHO cell. B-actin blot was used as a control.

3.2. siRNA reduce the total sialylation level in the total cell protein

CHO cells transfected with single, dual and triple siRNA combinations were lysed and the total sialylation levels analyzed using a Maackia amurensis lectin II enzyme-linked lectin absorption assay (MAL II ELLA), which recognizes glycoproteins with α2,3 sialylation linkages. All transfections showed a reduced level of α2,3 sialylation compared with the negative control (Fig. 2A) with the double transfection groups exhibiting generally lower sialylation levels than the single transfection group. In turn, the triple transfection group displayed the lowest MAL II levels, indicating each gene likely contributes some to α2,3 sialylation. Next, cell lysates were subjected to sialic acid content quantification with HPLC analysis. Similar trends were observed as in the previous ELLA analysis-in which the knockdown of all three genes is superior to knocking down two genes, which is on average lower than a single gene knockdown.

In both Fig. 2A and B, some double siRNAs transfection groups showed limited reductions in both MALII activity and sialic acid levels compared with the single siRNA groups. This suggests that, while all the genes play a role, certain sialylatransferases may be more significant in creating alpha2,3 linkages in CHO cells than others. . While the total cell lysate includes sialylation on both glycoproteins and lipids, subsequent analysis was undertaken to examine the role of these individual alpha2,3 sialyltransferase genes on a specific recombinant glycoprotein, erythropoietin (EPO).

3.3. siRNA reduce the sialylation level in the EPO protein

The same transfection strategy of using single, dual and triple siRNA combinations was applied to CHO cells stably expressing heterologous EPO. EPO was purified from all the transfection groups (single, dual and triple) and then subjected to MAL II ELLA

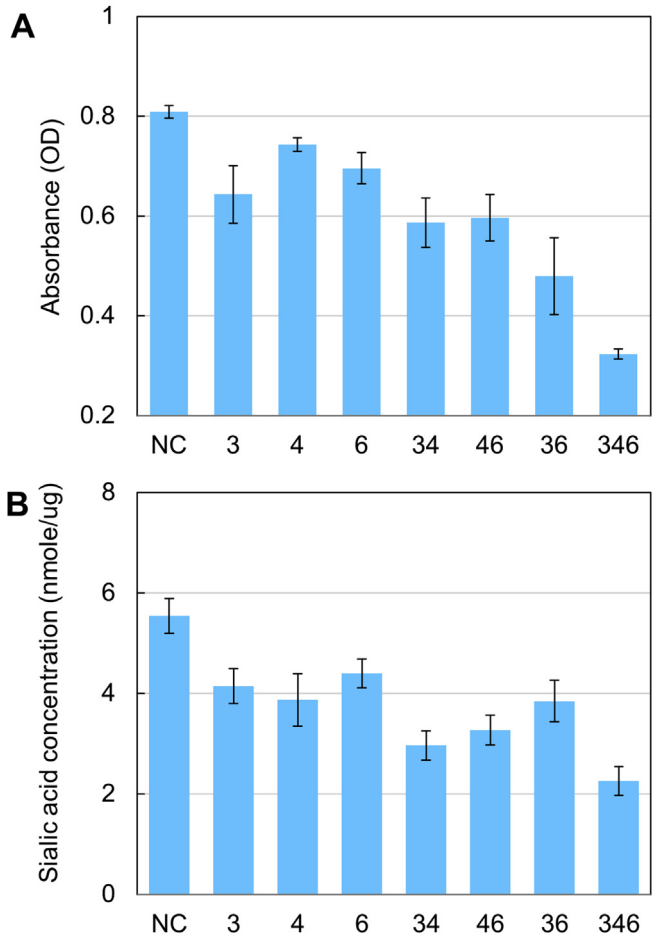

Fig. 2. Assessment of ST3GAL siRNAs' efficacies on reduction of α2,3 sialylation on total cell lysate. A: Enzyme-linked lectin assay (ELLA) of total cell lysate using MAL II lectin. CHOK1 cells were transfected with single, double and triple gene knockdown using siRNA transfection. The cells were lysed and total protein concentration of each group was measured by BCA assay. NC: transfected with negative control siRNA. 3: transfected with ST3GAL3 siRNA. 4: transfected with ST3GAL4 siRNA. 6: transfected with ST3GAL6 siRNA. 34: transfection with both ST3GAL3 and ST3GAL4 siRNAs. 46: transfected with ST3GAL4 and ST3GAL6 siRNAs. 36: transfected with ST3GAL3 and ST3GAL6 siRNAs. 346: transfected with ST3GAL3, ST3GAL4 and ST3GAL6 siRNAs at once. B: sialic acid content quantification by HPLC using total cell lysate from CHO transfected with different siRNAs.

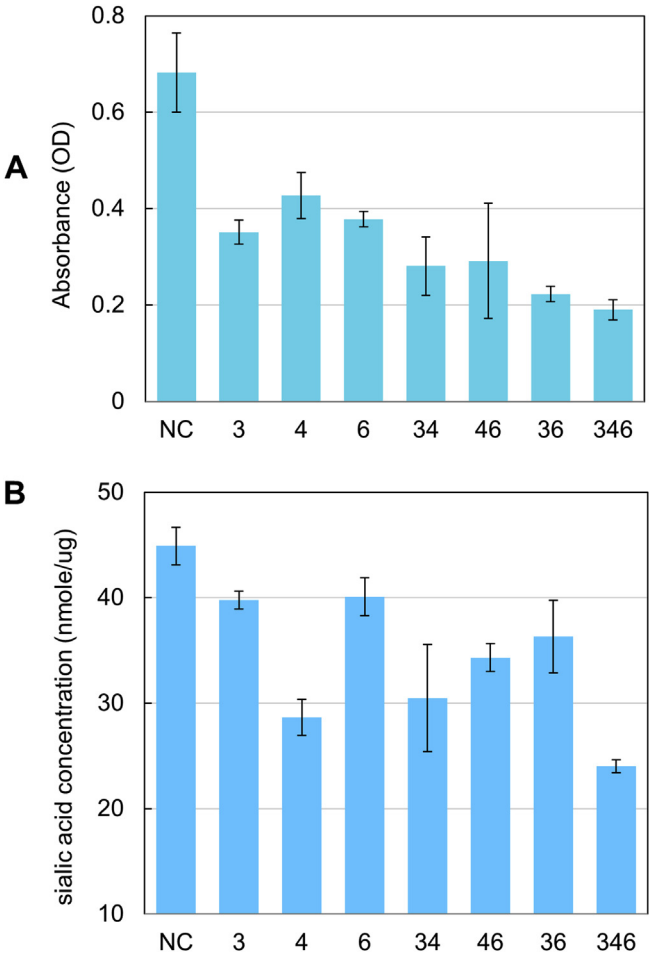

Fig. 3. Assessment of ST3GAL siRNAs' efficacies on reduction of EPO sialylation. A: EPO ELLA analysis: EPO harvested from CHO-EPO cells transfected with different siRNA groups were subjected to ELLA analysis. B: Sialic acid content quantification on the purified EPO protein from CHO-EPO cells transfected with different siRNA groups was subjected to sialic acid quantification using HPLC.

analysis. As shown in Fig. 3A, the EPO from ST3GAL3 siRNA transfection had the averagely lowest sialic acid content among the single transfection groups followed by EPO from ST3GAL6 and ST3GAL4 siRNA single transfection. Double transfection groups exhibited overall lower sialic acid content as opposed to the individual transfection groups. The triple transfection group rendered on average the lowest sialic acid content but did not exhibit a significant difference from the double transfection groups.

Interestingly, analysis of the sialic acid content on the purified EPO using HPLC exhibited slightly different results from the MAL II ELLA analysis (Fig. 3B). EPO from CHO cells transfected ST3GAL4 siRNA showed the lowest sialylation level among the single transfection groups. HPLC analysis showed siRNA combinations containing two siRNAs were only moderately reduced as compared with the single ST3GAL3 and ST3GAL6 transfections. Furthermore, the EPO from double siRNA transfections were not as effective at reducing the sialic acid content as the single transfection of STGAL4 alone. It is curious that using 2 siRNAs were less effective in at least one case than using the ST3GAL4 siRNA alone. Interestingly, a similar negative outcome was reported by Sriram et al. and Castanotto et al. [18,19]. They speculated that the reduced siRNA efficacies due to the competition between siRNAs to form the RISC complex or Ago2 favor a certain dsRNA configuration. Nonetheless, the combination of reducing all three STGALs was once again the most effective means for reducing total sialic acid activity as measured using HPLC analysis.

The results from the MALII ELLA analysis indicate that ST3GAL3 and ST3GAL6 can play roles in lowering the sialylation of EPO while the HPLC and MALII results together indicate that ST3GAL4 can also be relevant to EPO sialylation. While the MAL II lectin assay represents a ligand binding assay for glycans bearing α2,3 sialylation, the HPLC provides quantitative information about the sialic acid levels using chemical treatments. Furthermore, the importance of all three sialylatransferases is evident only when we consider both assays. All three genes is crucial to protein sialylation as further indicated by the fact that the lowest signals are reported for EPO when all three genes' expression are inhibited.

3.4. siRNA reduce the N-linked sialylation level in the EPO protein

CHO recombinant EPO contains three N-linked glycan sites and one O-linked glycosylation site. To elucidate the role of these enzymes on N-linked sialylation of EPO, a separate analysis was implemented. The N-glycans were released from EPO by treatment with PNGaseF and the sialic acid content of the free N-glycans was

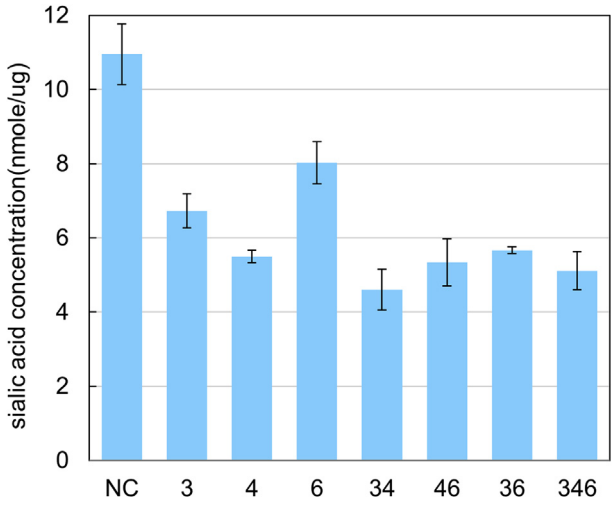

Fig. 4. Sialic acid content quantification of the N-glycans released from the CHO produced EPO.

then quantified. As shown in Fig. 4, a similar, but less extreme, trend was observed for the sialic acid content as detected without the treatment of PNGaseF. While all three STGAL siRNAs lowered the sialic acid content on the N-glycans relative to the negative control, the ST3GAL4 siRNA once again lowered the sialylation level the most, which is consistent with the overall EPO sialic acid content analysis.

Furthermore, combinations of different siRNAs including ST3GAL4 resulted in the lowest overall average N-glycan sialic acid content on EPO, although the difference compared with ST3GAL4 alone was not significant. Thus, the findings in both Fig. 4 and Fig. 3B indicate that ST3GAL4 is particularly relevant to dictating the final N-linked sialic acid content on heterologous EPO. The other alpha2,3 sialyltransferases contribute to EPO sialylation but play a less prominent role in the final N-linked sialic acid content levels.

The application of siRNA can be used to lower the CHO expression levels of ST3GAL3, 4, and 6, which are known to play roles in protein glycosylation. Indeed, our total cellular analysis indicated that all three enzymes are relevant to cellular glycosylation as indicated by both MAL II lectin binding and sialic acid analysis. Furthermore, some combinations of these siRNAs are more effective than using one siRNA alone. Similar trends were detected from the analysis of total EPO glycans and N-glycans in which each of the individual siRNAs lowered the MAL II lectin binding and sialic acid content. While the genes appear to differ in their importance to EPO sialylation, the application of siRNAs including all three genes was typically the most effective to indicate that ST3GAL3, ST3GAL4, and ST3GAL6 all partake in the sialylation, and in particular N-glycan sialylation, of the glycoprotein. In particular, ST3GAL4 appears to play a prominent role in EPO N-linked glycan sialylation which was consistent with the report that type II glycan is the most predominant glycan in EPO from CHO cells and ST3GLA4 were shown to exhibit highest substrate activity toward type II glycan [10]. It is also interesting that the MAL II lectin assays yielded different trends from those obtained with the sialic acid analysis using HPLC. These differential findings demonstrate the value in using multiple assays in order to assess the role of particular glycosylation enzymes in affecting protein glycosylation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We would like to acknowledge National Science Foundation (NSF) for support of this research (BES grant no. 1264802). Scholarship support to C.C provided by Taichung Toyu Rotary Club, Taichung,Taiwan ROC.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.023.

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