Estrogen-inducible transgene expression in CHO cells for producing recombinant proteins

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Contents

Chapter 1

Introduction

Biological research advanced in the last century identified biomolecules with therapeutic potential for their use in medical purposes. Among them, antibodies and insulin are collected by direct extraction from their native sources, but the yield was low for medical use. Moreover, advances in synthetic and semi-synthetic pathways showed large-scale production alternatives. However, technical shortages and costs made those initiatives impractical and unrealistic for some biochemical [1].

In the mid-1970s, hybridoma technology and genetic engineering were introduced. These technologies augmented the establishment of biopharmaceutical companies producing therapeutic proteins. The demand for therapeutic Biopharmaceuticals has dramatically increased in the last decades. The efficacy of Biopharmaceuticals makes it a treatment choice on the priority list. The Biopharmaceutical industry is looking to improve the volumetric productivity of recombinant proteins. Advancements in mammalian cell culture technology, especially the optimization of cell culture medium, cell engineering, and prolonged cell viability, enable the production titer to cross 10 g/L of recombinant antibody.

Chinese Hamster Ovary (CHO) cell lines are used as a working horse to improve productivity in most industrial production. CHO cells can be engineered to make mutants for selectively producing proteins [2]. Because of robust cell lines, many researchers are working on it. FDA and other regulatory bodies approved biological systems using CHO cell lines for producing therapeutic proteins. CHO cell lines evolved; many derivatives, such as CHO-S, CHO-DG44, and CHO-K1 variants, emerged and were used by others.

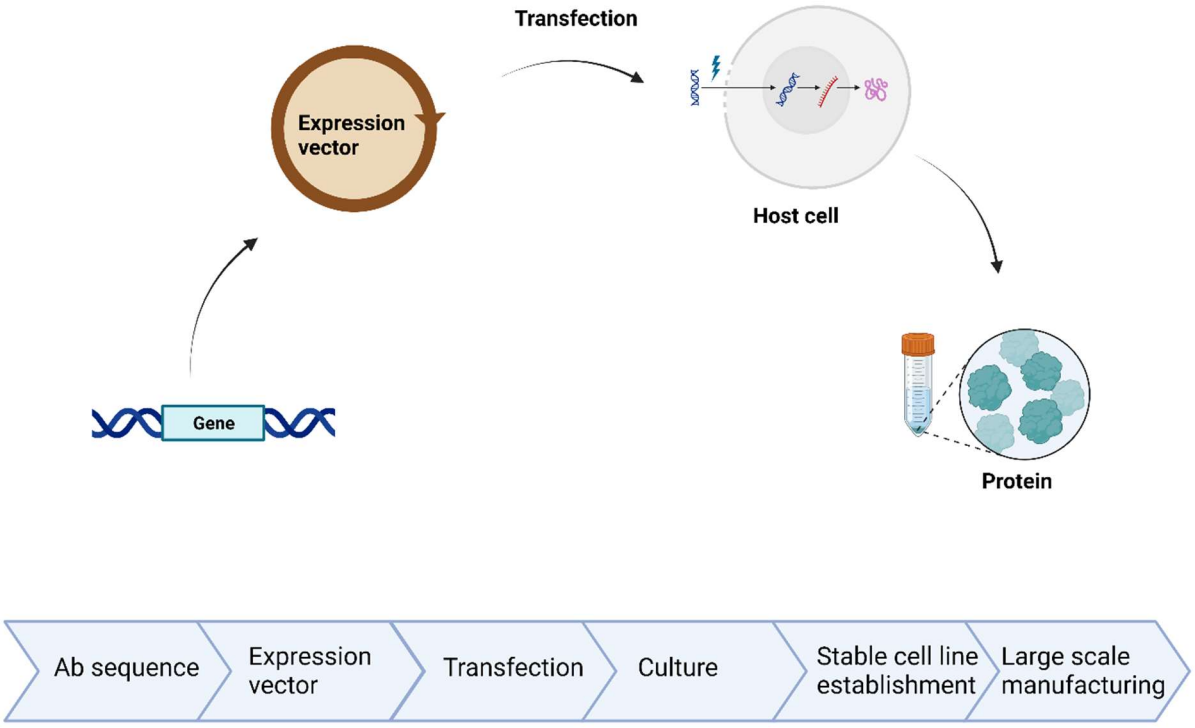

Figure 1 Steps of recombinant antibody production

Establishing stable cell lines is challenging because generating the expected cell clones takes time. Moreover, the growth rate of cells is a rate-limiting factor for volumetric productivity. In the initial growth stage, the cells' energy is utilized to proliferate the cells. So, the desired productivity cannot be achieved. Hence, controlling cell growth is a strategy as it can suppress cell proliferation for a specific time [3]. In the meantime, the energy previously used for the proliferation of the cells can be converted to increase productivity.

An inducible gene expression can be applied as a proliferation control strategy to control cell growth and productivity in the cell culture. In the inducible hormonal system, an exogenous drug binds with the transactivation system inside the cells. Upon drug application, the transactivator translocated from the cytoplasm to the nucleus of the cells and started transgene expression in an inducible manner [4]. Furthermore, this type of transgene expression system can regulate the tight control of the target

gene. The decoupling of cell growth and productivity in CHO cell lines is popularizing in the Biopharmaceutical industry as a proliferation control strategy. However, an ideal inducible transgene expression system may fulfill the current expectation.

1.1 Biopharmaceutical protein and its application

Biopharmaceutical is a subset of biological drugs manufactured using a biological process. In other words, the pharmaceuticals manufactured with the help of Biotechnology are called biopharmaceuticals [5]. Most biopharmaceutical drugs include proteins comprising hundreds or thousands of amino acids or nucleotides. Some examples of biopharmaceutical drugs are monoclonal antibodies (mAbs), hormones, clotting factors, enzymes, vaccines, nucleic acid-based, and engineering cell-based products. MAbs got the highest approvals from the regulatory bodies and entered the market in the last four years [6]. Some contain identical active ingredients but are commercialized under different trade names. The Biopharmaceutical market is expanding fast and has a great future because of enormous demands as the number of the older population is increasing daily, and the number of diabetes, cancer, and autoimmune disease patients has been raised. The high cost of developing biopharmaceuticals is a challenge to further expanding the market.

Biopharmaceutical drugs have transformed the existing treatment of different diseases. These drugs are widely used in all branches of medicine and proved to be effective treatment interventions for a broad spectrum of diseases [7]. The revolution of treatment becomes possible with the advancement of recombinant DNA and hybridoma technologies. Biopharmaceutical proteins are used in cancer, immune disorders, and infectious diseases [8]. Biopharmaceuticals and synthetic drugs differ in their identity, composition, manufacturing method, purification process and regulation for product approval. Usually, Biopharmaceuticals are produced by living cells, whereas chemical processes manufacture synthetic drugs. Due to their target specificity, biopharmaceuticals rarely showed side effects compared to conventional small-molecule drugs. Hence, it is recommended when traditional medicine responds poorly to patients.

Almost all synthetic drugs are small molecules. On the other hand, Biopharmaceutical is a larger molecule composed of many atoms. The process of identification and purification is more manageable in the case of synthetic drugs. Moreover, batch-to-batch variation is less in small molecular compounds. The biological expression system varies, so Biopharmaceutical production strictly monitors batch-to-batch variation. The mechanism of action is more complex in Biopharmaceutical drugs than in synthetic drugs.

In the last thirty years, Chinese hamster ovary (CHO) cells have been used extensively to produce therapeutic proteins, and these therapeutic proteins are produced in different cell lines of CHO cells, for example, CHO-S, CHO-K1, DX B11, DG44, and others [9,10]. Hence, CHO cells have become an established cell line for therapeutic protein production for the past three decades.

1.2 CHO Cell line as a host for Biopharmaceutical production

The mammalian cell line that produces recombinant therapeutic proteins is preferable in the biopharmaceutical market. The CHO cell lines draw the most attention among mammalian cells because they make complex therapeutic proteins with easy manufacturing adaptability. These cells can be grown as adherent or suspension cell culture conditions desirable in the industry due to the option of large volume can be applied for the bioreactors. The gene transfection, manipulation, and clonal selection in CHO cells are well known. Day by day, CHO cells become one of the preferred expression systems for therapeutic protein production compared to other systems because they can proliferate in a serum-free medium with high cell concentrations [11,12].

The choice of CHO cell lines relies upon some advantageous features. One advantage is that CHO can produce properly folded recombinant protein with human-like post-translational modification, which is compatible and bioactive in the human body [13]. Moreover, CHO cells are considered safe hosts as they can resist human viral infection because they do not express viral entry genes [14,15]. The safe host criteria enable regulatory bodies such as the FDA to approve the therapeutic protein to enter the markets.

Furthermore, because of the development of high-producer CHO cell lines, the biopharmaceutical industry has dramatically improved productivity in grams per liter. The specific productivity has increased several folds in pg/cell/day than the yields obtained in the late 1980s [16, 17]. This volumetric increase in productivity comes from the advancement in process control and the improvement in media composition. However, a CHO cell culture medium is added with 10% fetal bovine serum (FBS), a risk factor for contamination and protein purification [18]. All these characteristics make the CHO cell lines the choice of host cells for therapeutic protein production with maximum attainable yields.

1.3 Challenges of developing CHO cell line for producing Biopharmaceuticals

The increasing demand for biotherapeutics necessitates the continuous development of CHO cell line-associated production technologies. Numerous challenges exist to increasing the specific productivity of target proteins in CHO cell lines. The major obstacles are the vector construction, transfection efficiency and transgene integration into the specific chromosomal locus in the CHO cells. Another limitation is the availability of a high-producer cell line because it takes a long time and labor to establish it. Moreover, heterogeneity and instability of the CHO cell line hugely impact the improvement process. Therefore, targeted transgene integration into a specific genomic locus capable of high and stable transgene expression might be a solution to reduce the tedious screening process [19]. The site-specific integration with advanced gene-editing technology, such as CRISPR-Cas9, further helps develop CHO cell lines for transgene expression of the target protein [20]. In addition, high throughput screening and epigenetic regulation have been designed to overcome the limitation of CHO cell line development.

Moreover, the genome sequence data of CHO-K1 is now available, which can be used to understand clonal variability and further help to face the challenges imposed by the CHO cell line development process. The genomics data will identify the target site of the genome where the gene of interest will be integrated and will improve the problems caused by clonal heterogeneity. The

transcriptomic analysis will determine the cell lines' low productivity and instability characteristics [21]. These efforts will reduce the limitations and challenges recently faced by the CHO cell line technologies. That is why high-producer cell lines comply with market requirements, urging novel **research to fulfill the needs.**

1.4 Strategies for improving biopharmaceutical productivity in CHO cells

Several strategies have been taken to improve biopharmaceutical productivity in CHO cells. Reactive oxygen species are generated during respiration metabolism in large-scale biopharmaceutical production. Different approaches are established to decrease oxidative stress in CHO cells [22]. Biopharmaceuticals can be produced efficiently by modifying gene amplification and vector engineering [23]. Preventing programmed cell death could be a strategy to improve the specific productivity in CHO cells [24]. Recent advances, for example, developing culture media, using disposable bioreactors, and improving downstream processing, can increase biopharmaceutical production [25]. Site-specific integration is an efficient method for establishing a high-producing recombinant CHO cell line [13]. For example, an accumulative site-specific gene integration system has been developed [26]. Cell engineering used for cell line development, such as anti-autophagy, could increase cell productivity [27]. Metabolites added into cell culture media can increase monoclonal antibody production in the CHO cells [28]. CHO cell death due to apoptosis can reduce biopharmaceutical productivity. Hence, anti-apoptotic cell line engineering can decrease apoptosis in CHO cells [29]. Vitreoscilla hemoglobin expression in CHO-K1 cells can increase its specific growth rate in industrial production [30]. The output of non-monoclonal antibody production can be improved by a versatile target gene screening platform [31]. Anti-apoptotic genes Bcl-x L and Mcl-1 can enhance anti-PD1 antibodies in CHO cells by reducing apoptosis levels [32]. Recombinant scFv-Fc antibody production can be improved by introducing a production enhancer DNA element into the expression system [33]. Valproic Acid addition to the cell culture process can increase biopharmaceutical protein production in CHO cells [34]. Adding purines and pyrimidines can improve

the production of recombinant proteins in a CHO cell line [35]. Overall, the continuous demand for therapeutic protein requires a cost-effective manufacturing process.

1.5 Estrogen and its derivatives Tamoxifen action in CHO cells

Estrogens intensely affect various target cells' physiological functions, including the reproductive, cardiovascular, bone, liver, and brain [36-38]. The action of estrogen is interposed by estrogen receptors, namely, ERα and ERβ [39,40]. These effects are regulated when the estrogenic ligands bind with the estrogen receptor subtypes, further exerting gene expression [41-43]. Estrogens are responsible for the growth and advancement of breast cancer. However, if it is possible to inactivate estrogen receptors, the gene expression and cell proliferation mediated by the estrogen receptors will not be regulated. The antiestrogen drug Tamoxifen works following the mentioned mechanism of action [44,45]. Tamoxifen is used to treat breast cancer as one of the antiestrogens and antagonists of ER [46-48]. Tamoxifen binds to the DNA binding domain of ER of its activation, exerts estrogen-like effects [49], and shows cytotoxic effects in many cell types, such as melanoma cells [50] and prostate cells [51] erythrocytes [52]. Some studies indicated the impact of Tamoxifen on the human ovary [53,54] and rat uterus ovary [55,56]. Tamoxifen also has effects on the Chinese hamster ovary cell line. It is proved that Tamoxifen has increased [Ca2+] in CHO-K1 cells [57]. Estrogen receptor agonist butyl 4-(butyryloxy) benzoate can induce glucose transporter 4 (GLUT4) expression in CHO-K1 cells [58]. 4-Hydroxytamoxifen (4OHT) is an active metabolite of Tamoxifen that exerts anti-estrogenic activity [59]. 4OHT binds estrogen receptors (ER) and estrogen-related receptors (ERR) with estrogenic and anti-estrogenic effects. Cytochrome P450 2D6 forms this metabolite in the human liver and is a potent and selective estrogen receptor antagonist. It is proved that 4OHT has 1.6 times more binding affinity than E2 for the ER receptors [60]. 4OHT works as a mixed agonist/antagonist with ERα but pure antagonists with ERβ. The 4-OHT action in CHO cells has not been investigated yet. My research aims to see the 4-OHT inducible gene expression in the CHO-K1 cell line.

1.6 The aim of the research

Over the last decade, biopharmaceuticals produced using animal cell culture have increased due to the demand and efficacy of approved biopharmaceuticals, such as monoclonal antibodies [61, 62]. The advancement in animal cell culture technology for manufacturing biopharmaceuticals contributed to the success of biopharmaceutical products. Understanding cellular metabolism and physiology facilitates designing bioreactors that significantly improve the volumetric yield of the biopharmaceutical product. It is even possible to manufacture the product with serum-free media, which confirms the product is free from contamination. Many areas exist that still can be improved to increase productivity.

The biopharmaceuticals obtained from the CHO cell culture correlate with the cell numbers. Cell growth in a standard cell culture consists of four phases: lag, log, stationary, and decline. Unexpectedly, the lag phase is longer. Therefore, it is crucial to have high growth rates at the beginning phase to obtain maximum productivity [3]. Moreover, if cell proliferation is not checked, the cell density becomes so high that depletion of nutrients and oxygen, accumulation of toxic metabolite, cell death, and degradation of the products could occur [63-65]. In this regard, cell engineering [66, 67], cell cycle inhibitors [68], amino acid depletion [69], and temperature shift [70] have been investigated to control cell proliferation.

Since the culture cost is high in animal cell production, it is required to increase cell productivity. To do so, several cell engineering strategies, for example, increasing cell proliferation and protecting from apoptosis, have improved productivity. Usually, daily substances are produced simultaneously as cell proliferation in the current mainstream output by fed culture. However, the cells' energy is used only for multiplication in the proliferation phase to reach high cell density quickly. Though high cell density is a positive factor for increased productivity, uncontrolled proliferation is unacceptable. Consequently, controlling cell proliferation throughout the production process with targeted specific productivity could be a strategy in Biopharmaceutical production in CHO cells.

Currently, fed-batch culture is the most widely used method for producing biopharmaceuticals. Still, there is an urgent need to develop a production method incorporating continuous culture due to its various advantages, such as reducing the scale of facilities and costs. In the production of biopharmaceuticals, cell lines suitable for continuous culture are required to produce at high cell densities by controlling proliferation and substance production at arbitrary timings.

Synthetic biology has the significant advantage of offering an industrially suitable approach for decoupling growth and production. However, it has advanced in microbial systems while mammalian cells remain unexplored. The proliferation control strategy in mammalian cells is advantageous over conventional ones [71].

Our laboratory has previously found that adding estradiol (E2) suppresses the proliferation of CHO-K1 cells, which are widely used in biopharmaceutical production. Therefore, we thought that if it is possible to control the growth and production of CHO-K1 cells by administering E2, this cell line will be suitable for continuous culture in the production of biopharmaceuticals. In this study, we aimed to create a cell line suitable for continuous culture in which growth and production can be controlled by adding 17β-estradiol (E2) and its derivative 4-Hydroxytamoxifen (4-OHT) to CHO-K1 cells.

It is also possible to divert the energy after getting the target cells and then switch to the production stage to produce genes of interest with high efficiency. As a result, stable production of the target gene of interest or protein will be obtained as expected.

Proliferation ↔ Productivity

I focused on a production method that can switch between proliferation and production to produce highly efficient and stable biopharmaceutical protein. Constructing genetically engineered CHO cells that allow switching between cell growth and production can help efficiently produce biopharmaceuticals. In this study, we attempted to develop a production system that enables this concept in CHO-K1 cells. In my experiments, I used the hormonal drugs E2 and 4-OHT to control cell proliferation while maintaining cell productivity. E2 can suppress the growth of the CHO-K1 cell

line. I applied the UAS/GAL4 transgene expression system to describe an inducible gene expression system that expresses the Gal4 transcription factor fused to the estrogen-binding domain of the human estrogen receptor and the transcriptional activation domain from the viral VP16 protein. This study used Gal4-ERT2-VP16 (GEV) in CHO-K1 cells to increase cell proliferation and target substance production by adding 4-OHT. It is known that GEV is used as a control. GEV has ERT2, in which 4-OHT binds. Gene expression is controlled by adding 4-OHT by utilizing nuclear translocation only in the presence of 4-OHT. I hypothesized that GEV would confer rapid induction of transgenes following 4-OHT exposure in CHO-K1 cell lines. In my study, the proliferation control strategy with inducible transgene expression is applied in CHO-K1 cells to improve productivity.

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Chapter 2

The application of inducible gene expression systems in mammalian cells

2.1 Introduction

Inducible gene expression systems allow researchers to control the activity of genes in mammalian cells and animals. Inducible gene expression is a cellular process in which cells respond to external stimuli to produce new proteins. An inducible transgene expression system is a type of gene regulation in which chemical or environmental signals control the expression of a gene. Usually, the expression of a gene can be turned on or off depending on the presence or absence of the inducing agent. In the inducible gene expression, the genes rapidly activate in response to the inducer and back to the basal state when removed [1].

Several strategies have been developed to achieve inducible gene expression. One approach is based on components of the tetracycline resistance operon, which has been improved over time. Another promising approach involves non-mammalian steroid hormones and small molecules [2]. For effectiveness, inducible gene expression systems should meet specific criteria. They should not interfere with cellular pathways, have minimal basal activity in the inactive state, rapidly generate high levels of gene expression in the active state, be able to penetrate all tissues, including the placenta and blood-brain barrier, be specific to exogenous non-toxic drugs, and allow for rapid, robust, precise, and reversible induction of gene activity [2].

The inducible gene expression systems in mammalian cells have various applications in molecular biology research and biotechnology. The most common applications include studying gene function, producing recombinant proteins, gene therapy, drug screening and toxicity testing. Thus, inducible gene expression systems in mammalian cells provide a versatile platform for biological and biotechnological applications.

Numerous inducible systems to regulate gene expression in mammalian cells have been developed. For example, steroidal hormone inducible gene expression has been introduced in the mammalian cell [3-7]. Besides hormonal regulation, the tetracycline-based control system was applied in mammalian cells for gene expression [8]. Following the example of tetracycline, other antibiotics, such as streptogramin and macrolide-based gene control systems, were used in mammalian cells [9-11]. Furthermore, cumate and tamoxifen can trigger gene expression in mammalian cells [12-13]. In mammalian cells, inducible gene expression is often achieved using the tetracycline-inducible or hormone-inducible systems. In the tetracycline-inducible system, the expression of a target gene is controlled by the presence or absence of tetracycline or its analog, doxycycline. On the other hand, in the hormone-inducible system, the expression of a target gene is controlled by the presence or absence of a specific hormone.

These gene expression systems have unique characteristics that make them more suitable than others. An ideal system will be specific, non-interfering with other cellular pathways, rapidly inducible, reversible, and show dose-dependent induction of the target gene [2]. The ideal inducible system also acts as an endogenous system that will be activated upon applying an exogenous drug. Therefore, the regulated expression system will tightly control the transgene after integration into a cell line, activate or deactivate the gene transcription rapidly, and quickly adapt to cell cultures. Furthermore, the inducer will be readily available to all tissues, should be reversible, and should induce gene expression according to concentration.

Overall, inducible gene expression systems are valuable tools for researchers and clinicians to manipulate gene expression in a controlled and regulated manner. This review paper presents the available inducible gene expression systems in mammalian cells. The classification and characteristics of several inducible expression systems and their applications will be informative to those experts conducting research in the relevant field.

2.2 Small drug inducible gene expression system

2.2.1 Tetracycline inducible expression system

The Tetracycline-based system regulates gene expression in response to the presence or absence of the antibiotic tetracycline or its analog, doxycycline. This system is widely used in molecular biology and biotechnology applications to control gene expression in vitro and in vivo. The tetracycline-based transcriptional control is one widely used system for regulating transgene expression [8,14]. The tetracycline-inducible system has successfully controlled the gene in many cell cultures. Due to the pharmacological characteristics of tetracycline, it can also be used in gene therapy. This system consists of a "Tet-Off" and a "Tet-On" system. The system has a transactivator called tTA, which functions under the control of a promoter fused to the VP16 activation domain. This tTA transactivates a target gene of interest bound with a tet operator (tetO). For binding DNA, tTA necessitates tetracycline or its derivative, doxycycline. The terminated target gene expression is called the Tet-Off system when doxycycline is present.

On the other hand, the absence of doxycycline causes transcription of the gene of interest, which is regarded as the Tet-On system [15]. The specific interaction of TetR with tetO, and the massive inducibility with tetracycline derivative on and off mechanism makes this a desirable regulation system [8]. That is why this system was successfully applied in many cell and animal models. Transcriptional activation by the Tetracycline-based system is used in the mammalian cell to establish a regulatory system for tightly controlling gene expression [8, 16]. Then, it is widely used as an inducible gene regulation in mammalian cells. However, the tetracycline-based system has drawbacks of background leakage; tetracycline-derived contaminants can be present in the cell cultures [17]. The impurity can be reduced by using the tetracycline-free fetal bovine serum. The tetracycline-induced operator system also limits inducibility; during continuous cell culture, some cell lines can lose their inducibility [18]. Later, this regulatory system was modified and introduced in the mammalian cell. The emphasis was given to reducing the leakage of the system and making the system precise and controlled.

2.2.2 Cumate-inducible expression system

The demand for different regulatory systems for controlling gene expression in the mammalian cell paves the way to finding a new system. The need for various regulatory systems comes from controlling several genes independently. The limitation of background expression or leakiness of the inducible system, another operator system, and a cumate inducible system have been developed. Cumate is a non-toxic small molecule that can be added to the cell culture. In an ideal system, the inducer needs to be an inert molecule. The cumate inducible system works by three components: the cumate operator (CuO), its repressor (CymR), and the inducer cumate. CuO is found downstream of the promoter and upstream of the transgene. The regulation occurs whether the CymR binds with the CuO or not. When the cumate is added, it associates with the CymR, and the transgene transcription occurs. But without cumate application, CymR is tightly bound with CuO; hence, the transcription does not proceed [12]. A cumate system is desirable because it can be improved because of its modular nature. Thus, this system is further developed to knock down gene expression [19]. The Cumate inducible system offers tight control of gene expression, easy adaptability, and reversible gene expression.

2.2.3 IPTG inducible expression system

IPTG is one of the most used inducers for mammalian cells. The regulation of gene expression using this inducer offers excellent advantages that facilitate gene function studies. Currently, several methods have been developed for the regulation of gene expression in mammalian cells. Among them, one of the employed inducible systems is the Lac operon system induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG has a similar molecular structure to allolactose and a strong affinity for the corresponding repressor. Moreover, IPTG can quickly penetrate mammalian cells and exhibits low toxicity [20,21]. A research group provides information about developing and optimizing an IPTG inducible gene expression system and using lactose as an inducer in combination with IPTG to enhance gene expression [22]. They observed that co-treatment with IPTG and lactose could induce

higher GFP expression than treatment with IPTG or lactose alone. Another study describes the development of an IPTG-responsive lac repressor-operator-controlled RNA polymerase III system for inducible gene silencing in mammalian cells [23]. This system allows for conditional inhibition of gene expression in a dose- and time-dependent manner by administration of the inducing agent IPTG. It can be used in established stable clones and transient transfection cells, providing convenience and versatility in gene silencing experiments.

2.3 Hormone-inducible gene expression systems

2.3.1 Estrogen inducible expression system

The estrogen-inducible system is a method of regulating gene expression in response to the hormone estrogen. In this system, a target gene is placed under the control of an estrogen response element (ERE) in the promoter region. Without estrogen, the estrogen receptor (ER) is inactive and does not bind to the ERE. Upon estrogen binding, the ER becomes activated and recruits co-activators that increase gene transcription, leading to the induction of gene expression. The system provides a highly regulated and reproducible way to control gene expression in vitro and in vivo.

Estrogens tremendously affect reproductive cells, especially in females [24], and the skeletal, cardiovascular, and central nervous systems. Consequently, pharmaceuticals based on estrogens have been developed to treat infertility, breast cancer, and osteoporosis. A receptor protein mediates the biological activity of estrogen. At first, it was thought that only one ER is responsible for estrogens' physiological and pharmacological action. Later, a second estrogen receptor was discovered. So, by binding with two estrogen receptors, ERα and ERβ, estrogens can regulate the transcription of endogenous genes. The ERα and ERβ are members of the hormone superfamily of nuclear receptors, and they are ligand-dependent transcription factors that can control the expression of target genes. Synthetic estrogen and antiestrogen ligands have been developed to study estrogen actions [25]. The estrogen's mechanism of action consists of estrogen receptors with their ligands and effectors.

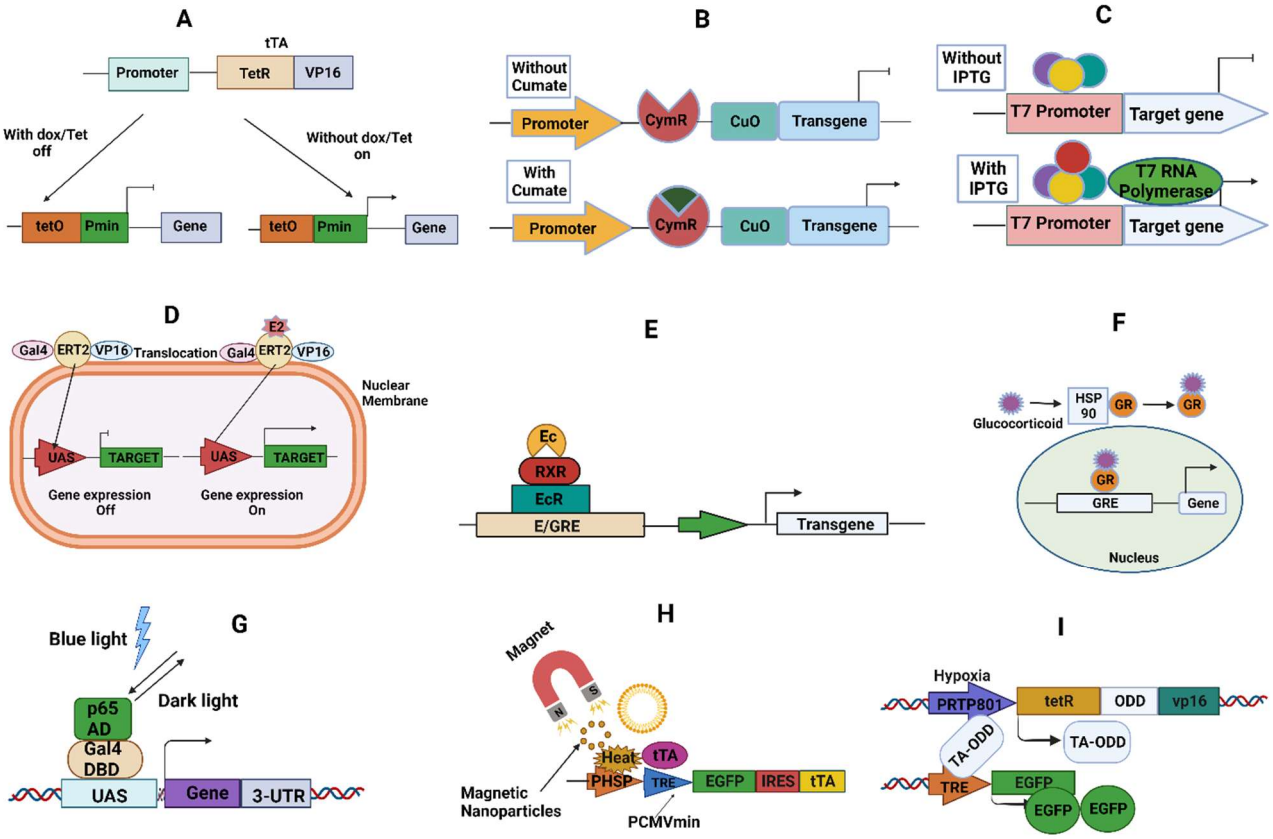

Figure 1 Inducible gene expression system. **(A)** Tetracycline (Tet)-inducible expression system **(B)** Cumate-inducible expression system **(C)** IPTG-inducible expression system **(D)** Estrogen inducible expression systems **(E)** Ecdysone-inducible expression system **(F)** Glucocorticoid-inducible expression system **(G)** Light-inducible gene expression system **(H)** Heat inducible expression system **(I)** Hypoxia-inducible expression system.

When the ligand binds with the receptor, it makes conformational changes, giving the responses [26-28]. The ligand-binding domain of the receptor binds with the ligand and directs the receptor to the active transcriptional sites. Endogenously, E2 acts as the ligand that binds with ERs, which interact with estrogen-response elements (ERE) DNA sequences. The ERE-bound ERs that transcripts target genes [29,30]. The ERE sequence has differential effects on the ERs. When the ligand is added to the mammalian cell, the different ERE sequences show differences in the interaction with the estrogen receptors for modulating the receptor-ligand actions [30].

2.3.2 Glucocorticoids inducible expression system

Steroid hormones play essential roles in the physiology and development of mammalian cells. After entering the target cell, steroidal hormones bind with their intracellular receptors, modulating the transcription of the gene of interest [31]. Steroidal hormone inducible gene expression is the regulation of gene expression by steroids, a type of hormone. Several hormonal-modulated systems were introduced to control the mammalian cell's gene transcription [32,33]. The most used steroid hormone in gene regulation is a glucocorticoid, which can bind to the glucocorticoid receptor. The glucocorticoid inducible system is commonly used in molecular biology research and provides a highly regulated and reproducible way to control gene expression in vitro and in vivo. These systems are active in both transiently and stably transfected cells. The hormone-modulated system consists of an inducible promoter, a receptor, and an inducer drug. A hormone-inducible gene expression system uses biologically active inducers to control the transcription of the target gene. The inducer hormone only transcripts when added to the system and terminates expression without it. One of the exciting points of hormone-inducible gene expression is that it does not alter cell growth and metabolism, unlike other inducible expression systems, which require heat shock proteins. In the steroidal hormone inducible gene expression system, the steroid hormone receptors (SHRs) control gene expression in response to ligands [34,35]. The SHRs are essential in cell differentiation, proliferation, and metabolism [36].

2.3.3 Ecdysone inducible expression system

Glucocorticoids and other steroidal hormones are usually used in mammalian cell culture to induce gene expression. However, the steroidal insect hormone, 20-OH ecdysone, can be used in an inducible system to regulate gene expression in the mammalian cell. The ecdysone-based system has low basal activity and high inducibility and can penetrate all tissues. It has good pharmacokinetics with easy clearance [6]. Compared to the tetracycline-based system, the ecdysone-based inducible system is better inducible, has fast clearance, and has no toxicity or teratogenic effects. However, the previous Ecdysone inducible system has limitations [6, 37-39]. After that, an improved ecdysone-based

regulatory inducible system was applied in the mammalian cells [40]. This format showed the lowest basal activity and highest inducibility.

2.4 External environment inducible gene expression system

2.4.1 Hypoxia-inducible expression system

The decrease of available oxygen in the cellular environment or hypoxia may induce gene expression in the mammalian cell. Lack of oxygen produces hormones, cytokines, and enzymes due to hypoxia, causing the expression of the respective genes [41]. One study found that hypoxia-inducible RTP801 promoter and transactivator RTP801/TA-ODD help induce reporter gene expression in an oxygen-concentration-dependent manner [42]. The hypoxia-responsive VEGF gene expression system has promising results for inducing angiogenesis during three-dimensional (3D) tissue construction [43]. Recently, a study was done where engineered macrophages express protein in hypoxic conditions [44].

2.4.2 Heat-inducible expression system

Controlling therapeutic genes with a suitable inducible system is essential in gene therapy. The existing inducible systems cannot switch off gene expression. Thus, efforts have been made to develop a new inducible system for controlling gene expression spatially and temporally. In doing this, a novel heat-inducible gene expression system has been developed [45, 46]. In these inducible systems, the thermal heat generated by magnetic nanoparticles under an alternating magnetic field has been used to activate gene expression. When hypothermia is provided to induce gene expression, it is considered a non-toxic and non-invasive alternative. Even liver function can be enhanced with heat-inducible gene expression, as shown in a study [47].

2.4.3 Light inducible expression system

Light has the advantage of manipulating or controlling cellular functions, for example, gene expression, because it can be switched on or off at the user's end. It can also be applied to a cell or group of cells. Using light to regulate biochemical reactions, photoactivatable (PA) compounds, which can be

obtained from plants, fungi, and bacteria, were developed. PA compounds can activate ion channels or signaling pathways at the cellular level. Channelrhodopsin-2 is a light-sensitive ion channel that activates under blue light illumination and allows ions to pass into the cell through the cell membrane, which depolarizes the cell membrane [48]. Light controllable gene expression system is a recent development in inducible gene expression that utilizes photoactivatable molecules to control gene expression [49-51]. The light-inducible strategy uses blue or red/far-red wavelengths that can precisely control temporal and spatial manipulation of targeted downstream gene expression [52]. For this purpose, a blue light-inducible Gal4/UAS gene expression system has been introduced in mammalian cells. This system consists of a cryptochrome 2 (Cry2) photoreceptor with its binding protein cryptochrome-interacting basic-helix-loop-helix 1 (CIB1). They optimized and developed a set of PA-Gal4 transcriptional activators that can precisely control gene expression in a spatiotemporal manner [53]. Compared with small molecule applications with unexpected pharmacological activity, light is non-toxic and inert to mammalian cells.

2.5 Combined inducible gene expression systems

DNA-specific site-specific recombinases (SSRs) are helpful in mammalian genetics to trigger excision, integration, inversion, and translocation of DNA substrates in living cells at precise recognition sites [54]. The two commonly used SSRs in genetic engineering are Cre recombinase, obtained from bacteriophage P1, and FLP recombinase, obtained from *S. cerevisiae*. Cre and FLP enzymes mediate site-specific recombination at defined target sites, called locus of crossover (loxP) and FLP recombinase recognition target (FRT), respectively [55]. One study demonstrated that >75% of recombination in fibroblasts and mice, as well as human embryonic stem (ES) cells, can be obtained by the engineering of a recombinant cell-permeant FLP protein (TAT-FLP) [56]. This FLP system could create an inducible transgene expression cassette (FLP-ON-Cre-OFF) that allows reversible and tightly controlled gene expression.

Cre-mediated site-specific recombination is another combination of inducible gene expression

systems. Cre recombinase is most widely found to inactivate gene expression conditionally [57]. Usually, ER is fused with Cre recombinase activated when translocated to the nucleus. Cre-ER combination admits induction of recombinase activity in target cells on time. Cre removes stop cassettes from transgenes during gene activation, allowing reporter gene expression [57]. Inducible Cre recombination systems have been developed to bypass initial embryonic lethal phenotypes and provide access to later embryonic or adult phenotypes [58]. They discussed the production of Cre transgenic mice in which tamoxifen-inducible excision occurs in a widespread mosaic pattern.

Rapamycin-inducible gene expression is used in engineered T cell therapies to selectively deplete T cells after administration. One approach is using a rapamycin-activated caspase 9 suicide gene, which allows for controlled cell death [59]. This suicide gene is designed to be activated by rapamycin, a safe and widely available immunosuppressive drug. The rapamycin-induced caspase 9 suicide gene, called rapaCasp9, consists of the fusion of the FKBP12 and FRB fragments of mTOR with the catalytic domain of caspase 9. This single protein rapaCasp9 is functional when expressed with a CD19 CAR in vitro and in vivo [59].

CRISPR/Cas9 is a gene-editing tool that utilizes the CRISPR RNA-guided system to target specific genes and make precise modifications. The CRISPR/Cas9 system has successfully edited genes in various organisms, including bacteria and human cells. Using this system, gRNA can direct the DNA binding specificity of dCas9 to activate the human endogenous gene [60]. A group of researchers developed a system for temporal regulation of multiple genes using CRISPR/Cas9 activators and chemically induced dimerizing (CID) proteins. This system allows simultaneous activation of multiple genes and orthogonal regulation of different genes in a ligand-dependent manner with minimal background [61].

2.6 Practical production by applying inducible gene expression system

2.6.1 Cumate-induced protein production (Fc fusion) in CHO cell lines

Several gene expression systems have been explored for therapeutic protein production in CHO cell

lines. They have unique characteristics to be applied to protein production. Moreover, a compatible system like a cumate gene expression system is needed [12]. Cumate gene switch may induce rapid protein production using CHO cell lines.

Table 1: Various inducible system with its application in mammalian cells

Name	Types	Advantages	Reference
Small drug inducible gene expression	Tetracycline inducible gene expression	The system is tightly regulated, robust and relatively non-toxic to cells in culture.	86
	Cumate inducible expression	It is a versatile inducible expression system with fewer side effects, such as low background expression levels.	12
	IPTG inducible expression	It is a widely used and well-established inducible gene expression system.	22
Hormone inducible gene expression	Estrogen inducible expression	Rapid and best reversible switching for turning on or off gene functions.	85
	Ecdysone inducible expression	It has low basal activity and high inducibility, can penetrate all tissues and has good pharmacokinetics with easy clearance.	6
	Glucocorticoid inducible expression	It is a good candidate for an efficient genetic switch, offering potential applications in various biotechnology research fields.	84
External environment inducible gene expression	Hypoxia-inducible expression	Offer precise control and potential applications in hypoxia detection and monitoring.	42
	Temperature inducible expression	It enables remote control of gene expression in a space and time-selective manner.	46
	Light inducible expression	Optogenetic strategies using blue or red/far-red wavelengths offer the advantage of controlling gene expression with high specificity.	52
Combination	Recombinase (Cre/Flp)	It provides a high recombination efficiency in fibroblasts, mice, and human embryonic stem cells.	56
	Cre-ER	Allow for the induction of recombinase activity in specific cells at defined time points, providing temporal control over gene expression.	57
	Rapamycin-activated caspase9	Selectively depleted engineered T cells with a safety profile are widely available.	59
	CRISPR/Cas9	It offers various advantages, including precision, versatility, efficiency, therapeutic potential, flexibility, and programmability, making it a valuable tool in genetic research and engineering.	61

One study showed that CHO$^{BRI/rcTA}$ cell line could inducibly express recombinant protein with the help of a cumate gene switch [62]. This system is based on binding the cumate reverse transactivator (rcTA)

to the CR5 inducible promoter in the presence of cumate, a small, non-toxic inducer. The cumate-responsive $CHO^{BRI/rcTA}$ cells obtained the maximum recombinant protein production when a cumate concentration of 1-2 μg/mL was used [62].

The $CHO^{BRI/rcTA}$ stable pool can produce up to 900 mg/L for an Fc fusion protein and up to 350 mg/L for an antibody during 14 days in fed-batch cultures after induction by cumate. It is also shown that volumetric protein production could be enhanced 3-4 times higher with the CR5 cumate-inducible promoter than with the human CMV or hybrid EF1α-HTLV constitutive promoters [62]. This phenomenon proved that the cumate-inducible $CHO^{BRI/rcTA}$ stable pool is an effective system that can rapidly produce recombinant proteins.

In another study, the lentiviral vector and cumate gene-switch system induced protein production in CHO cell lines. In this study, CD200Fc (CD200 fused to IgG Fc region) was effectively produced with the help of the system [63]. This system significantly improved protein expression during induced conditions. Thus, the cumate gene-switch system is robust and contributes to recombinant protein production in CHO cell lines.

2.6.2 Hypoxia-induced expression in CHO cell lines

Hypoxia, a condition of oxygen deficiency, affects various physiological processes, including energy metabolism, autophagy, cell motility, angiogenesis, and erythropoiesis [64]. When biopharmaceutical proteins are produced on a large scale, the production cell lines face adverse hypoxic conditions. Thus, hypoxia negatively impacts biopharmaceutical protein manufacturing, reducing cell growth and productivity [65,66]. Previously, CHO cell lines used in biopharmaceutical production were not explored whether hypoxia may induce productivity. During the normoxic condition, hypoxia-inducing factor 1α (HIF-1α) destructed due to hydroxylation [67] may escape the process in hypoxic conditions. HIF-1α and HIF-1β heterodimers comprise a transcriptional complex that binds to hypoxia response elements (HREs) at the genomic loci [68,69] and can induce target gene expression. Following this phenomenon, CHO cells have been studied under hypoxia conditions. In one study, they developed

hypoxia-responsive expression vectors, which improved recombinant protein productivity when production cells faced hypoxia [70]. With this strategy, bioprocessing challenges like hypoxia have been encountered by optimizing the CHO cell line for induced protein expression.

2.6.3 Cumate-induced recombinant adeno-associated virus (rAAV) production in HEK 293 cell

On behalf of in vivo gene therapy, rAAVs are superb vectors because they have broad-spectrum tropism, lack pathogenicity in humans, and have longstanding transgene expression stability [71,72]. The primary method of rAAV production in HEK293 cells is transfection, but it could be problematic during large-scale transfection [73]. Moreover, constitutive promoters used in HEK293 cells make it difficult to isolate packaging cell lines and get unstable clones. To overcome this challenge, a research group studied inducible promoters in HEK293 cells [74]. The study used HEK293SF cells with a gene expression regulation system induced when cumate and coumermycin were added to create REP-expressing AAV packaging cells. Therefore, cumate was used as an inducer to regulate Rep expression and induce rAAV production in HEK293 cells. The choice of Rep proteins for rAAV production in HEK293SF cells was Rep68 with Rep40, as it was the combination with the highest rAAV yields [74]. It was also found that the best clones that produce rAAV titers were stable for about seven weeks in continuous cultures [74].

2.6.4 Cumate-induced recombinant Lentiviral (LV) production in HEK 293 cell

Lentiviral vectors (LVs) derived from the lentivirus can integrate genes into the chromosomes of the target cell. Because of this capability, LVs are used in genetically modified cells for gene therapy [75]. Due to the growth of gene therapy products, the demand for high-quality and high-titer LVs is expected from the used ends. The conventional method of transfecting plasmids containing genetic elements for LVs into the HEK 293 is neither cost-effective nor labor-intensive. Therefore, an efficient method and stable clone for producing LVs was expected. A technique was developed to obtain efficient packaging cells and stable producer cell clones to get LVs [76]. They have demonstrated tight control of the expression of cytotoxic packaging elements required for generating LVs in HEK 293 cells using a gene

regulation system, which could be induced by combining the cumate and coumermycin induction systems. In this study, the genetic elements of packaging cells were transfected into HEK 293 cells, and finally, clones were isolated by limiting dilution into nanowell arrays [76]. The best-isolated clone can produce LVs expressing GFP at a titer of 2.3 × 10^8 transduction units (TU)/mL in the culture medium. In this method, the clone 3D4 transfected with LV-CMV-GFP was induced with cumate to produce LV [76], which was collected after 48 hours of induction and concentrated by ultracentrifugation. LV-CMV-GFP production was evaluated in suspension culture in six-well plates at different time points after induction with cumate [76].

2.6.5 CAR-T cell-based inducible gene expression

CAR-T cell refers to chimeric antigen receptor T cell, a cell-based therapy that uses genetically engineered T cells to target cancer cells. CAR-T cell therapy has shown promising responses in clinical trials for B cell cancers, with up to a 90% complete response rate in patients [77-79]. Inducible gene expression systems are being explored in CAR-T cell therapy to control the activation and function of CAR-T cells [80], minimizing potential toxicities and side effects. One example of an inducible gene expression system is the tetracycline regulatory platform, where the expression of CAR components can be triggered by exogenous tetracycline or doxycycline [80].

Inducible gene expression in CAR-T cells can be achieved using recombinase-based gene circuits. These circuits enable controlled and regulated expression of the CAR gene. Recombinase-based gene circuits allow one-time state switching in CAR-T cells to control when and how strongly the CAR gene is expressed [81]. These gene circuits exhibit memory, meaning that the changes induced in the CAR-T cells are maintained even after removing the drug inducer, avoiding prolonged drug inducer exposure and reducing potential side effects. One study demonstrated that these gene circuits can control anti-Her2-CAR expression for regulating CAR expression and T cell activity [81]. Computational and experimental work is needed to understand the combined parameter space of drug dosage and duration for optimal inducible gene expression in CAR-T cells.

2.7 Tet-transactivation system for designer cells

2.7.1 Hepatoma cell line with heat-inducible system

Hepa cells are a type of hepatoma cells genetically modified to enhance their liver function. In a study, a genetically modified hepatoma cell line called Hepa/8F5 was developed by transducing eight liver-enriched transcription factor (LETF) genes into mouse hepatoma Hepa1-6 cells using a drug-inducible transactivator system [82]. Transactivation system in the context of the research paper refers to the Tet-transactivator system involving rtTA, which is utilized to control the expression of the eight liver-enriched transcription factor (LETF) genes in the genetically modified hepatoma cell line, Hepa/8F5. These cells can proliferate actively under standard culture conditions and be easily prepared in large quantities. The overexpression of LETFs in Hepa/8F5 cells is induced by the addition of an inducer drug, which leads to a change in cell morphology and high expression of liver functions [82]. However, the liver functions of these cells depend on the continuous presence of the inducer drug. To remove the requirement for continual drug addition, a heat-triggered LETF expression system was constructed in which the artificial transactivator was transcribed and amplified under the control of a heat-shock protein promoter and introduced into the genome of Hepa/8F5 cells. This modified cell line, called Hepa/HS, exhibited high, heat-inducible liver functions, making it a potential cell source for hepatic studies and the construction of bioartificial liver systems [83]. Moreover, there is no need to add a small molecular drug for cell proliferation with Hepa/HS cells. The newly constructed Hepa/HS cells present a new cell source, which may be considered for hepatic drug screening.

The researchers also developed a heat-inducible system in HepG2 cells to enhance liver functions [47]. The genetically modified hepatoma cells, HepG2/8F_HS, actively proliferate under standard culture conditions and can be easily prepared in large quantities. When the liver-enriched transcription factor (LETF) gene expression was induced by heat treatment at 43°C for 30 minutes, the cells ceased proliferation and demonstrated enhanced liver functions. Three-dimensional spheroid cultures of HepG2/8F_HS cells showed a further increase in liver functions upon heat treatment [47]. The heat-

induced liver functions of HepG2/8F_HS cells were enhanced by forming spheroids, which could be sustained for 9 days.

2.7.2 C2C12 cell and Hela cell with hypoxia-inducible system

A synthetic biology approach developed a hypoxia-responsive transgene expression system [42]. This system combined a hypoxia-inducible RTP801 promoter with a tetracycline-responsive transactivator fused with an oxygen-dependent degradation domain (TA-ODD). The system demonstrated high induction of reporter gene expression in response to hypoxia, both in transfected cells and in a stable cell line. These cells showed potential as sensors to detect hypoxic conditions in a three-dimensional tissue culture in vitro. The activity of the RTP801 promoter was not as strong as virus-derived constitutive promoters, so it was combined with the tetracycline-responsive transactivator system to amplify transcription levels. This system allows for precise control of gene expression in response to hypoxia and can potentially be used in constructing cell-based hypoxia detection systems.

2.8 Conclusion

Precise control of gene expression is desired in gene function analysis, developmental studies in biological systems and gene therapy. Several inducible gene expression systems have been applied in mammalian cell cultures to tightly control the gene of interest. Small molecule drugs, hormones, and external environmental factors could induce those inducible systems, including light, heat, and hypoxia-inducible gene expressions. Moreover, combined inducible systems such as Cre/Flp recombinase, Cre-ER, FKBP12 FRB, CRISPR and rapamycin-activated caspase9 are notable. Some systems have limitations and were modified to overcome the previous disadvantages. The ideal system requires fast inducibility, low basal background, no cytotoxic effect of the ligand, penetration into all tissues, including the brain and clearance from the body. Overall, inducible gene expression systems provide a versatile platform for controlling gene expression in a controlled and regulated manner. Following identifying the inducible gene expression system, the researchers are looking for a new inducible system with favorable characteristics for large biopharmaceutical manufacturing.

Inducible gene expression is a method of controlling the expression of a specific gene in response to an external stimulus, such as a drug or chemical compound. Inducible gene expression can be achieved by placing the gene of interest under the regulation of an enhancer sequence, and then the gene can be induced by regulating the expression of a specific protein binding to the enhancer sequence. Several inducible gene expression systems have been employed in mammalian cell cultures to control the gene expression spatially and temporally. Among them, small drug molecules such as tetracycline, cumate, and IPTG-induced gene expression have been explored. Hormonal inducible systems such as estrogen, glucocorticoid and ecdysone inducible expression systems are notable. External environment-induced expression systems, for example, light-inducible gene expression, hypoxia-inducible gene expression, and heat-inducible gene expression systems, which have comparative advantages, were also explored.

Moreover, combined gene expression systems have been applied in mammalian cells. An ideal inducible gene expression should be induced quickly, with no deleterious effects on the cell and will be regulated tightly with no basal level of gene expression. Light-inducible gene expression has recently gained interest due to its feasibility and adaptability in mammalian cell cultures. Heat-inducible and hypoxia-inducible gene expression systems are also promising in mammalian cell culture technology. The various inducible gene expression systems in mammalian cells have multiple applications in molecular biology research and biotechnology. The most common applications include studying gene function, producing recombinant proteins, gene therapy, drug screening and toxicity testing. Thus, inducible gene expression systems in mammalian cells provide a versatile platform for biological and biotechnological applications. A more desirable inducible gene expression system with more promise and prospects is expected to be in the research soon.

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Chapter 3

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

3.1 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. CHO cells as hosts in production systems have become an established method for producing therapeutic proteins, primarily due to their ability to proliferate in high-cell-density serum-free cultures [4].

Like humans, CHO cells can produce properly folded proteins with post-translational modifications [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 in enhancing the specific productivity of target proteins. These include the optimizations of transgene expression units, transfection efficiency and transgene integration sites in the CHO genome. Another limitation is the identification 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 procedures, media composition, and culture optimization have been extensively examined due to biopharmaceuticals' demand for and regulatory requirements [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 particular cells during specific periods [12]. Switching between growth and production phases may 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. Especially when the production of a target product hampers cell growth, employing inducible production methods capable of distinguishing between cell growth and production phases has proven advantageous and has been implemented in microbial cultures [13].

Recently, gene expression control systems have become essential techniques for analyzing various biological functions of both prokaryotes and eukaryotes. Different gene expression control systems have been developed based on artificial transcription activators, such as the tetracycline repressor-based transcriptional activation system [14] and the CRISPR transcriptional activation system [15]. 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 [16-24]. 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 utilized to construct biopharmaceutical production systems that can switch between cell growth and production modes. We employed an artificial gene expression system using the chimeric protein Gal4-ERT2-VP16 (GEV), activated by adding 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 estrogen receptor ligands can control gene expression. 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 a semi-continuous suspension culture with high cell density, and antibody production stability was investigated.

3.2 Materials and Methods

3.2.1 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). 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) 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.

3.2.2 Plasmid construction

The GEV gene was amplified by PCR to construct a transposon-based GEV expression vector.

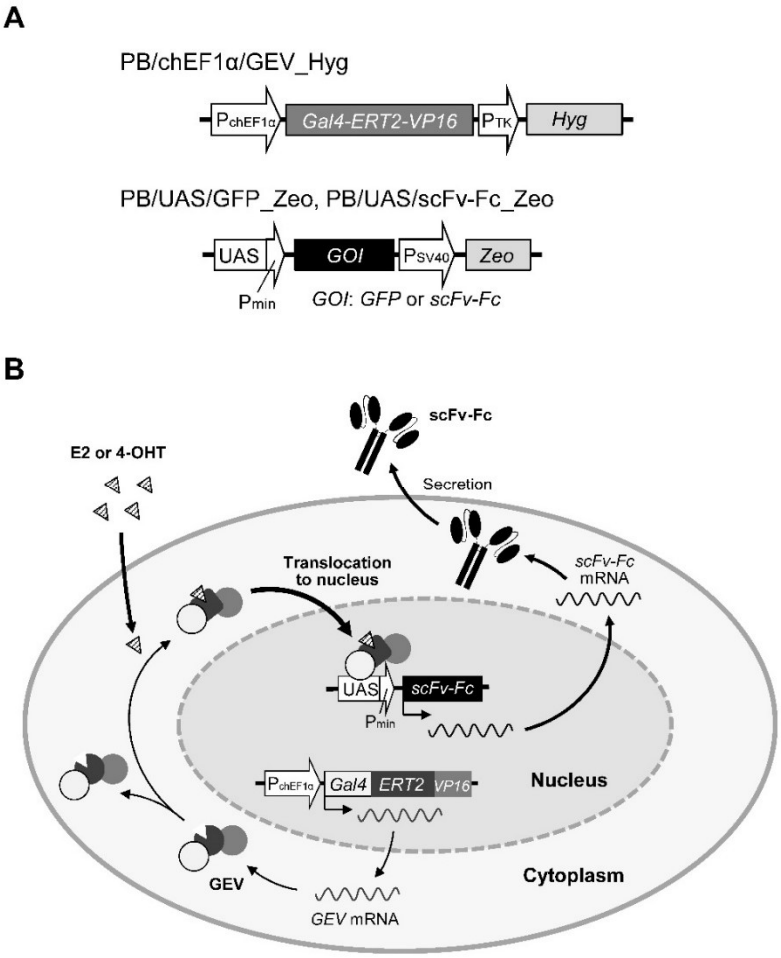

Figure 1 Estrogen receptor-based artificial gene expression system. **(A)** Vector constructs of the Gal4-ERT2-VP16 (GEV) expression vector (PB/chEF1α/GEV_Hyg) and gene-of-interest (GOI) expression vectors (PB/UAS/GFP_Zeo and PB/UAS/scFv-Fc_Zeo). $P_{chEF1\alpha}$, Chinese hamster-derived elongation 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.

pME-GAL4-ERT2-VP16 (#82588, Addgene, Watertown, MA) [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) 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 adenovirus major late promoter region 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 build 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 Table 1 were used for amplification. All PCR products were subjected to gene sequence analysis by Sanger sequencing to confirm correct sequences.

3.2.3 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 10^5 cells/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.

Table1 Primer lists for subcloning.

Target gene		Sequence (5′→3′)
GEV	FW	AAA GCT AGC ACC GGT GCC GCC ACC ATG AAG CTA CTG TCT TCT ATC GAA CAA GCA TGC
	RV	AAA TCG CGA TTA CCC ACC GTA CTC GTC AAT TCC AAG G
GFP	FW	AAA GAG CTC ATG CCC GCC ATG AAG ATC GAG TG
	RV	AAA TCG CGA TTA CAC ATT GAT CCT AGC AGA AGC ACA GGC
PTK-Hyg	FW	AAA GCG CGC TGC TTC ATC CCC GTG GCC CG
	RV	AAA GCG CGC TAT GGC AGG GCC TGC CGC
PSV40-Zeo	FW	AAA CTC GAG CTG TGG AAT GTG TGT CAG TTA GGG TGT GG
	RV	AAA ACC ATA CCA ATG GCA GAC ATG ATA AGA TAC ATT GAT GAG TTT GGA CAA ACC

Next, PB/UAS/GFP_Zeo was transiently introduced into CHO/GEV with pPBase using Lipofectamine 2000 as described above. Drug selection was performed for 14 days using 400 μg/mL Zeocin (#R25001, Invitrogen). Next, the cells were added 1 μM 4-OHT (#H6278, Sigma-Aldrich). 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 stably transformed cells, and cell clones (CHO/GEV_scFv-Fc) were obtained by limiting dilution.

3.2.4 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 a serum-free medium containing 0.1 μM 4-OHT, cells were adjusted to a 0.5 or 1.0 × 10^7 cells/mL density. After that, 10 mL of cell suspension was seeded in a bioreactor tube and cultured for 15 days. The culture medium was replaced with a 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 by dividing the amount of scFv-Fc produced within a predetermined period by the average cell number. Glucose and lactate concentrations were measured using commercially available kits (Glucose Assay Kit-WST, #G264, Dojindo, Kumamoto, Japan; Lactate Assay Kit-WST, #L256, Dojindo).

3.2.5 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-mercaptoethanol 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 a 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 the culture medium using a Protein A Sepharose column (rProtein A Sepharose Fast Flow, #17127901; Cytiva, Marlborough, MA) [31].

3.3 Results

3.3.1 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 the 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 (Figure 2A). The same PB/UAS/GFP gene introduction was performed for three selected cell clones. After the 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 (Figure 2B).

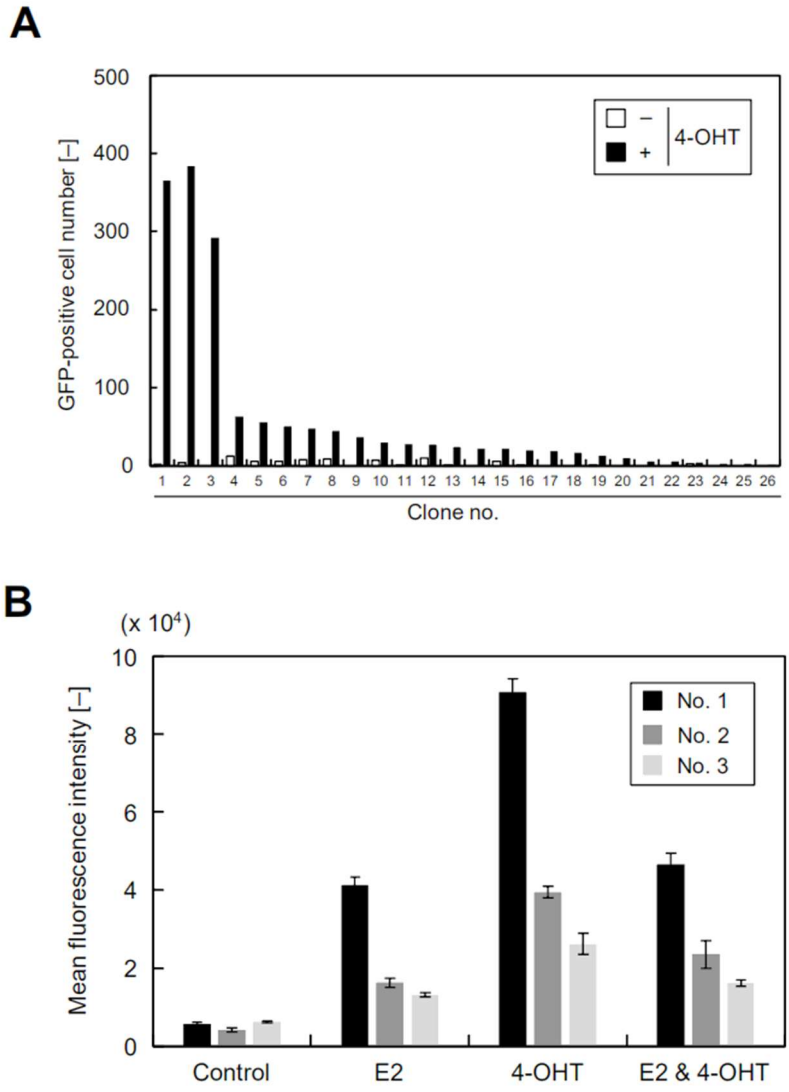

Figure 2 Screening of CHO/GEV cell clones. (A) GFP expression induction upon adding 4-OHT was assayed in 26 cell clones. (B) Mean GFP fluorescence intensity in three selected cell clones.

The mean fluorescence intensity of GFP was measured using a flow cytometer. The cell clone CHO/GEV_1 had the highest mean fluorescence intensity in all conditions. Therefore, this cell clone was chosen for subsequent experiments.

3.3.2 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. GFP expression was induced in all

cells after adding 1.0 μM 4-OHT to drug-selected 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 3). 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 3A). When 20 μM E2 was added, cell proliferation was suppressed entirely 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 3B).

However, the mean fluorescence intensity was only about twice that of the non-additive control, suggesting that the expression level was insufficient (Figure 3C). Next, we attempted gene expression induction using 4-OHT, an antagonist of E2. Cell toxicity was observed at 10 μM or higher (Figure 4).

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 could proliferate without toxicity (Figure 3D). 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 3E). 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 3F). 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.

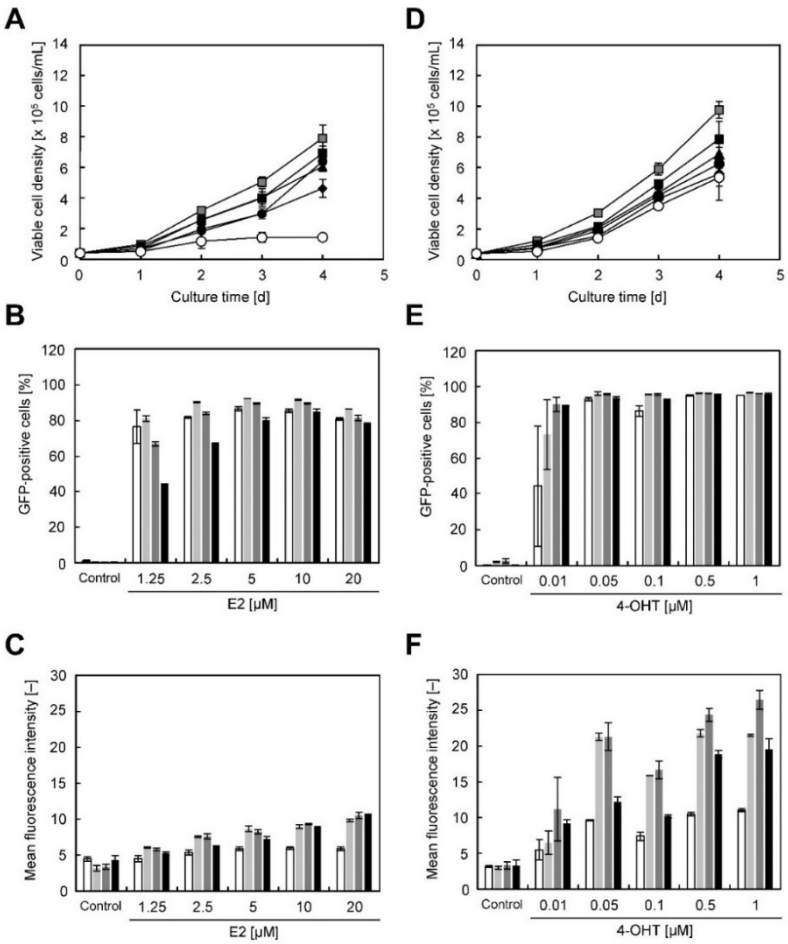

Figure 3 Inducible expression of reporter gene using E2 or 4-OHT. Growth responses of CHO/GEV_GFP cells upon the addition of E2 **(A)** or 4-OHT **(D)**. Symbols are indicated as follows. **(A)** Gray squares, no addition; closed squares, 1.25 μM; closed triangles, 2.5 μM; closed circles, 5 μM; closed 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 E2 (B) or 4-OHT (E) concentration. 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)**.

3.3.3 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 5). 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 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, 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 5A). Measurement of scFv-Fc production showed a maximum of 1.0 μg/mL at 5 μM (Figure 5B), with a specific productivity of 5 pg $cell^{-1}$ day^{-1} (Figure 5C). Next, we attempted to induce antibody production using 4-OHT. As 4-OHT effectively induced reporter gene expression and protein production levels, we also expected a high induction effect for scFv-Fc production. Addition of 4-OHT at concentrations of 0.01–0.1 μM resulted in proliferation comparable with the control (Figure 5D).

Evaluation of scFv-Fc production reveals that scFv-Fc gene expression was induced with 0.05 μM or higher 4-OHT concentrations (Figure 5E), 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 5F), which is 5-fold and 4-fold higher compared with E2.

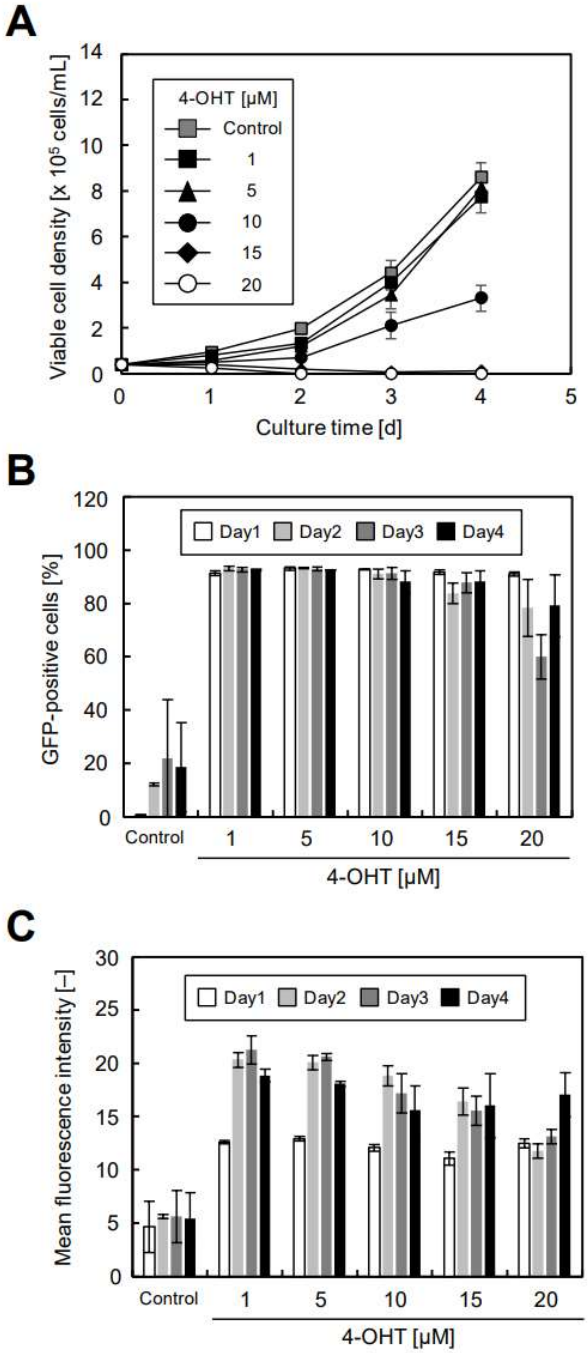

Figure 4 Expression induction of CHO/GEV_GFP cells under high 4-OHT concentration conditions. (A) Cell proliferation. (B) Percentage of GFP-positive cells to total cells. (C) Mean GFP fluorescence intensity.

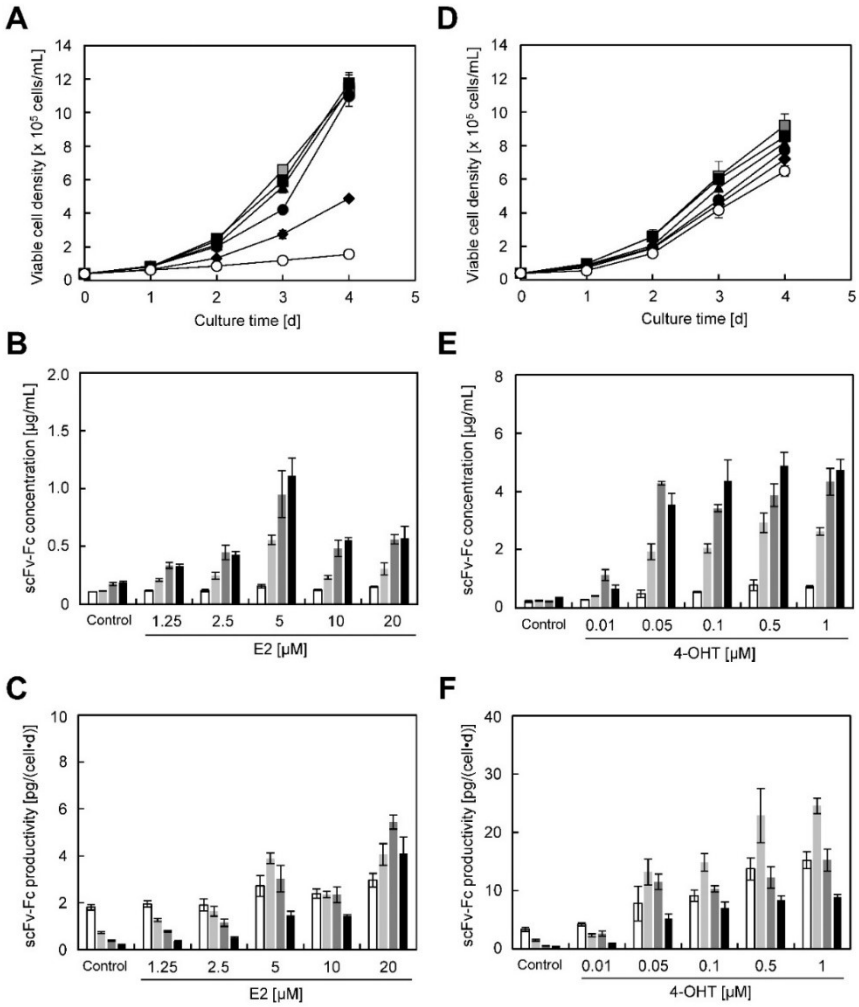

Figure 5 Inducible expression of scFv-Fc gene using E2 or 4-OHT. Growth response of CHO/GEV_scFv-Fc cells upon the addition of E2 **(A)** or 4-OHT **(D)**. scFv-Fc concentrations in media of cells cultured with each concentration of E2 **(B)** or 4-OHT **(E)**. Specific production rate at each E2 (C) or 4-OHT (F) concentration. The distinction between symbols **(A and D)** and columns **(B, C, E and F)** is the same as in Figure 3.

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 (Figure 6A and 6D). Overall induction levels were lower than 4-OHT alone (Figure 6B-C and 6E-F). Therefore, adding 4-OHT alone to induce production is suitable for antibody production in this artificial gene expression system.

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

To produce valuable 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 7).

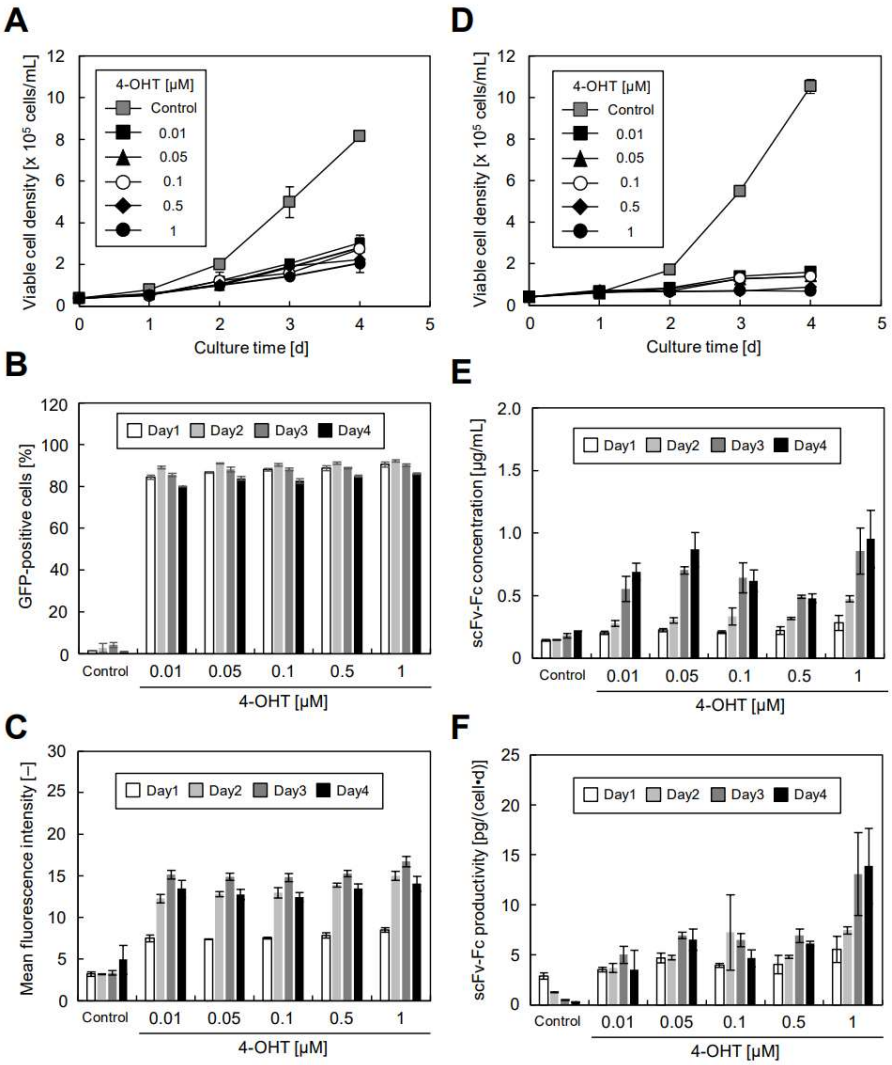

Figure 6 Responses in growth and transgene expression with co-addition of E2 (20 μM) and 4-OHT.

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. Figures 7A and 7B show cell proliferation and viability, 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 by 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 7C), 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 7D), 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 a serum-free medium, and the optimal concentration of 4-OHT is approximately 10-fold lower compared with serum-containing culture.

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

Next, using a bioreactor tube, we attempted continuous production under high-cell-density culture conditions with 4-OHT induction (Figure 8). 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.

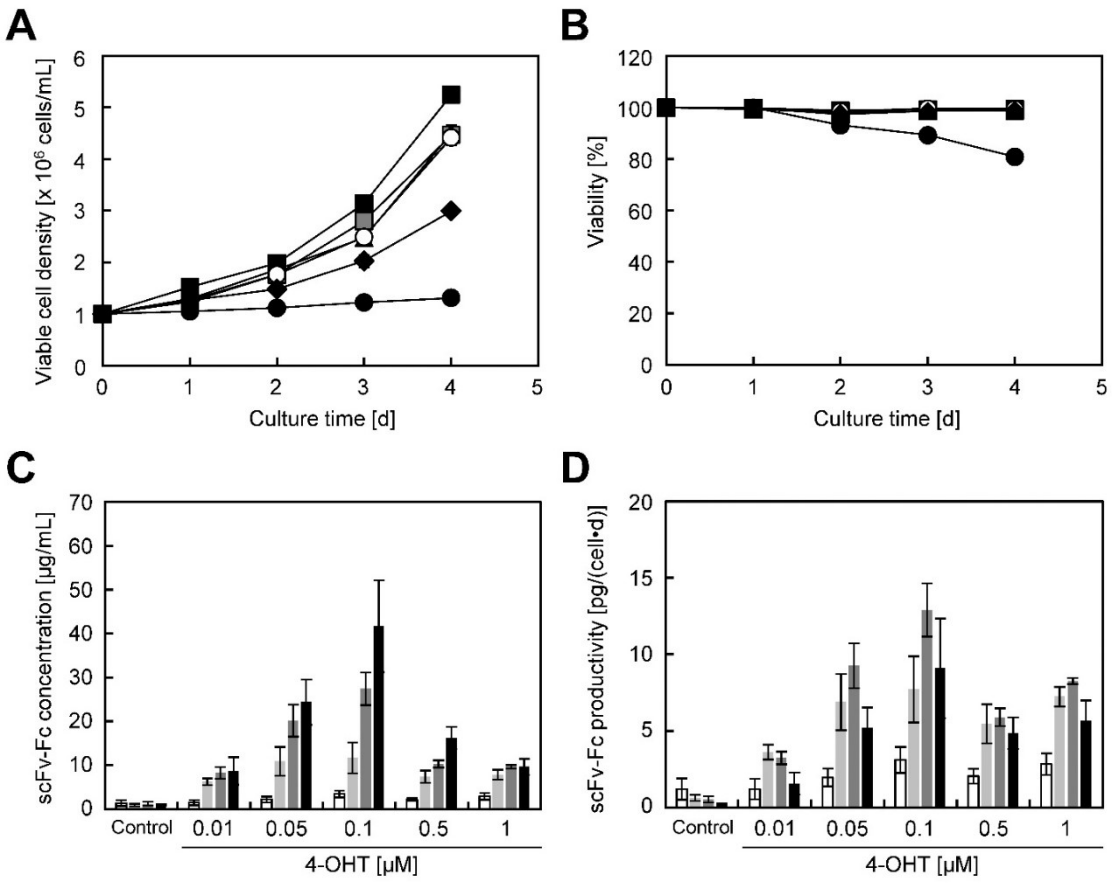

Figure 7 Serum-free culture of CHO/GEV_scFv-Fc cells with the addition of 4-OHT. Growth response **(A)** and viability **(B)** during culture. Symbols: gray squares, no addition; closed squares, 0.01 μM; closed triangles, 0.05 μM; open circles, 0.1 μM; closed diamonds, 0.5 μM; closed circles, 1 μM. scFv-Fc concentration **(C)** and specific production rate **(D)** during culture. The distinction of columns is the same as described for Figure 3.

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 a 4-OHT-containing medium. The semi-continuous culture was carried out for over two 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 8A), but a decreased level of cell viability was maintained

around 80% (Figure 8B). scFv-Fc was produced in the medium at an average concentration of 0.44 ± 0.05 g/L throughout the culture (Figure 8C), with a specific productivity of 37 ± 4 pg cell^{-1} day^{-1} (Figure 8D). In contrast, with a seeding density of 0.5 × 10^7 cells/mL, nearly 100% viability was maintained throughout the culture period (Figure 8B) and scFv-Fc production at a concentration of 0.31 ± 0.03 g/L (Figure 8C) and specific productivity of 57 ± 5 pg cell^{-1} day^{-1} (Figure 8D) was stably achieved. Analysis of metabolites in the spent medium indicates 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 8E). 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 8F). Conversion rates from glucose consumption to lactate production were estimated at 52% (0.5 × 10^7 cells/mL) and 100% (1.0 × 10^7 cells/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 (Figures 9A and 9C) and non-reducing (Figures 9B and 9D) conditions, indicating that the protein was produced intact. These results demonstrate that our estrogen receptor-based inducible expression system can be applied to high-cell-density semi-continuous culture to stably produce recombinant proteins for over two weeks.

3.4. Discussion

Recombinant protein production in *Escherichia coli* cells has been carried out using *lac*-based inducible promoters [13]. Although gene expression control systems have been extensively reported in animal cells, inducible expression systems are rarely employed for the 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 improve productivity per unit of time. In this study, to demonstrate the concept of separating the growth and production phases, we constructed a biopharmaceutical production system with inducible target gene expression using the estrogen-binding domain of an

estrogen receptor.

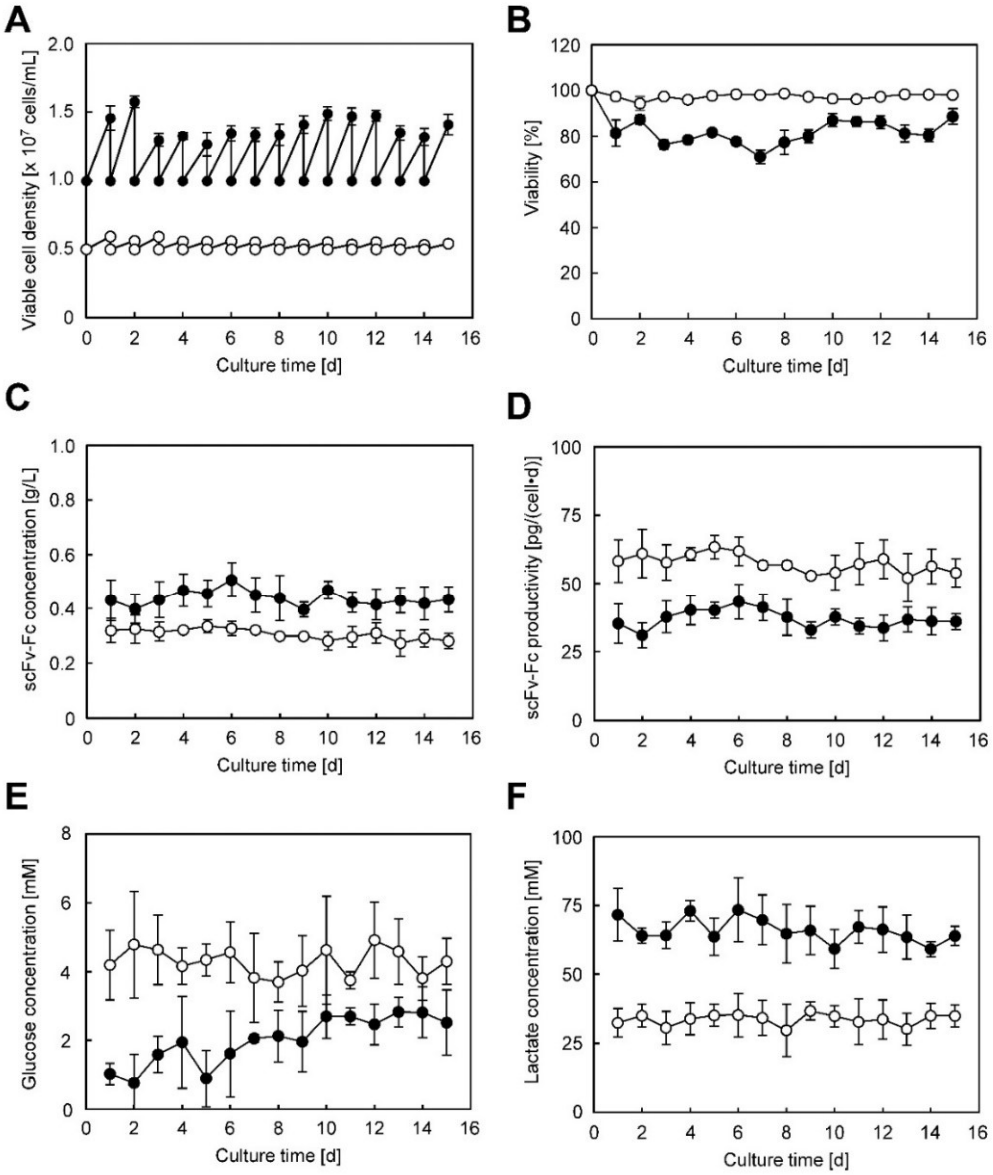

Figure 8 Continuous production of scFv-Fc in semi-continuous cultures with high cell density. CHO/GEV_scFv-Fc cells (10 mL) induced with 0.1 μM 4-OHT were seeded in 50-mL bioreactor tubes at cell densities of 0.5×10^7 cells/mL and 1.0×10^7 cells/mL. Cells were counted the day after seeding. The same 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.

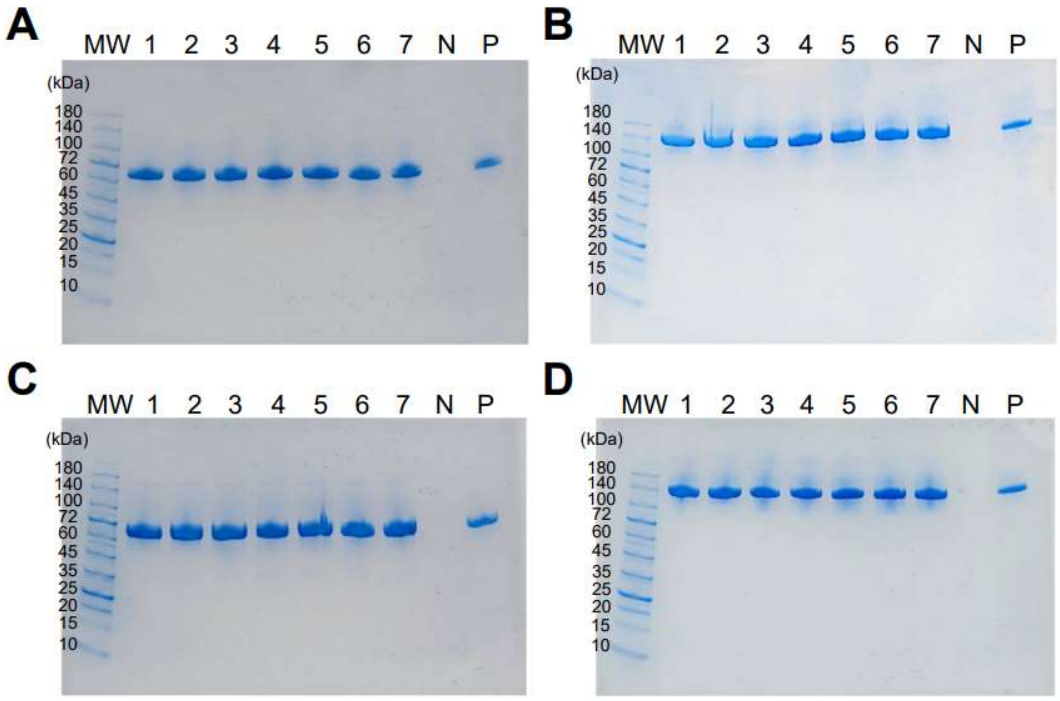

Figure 9 SDS-PAGE analysis of medium samples harvested from semi-continuous cultures. Medium samples (10 μL) from cultures seeded at cell densities of 0.5×10^7 cells/mL (A, B) and 1.0×10^7 cells/mL (C, D) were applied to SDS-PAGE analysis under reducing (A, C) and non-reducing (B, D) conditions. Lane MW, molecular weight markers; lanes 1-7, medium samples harvested at day 2 (lane 1), day 4 (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).

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 a serum-free medium, sensitivity to the inducer and responsiveness to gene expression induction increase. After achieving the required cell

number in a 4-OHT-free medium, production culture was performed under 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 the semi-continuous cultures, aeration was performed by shaking, and therefore, the enhanced antibody productivity may be an effect of improved oxygen supply and high cell density.

GFP-positive cells with over 95% efficiency were observed in this gene induction system after 0.5 μM 4-OHT addition from day one. Maximum GFP expression intensity or 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) [33] 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) [34] of human ERα in ERT2. Nuclear translocation of proteins can be controlled by the type of nuclear localization signal, mutations, or their placement [35,36]. Thus, screening for an optimal nuclear translocation signal for GEV may enhance nuclear translocation and rapid transgene expression induction following the inducer drug's addition.

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. 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 high-level induction and production while maintaining constant cell viability. Glucose in the medium was consumed at 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 the consumed glucose was converted to lactate. Cell viability was around 80%, indicating oxygen depletion 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 due to the improved oxygen supply, 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 [37,38]. Accordingly, improving the oxygen supply and developing a 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 [39], transcription factors [40] 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 continuous production, highlighting its effectiveness as a production system that switches between cell proliferation and production modes.

It has been reported that reducing culture temperature suppressed proliferation and improved productivity [41,42]. Applying such a method might further increase productivity. To minimize or avoid inducer drugs altogether, developing a single induction and an artificial gene circuit based on external environments [17,18,21] would be a helpful improvement to the inducible system for practical biopharmaceutical production.

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 [43,44] to produce 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 artificial gene expression, is expected to become an essential method in biopharmaceutical production.

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Chapter 4

4.1 Conclusion and Perspectives

The demand for Biopharmaceuticals, especially monoclonal antibodies, is increasing daily. Due to the efficacy of the therapeutic protein, the regulatory bodies approving many products have never been seen in the past decades. The advancement and improvement in mammalian cell culture technology facilitates higher productivity in CHO cell lines. The recent focus is on the proliferation control strategy to improve productivity. Hence, the concept of decoupling growth stage and productivity interests the researchers. Temperature shifts and applying chemical compounds to increase productivity are challenges in producing quality products. However, efforts have been made with the application of small chemical compounds. In this regard, estradiol and its derivative 4-OHT were chosen for my research ideas. These chemical compounds can be used as an inducer to make estrogen-inducible transgene expression systems. The UAS/Gal4 system is a binary gene expression system where estrogen drugs are applied as a proliferation control strategy. An artificial transactivation system was constructed by transactivator Gal4 fusing with human estrogen receptor mutant ERT2 and the activation domain of viral protein VP16, namely Gal4-ERT2-VP16 (GEV). After applying estrogen drugs, GEV translocates into the nucleus from the cytoplasm of the CHO cell, where it sits on the UAS-activating sequence. By binding GEV with UAS, it starts to transactivate the expression of transgene GFP in an inducible manner. The switching between cell growth and productivity using an estrogen-inducible gene expression system has been applied in the CHO-K1 cell line. Estrogen and 4-OHT can efficiently express GFP expression in the recombinant cells. The Gal4-ERT2-VP16 transactivator system is used in CHO-K1 recombinant cells. This system showed GFP expression in a drug-dependent manner. Without drug application, the cells do not fully respond to whether the cells are almost responded to the drug conditions. The recombinant CHO-K1 cells can efficiently produce target antibody scFvFc in serum-free medium for more than two weeks, which indicates stable

biopharmaceutical manufacturing. The drug-inducible gene expression system used has promising applications for producing recombinant proteins. These results suggest that CHO cells' estrogen-inducible transgene expression system may be promising for efficiently producing recombinant proteins. The concept of inducible production using the artificial transactivator with the estrogen-binding domain is hoped to provide new insight to research groups working in recombinant protein production using CHO cells.

4.2 Summary of the thesis

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 with cell growth. In this study, we developed a technology that controls cell growth and recombinant protein production to induce recombinant protein production with pre-determined timing. Expression of the 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 a synthetic 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. We used 4-hydroxytamoxifen, an estrogen antagonist, as an inducing agent that yielded high gene expression at a concentration more than 10-fold lower than that of β-estradiol. When scFv-Fc was produced under inducing conditions, continuous production was possible for more than two 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 the inducible production of biopharmaceuticals.

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