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Cell Line Development for Therapeutic Protein Production

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2.1 Introduction

Biopharmaceuticals are any pharmaceutical drugs manufactured from a biological source, and they include nucleic acids, blood, vaccines, and therapeutic proteins such as monoclonal antibodies (mAbs) and erythropoietin (EPO). Since the first approval of recombinant human insulin (Humulin, Eli Lilly) as a recombinant therapeutic protein in 1982, the size of the biopharmaceutical market has been growing rapidly. The number of approved biopharmaceuticals is more than 300 in the USA and the European Union by 2016, and many more are in the process of being approved [1]. Therapeutic proteins are mostly produced by microorganisms (microbial systems) and mammalian cells (mammalian expression systems), and in rare cases by insect cells and plant cells.

Microbial systems have the advantages of low cost, short timeline, easy control, and high productivity. The first approved recombinant human insulin was made from *Escherichia coli*. However, many other subsequent targets for therapeutic proteins were revealed to be more complex, and prokaryotes cannot express a large complex protein with multiple subunits, cofactors, and eukaryotic post-translational modifications. Posttranslational modifications such as glycosylation and disulfide bond formation are very important for biological function, stability, and pharmacokinetics of the products [2]. Yeast expression systems (e.g. *Saccharomyces cerevisiae* and *Pichia pastoris*), which also achieve rapid cell growth and high protein yields, can produce some proteins that cannot be produced from *E. coli* because of some folding and glycosylation problems [3]. However, yeasts still cannot modify proteins with human glycosylation structures.

Plant cells can be maintained in simple synthetic media, and they can synthesize complex multimeric proteins and glycoproteins with greater similarity to human counterparts [4]. However, plant cells do not necessarily produce proteins with the same 3D structure as that of humans. Furthermore, the glycosylation pattern occurring in the late Golgi apparatus is different between plants and mammals. Insect cells are mostly used for the development of viruslike particles and vaccines. They produce trimmed *N*-glycan precursors that do not develop further into terminal galactose and/or sialic acid residues [5].

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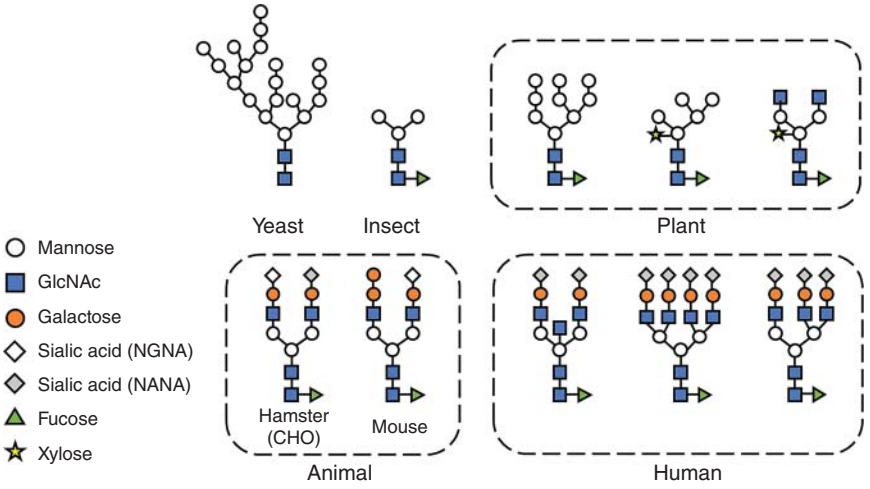

Figure 2.1 *N-linked glycosylation patterns in different organisms*. Although proteins produced from yeast, insect cells, or plant cells lack galactose and sialic acid in their N-linked glycosylation pattern, proteins produced from animal cells show quite a similar pattern to those produced from human cells.

Mammalian expression systems are, therefore, preferred for the production of many complex therapeutic proteins despite the fact that they are slower and more expensive than the other systems. Although a microbial system requires cell lysis and subsequent protein refolding, most proteins can be secreted with proper folding in mammalian cells. Unlike yeast, insect, and plants, mammalian cells have a glycosylation pattern that is highly similar to that of humans (Figure 2.1).

To produce therapeutic proteins from mammalian cells, an immortalized cell line is necessary. The cells should grow for prolonged periods to produce a large amount of therapeutic protein. There are several types of mammalian host cell lines used for the production of therapeutic proteins. Among them, Chinese hamster ovary (CHO) cells have been most widely used for the commercial production of biopharmaceuticals since 1987. Murine myeloma cell lines such as Sp2/0-Ag14 and NS0 have also been used but to a much lesser extent than CHO cells. Recently, there has been increasing interest in human cell lines such as human embryonic kidney (HEK293), human fibrosarcoma (HT-1080), and PER.C6.

The host mammalian cell lines are transfected with the expression vector(s) with a gene of interest (GOI). The protein expression can be transient or stable (Figure 2.2). In the transient expression system, transfected cells are cultivated until the end of the production phase and GOI does not necessarily integrate into the genome of the cells. In the stable expression system, the cells undergo a selection phase after transfection. The GOI is integrated into the chromosome of the surviving cells, and stable cell lines with high productivity are finally selected. Although the process of stable cell line generation is time-consuming and labor-intensive, it is a common practice to produce a large amount of therapeutic protein with consistent product quality.

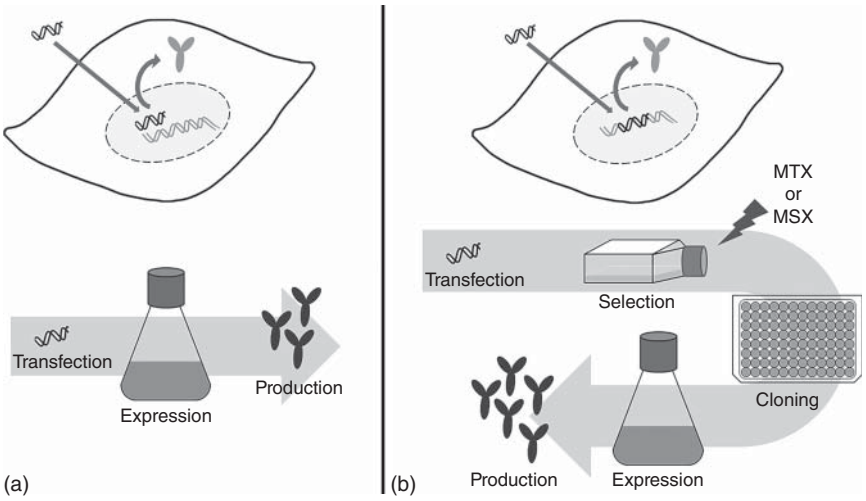

Figure 2.2 *Comparison between (a) transient expression system and (b) stable expression system.* Transient expression system produces target proteins within a relatively short timeline. The stable expression system requires a relatively longer timeline, but it can stably produce a larger amount of the target protein.

In this chapter, mammalian cell lines used for therapeutic protein production (especially focusing on CHO and human cell lines) will be introduced with their brief history and characteristics. Then, the cell line development process of CHO and human cells will be reviewed. Some important considerations during the cell line development process will also be discussed.

2.2 Mammalian Host Cell Lines for Therapeutic Protein Production

2.2.1 CHO Cell Lines

CHO cells have been used most widely for the commercial production of therapeutic proteins since the approval of the first CHO-derived tissue plasminogen activator (tPA) in 1987. Currently, more than half of the top 10 selling drugs, including both chemical and biological, are produced in CHO cells [6]. Such popularity is explained by several traits that CHO cells possess (i) the ability to grow in a serum-free suspension culture for large-scale production of therapeutic proteins, (ii) amplification systems that enable gene amplification leading to higher productivity, (iii) posttranslational modification capability that makes the quality of CHO-derived glycoproteins compatible with that of humans, and (iv) resistance to human viral infections because of the lack of genes responsible for human viral entry. In addition, CHO cells have been demonstrated as safe hosts for the past three decades, which makes the approval from regulatory agencies easier.

CHO cells were first established from the ovary of a Chinese hamster and immortalized by Theodore Puck in 1957 [7]. There are several CHO-derived

cell lines such as CHO-K1, CHO-S, DXB11, and DG44. The CHO-K1 cell line was cloned from the original CHO cells and has been used in many laboratories for decades, and the first genome sequence of CHO cells was published using CHO-K1 [8]. The CHO-S cell line was derived from the CHO-Toronto cell line (a sister cell line of CHO-K1) and adapted to grow in suspension culture. The DXB11 cell line was generated from the CHO-K1 cell line for the metabolic studies relating to cancer chemotherapy by deleting the dihydrofolate reductase (DHFR) activity, resulting in a deletion of one locus for DHFR and a missense mutation of the other DHFR locus [9]. The DXB11 cell line became the starting point of the DHFR system and became the host cell line for the production of human tPA. Because there was a detectable rate of reversion to DHFR activity in the DXB11 cell line, DG44 cell line was generated from the Toronto cell line with full deletion of the two DHFR loci on chromosome 2 [10].

2.2.2 Human Cell Lines

Several human cell lines are used for the production of therapeutic proteins, and they can produce therapeutic proteins that are most similar to the proteins naturally synthesized by humans. One concern with the use of a human cell line is the increased risk of transfer of adventitious agents because of lack of a species barrier [11].

HEK293 cell lines overexpressing the adenovirus E1A and E1B genes were generated in 1977 by the transfection of HEK cells with adenovirus type 5 DNA [12] and have been used for transgene expression since then. Because of their ease of cultivation and transfection, HEK293 cell lines have been the most widely used host cell lines for transient expression. Recently, several therapeutic proteins produced in the HEK293 cell lines such as recombinant factor VIII Fc fusion protein (rFVIIIFc) and dulaglutide have been approved by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA) [13]. The eHEK293T and HEK293E cell lines were generated by stable transfection of the parental line with the simian virus 40 (SV40) large T antigen gene and the Epstein–Barr virus nuclear antigen 1 (EBNA1) gene, respectively, to support the episomal replication and maintenance of the transfected plasmid DNAs [14, 15]. The HEK293H and HEK293F cell lines were clonally isolated for fast growth in serum-free medium, high transfection efficiency, and high level of protein production.

One disadvantage of the HEK293 cell lines is the formation of large aggregates in suspension cultures. Therefore, to solve the aggregation problem, a hybrid of the kidney and B cell line (HKB-11) was developed by fusion of the HEK293S cell line with the human suspension cell line, 2B8 (a Burkitt's lymphoma derivative) [16]. Although the HKB-11 cell line showed several characteristics suitable for producing therapeutic proteins, it showed unstable expression of EBNA1 during long-term cultivation because of a loss of EBV genome. To overcome the instability of the EBNA1 genome, the F2N78 cell line was established by the fusion of HEK293 cells with Namalwa cells [17]. In the F2N78 cell line, the EBV genome was inserted into the chromosome rather than existing as an episome.

The PER.C6 cell line was created by immortalizing human embryonic retinoblasts with the E1 gene of adenovirus [18]. The production platform of

PER.C6 showing high productivity was established by Crucell and DSM biologics [19]. The PER.C6 cell line has been used for the production of classical vaccines as well as therapeutic proteins [20]. CEVEC's amniocyte production (CAP) system was developed by immortalization of primary human amniocytes using adenoviral genetic E1/pIX functions [21]. In the CAP system, amniocytes were cotransfected with the E1-expressing plasmid containing the E1A-, E1B-, and pIX-function and the plasmid expressing the target protein. Only transfected cells can grow and divide while nontransfected cells cease to grow at an early passage. This method is novel in the way that it uses a nontumorigenic cell line, and the selection process does not require antibiotics.

2.2.3 Other Mammalian Cell Lines

Although not as popular as CHO cell lines and human cell lines, murine myeloma cell lines such as Sp2/0-Ag14 and NS0 have been used as host cell lines for the production of therapeutic proteins. The Sp2/0-Ag14 cell line is a non-Ig-secreting cell line derived from a fusion of a BALB/c mouse spleen and the mouse myeloma cell line [22]. NS0 was cloned to establish a nonsecreting cell line and has been well studied in combination with the glutamine synthetase (GS) expression system [23]. However, murine cell lines express considerably higher levels of *N*-glycolylneuraminic acid (NGNA) and Galα1-3Galβ1-GlcNAc-R (α-Gal) compared to hamster cell lines. There was a case of severe anaphylactic reaction to the nonhuman glycan epitopes of an Sp2/0-derived antibody in patients [24]. Additionally, the baby hamster kidney (BHK21) cell line has been used in the production of some coagulation factors such as factor VIIa and factor VIII [25]. In addition, the COS cell line (fibroblast-like cell line derived from monkey kidney tissues) has been used mostly for transient expression [26].

2.3 Development of Recombinant CHO Cell Lines

CHO cells are used for both transient and stable expression systems. A transient expression system is usually adopted when a large number of various proteins are prepared in a quick manner. The advantages of a transient expression system include a simpler expression vector with no selective marker, a shorter timeline for therapeutic protein production, applicability to a wide range of host cell lines, and suitability to multiple processing at the same time [27]. However, several drawbacks still limit the utility of transient expression systems for large-scale production of therapeutic proteins because (i) the transient expression experiments require a large quantity of the transfection reagent and plasmid DNA that are economically burdensome and (ii) the protein yield from a transient system is dependent on the transfection efficiency, which may vary among the experiments. In addition, although the volumetric productivity in transient expression systems has been improved lately [28–30], it is still lower than that in stable gene expression systems.

A stable expression system, on the other hand, requires a longer timeline because of the selection of the transfected cell pool followed by gene

amplification and/or clone selection processes. However, GOI is integrated into the chromosome of the host cells during the cell line generation process. Once stable clones with high productivity are established, those clones show consistent and high production of the target protein. High producing clones can be cultivated repeatedly under optimal culture conditions leading to maximum yield. Therefore, to produce a large amount of therapeutic proteins with consistent product quality, stable expression systems are inevitably used in the biopharmaceutical industry.

2.3.1 Expression Systems for CHO Cells

To establish a stable recombinant CHO cell line with high productivity, two expression systems are dominantly used: the DHFR system and the GS system. DHFR catalyzes the conversion of folic acid to tetrahydrofolate, which is required for biosynthetic pathways that produce glycine, purine, and thymidylic acid. The DHFR system can be applied to mutant strains of CHO cells such as DXB11 and DG44, in which the *dhfr* gene was either mutated or deleted. To survive, such auxotrophic cell lines require a culture medium containing glycine, hypoxanthine, and thymidine (GHT). In a DHFR system, a GOI is transfected into host cells with the *dhfr* gene either in the same expression vector or in a different expression vector. The transfected cells undergo selection in the media without GHT, and the surviving cell pool will have the GOI together with the *dhfr* gene in their genomes. The inserted genes can be amplified with the use of MTX, a DHFR inhibitor, and the DHFR system is frequently used because of its high efficiency of gene amplification.

The GS system uses the *gs* gene as a selectable marker. GS catalyzes the conversion of ammonia and glutamate into glutamine, and MSX inhibits the activity of the GS protein. Although the DHFR system can be used only with DHFR-deficient cell lines, the GS system can be used with cell lines having an endogenous *gs* gene. NS0 murine myeloma cells are auxotrophic for glutamine and can be selected in glutamine-free medium using the GS system. In contrast, CHO cells possess an endogenous *gs* gene and can be selected with the concurrent addition of MSX at a low level [31, 32]. The CHO-K1 cell line was first used for the GS system [33]. To improve the efficiency of the cell line generation, a GS knockout cell line was also developed [34]. Compared to the DHFR system that requires a long timeline for gene amplification through the gradual increment of MTX, the GS system can achieve sufficient expression level with a single round of selection and amplification, thereby shortening the total timeline required for cell line generation [23, 33]. Furthermore, the GS system mitigates ammonia accumulation in culture medium because the overexpressed GS protein catalyzes the conversion of glutamate and ammonia into glutamine [35].

2.3.2 Cell Line Development Process Using CHO Cells Based on Random Integration

Both the DHFR and GS systems share a concept that a selection gene (*dhfr* or *gs*) is transfected with a GOI, providing a selective advantage to the cells in the selective

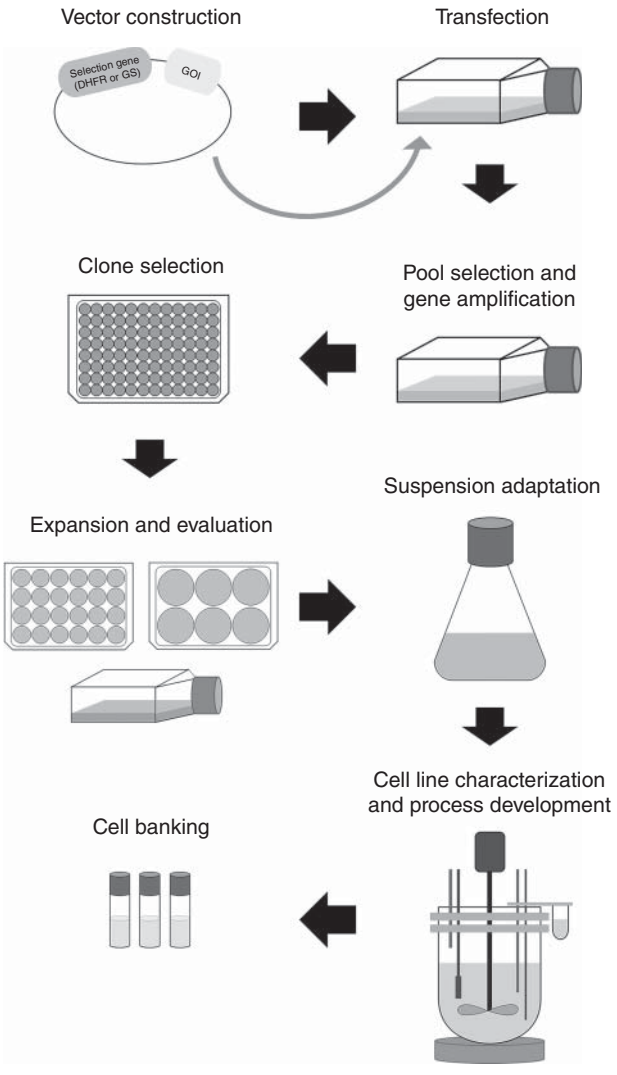

Figure 2.3 *Schematic representation of cell line development process*. Expression vector(s) with selection gene and GOI is transfected to host cells. The transfected host cells undergo pool selection process and, if necessary, repeated gene amplification process. Single-cell-derived clones are selected from the pool and clones are expanded and adapted to grow in suspension culture. Final cell lines are characterized and undergo process development to achieve maximum production.

medium. A schematic representation of the development of a stable recombinant cell line is shown in Figure 2.3.

2.3.2.1 Vector Construction

The first step of the cell line development process is the molecular cloning of a GOI in a mammalian expression vector. In most cases, a selection gene (*dhfr*

or *gs*) is in the same expression vector but sometimes can be in a separate vector because they can be linked upon gene transfer by intracellular ligation or cointegration at the same site [36]. Antibiotics such as zeocin, geneticin (G418), hygromycin B, blasticidin, and puromycin can be used for dual selection and a relevant resistance gene can be inserted into the expression vector.

Protein expression can be improved through vector design by providing the appropriate promoter, signal peptide, and codon optimization [37]. To increase the probability of obtaining a high-level producer, the selection gene is driven from a weak promoter, and the GOI is driven from a strong promoter such as the CMV promoter. Several cis-acting elements might also affect cellular expression. For example, the Kozak sequence has a role in translation initiation [38], and the viral posttranscriptional regulatory sequence from the woodchuck hepatitis virus (WPRE) enhances transgene expression when placed downstream of an open reading frame [39]. Moreover, signal peptides can improve the secretion efficiency of a therapeutic protein, and the optimization of signal peptides has been well studied [40]. Codon optimization can improve the expression level of GOI because various organisms show a preference for certain codons [41]. In CHO cells, protein expression was improved by codon optimization [42, 43].

2.3.2.2 Transfection and Selection

The expression vector(s) are then transfected into host cells. Many transfection methods for mammalian cells have been developed, which include biological, chemical, and physical methods [44]. The biological method usually involves virus-mediated transfection (transduction), and it is highly efficient and easy to use. However, because of the risk of immunogenicity and the requirement for higher levels of safety containment, a nonviral gene transfer is rather preferred for manufacturing therapeutic proteins. A chemical method was the first method to be used for mammalian cells, and it uses a cationic polymer, calcium phosphate, or cationic lipid. The chemical method has the merits of less induction of the immune system, and there are many commercially available products with good efficiency [45]. A physical method has been developed most recently, and it includes direct microinjection, electroporation, and laser irradiation. Electroporation is the most widely used method because it can easily and rapidly transfect a large number of cells once the optimal conditions for transfection are determined [44].

The transfected cells, 24–48 hours after the transfection, are transferred to a selection medium to undergo pool selection. In the case of DHFR system, the selection medium will be deficient in GHT [46]. In the case of GS system, the selection medium will be deficient in glutamine, and 25–50 μM of MSX may be added to suppress the endogenous GS protein of the host cells [34]. The resulting pool of cells surviving in the selection medium will have the *dhfr* or *gs* gene and GOI that are randomly integrated into their genomes at various regions. Each cell will exhibit different levels of protein expression because the number of transgene(s) and the surrounding gene sequence of the integration sites vary.

2.3.2.3 Gene Amplification

When using the DHFR system, it is common to amplify the GOI during the cell line development process. Cells develop resistance to the increased level of MTX

by amplifying the *dhfr* gene. Because the size of the amplification unit is much larger than that of the *dhfr* gene (130–3000 kilobases), the GOI is coamplified together with the *dhfr* gene [47]. The initial pools are exposed to a low concentration of MTX so that only the cells containing at least several copies of the *dhfr* gene and GOI may survive. Such a procedure is repeated using stepwise increasing concentrations of MTX because a single-step exposure to a high concentration of MTX may result in MTX-resistant DHFR mutants or cells with altered MTX transport properties [48]. This amplification process is often a bottleneck during the cell line development process because it requires several months to reach the high MTX level. The gene copy number of the GOI increases with the increased level of MTX, but there exists a saturation limit after which the gene copy number does not increase any more even with a higher level of MTX [49].

When using the GS system, the timeline for gene amplification is relatively short (Figure 2.4). A single round of amplification (100–500 μM) is sufficient to achieve efficient expression, and further rounds do not seem to result in higher product titers [33]. To minimize the time required for cell line generation, no amplification is even used. In most cases, a low level of MSX (25–50 μM) used during the first selection after transfection is sufficient enough to achieve a desired level of protein production [50].

2.3.2.4 Clone Selection

Another time-consuming step in the cell line development process is the selection of clones that exhibit the best productivity, desired product quality, and

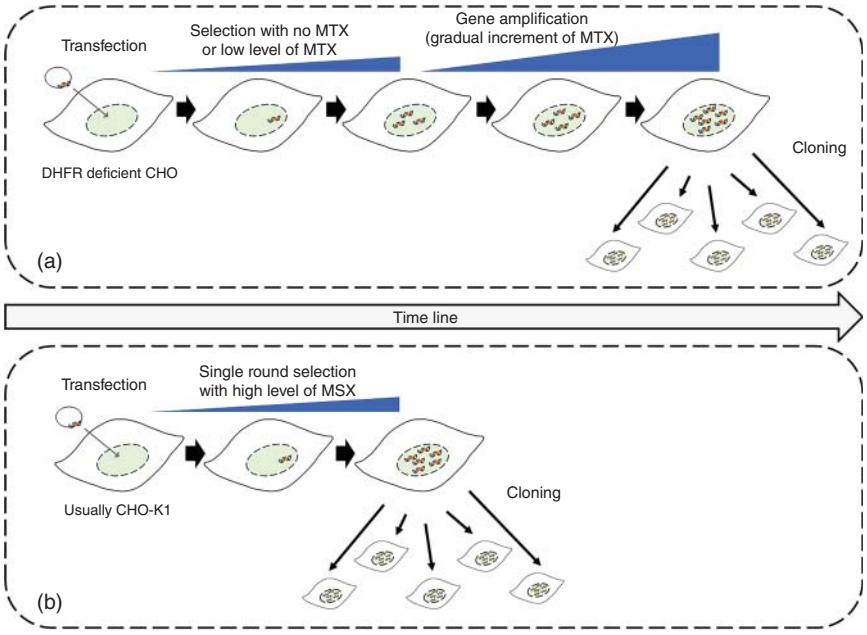

Figure 2.4 *Comparison between (a) DHFR system and (b) GS system*. DHFR system requires repeated gene amplification process with a gradual increment of MTX and it makes the cell line development timeline longer. GS system does not require repeated amplification process and the cell line development timeline is shorter.

good growth profile. A traditional way of clone selection is "limiting dilution" in which cells are transferred to multiwell plates such that on an average only one cell is present per two to three wells and forms a colony of a single cell. Each clone is then scaled up and evaluated for its growth, productivity, and product quality. Because the traditional methods are time-consuming and limited by the number of clones that can be screened, alternative high-throughput methods (i.e. fluorescence-activated cell sorting (FACS), the ClonePix™ system (Genetix), and the Cell Xpress™ system (Cyntellect)) have been developed and are widely used in industry.

FACS is the most widely used method for high-throughput screening. After staining cells with antibodies conjugated with fluorescent molecules, high producing clones can be sorted out based on the fluorescence intensity of cells by FACS [51–53]. The ClonePix system is an automated colony picker that enables screening a large number of clones in a semisolid medium. Therapeutic proteins secreted from the colony are captured by antibodies conjugated with fluorescent molecules, and the colonies with high fluorescent intensity are then picked [54, 55]. The Cell Xpress system identifies high producing clones in a semisolid medium by live cell imaging with a detection reagent, and non- or low-producing clones are eliminated by laser-induced apoptosis [56].

In the case of the DHFR system, there exist two different strategies for clone selection according to the clone selection timing in the gene amplification process. The first strategy is selection based on individual clones in which cell cloning is performed once at the time of the first selection of parental clones and once again after obtaining high-producer clones through gene amplification. The second strategy is a selection based on cell pools in which cell cloning is performed only once at the final selection stage after gene amplification is completed. Although the first strategy is labor-intensive and requires a longer time, it has been shown to be more efficient in providing higher producers [57].

After the isolation of the clones with high productivity, their stability in regard to productivity is evaluated by subculturing them for a couple of months. Culture conditions such as temperature, pH, dissolved oxygen tension (DOT), and osmolality can affect the productivity of the clones [58–64]. Therefore, the culture conditions of selected clones are optimized in a small-scale bioreactor with a working volume of 1–2 l using cell culture media used for large-scale cultures. Nowadays, a microscale cell culture system that uses cost-effective, disposable, multiple micro-bioreactors and is controlled by an automated workstation is also widely used for culture process optimization in industry.

2.3.3 Cell Line Development Process Using CHO Cells Based On Site-Specific Integration

Random integration of a GOI has some drawbacks such as variation in productivity and a laborious selection process. To overcome these drawbacks, site-specific integration of a GOI has emerged as an alternative method of transgene integration. By adopting a targeted integration method, the GOI can be inserted into a site that shows a higher expression level of the therapeutic protein and stability on a long-term basis. For site-specific integration of a GOI, the information

on a high-expressing site, known as a hot spot, is first needed [65]. Although the exact mechanism explaining the different levels of gene expression at different integration sites is still under study, several mechanisms are considered as being responsible, such as cis-regulatory elements, DNA and histone modifications, and chromosome structure [66–68].

A recombinase-mediated cassette exchange (RMCE) method is capable of targeted integration using site-specific recombinases and distinct recombinase-recognition sequences. After specific sites are targeted by random integration of heterologous genes flanked by recombinase-recognition sequences, those sites are confirmed by different levels of gene expression. Then, the sites that show a higher level of expression are exchanged with the GOI by the corresponding recombinase. The Cre/loxP system, consisting of the Cre recombinase and the loxP sequence, was first used in the CHO-DG44 cell line to obtain high-producing clones selected by measuring the GFP expression level [69]. This system has been applied to different CHO cell lines in modified forms, including accumulative integration, mutated loxP sequences, and epigenetic modifier elements [70–72]. Another example of RMCE is the Flp/FRT system, and it used the Flp recombinase and the FRT sequence in the CHO DG-44 cell line producing EPO to screen for a stable EPO producer [73]. Finally, PhiC31 integrase was exploited in the CHO-S cell line producing a humanized IgG1 antibody to reduce the possibility of a reversible reaction in other systems (Cre/loxP and Flp/FRT) by irreversible integration of the GOI into the genome [74]. One of the drawbacks of the targeted integration methods by a recombinase-mediated system is that they need to prepare the tagged sites containing reporter sequences flanked by FRT or loxP before GOI integration into specific sites of the host cells [75].

An endonuclease-mediated targeted integration method has also been used for site-specific integration by introducing a DNA double-strand break (DSB) and then being repaired by DNA damage repair pathways. There are three major kinds of targetable nucleases: zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR-associated protein (Cas) RNA-guided nuclease [76]. These nucleases have a DNA-binding domain or guide RNA and cleave the targeted sequence by the *Fok* I cleavage domain or the Cas9 protein.

Each zinc-finger motif interacts with a 3-bp DNA sequence, and two ZFNs need to form a dimer to function properly [77]. TALENs also contain the *Fok* I domain as a nuclease domain but use another type of DNA-binding domain, called transcription activator-like effectors (TALEs) [78]. Unlike ZFNs, TALENs can recognize DNA sequences by pairing one TALE repeat with one base, which guarantees a higher specificity and lower off-targeting compared to ZFNs [79]. It was demonstrated that ZFN- and TALEN-mediated targeted integration is applicable in mammalian cells by introducing the IgG gene into the FUT8 locus in the CHO-K1 cell line [80]. In addition to the ZFNs and TALENs, CRISPR/Cas9 has recently emerged as a genomic engineering tool. It is composed of a single-chain guide RNA targeting specific DNA sequence and the Cas9 protein acting as an active nuclease in the presence of the guide RNA [81]. The CRISPR/Cas9 system has many advantages, including ease of design and preparation, lower

cytotoxicity, higher efficiency, and lower cost compared to the other two methods mentioned above [82–85]. CRISPR/Cas9-mediated gene integration has been attempted in human cells by means of CRISPR/Cas9-mediated precise integration into a target chromosome (PITCh) system dependent on the microhomology-mediated end-joining (MMEJ) mechanism [86]. In CHO cells, CRISPR/Cas9 system can integrate a GOI into specific sites by exploiting the homology-directed repair (HDR) pathway [87].

Transposons are mobile genetic elements found in various organisms and classified by their transition mechanism. DNA transposons are composed of inverted repeats, directed repeats, DNA-binding domain, nuclear localization signal (NLS), and catalytic amino acids [88]. DNA transposons need a specific transposase enzyme that is encoded between inverted terminal repeats (ITRs) to transfer by a mechanism of copy-and-paste, and several kinds of DNA transposon have been used as a genetic engineering tool in many studies [89]. Two vectors, a donor plasmid with a GOI flanked by ITRs and a helper plasmid with a transposase sequence, are necessary for transgene integration [90]. Transposition activities of Sleeping Beauty, Tol2, Mos1, and PiggyBac transposons were measured in different mammalian cell lines including CHO cells [91]. Although the transposon-mediated gene integration method has no intended target specificity, some transposons have the tendency to integrate into specific regions, such as transcriptional start sites (TSSs), CpG islands, and DNase I hypersensitive sites [92]. It was observed that the PiggyBac transposon has a preference for transposition into specific sites including the TTAA sequence as well as TSSs and CpG islands in primary human T cells [93]. PiggyBac transposon-mediated integration was proved as an efficient tool and showed higher productivity and stability compared to the random integration method due to insertion into actively transcribed regions [94]. Recently, cell pools generated by the PiggyBac transposon yielded high productivity up to 7.6 g/l [95]. Furthermore, the product quality of the cell pools generated by the PiggyBac transposon was comparable to the control, and the higher productivity was reproducible in 36 l bioreactor [96].

2.4 Development of Recombinant Human Cell Lines

2.4.1 Necessity for Human Cell Lines

Although CHO cells mimic many of the human glycosylation patterns, proteins produced by CHO (and other nonhuman) cells sometimes show nonhuman glycan structures such as NGNA and α-Gal. Because humans have circulating antibodies against NGNA and α-Gal, proteins produced from a nonhuman cell line can be highly immunogenic and/or rapidly cleared from circulation [97]. In addition, CHO and other nonhuman cells lack some sugar-transferring enzymes such as α2-6 sialyltransferase and α1-3/4 fucosyltransferase [98].

There are several cases in which human cells must be used as a host cell line. Drotrecogin alfa, a recombinant activated protein C showing antithrombotic,

anti-inflammatory, and profibrinolytic properties, is not produced in CHO cells with adequate efficiency because CHO cells have less capacity for γ-carboxylation than that of human cell lines [99]. Dimeric cartilage matrix protein-angiopoietin-1 (CMP-Ang1), a potential growth factor for therapeutic angiogenesis and vascular stabilization, requires specific N-glycosylation. Dimeric CMP-Ang1 produced by CHO cells showed no activity while that produced by HEK293 cells showed reproducible activity [100]. Therefore, the necessity of human cell lines for the production of therapeutic proteins cannot be disregarded, and efforts in using human cell lines should be pursued.

Human cell lines, especially the HEK293 cell line, are widely used for transient expression systems. In general, the production yield of HEK293 cell lines is relatively higher than that of CHO cell lines when therapeutic proteins are transiently expressed [101]. However, large-scale production of therapeutic proteins using a transient expression system requires a large amount of purified DNA and a large number of cells to be prepared on the day before transfection, possibly causing economical and technical problems. Another major concern with transient expression systems is the batch-to-batch variability in regard to protein yield and quality, especially protein glycosylation, although there are studies showing little batch-to-batch variation [102].

2.4.2 Stable Cell Line Development Process Using Human Cell Lines

Human cell lines are also used for establishing stable cell lines but in fewer cases than that of CHO cell lines. Basically, the protocol for stable cell line development for human cell lines is similar to that used for CHO cell lines. In most cases for human cell lines, however, only antibiotics are used for the selection, and no gene amplification is available. Accordingly, the specific productivity of human stable cell lines is relatively low compared to stable CHO cell lines. Although not as much as CHO cell lines, hundreds of milligrams per liter of therapeutic proteins were stably produced in human cells [51, 103].

A stable HEK293 cell line producing human recombinant IFNα2b was established in the presence of blasticidin, and the product titer exceeded 200 mg/l in a batch culture [103]. In addition, up to a 655 mg/l titer was achieved using the antibody-producing stable HEK293 cell line screened with high-throughput FACS [51]. To increase the productivity of HEK293 cell lines, the GS-mediated gene amplification system was applied to HEK293 cells. However, a high level of expression of endogenous *gs* gene in HEK293 cells resulted in elevated resistance to MSX and therefore hampered the GS-mediated selection and gene amplification by MSX [104].

A stable F2N78 cell line producing IgG was established using the puromycin gene as a selection marker, and it showed an antibody production up to 340 mg/l in fed-batch cultures [105]. A PER.C6 cell line producing an antibody was established in the presence of neomycin (G418), and it showed a high level of antibody production (300–500 mg/l) in a batch culture [19]. With the modified perfusion/fed-batch system, yields of alpha-1-antitrypsin (A1PI) over 2.5 g/l and of human IgM antibodies up to 2 g/l were achieved [106, 107].

The CAP system of CEVEC does not require the use of antibiotic selection. Cotransfection of the primary human amniocytes with a plasmid expressing adenoviral E1 functions and a plasmid containing a GOI resulted in stable cell lines expressing a fully glycosylated and sialylated protein. In an optimized fed-batch culture, 200–250 mg/l of C1-inhibitor was produced [108].

2.5 Important Consideration for Cell Line Development

In addition to the productivity, there are several factors that should be considered during the cell line development process, and these include clonality, stability, and product quality.

2.5.1 Clonality

Once cells are transfected with expression vectors, clonal variations occur because of the differences in the chromosomal context of the randomly integrated genes and/or a disruption of the endogenous genes caused during the gene integration and amplification process. Clones, even if they are derived from the same parental cells, react differently to culture conditions such as culture temperature, osmolality, and media additives [109–112]. Such clonal variation, therefore, should be considered in clone selection during the cell line development process.

Recently, regulatory agencies such as the FDA and EMA have required "proof of clonality" for the approval of biopharmaceuticals, which is an important issue especially in the industry [113]. Clonality means that the cell population is arisen from a "single cell," which is often assured by several rounds of limiting dilution. Such a regulatory request was established to ensure the purity and homogeneity of the product. To confirm the clonality, visual monitoring of plates is usually done using automatic image devices such as CloneSelect™ Imager (Molecular Devices) and Cell Metric™ imager (Solentim). Using such devices, 96-well plates are automatically imaged to identify the formation of colonies derived from a single cell. However, the viewpoint of the industry on the importance of clonality seems to be different from that of regulatory agencies [114]. Because the host cells used for the manufacture of biopharmaceuticals are immortalized cell populations that have escaped from normal control of cell division, the rate of genetic drift and chromosomal instability are inevitably very high [115]. Even after single cell cloning, genomic heterogeneity caused by inherent DNA replication errors, error-prone DNA repair process, and Darwinian selection occurs during cell growth and expansion. Therefore, although strict cloning is conducted during the cell line development process, the heterogeneity of the producing cells cannot be avoided and more focus should be on the consistency of the manufacturing process and on the quality of the product rather than clonality itself.

2.5.2 Stability

The productivity of recombinant cells often declines as cell age increases during prolonged culture. Unstable clones are not suitable for large-scale production

because cell age inevitably increases during the scale-up process. Thus, cell line stability is an important factor to be considered in clone selection.

Although the underlying causes and the precise molecular mechanisms behind cell line instability have yet to be fully elucidated, possible reasons are loss of gene copy number, transcriptional regulation, and posttranscriptional regulation. The decrease in protein productivity is in many cases caused by a loss in gene copy numbers [49, 116–118]. In some cases, the gene copy number did not change, and only the mRNA level was decreased [119, 120]. Methylation-induced transcriptional silencing has been pointed out as one of the epigenetic reasons for production instability [121, 122]. In contrast, there was a case in which antibody productivity decreased without any decrease in both gene copy number and mRNA level. In this case, decreased cell growth was responsible for the decreased antibody yield [123].

In the case of a CHO cell line with DHFR-mediated gene amplification, there are a number of reports that have shown instability of production during long-term culture in the absence of MTX [49, 116, 124]. Production instability was observed even in the presence of MTX but at a lesser extent than in the absence of MTX. In addition, there is a report that MTX did not show any benefit in product stability while the addition of hypoxanthine and thymidine demonstrated better maintenance of genetic stability [125]. Compared with the DHFR expression system, less information is available on the production stability of rCHO cells with GS-mediated gene amplification. Humanized antibody productivity of GS-CHO cells decreased during long-term culture regardless of the presence of MSX [126]. In contrast, antibody productivity of GS-CHO cells was kept constant in the presence of MSX [32]. Conflicting reports on the production stability are likely due to clonal variations. The clones derived from the same cell line generation process showed different production stabilities [122, 127].

Generation of clones with improved production stability facilitates large-scale production of therapeutic proteins. The location of a GOI in the chromosome may affect the production stability. It was reported that clones with amplified genes located near the telomeric regions are more stable than those located in other regions [128]. In addition, some chromosomes were revealed to be genetically more conservative among different CHO cell lines [129]. Such information at the chromosomal level will enable identification of potential target sites for site-specific integration to generate producing cell lines with improved production stability. Furthermore, gene engineering of CHO host cells would also lead to the isolation of more clones with improved production stability as observed with the knockout of the gene *Fam60A* in CHO-K1 [130].

2.5.3 Quality of Therapeutic Proteins

Maintaining consistent and comparable product quality is one of the most important and challenging issues in the production of therapeutic proteins. During the cell line development process, product quality attributes vary significantly among the clones. Therefore, it is important to select the clones producing the therapeutic protein with the desired quality using various analytical assays and quality

assessment criteria. In particular, for the production of biosimilar candidates, the extent of comparability to original drugs, which is the most important concern during the approval process, is an important criterion to select the clones. To determine whether the quality of a biosimilar candidate sits within the "goal-posts" of acceptable features, state-of-the-art liquid chromatography and mass spectrometry technologies are used to compare intact masses, protein sequences, posttranslational modifications, and microvariants [131].

Methods based on chromatographic or electrophoretic separation such as size-exclusion chromatography (SEC), high-performance liquid chromatography (HPLC), and isoelectric focusing (IEF) have been used traditionally to assess the quality attributes such as molecule integrity, aggregation, glycosylation, and charge heterogeneity. Recently, rapid and high-throughput analysis of sequence variants as well as glycosylation patterns during the early stage of cell line development process has become available [132–134].

2.6 Conclusion

Mammalian cells are preferred for the production of complex therapeutic proteins. Among them, CHO cells have been dominantly used in the industry for the production of therapeutic proteins including antibodies. To a lesser extent, human cells such as HEK293, HT-1080, and PER.C6 have also been used for commercial production of therapeutic proteins.

Currently, the generation of recombinant cell lines is the most time-consuming step for developing the process for commercial production of therapeutic proteins. However, recent progress in gene editing technology such as CRISPR/Cas9 will make cell line generation more efficient and faster. As the demand for antibodies and other therapeutic proteins continue to increase, the popularity of mammalian cells, particularly CHO cells, is likely to persist.

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