Inducible transgene expression in CHO cells using an artificial transcriptional activator with estrogen-binding domain

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Abstract

Biopharmaceuticals, including therapeutic antibodies, are rapidly growing products in the pharmaceutical market. Mammalian cells, such as Chinese hamster ovary (CHO) cells, are widely used as production hosts because recombinant antibodies require complex three-dimensional structures modified with sugar chains. Recombinant protein production using mammalian cells is generally performed in conjunction with cell growth. In this study, we developed a technology that controls cell growth and recombinant protein production to induce recombinant protein production with arbitrary timing. Expression of green fluorescent protein (GFP) gene and a single-chain antibody fused with the Fc-region of the human IgG1 (scFv-Fc) gene can be induced and mediated by the estrogen receptor-based artificial transcription factor Gal4-ERT2-VP16 and corresponding inducer drugs. We generated CHO cells using an artificial gene expression system. The addition of various concentrations of inducer drugs to the culture medium allowed control of proliferation and transgene expression of the engineered CHO cells. Use of 4-hydroxytamoxifen, an antagonist of estrogen, as an inducing agent yielded high gene expression at a concentration more than 10-fold lower than that of β-estradiol. When scFv-Fc was continuously produced under inducing conditions, stable production was possible for more than 2 weeks while maintaining high specific productivity (57 pg cell^{-1} day^{-1}). This artificial gene expression control system that utilizes the estrogen response of estrogen receptors can be an effective method for inducible production of biopharmaceuticals.

1 INTRODUCTION

Biopharmaceuticals are high-molecular-weight biological drugs manufactured using biological processes.[1] Many biopharmaceuticals include proteins or nucleotides consisting of hundreds or thousands of amino acids or nucleosides, such as monoclonal antibodies, cytokines, enzymes, viruses, and nucleic acid-based products. Among them, monoclonal antibodies have received the most approvals from regulatory agencies and entered the market.[2] The biopharmaceutical market is rapidly expanding and has promising prospects due to enormous demand.[3] In the biopharmaceutical market, mammalian cell lines are preferred for producing recombinant therapeutic proteins. Chinese hamster ovary (CHO) cell lines are particularly notable. The use of CHO cells as hosts in production systems has become an established method for producing therapeutic proteins, primarily due to their ability to proliferate in high-cell-density serum-free cultures.[4]

CHO cells can produce properly folded proteins with post-translational modifications similar to those in humans.[5,6]Additionally, CHO cells are recognized as safe hosts with resistance to human viral infections because they do not express genes for viral entry.[4,7] There are several challenges to improve the specific productivity of target proteins. The main barriers include vector construction, transfection efficiency, and integration into specific loci on the transgenic chromosome. Another limitation is the availability of high-producing cell lines. Establishing high-producing cell lines is time-consuming and labor-intensive, so targeted

integration of the desired transgene into specific genomic loci may be a potential solution to streamline the complex screening process.[8,9] Cell line development, media composition, and culture optimization have increased due to the demand for and regulatory requirements of biopharmaceuticals.[10] However, novel research is needed to develop high-producing cell lines that meet market requirements.

In the current mainstream production method, fed-batch cultivation, the desired substance is produced simultaneously with cell proliferation. For biotechnology applications, it is expected that the target gene will be expressed in a controllable manner.[11] This can be achieved with the help of inducible systems, which allow for specific control of transgene expression in certain cells during specific periods.[12] By switching between growth and production phases, it may be possible to reduce the burden on cells during proliferation while maintaining a high proliferation capacity and achieving high productivity by inducing production after reaching high cell density in a short period of time.

In recent years, gene expression control systems have become important techniques for analyzing various biological functions of both prokaryotes and eukaryotes. Various gene expression control systems based on artificial transcription activators have been developed, such as the tetracycline repressor-based transcriptional activation system[13] and CRISPR transcriptional activation system.[14] We previously developed an artificial gene expression system that responds to external environments such as heat treatment and hypoxia in mammalian cells derived from liver and muscle.[15–24] Using these systems, we succeeded in inducing artificial gene expression and making it functional by autonomously responding to the environment.

In this study, we aimed to construct a biopharmaceutical production system that utilizes the estrogen response of estrogen receptors to control target gene expression. Estrogen receptors translocate to the nucleus upon binding to their ligand estrogen and function as transcriptional activators.[25,26] Therefore, inducible expression systems using estrogen as a switch can be used to construct biopharmaceutical production systems that can switch between cell growth and cell production modes. We employed an artificial gene expression system using the chimeric protein Gal4-ERT2-VP16 (GEV), which is activated by the addition of estrogen receptor ligands. GEV is a transcriptional activator that can induce target gene expression in response to the addition of estrogen, β-estradiol (E2), or the E2 antagonist 4-hydroxytamoxifen (4-OHT) to the culture medium.[27,28] We generated CHO cells constitutively expressing GEV in which gene expression can be controlled by estrogen receptor ligands. First, a reporter gene expression system capable of responding to GEV activated in the presence of a ligand was introduced into cells and the induced expression behavior was analyzed. Subsequently, a gene expression cassette for producing an anti-prion single-chain antibody fused with the Fc-region of human IgG1 (scFv-Fc), as a model antibody, was incorporated into cells and the induced antibody production in response to ligand drugs was evaluated using serum-free medium. Finally, continuous production of scFv-Fc using this artificial gene expression control system was attempted by semi-continuous suspension culture with high cell density, and antibody production stability was investigated.

2 MATERIALS AND METHODS

2.1 Cells and media

CHO-K1 (RIKEN Cell Bank, Tsukuba, Japan) and recombinant CHO cells were cultured in F12 medium (#N6760; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum (BioWest, Nuaillé, France) and antibiotics (Penicillin-Streptomycin, #15140122; Invitrogen, Waltham, MA, USA). Cells were cultured in a 5% CO_2 incubator at 37°C. For serum-free cultures, CD CHO AGT Medium (#12490025, Invitrogen) containing 4 mM L-glutamine (#074-00522; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 0.2% anti-clumping reagent (#0010057AE, Invitrogen), and antibiotics (penicillin-streptomycin) was used. When cells were cultured in serum-containing medium, 100-mm cell culture dishes (BioLite #130182; Thermo Scientific, Waltham, MA, USA) or 24-well tissue culture plates (BioLite #130186, Thermo Scientific) were used. For serum-free suspension culture, bioreactor tubes (#87050; TPP Techno Plastic Products AG, Trasadingen, Switzerland) placed in a shaker (Model #0081704-000; Taitec, Koshigaya, Japan) with a 45° angle were incubated at 37°C in a 5% CO_2 incubator.

2.2 Plasmid construction

To construct a transposon-based GEV expression vector, the GEV gene was amplified by PCR using pME-GAL4-ERT2-VP16 (#82588, Addgene, Watertown, MA, USA)[28] as a template. A DNA fragment of the Chinese hamster-derived EF1α promoter[8] was chemically synthesized. A hygromycin-resistant gene expression unit (selection marker) was obtained by PCR using pCEP4 (#V04450, Invitrogen) as a template. These DNA fragments were inserted into a PiggyBac transposon vector plasmid (#PB513B-1; System Biosciences, Palo Alto, CA, USA) to generate PB/chEF1α/GEV_Hyg (Figure 1A).

Next, to generate the UAS/scFv-Fc expression vector, a DNA fragment of scFv-Fc gene was obtained by cutting plasmid R2[8]with *Eco* RI and *Spe* I. A DNA fragment of the UAS response element and minimal region of the adenovirus major late promoter were chemically synthesized. A zeocin-resistant gene expression unit was amplified by PCR using pcDNA4/GP[29] as a template. By inserting these DNA fragments into the PiggyBac transposon vector plasmid, PB/UAS/scFv-Fc_Zeo was constructed (Figure 1A). To construct the UAS/GFP expression vector, a *GFP* gene was amplified by PCR using pGreenFire1-RARE (#TR037PA-P, System Biosciences) as a template. PB/UAS/GFP_Zeo was constructed by replacing the scFv-Fc gene region in PB/UAS/scFv-Fc_Zeo with the resulting fragment (Figure 1A).

For PCR, KOD plus Neo DNA polymerase (#KOD-401; Toyobo, Tsuruga, Japan) and the primers shown in Supplementary Table S1 were used for amplification. All PCR products were subjected to gene sequence analysis by Sanger sequencing to confirm correct sequences.

2.3 Generation of recombinant CHO cells

To generate CHO cells expressing the GEV gene (CHO/GEV), CHO-K1 cells were seeded in a 24-well plate at 1.0 x 10^5cells/well. The next day, PB/chEF1α/GEV_Hyg and PiggyBac transposase expression vector plasmids (pPBase; #PB210PA-1, System Biosciences) were transiently transfected into CHO-K1 cells using Lipofectamine 2000 (#11668019, Invitrogen) according to the manufacturer's procedure. After 48 h, cells were plated in six-well plates (BioLite #130184, Thermo Scientific), Hygromycin B (#008-07683, Wako) was added at a concentration of 400 μg/mL, and drug selection was performed for 14 days. Stable transformed cells expressing GEV were obtained. Cell clones (CHO/GEV) were established by limiting dilution.

Next, PB/UAS/GFP_Zeo was transiently introduced into CHO/GEV together with pPBase using Lipofectamine 2000 as described above. Drug selection was performed for 14 days using 400 μg/mL Zeocin (#R25001, Invitrogen). Next, 1 μM 4-OHT (#H6278, Sigma-Aldrich) was added to the cells. The day after drug addition, single-cell cloning of GFP-positive cells was performed using a cell sorter (SH800; Sony, Tokyo, Japan) to establish drug-inducible GFP-expressing cell clones (CHO/GEV_GFP).

To generate drug-inducible scFv-Fc-producing cells, PB/UAS/scFv-Fc_Zeo linearized with *Fsp* I was introduced into CHO/GEV using Lipofectamine 2000. Selection with Zeocin was performed in the same manner as described above to establish stable transformed cells, and cell clones (CHO/GEV_scFv-Fc) were obtained by limiting dilution.

2.4 Induction of target genes

Cells were seeded with 1 mL per well in 24-well plates at a seeding density of 1.0 × 10^5 cells/mL in serum-containing F12 medium or 1.0 × 10^6 cells/mL in serum-free medium, and cultured for 4 days. During culture, the medium was changed daily to medium containing E2 (#E2758, Sigma-Aldrich) or 4-OHT at various concentrations. Cell numbers were counted using the trypan blue dye exclusion method.

High-cell-density serum-free culture was performed as follows. After culturing CHO/GEV_scFv-Fc for 3 days in serum-free medium containing 0.1 μM 4-OHT, cells were adjusted to a density of 0.5 or 1.0 × 10^7 cells/mL. Thereafter, 10 mL of cell suspension was seeded in a bioreactor tube and cultured for 15 days. The culture medium was replaced with fresh medium every day, and the number of cells was counted at that time.

Drug-induced GFP expression in CHO/GEV_GFP was analyzed using a flow cytometer (SH800). scFv-Fc secreted into the culture medium was quantified using an enzyme-linked immunosorbent assay method[30]. The scFv-Fc specific production rate (pg $cell^{-1}$ day^{-1}) was calculated from scFv-Fc concentrations in the spent

medium and numbers of viable cells. Glucose and lactate concentrations were measured using commercially available kits (Glucose Assay Kit-WST, #G264, Dojindo, Kumamoto, Japan; Lactate Assay Kit-WST, #L256, Dojindo).

2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Medium samples (10 μL) harvested from semi-continuous cultures were mixed with SDS sample buffer (5 μL) with or without 2-melcaptoethanol and boiled for 5 min. Sample solutions were electrophoresed on 4%–20% precast polyacrylamide gels (#4561094; BioRad, Hercules, CA, USA). Protein bands were visualized by incubating gels in protein staining solution (#LC6060, SimpleBlue SafeStain, Invitrogen) for 1 h. Next, gels were immersed in distilled water for 2 days to remove excess dye. Purified scFv-Fc was prepared from culture medium using a Protein A Sepharose column (rProtein A Sepharose Fast Flow, #17127901; Cytiva, Marlborough, MA, USA).[31]

3 RESULTS

3.1 Establishment of founder CHO cells expressing GEV transactivator

The GEV, designed as an artificial transcription factor, is a fusion protein consisting of the DNA-binding domain Gal4, ligand-binding domain ERT2 of the estrogen receptor, and transcription activation domain VP16 derived from herpes simplex virus. In the absence of E2 or 4-OHT, GEV remains in the cytoplasm; however, upon the addition of E2 or 4-OHT, GEV translocates to the nucleus through ligand binding to the ERT2 domain. Subsequently, GEV binds to the Gal4-binding sequence (UAS) of an artificial promoter (UAS-P_{min}) and induces expression of the target gene under control of the artificial promoter using the activity of VP16 (Figure 1B). Initially, to establish CHO cells constitutively expressing GEV, the GEV gene was incorporated into a PiggyBac transposon vector and introduced into CHO cells along with a transposase expression vector. Stable expression cell lines were obtained by selection with the corresponding drug. As a preliminary investigation, a GFP reporter vector plasmid (PB/UAS/GFP) was transiently transfected into stable GEV-expressing cell lines, and drug-dependent GFP expression was confirmed for cells cultured in the presence of 20 μM E2 or 1.0 μM 4-OHT. Subsequently, PB/UAS/GFP was transiently introduced into these cells and cloning was performed by sorting the cell fraction induced for GFP expression under 1.0 μM 4-OHT using a cell sorter. For the obtained 26 cell clones, transient gene introduction of PB/UAS/GFP was performed, followed by culture in the presence of 1.0 μM 4-OHT. After 48 h, cell clones with high GFP expression were selected by observation under a fluorescence microscope (Supplementary Figure S1A). The same gene introduction of PB/UAS/GFP was performed for three selected cell clones. After introduction, cells were evaluated under conditions of 20 μM E2, 1.0 μM 4-OHT, and co-addition of 20 μM E2 and 1.0 μM 4-OHT (Supplementary Figure S1B). The mean fluorescence intensity of GFP was measured using a flow cytometer. In all conditions, the cell clone CHO/GEV_1 had the highest mean fluorescence intensity. Therefore, this cell clone was chosen for subsequent experiments.

3.2 Inducible expression of *GFP* reporter gene in CHO/GEV_GFP cells

We next investigated proliferation and gene expression responsiveness using a *GFP* reporter gene. A transposon vector containing the GEV-responsive GFP expression unit was introduced into CHO/GEV cells and integrated into the cell genome through transposition. Upon the addition of 1.0 μM 4-OHT to drug-selected cells, GFP expression was induced in all cells. Therefore, cloning was performed using a cell sorter. All six tested clones showed equivalent GFP induction and proliferation responsiveness upon 4-OHT addition. Based on these results, a representative cell clone (CHO/GEV_GFP) was used for further analysis.

CHO/GEV_GFP cells were seeded in a 24-well plate to evaluate cell proliferation and drug responses (Figure 2). Various concentrations of E2-containing medium were used, and the medium was changed daily. As a result, a concentration-dependent decrease in proliferation was observed (Figure 2A). When 20 μM E2 was added, cell proliferation was completely suppressed without decreasing cell viability. In our analysis of *GFP* expression induction, GFP-positive cells were observed from the day after the addition of E2, and 80%–90% of GFP-positive cells were observed throughout the culture period at E2 concentrations of 5 μM or

higher (Figure 2B). However, the mean fluorescence intensity was only about twice that of the non-additive control, suggesting that the expression level was insufficient (Figure 2C). Next, we attempted gene expression induction using 4-OHT, an antagonist of E2. Cell toxicity was observed at concentrations of 10 μM or higher (Supplementary Figure S2). Therefore, we evaluated CHO/GEV_GFP cells with a low concentration range of 0.01–1 μM 4-OHT. As a result, a decreased proliferation rate was observed and cells were able to proliferate without toxicity (Figure 2D). Analysis of GFP expression inducibility reveals that almost all cells became GFP-positive from day 1 of culture at concentrations of 0.5 μM 4-OHT or higher (Figure 2E). The mean fluorescence intensity of GFP reached its maximum on day 3 of culture, and a 2.5-fold higher expression was observed with 4-OHT compared with E2 (Figure 2F). This is attributable to the high affinity of 4-OHT for ERT2 compared with E2.[32] Thus, it was found that 4-OHT could induce higher expression at concentrations more than 10-fold lower than E2.

3.3 Inducible expression of scFv-Fc antibody gene in CHO/GEV_scFv-Fc cells

The functionality of our artificial gene expression system mediated by a transcription factor containing the estrogen-binding domain of the estrogen receptor can be confirmed using a reporter gene. Thus, we attempted to induce the production of a model antibody by adding E2 or 4-OHT (Figure 3). Plasmids containing the UAS/scFv-Fc expression unit were linearized and introduced into CHO/GEV cells. After selection with appropriate drugs, we obtained a pool of transformed cells and performed cloning using the limiting dilution method. Among the 22 clones obtained, we evaluated six clones that functioned correctly in response to 0.1 μM 4-OHT and selected the one with the best cell proliferation and scFv-Fc production responsiveness. This cell line, designated as CHO/GEV_scFv-Fc cells, was further investigated for proliferation and production induction analysis.

Evaluation of CHO/GEV_scFv-Fc cell proliferation in the presence of 1.25–5 μM E2 revealed no significant impact compared with the control (Figure 3A). Measurement of scFv-Fc production showed a maximum of 1.0 μg/mL at 5 μM (Figure 3B), with a specific productivity of 5 pg cell^{-1} day^{-1} (Figure 3C). Next, we attempted to induce antibody production using 4-OHT. As 4-OHT effectively induced reporter gene expression and protein production levels, we expected a high induction effect for scFv-Fc production as well. Addition of 4-OHT at concentrations of 0.01–0.1 μM resulted in proliferation comparable with the control (Figure 3D). Evaluation of scFv-Fc production reveals that scFv-Fc gene expression was induced with 0.05 μM or higher 4-OHT concentrations (Figure 3E), and the concentration of scFv-Fc increased over the culture period. Following the addition of 0.5 μM or 1.0 μM 4-OHT, the scFv-Fc concentration and specific productivity reached 4.9 μg/mL and 24 pg cell^{-1} day^{-1}, respectively (Figure 3F), which is 5-fold and 4-fold higher compared with E2. We next examined the combined effect of E2 and 4-OHT addition. With a fixed E2 concentration of 20 μM, we attempted induction of both the reporter gene and scFv-Fc gene with various 4-OHT concentrations under conditions of cell growth inhibition (Supplementary Figure S3A and S3D). Overall induction levels were lower compared with conditions with 4-OHT alone (Supplementary Figure S3B-C and S3E-F). Therefore, the addition of 4-OHT alone to induce production is suitable for antibody production in this artificial gene expression system.

3.4 Suspension culture of CHO/GEV_scFv-Fc cells in serum-free medium

For production of useful substances using CHO cells, suspension culture with serum-free medium is widely employed. Due to the absence of serum components, the responsiveness of cell proliferation and exogenous gene expression to the inducing agent 4-OHT may vary. As the drug response may become more sensitive compared with conditions using serum-containing medium, we investigated the impact of 4-OHT concentration on scFv-Fc production for CHO/GEV_scFv-Fc cells cultured in a commonly used serum-free medium (Figure 4). CHO/GEV_scFv-Fc cells were seeded at a concentration of 1.0×10^6 cells/mL in 2 mL of serum-free medium containing various concentrations of 4-OHT and cultured in a 24-well cell culture plate. The medium was changed daily to fresh medium containing 4-OHT, and cells were cultured for 4 days. Cell proliferation and viability are shown in Figure 4A and 4B, respectively. Only a slight decrease in cell proliferation was observed at 4-OHT concentrations below 0.1 μM, and the viability remained close to 100% throughout the culture period. In contrast, significant decreases in cell proliferation were observed, particularly at 0.5

μM and 1.0 μM. Additionally, cell viability was affected with the 1.0 μM addition condition, dropping to around 80% by day 4 of culture. To evaluate scFv-Fc production over the culture period, we increased the seeding cell density 10-fold compared with the serum-containing culture. Under this condition, the scFv-Fc concentration reached 42 μg/mL (Figure 4C), which is 8.6-fold higher than that of serum-containing culture. However, specific productivity was decreased in conditions with 0.5 μM and 1.0 μM 4-OHT, which showed good values in serum-containing cultures. With 0.1 μM 4-OHT, the maximum specific productivity was 13 pg cell^{-1} day^{-1} (Figure 4D), comparable to the production level observed in serum-containing cultures with the same 4-OHT concentration (15 pg cell^{-1}day^{-1}). These results confirm that drug-inducible transgene expression can be achieved even with serum-free medium, and the optimal concentration of 4-OHT is approximately 10-fold lower compared with serum-containing culture.

3.5 scFv-Fc production by CHO/GEV_scFv-Fc cells in semi-continuous culture at high density

Next, we attempted continuous production under high-cell-density culture conditions with 4-OHT induction using a bioreactor tube (Figure 5). As described above, our results reveal that antibody production could be induced without significantly impacting cell proliferation under the 0.1 μM 4-OHT condition. We also found that the maximum concentration and specific productivity of scFv-Fc could be achieved within 3 to 4 days after adding 4-OHT. Following pre-culture for cell proliferation without 4-OHT, we initiated suspension cultures with high cell density by switching to 4-OHT-containing medium. Semi-continuous culture was carried out for more than 2 weeks to evaluate the stability of induced scFv-Fc production under the 0.1 μM 4-OHT condition. When seeded at a density of 1.0×10^7 cells/mL, cell proliferation was observed (Figure 5A) but a decreased level of cell viability was maintained around 80% (Figure 5B). scFv-Fc concentrations demonstrate stable production in the medium at an average of 0.44 ± 0.05 g/L over the duration of culture (Figure 5C), with a specific productivity of 37 ± 4 pg cell^{-1} day^{-1} (Figure 5D). In contrast, with a seeding density of 0.5×10^7cells/mL, nearly 100% viability was maintained throughout the culture period (Figure 5B) and scFv-Fc production at a concentration of 0.31 ± 0.03 g/L (Figure 5C) and specific productivity of 57 ± 5 pg cell^{-1} day^{-1} (Figure 5D) was stably achieved. Analysis of metabolites in the spent medium indicate glucose concentrations of 4.3 ± 0.9 mM and 2.0 ± 0.7 mM for seeding densities of 0.5×10^7 cells/mL and 1.0×10^7 cells/mL, respectively (Figure 5E). Lactate concentrations were measured to be 34 ± 6 mM (0.5×10^7 cells/mL) and 66 ± 7 mM (1.0×10^7 cells/mL) (Figure 5F). Conversion rates from glucose consumption to lactate production were estimated to be 52% (0.5×10^7 cells/mL) and 100% (1.0×10^7cells/mL).

scFv-Fc protein produced in the medium of semi-continuous cultures was analyzed using SDS-PAGE. The results show that bands of the expected molecular weight were detected under both reducing (Figure 6A and 6C) and non-reducing (Figure 6B and 6D) conditions, indicating that the protein was produced in an intact form (Figure 6).

Taken together, these results demonstrate that our estrogen receptor-based inducible expression system can be applied for high-cell-density semi-continuous culture to stably produce recombinant proteins for over 2 weeks.

4 DISCUSSION

Recombinant protein production in *Escherichia coli* cells has been carried out using *lac* -based inducible promoters.[33] Although gene expression control systems have been extensively reported in animal cells, inducible expression systems are rarely employed for industrial-scale production of recombinant proteins. Cell growth inhibition is often observed in cell lines that produce recombinant proteins at extremely high levels. In such cases, a production system that switches between cell growth and protein production phases may effectively improve productivity per unit time. In this study, to demonstrate the concept of separating growth and production phases, we constructed a biopharmaceutical production system with inducible target gene expression using the estrogen-binding domain of an estrogen receptor. As a gene expression inducer, 4-OHT can bind to the estrogen receptor with high affinity compared with the natural ligand E2,[32] enabling expression induction at low concentrations. Using 4-OHT as an induction switch, scFv-Fc protein production could be induced with 4.4-times higher concentration and 5.5-times higher specific productivity compared

with E2, under lower induction concentrations. The model antibody scFv-Fc was produced by inducing expression in CHO cells using a serum-free medium commonly used for biopharmaceutical production. In serum-free medium, sensitivity to the inducer and responsiveness to gene expression induction are increased. After achieving the required cell number in 4-OHT-free medium, production culture was performed under the condition of 0.1 μM 4-OHT. Within 3 days, cells were induced to a maximum production level. scFv-Fc production in CHO/GEV_scFv-Fc cells with high cell density was stably maintained during semi-continuous culture for over 2 weeks, with specific productivity ranging from 37 to 57 pg cell^{-1}day^{-1} and secretion levels of 0.31–0.44 g/L. Interestingly, the specific productivity of cells at high density was 2.8 to 4.4-fold higher compared with the growth state at low cell density (13 pg cell^{-1} day^{-1}).

In this gene induction system, GFP-positive cells with over 95% efficiency were observed from day 1 after 0.5 μM 4-OHT addition. Maximum GFP expression intensity or maximum scFv-Fc production was generally observed 2–3 days after adding the inducer drug. In ERT2 fusion proteins, including ERT2-Cre, the mutant ERT2[32]derived from the ligand-binding domain of human ERalpha (amino acids 282–595; Accession No. NP_000116)[34] binds to the molecular chaperone HSP90 in the cytoplasm. In the presence of 4-OHT, HSP90 is replaced by 4-OHT and undergoes nuclear translocation.[25,26] GEV utilizes the same region as the ligand-binding domain of Cre-ERT2 but lacks the nuclear localization signal region (243-RKC YEV GMM KGG IRK DRR GGR MLK HKR QRD-272)[35] of human ER[?] in ERT2. Nuclear translocation of proteins can be controlled by the type of nuclear localization signal, mutations, or their placement.[36,37] Thus, screening for an optimal nuclear translocation signal for GEV may enhance nuclear translocation and rapid induction of transgene expression following addition of the inducer drug.

When using a seeding cell density of 2.0 × 10^{7}cells/mL in suspension culture under high-cell density conditions, a significant decrease in cell viability was observed from day 2 of culture (data not shown). Therefore, the initial seeding cell density was set to 0.5 or 1.0 × 10^7 cells/mL, and daily medium changes were performed. This strategy allowed for stable induction and production while maintaining constant cell viability. Glucose in the medium was consumed at a rate of 86%–93% and the specific consumption rate was calculated as 0.4–1.0 ng cell^{-1}day^{-1}. With a seeding cell density of 1.0 × 10^7 cells/mL, a lactate concentration of 66 mM was measured, corresponding to consumption of approximately 33.5 mM glucose per day. This suggests that almost all of the consumed glucose was converted to lactate. Cell viability was around 80%, indicating that oxygen depletion occurred in this culture. With a seeding cell density of 0.5 × 10^7 cells/mL, the glucose consumption rate was 31.3 mM and lactate accumulation was approximately 34 mM, almost half the amount of the consumed glucose. This suggests that although most glucose was consumed, lactate accumulation was suppressed, resulting in cell viability being maintained at nearly 100% and a high specific production rate (57 pg cell^{-1}day^{-1}). In this experiment, a commonly used serum-free medium was employed for evaluation; however, in recent years, data-driven medium development and feed development in CHO cell metabolism have been actively pursued.[38,39]Accordingly, improving the oxygen supply and developing medium suitable for this inducible gene expression system may further enhance productivity.

A drug-inducible nuclear translocation system based on estrogen receptors enables spatiotemporal control of gene function. Numerous applications have been reported using recombinase,[32]transposase,[40] transcription factors,[41] and other approaches. In this study, we constructed a gene expression system incorporating an estrogen-responsive domain into an artificial transcription factor designed for target gene expression and applied it to the inducible production of scFv-Fc antibody. The system demonstrated high-level induction of expression and long-term stable production, highlighting its effectiveness as a production system that switches between cell proliferation and production modes. Due to its anticipated versatility, this gene induction system is considered applicable to CHO cells as well as HEK293 cells, which are commonly used for viral production, and newly developed Chinese hamster-derived cells[42,43] for the production of valuable substances.

In conclusion, we developed an inducible gene expression system for biopharmaceutical production utilizing the estrogen-responsive property of the estrogen receptor. Using this system, we demonstrated an efficient recombinant protein production system in CHO cells that effectively separates the cell proliferation phase from the production phase. The production system, built upon a synthetic biology-based approach for an

artificial gene expression, is expected to become an important method in biopharmaceutical production.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare no commercial or financial conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, M.K.; methodology, M.R.R., Y.K., and M.K.; validation, Y.K. and M.K.; formal analysis, M.R.R., Y.K., K.S., and M.K.; investigation, M.R.R., K.S., S.C. and Y.A.; resources, Y.K. and M.K.; data curation, Y.K. and M.K.; writing—original draft preparation, M.R.R., Y.K., and M.K.; writing—review and editing, M.R.R., Y.K., and M.K.; visualization, M.R.R., Y.K., and M.K.; supervision, M.K.; project administration, M.K.; funding acquisition, M.K. All authors have read and agreed to the published version of the manuscript.

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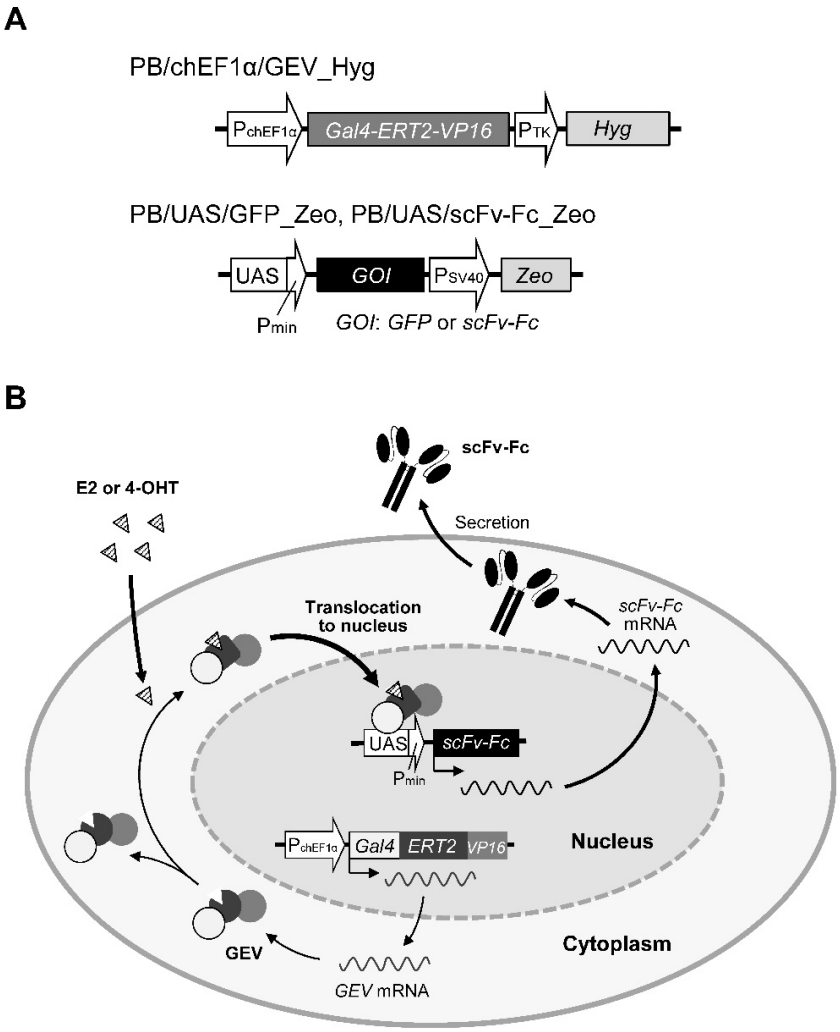

Figure 1 Estrogen receptor-based artificial gene expression system.**(A)** Vector constructs of the Gal4-ERT2-VP16 (GEV) expression vector (PB/chEF1α/GEV_Hyg) and gene-of-interest (GOI) expression vectors (PB/UAS/GFP_Zeo and PB/UAS/scFv-Fc_Zeo). $P_{\varsigma\eta E\Phi 1\alpha}$, Chinese hamster-derived elongation factor 1α promoter; Gal4-ERT2-VP16 (GEV), estrogen receptor-based artificial transcription factor; Gal4, DNA binding domain of yeast transcription factor; ERT2, ligand binding region of estrogen receptor mutant; P_{TK}, thymidine kinase promoter; Hyg, hygromycin resistance gene; UAS, Gal4-linked upstream activation sequence; P_{min}, Ad major late promoter minimal region; GOI, target gene; GFP, green fluorescent protein; anti-prion single-chain antibody fused with the Fc-region of human IgG1 (scFv-Fc); P_{SV40}, SV40 promoter; Zeo, zeocin resistance gene. **(B)** GEV expressed under a constitutive promoter accumulated in the cytoplasm. In the presence of β-estradiol (E2) or 4-hydroxytamoxifen (4-OHT), GEV translocates into the nucleus and binds to UAS-responsive elements. The activated GEV induces expression of the target gene (scFv-Fc) under control of the UAS/P_{min} artificial promoter. Translated scFv-Fc protein is extracellularly secreted.

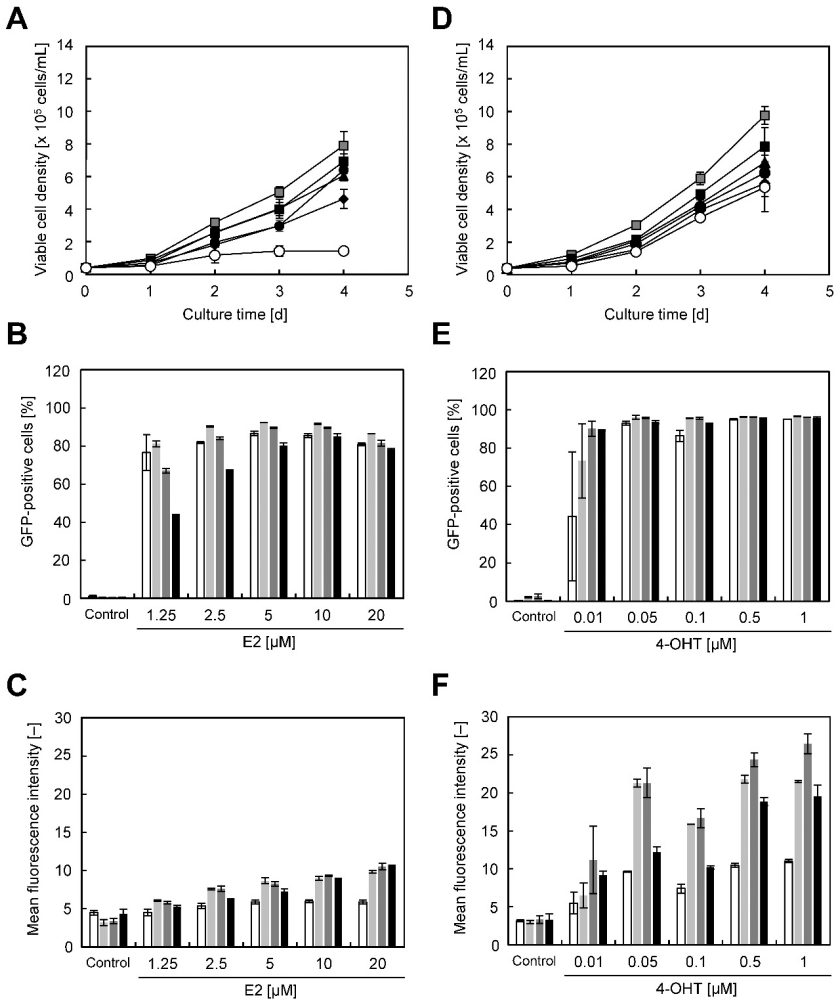

Figure 2 Inducible expression of reporter gene using E2 or 4-OHT. Growth responses of CHO/GEV_GFP cells upon the addition of E2 **(A)** or 4-OHT **(D)** . Symbols are indicated as follows. **(A)** Gray squares, no addition; closed squares, 1.25 μM; closed triangles, 2.5 μM; closed circles, 5 μM; closed diamonds, 10 μM; open circles, 20 μM.**(D)** Gray squares, no addition; closed squares, 0.01 μM; closed triangles, 0.05 μM; closed circles, 0.1 μM; closed diamonds, 0.5 μM; open circles, 1 μM. Percentage of GFP-positive cells at each concentration of E2 **(B)** or 4-OHT **(E)** . Open columns, day 1; light gray columns, day 2; dark gray columns, day 3; closed columns, day 4. Mean green fluorescence intensity at each concentration of E2 **(C)** or 4-OHT **(F)** . The distinction of columns is the same as **(B)** and **(E)** .

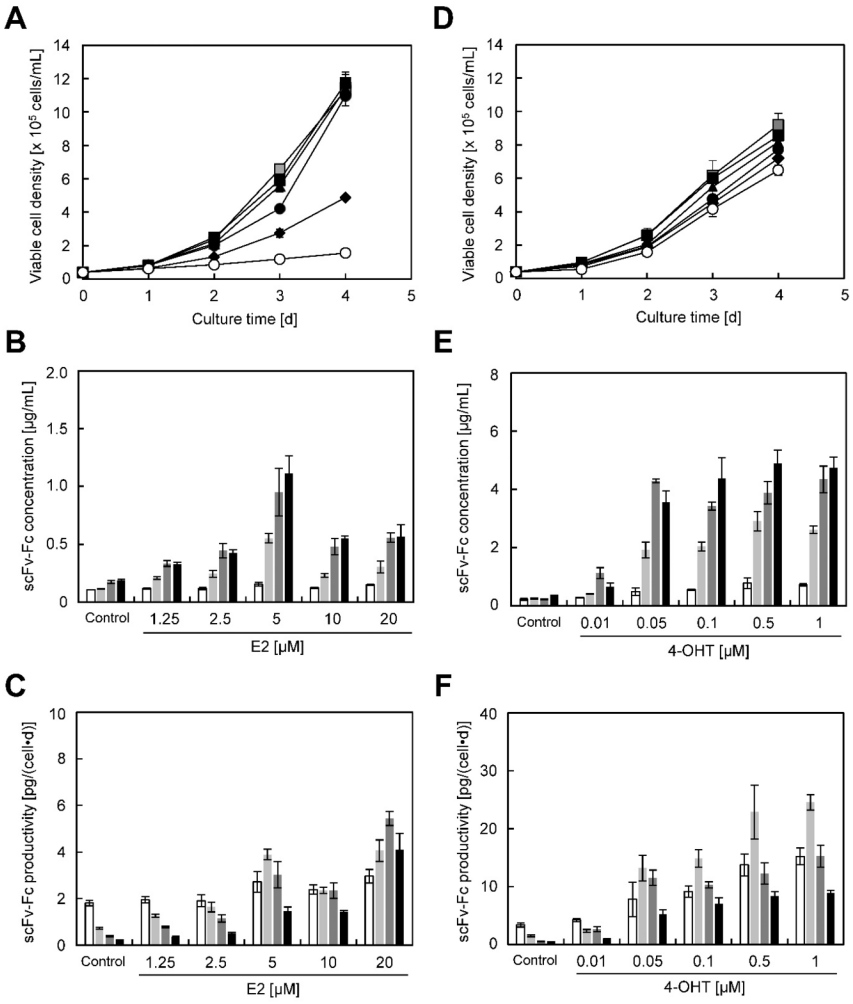

Figure 3 Inducible expression of scFv-Fc gene using E2 or 4-OHT. Growth response of CHO/GEV_scFv-Fc cells upon the addition of E2 **(A)**or 4-OHT **(D)** . scFv-Fc concentrations in media of cells cultured with each concentration of E2 **(B)** or 4-OHT **(E)** . Specific production rate at each concentration of E2 **(C)** or 4-OHT **(F)** . The distinction between symbols **(A and D)**and columns **(B, C, E and F)** is the same as described for Figure 2.

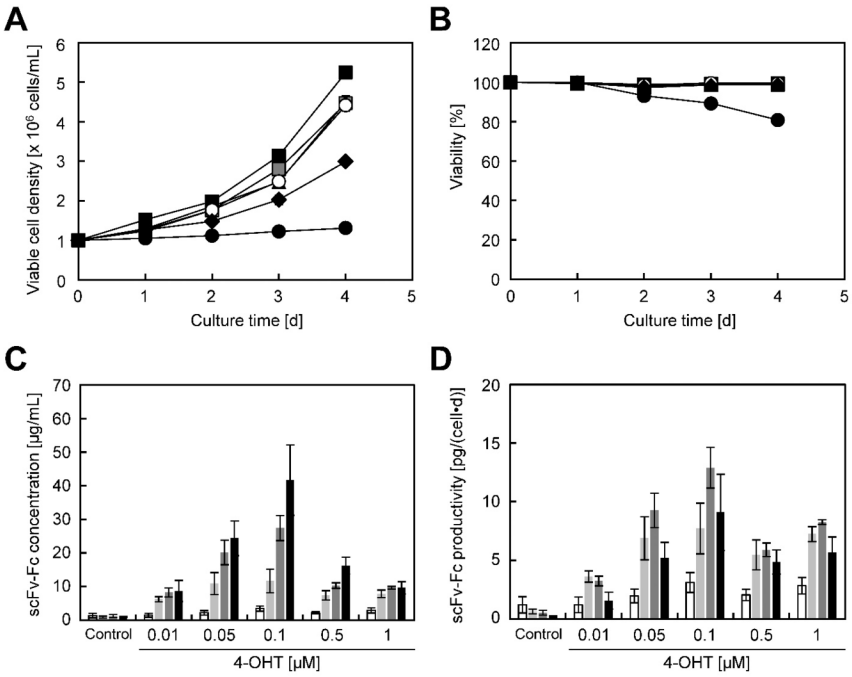

Figure 4 Serum-free culture of CHO/GEV_scFv-Fc cells with the addition of 4-OHT. Growth response **(A)** and viability**(B)** during culture. Symbols: gray squares, no addition; closed squares, 0.01 µM; closed triangles, 0.05 µM; open circles, 0.1 µM; closed diamonds, 0.5 µM; closed circles, 1 µM. scFv-Fc concentration**(C)** and specific production rate **(D)** during culture. The distinction of columns is the same as described for Figure 2.

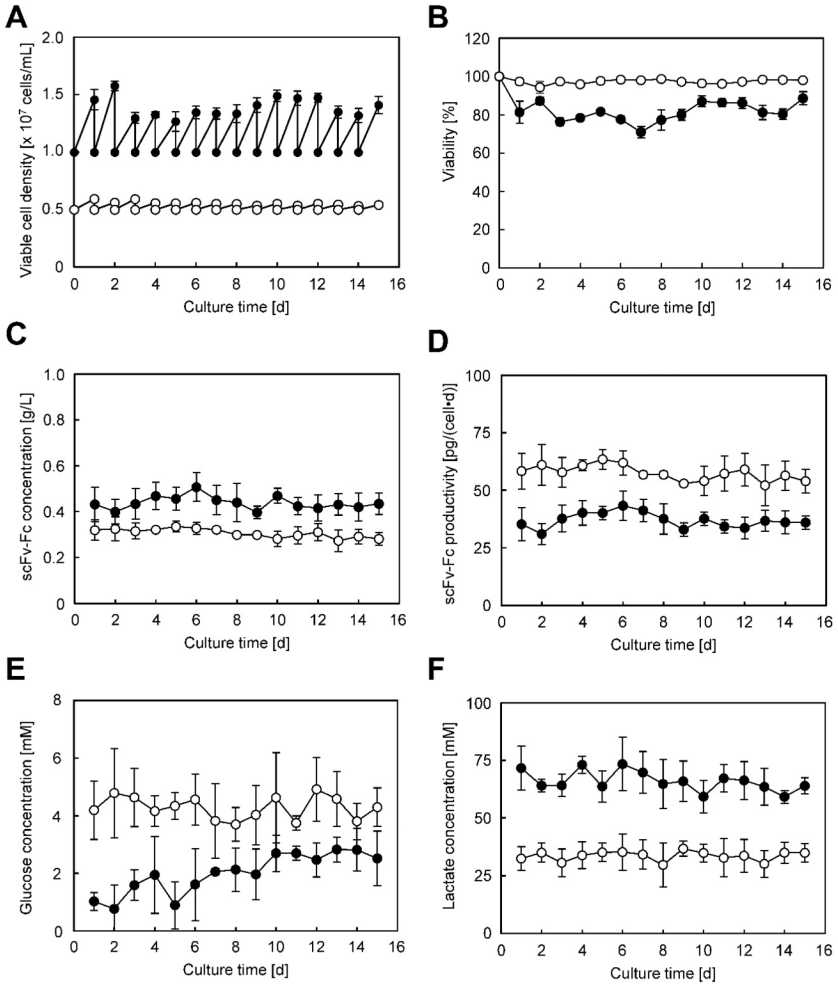

Figure 5 Continuous production of scFv-Fc in semi-continuous cultures with high cell density. CHO/GEV_-scFv-Fc cells (10 mL) induced with 0.1 μM 4-OHT were seeded in 50-mL bioreactor tubes at cell densities of 0.5×10^7 cells/mL and 1.0×10^7 cells/mL. Cells were counted the day after seeding. The same seeding density was prepared in fresh medium containing 0.1 μM 4-OHT and then reseeded. **(A)** Viable cell density. **(B)** Viability. **(C)** scFv-Fc production concentration. **(D)** Specific production rate. **(E)**Glucose concentration. **(F)** Lactate concentration. Symbols: open circles, 0.5×10^7 cells/mL; closed circles, 1.0×10^7 cells/mL.

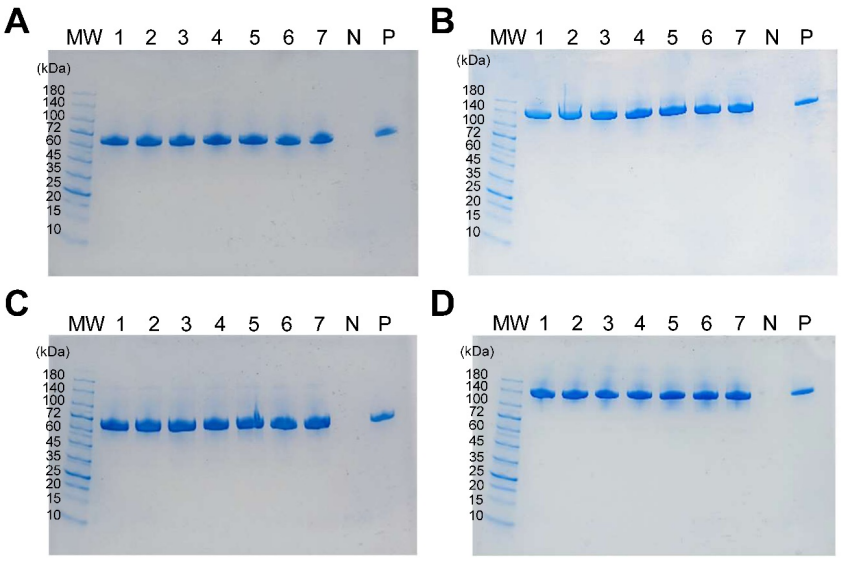

Figure 6 SDS-PAGE analysis of medium samples harvested from semi-continuous cultures. Medium samples (10 μL) from cultures seeded at cell densities of 0.5×10^7 cells/mL **(A, B)**and 1.0×10^7 cells/mL **(C, D)** were applied to SDS-PAGE analysis under reducing **(A, C)** and non-reducing**(B, D)** conditions. Lane MW, molecular weight markers; lanes 1–7, medium samples harvested at day 2 (lane 1), day4 (lane 2), day 6 (lane 3), day 8 (lane 4), day 10 (lane 5), day 12 (lane 6), and day 14 (lane 7); lane N, fresh culture medium; lane P, purified scFv-Fc (2 μg).