



Establishment of serum-free adapted Chinese hamster ovary cells with double knockout of GDP-mannose-4,6-dehydratase and GDP-fucose transporter

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Abstract Although antibodies have attracted attention as next-generation biopharmaceuticals, the costs of purifying the products and of arranging the environment for cell cultivation are high. Therefore, there is a need to increase antibody efficacy and improve product quality as much as possible. Since antibodies are glycoproteins, their glycan structures have been found to affect the function of antibodies. Especially, afucosylation of the *N*-linked glycan in the Fc region is known to significantly increase antibody-dependent cellular cytotoxicity. In this study, we established a double-mutant Δ GMD Δ GFT in which GDP-mannose 4,6-dehydratase and GDP-fucose transporter were knocked out in Chinese hamster ovary cells, a platform for biopharmaceutical protein

production. By adapting Δ GMD Δ GFT cells to serum-free medium and constructing suspension-cultured cells, we established host CHO cells with no detected fucosylated glycans and succeeded in production of afucosylated antibodies. We also demonstrated that, in culture in the presence of serum, fucosylation occurs due to contamination from serum components. Furthermore, we found that afucosylation of glycans does not affect cell growth after adaptation to serum-free medium as compared to wild-type CHO cells growth and does not significantly affect the expression levels of other endogenous fucose metabolism-related enzyme genes.

Keywords Chinese hamster ovary cell · *N*-glycan · Fucosylation · GDP-fucose · Serum-free medium adaptation

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Abbreviations

GMD	GDP-mannose 4,6-dehydratase
GFT	GDP-fucose transporter
Man	Mannose
Fuc	Fucose
GlcNAc	<i>N</i> -Acetylglucosamine
Fmoc	9-Fluorenylmethyloxycarbonyl

Introduction

Protein-based pharmaceuticals are representative of the biopharmaceutical industry, and the market for them, with billions of dollars in annual sales, is growing (Aggarwal 2014; Walsh 2018). These products include various proteins such as antibodies, enzymes, hormones, cytokines, growth factors, and coagulation factors (Dumont et al. 2016; Walsh 2018). In addition, biopharmaceuticals to treat diseases such as cancer, immune diseases, infectious diseases, genetic diseases, Alzheimer's disease, and Parkinson's disease are being widely developed. In particular, antibody drugs occupy a large place in the current pharmaceutical market. In 2016, antibody drug sales accounted for approximately 66% of all biopharmaceuticals excluding vaccines, with 8 of the top 10 sales being antibody-related (Kesik-Brodacka 2018).

Chinese hamster ovary (CHO) cells are the main platform for producing these recombinant biopharmaceuticals. However, antibody drugs are much more expensive than conventional chemotherapeutic drugs. This is because culturing animal cells requires expensive culture media and culture facilities, and purifying the produced protein is expensive. In addition, antibody drugs are administered in higher dose than conventional protein drugs, resulting in higher treatment costs. Therefore, it is necessary to enhance the efficacy of antibody drugs as much as possible.

Many protein-based biopharmaceuticals, including antibodies, are glycoproteins. Added to these glycoproteins are oligosaccharides and sugar chains that contribute to the quality of the product by post-translational modification. Especially, it is important to add *N*-linked glycans to many recombinant biopharmaceutical proteins. The properties of glycosylation play major roles in altering protein stability, including factors such as half-life, folding, targeting, transporting, immunogenicity, and biological activity (Runkel et al. 1998; Walsh and Jefferis 2006; Jefferis 2016; Kuriakose et al. 2016). Glycosylation results in broad changes in the function of a single protein. Therefore, modification of the glycan structure can be a means to improve the quality of CHO-derived recombinant biopharmaceuticals.

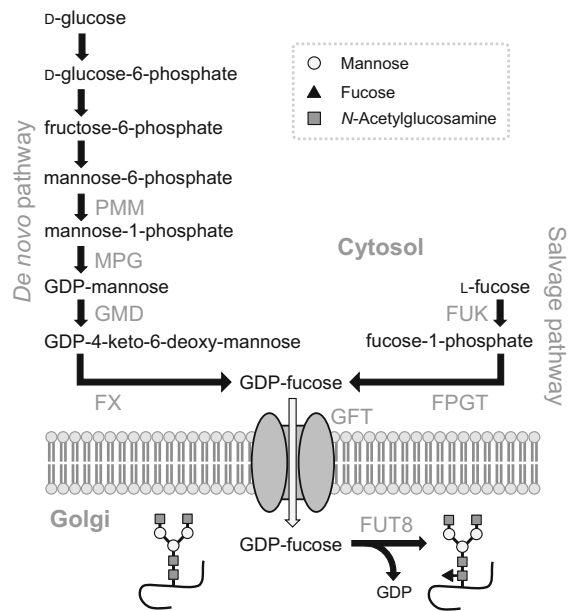
Afucosylated *N*-glycan of the human IgG1 Fc region was reported to increase the ability of natural killer cells to bind to the Fc γ RIIIa receptor (Shields et al. 2002; Shinkawa et al. 2003; Niwa et al. 2004;

Iida et al. 2006). This property has been used to further improve the cancer therapeutic performance of monoclonal antibodies via antibody-dependent cellular cytotoxicity (ADCC) (Chung et al. 2012). α 1,6-Fucosyltransferase (FUT8) adds fucose (core fucose) with α 1,6-linkage to the reducing terminal *N*-acetylglucosamine (GlcNAc) of the *N*-linked glycan. The production of the donor substrate GDP-fucose of FUT8 takes place via the de novo synthetic or salvage pathway (Fig. 1). The de novo synthetic pathway is the main synthetic route of GDP-fucose catalyzed by GDP-mannose-4,6-dehydratase (GMD) and GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase. In contrast, the salvage pathway is catalyzed by L-fucose kinase (FUK) and fucose-1-phosphate guanylyltransferase (FPGT) using fucose as a substrate. The produced GDP-fucose is then transported by the GDP-fucose transporter (GFT) into the Golgi apparatus to be catalyzed by FUT8. At present, research to produce low-fucosylated or nonfucosylated *N*-glycans of monoclonal antibodies by targeting these genes is intensifying (Mori et al. 2004; Imai-Nishiya et al. 2007; Kanda et al. 2007; Malphettes et al. 2010; Haryadi et al. 2013; Louie et al. 2017).

Transfer of fucose residue to the *N*-glycans was still remaining in CHO-DG44 cells under single *GMD*-knockdown (Imai-Nishiya et al., 2007). In contrast, serum-free fed-batch culture made no fucosylation of antibodies produced in *GMD*-knockout CHO cells (Kanda et al. 2007). However, even if only the expression of *GMD* is suppressed, there is a strong possibility that core fucosylation to the glycans may occur in the presence of extracellular GDP-Fuc contamination present in the culture medium containing serum. Thus, to solve this problem, we further knocked out *GFT* that transport GDP-Fuc into the Golgi lumen, which is the site of glycosylation. According from a previous report, knocking down or knocking out *GFT* expression markedly suppressed the fucosylation of endogenous and recombinant proteins (Omasa et al. 2008; Zhang et al. 2012; Chan et al. 2016). However, there have been few reports of double knockout of fucosylation-related enzyme genes. Although more potent inhibition of fucosylation to total intracellular glycans is expected, effects of the multi-mutations on cell growth, the adaptation to serum-free medium, the expression levels of other glycosylation-related enzyme genes, and *N*-linked glycan structure of a recombinant biopharmaceutical

Fig. 1 Synthetic pathway of fucosylated *N*-glycans and GDP-fucose via the de novo and the salvage pathway.

PMM phosphomannomutase, *MPG* mannose-1-phosphate guanylyltransferase (GDP-mannose pyrophosphorylase), *FX* GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (GDP-L-fucose synthase), *FUK* fucose kinase, *FPGT* fucose-1-phosphate guanylyltransferase



Establishment of suspension-cultured Chinese hamster ovary cells with double knockout of GDP-mannose-4,6-dehydratase and GDP-fucose transporter
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proteins still remain unclear. Hence, we examined these effects using CHO-K1 cell lines producing recombinant antibodies.

Mutation of FUT8 is a simple and attractive approach for inhibiting core fucosylation of *N*-linked glycans. In contrast, the FUT8 remains active in the mutant cells established in this study. This means that extracellular supplementation of monosaccharides or sugar nucleotides can exchange the α 1,6-fucosylation to other core-glycosylation. A previous study showed that, using sugar nucleotides synthesized by adding an excess of L-fucose structural-like sugars such as L-arabinose as a donor substrate, FUT8 transfers those L-fucose-like sugars to the *N*-linked glycans of antibodies in competition with GDP-Fuc, resulting in an altered ADCC activity (Hossler et al. 2017). The mutant cell line established in this study could be used in the future to efficiently transfer an L-fucose-like sugar in place of the core fucose residue in *N*-linked glycans of the recombinant antibodies and confer high ADCC activity.

Materials and methods

Cells

Adherent CHO-K1 cells from ATCC were cultured in 5% CO₂ at 37 °C in Ham's F12 Nutrient Mixture (Nacalai tesque, Kyoto, Japan) containing 10% fetal bovine serum (FBS) or ProCHOTM AT serum-free medium (Lonza, Basel, Switzerland). Suspension-cultured cells were cultured in EX-CELL® CD CHO serum-free medium (Sigma-Aldrich, St. Louis, MO, USA). The number of viable cells was measured using cells cultured in 10 mL medium on 10-cm dishes. Each initial cell number was 1.4×10^5 cells and the sampling was conducted at every 48 h from 1-day culture. Immunoglobulin G (IgG) was produced in a 350-mL culture bag A-350NL (NIPRO, Osaka, Japan) without shaking. Viable cells of each cell line on a 10 cm-dish were stained by trypan blue and the number of cells were then counted using hemocytometers.

sgRNA design, plasmid construction, and screening of single cell lines

The target sequences of single guide RNA (sgRNA) for genome editing were designed as follows: 5'-CACCGTTCGACCTGTATTAAATGAAC-3' (sense 1 for *GMD*), 5'-CAGCTGGACATAATTTACTTGCAA-3' (anti-sense 1 for *GMD*), 5'-CACCGACATT-TATATAAGAATCCAC-3' (sense 2 for *GMD*), 5'-CTGTAAATATATTCTTAGGTGCAAA-3' (anti-sense 2 for *GMD*), 5'-CACCGTGCAGCGCCCG-CAGCAGAAA-3' (sense 1 for *GFT*), 5'-CACGTCG CGGGCGTCGTCTTTCAA-3' (anti-sense 1 for *GFT*), 5'-CACCGCGCTGGTCGTCTCTCTCTAC-3' (sense 2 for *GFT*), 5'-CGCGACCAGCAGAGAGA-GATGCAA-3' (anti-sense 2 for *GFT*). These sense oligonucleotides were annealed to their pair antisense oligonucleotides, respectively. The annealed double strand DNAs were then introduced to pSpCas9n(BB)-2A-Puro (PX462) (Addgene, Cambridge, MA). Transfection to cells grown on 6-well plates was performed using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The transfected cells were sorted to 24-well plates as single cell clones.

Sequencing analysis

Genomic DNA of the transfectants was extracted by Kaneka Easy DNA Extraction Kit version 2 (Kaneka, Osaka, Japan) according to the manufacturer's instructions. Mutated regions were amplified by PCR using the primer sets shown in Supplementary Table 1. The PCR products were subcloned to pGEM®-T Easy vector and sequenced using M13 forward or reverse primers.

Lectin blotting

Proteins were extracted from cultured CHO cells using CytoBuster™ Protein Extraction Reagent (Merck Millipore, Billerica, MA), separated in 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing condition, and transferred to a polyvinylidene difluoride membrane. The membrane was then washed with lectin buffer (10 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20) and incubated with biotin-conjugated *Aleuria aurantia* lectin, AAL (J-Oil Mills, Tokyo, Japan). Fucosylated glycans were

visualized by peroxidase-conjugated streptavidin (Merck Millipore) and Luminata™ Forte Western HRP Substrate (Merck Millipore).

Adaptation of cells to serum-free medium and preparation of suspension-cultured cells

Serum concentration in medium was reduced stepwise. Cells were cultured in Ham's F12 Nutrient Mixture containing 2.5% or 1.0% FBS and ProCHO™ AT containing 0.5% or 0.25% or 0.1% FBS, respectively. Detached cells under the 0.1% FBS were harvested and adapted to EX-CELL® CD CHO serum-free medium.

Construction of expression vectors of IgG and establishment of cells stably producing the recombinant IgG

Genes encoding variable region of the heavy (H)- and light (L)-chains of human anti-dengue virus monoclonal antibody, D23-4F5E1, were cloned as previously reported (Sasaki et al. 2013). The coding region of H- and L-chains were introduced to pQCXIP (TaKaRa Bio Inc., Shiga, Japan) and pQCXIH (TaKaRa Bio Inc.) with a human H-chain and L-chain constant regions, respectively, as previously performed (Misaki et al. 2016). Transfection of the expression vectors to suspension-cultured *GMD/GFT* double-knockout CHO-K1 cells was conducted using Neon™ Transfection System (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells stably expressing both H- and L-chains were selected in EX-CELL® CD CHO serum-free medium containing 2 µg/mL puromycin dihydrochloride and 200 µg/mL hygromycin B.

Purification of IgG

The 2-week cultured medium in a batch culture was centrifuged at 1500×g for 5 min and the supernatant was applied to a Protein G Sepharose™ 4 Fast Flow column (GE Healthcare UK Ltd., Buckinghamshire, UK) equilibrated with a binding buffer, 20 mM sodium phosphate buffer, pH 7.0. The IgG was eluted by 100 mM glycine buffer, pH 2.7 after washing with the binding buffer, and a neutralization buffer, 1 M Tris–HCl buffer, pH 9.0 was rapidly added and mixed with the eluted fractions.

Preparation of *N*-glycans

1.0×10^8 cells were washed with PBS(–) twice and lysed in cold-Radio-Immunoprecipitation Assay buffer. The cells lysate was then incubated on ice for 30 min and centrifuged at 4 °C at $1000 \times g$ for 5 min. The supernatant containing the total soluble proteins was dialyzed against ultrapure water and lyophilized. Purified IgGs were also dialyzed against ultrapure water and lyophilized. *N*-Glycans were released from the total soluble proteins or the purified IgGs by Glycopeptidase F (Takara Bio Inc.) according to the manufacturer's instructions. The obtained *N*-glycans were lyophilized and labeled with 2-pyridylamine (PA) as described (Kondo et al. 1990). The PA-labeled *N*-glycans were separated from unreacted PA by phenol/chloroform extraction.

High-performance liquid chromatography (HPLC)

PA-labeled *N*-glycans were monitored on a HPLC apparatus (Hitachi 7000 HPLC system, Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 310 nm and 380 nm, respectively. The PA-labeled *N*-glycans were fractionated using a 4.6×250 mm column (Cosmosil 5C18-P column, Nacalai Tesque, Kyoto, Japan) and eluted by linearly increasing the acetonitrile concentration in 0.02% trifluoroacetic acid from 0 to 7% for 35 min at a flow rate of 1.2 mL/min in a reversed-phase HPLC.

LC–MS/MS analysis of *N*-glycans

The molecular mass and composition of the PA-labeled *N*-glycans were monitored by using an LC–MS/MS system (1200 series, Agilent Technologies, Santa Clara, CA) equipped with HCT plus (Bruker Daltonics, Bremen, Germany). The mobile phase in the LC consisted of a mixture of solvent A (acetonitrile:acetic acid = 98:2, v/v) and solvent B (water:acetic acid:triethylamine = 92:5:3, v/v/v). The PA-labeled *N*-glycans were separated using a 2.0×150 mm column (Shodex Asahipak NH2P-50, Showa Denko, Tokyo, Japan) by linearly increasing the solvent B concentration from 20 to 55% for 35 min at a flow rate of 0.2 mL/min. The parameter of the MS/

MS analysis was as follows: scan 350–5000 m/z , 5.0 psi nebulizer flow, 3.0 L/min dry gas flow rate, 300 °C dry temperature, 200,000 target count, and the MS/MS Frag. Ampl. of 1.0 V in the positive-ion mode. The relative amount of detected PA-labeled *N*-glycans was calculated on the basis of the peak area of the LC.

Estimation of intracellular GDP-fucose amount

Cultured 5.0×10^7 cells were homogenized in phosphate buffered saline (PBS) and centrifuged. The obtained supernatant was lyophilized as the donor substrate including GDP-fucose for the next enzymatic reaction. Recombinant mouse FUT8 was prepared using a baculovirus expression system as previously described (Ohashi et al. 2017). The mixture of cell extracts and FUT8 was incubated in PBS, 0.02 mg/mL GlcNAc₂Man₃GlcNAc₂-Asn-Fmoc at 37 °C for 16 h. The mixture was then incubated at 100 °C for 3 min and centrifuged. The supernatant was subjected to HPLC, and the HPLC analysis was performed as described (Ohashi et al. 2017). The amount of GDP-fucose was calculated from the peak area of synthesized GlcNAc₂Man₃FucGlcNAc₂-Asn-Fmoc.

Reverse transcription-PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from 3-day cultured cells using ISOGEN II (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The cDNA was synthesized using SuperScript™ VILO™ MasterMix (Thermo Fisher Scientific) according to the manufacturer's instructions. The expression of *GMD* and *GFT* genes was confirmed by RT-PCR. β -Actin (*ACTB*) and *FUT8* genes were also amplified as internal controls. The expression levels of enzymes related to the synthesis of fucosylated glycans, α -L-fucosidase 1 (*FUC1*), α -L-fucosidase 2 (*FUC2*), fucosyltransferase 4 (*FUT4*), *FUT8*, fucosyltransferase 9 (*FUT9*), and fucose-1-phosphate guanylyltransferase (*FPGT*), were evaluated by qRT-PCR. All primer sequences used in these PCR are shown in Supplementary Table 1.

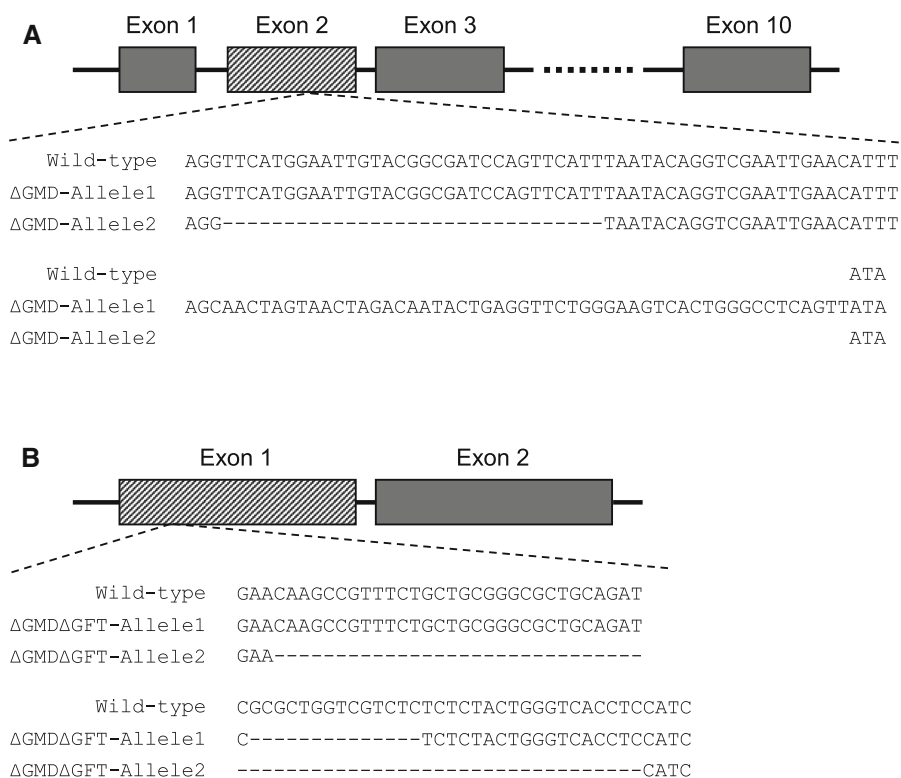
Results

Establishment of GMD single-mutated and GMD/GFT double-mutated CHO cell lines

Sequencing analysis of each single cell line resulted in the establishment of *GMD*-knockout CHO-K1 (Δ GMD) with insertion and deletion of nucleotides on its alleles in exon 2 as shown in Fig. 2A. *GMD/GFT* double-knockout CHO-K1 (Δ GMD Δ GFT) was succeeded by the deletion of nucleotides in exon 1 of the *GFT* gene in Δ GMD cells (Fig. 2B). The *GMD* and *GFT* gene expression in wild-type CHO-K1 and Δ GMD Δ GFT cells was confirmed by RT-PCR (Supplementary Fig. 1). The *GMD* gene was amplified

using cDNA synthesized from Δ GMD Δ GFT-mRNA. The gene was longer compared to wild-type cells because of an insertion of nucleotide on one allele in Δ GMD Δ GFT described above. The *GFT* gene was correctly amplified from wild-type- but not Δ GMD Δ GFT-cDNA because the Δ GMD Δ GFT-cDNA lacks nucleotide regions on its allele 1 and 2 which the PCR antisense primer anneals to (Fig. 2B and Supplementary Table 1). The internal control *ACTB* and *FUT8* genes were amplified using both wild-type- and Δ GMD Δ GFT-cDNAs.

We then examined the reduction of fucosylation in total soluble proteins from Δ GMD and Δ GMD Δ GFT cells by lectin blotting using AAL. Although total soluble proteins from Δ GMD cells showed a slight



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Fig. 2 Sequence of genomic DNA around the mutated region. Exon 2 of *GMD* in wild-type CHO-K1 cells, DNA-inserted allele 1 and -deleted allele 2 of Δ GMD cells (a); Exon 1 of *GFT* in wild-type CHO-K1 cells and both DNA-deleted alleles of Δ GMD Δ GFT cells (b)

reduction of AAL-positive bands compared with the wild type, the Δ GMD Δ GFT double mutation significantly decreased the reactivity of cell extracts to the AAL (Supplementary Fig. 2). The adherent wild-type CHO-K1 and both mutant cells were cultured in serum-reduced medium for 6 months, and finally their suspension cells adapting to serum-free medium were prepared.

N-glycan analysis of Δ GMD and Δ GMD Δ GFT cells

PA-labeled *N*-glycans from adherent cells were fractionated on RP-HPLCs (Fig. 3A). In these chromatograms, all PA-labeled *N*-glycans were eluted from 20 to 35 min on HPLC. The HPLC profiles after 25 min, which contains large amounts of fucosylated *N*-glycans, different between the wild type and the

mutants. The detailed structures and ratios of the *N*-glycans are shown in Table 1. GN, M, Gal, NA, NG, and GL show *N*-acetylglucosamine, mannose, galactose, *N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, and glucose, respectively. The italicized structures show fucosylated glycans. *GMD* knockout decreased the proportion of fucosylated *N*-glycans in wild-type CHO-K1 cells from 18.7 to 10.1% of the total amount of *N*-glycans. The *N*-glycan in Δ GMD Δ GFT cells still contained 0.9% fucosylation even if the double knockout of *GMD* and *GFT* produced a significant reduction. The amounts of high-mannose-type and complex-type *N*-glycans in both mutants were almost the same as those in the wild-type cells. The occupancy of galactosylated *N*-glycans, which contribute to the increase in ADCC activity, also showed no difference among the wild-type, Δ GMD, and Δ GMD Δ GFT cells. The RP-HPLC

Fig. 3 RP-HPLC profiles of PA-labeled *N*-glycans from total soluble proteins of adherent wild-type CHO-K1 cells, Δ GMD cells, and Δ GMD Δ GFT cells (a); of suspension-cultured wild-type CHO-K1 cells, Δ GMD cells, and Δ GMD Δ GFT cells (b)

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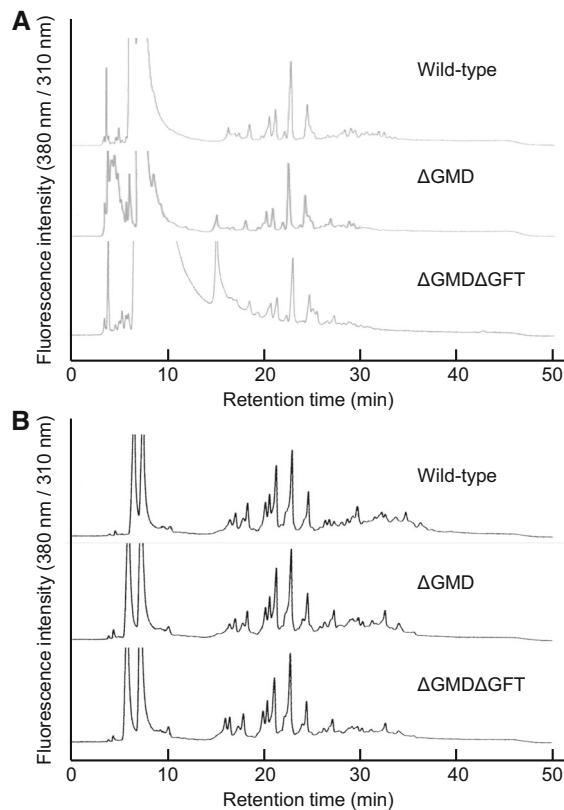


Table 1 *N*-glycan structures in adherent cells

Structure	Wild-type			Δ GMD			Δ GMD Δ GFT		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
GLM9GN2				0.1	0.7		–	–	–
M5–9GN2	63.3	63.4	68.8	61.7	61.9	53.8	60.8	68.6	55.7
<i>M5FGN2</i>	–	<i>0.1</i>	–	–	–	–	–	–	–
M4GN2	2.3	3.8	4.3	3.5	4.0	5.1	3.3	3.4	3.5
<i>M4FGN2</i>	–	<i>0.5</i>	–	–	–	–	–	–	<i>0.2</i>
M3GN2	0.9	1.6	3.5	3.8	4.9	5.1	7.5	5.2	7.0
<i>M3FGN2</i>	<i>3.0</i>	<i>1.8</i>	<i>2.3</i>	<i>0.7</i>	<i>0.8</i>	<i>1.5</i>	<i>0.2</i>	–	–
M2GN2	0.7	4.9	2.7	4.3	4.2	6.2	10.5	7.1	4.9
<i>M2FGN2</i>	<i>6.1</i>	<i>5.2</i>	<i>5.3</i>	<i>1.5</i>	<i>2.6</i>	<i>4.1</i>	–	–	–
GNM3GN2	–	0.1	–	0.2	0.4	0.4	0.8	0.6	3.1
<i>GNM3FGN2</i>	<i>0.9</i>	<i>0.7</i>	<i>1.5</i>	<i>0.3</i>	<i>0.7</i>	<i>1.0</i>	–	–	<i>0.6</i>
GNM5GN2	2.4	0.4	1.6	1.3	0.8	1.6	1.2	0.5	0.2
GN2M3GN2	–	0.1	0.1	0.9	0.7	0.8	1.5	1.8	2.5
<i>GN2M3FGN2</i>	<i>4.1</i>	<i>1.8</i>	<i>1.8</i>	<i>1.6</i>	<i>1.1</i>	<i>1.9</i>	<i>0.9</i>	<i>0.6</i>	–
GN3M3GN2	–	–	–	0.4	0.3	0.4	1.3	0.9	0.4
<i>GN3M3FGN2</i>	<i>1.1</i>	<i>1.1</i>	<i>0.7</i>	<i>0.6</i>	<i>0.8</i>	<i>0.7</i>	–	<i>0.1</i>	–
GN4M3GN2	–	–	–	–	–	–	0.1	0.1	–
GalGNM3GN2	1.8	0.2	0.5	0.5	0.2	0.9	1.1	0.5	2.4
<i>GalGNM3FGN2</i>	<i>0.6</i>	<i>0.3</i>	<i>0.9</i>	<i>0.2</i>	<i>0.4</i>	<i>0.4</i>	–	–	<i>0.2</i>
GalGNM5GN2	–	–	0.1	–	–	–	–	–	–
GalGN2M3GN2	–	0.2	0.1	0.4	0.3	0.4	0.7	0.8	1.6
<i>GalGN2M3FGN2</i>	<i>0.5</i>	–	<i>0.5</i>	<i>0.4</i>	<i>0.2</i>	<i>0.5</i>	–	–	–
GalGN3M3GN2	1.8	0.4	0.9	0.6	–	0.9	1.1	1.0	0.6
GalGNM6GN2	–	–	–	–	–	0.5	–	–	–
Gal2GN2M3GN2	2.4	2.4	0.4	5.7	5.6	2.2	6.1	5.1	10.4
<i>Gal2GN2M3FGN2</i>	<i>5.9</i>	<i>3.7</i>	<i>2.4</i>	<i>2.3</i>	<i>1.7</i>	<i>1.1</i>	–	–	–
Gal2GN3M3GN2	–	0.1	–	0.1	–	0.2	–	0.1	2.9
<i>Gal2GN3M3FGN2</i>	–	–	<i>0.1</i>	–	<i>0.2</i>	<i>0.1</i>	–	–	–
Gal3GN3M3GN2	–	5.9	0.1	2.6	3.8	1.7	0.3	1.8	–
<i>Gal3GN3M3FGN2</i>	<i>1.6</i>	<i>0.5</i>	<i>0.3</i>	<i>0.8</i>	<i>0.1</i>	<i>0.4</i>	–	–	–
Gal4GN4M3GN2	–	–	–	0.9	–	–	–	0.8	1.6
<i>Gal4GN4M3FGN2</i>	–	<i>0.2</i>	–	<i>0.3</i>	–	<i>0.5</i>	–	–	–
NAGalGNM3GN2	–	–	–	–	–	–	–	0.1	–
NAGalGN2M3GN2	–	–	0.1	0.5	–	0.2	–	–	0.8
<i>NAGalGN2M3FGN2</i>	–	–	<i>0.1</i>	–	–	–	–	–	–
NAGalGN4M3GN2	–	–	–	–	–	–	–	–	0.2
NAGal2GN2M3GN2	–	–	0.4	1.0	0.8	0.8	1.1	0.7	–
<i>NAGal2GN2M3FGN2</i>	<i>0.5</i>	–	–	<i>0.2</i>	<i>0.2</i>	<i>0.1</i>	–	–	–
NAGal2GN3M3GN2	–	–	0.1	–	–	0.4	–	–	–
NAGal3GN3M3GN2	–	0.3	0.1	1.2	3.1	3.1	0.8	0.3	0.2
NGGal2GN2M3GN2	–	–	–	0.1	0.2	0.1	0.3	–	–
<i>NGGal2GN2M3FGN2</i>	–	–	–	<i>0.2</i>	–	<i>0.2</i>	–	–	–
NGGal2GN3M3GN2	–	–	–	–	–	–	–	–	0.2
NA2Gal2GN2M3GN2	–	–	–	0.2	–	0.6	–	–	–
NA2Gal3GN3M3GN2	–	–	–	0.8	–	1.8	0.3	–	0.6

Analysis was conducted three times for each cell line (1st, 2nd, 3rd)

The italicized area shows the data of fucosylated glycans

profiles of PA-labeled *N*-glycans from suspension cells that were adapted to serum-free medium are also shown in Fig. 3B. All PA-labeled *N*-glycans were eluted from 20 to 35 min in the RP-HPLC as those from adherent cells, and the HPLC profiles after 25 min from the mutants differed from those of the wild type. The distribution of the *N*-glycan structures from suspension cells is summarized in Table 2. Fucosylated *N*-glycans in wild-type cells made up 25.1% of the total amount of *N*-glycans, and the *GMD* single knockout reduced the proportion significantly, to 0.2%. Moreover, no fucosylated glycans were detected in Δ GMD Δ GFT cells. Compared with the adherent cells, the suspension-cultured cells had almost the same amounts of high-mannose-type structures. On the other hand, complex-type structures increased by approximately 1.2- to 1.7-fold and, accordingly, the amounts of galactosylated *N*-glycans increased in the suspension cells.

Evaluation of cell growth

Figure 4 shows the number of viable wild-type CHO-K1, and Δ GMD Δ GFT cells. During a 7-day cultivation, adherent Δ GMD Δ GFT cells grew at a rate similar to that of adherent wild-type CHO-K1 cells. The total number of adherent cells increased from 1.4×10^5 to approximately 1.2×10^7 until day 5, and the growth rate decreased during the last 2 days. The growth rate of suspension-cultured Δ GMD Δ GFT cells was also similar to that of the wild type. Unlike the adherent cells, however, their growth rate significantly increased after day 5.

Expression levels of enzyme genes relating to fucosylated glycan synthesis

The gene expression levels of *FUT8*, *FPGT*, *FUC1*, and *FUC2* in the wild-type and both mutant cell lines were estimated by qRT-PCR (Fig. 5). All gene expression levels in adherent wild-type CHO-K1 cells were normalized to 1. Expression of *FUT4* and *FUT9* was not found in CHO-K1 cells (data not shown). In both adherent and suspension cells, expression of *FUT8* was reduced by *GMD* knockout but not by *GFT* knockout. *GMD* knockout significantly increased *FPGT* expression in adherent and suspension cells compared with the wild-type cells, though the expression levels decreased by approximately 50% by the

adaptation to serum-free medium. Expression of *FUC1* was not affected by the knockout of *GMD* or *GFT*, though the adaptation to serum-free medium decreased it in the suspension cells. *GMD* knockout also decreased *FUC2* expression, but *GFT* knockout improved *FUC2* expression in adherent and suspension-cultured mutant cells.

Analysis of amounts of intracellular GDP-fucose

The amounts of GDP-fucose in adherent wild-type CHO-K1, Δ GMD, and Δ GMD Δ GFT cells cultivated under serum were estimated (Fig. 6A). An enzyme reaction using extracts of wild-type CHO-K1 cells resulted in the appearance of a new peak whose retention time corresponded to that of fucosylated GN2M3FGN2-Asn-Fmoc on RP-HPLC. The amount of GDP-fucose in 1.0×10^7 cells worked out to 87.9 pmol from the peak area of newly synthesized GN2M3FGN2-Asn-Fmoc. Although the amount of enzyme reaction product was significantly reduced by *GMD* following *GFT* knockout, 15.1 pmol and 8.5 pmol of GDP-fucose were still observed in 1.0×10^7 cells of Δ GMD and Δ GMD Δ GFT, respectively. The results of the enzyme reaction using suspension cells are shown in Fig. 6B. As estimated from the peak area of GN2M3FGN2-Asn-Fmoc on RP-HPLC, 1.0×10^7 wild-type cells contained 130.5 pmol of GDP-fucose. In contrast, GN2M3FGN2-Asn-Fmoc was not detected by enzyme reaction using extracts of mutant, Δ GMD, or Δ GMD Δ GFT cells.

N-glycan analysis of recombinant human IgG produced in Δ GMD Δ GFT cells

PA-labeled *N*-glycans prepared from recombinant human IgG produced in Δ GMD Δ GFT suspension-cultured cells were fractionated on an RP-HPLC (Fig. 7A). Peaks containing PA-glycans are indicated with their abbreviations. A glycan structural analysis using an LC-MS/MS resulted in no detection of any fucosylated *N*-glycans (Fig. 7B). High-mannose-type, hybrid-type, asialo-type, and sialo-type *N*-glycans accounted for 4.0%, 69.3%, 26.0%, and 0.7% of the total *N*-glycan content, respectively.

Table 2 N-glycan structures in suspension cells

Structure	Wild-type			Δ GMD			Δ GMD Δ GFT		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
GLM9GN2	0.1	0.8	0.4	1.5	0.8	0.9	0.2	0.7	0.6
M5-9GN2	73.4	57.7	61.8	78.0	53.1	63.8	75.4	71.6	50.9
<i>M6FGN2</i>	0.1	–	–	–	–	–	–	–	–
<i>M5FGN2</i>	0.4	–	0.2	–	–	–	–	–	–
M4GN2	0.4	1.1	1.4	0.5	2.0	0.8	0.8	1.1	1.1
<i>M4FGN2</i>	–	–	0.2	–	–	–	–	–	–
M3GN2	0.3	1.2	1.2	1.1	9.1	5.1	1.6	4.9	5.3
<i>M3FGN2</i>	0.7	4.7	3.4	–	0.1	–	–	–	–
M2GN2	–	–	–	–	2.8	0.7	–	–	1.1
<i>M2FGN2</i>	–	0.4	0.5	–	–	–	–	–	–
GNM3GN2	0.1	0.9	0.1	0.6	7.1	5.4	0.5	3.0	5.0
<i>GNM3FGN2</i>	0.4	3.6	3.9	–	–	–	–	–	–
GNM5GN2	0.3	0.6	0.5	0.3	0.3	0.5	0.2	0.7	0.9
GN2M3GN2	–	0.7	0.4	0.8	4.4	5.7	1.6	3.9	6.0
<i>GN2M3FGN2</i>	0.4	2.3	3.3	–	–	0.1	–	–	–
GN3M3GN2	0.1	0.3	0.1	0.3	1.5	2.7	0.5	0.9	2.3
<i>GN3M3FGN2</i>	0.2	1.5	1.4	–	–	–	–	–	–
GN4M3GN2	–	–	–	0.2	0.1	0.2	0.2	0.1	0.1
<i>GN4M3FGN2</i>	–	0.1	0.1	–	–	–	–	–	–
GN5M3GN2	–	–	–	–	0.1	–	–	–	–
GalGNM3GN2	–	0.6	0.7	0.4	2.7	1.4	0.4	2.0	1.1
<i>GalGNM3FGN2</i>	0.3	1.1	1.0	–	–	–	–	–	–
GalGNM5GN2	–	0.4	0.1	0.4	0.2	0.1	0.3	–	0.3
GalGN2M3GN2	–	0.1	0.1	1.3	2.1	0.7	1.0	3.5	1.5
<i>GalGN2M3FGN2</i>	0.5	0.7	1.2	–	–	–	–	–	–
GalGN3M3GN2	1.4	1.7	1.9	0.5	1.3	1.8	0.8	1.1	1.0
<i>GalGN3M3FGN2</i>	–	–	–	0.1	–	–	–	–	–
GalGN4M3GN2	–	–	0.1	–	0.1	0.1	–	–	0.4
GalGNM6GN2	–	–	–	–	–	0.1	–	–	–
Gal2GN2M3GN2	0.9	1.6	2.7	9.3	4.9	3.3	8.6	3.4	10.1
<i>Gal2GN2M3FGN2</i>	3.7	7.5	2.8	0.2	–	–	–	–	–
Gal2GN3M3GN2	–	0.1	0.3	0.3	0.9	0.6	0.4	0.8	0.6
<i>Gal2GN3M3FGN2</i>	0.1	–	0.1	–	–	–	–	–	–
Gal2GN4M3GN2	–	–	–	–	0.1	–	–	–	–
Gal3GN3M3GN2	0.1	0.9	0.9	2.7	2.9	1.3	2.0	1.2	3.2
<i>Gal3GN3M3FGN2</i>	1.4	1.6	1.4	–	–	–	–	–	–
Gal3GN4M3GN2	0.3	–	–	–	–	–	0.2	–	–
<i>Gal3GN4M3FGN2</i>	0.3	–	–	–	–	–	–	–	–
Gal4GN4M3GN2	–	–	–	0.8	1.3	1.4	1.9	0.5	2.3
<i>Gal4GN4M3FGN2</i>	2.0	1.7	1.3	–	–	–	–	–	–
NAGalGNM3GN2	0.1	–	0.2	–	–	–	0.3	0.1	0.1
NAGalGNM4GN2	–	–	0.2	–	–	–	0.2	–	–
NAGalGNM5GN2	0.1	0.1	–	–	–	–	0.2	–	0.2

Table 2 continued

Structure	Wild-type			Δ GMD			Δ GMD Δ GFT		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
NAGalGN2M3GN2	0.1	0.1	–	0.1	–	–	–	–	0.2
<i>NAGalGN2M3FGN2</i>	<i>0.3</i>	–	–	–	–	–	–	–	–
NAGalGN4M3GN2	–	0.1	–	–	–	–	–	–	–
NAGal2GN2M3GN2	0.5	1.0	1.7	0.7	0.4	1.4	2.2	0.3	1.8
<i>NAGal2GN2M3FGN2</i>	<i>5.6</i>	<i>2.1</i>	<i>1.7</i>	–	–	–	–	–	–
NAGal2GN3M3GN2	–	0.4	–	–	0.1	–	–	0.1	0.1
NAGal3GN3M3GN2	–	–	–	0.1	0.6	0.6	0.6	0.1	1.9
<i>NAGal3GN3M3FGN2</i>	<i>1.4</i>	<i>1.2</i>	<i>1.4</i>	–	–	–	–	–	–
NAGal4GN4M3GN2	–	–	–	–	0.2	0.3	–	–	0.9
NGGalGN2M3GN2	–	–	–	–	0.3	0.2	0.1	–	0.1
NGGal2GN2M3GN2	–	–	–	–	0.3	0.2	0.3	–	0.1
<i>NGGal2GN2M3FGN2</i>	–	<i>0.2</i>	–	–	–	–	–	–	–
NGGal3GN3M3GN2	–	–	–	–	0.1	–	–	–	–
NA2Gal2GN2M3GN2	1.1	0.2	–	–	0.3	0.6	1.1	–	0.9
<i>NA2Gal2GN2M3FGN2</i>	<i>2.9</i>	<i>0.8</i>	<i>1.1</i>	–	–	–	–	–	–

Analysis was conducted three times for each cell line (1st, 2nd, 3rd)

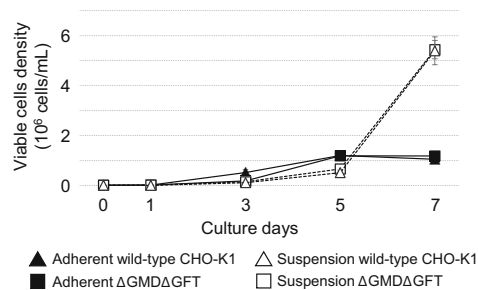
The italicized area shows the data of fucosylated glycans

Discussion

In this study, we succeeded in establishing CHO-K1 cells that could synthesize afucosylated glycans by knockout of *GMD* and *GFT*, and examined the effects of afucosylation on serum-free adaptation of cells and

the gene expression levels of other endogenous enzymes related to the synthesis of fucosylated glycans.

First, we destroyed the de novo synthetic pathway of GDP-fucose, which is a donor substrate of fucosyltransferases, by *GMD* knockout in CHO-K1 cells.



Establishment of suspension-cultured Chinese hamster ovary cells with double knockout of GDP-mannose-4,6-dehydratase and GDP-fucose transporter

Cytotechnology

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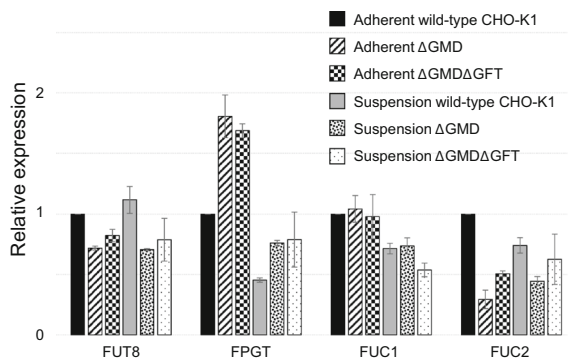
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Fig. 4 Growth curve of wild-type CHO-K1 cells and Δ GMD Δ GFT cells. Closed triangle: adherent wild-type CHO-K1 cells, closed square: Adherent Δ GMD Δ GFT cells, opened triangle: wild-type CHO-K1 suspension cells, opened square:

Δ GMD Δ GFT suspension cells. Vertical and horizontal axis shows the cells density and culture days, respectively. Initial number of cultured cells was 1.4×10^5 cells. Mean (SE), n = 3

Fig. 5 Relative gene expression of endogenous enzymes relating to the fucose metabolism. Bars mean adherent wild-type CHO-K1 cells, adherent Δ GMD cells, adherent Δ GMD Δ GFT cells, suspension-cultured wild-type CHO-K1 cells, suspension-cultured Δ GMD cells, suspension-cultured Δ GMD Δ GFT cells from the left, respectively. The expression amount of each gene from the wild-type CHO-K1 cells was normalized to 1. Mean (SE), $n = 3$



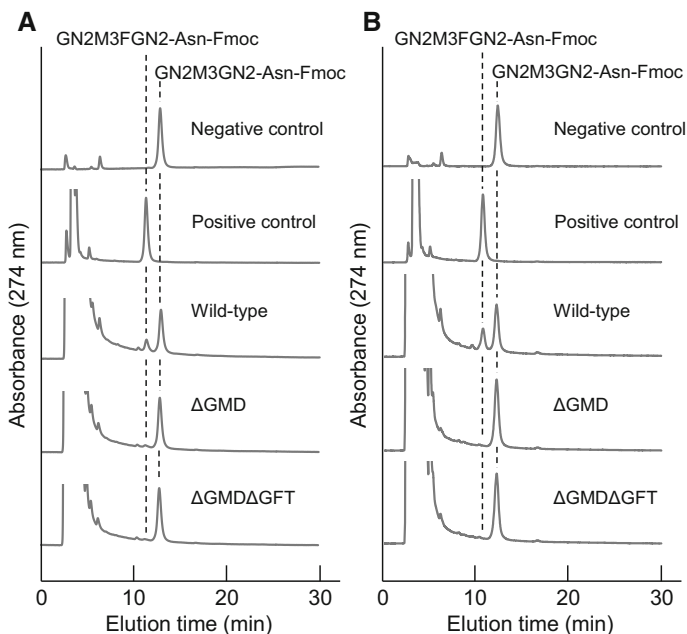
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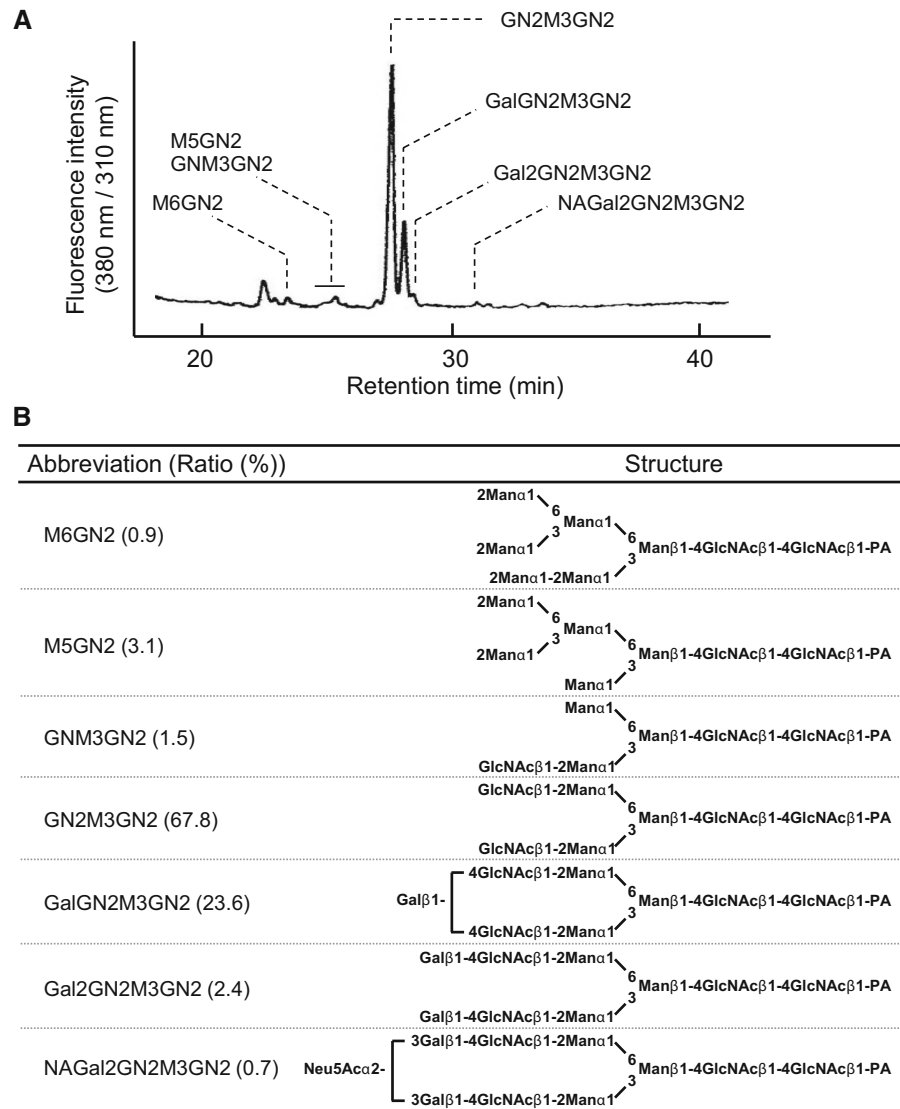
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Fig. 6 Estimation of intracellular GDP-fucose in adherent cells (a); in suspension cells (b). Enzymatically produced GN2M3FGN2-Asn-Fmocs were eluted by HPLCs. Negative control: Fut8+GN2M3GN2-Asn-Fmoc, positive control: GDP-

Fuc+Fut8+GN2M3GN2-Asn-Fmoc, wild-type: CHO-K1 extracts+Fut8+GN2M3GN2-Asn-Fmoc, Δ GMD: Δ GMD extracts+Fut8+GN2M3GN2-Asn-Fmoc, Δ GMD Δ GFT: Δ GMD Δ GFT extracts+Fut8+GN2M3GN2-Asn-Fmoc

Fig. 7 *N*-Glycan analysis of recombinant human IgGs produced in Δ GMD Δ GFT cells. RP-HPLC profiles of PA-labeled *N*-glycans of recombinant human IgGs produced in suspension-cultured Δ GMD Δ GFT cells (a); *N*-glycan structure determined by HPLC and LC-MS analysis (b)

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The adherent Δ GMD cells showed a 54% reduction of fucosylated *N*-glycans compared with adherent wild-type cells, but 10% of fucosylated glycans were still found in Δ GMD. Galactosylation, which contributes to ADCC activity, was not affected by the *GMD* knockout. The knockout affected the amounts of high-mannose-type and complex-type *N*-glycans only slightly. Almost all fucosylation of *N*-glycans was lost by the double mutation of *GMD* and *GFT*, as 0.9% of fucosylation was detected. These results suggest

that both the inactivation of the GDP-fucose de novo synthetic pathway and the loss of transport of the GDP-fucose from cytosol to the Golgi apparatus specifically suppressed the fucosylation of *N*-glycans. However, Δ GMD cells cultured in FBS contain approximately 10% fucosylated *N*-glycans, though a previous work mentioned that depletion of *GMD* produced no fucosylated glycans in CHO cells cultured in dialyzed FBS (Kanda et al. 2007). GDP-

fucose should be synthesized only through the salvage pathway in Δ GMD cells because of the lack of the de novo synthetic function. In the salvage pathway, L-fucose kinase (FUK) and FPGT synthesize GDP-fucose from L-fucose via L-fucose-1-phosphate. Δ GMD cells cannot produce fucose. Therefore, L-fucose and GDP-fucose are thought to come from extracellular source, such as medium and FBS. Then, the internalized or FUK/FPGT-synthesized GDP-fucose was transported to the Golgi lumen by GFT and used to synthesize fucosylated glycans. Internalized fucosylated glycoproteins are degraded in lysosomes (Becker and Lowe 2003), and released free fucose residues may then be reused to synthesize GDP-fucose in Δ GMD cells. Similarly, fucosylated glycans of exogenous proteins from extracellular sources may have been detected in small amounts because GDP-fucose is not transported into the Golgi apparatus by *GFT* knockout. This means that at least the remove of serum from culture medium is required for the construction of cells without any fucosylated glycans. FBS contains many glycosylated enzymes and hormones, such as alkaline phosphatase, thyroid-stimulating hormone, and so on (Endo et al. 1988; Ikegami et al. 2014). Next, we developed wild-type CHO-K1, Δ GMD, and Δ GMD Δ GFT cells adapted to serum-free medium, to which the current host cells should be adapted from the standpoints of both the effectiveness and safe industrial production of biopharmaceutical proteins. These cell lines were then established as suspended cells in the process of serum-free adaptation. The *N*-glycosylation pattern except for fucosylation among these cell lines were not affected by the serum-free adaptation. The adaptation of wild-type CHO-K1 cells to serum-free medium also produced no differences in the amounts of fucosylated *N*-glycans. However, knockout of *GMD* produced only 0.2% fucosylation in Δ GMD suspension cells, which is less than that in adherent Δ GMD Δ GFT cells. Moreover, fucosylated *N*-glycans were not detected in Δ GMD Δ GFT suspension cells. These results suggest that inactivation of *GFT* and adaptation to serum-free medium are required for the complete removal of fucosylated glycans from CHO cells. On the other hand, even suspension Δ GMD cells under serum-free were able to greatly suppress glycan fucosylation (0.7% relative to the suspension wild-type cells) compared to adherent Δ GMD Δ GFT cells cultured in the presence of serum (5% relative to the adherent

wild-type cells). Although the Δ GMD cells were not completely defucosylated in serum-free medium as previously reported (Kanda et al. 2007), a single mutation of *GMD* can be a strong way to effective production of antibodies with high ADCC activity under serum-free.

It was thought that the amount of fucosylated glycans depended on that of intracellular GDP-fucose. As shown in Fig. 6A, the amounts of GDP-fucose in 1.0×10^7 adherent wild-type and Δ GMD cells were 87.9 and 15.1 pmol, respectively. It was thought that the GDP-fucose contained in Δ GMD cells was internalized from medium with serum or synthesized using internalized L-fucose by FUK and FPGT, and the GDP-fucose was then used as a substrate for the production of approximately 10% fucosylated *N*-glycans. No remaining GDP-fucose was found in extracts of Δ GMD suspension cells, as shown in Fig. 6B. This means that these exogenous substrates were removed by the adaptation of Δ GMD cells to serum-free medium. Actually, we detected GDP-fucose from medium containing 10% FBS without any cells (data not shown). Similarly, 1.0×10^7 of adherent Δ GMD Δ GFT cells contained 8.5 pmol of GDP-fucose, and the adaptation of Δ GMD Δ GFT cells to serum-free medium resulted in the total absence of fucosylated *N*-glycans. In contrast, adaptation of wild-type CHO-K1 cells to serum-free medium increased the amount of intracellular GDP-fucose. Probably, suspension-cultured cells need supplementation with L-fucose or GDP-fucose and positively produce GDP-fucose using active *GMD*, FUK, and FPGT because serum-free medium contains no L-fucose. We further examined a potential of the suspension Δ GMD Δ GFT cells for industrial biopharmaceutical protein production by expression of a recombinant human IgG under serum-free cultivation. Fucosylation was not detected in the purified IgGs as shown in Fig. 7 and the distribution of detected *N*-glycan structures was quite similar without the abnormal structures compared to trastuzumab produced in *GFT*-mutated CHO-DG44 cells reported by Chan et al. (2016).

Although Kanda et al. (2007) and Chan et al. (2016) demonstrated that *GMD*- and *GFT*-knockout did not affect cell growth of CHO-DG44 cells, respectively, there are no information about the effect of the *GMD*-*GFT*-double knockout on cell characteristics. First, the effects of the knockout of *GMD* and *GFT* on the expression of genes encoding other enzymes relating

to fucosylation was analyzed. Overall, the expression level of each target gene except for FUT8 in suspension cells was lower than that in adherent cells. As shown in Fig. 4, the suppression of fucosylated *N*-glycans did not affect cell growth among the wild type or the mutants. Although increase of the number of both adherent Δ GMD Δ GFT and wild-type cells was suppressed by the high-confluency, the number of suspension Δ GMD Δ GFT and wild-type cells increased dramatically as well after the 5-day culture.

In this study, we succeeded in using *GMD* and *GFT* knockout to establish a novel CHO cell line, Δ GMD Δ GFT, in which fucosylated *N*-glycans are undetectable and production of fucose-free human IgGs using the Δ GMD Δ GFT cells. Moreover, as demonstrated in previous studies with *GMD* or *GFT* single knockout cell line (Kanda et al. 2007; Chan et al. 2016), we demonstrated that adaptation to serum-free medium did not affect the growth of Δ GMD Δ GFT cells compared to that of wild-type CHO-K1 cells. This means that Δ GMD Δ GFT cells can be used for the industrial production of recombinant biopharmaceutical proteins as a good host cell line. Especially, the production of the next generation of recombinant monoclonal antibodies has received a lot of attention, and the market is growing significantly every year. Not only improved productivity but also highly value-added antibody production are required. Δ GMD Δ GFT cell with afucosylated *N*-glycans can be a useful host for IgGs because the removal of the core-fucosylation of IgG brings a huge increase of ADCC activity described above. On the other hand, Ohashi et al. and Hossler et al. reported that FUT8 can add D-arabinose and L-galactose instead of L-fucose as the core glycosylation (Ohashi et al. 2017; Hossler et al. 2017). Intriguingly, the IgG with arabinosylated *N*-glycan showed a high affinity to Fc γ RIII similar to or slightly higher than that of the IgG with afucosylated *N*-glycans. A previous study suggested that fucose residues are internalized from extracellular compartment via a specific plasma membrane transporter system (Wiese et al. 1994). Accumulation of fucose in HepG2 cells was facilitated by existence of other monosaccharides including D-arabinose except for glucose (Wiese et al. 1997). Although there is no information about uptake of D-arabinose via the same transporter, Hossler et al. 2017 showed that addition of high D-arabinose into cultured medium achieved its uptake to CHO cells. One research group

demonstrated that combination of FUK and FPGT can produce GDP-D-arabinose from the substrate D-arabinose (Park et al. 1997). Substrate specificity of GFT is still unclear. For instance, *Leishmania LPG2* is known to transport not only GDP-mannose but also GDP-fucose (Ma et al. 1997). However, endogenous GFT may have transported D-arabinose from cytosol to the Golgi lumen in the case of Hossler et al. 2017. Therefore, Δ GMD Δ GFT cells may have a reduced capacity for D-arabinose uptake because of lack of GFT function. On the other hand, D-arabinose is efficiently transferred to *N*-glycans without competing with fucose under depletion of *GMD*. Low concentration of D-arabinose in cell cultured medium may economically achieve the production of arabinosylated *N*-glycans. Δ GMD cells in this study or previously established *GMD*-knockout cells (Kanda et al. 2007) are promising host cells for effective production of antibodies with arabinosylated *N*-glycans.

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