Chapter 7

Cell Line Development

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7.1 INTRODUCTION

Cell lines suitable for stable production of recombinant proteins must be possible to scale up for commercial GMP production. In particular, the cell line should: (i) be adapted to serum-free and suspension growth conditions, (ii) be able to grow at a high rate, and (iii) have a high secretory capacity. It must also exhibit an efficient energy metabolism without excess secretion of by-products and should have well-tuned protein secretion and glycosylation machinery. Furthermore, it must be stable with respect to product quality and productivity over time, and the timelines and population doublings from gene transfection to production cell line should be as short as possible.

Additional points must also be critically evaluated in order to ensure a safe cell line development process. This includes monitoring each raw material that is supplemented into the culture medium at any step during cell line development (CLD) and thus interacts with final production cells. Since the mad cow disease crisis in the 1990s, each culture medium component has to be certified animal component-free (ACF) to ensure maximum safety profiles.

In this chapter, we focus on the development of cell lines for production of biopharmaceuticals, exemplified by CHO cells, which is the most widely used cell type for production of complex biopharmaceuticals such as monoclonal antibodies. We describe how stable cell lines are generated, how the requirement for clonality can be fulfilled and which approaches can be applied to engineer the host cell line.

7.2 GENERATION OF STABLE CHO CELL LINES

7.2.1 Vector Design and Production of Recombinant DNA

The process of cell line development starts with introduction of plasmid DNA encoding for the gene of interest, gene regulatory elements, and a selectable marker into the production host cell line a process called transfection [1]. A plasmid expression vector is composed of many different elements. An example of a vector for expression of a mAb by random integration is shown in Fig. 7.1. The elements outlined in the figure are the coding sequences for the light and heavy chain of the antibody, the selection marker (GS in this case), promoters, signal peptides, and sequences related to the production of the vector in *E. coli* (Ori and antibiotic resistance). The vector elements are usually produced in *E. coli* and bacterial plasmids. To increase the yield of DNA from the bacterial cultures, high copy number plasmids are used. After the bacterial culture, the plasmid DNA is prepared and purified. The construction of the recombinant DNA vector is achieved through manipulation of the DNA with purified enzymes, e.g., nucleases, ligases, and polymerases.

7.2.2 Gene Delivery

High transfection efficiency represents a cornerstone in generating an industrial manufacturing cell line and thus is a fundamental part of cell line development. Generally, there are several transfection methods that differ in their mode of action: the application of cationic lipids, cationic polymers (polycations), calcium phosphate, or electroporation [2,3].

A plethora of cationic lipids and polymers are commercially available by different vendors, but transfection efficiency and functionality of these reagents can vary tremendously depending on a multitude of different factors such as cell type, culture media, reagent-to-DNA ratio, DNA purity, complexation buffer, complex formation time, and volume [2,4,5].

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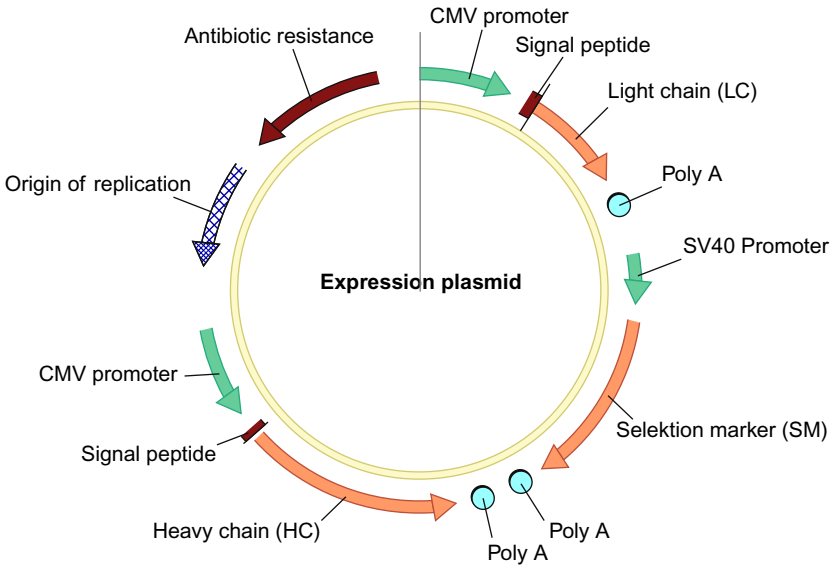

FIG. 7.1 Example of a mAb expression vector.

The advantages of cationic lipids and polycations are their ease of use and the fact no special equipment is required for transfection. Major disadvantages of cationic lipids and polymers include possible incompatibility of the reagent with culture medium compositions (leading to impairment of transfection efficiency), tedious transfection optimization procedures, high costs, and the requirement for a certified animal component-free (ACF) manufacturing process. However, CHO cells are inherently difficult to transfect and are maintained in complex production media, which challenges gene delivery efforts, and therefore chemical-based delivery vehicles often exhibit low transfection efficiency. Fortunately, continuing refinement in electroporation instruments, parameters, and electrolyte buffer compositions could further improve transfection efficiency and tolerability by the cells. In addition, since electroporation events may induce DNA double-strand breaks, it might facilitate genomic integration of exogenously introduced expression plasmids during DNA repair. Developmental strategies of electroporation processes considerably increased efficiency of transgene delivery into CHO cells. Furthermore, successful electroporation events are independent of parameters such as culture media composition or the requirement for an ACF manufacturing process of reagents used. However, optimization of transfection conditions is still required, as is purchase of cost-intensive electroporation devices and transfection kits. Nonetheless, reproducibly high transfection efficiencies of >80% are routinely achieved with CHO cells using optimized electroporation protocols. Because such high transfection efficiencies have not yet been reproducibly achieved using chemical transfection reagents, electroporation still represents the current gold standard for the development of recombinant CHO cell lines [6–9].

Another option to introduce genes is through retroviral vectors that can be used to stably insert single copies of genes at multiple genomic locations into dividing cells. One commercially available technology using retrovectors is the GPEx technology from Catalent.

Retrovectors deliver genes coded as RNA that, after entering the cell, are reverse- transcribed to DNA and integrated stably into the host cell genome. Two enzymes, reverse transcriptase and integrase, provided transiently in the vector particle, perform this function. These integrated genes are maintained through subsequent cell divisions as if they were endogenous cellular genes. By controlling the number of retroviral particles accessing the cell, multiple gene insertion (desirable for high-yielding cell cultures) is achieved without any of the traditional amplification steps. The retroviral vectors have been shown to preferentially insert into or around the transcription start point of genes. This preference for transcriptionally "active" regions of the genome allows for higher, more consistent levels of expression per copy of the gene inserted, as compared to other methods of gene insertion. Protein-producing cell clones generated by this method are regarded as consistent, and only a few hundred clones are screened to identify high-expressing master cell bank candidate clones. Due to the high gene-insertion efficiency of the technology process, no selectable markers (e.g., neomycin, blasticidin, hygromycin, or puromycin resistance genes) are needed for cell line generation. The system can be used together with any mammalian host cell line

TABLE 7.1 Natural Recombinases and Recombination Sites Broadly Used for Site-Directed Integration

Recombinase	Recognition Site	Origin	Type of Recombinase
Cre	Lox	P1 phage	Tyrosine
Dre	Rox	D6 phage	Tyrosine
Flp	FRT	*S. cerevisiae*	Tyrosine
φC31	att	φ31 phage	Serine

Many gene delivery systems resulting in random integration of the transgene are impacted by and have the drawback of positional effects of the integration. The transgene can theoretically be integrated in any region of the host genome. However, the vast majority of the genome is not transcriptionally active. Insertion in inactive regions is unlikely to result in gene expression. Random integration in transcriptionally active and critical genes may disrupt the gene expression and have serious consequences for cell growth. To avoid these effects, the transgene needs to be inserted in the host genome in a way that does not disturb endogenous gene expression, but which places the transgene in transcriptionally active locations, called hot spots [10]. The use of site-specific integration sites has also promised more predictable protein production [11,12].

Two approaches can be used to identify hot-spot sites to build an expression platform for site-directed integration: first, by using a high-producing cell clone from a traditional random integration CLD process as starting point and determining the exact genomic sequence and location of the insertion site so that they may be retargeted with other transgenes, and second, by transfecting a host cell line with an expression cassette tagged with a reporter gene and a selectable marker (e.g., GFP) and identifying high-producing clones with flow cytometry. The landing pad cassette is inserted into the chromosomal hot spot by means of a gene editing technology discussed before (e.g., meganucleases), transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), CRISPR-Cas systems, or by homologous recombination. The landing pad includes the recombinase-specific recognition site and at least a selective marker, which could be a fluorescent protein. The selective marker can be used to select for cells with the landing pad, demonstrate the landing pad activity, and allow for selection of transfected cells once the integration plasmid is transfected and the selection marker is flipped out.

Four major recombinases and their recognition sites are used for recombinant transgene insertion and are listed in Table 7.1.

The first generation of chromosomal gene modifications using recombinases were targeted gene deletions using Cre/loxP and Flp-FRT systems. Although these recombinases can catalyze integration reactions into each target site, the deletion reaction is kinetically favored over the integration reaction. Thus, these systems have been frequently employed as a method of conditional knockout of the integrated gene. The method of recombinase-mediated targeting was significantly improved by flanking an initial tagging cassette with a set of non-interacting recombinase recognition sites. Upon integration, such cassettes can be precisely exchanged for an incoming vector flanked with the same set of recombinase recognition sites [11–15]. Hence, the term recombinase-mediated cassette exchange (RMCE) was coined [16]. Basically, RMCE relies on two heterologous recombinase target sites (spacer mutants) that resist site-specific recombination between each other but still undergo recombination with their respective homologous counterparts. Mutants that can be exploited in this respect have been identified both for the Flp [15] and the Cre systems (reviewed in Refs. [11,12]). The main advantage of RMCE is the lack of excision, which reduces the targeting efficiency in simple first-generation, targeted integration approaches. Upon implementation of stringent selection strategies, the frequency of targeting can be increased [17], even up to 100% [18,19].

Another phenomenon impacting the stability of the gene expression is gene silencing, the effect of negative positional effects of the random integration of the transgene into the host cell genome. Gene silencing by negative positional effects can be overcome by integration of the transgene in defined loci (by SDI), or by flanking the vector with insulating sequences that avoid or minimize negative positional effects of the chromatin (matrix attachment regions, MARs etc) [20] or ubiquitous chromatin opening elements (UCOEs) [21].

7.2.3 Pool Selection and Enrichment/Bulk Sorting

Once a production host cell line has been transfected with the respective expression plasmid(s), cells are subjected to selection pressure in order to obtain stable recombinant cell populations with genomic integration of the expression cassette.

Regarding DHFR-deficient CHO cells (CHO-DXB11 and CHO-DG44), the selection medium lacks thymidine and hypoxanthine, resulting in the survival of cells expressing sufficient quantity of exogenously introduced DHFR gene copies. CHO cells expressing low levels of or lacking functional GS (CHOK1SV and or other GS-knockout cells) are selected in medium supplemented with L-glutamic acid instead of L-glutamine in order to obtain cell populations having the ectopically delivered GS stably integrated into their host cell genomes. Notably, in terms of monoclonal antibody production, required DNA sequences are often provided on two separate expression plasmids, one coding for the heavy chain (HC) while the other encoding the light chain (LC) sequence. One of the two antibody chains is normally expressed in conjunction with a functional DHFR (or GS) gene copy, while the other chain is either co-expressed with the same or using another selective marker. It is also possible to integrate the expression cassettes of both HC and LC gene copies on the same expression plasmid. Although this simplifies handling and increases the probability of a combined genomic integration of both antibody chains, it also increases plasmid size and thus limits space for other advantageous genetic elements. Furthermore, putting heavy and light chain sequences on two separate plasmids enables transfection of different plasmid ratios, which can be utilized to influence final expression levels [22]. However, it is still controversial whether positive effects on productivity observed using particular HC:LC ratios in transient transfection experiments can be translated into stable expression. Considering that the final production clone will have dozens to thousands of GOI copy numbers genomically integrated, it is questionable whether the previously determined optimal plasmid rations are finally achieved. The stable integration of transgenes and selective markers into the host cell genome generally occurs in a randomized way which cannot be properly predicted. Consequently, stably selected cell populations consist of a heterogeneous mixture of cells which substantially differ in the number and localization of integrated transgenes and are thus considerably divergent in cell-specific productivity. Therefore, these cells are considered cell pools.

To enhance the probability of establishing as many high-yielding production cells as possible, stable cell pools can be enriched for cell populations exhibiting increased recombinant protein productivity, in a method known as bulk sorting. In this context, stable cell pools can be incubated with fluorescently labelled affinity molecules or specific anti-IgG antibodies in order to indicate secreted recombinant antibodies on the cell surface of the production cell. As the cell-specific productivity has been described to correlate with the number of antibodies displayed on the cell surface during secretion [23], fluorescent-activated cell sorting (FACS) based discrimination of cells showing elevated fluorescence intensity allows for enrichment of high-producing cell populations [24]. Regulatory authorities only accept biopharmaceuticals produced by clonal cell lines, meaning that production cells have to be cloned from a single cell progenitor [25]. Therefore, the next chapter will focus on the generation and selection of a suitable production cell clone.

7.2.4 Single Cell Cloning

Transfection of a plasmid encoding a GOI, together with a selectable marker and subsequent selection of stably transfected cells, generates a heterogeneous cell population where each cell exhibits unique genetic and phenotypic characteristics. Unfortunately, there are several issues arising from an insufficient monoclonality, such as discrepancies in product quality, stability of recombinant protein production, metabolic profile, and growth rate. For instance, if a cell population consists of cells exhibiting even slight differences in specific productivity (and thus also in growth rate), the less-producing cell population showing accelerated growth characteristics will gradually outgrow the high-producing population. In this conjunction, it has been reported that a growth advantage of as little as 9% is sufficient to overgrow a cell population within 25 passages [26]. This implies that an industrial manufacturing cell line has to be genetically and phenotypically identical [27]. This requirement for monoclonality comes along with several technical challenges to discriminate single cells from a heterogeneous population of stably transfected and selected cells, as well as to ensure that all subsequently established cell lines originally stemmed from a single cell. Hence, for regulatory safety, it is required that biopharmaceuticals which are intended to be used for treatment of a human disease have to be produced by a monoclonal cell line that has been derived from a single cell [28].

Limiting Dilution

For many years, limiting dilution (LD) represented the gold standard for generating single cell clones from a heterogeneous cell population, such as stably transfected cell pools [29]. By limiting dilution, a pool of selected recombinant cells is highly diluted, and the resulting low cell density culture is distributed into several 96- or 384-well microplates. The final cell density of the diluted culture is adjusted in a way that, on average, every well of a microplate statistically contains less than one cell per well. Consequently, (other than empty wells or wells containing several cells) many microplate wells will finally contain a single cell clone which can be propagated to form a monoclonal cell population. Each well has to be observed

microscopically, then wells containing single cells are marked, and the remaining cells containing none or several cells will not be considered further [27]. Of note, to ensure survival of cells at low cell densities, special single cell cloning media formulations are required that are rich in (paracrine and autocrine) growth factors to mimic a greater number of cells in order to support cell growth and to prevent loss of high-productive clones [30,31]. However, although limiting dilution represents a rather simple cloning method, it is also inefficient and labor-intensive and exhibits a higher probability to generate cell lines which are not monoclonal [27,32,33]. Note that, in order to establish a high-producing, single cell derived production clone, several rounds of LD together with appropriate statistical background are required by regulatory authorities to ensure sufficient degree of clonality. However, this strategy is time-consuming and results in a considerable number of additional cell divisions, which is clearly not desired since it increases cellular aging before GMP cell banking. Furthermore, due to remarkable advances in (automated) single cell isolation technologies, limiting dilution might gradually be replaced by these novel techniques in the future.

Automated Colony-Picking Systems

Integration of automated systems for single cell cloning and detection such as clone-picking robots have decreased hands-on times while increasing throughput and success rates in cell line development. Automated colony-picking systems such as ClonePix FL from Molecular Devices or CellCelector from Aviso are the most frequently applied devices [27]. Low cell density cultures are seeded in semi-solid growth medium and incubated at 37°C to allow for formation of colonies which are ideally derived from single clones. Due to the presence of a matrix in the semi-solid media, secreted proteins accumulate in close proximity to the production cell [34,35]. Fluorescently labelled detection antibodies can be used to identify the concentrated product, leading to a fluorescent halo around the cells where the fluorescence intensity correlates with the product concentration [34,35]. Colonies exhibiting the brightest fluorescence are detected as high-producers and can be picked automatically in a sterile environment a few days after seeding [27]. However, there is accumulating evidence that automated colony-picking systems still exhibit a considerable risk of establishing cell lines which are not derived from a single cell clone. Consequently, production cell lines generated using this system must also be subjected to at least one additional round of cloning to increase statistical probability of generating a monoclonal cell line.

Fluorescent Activated Cell Sorting

In the past decade, Fluorescent Activated Cell Sorting (FACS) technology has become the widely used standard technique for single cell isolation and cloning within the biopharmaceutical industry [36–39]. This technology enables the rapid screening of millions of cells for particular cellular parameters such as cell size, granularity, viability, apoptosis, transfection efficiency, cell surface protein expression, and much more [40]. Refinements in sorting capacity and accuracy, in combination with the ability to deposit single cells into 96- and 384-well microplates, represent significant progress in cell line development. FACS-based sorting technologies enable high-throughput single cell cloning, allowing for establishment of thousands of individual cell clones concomitantly. In addition, single cell deposition and doublet discrimination procedures have been used in the flow cytometry field for decades [41]. These techniques allow one to faithfully identify single cells and deposit droplets containing only one cell at a dedicated place, e.g., a well within a multiwell plate.

After deposition into microplates, the sorted cells divide to form monoclonal cell populations which are considered to be genetically and phenotypically identical. In order to enhance sorting efficiency, various methods have been established to increase the probability of identifying a high-producing production cell line and reducing the number of clones to be characterized. These methods take advantage of a correlation between the concentration of the recombinant protein on the cell surface and the cell-specific productivity [42]. For instance, by using fluorescently labelled product-specific or non-specific affinity molecules, cell surface staining of the secreted therapeutic protein could be enabled [36,37,43–45]. Integration of an immunoglobulin transmembrane anchor downstream of a leaky stop codon terminating the coding sequence of a therapeutic protein leads to stable cell surface display of a small proportion of the expressed biotherapeutic [46]. These modifications can significantly improve stability and efficiency of cell surface staining-based single cell sorting capacity. Another group linked the mRNA of the recombinant protein to a cell-surface protein which is usually not expressed in CHO cells (e.g., CD20) using an internal ribosome entry site (IRES), and sorted cells based upon concentration of the cell surface protein [47]. Employing novel genome editing tools, such as zinc finger nucleases (ZFNs) or the Cas9-mediated clustered regularly interspaced short palindromic repeats (CRIPSR/Cas9) system, subsequently allows for genomic deletion of the cell surface protein in the final production clone to reduce translational burden. Yoshikawa et al. developed an intracellular staining procedure employing fluorescently labelled MTX in order to visualize DHFR protein abundance and activity [48].

The fluorescently labelled MTX quantitatively binds DHFR within the cytosol and thus can indicate cell clones exhibiting enhanced DHFR expression levels which normally well correlate with cell specific productivity [48–50]. Note that this staining method will only be applicable to cases where DHFR has been used as a selection marker.

Automated High-Throughput Microscopy

Regardless of whether limiting dilution, FACS, or other methods are used for deposition of single cells into multiwell plates, automated microscopy has recently been increasingly implemented in the biopharmaceutical industry in order to confirm that only a single cell had been deposited into the respective wells [51]. Imaging solutions from SynenTec Bio Services or Solentim such as the Cellavista/NyOne and Cell Metric high-throughput microscopic systems, respectively, are the most frequently applied instruments for cell analysis after single cell sorting and offer the opportunity to take both bright-field as well as fluorescent images. In this context, a further advantage of the above-mentioned viability staining method would be that fluorescence signals can be exploited to increase the content and accuracy of the subsequently conducted fluorescence microscopy. Overlay of bright-field and fluorescent micrographs can markedly facilitate identification of single cell clones. Consequently, wells which do not contain single cells will not be further considered as putative production cell lines, and can therefore be discarded.

7.2.5 Importance of Clonality of Production Cell Lines

The purpose of a cloning step is the isolation and generation of homogenous cell populations derived from single cells. A heterogeneous pool of cells is not suitable for biopharmaceutical production, since the relative contribution of different clones in a population is extremely likely to differ between different cell ages and between different production conditions (in the case of process changes). The demonstration of clonality of a production cell line has therefore been in the focus of regulatory agencies for some time, and the notion of production clones within the industry has changed from “a cell line having undergone a cloning step” to “a cell line for which the derivation from one single cell has been demonstrated.”

However, non-clonality is not the only possible root cause for heterogeneities in production cell banks. CHO cells are well known for their genetic flexibility [52,53], and the likelihood of mutations, chromosomal aberrations, and epigenetic alterations upon prolonged culture is high. Given that in order to reach the ~10^{10} cells needed for MCB/WCB manufacture, 33 cell doublings are needed, the likelihood of different cells being present in a given MCB/WCB is high [54]. Therefore, the thorough genetic characterization of the production cell line, as well as the demonstration and thorough characterization of a robust production process, can be considered at least as important as a formal demonstration of clonality for existing cell banks [55].

To fulfill the expectation of demonstration of clonality for new production cell lines, a thorough understanding and characterization of the cloning method used is needed. For limiting dilution and soft agar-based techniques, the likelihood for clonality depends on the dilution of the cells [56]. If no other controls are performed, typically two sequential cloning steps are performed, and the overall probability of clonality is obtained by multiplication of the probabilities for the individual steps.

Since two sequential steps are time consuming, labor-intensive, and lead to prolonged culture of the cell (and therefore increased risk of accumulating mutations and other alterations), a single step cloning approach is preferable. This can be obtained by increasing the dilution factor in the case of limiting dilution or soft agar-based techniques. However, this will be very impractical, as the number of clones obtained per multiwell plate will be very low. Also, most if not all CHO suspension cell lines form aggregates of two or more cells, at least to the extent of low double digit percentages. Since these doublets or aggregates are not depleted by simple dilution, they represent a risk for clonality of the derived cell lines. For flow cytometry based systems, this risk is considerably lower. The principle of doublet discrimination by pulse processing has been used for decades, and the accuracy of deposition of droplets containing single cells is high [57]. Therefore, flow cytometry can be considered the most reliable approach with respect to robust generation of clonal cell lines. However, all these approaches have to be experimentally validated by the user with respect to likelihood of clonality, since important parameters such as cell size, aggregation rate, and behavior in highly diluted cultures vary between labs, and may also vary between different production cell lines within one lab.

Both flow cytometry and limiting dilution approaches result in individual cells in 96- or 384-well plates. These often are documented by bright-field or automated fluorescence microscopy, and only wells which show one single cell after deposition are further evaluated. However, also in this case, the method itself needs to be validated experimentally by the user [58].

One single cell on an image primarily demonstrates that one single cell was present in the focal plane of the microscope at the time the image was taken. The presence of a second cell elsewhere in the well can only be excluded if the overall procedure, including timing in between deposition and imaging, sedimentation of the cells, etc., has been demonstrated to be sufficiently robust and sensitive.

7.2.6 Clone Development and Characterization

Once the single cell clones have been generated, these need to be scaled up through the use of various cultivation systems with increasing size. Also, during scale-up, cells need to be transferred from the initially used static cultivation systems (multiwell plates) to growth in suspension in shaken systems to generate high viability and high cell density cultures. During static culture in multiwell plates, cell confluence can be regularly determined using the above-mentioned automated high-throughput microscopy solutions. This allows for rapid monitoring of cell growth and determination of ideal time points for a transfer of the cells into the following microplate format.

Furthermore, screening for desirable properties of the clones, such as productivity, growth and product quality attributes needs to be performed. Typically, this is done in a step-wise manner, with the application of increasingly more refined and sophisticated methods along the way, allowing for the reduction of the number of clones from initially several thousands to a few hundred, until the final clone can be selected. In order to rapidly decrease the number of cell clones to be expanded, cellular productivity should be determined as early as possible. In the past, enzyme-linked immunosorbent assay (ELISA) systems have been the predominantly used quantification method for product titer determination. However, to increase the probability to establish as many as high-producing cell clones as possible a high number of cell clones must be analyzed exceeding the capacity of tedious ELISA-based protein quantification. Other analytical detection and quantification methods for recombinant proteins include, e.g., homogeneous time-resolved fluorescence (HTRF) based quantification or nephelometry [59,60]. In the meantime, high-throughput product quantification methods such as the Octet System from FortéBio/Pall, which employs bio-layer interferometry to directly quantify the secreted protein in the culture supernatant, have become standard in industrial cell line development facilities.

In a next step, the top few hundred cell clones exhibiting the highest productivity are selected and sequentially expanded over microplate format to shake flasks. For long time, this clone development process has been conducted solely in microplates cultivated under static conditions. However, at a very early stage where the cell clones are transferred to 96-well microplates, it would be also possible to change the cultivation mode from static to agitated culture. This switch may allow for identification of cell clones which robustly grow in suspension culture. Furthermore, cells cultivated in agitated cultures will not grow anymore as monolayer, which enables a three-dimensional space for cell growth. Therefore, cell cultures can be directly transferred, for example, from 96-well microplate to 6-well macroplate format. This can markedly reduce handling times and material costs, which eventually increases overall process efficiency. Regardless of the cultivation mode, cellular productivity and growth behavior of the cells should be monitored during the entire clone expansion process in order to gradually decrease the number of clones to be further evaluated. Final transfer and expansion into shake flasks is usually conducted using the top few dozen clonal cell lines to generate safety cell banks (SCBs).

Technical achievements in miniaturizing disposable bioreactor systems such as the Advanced Microscale Bioreactor (ambr) instruments, which have been developed by TAP Biosystems to mimic characteristics of classical bioreactors, offer accelerated and careful production clone examination at a very early stage during cell line development [61–64] (Chapter 28). This allows for parallel investigation of different production clones which can be analyzed with biological replicates in order to increase statistical content. Another key advantage of these micro bioreactor systems is that they are much better suited as scale-down models compared to classical shake flask or tube spin bioreactor cultivation, as they are capable of more accurately predicting cell culture behavior of the newly established cell lines as well as critical product quality attributes in larger bioreactor scales [61,64]. This allows for a further reduction in the number of top cell line candidates down to three to five which are finally characterized in more detail for qualities such as bioprocess performance, metabolic profile, and product quality. Finally, it is very important to assess the stability of cellular productivity prior to the nomination of final production clones and to monitor product quality and integrity throughout the entire production run. The duration of a production campaign depends upon the final production scale as well as the desired process mode, but usually takes 60–90 days. Hence, transgene expression stability and stability of product quality need to be investigated for a period equal to the time from thawing a WCB vial to harvest. Only clones exhibiting a stable phenotype will be acceptable for commercial manufacturing, from both economical and regulatory points of view. A schematic overview on the entire cell line generation and characterization process is illustrated in Fig. 7.2.

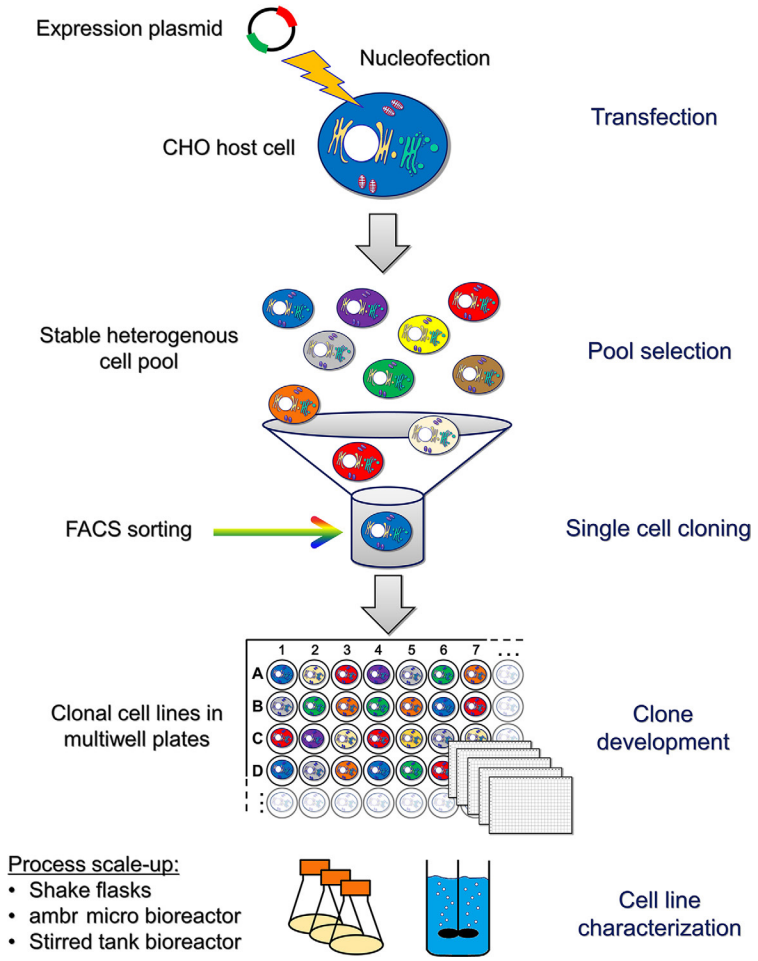

FIG. 7.2 Schematic representation of a state of the art cell line development process designed for the generation of high-producing CHO manufacturing cell lines. Host cells are transfected with DNA encoding a recombinant protein. After stable transfectants (pools) have been selected single cell cloning (e.g., by FACS, limiting dilution series etc) is performed to generate a monoclonal cell line which is derived from an individual cell. This is achieved by depositing hundreds of different single cells individually into 384- or 96-well microplates, followed by clonal expansion until a sufficient number of cells can be cryopreserved. During clone development cells are assessed for cellular productivity and growth performance to select optimal clones. Eventually, the best clones are characterized in detail using representative scale-down cultivation models for bioprocess performance in fed-batch or perfusion cultivation and product quality before the final production clones are selected.

7.3 HOST CELL ENGINEERING

Compared to bacterial or yeast-based expression systems, mammalian cell factories still exhibit some bottlenecks in terms of cell-specific productivity or maximum viable cell densities [65]. However, constant optimization efforts have increased cellular productivity of mammalian production hosts more than a hundredfold within the last two decades [50,66]. Besides bioprocess developments or improvements in expression vectors and CD media compositions [31], host cell engineering represents a powerful approach to enhance production cell performance for biopharmaceutical manufacturing [68,69]. In recent years, novel discoveries in genetic engineering such as precise genome editing or exploitation of non-coding RNAs had remarkable impact on CHO cell line development [70]. Fig. 7.3 provides an overview of the most important host cell engineering strategies using functional genomics tools.

Even though current state-of-the-art cell line generation processes are capable of routinely producing manufacturing cell lines exceeding volumetric productivities of 5 g/L, further improvements of CHO cell factories will still be required in the future. For instance, increasing overall production or secretion capacity of host cell lines can markedly reduce the number of clones to be screened to identify a high-producing cell line. In addition, engineering cell growth and proliferation supports accelerated and more robust clonal recovery rates after single cell cloning which shortens cell line development timelines. Suppressing susceptibility to apoptosis, a major cause of cell death during bioprocessing, can either prolong cultivation timelines thus increasing volumetric titers [71,72], or can increase cell viability at the time of harvest, which minimizes host cell protein (HCP) content and therefore impurities in the culture supernatant. Removal of intracellular

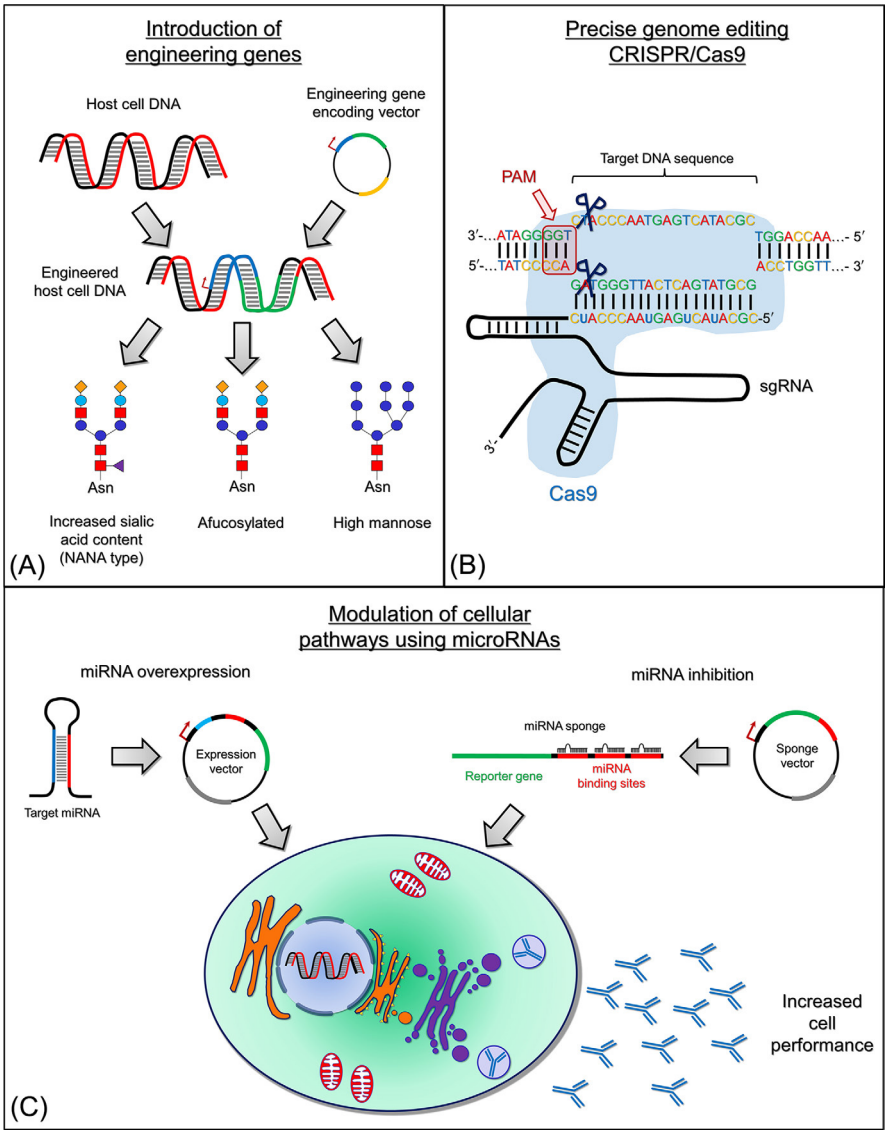

FIG. 7.3 CHO cell engineering. Cellular performance can be optimized by host cell optimization using genetic engineering. (A) Beneficial genes which improve bioprocess performance can be introduced into the host cell genome or (B) genes which negatively affect bioprocess relevant phenotypes can be knocked-out by genome editing technologies such as CRISPR/Cas9 in order to prevent their activity. (C) An innovative approach to regulate CHO cell phenotypes without adding a translational burden to the host cell represents the overexpression of advantageous or repression of disadvantageous microRNAs (miRNAs). Thereby, modulation of expression of hundreds of different endogenous genes can be achieved by small non-coding RNAs.

bottlenecks hindering efficient production of difficult-to-express proteins, which currently appear more often in biopharmaceutical development, would be another issue to be addressed by genetic engineering in order to elevate production titers up to levels that meet the requirements for commercial manufacturing. Finally, there is an increasing demand for recombinant proteins exhibiting tailored forms of post-translational modifications such as afucosylated mAbs which are used as therapeutics in cancer therapy. Production of recombinant proteins with pre-defined properties have already become feasible using genetically engineered CHO cell lines [73–78]. Finally, inhibiting expression of endogenous proteins which might be dispensable for a production cell and which additionally contribute to HCP content would be another ultimate goal for host cell engineering. Besides releasing energetic resources to be used for recombinant protein expression, knockout of unnecessary genes may result in a reduction in HCP content in the culture supernatant to significantly increase convenience for downstream and analytical development.

Strategies for host cell engineering can include methods such as the overexpression of endogenous genes [79–82], or the introduction of exogenous gene products (originated from other species) that induce superior cell phenotypes [83–86]. In contrast, suppression of disadvantageous gene products which promote apoptotic cell death [87,88], impair product quality [89,90,92], or negatively affect cell metabolism [93,94], can be erased by gene knockout or RNA interference (RNAi)

mediated gene silencing. Furthermore, recent transfer of the novel CRISPR/Cas9 genome editing tool into the CHO cell research area has paved the way for establishing cell lines showing multiple knockout phenotypes [95,96]. After identification of dispensable genes which are not required for a high-yielding CHO cell factory, the CRISPR/Cas9 technology can be employed to gradually establish a minimal CHO host cell. Consequently, genomic elimination of unnecessary genes might relieve protein translation capacity and increase the availability of free energy which can be used for the production of the transgene as well as may decrease the number of HCPs to contaminate the culture supernatant. Finally, a novel class of small non-coding RNA molecules termed microRNAs (miRNAs) has recently entered the field of CHO host cell engineering [97–99]. These crucial endogenous regulators of gene expression are considered to be next-generation cell engineering tools, as individual miRNAs can control entire cellular pathways, and miRNA overexpression does not add translational burden to the host cells [70,100]. All these advances have contributed considerably to the development of the CHO expression platform in the past, and continuing efforts in CHO host cell engineering will certainly provide an avenue for further optimizations in the future.

7.3.1 Strategies to Develop Biosuperiors/Biobetters (Glyco-Engineering)

The use of engineered cell lines for the development of "biosuperior" mAbs with enhanced pharmacological properties has shown great promise, with at least 16 glycoengineered mAbs having entered clinical trials [101]. The main target for glycoengineering has been to increase ADCC activation by either reducing or removing core fucosylation of N-linked oligosaccharides.

One approach has been to knock out intrinsic α-1,6-fucosyltransferase (FUT8) enzyme activity, which is responsible for core fucosylation (POTELLIGENT technology; [90]). Other recombinant DNA-based glycoengineering approaches have been achieved through overexpression of heterologous β-1,4-*N*-acetylglucosaminyltransferase III (GnT-III); GnT-III adds a bisecting GlcNAc to an oligosaccharide, which sterically blocks core-fucosylation (GlycoMAb;[102]), and overexpression of heterologous GDP-6-deoxy-D-lyxo-4-hexulose reductase (GlymaxX; [103]). A different approach to produce glycoengineered MAbs is to enhance CDC activity by, for example, feeding uridine, manganese chloride, and galactose during a fed-batch process, which promotes antibody galactosylation and therefore enhances CDC activity [104].

7.4 CONCLUSIONS AND FUTURE PERSPECTIVES

This chapter has comprehensively summarized the most critical factors important for creating an economic and productive manufacturing CHO cell line. During the past 25 years, CHO cell line development processes have gone through a remarkable progress reflected in a hundredfold improvement in cellular productivity in terms of monoclonal antibodies manufacturing. This success story was mainly driven by technical achievements, but also by a more in-depth understanding of CHO cell biology, which is displayed by a considerable track record of scientific publications. However, considering that the natural limit of heterologous protein expression has not been entirely reached yet, especially in case of novel biopharmaceutical product formats (which have not undergone millions of years of evolution), state-of-the-art cell line engineering strategies are crucial to steadily improve mammalian cell factories for biologics production. The increasing numbers of sophisticated protein formats of drug pipelines of most pharmaceutical companies, combined with the emergence of novel therapeutic formats (such as viral vectors used for gene and oncolytic therapy) raise the question of whether the human expression system will become more interesting in the future. However, most of the cell line development processes outlined above will likely also apply to novel production host cell systems and will thus still provide practical suggestions for a broad range of users in the biopharmaceutical sector. The ideal cell line development approach requires balancing a multitude of factors. Thoroughly considering the described technical factors and using the appropriate data for informed decision-making contributes to the successful development of a biopharmaceutical production cell line. The most important technical factors are further summarized as follows:

(i) The use of a robust and fast-growing, well-characterized host cell line adapted to a suitable medium, allowing the cultivation from host cell to final clone in a comparable environment without the need of extensive adaptation.
(ii) Expression of the GOI using a vector system ensuring high levels of mRNA transcription, mRNA stability, translation initiation, and protein translocation.
(iii) A transfection procedure highly compatible with the host cell and media system, carefully optimized and titrated for performance of gene transfer and recovery of high-producing cells.
(iv) A combination of selectable markers and selection procedures suitable to balance the need for stringent selection and timely recovery of high producing cells, often requiring labor-intensive and time-consuming optimization before establishment of a robust and high performing standard procedure.
(v) The use of a well-characterized system for the generation and documentation of single cell clones, including an optimized medium composition to allow for efficient recovery and upscale of cell clones.

(vi) A rapid and robust scale-up of clones until a first cell bank can be prepared. This also includes a thorough stability assessment of the final production clone.

(vii) The use of relevant assays, ideally applicable at miniaturized scale, to determine as early as possible the performance of the selected cell clone in a production process.

(viii) The use of analytical methods to monitor the quality of the recombinant protein already at very small scale.

(ix) The availability of a robust and scalable platform process to allow for the fast and smooth scale-up from benchtop scale to toxicity and clinical material production.

(x) In case when specific, tailored product quality attributes are needed, the use of an engineered and well characterized host cell capable of introducing the necessary post-translational modifications.

APPENDIX I BIOSAFETY ANALYSIS FOR CELL BANKS

Biosafety testing and characterization of eukaryotic and prokaryotic cell-lines used for the development/manufacturing of therapeutic products intended for human use falls under the framework of recommendation/guidelines issued by the international regulatory authorities.

Of special importance are the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH) guidelines Q5A [105], Q5B [106], Q5C [107], Q5D [25], and the Q7A [108].

The FDA has published a number of relevant guidance documents and points to consider: [109], [110], [111], [112], and [113].

The European authorities (EMA) have published as well several guidance documents: [114,115], [116], [117] and [118].

Below are proposed testing schedules for Master Cell Banks for eukaryotic and prokaryotic cell-lines:

	Rodent cell lines	**Human cell lines**	***E. coli* cell strains**
Purity and identity testing of host cell	• Sterility testing by direct inoculation method • Qualification of test article material for sterility by direct inoculation method • Mycoplasma detection, conducted according to EP or PTC guidelines • Identity and characterization of parental cell origin by analysis of isoenzymes or fingerprint assay (PCR)	• Sterility testing by direct inoculation method • Qualification of test article material for sterility by direct inoculation method • Mycoplasma detection, conducted to EP or PTC guidelines • Identity and characterization of HEK / PER.C6 cell origin by analysis of isoenzymes or fingerprint assay (PCR)	• Identification of Enterbacteriaceae and other gram negative rods using API-20 • Identity of E. coli • Detection of bacteriophage in E. coli or material used in the propagation of E. coli cultures • Purity testing of bacterial cell banks: Presence of bacterial and fungal contaminants • (Determination of purity of the bacterial strain by gram staining)
Viral testing	The focus point for the testing strategy is the MCB (and EPC). An example strategy is outlined below for the various virus test-assays. • Reverse transcriptase assay, PCR based, followed by S+L- assay if positive • Direct/extended S+L- focus assay: In vitro detection of murine retroviruses alt. • Transmission Electron Microscopy (TEM) • Mouse antibody production assay (MAP) • Hamster antibody production assay (HAP) • In vitro adventitious virus assay 28 days (3 detector cell lines) • In vivo adventitious virus assay using suckling mice, adult mice and embryonated eggs (consider FDA and EMEA demands if an additional assay is requested)	The focus point for the testing strategy is the MCB and later EPC. An example strategy is outlined below for the various virus test-assays. • Transmission Electron Microscopy (TEM) • Reverse transcriptase assay, PCR based, followed by S+L- assay if positive • Direct/extended S+L- focus assay: In vitro detection of human retroviruses alt. • Human virus PCR screen (HIV 1&2, HTLV 1&2, CMV, EBV, HHV 6,7&8, HAV, HBV, HCV, human parvovirus B19, HpoV etc) • In vitro adventitious virus assay 28 days (3 detector cell lines) • In vivo adventitious virus assay using suckling mice, adult mice and embryonated eggs (consider FDA and EMEA demands if an additional assay is requested)	N/A

Continued

	Rodent cell lines	Human cell lines	*E. coli* cell strains
	• Assay for detection of Mouse Minute Virus (PCR) • In vitro bovine virus screen according to 9CFR (BVDV, BAV, BRSV, BPV, Reovirus, Bluetongue and bovine polyoma virus) by PCR • In vitro porcine virus screen for parvovirus • Detection of Cache Valley Virus and West Nile Virus	• Detection of adeno-associated virus (AVV) by PCR • Note: only recommended when expression system is based on recombinant adeno virus • In vitro porcine virus screen for parvovirus • In vitro bovine virus screen according to 9CFR (BVDV, BAV, BRSV, BPV, Reovirus, Bluetongue and bovine polyoma virus) by PCR • Detection of Cache Valley Virus and West Nile Virus	
Genetic characterization	Only applicable with GOI inserted • Restriction enzyme map on plasmid vector, "for information" generated in house or equivalent • Sequence data of GOI from plasmid, "for information" generated in house or equivalent	Only applicable with GOI inserted • Restriction enzyme map on plasmid vector, "for information" generated in house or equivalent • Sequence data of GOI from plasmid, "for information" generated in house or equivalent	Stability and genetic characterization only applicable with GOI inserted • Plasmid Retention Testing for Bacterial Cell banks • Viability Testing for Bacterial Cell Banks • Genetic stability and plasmid identity by restriction enzyme map analysis • Sequencing of the recombinant plasmid expression unit and flanking sequences. • Sequencing of a recombinant plasmid expression vector • Gene Copy number

APPENDIX II TRANSIENT TRANSFECTION

Transient transfection is used to produce mg to g amounts of material for early development activities. This is achieved in working volumes ranging from the liter scale up to 100 L [2,119]. The transgene is not stably integrated into the host cell genome. As a result, no clone selection and screening is needed. Traditionally, transient transfection has been used with HEK-293 cells due to higher titers compared to other cell lines [120]. However, the product quality might differ from CHO derived material [121] which could be important when the material is used for preclinical assessment of lead candidates. The main challenge and limitation with transient transfection is the rapid plasmid copy number dilution during cell division, resulting in low productivities in the later stage of the process.

To improve titers in transient transfection, technologies to maintain the plasmid copy number in transient transfection have been developed. Most technologies incorporate viral elements to maintain the plasmid copy number and give better titers, e.g., Epstein-Barr virus nuclear antigen 1 (EBNA-1) with plasmids containing an Epstein-Barr virus latent origin of replication (OriP) to facilitate plasmid maintenance [122]. This system is even used for transient expression in CHO and high titers have been reported by the coexpression of genes encoding EBNA-1 and GS [123]. Furthermore, elements from murine polyomavirus can also be added to the vector to allow plasmid replication and maintenance [124]. However, even stable transfected pools can be used for initial material production [66].

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