Bioengineered 4:2, 90–94; March/April 2013; © 2013 Landes Bioscience

CHO-gmt5, a novel CHO glycosylation mutant for producing afucosylated and asialylated recombinant antibodies

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Keywords: CHO cells, CMP-sialic acid transporter, GDP-fucose transporter, zinc-finger nucleases (ZFNs), recombinant antibodies, antibody-dependent cell-mediated cytotoxicity (ADCC)

Engineered zinc-finger nucleases (ZFNs) are powerful tools for creating double-stranded-breaks (DSBs) in genomic DNA in a site-specific manner. These DSBs generated by ZFNs can be repaired by homology-directed repair or nonhomologous end joining, in which the latter can be exploited to generate insertion or deletion mutants. Based on published literature, we designed a pair of zinc-finger nucleases and inactivated the GDP-fucose transporter gene (*Slc35c1*) in a previously reported CHO mutant that has a dysfunctional CMP-sialic acid transporter gene (*Slc35a1*). The resulting mutant cell line, CHO-gmt5, lacks functional GDP-fucose transporter and CMP-sialic acid transporter. As a result, these cells can only produce asialylated and afucosylated glycoproteins. It is now widely recognized that removal of the core fucose from the *N*-glycans attached to Asn^{297} of human IgG1 significantly enhances its binding to its receptor, FcγRIIIa, and thereby dramatically improves antibody-dependent cellular cytotoxicity (ADCC). Recent reports showed that removal of sialic acid from IgG1 also enhances ADCC. Therefore, CHO-gmt5 may represent a more advantageous cell line for the production of recombinant antibodies with enhanced ADCC. These cells show comparable growth rate to wild type CHO-K1 cells and uncompromised transfection efficiency, which make them desirable for use as a production line.

Recombinant human IgG1 antibodies have been successfully used as therapeutic drugs to target malignant cells in cancer patients. Upon binding to the target molecule expressed on cancer cells, the Fc region of the antibody recruits the effector cells such as natural killer (NK) cells to kill cancer cells by antibody-dependent cellular cytotoxicity (ADCC). Studies have shown that the major interaction sites of the Fc region by the FcγRIII are located in the hinge region and the CH2 domains of the antibody.[1,2] The binding of the Fc and the Fc receptor is known to be dependent on the structures of the *N*-glycans attached to the conserved glycosylation site at Asn^{297} in each of the CH2 domains.[3]

It has been demonstrated clearly that removal of the fucose residue from the *N*-glycan attached to Asn^{297} of human IgG1 significantly enhances its binding to FcγRIIIa and thereby dramatically improves ADCC.[4,5] Detailed binding analyses indicated that removal of fucose enhanced binding enthalpy and increased binding constant of IgG1 for FcγRIIIa.[6] The molecular mechanism underlying the enhanced affinity between Fc and FcγRIIIa was further investigated. The structural differences between fucosylated and afucosylated Fc fragments of human IgG1 were compared in X-ray crystallographic and NMR spectroscopic studies. The overall conformations of the fucosylated and afucosylated Fc fragments are similar except for hydration mode around Tyr^{296}.[7] The conformation of Tyr^{296} is more flexible for FcγRIIIa in afucosylated Fc than in fucosylated Fc.[7] Tyr^{296} has already been indicated in the interaction with FcγRIIIa.[2,8]

Enhanced ADCC was not only observed for the afucosylated antibody in in vitro assays, it was also confirmed in vivo in patients or animal models.[9-12] These data have convincingly shown that removal of fucose from human IgG1 can be a general method for treating cancer patients with antibodies through improved ADCC.

In addition to fucosylation, sialylation of the Fc *N*-glycan may also affect the cell-killing activity of the antibody. Increased sialylation of the Fc *N*-glycan was shown to reduce the binding of Fc to FcγRIIIa and consequently, decrease ADCC.[13,14] We have previously reported the isolation of a CHO mutant (CHO-gmt1) that produces asialylated glycoproteins due to the lack of a functional CMP-sialic acid transporter.[15] Using zinc-finger nuclease technology we have inactivated the GDP-fucose transporter in CHO-gmt1.[16] The resulting mutant, CHO-gmt5, is able to produce afucosylated and asialylated recombinant antibodies. The efficacy in ADCC by antibodies produced in CHO-gmt5 cells will be evaluated.

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Submitted: 07/27/12; Revised: 09/17/12; Accepted: 09/17/12
http://dx.doi.org/10.4161/bioe.22262

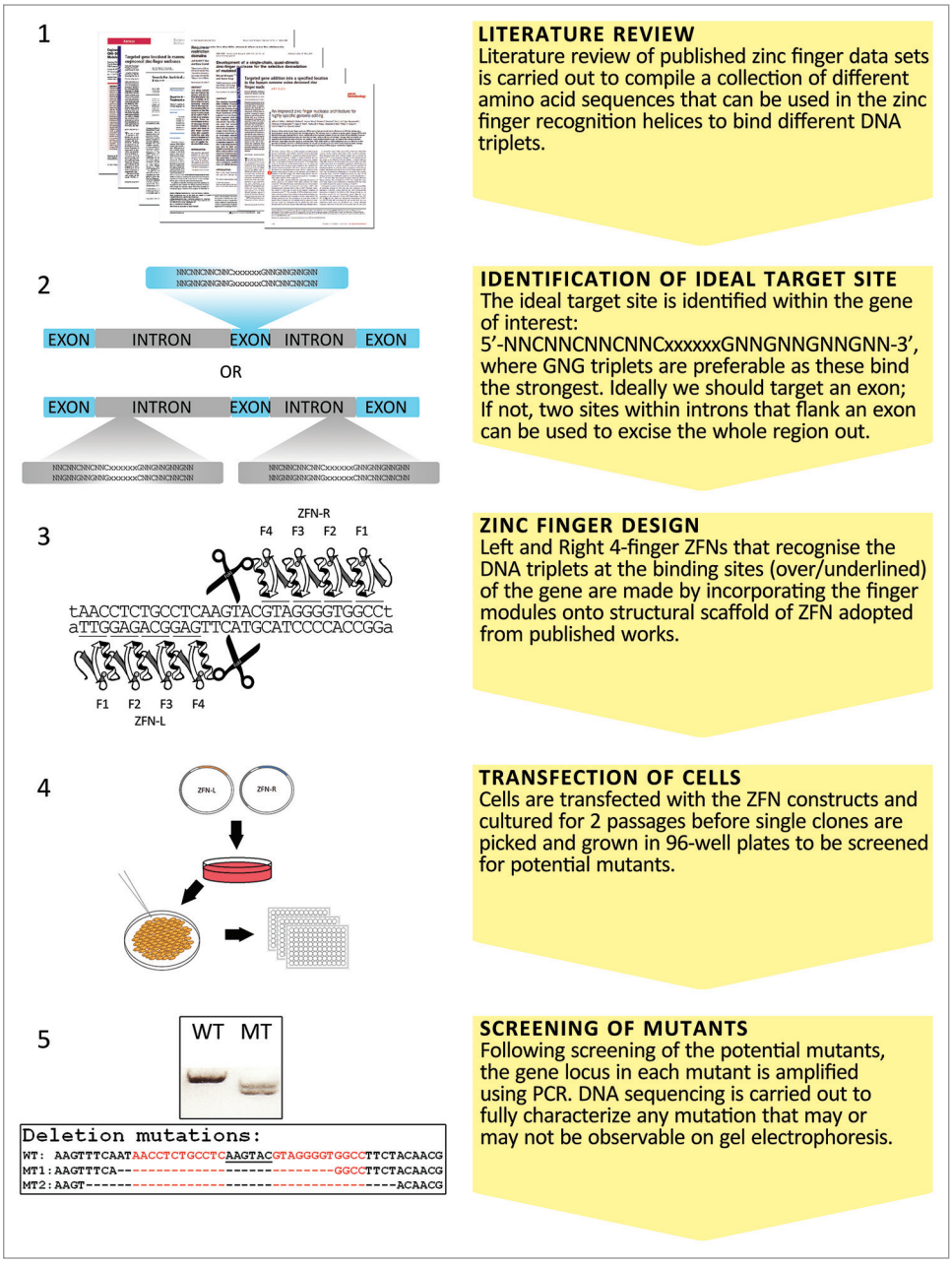

Figure 1. Outline for the interruption of a target gene using zinc-finger nucleases designed by the "modular assembly" strategy.

CHO-gmt1 Cells

CHO-gmt1 cells (originally called MAR-11) were isolated from CHO-K1 cells treated with *Maackia amurensis* agglutinin (MAA) which is specific for Neu5Acα2,3Gal structure. The cells have a dysfunctional CMP-sialic acid transporter (CMP-SAT).[15] A point mutation in the CMP-SAT gene in CHO-gmt1 cells results in a premature stop codon. As a result, these cells express a truncated version of CMP-SAT which contains only 100 amino acids, rather than the normal CMP-SAT which consists of 336 amino acids. As a result, glycoproteins produced by this cell line are completely free of sialic acid. These cells have been used to study the structure-function relationships of CMP-SAT.[17]

A Simplified "Modular Assembly" Strategy to Design Zinc-Finger Nucleases (ZFNs) Based on Publically Available Information

Zinc-finger nucleases (ZFNs) are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to the cleavage domain of restriction enzyme FokI. The zinc finger DNA-binding domain of ZFNs consists of three or four zinc finger units. Each of these recognizes a 3-bp motif in the chromosomal DNA. The specificity of the ZFNs is determined by 7 amino acids within each zinc-finger unit that interact with the DNA. In order to allow the two FokI cleavage domains to dimerize and cleave DNA, the two ZFNs must bind opposite strands of DNA and the two binding sites have to be separated by 5–7 bps. The double-stranded-breaks (DSBs) in genomic DNA created by ZFNs can be repaired by nonhomologous end joining (NHEJ). During NHEJ, cells often create insertion or deletion mutations.

ZFNs generated by the combinatorial selection methods may have high DNA-binding affinity and low toxicity.[18] Sangamo Biosciences has used its own proprietary information to create highly specific ZFNs.[19-21] However, most laboratories do not have the randomized libraries or the selection expertise to do so. Alternatively, a modular assembly strategy can be used based on publically available information in the literature.[22,23] We also used the modular assembly strategy to design ZFNs to interrupt the GDP-fucose transporter gene in CHO cells. To target a gene with ZFNs, the first step is to identify an ideal target site in the gene of interest. The open reading frame of the cDNA can be analyzed by the web-based ZiFiT program provided by the Zinc Finger Consortium (ZiFiT: software for engineering zinc finger proteins (V3.0)) at: http://bindr.gdcb.iastate.edu/ZiFiT/.[24] The ZiFiT output will suggest a few potential target sites. The fingers that bind the 5'-GNN-3' sequences are the best studied and strongest DNA-binding fingers.[25-27] Two binding sites for ZFNs should be separated by 5–7

bps which is the optimal distance for the two FokI domains to dimerize and cleave the targeted site. Therefore, an ideal target sequence for two 4-fingered ZFNs should be: 5'-NNC NNC NNC NNC xxx xxx GNN GNN GNN GNN-3'. This sequence ensures each zinc finger binds a 5'-GNN-3' sequence. The 5'-GNG-3' sequences are better binding sites compared with other 5'-GNN-3' sequences. In addition to the 5'-GNN-3' sequences, other sequences have also be successfully used in the literature. These include CTG, TGG, AAG and AAA triplets. It is generally believed that 3-fingered ZFNs should work as well as the 4-fingered ZFNs. In the event that there is no ideal site in the open reading frame, one can try to find two ideal sites that flank an exon in the open reading frame of the targeted gene. ZFNs designed to target two different sites can introduce two concurrent DNA double-strand breaks in the chromosome and create deletions of the genomic segment between the two sites.[28] In this situation, cells will be transfected with two pairs of ZFNs simultaneously in order to generate targeted deletions of genomic segments. A simplified "modular assembly" strategy to design zinc-finger nucleases (ZFNs) based on publically available information is outlined in **Figure 1**.

The structural scaffold for the ZFNs can be adopted from previous publications.[19,20] To eliminate unwanted homodimerization of FokI cleavage domain, the high-fidelity FokI-KK and FokI-EL variants can be used.[29] The amino acid sequences of the DNA-binding domains in the ZFNs are assembled using an archive of zinc-finger motifs collected from previous publications[25-27] and many other related publications which are not listed here.

Using ZFNs to Inactivate GDP-Fucose Transporter Gene in CHO Cells and Fluorescence-Activated Cell Sorting to Rapidly Isolate Mutant Cells

Analysis of the open reading frame of Chinese hamster GDP-fucose transporter gene using the ZiFiT program[24] identified one potential target site in the first exon of the GDP-fucose transporter coding region (5'-tAA CCT CTG CCT CAA GTA CGT AGG GGT GGC Ct-3'). As discussed earlier, this sequence allows each zinc finger in the left ZFN and the right ZFN to bind to a 5'-GNN-3' DNA triplet.

CHO cells were transiently transfected with plasmids expressing the left and right zinc-finger nucleases as described previously.[16] In our previous report, two days after transfection, single cells were seeded in a 96-well plate for clone isolation. Genomic DNA from each single clone was isolated and the targeted GDP-fucose transporter locus was amplified by two specific PCR primers flanking the targeted site in the gene. The PCR products were sequenced in order to identify the mutation. However, this is a labor intensive and time-consuming process and we identified only one mutant.[16] To increase the selection efficiency, a fucose-specific lectin combined with fluorescence-activated cell sorting (FACS) strategy was employed. After transfection with the ZFN constructs, the cells were cultured for several generations to allow the daughter cells of successful knockout mutants to express afucosylated glycoproteins on their surface. Mutant and the wild type CHO cells in the transfected pool were stained with biotinylated *Aleuria Aurantia* lectin (AAL) and Cy3-conjugated streptavidin. Stained cells were sorted by FACS using a Becton Dickenson FACSAria IIu SORP cell sorter. The negatively stained cells (AAL-ve) were pooled and cultured (**Fig. 2A**). When this population was subsequently stained with FITC and analyzed by FACS, a significant number of the cells were AAL-negative (**Fig. 2B**). Following two more cycles of this lectin-based cell sorting essentially all the cells in the population became AAL negative cells (data not shown).

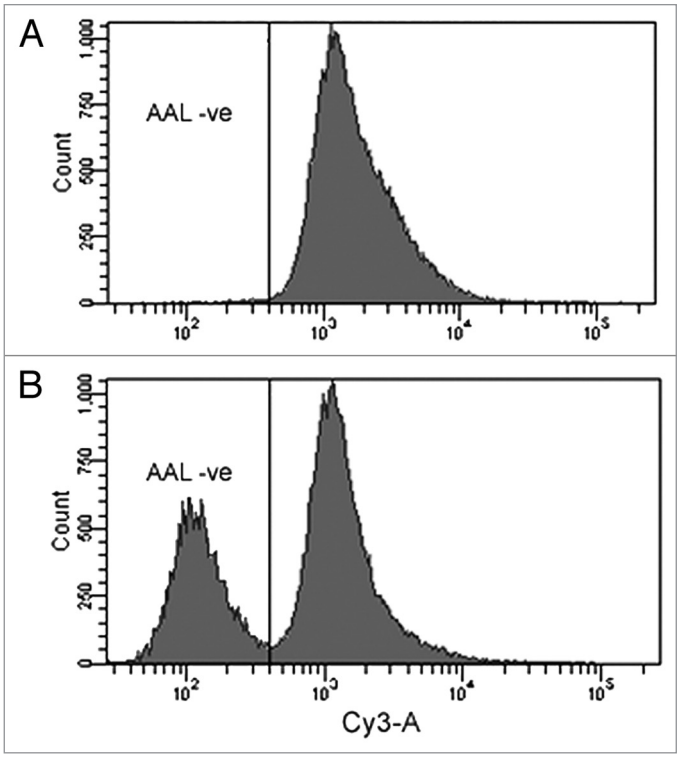

Figure 2. Using FACS to rapidly isolate cells with inactivated GDP-fucose transporter. Cells transfected with constructs expressing the ZFNs were cultured to near confluence and subcultured at 1:6 ratio for 2 passages. Ten million of the resulting cells were labeled with biotinylated AAL and Cy3-conjugated streptavidin. Stained cells were sorted on a Becton Dickenson FACSAria IIu SORP cell sorter. (**A**) FACS histogram for transfected cells at the first round of sorting. The sorting gate for AAL-negative (AAL-ve) cells was set to collect the lowest 0.5% of AAL-stained cells. Approximately 12,000 cells were collected from a total of 3.5 million cells sorted. These cells were cultured for 2 weeks before being subjected to a second round of sorting. (**B**) Second round of FACS shows that more than 30% of the cells are AAL-ve cells after the first round of sorting. Single AAL-ve cells were isolated from this pool for further characterization.

In order to confirm that the phenotypic change observed in the flow cytometry was indeed due to the disruption of the GDP-fucose transporter gene, the cells were transfected with a construct that expressed wild type GDP-fucose transporter in a rescue assay. The conversion of the AAL-negative phenotype to AAL-positive following this assay is indicative of a mutated GDP-fucose transporter. The gene locus in the mutants was then amplified by PCR and sequenced. The results further

confirmed the genetic mutation at the targeted site. Taken together, the fucose-specific lectin in combination with FACS is a successful method to rapidly isolate mutants.

CHO-gmt5 Cells

The GDP-fucose transporter gene was successfully inactivated by ZFNs in CHO-gmt1 cells.[16] The resultant CHO-gmt5 cells lack functional CMP-sialic acid transporter and GDP-fucose transporter. They can be used to produce afucosylated and asialylated recombinant antibodies such as Rituximab. The ability of fucose-free and sialic acid-free antibodies to enhance ADCC will be assessed. We have also shown that CHO-gmt5 cells have comparable growth rate to wild type CHO-K1 upon adaptation to suspension culture in chemically defined medium, as well as uncompromised transfection efficiency when transfected with GFP and recombinant Rituximab constructs.

Perspectives

This work demonstrated that the "modular assembly" strategy can be successfully utilized by labs without special expertise in zinc finger design. Utilizing the archive of zinc-finger motifs collected from all the zinc finger work published in the literature, researchers will be able to design better zinc fingers.

FACS is a simple method to rapidly isolate mutant cells provided the mutant cell can be identified by a fluorescent tag. In our case, fucosylated proteins on the wild type cell surface can be positively stained by AAL, which consequently excludes the mutant cells. Based on this feature we were able to enrich and isolate the cells with inactivated GDP-fucose transporter gene. FACS can also rapidly enrich and isolate mutant cells in which the dihydrofolate reductase (DHFR) gene is interrupted by ZFNs. Wild type cells that express DHFR bind fluorescent methotrexate.[30] Mutants that are no longer able to bind fluorescent methotrexate can be isolated by FACS. We have successfully used methotrexate to isolate DHFR negative CHO cells using previously reported ZFNs.[21] Thus, the FACS strategy can be applied to the isolation of a variety of mutants, including mutations in the genes that encode cell surface proteins, provided antibodies specific for the proteins are available.

CHO-gmt5 cells lack functional CMP-sialic acid transporter and GDP-fucose transporter. These cells can be used to study structure-function relationships of these two receptors.[16,17] They can also be used to produce afucosylated and asialylated recombinant antibodies. It is of great interest for us to investigate whether these antibodies will have enhanced ADCC against the target cells.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

Acknowledgments

The authors would like to thank Ms Natasha A. Pereira for critically reviewing the manuscript. This work was funded by the Agency for Science, Technology and Research (A*STAR), Singapore.

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