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Establishment of *DHFR*-deficient HEK293 cells for high yield of therapeutic glycoproteins

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Since the use of protein therapeutics is effective for treating intractable human diseases, the production of biologic therapeutic agents has dramatically increased over the past three decades. The Chinese hamster ovary (CHO) cell lines are the most commonly used host cell expression system for recombinant protein production. High productive and stable clonal cell lines for recombinant protein production have been established from the *DHFR*-deficient CHO cell using the dihydrofolate reductase/methotrexate (DHFR/MTX) selection methods. Human embryonic kidney 293 (HEK293) cells are alternative host cells widely used for protein production. In most case, however, the cells are used for the transient expression, and there is no gene amplification system in HEK293 cells. In this study, we established a *DHFR*-deficient HEK293 cell line for the high yield of recombinant proteins. We doubly knocked out *DHFR* and *DHFR2* in the *MAN1A1*/*A2*/*B1*/*C1*-quadruple knockout HEK293 (QD-KO) cells, using the CRISPR/Cas9 system. The *DHFR*-deficient QD-KO cells were used to overexpress two proteins, lysosomal acid lipase and the constant fragment of human immunoglobulin G_1 by the DHFR/MTX gene-amplification method. This method resulted in a dramatic increase in the two protein expressions in the *DHFR*-deficient QD-KO cells by increasing MTX concentration. Our system could be adopted in the production of several recombinant proteins including therapeutic proteins.

[**Key words:** HEK293 cells; Dihydrofolate reductase; Methotrexate; Hypoxanthine-thymidine; Protein expression; Biotherapeutics]

There has been a quantum rise in the production of biotherapeutics throughout the past three decades. The application of these therapeutic proteins in the clinical setting has also increased during these years (1). The use of therapeutic proteins has become an alternative transformative approach to treating human diseases. Several host cell expression systems have been developed for the production of therapeutic proteins. Depending on the proteins to be expressed, host cell expression systems derived from mammalian cells, non-mammalian cells, and transgenic animals are appropriately used (2). Among the mammalian cell expression systems are Chinese hamster ovary (CHO) cell lines, human embryonic kidney 293 (HEK293) cells, and mouse myeloma cells (NS0), to mention a few, have been widely used in the production of therapeutic proteins (2,3). Currently, 100 and more recombinant therapeutic proteins produced in mammalian cells have been approved by the United States Food and Drug Administration (FDA) (4). With about US$120 billion worldwide sales every year, the therapeutic protein production has become a lucrative field for both industry and academic research groups, to further come up with advance and better methods than previous ones (5).

Since mammalian cell lines have capacities to fold and assemble proteins and carry out human-like post-translational modifications, more than half of recombinant therapeutic proteins are produced in those cells (6). CHO cells are presently the most widely used cell expression system (7–9) for the production of most commercially available biotherapeutics. It is because the CHO cells are a well-characterized system of operation (10), easy to manipulate, have low transmission rate of human diseases due to species barrier, early availability of selective markers [dihydrofolate reductase (DHFR) or glutamine synthetase (GS)] and the long track record of products accepted by FDA (11). Also, the majority of human pathogens could not replicate in CHO cells, making them an excellent vehicle for human therapeutic protein production (12). One of the downsides of the production of recombinant proteins in CHO cells is the incorporation of non-human glycan moieties which instigates the recombinant protein to be immunogenic in the human body (13). Accurately, the addition of the non-human *N*-glycolylneuraminic acid (Neu5Gc) instead of *N*-acetylneuraminic (Neu5Ac) to glycans on proteins renders the proteins immunogenic (14).

The HEK293 cells are recognized as host cells mainly for their efficient transient production of therapeutic proteins at research laboratories (15). Like the CHO cell line, the HEK293 cells are easy to maintain, rapid in reproduction, and show high transfection efficiency and protein productivity. Several other HEK293 variants such as the HEK293T, HEK293S, HKB11 (a hybrid between HEK293S and human B cell line) have been developed to have additional features (e.g., suspension growth, growth under serum-free conditions, high protein yield) to that of the original cell line (16,17). Moreover, the HEK293 cells can produce intact proteins as compared with the CHO cell lines (18). Since HEK293 cells are human-derived cells, produced recombinant proteins possess the

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exact glycosylation patterns found in humans, eliminating the possibility of the immunogenicity (11). Currently, there are two commercially available biopharmaceutics, Eloctate (rh B-domain deleted factor VIII fused to a human IgG_1 Fc domain) and Alprolix (rh factor IX fused to a human IgG_1 Fc domain), which are produced in the HEK293 cell lines (19,20).

There are several selection systems applied alongside the different host cell lines for the production of protein therapeutics. The DHFR/methotrexate (MTX) selection is commonly used for multiple amplification to isolate high therapeutic and recombinant protein producing clones (21,22). DHFR is a reductase that converts dihydrofolate to tetrahydrofolate, an essential precursor in the *de novo* nucleotide synthesis pathway for cell proliferation and growth (Fig. 1) (23). When the *DHFR* gene is mutated or inhibited by MTX, the cells cannot proliferate without the supplement of hypoxanthine and thymidine (HT), which are utilized in the salvage pathway of nucleotide biosynthesis. Integration of plasmids having a gene of interest together with the *DHFR* gene into the genome of *DHFR*-deficient cells can be selected with the medium without HT (Fig. S1). By the increase of MTX concentration, the copy number of the *DHFR* gene together with the gene of interest is increased in the genome, resulting in higher gene expression. Even though this system is widely established in CHO cells, little is known about its presence in the human-host cell expression system. To our knowledge, *DHFR*-deficient HEK293 variant has still not been made for high yield of recombinant proteins.

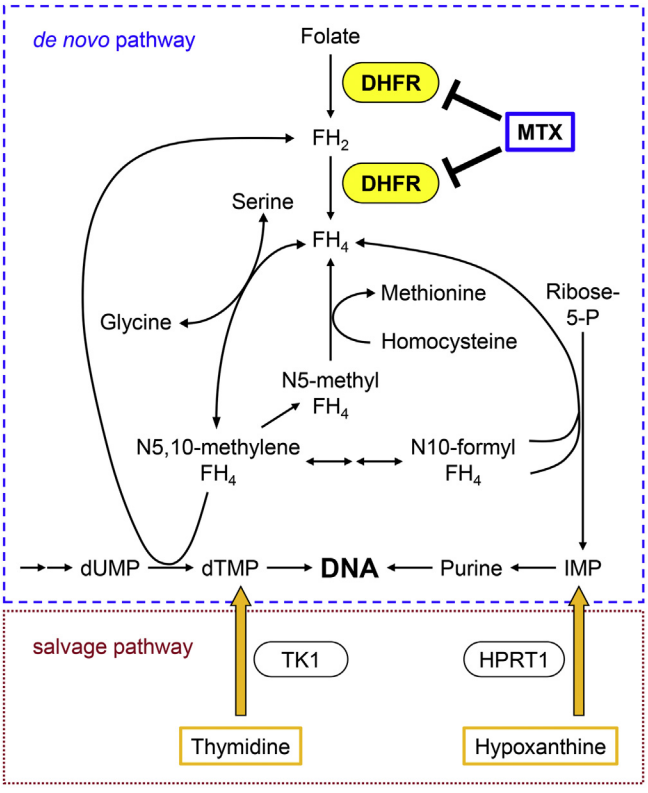

FIG. 1. Schematic view of the *de novo* and salvage nucleotide biosynthesis pathway. Dihydrofolate reductase (DHFR) converts folate to dihydrofolate (FH_2) and subsequently to tetrahydrofolate (FH_4) using NADPH as a proton donor in the *de novo* pathway of nucleotide biosynthesis. N5,10-methylene-FH_4 and N10-formyl-FH_4 are utilized to shuttle carbon in the dTMP and inosine monophosphate (IMP) formation, respectively. Methotrexate (MTX) inhibits DHFR activity to block the *de novo* nucleotide biosynthesis. Hypoxanthine and thymidine supplements are converted to dTMP and IMP by TK1 and HPRT1, respectively, for the salvage formation of nucleotides in the blocked *de novo* condition. dUMP, deoxyuridine monophosphate; Ribose-5-P, ribose-5-phosphate; DNA, deoxyribonucleic acid.

We previously reported the establishment of multiple mannosidase-I gene knockout (KO) cell lines (*MAN1A1/A2/B1* triple-KO), which can produce recombinant proteins with high-mannose-type N-glycans. In this study, we constructed a *DHFR*-deficient *MAN1A1/A2/B1/C1* quadruple-KO (QD-KO) HEK293 cell line for high production of therapeutic glycoproteins. *DHFR*-deficient cells were used as host cells for the overexpression of lysosomal acid lipase, LIPA and the constant fragment of human immunoglobulin G_1 (Fc-$hIgG_1$) as model proteins. Overexpression of *DHFR* rescued the growth defect in the *DHFR*-deficient QD-KO cells. Selection of these clones with the MTX enhanced the overexpression of DHFR, LIPA, and Fc-$hIgG_1$. The increase of protein overexpression in the clones was achieved by increasing the MTX concentration. This production system hence provides an insight into an alternative production system for recombinant protein in the industry.

MATERIALS AND METHODS

Cell lines, antibodies, and reagents The *MAN1A1/A2/B1/C1*-quadruple knockout (QD-KO) HEK293 cells were cultured in DMEM containing 10% Fetal bovine serum (FBS) (Biological Industries, Kibbutz Beit Haemek, Israel). The *DHFR*-deficient QD-KO cells were maintained in DMEM containing 10% FBS plus 1× HT supplement (H0137-1VL; Sigma–Aldrich, St. Louis, MO, USA). The appropriate antibiotic concentrations were used when necessary: hygromycin (400 μg/mL), puromycin (0.5 μg/mL), penicillin (100 U/mL)/streptomycin (100 U/mL). A rabbit polyclonal anti-DHFR antibody (15194-1-AP: Proteintech, Rosemont, IL, USA), a mouse monoclonal anti-FLAG antibody (HT201: TransGen Biotech, Beijing, China) and a mouse monoclonal anti-β-tubulin antibody (HC101: TransGen Biotech) were used as the primary antibodies. HRP-conjugated anti-mouse IgG (HS211-01; TransGen Biotech) and anti-rabbit IgG (HS101-01; TransGen Biotech) were used as the secondary antibodies. Methotrexate hydrate (MTX, A6770, 133073-73-1; Sigma–Aldrich) was used for drug treatment. Fluorescein-conjugated MTX (F-MTX) (M1198MP: Thermo Fisher Scientific, Waltham, MA, USA) was used to check the DHFR activity using flow cytometry. All the restriction enzymes except *Bbs*I (New England Biolabs, Ipswich, MA, USA) and *Eam1105*I (Thermo Fisher Scientific) were purchased from Takara (Shiga, Japan).

Plasmids The pX330-EGFP-DHFR-cr3,-cr4 and pX330-EGFP-DHFR2-cr1,-cr2 plasmids were constructed by restricting the plasmid pX330-EGFP with *Bbs*I restriction enzyme (24). The KO target sequences were designed using the E-CRISP website (http://www.e-crisp.org/E-CRISP/) and ligated into the *Bbs*I restriction site in the pX330-EGFP plasmid. All the KO oligonucleotides used are listed in the Table S1. To construct pME-Hyg-HPRT1 and pME-Puro-TK1, DNA fragments coding for *HPRT1* and *TK1* were amplified by PCR from the Invitrogen human cDNA clone plasmids containing *HPRTI* and *TKI* (Thermo Fisher Scientific), and ligated into the *EcoR*I and *Not*I restriction sites in the vectors pME-Hyg and pME-Puro (25), respectively. The pHEK293-Ultra-sHF-LIPA was constructed by amplifying the sHF-LIPA coding sequence from the pME-Hyg-sHF-LIPA (26) by PCR and ligated into the *Sma*I and *Sph*I restriction sites in the pHEK293-Ultra-expression-I vector (Takara) by infusion cloning technique. To construct pHEK293-Ultra-sHF-LIPA-mDhfr (Fig. S3) and pHEK293-Ultra-mDhfr, a DNA coding for mouse Dhfr (mDhfr) was amplified by PCR and ligated into the *Ssp*I restriction site in pHEK293-Ultra-sHF-LIPA and pHEK293-Ultra-expression-I vector by infusion cloning technique. The pME-Puro-sHF-Fc-$hIgG_1$(LD) was constructed by substituting the GPI gene in pME-Puro-sHF-GPI with the DNA coding the constant fragment of human IgG_1(LD) from the pME-CD85j-IgG_1(LD) using *Xho*I and *Not*I restriction enzymes. The pHEK293-Ultra-sHF-Fc-$hIgG_1$(LD)-mDhfr (Fig. S3) was constructed by amplifying the DNA fragment coding the Fc region from the pME-Puro-sHF-Fc-$hIgG_1$(LD) and was ligated into the *BamH*I and *Xba*I restriction sites in the pHEK293-Ultra-mDhfr plasmid. The plasmids pHEK293-Ultra-sHF-LIPA-mDhfr and pHEK293-Ultra-sHF-Fc-$hIgG_1$(LD)-mDhfr were digested by *Eam1105*I to obtain the linearized forms.

***DHFR/DHFR2* knockout in *MAN1A1/A2/B1/C1* QD-KO cells** The *MAN1A1/A2/B1/C1* QD-KO HEK293 cells were transiently transfected with pX330-EGFP having the respective guide RNA constructs. EGFP positive cells were sorted using a cell sorter S3e (Bio-Rad, Hercules, CA, USA). The sorted cells were cultured for >12 d and further subjected to limiting dilution to obtain clonal KO cells. The genes of the clonal KO cells were confirmed by PCR and sequencing. The DHFR protein level was checked by western blotting. A clonal *DHFR*-deficient QD-KO cell line AA (clone AA) and a *DHFR/DHFR2*-double-deficient QD-KO clonal cell line 53 (QK53) were named as QDd1-KO and QDd1d2-KO cell lines, respectively.

Measurement of cell growth The growth of the KO cells was measured with a cell counting-kit-8 (CCK8) (HY-K0301; MedChemExpress, Monmouth Junction, NJ, USA). Two thousand cells were seeded in each well of 96-well plates (in triplicates)

for each cell line along with a blank (medium without cells) and were cultured in 100 μL DMEM containing 10% FBS with or without HT supplement in the presence or absence of MTX over a 4 d period. A 10 μL of CCK8 solution was added to each well, and cells were incubated for 1 h. The absorbance of each well was analyzed by a microplate reader (Bio-Rad) at a wavelength of 450 nm. The cell number was extrapolated based on a standard curve.

Stable transfection of plasmids to DHFR-deficient cell lines The QDd1d2-KO cell lines were transfected with the plasmids pHEK293-sHF-LIPA-mDhfr or pHEK293-sHF-Fc-hIgG$_1$(LD)-mDhfr using PEI-MAX MW-25000 (23,966, Polyscience, Niles, IL, USA), driving the expression of exogenous mDhfr as a marker, and LIPA or Fc-hIgG$_1$ recombinant proteins as model proteins. After 1 d of transfection, the HT supplemented medium was withdrawn, and cells were cultured in a medium without HT at least for 14 d. QDd1d2-KO cells overexpressing the exogenous mDhfr together with LIPA or Fc-hIgG$_1$ were subjected to MTX treatment at different concentrations. Cells were cultured for 14 d in DMEM/10% FBS with 5 nM MTX as the initial concentration (27). The concentration was subsequently increased to 50 nM, and 500 nM in a stepwise fashion. The medium was replaced with a fresh MTX-containing medium after every 3 d of culturing.

The plasmids, pME-Hyg-HPRT1, and pME-Puro-TK1 were stably transfected into QDd1d2-KO cells. After 1 d of transfection, the cells were selected in medium containing hygromycin (400 μg/mL) and puromycin (0.5 μg/mL) for >14 d. The cell growth in a 10% FBS DMEM medium with 1× HT supplement was measured using the CCK8 kit.

Western blotting To detect the expression of recombinant sHF-LIPA and sHF-Fc-hIgG$_1$ proteins, cells (5×10^5) were plated and cultured in 2.5 mL of medium without MTX. After 72 h, 1.4 mL medium was collected. The medium was centrifuged at 10,000 ×*g* for 5 min, and 1 mL of the supernatant was collected in a new tube. Afterward, 20 μL of prewashed anti-FLAG beads (A2220: M2 affinity gel, Sigma–Aldrich) was added. The samples were rotated for 2 h at 4°C and were then washed for 3 times with cold PBS. The sHF-LIPA and sHF-Fc-hIgG$_1$ were eluted with 50 μL of FLAG peptide (500 μg/mL). Eluted proteins (40 μL) were denatured by Laemmli sample buffer at 95°C for 5 min.

For the protein detection in cell lysates, about 7×10^5 cells were harvested with the trypsin/EDTA and washed using cold PBS at 4°C. A 50 μL lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, a protein inhibitor, 1 mM PMSF) was added to the cell pellets, and the mixture was incubated for 30 min on ice. The tube was centrifuged at 10,000 ×*g* for 15 min at 4°C. A 40 μL supernatant was collected, and a 10 μL sample buffer was added, with the mixture boiled at 95°C for 5 min. A mouse monoclonal anti-FLAG antibody (5000-fold dilution) and HRP-conjugated goat anti-mouse IgG (5000-fold dilution) were used as the primary and secondary antibodies respectively, for the detection of FLAG-tagged proteins. A rabbit polyclonal anti-DHFR antibody and a mouse monoclonal anti-β-tubulin antibody were also used as primary antibodies to detect DHFR and β-tubulin, respectively.

Quantification of volumetric protein productivity Cells (5×10^5) from 500 nM MTX pools of QDd1d2-KO overexpressing LIPA or Fc-hIgG$_1$ were cultured in a DMEM plus 1% FBS in a 6-well plate in triplicate for 5 d. After the 5 d culture, 1.4 mL of medium was collected from the cultures of QDd1d2-KO overexpressing LIPA or Fc-hIgG$_1$. The media were centrifuged at 10,000 ×*g* for 5 min, and 1 mL of the supernatant was collected in a new tube. A 10 μL of the medium from QDd1d2-KO 500 nM MTX pools expressing LIPA or Fc-hIgG$_1$ plus a 2.5 μL sample buffer was loaded on a 12% SDS-PAGE in triplicates. The purified His-Flag-green fluorescence protein-tagged CD59 (HF-GFP-CD59), which was expressed in *E. coli*, was used as a standard for the SDS-PAGE/western blotting. A standard curve was drawn based on the band intensities from the standard protein. The volumetric protein productivity of LIPA and Fc-hIgG$_1$ was extrapolated.

RESULTS

Generation of *DHFR/DHFR2* double-KO in *MAN1A1/A2/B1/C1* quadruple-KO HEK293 cells We previously constructed multiple α-mannosidase-I gene KO cells (*MAN1A1/A2/B1*-triple KO (T-KO)), in which the majority of N-glycan structures became high-mannose-type (26). To remove the hybrid-type and complex-type N-glycan structures completely, we further knocked out *MAN1C1*, which encodes another Golgi α-mannosidase-I, in T-KO, generating *MAN1A1/A2/B1/C1* quadruple-KO (QD-KO) cells (Ren et al., manuscript submitted). Those cells would be useful to produce recombinant proteins only having high-mannose-type N-glycan structures. To increase the protein productivity, we tried to introduce the DHFR/MTX selection system into the QD-KO HEK293 cells. In the human genome, there are two genes encoding dihydrofolate reductase, *DHFR*, and *DHFR2* (28). In HEK293 cells, both DHFR and DHFR2 are expressed (transcripts per million (TPM) value was 121 for *DHFR* and 1.6 for *DHFR2*, based on data in the Human Protein Atlas (https://www.proteinatlas.org/). Since it would be necessary to knockout the endogenous *DHFR* genes completely, we first knocked out both *DHFR* and *DHFR2* in QD-KO cells.

To knockout *DHFR* gene, two KO targets cr3 and cr4 were designed and used to instigate a double strand break at the *DHFR* target region in the genome (Fig. 2A). After transfection of two KO target plasmids, cells with transfected plasmids were enriched, cultured and limiting diluted to obtain clonal cells. The *DHFR*-KO target region on the genome was analyzed in the clonal cells. We selected one clone, named QDd1-KO, in which *DHFR*-KO target region was removed out from the genome (Fig. 2B). Compared with 537 bp in the wild-type HEK293 cells, there was a reduction in band size. The sequencing result showed that deletion of 195 bp including the exon 2 for the *DHFR* gene occurred as expected and an insertion of a single base.

We next knocked out *DHFR2* gene in the QDd1-KO isolated clonal cells. Two KO targets (cr1 and cr2) was designed (Fig. 2A) as same as targets for *DHFR*. Compared to the knockout of *DHFR*, we could not obtain the clear deletion of *DHFR2* at the expected site. After the selection, one clone 53, named QDd1d2-KO, having mutations in *DHFR2* was isolated. The region around the KO targets in the genome was analyzed. Compared with the WT band, the QDd1d2-KO had two bands (Fig. 2C). The lower band arose from the single base insertion at both target 1 (cr1) and target 2 (cr2) resulting in a point mutation of the *DHFR2* gene. The upper band represented a 191 bp insertion at the cleavage from target 1 (cr1) and a single base deletion at the target 2 (cr2). The target 2 (cr2) is located in the exon 2 on the *DHFR2* coding sequence, hence the 1-bp indel mutations at the target 2 cause frameshifts of the *DHFR2* gene. We concluded that both *DHFR* and *DHFR2* were successfully knocked out in QDd1d2-KO cells. Western blotting was carried out to further confirm the complete KO of *DHFR* in QDd1-KO and QDd1d2-KO cells (Fig. 2D). No band was detected for DHFR in the samples prepared from QDd1-KO and QDd1d2-KO cells, suggesting the complete KO of the *DHFR* gene.

To confirm whether endogenous dihydrofolate reductase activity is diminished in QDd1-KO and QDd1d2-KO cells, we used the fluorescein-conjugated MTX (F-MTX), which is used to ascertain the cellular DHFR activity (Fig. 2E). Once F-MTX is incorporated into the cells, it is bound to DHFR and retained in the cells. If there is no DHFR activity in the cells, F-MTX cannot be kept in the cells. When the QD-KO cells were treated with the F-MTX, the fluorescence intensity of the cells was detected by flow cytometric analysis. In contrast, the fluorescence intensity of F-MTX was diminished in both QDd1-KO and QDd1d2-KO cells, indicating that DHFR is disrupted (Fig. 2E). It is noted that there was no significant difference between the QDd1-KO and QDd1d2-KO cells. It would be because DHFR is responsible for the major activity of cellular dihydrofolate reductase activity in the cells (28).

HT supplement supports the growth of *DHFR*-deficient *MAN1A1/A2/B1/C1* QD-KO cells As described above, the HT supplement is vital for the growth of the cells lacking *DHFR* genes (29). To validate the loss of functional *DHFR* expression, we checked the growth and sensitivity of QDd1-KO, and QDd1d2-KO cells along with the parental strain in DMEM/10% FBS supplemented with or without HT (Fig. 3A, B). Although the growth of *DHFR*-deficient QDd1-KO and QDd1d2-KO cells decreased compared to the parental QD-KO and WT HEK293 cells, the QDd1-KO, and QDd1d2-KO cells still continuously grew in the presence of HT. In contrast, the growth of those two cells was significantly reduced in a medium without HT as compared with the parental QD-KO and WT HEK293 cells.

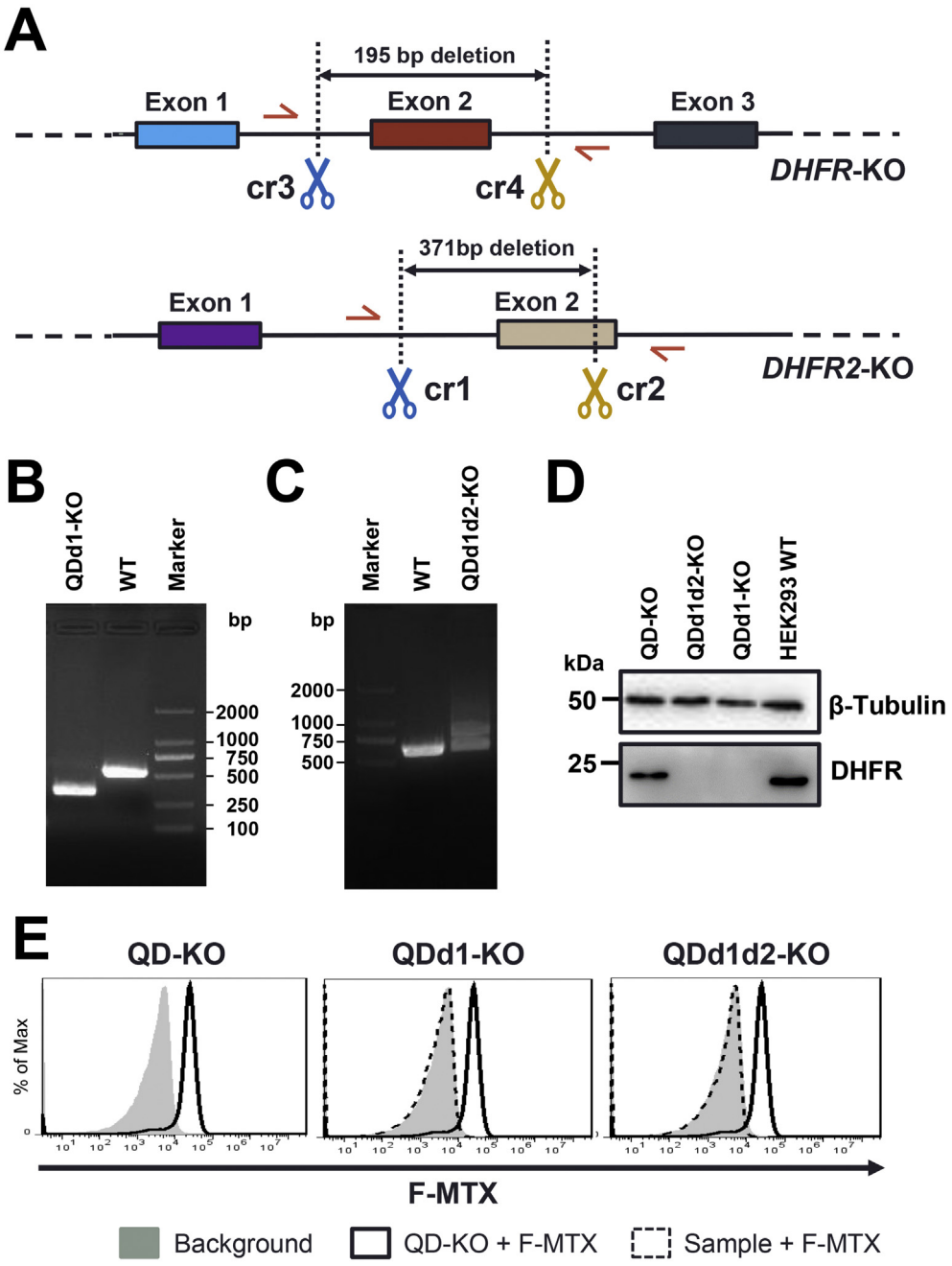

FIG. 2. Generation of *DHFR* and *DHFR2* knockout in QD-KO HEK293 cell line. (A) Schematic representation of *DHFR* and *DHFR2* knockout. The black line shows the genome of *DHFR* and *DHFR2*. Knockout targets cr3 and cr4 were designed to cause a 195 bp deletion including the Exon 2 of the *DHFR* gene in QD-KO HEK293 cells. Similarly, KO targets cr1 and cr2 were designed to delete 371bp including a part of Exon 2 of *DHFR2* gene in QDd1-KO cells. The red arrows represent the primer pairs used for the KO analysis by PCR. (B) After the knockout of *DHFR* in QD-KO HEK293 cells, QDd1-KO cell line was obtained, and the genomic DNA extracted, as well as that of HEK293 (WT) and were analyzed by PCR and agarose gel electrophoresis. Compared with the WT, the KO band for QDd1-KO was decreased. The sequencing result is shown in Fig. S2. (C) After the knockout of *DHFR2* in QDd1-KO, the region around the *DHFR2*-KO targets in clone QDd1d2-KO was amplified and analyzed by agarose gel electrophoresis. Two *DHFR2*-KO patterns were obtained, and both were confirmed by the sequencing result to have mutations in the *DHFR2* gene. Sequencing results are included in Fig. S2. (D) Cell lysates were prepared from parental QD-KO, wild-type (WT) HEK293, QDd1-KO and QDd1d2-KO cells, and DHFR was detected by western blotting. β-Tubulin was used as a loading control. (E) Analysis of DHFR activity using fluorescein-MTX (F-MTX). Cells were cultured overnight at 37°C in the presence of 1 μM fluorescein-MTX. Cells were harvested with Trypsin/EDTA and washed and resuspended in cold PBS for analysis by flow cytometry. The fluorescence intensity of QDd1-KO and QDd1d2-KO significantly decreased to the background level compared with the fluorescence intensity of the QD-KO.

When dialyzed FBS was used as the serum, QDd1-KO and QDd1d2-KO cells could not grow at all (Fig. 3C), suggesting the strict requirement of HT for the growth of *DHFR*-deficient *MAN1A1/A2/B1/C1* QD-KO cells.

Once HT are incorporated into cells, they are used for the salvage pathway of nucleotide synthesis. The hypoxanthine phosphoribosyltransferase (HPRT1) and thymidine kinase (TK1) are significant players in the salvage pathway (Fig. 1) (30,31). Since the growth of QDd1-KO and QDd1d2-KO cells was slower than parental QD-KO even in the presence of HT supplement, we hypothesized that slow growth of the *DHFR*-deficient QD-KO cells could be as a result of inefficient salvage pathway. To check the hypothesis, the effect of the overexpression of *HPRT1* and *TK1* in the QDd1-KO and QDd1d2-KO cells was analyzed. However, no significant growth

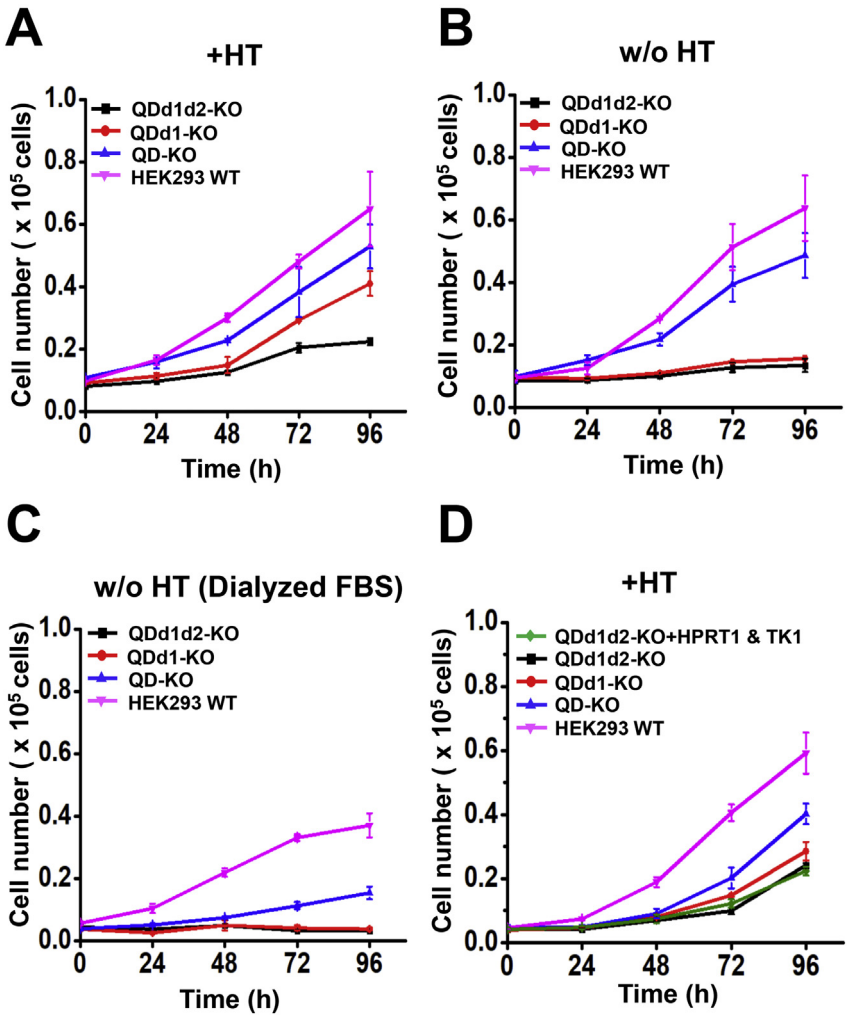

FIG. 3. Cell growth of DHFR-deficient QD-KO cells with or without HT supplement. (A, B) HT supplement supports the growth of *DHFR*-deficient QD-KO HEK293 cells. The growth of QDd1d2-KO and QDd1-KO cells was analyzed in the presence or absence of HT along with the QD-KO and parental cells. All the values shown are means ±SE of triplicate determinants. (C) The cell growth of QDd1d2-KO, QDd1-KO, and the parental cell lines were examined in DMEM containing 10% dialyzed FBS without HT over 4 d. (D) The growth of cells in panel A and QDd1d2-KO cells stably expressing HPRT1 and TK1 was analyzed in an HT supplemented medium over 4 d.

enhancement in the QDd1d2-KO cells overexpressing both *HPRTI* and *TKI* was observed compared with the original cells (Fig. 3D).

Transfection of a *Dhfr* containing vector rescues the growth of the *DHFR*-deficient *MAN1A1/A2/B1/C1* QD-KO cells To analyze whether the growth defects of QDd1d2-KO cells in a medium without HT supplement are due to defects in *DHFR* genes, the QDd1d2-KO cells were transiently transfected with the vector harboring the mouse *Dhfr* (*mDhfr*) gene along with a gene encoding lysosomal acid lipase (LIPA) (Fig. S3). The introduction of the *Dhfr*-containing plasmid significantly restored the growth defects of the QDd1d2-KO cells to the level of the parental QD-KO cells (Fig. 4A). This rescue of the slow growth phenotype strongly confirms the essence of the DHFR function in the growth of *DHFR*-deficient QD-KO cells.

DHFR/MTX-based selection and amplification of transgene expression in *DHFR*-deficient *MAN1A1/A2/B1/C1* QD-KO cells To analyze the capacity of the QDd1d2-KO cells to support the DHFR/MTX-based selection, we first checked the sensitivity of QD-KO HEK293 cells against MTX. The cell number of QD-KO and QDd1d2-KO cultured for 4 d in a medium with or without HT supplement was analyzed (Fig. 4B, C). The QDd1d2-KO cells showed slow growth in the presence of HT supplement compared to the QD-KO cells. On the other hand, QDd1d2-KO cells showed resistance to MTX (Fig. 4B). Even in the medium containing 2000 nM MTX, QDd1d2-KO cells could grow in the presence of HT supplement. The absence of DHFR rendered the MTX retained less in the cells for long since MTX has a high affinity for DHFR (32). QDd1d2-KO cells had minimal growth in the −HT medium because of the strict requirement of HT for growth. The parental QD-KO cells could grow in both the presence and absence of HT supplement. However, the QD-KO cells showed sensitivity to MTX in a dose-dependent manner in both medium with or without HT (Fig. 4B, C), suggesting that the QD-KO cells mainly utilize the *de novo* nucleotide synthetic pathway. The DHFR present in these cells tightly bound to the MTX to retain it in the cells to elicit its cytotoxic action of cell killing.

Next, the DHFR/MTX gene amplification was applied to overexpress two recombinant proteins, LIPA and the constant fragment of human immunoglobulin G_1 (Fc-hIgG1) in QDd1d2-KO cells. After the QDd1d2-KO cells were transfected with the linearized plasmid containing *mDhfr* and *LIPA*, cells were cultured in a medium without HT supplement. Cells were further cultured in a medium without HT and with MTX. MTX concentration was gradually increased from 5 nM to 500 nM when the cell number increased. The expression level of Dhfr in the corresponding gene-amplified

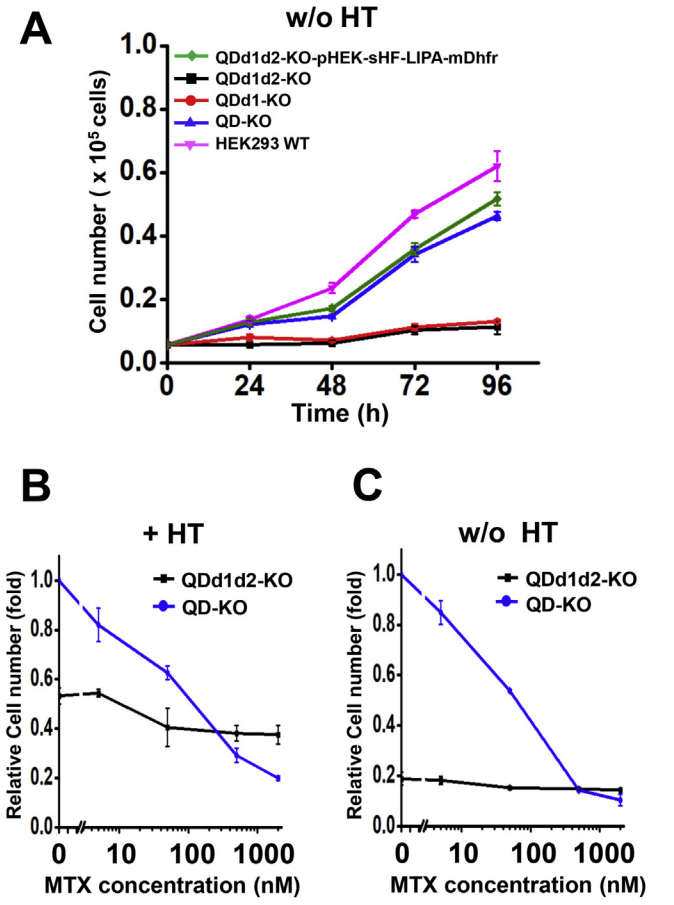

FIG. 4. Restoration of growth by DHFR expression and MTX sensitivity. (A) The QDd1d2-KO cells transiently transfected with pHEK293-sHF-LIPA-mDhfr rescues the growth defects in a medium without HT. The transiently transfected QDd1d2-KO cells along with the original and the parental cell line were analyzed for their growth in DMEM/10% FBS medium without HT for 4 d. Values shown are means ± SE of triplicate determinants. (B, C) MTX sensitivity of QD-KO and QDd1d2-KO cells. The sensitivity of QD-KO and QDd1d2-KO in a medium with HT (B) or without HT (C) at MTX concentrations of 5–2000 nM over 4 d culture was analyzed. The cell number of QD-KO cells cultured in a medium with HT (B) or without HT (C) in the absence of MTX was set as 1. Relative cell number was calculated. Values shown are means ± SE of triplicate determinants.

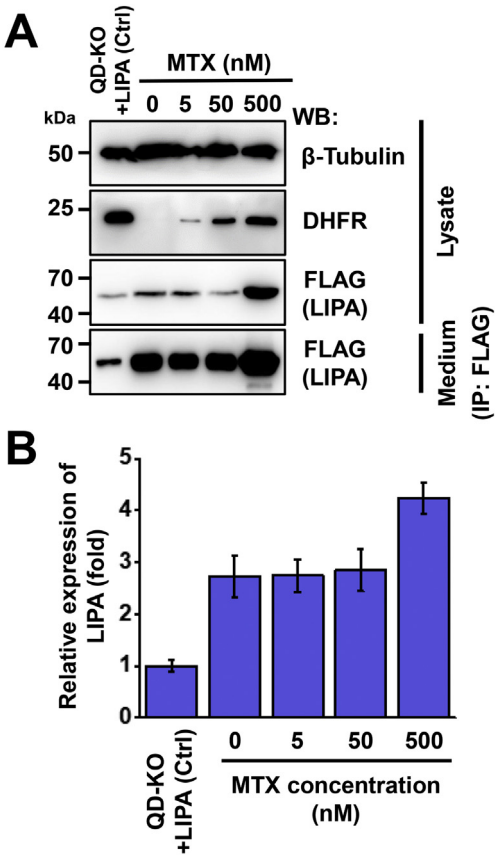

FIG. 5. Overexpression of LIPA and Fc-hIgG$_1$ in QDd1d2-KO using DHFR/MTX system. (A) Overexpression of LIPA and mDhfr by increasing MTX concentration. The QDd1d2-KO cells transfected with pHEK293-Ultra-sHF-LIPA-mDhfr were selected with MTX. The MTX concentration was gradually increased from 0 to 500 nM. The selected cells were cultured in a -HT medium for 3 d. DHFR and LIPA in the cell lysates were detected. β-Tubulin was used as a loading control. The LIPA secreted in the medium was immunoprecipitated by anti-FLAG beads and eluted by FLAG peptides. The precipitated proteins were analyzed for LIPA using anti-FLAG antibody. Cell and medium from QD-KO stably expressing LIPA selected by hygromycin (QD-KO+LIPA) were analyzed as a control. WB, western blotting; IP, immunoprecipitation. (B) The amount of secreted LIPA from the control cells was set as 1. The relative expression of LIPA from the IP of two separate experiments was plotted. The error bars shown are means ± error of two IP results.

pools increased sequentially with stepwise increases in MTX concentration (Fig. 5A). Although there was no significant difference in the LIPA expression level for the 0 nM–50 nM amplified pools both in the medium and the cell lysate, there was a dramatic increase in the LIPA expression level at 500 nM MTX concentration (Fig. 5B), which agrees with the underlying principle of yielding high protein expression with high MTX concentration.

Similarly, the expression levels of both the Dhfr and the Fc-hIgG1 sequentially increased with increasing MTX concentration of the amplification pools, with the highest Fc-hIgG1 expression at 500 nM MTX concentration (Fig. 6A). As the control, we used QD-KO cells that were selected with conventional antibiotics to express recombinant LIPA and Fc-hIgG$_1$. The expression levels of LIPA and Fc-hIgG$_1$ in MTX selected cells were higher than the control (Figs. 5B and 6B). These results indicate the capacity of the QDd1d2-KO cells to overexpress different recombinant proteins using the DHFR/MTX gene amplification and show the high efficiency of the DHFR/MTX-base gene amplification system.

We next analyzed the volumetric productivity of LIPA and Fc-hIgG1 from the QDd1d2-KO using the DHFR/MTX gene amplification. The productivity of LIPA and Fc-hIgG1 was 443 ± 6 mg/L and 700 ± 67 mg/L (mean ± standard error of triplicate experiments) for 5 d culture. To check the stability of the protein expression using the DHFR/MTX system, we compared the expression of recombinant proteins from cells selected and cultured with MTX (500 nM) over 60 d and those selected with conventional methods. The protein expression of both LIPA and Fc-hIgG1 produced by DHFR/MTX system was higher than that by conventional methods (Fig. S4), suggesting that the protein expression using DHFR/MTX systems in QDd1d2-KO cells is stable and could maintain high productivity over a long period.

DISCUSSION

There is a constant emergence of improved alternative production systems to meet the growing recombinant protein production field. CHO cells are widely utilized for the production of most recombinant biotherapeutics as well as other proteins (4,8,12). Several underlying mechanisms of operation in these host cells have been implemented including the most frequently used DHFR/MTX-based gene amplification system. This efficient system is usually used in the DHFR-negative CHO cells for stable gene amplification and high production of recombinant proteins in the

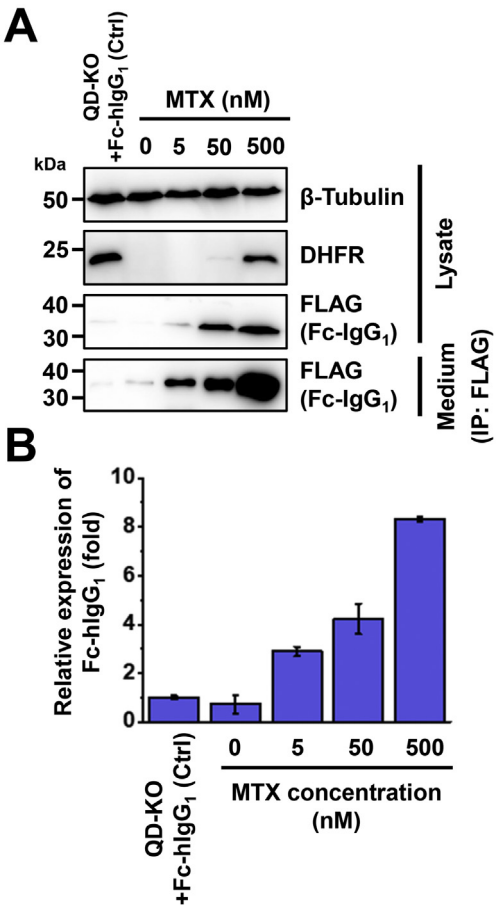

FIG. 6. Overexpression of Fc-hIgG$_1$ in QDd1d2-KO using DHFR/MTX system. (A) Overexpression of Fc-hIgG$_1$ and mDhfr by increasing MTX concentration. The QDd1d2-KO cells transfected with pHEK293-Ultra-sHF-Fc-hIgG1-mDhfr were selected with MTX as described in Fig. 5. DHFR, Fc-hIgG$_1$, and β-tubulin in the cell lysates and Fc-hIgG$_1$ secreted to the medium were detected. (B) The relative expression of Fc-hIgG$_1$ from the IP of two separate experiments was plotted as same as Fig. 5. The error bars shown are means ± error of two IP results.

industries and laboratories. However, some human recombinant proteins produced in the CHO cells tend to be immunogenic when introduced in the human body (33). Also, the genetic instability of the CHO cells makes them problematic. Yet, CHO cells remain the most currently used host cells for the production of recombinant proteins for its numerous benefits.

So far, several studies employing either the DHFR/MTX- or glutamine synthetase/methionine sulfoximine (GS/MSX) -mediated gene amplification for foreign gene amplification in the HEK293 cell variants have been previously reported (34–36). In these studies, however, the DHFR/MTX gene amplification was performed in a HEK293 cell in the presence of intact endogenous *DHFR* genes (34). The gene amplification was hindered by the action of the endogenous DHFR. Similarly, there was an obstruction of the GS/MSX gene amplification system by the highly functional endogenous GS in HEK293 cells (36). These studies, therefore, support the usefulness of the knockout of endogenous *DHFR* or *GS* in HEK293 cells for the implementation of either the DHFR/MTX or GS/MSX gene amplification system. This study successfully established a *DHFR*-deficient HEK293 cell for the overexpression of recombinant proteins using the DHFR/MTX gene amplification method. To our knowledge, it is the first time to construct the system in HEK293 cells.

In this study, we expressed two different types of proteins, LIPA and Fc-hIgG$_1$ in the QDd1d2-KO HEK293 cells using DHFR/MTX selection. The protein amounts of LIPA and Fc-hIgG$_1$ expressed with the MTX selection was 3.2-fold and 7.3-fold higher than those used by a conventional method selected with antibiotics, respectively. The increase in protein overexpression correlated to the expression levels of mDhfr, which increased upon the MTX concentration. The overexpression of the proteins was stable at least for over 60 d in the presence of MTX with high volumetric protein productivity. The phenomena indicate that the DHFR/MTX gene amplification system can be adopted in HEK293 cells similar to CHO cells.

Stable overexpression of the HPRT1 and TK1 in the *DHFR*-deficient cells failed to enhance the utilization of HT supplement. The slow growth of QDd1-KO and QDd1d2-KO cells in the presence of HT is not due to the conversion by those enzymes. One possible explanation is that glycine might not be sufficient, although DMEM media that were used in HEK293 cell culture contained glycine. When DHFR is disrupted, glycine cannot be produced in the cell and it is essential for cell growth in addition to HT. Another possibility is that, uptake of HT from outside to the cells is weakened in HEK293 cells. Overexpression of nucleoside transporters such as SLC29A2, which mediates transport HT at the plasma membrane, would be one of the targets to improve the growth of the *DHFR*-deficient HEK293 cells in a medium with HT supplement in the future analysis.

In this study, *MAN1A1*/*A2*/*B1*/*C1* QD-KO HEK293 cell line was used as the parental strain. It is because the cell line can produce glycoproteins containing high-mannose-type N-glycans like the *MAN1A1*/*A2*/*B1* T-KO cell line as we previously reported (26). In the QD-KO cells, N-glycan structures on recombinant proteins were changed from heterogeneous mixtures of complex-type, hybrid-type and high-mannose-type to only high-mannose-type. The property is useful to express some therapeutic proteins such as lysosomal enzymes with homogenous high-mannose-type N-glycans. Recombinant lysosomal enzymes are used in enzyme replacement therapy for lysosomal storage diseases. Intravenously injected proteins are incorporated into the cells through the mannose receptors and mannose-6-phosphate receptors on the cell surface. Establishment of the DHFR/MTX system in the *MAN1A1*/*A2*/*B1*/*C1* QD-KO cells will maximize the production of homogenous glycoproteins like the lysosomal proteins, leading to cut down the cost of lysosomal enzymes for enzyme replacement therapy when applied in the industry.

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