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Short Communication

Functional knockout of FUT8 in Chinese hamster ovary cells using CRISPR/Cas9 to produce a defucosylated antibody

We report the adaptation of the new CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9) system to disrupt the gene encoding fucosyltransferase 8 (FUT8), an α1,6-fucosyltransferase that directs fucose addition to derived antibody Fc region asparagine 297, in Chinese hamster ovary (CHO) cells. Compared to previously reported homologous recombination or zinc-finger nucleases (ZFNs) applications in CHO cells, CRISPR/Cas9 demonstrated higher targeting efficiency and easier customization. FUT8 disruptive clones (FUT8$^{-/-}$) were obtained within 3 weeks at indel frequencies ranging from 9 to 25%, which could be enhanced to 52% with *Lens culinaris* agglutinin (LCA) selection. Based on the lectin blot method, the derived FUT8$^{-/-}$ clone had the ability to produce defucosylated therapeutic mAb with no detrimental effects on cell growth, viability, or product quality. The clone had the potential of industrial application for therapeutic antibodies manufacturing. We have demonstrated functionally that a gene related to product synthesis could be mutated using CRISPR/Cas9 technology, and consequently the glycan profile of expressed mAb was alternated. We believe that with its robustness and effectiveness, CRISPR/Cas9 can be widely applicable in cell line development leading to higher productivity and better quality of mAbs and other biological therapeutics.

Keywords: Antibody / Antibody-dependent cellular cytotoxicity / CHO-K1 cells / CRISPR/Cas9 / Fucose

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1 Introduction

mAb therapeutics is increasing rapidly in treatment of various diseases [1]. There are more than 30 antibody therapeutics approved by regulatory agencies for the worldwide market, and many more mAb candidates are in clinical trials [2]. Antibodies execute their function by destroying targets through two pathways: complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) [3]. ADCC is mediated by antibody Fc region binding to lymphocyte receptors [4]. *N*-oligosaccharide is present on asparagine 297 of IgG heavy chain Fc region, whereas fucose is commonly found in the oligosaccharide structure, which could hinder Fc binding to lymphocyte receptors [5]. Compared to fucosylated antibodies derived from wild-type Chinese hamster ovary (CHO) cells, defucosylated antibodies achieved 100-fold higher ADCC activity in vitro [6]. Therefore, antibody defucosylation is considered to be a powerful approach to enhance ADCC activity.

The majority of commercial antibodies are produced from CHO cells, with advantages of posttranslational modifications,

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; **CHO**, Chinese hamster ovary; **CRISPR/Cas9**, clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9; **FUT8**, fucosyltransferase 8 (α1,6-fucosyltransferase); **LCA**, *Lens culinaris* agglutinin; **sgRNA**, single guide RNA; **TALEN**, transcription activator-like effector nuclease; **ZFN**, zinc-finger nuclease

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such as glycosylation, phosphorylation, acetylation, etc [7]. However, it is notable that core fucosylation commonly found in mAbs derived from CHO cells would hinder antibodies binding to their receptors [8]. In CHO cells, fucosyltransferase 8 (FUT8) is an α1,6-fucosyltransferase that directs fucose addition to asparagine-linked *N*-acetylglucosamine moieties, a common feature of *N*-linked glycan core structures [9]. So far, the effect of disrupting FUT8 gene to enhance ADCC has been reported by several groups using different approaches, such as homologous recombination [10] or zinc-finger nucleases (ZFNs) [11], both achieved increased ADCC activity (approximately 100-fold).

Traditionally, homologous recombination was used for genome editing in cell lines and animal models, though it was less efficient and was a time-consuming process due to technical complexity [12]. This was particularly true in the case of FUT8 disruption, as approximately 120 000 clones were screened to obtain only three FUT8$^{-/-}$ clones with a normal growth profile [13]. To improve the efficiency of disrupting a coding gene, technologies such as ZFNs and transcription activator-like effector nucleases (TALENs) were developed and reported [14]. Compared to traditional homologous recombination, ZFNs and TALENs demonstrated higher genome editing efficiency [15]. These nucleases could introduce double-strand breaks, which would be repaired by one of the following two major mechanisms: the error-prone nonhomologous end-joining or the high-fidelity homology-directed repair pathway [16]. Nonhomologous end-joining can be harnessed to mediate gene functional disruptions, as indels occurring in a coding region could lead to frameshift mutations and protein function loss [17]. ZFNs and TALENs were extremely expensive and difficult to design, which limited their widespread use. More recently, CRISPR (clustered regularly interspaced short palindromic repeat)/Cas9 (CRISPR-associated protein 9) system, which was derived from the microbial adaptive immune system [18], has been developed as an efficient genome editing tool and applied to diverse species such as plants, animals, bacteria, and yeast [19], as well as in CHO cells [20]. This system required only Cas9 and single guide RNA (sgRNA) [21], giving it several advantages including ease of customization, higher targeting efficiency, and ability to facilitate multiplex genome editing [22].

Here, we reported a new procedure to disrupt FUT8 gene in CHO-K1 cell line using CRISPR/Cas9 system. *Lens culinaris* agglutinin (LCA) based phenotypic screen was used to enrich clones with exclusively doubly modified alleles. mAbs produced in the CHO FUT8$^{-/-}$ cells were defucosylated. Further, we demonstrated that the gene modification had no detrimental effects on cell growth, viability, or product quality.

2 Materials and methods

2.1 Construction of FUT8 disruptive vector

The exon 10 of FUT8 (GeneBank ID: 100751648) was selected as the target site for mutagenesis because it encoded for the catalytic site of the enzyme. Five sgRNAs were designed, based on previously described design rules [19] to obtain satisfied indel frequencies (Supporting information Tables S1–S3 and Supporting information, Fig. S1). Exon 10 and adjacent region (685 bp) was amplified by PCR using primers FUT8-F (5'-CTGTTGATTCCAGGTTCCCATATA-3′) and FUT8-R (5′-TTGAATGATGACTGCTA GTGATGCT-3′). The plasmid pX330 was kindly provided by Dr. Zhang Feng (MIT) [19] and digested with BbsI (New England Biolabs, MA, USA); a pair of annealed sgRNA was ligated to the linear plasmid. All cloning steps were confirmed by DNA sequencing at Invitrogen (Shanghai, China), derived plasmids were addressed as pX330-sgRNA1∼5.

2.2 Analysis of Cas9 activity in CHO-K1 cells

Cells were incubated at 37°C with 5% CO_2 and transfected using Lipofectamine 2000 reagent (Invitrogen, CA, USA). Forty-eight hours posttransfection cells were harvested and genome DNA was extracted using Genome DNA Extraction Kit (Axygen, CA, USA). Approximately 200 ng PCR product of FUT8 exon 10 region (685 bp) was annealed using the following program: heated to 95°C for 3 min and then ramped down to 25°C at 5°C/min. Annealed PCR fragment was treated with 0.3 μL T7 endonuclease at 37°C for 45 min, followed by analysis on 1% agarose gel. Related indels were stained with ethidium bromide and detected under gel imaging systems (Tanon, Shanghai, China). Percentage of indel frequency was calculated using ImageJ software as previously described by Hwang et al. [23].

2.3 Generation of CHO FUT8$^{-/-}$ clones

Cells were exposed to 100 μg/mL LCA (Vector Labs, CA, USA) 2 days posttransfection. After 6-day growth in the presence of 100 μg/mL LCA, clones morphologically similar to the wild-type CHO-K1 were expanded. Limiting dilution was performed to screen doubly modified alleles' clones. When clones reached an 80% confluence, genome DNA extraction and T7 endonuclease digestion were performed as described above. To identify both alleles' modification in LCA-resistant clones, PCR product from each T7E1-positive clone was TA-cloned using TA cloning kit (Takara, Tokyo, Japan). At least 10 colonies were picked from each transformation for DNA sequencing as CHO cells were aneuploids.

2.4 LCA reactivity and genetic stability analysis

FITC-LCA binding assay was performed to test FUT8$^{-/-}$ clones phenotypic profiles. Briefly described, cells were seeded in 12-well plates and incubated in the presence of 20 μg/mL FITC-LCA (Vector Labs) for 30 min. Cells were washed three times and visualized under fluorescence microscope (Olympus, Tokyo, Japan). An LCA-binding test based on flow cytometer analysis was also performed as previously described [13]. Clones were cultured for 10 passages to confirm the genetic stability of the modified clones, both alleles' DNA sequencing was performed as described above.

2.5 Anti-CD20 mAb expression and purification

Chimeric anti-CD20 mAb (Rituximab, drug bank: DB00073) was chosen as a model antibody [24]. The heavy chain and light chain were separately subcloned into pcDNA3.1 (Invitrogen)

as expression vectors (heavy chain and light chain sequences are shown in Supporting information). All cloning steps were confirmed by DNA sequencing. Stable transfected cell pools of CHO-K1 cells and FUT8$^{-/-}$ clones were selected via 800 μg/mL G418 pressure for 10 days. Serum-free adaptationwas performed to eliminate the influence of FBS presence. Cell count and viability were determined by Trypan blue method. AKTA purifier system (GE Healthcare, CT, USA) was used to purify antibodies from culture supernatants as previously described [25].

2.6 SDS-PAGE, western blot, and lectin blot

Protein fractions from protein A chromatograph were analyzed by 10% SDS-PAGE to confirm that purified glycoproteins were antibodies. Protein loading was normalized to 500 ng/well for SDS-PAGE, 50 ng/well for lectin blot, and 5 ng/well for western blot. For lectin blot, the membrane was incubated with 10 μg/mL FITC-LCA for 30 min in the dark, and the green fluorescence signal was scanned by multiple function laser scanner (Typhoon FLA 9500, GE Healthcare).

3 Results

3.1 FUT8 modification generated by CRISPR/Cas9

The exon 10 encoded for the catalytic site inverting substrate GDP-fucose, therefore was chosen as the target for CRISPR/Cas9 editing. Plasmid containing a chimeric guide RNA structure had higher genome editing efficiency as compared to plasmid containing a crRNA-tracrRNA guide RNA [19], hence plasmid pX330 containing human U6 polymerase promoter generating chimeric sgRNA was selected to perform gene perturbation, which was confirmed to be effective in multiple species as well as Chinese hamster [20]. Two days posttransfection, T7 endonuclease digestion tests were performed in cells transfected with diverse pX330-sgRNAs. Indels were stained with ethidium bromide and visualized under DNA gel imaging system, indel frequencies (%) were analyzed using ImageJ software with the value ranging from 9 to 24% as indicated in Fig. 1A. No indels were visualized without pX330-sgRNA treatment.

3.2 FUT8 functional disruptive clones generated by limiting dilution

To produce defucosylation mAbs in CRISPR/Cas9 derived clones, both alleles of FUT8 gene should be disrupted. LCA is a plant lectin with high specificity to bind IgG *N*-glycan core fucose, which can lead to cell death [26]. CHO FUT8$^{-/-}$ clones are able to survive in the presence of LCA as cells fail to synthesize fucosylated glycoproteins. In our experiment, cells were transferred to 12-well plates 2 days posttransfection and LCA was added to each well at a final concentration of 100 μg/mL (day 2). Eighteen hours later, most cells became round and got detached from the plate, the phenotype similar to wild-type clones appeared 2 days after LCA treatment (day 4, Fig. 1B). Cells were LCA selected for 6 days and expanded after fresh medium replacement (day 8). As shown in Fig. 1A, indel frequencies of LCA-resistant clones were ranging from 24 to 52%, indicating that LCA treatment enriched FUT8$^{-/-}$ clones. No LCA-resistant clones were detected in the absence of pX330-sgRNA transfection within 6 days LCA exposure.

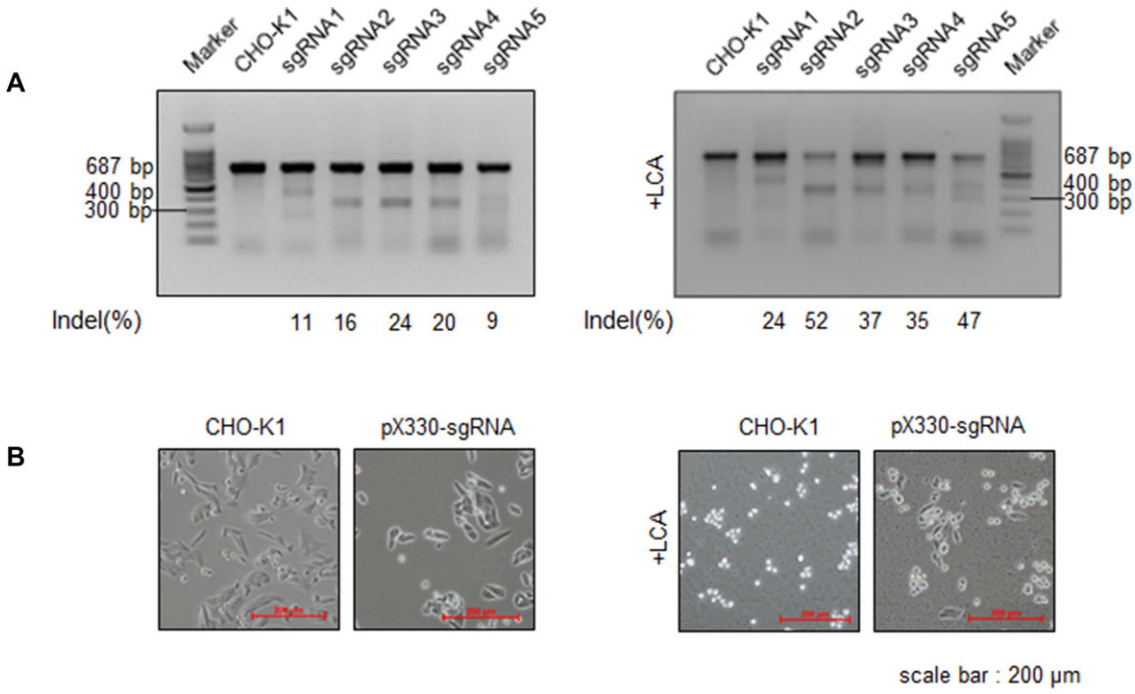
LCA-selected clones derived from pX330-sgRNA1 transfection were subcloned by limiting dilution. Three clones, named D5, C9, and C10 showing positive T7E1 digestion activity were chosen for further research. Exon 10 region was amplified by PCR from these clones, both alleles' genotypes of clone D5, C9, and C10 are listed in Fig. 1C. These modifications were considered to lead FUT8 truncation and frameshift mutations in these clones. C9 clone was morphologically different from wild-type CHO cells (Supporting information, Fig. S2), which indicated that other alternations in cell metabolism might occur besides the FUT8 disruption. C10 clone was successfully adapted to serum-free medium and had the best cell growth among the three clones, therefore was selected for further studies.

There was no available commercial antibody that could distinguish FUT8 exon 10 functional disruption. We therefore tested the FUT8 activity by LCA-resistance phenotype method. It was confirmed by fluorescence microscopy images and flow cytometer analysis that these clones lacked the ability to bind FITC-LCA. As indicated in Fig. 2A and B and Supporting information Fig. S3, FITC-LCA could bind to wild-type CHO-K1 cells but not the FUT8$^{-/-}$ clones. Genetic stability of C10 clone was also evaluated for 10 passages. No alternation was found in genotype or LCA-resistance ability compared to the parental clone (data not shown).

3.3 Antibodies expression and lectin blot based fucosylation detection

Prior to express an antibody in the cells, growth profiles of C10 clone and wild-type CHO-K1 cells were compared side by side in 125-mL shaker flasks with 20 mL working-volume medium. During 8 days incubation, both CHO-K1 cells and C10 clone cell densities reached greater than 6×10^6 cells/mL and maintained viability above 90%. As shown in Fig. 2C and D, C10 clone had comparable viable cell counts and culture viability compared to wild-type CHO-K1 cells throughout three parallel experiments.

Cell pools stably expressing anti-CD20 antibody were derived from both wild-type CHO-K1 cells and C10 clone using G418 selection. Each cell pool was cultured in a 1000-mL shake flask containing 300 mL CD-CHO medium supplemented with HT and glutamine, supernatants were collected when cell viabilities were below 30%. Proteins of interest in the supernatants were purified by AKTA purifier system and analyzed by SDS-PAGE and western blot (Fig. 3A and B). Roughly equivalent quantity of purified IgGs were obtained from C10 clone and CHO-K1 cells pools under the same cell incubation condition based on bicinchoninic acid assays, with expression levels around 1.2 mg/L. FITC-LCA lectin blot was chosen as an alternative method to examine whether antibody derived from the C10 clone contained fucose [27]. As expected, antibody derived from C10 clone showed no visible fluorescence compared to antibody from parental CHO-K1 cells under both nonreducing and reducing conditions in three



C Both alleles genotypes of three LCA-resistant clones

Wild-type	AAAAAAAGAGTGTATCTGGCCAC**TGATGACCCTTCTTTGTT–AA**AGGAGGCAAAGACAAAGT	
Clone D5-A	AAAAAAAGAGTGTATCTGGCCACTGATGACCCTTCTTTGTTTAAAGGAGGCAAAGACAAAGT	+1
Clone D5-B	AAAAAAAGAGTGTATCTGGCCACTGATGACCCTTCTTTGT––AAAGGAGGCAAAGACAAAGT	Δ1
Clone C9-A	AAAAAAAGAGTGTATCTGGCCACTGATGACCCTTCTTTG–––AAAGGAGGCAAAGACAAAGT	Δ2
Clone C9-B	AAAAAAAGAGTGTATCTGGCCACTG----------35----------GT	Δ35
Clone C10-A	AAAAAAAGAGTGTATCTGGCCACTGATGACCCTTCTTTGT––AAAGGAGGCAAAGACAAAGT	Δ1
Clone C10-B	AAAAAAAGAGTGTATCTGGCCACTGATGACCCTT----------20---------- ACAAAGT	Δ20

Figure 1. Genome editing in CHO-K1 cells by CRISPR/Cas9. (A) T7E1 digestion for Cas9 activity in CHO cells. Two hundred nanograms PCR product was digested and loaded for each lane. Left panel, genomic DNA was extracted from CHO-K1 cells 2 days after transfected with different pX330-sgRNAs. Right panel, genomic DNA was extracted from expanded clones by LCA selection. Note that indel frequency was not completely equivalent to disruption efficiency. (B) Wild-type CHO-K1 cells were round and detached from the plate when treated with 100 μg/mL LCA for 18 h, while pX330-sgRNAs transfected clones with wild-type morphology appeared under the same condition. LCA precipitate was visible. (C) Sanger sequences of FUT8 exon 10 mutated alleles obtained from three CHO FUT8$^{-/-}$ clones. Alleles were designated as A and B. The gap shown in the reference sequences has been included for better representation of alignment results. All modifications could lead to FUT8 frameshift mutations.

parallel experiments (Fig. 3C). We concluded that CHO FUT8$^{-/-}$ clone generated by CRISPR/Cas9 was able to express mAb with significantly reduced fucosylation.

4 Discussion

In this report, we presented successful FUT8-functional disruption in CHO-K1 cells using CRISPR/Cas9 system. Normally, the 20–30 bp region downstream of the start codon ATG was chosen as the target [28], while catalytic site of FUT8 protein was chosen for successful gene perturbation in CHO-K1 cells as previously reported [8]. Off-target mutagenesis was always an undesirable effect induced by Cas9 and hard to avoid, enhancement of Cas9 targeting specificity seemed to be promising as many researchers had fruitful achievements of improved Cas9 target recognition fidelity [29, 30]. A successful gene disruption in COSMC and FUT8 with high indel frequency was reported, in addition to gene disruption results that showed consistency as reported by Ronda et al. [20], we were able to demonstrate the final product without fucose.

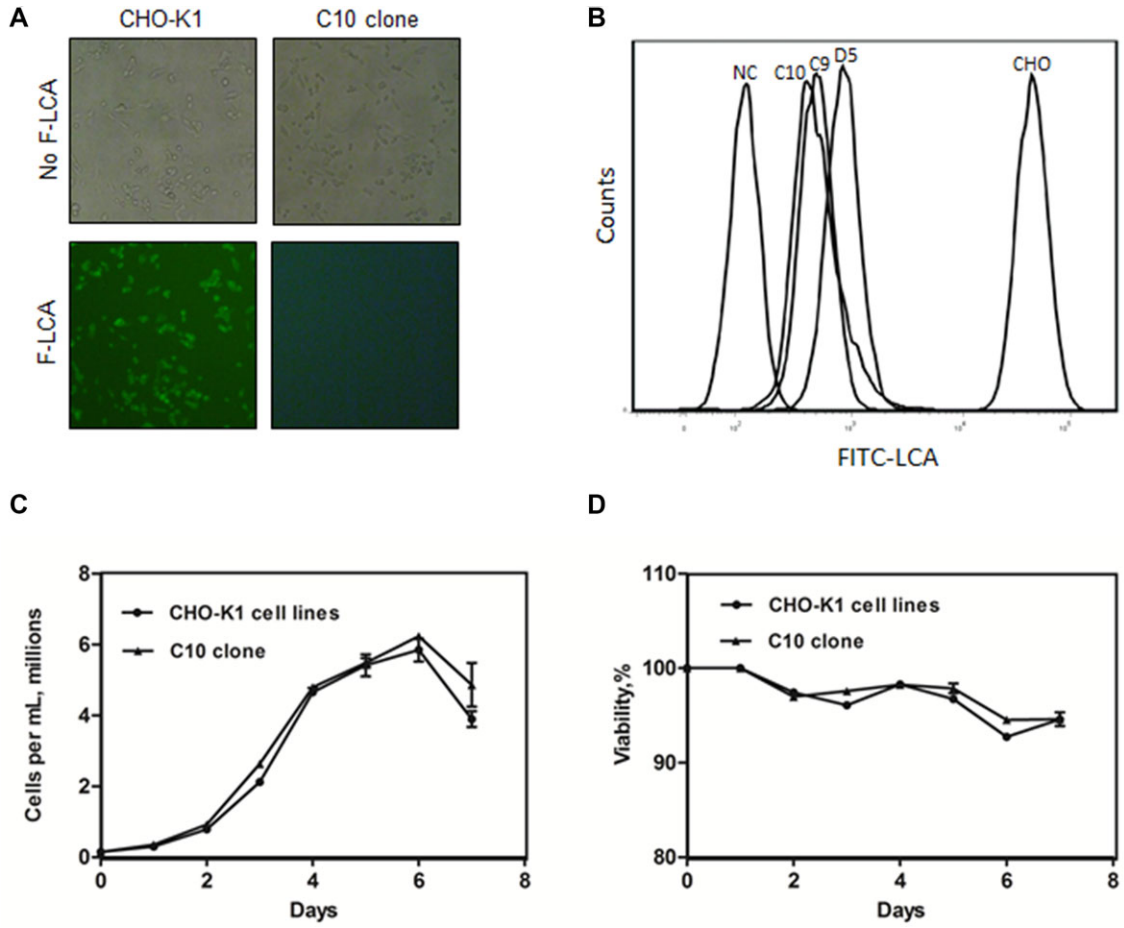


Figure 2. LCA reactivity and cell growth of CHO FUT8$^{-/-}$ C10 clone. (A) FITC-LCA failed to bind C10 clone membrane glycoproteins. Under panels, cells were treated with 20 μg/mL FITC-LCA, fluorescence microscopy images were subsequently acquired at a green fluorescent protein channel. (B) FACS paragraphs of FITC-LCA stained cells. *x*-axis, fluorescence intensity; *y*-axis, cell counts. NC refers to negative control wild-type CHO-K1 cells without FITC-LCA treatment. For details see Supporting information Fig. S3. Cell growth (C) and viability (D) of serum-free adapted C10 clone and CHO-K1 cells. Cell density and cell viability were examined in 125-mL shaker flasks (20 mL working volume) during 8 days incubation. Error bars represent the SD from three parallel experiments.

LCA selection was conducted to obtain stable FUT8$^{-/-}$ clones within a short duration. Another gene related to core fucose synthesis pathway named GDP-fucose transporter was also disrupted in our lab (data not shown), which was responsible for transporting GDP-fucose into Golgi apparatus [31]. GDP-fucose transporter modified clones showed no resistance to LCA, indicated that other GDP-fucose transporting pathways could play complementary roles.

A new host cell line to produce completely defucosylated antibodies, needs to be characterized in terms of growth, viability, and antibody productivity [32]. In this study, we compared the C10 clone and wild-type CHO-K1 cells in a shake flask system. Cell cultures were maintained for 8 days before significant clumping occurred. For further comparison, feb-batch cultivation in a bioreactor system would be preferable. Our data showed CHO FUT8$^{-/-}$ C10 clone had comparable viable cell counts and viability throughout three parallel experiments compared to the wild-type cell line.

To detect the presence of fucose in the mAb glycan structure, we employed a simple and quick FITC-LCA lectin blot method, whereas other approaches such as MALDI-TOLF-MS and high pH anion exchange chromatography with pulsed amperometric detection could be utilized [33]. FITC-LCA showed very high specificity binding to α1,6-fucose and was a widely accepted approach to confirm *N*-glycan fucosylation of glycoproteins [27, 34]. As expected, antibody produced in C10 clone showed no detectable fluorescence compared to antibody from the parental CHO-K1 cells under both nonreducing and reducing conditions, indicating that the C10 clone produced defucosylated antibodies.

In summary, our derived CHO FUT8$^{-/-}$ clone had the ability to produce defucosylated antibody with no detrimental effects in cell growth, viability, and the potential for industrial utilization. We demonstrated CRISPR/Cas9 application in CHO-K1 cells genome editing as well as its effect on product glycosylation alternation, which would be potentially useful in industrial purposes [35, 36]. To develop the potential of CRISPR/Cas9

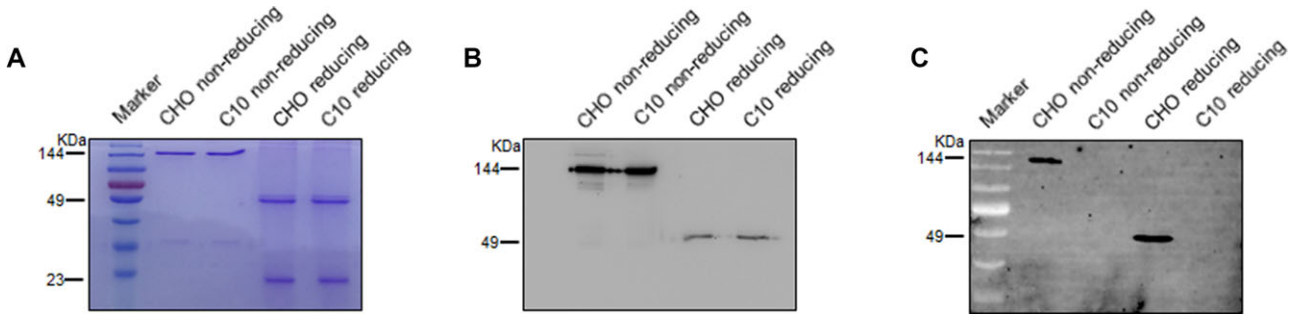


Figure 3. Biochemical analysis of IgG purified from C10 clone and CHO-K1 cells transfected with anti-CD20 mAb expression vectors. (A) SDS-PAGE and Coomassie brilliant blue staining of the purified IgG. (B) Western blot of the purified IgG. Anti-CD20 mAbs were detected by HRP-labeled goat antihuman Fc antibody and ECL. (C) The PVDF membrane was incubated with 10 μg/mL FITC-LCA, and subsequently detected using multifunctional laser scanner after washing three times under a 488 nm channel. Note that proteins were normalized to 500 ng/well for SDS-PAGE, 50 ng/well for lectin blot, and 5 ng/well for western blot.

application in bioprocess, other genes related to recombinant protein productivity or quality enhancement are currently being investigated in our labs. We believe that as a robust genome editing tool, CRISPR/Cas9 system can be widely applicable in cell line development for manufacturing high-quality mAbs or other biological therapeutics.

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The authors have declared no conflict of interest.

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