CRISPR-Cas13d for CHO Cell Engineering and Antibody Production

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Abstract

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Running Title: CRISPR-Cas13d for CHO engineering

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Abstract

Chinese hamster ovary (CHO) cell is the predominant cell factory to produce biopharmaceuticals such as immunoglobulin G (IgG), but in CHO cells lactate accumulation and cell death compromise IgG production while fucosylation mitigates the antibody-dependent cellular cytotoxicity (ADCC) of IgG. To tackle these problems, we harnessed a burgeoning gene silencing system, CRISPR-Cas13d, to effectively suppress the endogenous genes governing lactate production (LDHA), fucosylation (GFT) and cell death (DDIT3), hence repressing lactate accumulation and core fucosylation. We further exploited the *Sleeping Beauty*system to integrate the CRISPR-Cas13d module co-targeting these 3 genes and generated a CHO cell platform for IgG production. The new platform exhibited simultaneous knockdown of LDHA, GFT and DDIT3, accumulated less lactate, had prolonged longevity, produced more IgG with less fucosylation and stronger ADCC efficacy. These data collectively warrant the potentials of CRISPR-Cas13d for CHO cell engineering and improving antibody production with regard to quantity and quality.

Keywords: antibody production, Cas13d, CRISPR, CHO, gene knockdown

Introduction

Monoclonal antibody (mAb) is the predominant type of biopharmaceutical anti-cancer drug (*1*). Most mAb in clinical use are immunoglobulin G (IgG) whose glycosylation pattern dictates the efficacy and safety of IgG as an anti-cancer drug. Such requirements for glycosylation render Chinese hamster ovary (CHO) cell the predominant cell chassis for IgG production (*2*). During the CHO cell culture and IgG production, however, lactate is produced and accumulates, thus negatively impacting on protein production (*3, 4*). Meanwhile, massive production of misfolded or unfolded IgG provokes endoplasmic reticulum (ER) stress and hence triggers CHO cell death (*2*). Conversely, antibody-dependent cellular cytotoxicity (ADCC) is a key mechanism for antibody-mediated killing of cancer cells (*5*) and requires interactions between the fragment crystallizable (Fc) region of IgG and FcγIIIa receptors on immune cells (e.g. natural killer cells). Fucosylation of IgG at the Fc region hinders the interactions and compromises ADCC activity (*6*). However, recombinant IgG produced from CHO cells are heavily fucosylated (*7*). Removal of fucose from these oligosaccharides enhances ADCC of antibodies such as Herceptin?(*8*).

LDHA encodes the subunit A of lactate dehydrogenase which converts pyruvate to lactate, resulting in lactate accumulation along CHO cell culture and reducing recombinant protein yield (*9*). Fucosylation requires the transport of GDP-fucose into Golgi apparatus by GDP-fucose transporter (GFT) (*6*). Damage-inducible transcript 3 (DDIT3) encodes C/EBP homologous protein and is induced by ER stress, hence triggering cell apoptosis (*10*). As such, inhibiting endogenous expression of LDHA, GFT and DDIT3 may offer a new strategy to improve antibody production in CHO cells.

CRISPR is the innate immune system in prokaryotic cells to defend against invading nucleic acids, and has been repurposed for DNA and RNA editing (*11, 12*). Type II CRISPR system comprising Cas9 and guide RNA (gRNA) has been leveraged to target sequence-specific DNA for genome engineering in CHO cells (*13, 14*). Recently, type VI CRISPR system encompassing such effectors as Cas13a, Cas13b and Cas13d (*15-18*) was also discovered. These effectors coordinate with the single CRISPR RNA (crRNA) to target specific single stranded RNA (ssRNA) for cleavage (*19*) and have been harnessed for gene knockdown (*15-18*). Among these effectors, Cas13d from *Ruminococcus flavefaciens* strain XPD3002 is the most effective for gene silencing (*18*) and can process crRNA and ssRNA. Cas13d binds to precursor crRNA transcripts and cleaves them to produce mature crRNA composed of a 5' direct repeat and a 3' spacer that targets the ssRNA. Cas13d associates with crRNA and specifically binds the ssRNA with sequence complementary to the spacer, leading to Cas13d-mediated ssRNA cleavage (*20*). Ectopic co-expression of Cas13d and the engineered mature crRNA (denoted as gRNA) knocks down target mRNA with high efficiency and high specificity without significant off-target effects in HEK293 cells (*18*). Very recently, CRISPR-Cas13d-mediated gene downregulation was exploited for neural cell fate manipulation (*21*), liver metabolism regulation (*22*) and neovascularization prevention (*23*) in mice.

Due to the roles of LDHA, GFT and DDIT3, we hypothesized that simultaneous inhibition of these 3 genes can prolong the cell longevity along CHO cell culture and improve the antibody production. We chose to

explore CRISPR-Cas13d to knockdown these 3 genes, individually or in combination. Although CRISPR-Cas13d has been exploited in other mammalian cells, whether CRISPR-Cas13d can efficiently and stably suppress endogenous gene expression and alter the CHO cell performance remains to be explored. In this study we demonstrated that CRISPR-Cas13d was able to efficiently knockdown these 3 genes. We further showed that CRISPR-Cas13d-mediated silencing altered the CHO phenotype, as well as augmented the antibody production with regard to quantity and quality.

Results

To explore the feasibility of using CRISPR-Cas13d to silence LDHA, GFT and DDIT3 in CHO cells, we first tested the gRNA design and constructed a series of pCas13d-gRNA plasmids that co-expressed Cas13d and one gRNA for each gene (Fig. 1A). The gRNA was composed of the 5' direct repeat and 3' spacer that targeted different sites on the coding sequences (Fig. 1B). We predicted 3 sites with the highest targeting scores on the transcripts of each gene (Fig. S1) and designed 3 gRNA for LDHA and DDIT3 (Figs. 1B-1C). Yet the 3 predicted target sites for GFT were in close proximity (Fig. S1), thus we designed only one gRNA for GFT. As a control, we also constructed pCas13d-gRNA$_{\emptyset}$ that expressed Cas13d and the scramble gRNA$_{\emptyset}$ consisting of the direct repeat only (Fig. 1B).

We transfected the plasmids separately into CHO cells and analyzed the gene expression at 2 days post-transfection. Compared with gRNA$_{\emptyset}$, the 3 gRNA for LDHA (gRNA$_{\text{LDHA-g1}}$to gRNA$_{\text{LDHA-g3}}$) significantly repressed LDHA expression, with gRNA$_{\text{LDHA-g1}}$ most efficiently knocking down the mRNA level to [?]12% (Fig. 1D). The gRNA targeting GFT also enabled effective knockdown of GFT to 27% (Fig. 1E). For DDIT3, only gRNA$_{\text{DDIT3-g3}}$ suppressed the expression to 72% while gRNA$_{\text{DDIT3-g1}}$ and gRNA$_{\text{DDIT3-g2}}$ failed to knock down DDIT3 (Fig. 1F), thus underscoring the importance of gRNA design. These data demonstrated the feasibility of CRISPR-Cas13d for gene knockdown in CHO cells. The gRNAs conferring the most effective knockdown were selected for subsequent experiments.

Chromosomal integration of CRISPR-Cas13d module conferred stable gene knockdown and altered CHO cell phenotype

We next aimed to integrate the entire CRISPR-Cas13d system into the chromosome to generate stable cells. CRISPR-Cas13d system comprises Cas13d, gRNA and the selectable marker. However, pCas13d-gRNA plasmids (Fig. 1) did not guarantee the co-integration of Cas13d and gRNA with the selectable marker, which could result in a heterogeneous population of cells with incomplete CRISPR-Cas13d module. To ensure the co-integration of Cas13d and gRNA, we exploited the *Sleeping Beauty* (SB) system, which uses the transposase SB100X to transpose the gene cassette flanked by IR/DR (inverted repeat/direct repeat) sequences into the chromosome (*24*). We constructed pIS-gRNA plasmids (Fig. 2A) that expressed the codon-optimized SB100X and CRISPR-Cas13d module (gRNA, Cas13d and Zeocin resistance gene (Zeo$^{\text{R}}$) that were linked by a P2A sequence). The CRISPR-Cas13d module was flanked by a pair of IR/DR sequences to ensure co-integration of the system. The plasmids expressing the scramble gRNA$_{\text{O}}$ (pIS-O) or gRNA targeting LDHA (pIS-LDHA), GFT (pIS-GFT) or DDIT3 (pIS-DDIT3) were separately transfected into CHO cells, followed by selection using Zeocin for 7 passages to generate stable cells.

Compared with the control pIS-O, pIS-LDHA significantly knocked down LDHA expression to 55% (Fig. 2B) and significantly ($p<0.05$) reduced lactate production (Fig. 2C). Meanwhile, pIS-GFT significantly repressed GFT expression to 47% (Fig. 2D). To attest whether GFT knockdown reduced fucosylation, we performed *Lens culinaris*agglutinin (LCA) assay because LCA lectin preferentially binds to the core fucose of N-glycans and causes cell death if the cell is expressing core-fucosylated proteins (*25, 26*). As shown in Fig. 2E, pIS-GFT gave rise to significantly ($p<0.05$) higher viability than pIS-O at high LCA concentrations, indicating that GFT knockdown repressed core fucosylation and enhanced the cell tolerance to LCA. Moreover, pIS-DDIT3 significantly repressed the DDIT3 mRNA levels to 22% (Fig. 2F) and reduced the DDIT3 protein levels at 2 and 6 days after subculture, as demonstrated by intracellular immunostaining (Fig. 2G). These data altogether confirmed that the SB system, in conjunction with CRISPR-Cas13d, enabled the generation of stable pools which knocked down selected gene and conferred desired cell phenotype. Intriguingly, the

stable selection resulted in lower degrees of LDHA and GFT inhibition than transient transfection (Fig. 1), probably because the copy number of CRISPR-Cas13d modules was higher at 2 days after transfection than after Zeocin selection and integration.

CRISPR-Cas13d crRNA array enabled multiplexing knockdown

To assess the efficacy of simultaneous knockdown of LDHA, GFT and DDIT3, we constructed pIS-LGD that resembled pIS-gRNA but expressed a crRNA array that co-targeted LDHA, GFT and DDIT3 (Fig. 3A). pIS-LGD and pIS-O were individually transfected into CHO cells, and selected with Zeocin for 7 passages to yield the stable cells IS-LGD and IS-O, respectively. qRT-PCR analysis revealed that, in comparison with IS-O, IS-LGD expressed similar levels of Cas13d (Fig. 3B), but significantly and concurrently repressed LDHA, GFT and DDIT3 (Figs. 3C-3E).

To examine whether this engineered cell line can serve as a platform for enhanced IgG production, we used a plasmid expressing a biosimilar IgG that resembled Herceptin[?], an IgG drug for the treatment of HER2-rich breast cancer (*26*). The plasmid was transfected into the parental CHO cells, IS-O or IS-LGD, followed by selection for 10 passages to yield different stable cells expressing the Herceptin[?]-like IgG (designated as Parental-H, IS-O-H and IS-LGD-H, respectively). After subculture, the cells were cultured for 3 days and the IgG titers were measured for the analysis of specific production rate (SPR). As shown in Fig. 3F, the SPR of IS-O-H was slightly higher than that of Parental-H but the difference was insignificant ($p>0.05$), suggesting that expression of Cas13d and $gRNA_O$ did not deter the IgG production. Instead, the SPR of IS-LGD-H was significantly ($p<0.05$) higher than those of Parental-H and IS-O-H, attesting that CRISR-Cas13d-mediated knockdown of LDHA, GFT and DDIT3 enhanced the IgG production.

CRISPR-Cas13d-mediated knockdown improved the cell phenotype and IgG production

To assess how the CRISPR-Cas13d-mediated gene knockdown affected the cell response to survival stress, we cultured the engineered (IS-LGD-H or IS-O-H) and parental (Parental-H) CHO cells in a batch mode until the viability dropped below or near 50%. As shown in Fig. 4, the Parental-H cells underwent exponential growth from day 0 to day 5 as the viable cell density (VCD) increased exponentially (Fig. 4A) and the viability remained higher than 90% (Fig. 4B). In parallel, the glucose concentration decreased sharply (Fig. 4C) and lactate rapidly accumulated (Fig. 4D). From day 5 to day 7, the Parental-H cells entered the stationary phase as judged from the stagnant VCD and decreasing viability, which was concurrent with the depletion of glucose and decreasing lactate concentration, indicating that the cells were using lactate as an alternative carbon/energy source in lieu of glucose (*9*). At day 8, the viability precipitously dropped to [?]5% and both glucose and lactate concentrations were low, thus the culture was terminated.

For the IS-O-H cells, the exponential growth phase lasted until day 4 after which time the VCD (Fig. 4A) and viability (Fig. 4B) dropped sharply. Due to the cell death, the culture was terminated at day 7. In comparison with the Parental-H cells, the glucose level decreased similarly fast (Fig. 4C) and was accompanied by lactate accumulation. The lactate level only slightly decreased from day 5 to day 7, due to cell death and retarded cell metabolism. These data indicated that the culture condition became more adverse along culture time due to nutrient depletion and lactate accumulation, hence resulting in cell death.

In contrast, the IS-LGD-H cells grew exponentially in the first 5 days and then entered the stationary phase from day 5 to day 8 at which time the viability remained higher than 85% (Figs. 4A-4B). Compared with the Parental-H and IS-O-H cells, the IS-LGD-H cells grew more slowly (Fig. 4A), but had a longer stationary phase and the viability remained higher than 50% even at day 10 (Fig. 4B), indicating that the CRISPR-Cas13d-mediated silencing increased the longevity of CHO cells. In parallel, the glucose consumption was slower (Fig. 4C) and the lactate concentration of the IS-LGD-H cells was significantly lower than the control IS-O-H cells at days 2 and 3 (Fig. 4D). The lactate concentration remained steady from day 4 to day 10 probably because the glucose was not depleted until day 9, thus the cells continued to use glucose as the major carbon source and did not consume lactate during the stationary phase (*9*).

Despite the slower growth, IS-LGD-H cells gave rise to [?]47% and 59% higher IgG titer than IS-O-H and

Parental-H cells, respectively (Fig. 4E), demonstrating that simultaneous silencing LDHA, GFT and DDIT3 improved the IgG production.

CRISPR-Cas13d-mediated knockdown improved the IgG quality

To ensure the IgG was correctly glycosylated and functional, we first performed LCA sensitivity assay (Fig. 5A) as LCA kills cells with core fucosylation (*27*). At high LCA concentrations (25 and 50 μg/ml), the viability of IS-LGD-H was significantly higher than those of IS-Ø-H and Parental-H, indicating that IS-LGD-H conferred less core fucosylation of IgG and hence was more resistant to LCA.

Moreover, the IgG was purified and subjected to ADCC reporter assay to validate ADCC efficacy of IgG produced from the engineered cells. In the ADCC reporter assay, more efficient IgG binding to the target cells recruits the recombinant effector cells and triggers higher luciferase expression. As shown in 5B, the IgG from IS-LGD-H gave rise to higher luminescence than those from IS-Ø-H and Parental-H at all IgG concentrations. Fig. 5 collectively attested that the IgG produced from IS-LGD-H was less fucosylated and exhibited better ADCC effects.

Discussion

Improving CHO cell productivity and protein quality is highly desired for the biopharmaceutical industry, and may be achieved by engineering the cells to reduce lactate production (*28*), to improve glycosylation patterns (*29*) or to resist apoptosis (*30*). To reduce lactate accumulation and IgG fucosylation, we chose to inhibit LDHA and GFT because LDHA catalyzes lactate production (*9*) while GFT is essential for fucose-GDT transportation into Golgi for IgG fucosylation (*6*). In addition, we chose to inhibit DDIT3 because DDIT3 is implicated in the death of many cells (*31*), but its roles in CHO cell physiology and recombinant protein production have yet to be explored. In our preliminary CHO cell culture study, we found that DDIT3 was upregulated at the late stage of batch culture (Fig. S2). The DDIT3 upregulation agreed with the notion that ER stress induces DDIT3 expression, which subsequently results in reactive oxygen species (ROS) response and triggers cell death (*10*). Therefore, we surmised that inhibiting DDIT3 may promote the longevity of CHO cells during cell culture.

To suppress these 3 genes, we initially selected CRISPR interference (CRISPRi) system, which uses deactivated Cas9 (dCas9) fused with a transcription repressor KRAB (Krüppel-associated box) and the gRNA to specifically inhibit gene transcription (*19*), because CRISPRi was recently harnessed for gene repression in CHO cells (*32, 33*). However, the magnitude of LDHA suppression was less than 40% even though we screened a large panel of gRNA (Fig. S3), suggesting that CRISPRi approach may be ineffective in repressing certain endogenous genes in CHO cells. Since CRISPRi suppresses gene expression by blocking transcription, whose efficiency may be affected by the local chromatin structure surrounding the gene (*19*), we therefore exploited a different mechanism by silencing gene translation using the newly developed CRISPR-Cas13d system.

We showed that transfection of CRISPR-Cas13d system into CHO cells effectively knocked down LDHA, GFT and DDIT3, while the efficiency was highly dependent on the gRNA design (Fig. 1). With the aid of SB system-mediated integration and Zeocin selection, stable pools of cells with individual gene knockdown were readily obtained (Fig. 2). The stable LDHA knockdown alleviated the lactate accumulation (Figs. 2B-2C); GFT suppression reduced the core fucosylation (Figs. 2D-2E); and DDIT3 knockdown decreased the intracellular DDIT3 protein level (Figs. 2F-2G).

Given these results, we integrated the Cas13d cassette and the gRNA array co-targeting LDHA, GFT and DDIT3 into the chromosome and achieved simultaneous knockdown of all 3 genes (Fig. 3A-E), hence establishing a CHO cell platform for protein production. By integrating the Herceptin$^{?}$-like IgG gene into the new cell platform, the engineered cell (IS-LGD-H) conferred significantly higher specific production rate than the parental CHO cell line (Fig. 3F).

Notably, the negative control IS-O-H exhibited faster cell death (Figs. 4A-4B), similar glucose consumption and accumulated more lactate than the Parental-H cells, probably because simultaneous overexpression of

IgG, Cas13d and the scramble gRNA resulted in aberrantly high ER stress and metabolic burden to the cells. Nonetheless, IS-O-H cells conferred similar IgG titer when compared with the Parental-H cells. In stark contrast, by expressing the gRNA array targeting LDHA, GFT and DDIT3, IS-LGD-H cells exhibited improved longevity (Fig. 4A-4B) and triggered lower degrees of ROS response (Fig. S4) than the parental cell (Parental-H) and the negative control (IS-O-H). Since DDIT3 induction triggers subsequent ROS response and cell death (*34*), the increased tolerance of IS-LGD-H to the adverse culture environment at the late stage (e.g. at day 10) could be attributable to the efficient knockdown of DDIT3. Moreover, IS-LGD-H exhibited lower glucose consumption and lactate accumulation (Figs. 4C-4D), thanks to the effective silencing of LDHA. The silencing of these two genes allowed for rewiring of the intrinsic metabolic pathway and enhanced the cell's tolerance to environmental stress. As a consequence, the IS-LGD-H conferred significantly higher IgG titer than Parental-H and IS-O-H (Fig. 4E). Equally importantly, the repression of GFT concomitantly attenuated the core fucosylation (Fig. 5A) and the resultant IgG exhibited more potent ADCC efficacy than the IgG produced from the parental CHO cells (Fig. 5B). These data altogether demonstrated the feasibility of using CRISPR-Cas13d to engineer CHO cells to improve antibody production. In addition, one problem in large-scale CHO cell culture is clumping. We found that the gene encoding clusterin (CLU), which is implicated in cell aggregation in somatic cells (*35*), was upregulated along CHO cell culture (Fig. S2). We also exploited CRISPR-Cas13d to knockdown CLU and hindered CHO cell clumping (Fig. S5), which further substantiates the potentials of CRISPR-Cas13d for CHO cell engineering.

CHO cells are commonly engineered by altering the cellular processes, which can be achieved using different strategies, such as deleting, silencing or over-expressing individual genes in a cellular pathway. However, deletion of essential genes such as LDHA is lethal to CHO cells (*36*). Conversely, gene silencing may be achieved by small interfering RNA (siRNA) or short hairpin RNA (shRNA). However, siRNA and shRNA are known to induce serious off-target effects (*37, 38*). Additionally, high degree of siRNA-mediated knockdown may require multiple rounds of selection (*39*) as well as additional phenotypic selection using agents such as LCA (*6*). In comparison, CRISPR-Cas13d was shown to exhibit more potent knockdown of various endogenous genes with less off-target effects in multiple cell types (*18, 21, 22, 38, 40*). The intrinsic ability of Cas13d to process its pre-crRNA into separate crRNA also allows CRISPR-Cas13d to simultaneously target multiple mRNA involved in different pathways, thus enabling concurrent knockdown LDHA, GFT and DDIT3.

In conclusion, we present a novel CRISPR-Cas13d-based approach to engineering CHO cells in the post-transcriptional level. By efficient and multiplex knockdown of LDHA, GFT and DDIT3, the engineered CHO cell accumulates less lactate and is more resistant to cell death, thus producing more IgG. Furthermore, antibody fucosylation in the engineered CHO cells is mitigated, thus enhancing the ADCC efficacy. These data collectively warrant the potentials of CRISPR-Cas13d for CHO cell engineering and antibody production.

Materials and methods

Design of gRNA spacer

The RNA cleavage efficiency of CRISPR-Cas13 depends on the sequence and secondary structure of the targeted RNA. We used the RNAfold software (*https://zlab.bio/cas13*) to predict the RNA secondary structure and used siRNA design software, RNAxs (*https://zlab.bio/cas13*), to locate regions with good accessibility of RNA for gRNA spacers design. For each targeted RNA, we designed and tested 3 gRNA spacers with the highest scores for gene repression (Fig. S1).

Plasmid construction

The Cas13d gene, CasRx from *Ruminococcus flavefaciens* strain XPD3002, and its upstream EF1α promoter were cloned from pXR001 (Addgene) and subcloned into pUSEamp(+) (Merck Millipore) using*Pac* I/*Nhe* I. Zeocin resistance gene (Zeo^R)-BGH poly A sequence was amplified from pSecTag2b (Thermo Fisher) and ligated with a self-cleavable 2A sequence derived from porcine teschovirus-1 (P2A) to form the P2A-Zeo^R-polyA fragment. This fragment (P2A-Zeo^R-polyA) was sublconed into pUSEAmp(+) to yield pEF1α-

Cas13d-P2A-Zeocin (pCas13d).

Meanwhile, U6 promoter, gRNA backbone and termination sequence were amplified from pXR003 (Addgene) and subcloned into pTA vector (Invitrogen) to form a pU6-gRNA vector which expressed the scramble $gRNA_{\emptyset}$ containing the 30 nt direct repeat without spacer. A duplex containing 22 bp gRNA spacer for specific gene targeting was cloned into pU6-gRNA using *Bbs* I to form a complete pU6-gRNA. These gRNA cassettes were digested using *Mlu* I/*Hind* III and subcloned into pCas13d to yield different pCas13d-gRNA. For gRNA array co-targeting LDHA, GFT and DDIT3, we first subcloned the gRNA cassette targeting GFT into pU6-gRNA that targeted LHDA to yield pU6-$gRNA_{LG.}$ Subsequently, the gRNA cassette targeting DDIT3 was subcloned into pU6-$gRNA_{LG}$ using*Bgl* II/*Hind* III to yield pU6-$gRNA_{LGD}$.

To ensure the co-integration of Cas13d and gRNA, we exploited the*Sleeping Beauty* (SB) system, which uses the transposase SB100X to transpose the gene cassette flanked by IR/DR (inverted repeat/direct repeat) sequences into the chromosome (*24*). Two IR/DR sequences and the codon-optimized SB100X gene together with the upstream CMV promoter were chemically synthesized and cloned into pUSEamp(+) to yield pIS plasmid. The Cas13d-P2A-Zeo^R sequence from pEF1α-Cas13d-P2A-Zeocin was cloned into pIS using*Mlu* I/*Pvu* II to yield pIS-Cas13d. gRNA under U6 promoter from pU6-$gRNA_{\emptyset}$ or various pU6-gRNA targeting different genes were cloned into pIS-Cas13d to yield various pIS-gRNA. In parallel, the gRNA array co-targeting LDHA, GFT and DDIT3 was subcloned from pU6-$gRNA_{LGD}$ into pIS-Cas13d to generate pIS-LGD. The plasmid ph4D encoding the complete Herceptin[?]-like anti-HER2 IgG1 was a gift from Dr. Min-Yuan Chou (Industrial Technology Research Institute, Taiwan).

Cell culture, transfection and selection

CHO-K1 cells (referred to as CHO cells thereafter) were routinely cultured using F12 medium (#11765, Gibco) containing 10% fetal bovine serum (FBS). For transient transfection, CHO cells were seeded to 12-well culture plates ($1x10^5$ cells/well) and cultured for 48 h, followed by transfection of 2 μg plasmid DNA using lipofectamine 3000 transfection reagent (#L3000-015, Invitrogen). At 2 days post-transfection, total RNA was isolated for qRT-PCR assay.

For chromosomal integration into CHO cells, the plasmids (pIS-gRNA, pIS-LGD or ph4D) were transfected. At day 2, the medium was replaced with F12 medium containing 10% FBS and 200 μg/ml Zeocin (Invitrogen), followed by subculture every 4 days. After selection for 7 passages, stable pools of cells were obtained.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from CHO cells using Quick-RNA Miniprep Kit (#R1055, ZYMO Research) and reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (#4368813, Applied Biosystems). For quantitative real-time PCR, the reaction was performed with FastStart Essential DNA Green Master reagents using gene specific primers (Table S1) on LightCycler96 real-time quantitative PCR system (Roche Life Science). To analyze the relative expression level of each target gene, β2-microglobulin (B2M) was used as the internal control gene and the gene expression levels were normalized to those of control cells.

Glucose and lactate detection using CuBiAn bioanalyzer

Cell culture supernatant was collected by centrifugation at 1500×g for 5 min and stored at -20°C until analysis. The glucose and lactate concentrations were analyzed using CuBiAn Biochemical Analyzer (#C125, Optocell) with a Glucose assay (#200106, 4BioCell) and a Lactate assay (#200115, 4BioCell).

Lens culinaris agglutinin (LCA) sensitivity assay

Cells were seeded into 96-well plates (1×10^4cells/well) and cultured at 37°C overnight. The culture medium was replaced with fresh culture medium containing LCA (Vector Laboratories) of different concentrations. The cells continued to be cultured at 37°C for 48 h and the viability was detected using the PrestoBlue assay (#A13262, Invitrogen) according to user's manual. The Resazurin in PrestoBlue can be converted to

fluorescent signal in live cells and the fluorescence was recorded using a 560 nm excitation /590 nm emission filter set in ELISA reader (SpectraMax® M2, Molecular Device).

Intracellular immunostaining

Cells were washed with the wash buffer (phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA)) and then fixed with 2% paraformaldehyde at room temperature for 10 min, followed by washing with the wash buffer. The cells were permeabilized with iced-cold 90% methanol at room temperature for 10 min and was washed again with the wash buffer. Cells were immunostained with anti-DDIT3 antibody (#AP11955b, ABGENT) at a concentration of 0.025 mg/ml for 90 min at room temperature. After wash, the cells were stained with the secondary antibody, Goat Anti-Rabbit IgG H&L conjugated with Alexa Fluor® 488 (#ab150077, Abcam), at a concentration of 0.005 mg/ml. After the staining, DDIT3 expression was analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

IgG quantification using enzyme-linked immunosorbent assay (ELISA)

For antibody titer analysis by ELISA, 96-well plates were coated with 100 µl/well of recombinant HER2 antigen (2 µg/ml) in 0.1 M $NaHCO_3$ (pH 9.6) at 4°C for 16 h and washed with PBST (PBS containing 0.1% Tween 20) three times. The wells in 96-well plates were blocked by adding StartingBlock^{TM} blocking buffer (Thermo Scientific) and incubated at 37°C for 1 h, followed by three PBST washes. The sample or serially diluted standard (anti-HER2 IgG) were added to the StartingBlock^{TM} buffer and added to the wells (100 µl/well). After incubation at 37°C for 1 h and three PBST washes, the secondary antibody (Goat Anti-Human Igκ chain antibody conjugated with horseradish peroxidase (HRP), Millipore) was diluted 10000 times with StartingBlock^{TM} and added to the wells. After incubation at 37°C for 1 h, HRP detection was performed using Tetramethylbenzidine (TMB) (#01016-1, Clinical Science Product) at room temperature for 15 min. Finally, 2 N HCl (50 µl/well) was added to stop the reaction and the signal was read at OD_{450}using an ELISA reader.

Specific production rate (SPR) determination

CHO cells were seeded into 6-well plates (1×10^5cells/well) and cultured with 2 ml growth medium. After 3 days, the cell numbers were countered and the anti-HER2 IgG titers were determined using ELISA. The SPR (pg/cell/day) was calculated as described (*41*).

Adaptation of CHO cells to serum-free medium

After generation of CHO cells capable of producing Herceptin?-like anti-HER2 IgG, the engineered CHO cells cultured in F12 containing 10% FBS were adapted to chemically defined serum-free medium (CDM4PERMAb medium, GE Healthcare). Briefly, the cells were cultured in T-flasks with F12 containing 10% FBS. When the confluence reached 80%, the medium was replaced with CDM4PERMAb medium. After 4 days, most cells became detached and the cells were transferred to the shaker flask (Corning) and cultured at a shaking speed of 120 rpm.

Production of IgG

After adaption to serum-free medium, the CHO cells expressing the Herceptin?-like anti-HER2 IgG were inoculated into 30 ml of CDM4PERMAb medium containing 4 mM L-glutamine ($3x10^5$ cells/ml). Cells underwent continuous culture with a shaking speed of 120 rpm at 37degC for 7-10 days. Cell numbers were countered and glucose and lactate concentrations were monitored every day. Once the cell viability dropped below 50%, the culture was terminated.

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

ADCC effect of IgG was evaluated with an ADCC reporter bioassay (#G9790, Promega). The assay uses hFcγRIIIa$^+$ Jurkat T cell that expresses a luciferase reporter driven by an NFAT-response element as the effector cell and HER2-rich SKBR3 breast cancer cell as the target cell. The HER2 receptor on SKBR3 is

recognized by the Herceptin®-like IgG whose Fc region binds the FcγRIIIa receptor and triggers the luciferase expression in the effector cells.

The IgG was harvested when the viability dropped below or near 50% and was first purified using Mabselect SuRe® resin (#17-5474-021, GE Healthcare) following the manufacturer's instructions. In brief, the culture supernatant was collected, centrifuged (4500×g, 10 min) and loaded to a column packed with 0.2 ml Mabselect SuRe® resin. After washing with Dulbecco's PBS and elution with Arginine-HCl (25 mM, pH 2.5), the purified IgG was neutralized with minute amount of Tris-HCl (1 M) and stored at 4°C until analysis.

For the ADCC reporter assay, the purified IgG was 10-fold serially diluted using RPMI-1640 medium containing 0.5% FBS (low IgG). SKBR3 cells (100 μl) were seeded to each well of 96 well plates (1×10^4 cells/well) and cultured overnight. After removal of supernatant, we added the serially diluted IgG and added 25 μl Jurkat-hFcγRIIIa-NFAT effector cells (1.5×10^5cells/well). After 6 h of reaction at 37°C, 75 μl Bio-Glo Luciferase Assay Buffer was added to detect the luciferase activity in the effector cells. The luciferase activity was read using GloMax®Navigator Microplate Luminometer for the quantification of ADCC effect of IgG.

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Conflicts of Interests

The authors declare no conflicts of interests.

Supporting Information : Fig. S1, Fig. S2, Fig. S3, Fig. S4.

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Figure Captions

Fig. 1. Design of CRISPR-Cas13d for gene knockdown in CHO cells. (A) Schematic illustration of pCas13d-gRNA that expressed Cas13d under EF-1α promoter and gRNA under U6 promoter. (B) Sequences of the gRNA composed of 5' direct repeat and 3' spacer to target the complementary mRNA sequence of LDHA, GFT and DDIT3. (C) Targeting positions on the coding sequences of LDHA, GFT and DDIT3. (D) Relative mRNA levels of LDHA. (E) Relative mRNA levels of GFT. (D) Relative mRNA levels of DDIT3. We designed 3 gRNA for LDHA and DDIT3 but only 1 gRNA for GFT because the predicted spacers were in very close proximity (Fig. S1). We constructed a set of pCas13d-gRNA expressing individual gRNA and transfected the plasmid into CHO-K1 (referred to as CHO thereafter) cells. Gene expression was analyzed at 2 days post-transfection and normalized to that in the cells transfected with the plasmid expressing the scramble $gRNA_{Ø}$.

Fig. 2. Chromosomal integration of CRISPR-Cas13d aided by *Sleeping Beauty* (SB) system enabled individual gene knockdown and altered CHO cell phenotype. (A) Schematic illustration of pIS-gRNA plasmids that incorporated the SB system and CRISPR-Cas13d. The plasmid expressed SB100X, Zeo^R, Cas13d and the gRNA targeting either LDHA, GFT or DDIT3. The CRISPR-Cas13d module and Zeo^R were flanked by a pair of IR/DR sequences for SB100X recognition. (B) Relative LDHA mRNA level. (C) Lactate production. (D) Relative GFT mRNA level. (E) LCA assay. (F) Relative DDIT3 mRNA level. (G) Intracellular DDIT3 protein levels. The cells were transfected with different pIS-gRNA (including pIS-Ø) plasmids and selected with Zeocin to yield stable pools of cells. The mRNA expression levels were analyzed by qRT-PCR and normalized to those of the cells transfected with pIS-Ø. The cells were subjected to lactate assay and LCA assay at day 2 after subculture. The DDIT3 protein levels were analyzed at day 2 and 6 by intracellular immunostaining and analyzed by flow cytometry.

Fig. 3. Establishing a stable CHO cell platform with simultaneous knockdown of LDHA, GFT and DDIT3. (A) Illustration of pIS-LGD plasmid that was similar to pIS-gRNA except that the gRNA was an array co-targeting LDHA, GFT or DDIT3. (B) Relative Cas13d mRNA level. (C) Relative LDHA mRNA levels. (D) Relative GFT mRNA levels. (E) Relative DDIT3 mRNA levels. (F) Specific production rate (SPR). The CHO cells were transfected with pIS-LGD and selected with Zeocin to yield stable pools of cells (IS-LGD). The gene expression levels were analyzed by qRT-PCR and normalized to the control transfected with pIS-Ø (IS-Ø). The SPR was calculated by measuring the IgG titer and cell number for 3 days.

Fig. 4. CRISPR-Cas13d-mediated knockdown improved the cell phenotype and IgG production. (A) Viable cell density (VCD). (B) Viability. (C) Glucose concentration. (D) Lactate concentration. (E) IgG titer. The cells were adapted to serum-free medium and cultured in shaker flasks in a batch mode. The VCD, viability, glucose and lactate were measured daily until the viability dropped below or near 50%. The IgG titer was determined at the end of culture by ELISA.

Fig. 5. Quality analysis of IgG produced the engineered cells. (A) LCA assay. (B) ADCC reporter assay.

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