Cho Cell Line Development & Engineering: Impact On Biopharmaceutical Production

1. Introduction

The approval of Chinese hamster ovary (CHO) cell cultures for the manufacturing of protein therapeutic products modernized conventional medicine. CHO cells remained the workhorse for the production of monoclonal antibodies (mAbs) for the last at least 20 years for different reasons. First, CHO cells are easily adapting and growing in suspension cultures which are ideal for large scale manufacture. Moreover, very few human viruses are able to proliferate in them. CHO cells can be submitted to post-translational modifications to make them compatible and bioactive in humans. Finally, gene amplification systems can take advantage of the genome instability of CHO cells and result in higher yields of recombinant proteins (Lai et al., 2013).

Biopharmaceutical companies spend a lot of funds to improve their manufacturing processes on two key aspects. Cell line development and engineering (CLD&E) and culture processes. Their main focus is the development of cost-effective methods and high yielding expression hosts to increase profits (Hong et al., 2018). A lot of host cells have been used to produce therapeutic mAbs with the final product being different regarding immunogenicity and clinical efficacy, indicating that host cells have a great impact on how biological active are the recombinant mAbs (Li et al., 2018). Enhancing the performance of CHO host cells in order to meet specific product quality criteria is one of the main targets of CLD&E while recently there have been many tries to establish a stable CHO cell line which produces high titers even in continues cultures (Hong et al., 2018).

2. Impact on Biopharmaceutical production

The preferred platform for biopharmaceutical manufacturing is the mammalian expression systems. These cell lines can produce large, perplexed proteins with post-translation modifications (PTMs) with a high resemblance to those in humans. Additionally, most proteins can be secreted instead of requiring cell lysis for extraction which can result in the refolding of the protein (Dumont et al., 2016). Nevertheless, PTMs, such as galactose-a1,3-galactose (a-gal), which are not expressed in humans can be produced by non-human mammalian cell lines. Consequently, cell lines have to be inspected and the clones with the acceptable glycan profiles to be identified (Dumont et al., 2016).

Since 2001, biopharmaceutical market is showing a constant increasing trend with an average growth of 35% per year. CHO cells will remain the main host for the production of therapeutic proteins, as an improvement of titers (~100-fold) has been observed in the last years. This improvement has been linked to the development of serum-free medium and the optimisation of feed strategies (Kim et al., 2012). However, the global market demand cell to be more productive and to be seeded and grown in, rigorously optimised, bioreactors resulting in increased cell densities (Kim et al., 2012).

Recombinant proteins produced by mammalian cells can be used in treatments against cancer,

infectious diseases and diseases like haemophilia. Nowadays, increasing yields of titers up to 10 g/L have been reported while the global sales of monoclonal antibodies (mAbs) were US$140 billion in 2017 (Walsh, 2018, EvaluatePharma, 2019) and are projected to reach up to US$200 billion by the year 2022 (Grilo and Mantalaris, 2019). The large-scale production of recombinant proteins is based on bioprocesses normally performed in dimensions up to 20,000 L. However, in order process optimization to be achieved; a scaled-down process is necessary which demands less materials thus reducing the overly large cost associated with large volumes.

The most used bioreactor in Biopharma is the fed-batch operated one, where the selection of the culture system can significantly influence the final product's yield and quality as well. The increasing demand on both quality and quantity ¬¬highlights that new strategies targeting towards both attributes must be developed. Hence cell line engineering has been employed to look upon other important features such as growth behavior, apoptosis and stress resistance, glycosylation pattern, viability and improved secretion of products too.

Apart from the process development, a major cost derives from clinical trials in order for the new protein to be approved by either FDA or EMA. During clinical trials the product is thoroughly investigated for potential side-effects and efficacy. Overall development cost of just one biopharma product is estimated to be around US$1 billion including development, pre-clinical and clinical trials (DiMasi and Grabowski, 2007). There are many saving potentials by implementing genetic engineering strategies aiming the minimization of development and process associated costs.

3. Review of current state of technology

Cellular and genetic engineering methodologies have been used to modify the host cells, including novel ways to regulate the growth of cell, prevent cell death and stimulate PTMs (Dangi et al., 2018a). This is mainly accomplished by controlling apoptosis, metabolic engineering, engineering cells for growth at lower temperature, chaperone engineering and glyco-engineering.

Traditionally, CHO CLD includes the transfection of a gene of interest, adaptation to specific conditions, gene amplification and selection of the transfected clones. After transfecting the cells with the gene of interest (GOI) high producers are created and identified with gene amplification systems such as DHFR-MTX. Next step is the isolation and selection of the high producer cells which can be done with an antibiotic to which the cells with the GOI are resistant to, or with Flow cytometry based on florescence. The technique to create a high producing cell line remained intact but the use of microfluid machinery, automated liquid handling systems and high-throughput analytics for single cell cloning, clone expansion, deep well plate cultivation and analysis of product quality attributes can lead to a more efficient and integrative process (Hong et al., 2018).

3.1. CHO cell line development

3.1.1. Ribosome engineering

MicroRNAs (miRNAs) are short non-coding RNAs with abilities to regulate entire cellular

pathways by controlling the expression of several genes post-transcriptionally. They have been used by industry to resolve issues arising during cell line development, providing simultaneous expression of different genes of interest rather than just a target gene. Their advantage over cellular engineering is that they don't require to be translated into proteins hence there is no translational limitation for the establishment of the new cell line (Dangi et al., 2018a)

3.1.2. CRISPR-Cas9

It is known that gene manipulation can be achieved through genome editing, a powerful tool for biomedical research, targeting various diseases. CRISPR was discovered in 1987 in the E. Coli genome as a series of repeated fragments of 29 nucleotides (nt) in length interspaced with variable sequence fragments of 32 nt. CRISPR-Cas9 is the most used genome editing system, which facilitates the fact that mature crRNA binds with a tracrRNA and forming a tracrRNA:crRNA complex. The guide RNA has complementary bases to those of the target sequence in the genome. This complex leads the Cas9 to the target site, where two DNA strands are split by the nuclease domain of Cas9. When the cut across both strands of DNA happens, cell identifies that its DNA is damaged and consequently deploys a series of steps to repair it. That can be used in order changes to one or more genes to be introduced into the genome of the cell (Rodríguez-Rodríguez et al., 2019).

3.1.3. Zinc Finger Nucleases (ZFNs)

Zinc Finger Nucleases (ZFNs) are synthetic restriction enzymes created by combining a zinc finger DNA-binding domain to a DNA-cleavage domain, engineered to aim at a desired DNA sequence. This allows ZFNs to find unique sequences within complicated DNA genomes. Leveraging endogenous DNA repair systems ZFNs can be used to modify with high precision the genomic DNA (Dangi et al., 2018b).

Until recently ZFNs have been widely used for HDR-mediated knock-in (KI) and knock-out (KO) of genes into CHO cells. CHO genome was altered by KI of GS and dihydrofolate reductase (DHFR) genes, establishing them as a high producer of recombinant proteins. However, ZFNs are not preferable within the industrial community due to certain constraints. It is not feasible all nucleotide genomic sequences to be be targeted. Specificity of sequence can be interrupted by neighboring protein domains. Additionally, constructing ZFPs is very complicated and along with their cost they are a less favored practice (Dangi et al., 2018b).

3.1.4. Transcription Activator-Like Effector Nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are engineered restriction enzymes, targeting to cut chosen DNA sequences. TAL proteins are the main part of TALENs, secreted from Xanthomonas bacteria. Their capability is to recognize a nucleotide not influenced by other domains present around it. TALENs are most commonly used for knock-in and knock-out of genes. Just like ZFNs, they also consist of two-site protein domains, whose work is to cut while TAL leads proteins to selected sites. TALENs used for KO have an effectiveness of 30-100%, while those used for KI 1-10% (Dangi et al., 2018b).

3.2. CHO cell engineering strategies

3.2.1. Regulating Cell Cycle Progression

Regulating the progression of the cell cycle can lead to higher cell density, viability and specific productivity in mammalian cells. The use of a small molecule, cell cycle inhibitor (CCI) was able to induce complete G0/G1 arrest in CHO cell cultures through selective inhibition of cyclin CDK 4/6 and led to improved specific productivity by twofold to threefold (Dangi et al., 2018b).

3.2.2. Engineering of Chaperones

Chaperones and foldases play an important role in the mediation of the mAb's folding. Hence, engineering those may influence the production levels of recombinant proteins by regulating the translational capacity of the antibody. Molecular chaperones are a group of proteins facilitating the folding and targeting of proteins in normal and stressed cells. The handling of chaperone levels in a cell line can significantly increase both the achievable steady-state levels and authenticity of a wide range of recombinant proteins. Large-scale production of mAbs from CHO cells for the pharmaceutical industry lead to studies on how the proteins fold and on the optimization of chaperone network (Jossé et al., 2012).

3.2.3. Regulating Apoptosis

The programmed cell death due to high stress circumstances is called apoptosis. Finding ways to inhibit apoptosis can lead to increased cell viabilities and expand cell life and productivity. Strategies to achieve that include the overexpression of anti-apoptotic and down-regulate pro-apoptosis genes. Postponing apoptosis can also be achieved with periodic nutrient feeding and the use of different carbon sources in the culture media such as galactose instead of glucose.

3.2.4. PTMs

Antibodies derived from CHO cells are described by low levels of bisecting-N-acetylglucosamine (GlcNAc) and high levels of core fucosylation. Non-fucosylated mAbs have also been advertised as the next generation of therapeutic antibodies. One of the methods used, involves knocking out the fucosyltransferase gene (FUT 8) from CHO cells resulting in the production of non-fucosylated molecules (Dangi et al., 2018b).

3.2.5. Metabolic Engineering

Culturing CHO cells can result in the accumulation of lactate and ammonia produced by the consumption of glucose and glutamine in the medium. Metabolic engineering methodologies have been deployed to deal with the such toxic metabolic by-products. One way to handle the increased level of ammonia in the culture is to over-express the glutamine synthetase (GS) gene in CHO cells, which will enable them to grow in the absence of glutamine into the medium serum. Similarly, the overexpression of pyruvate carboxylase can be used in order to reduce the accumulation of lactic acid (Dangi et al., 2018b).

3.2.6. Engineering Cells for Hypothermic Growth

Reducing the temperature throughout cell culture of CHO cell can lead to improved mAb production, due to the prolonging of the growth phases. As a result, there have been observed

that cell remain viable for a longer period. Developing genetic engineering methods for CHO cells to be cultured in low temperatures have been very promising. Cell line engineering has many potentials as well, including not only improved antibody production but production of other biopharmaceutical products (i.e. vaccines, fusion proteins, growth factors) as well. In addition, obtaining a desired quality in the final product and decreasing the impurities which can have a negative impact on the downstream process have also been attributes which can be dealt with such strategies (Dangi et al., 2018b).

4. Future developments

The global demand of the biopharmaceutical industry is constantly increasing throughout the last years, leading to a need for new CHO cell engineering strategies aiming towards process optimization. It is expected that ordinary-manufactured monoclonal antibodies will be eventually replaced by novel molecule formats such as antibody fragments or artificial scaffolds, requiring new CHO cell line development. Recently, there have been extraordinary advances in genome editing strategies such as the CRISPR-Cas9 tool outlining the potentials of novel engineering strategies (Fischer et al., 2015). Another very promising cell engineering strategy is the use of synthetic gene circuits from yeasts.

4.1.1. Synthetic gene circuits

It is reported that gene circuits could have various health care applications (Nevozhay et al., 2013). It is common that mammalian gene expression is regulated by gene expression systems which consist of a regulator whose control in the expression of a specific gene relies on the inducer lever in the culture medium. Certain limitations also arise, which can be dealt with precise mammalian gene expression control strategies. A promising solution can be a negative feedback-based linearizer gene circuit, utilized in yeast which has two appealing characteristics: (i) linear dependence between gene expression and extracellular inducer concentration and (ii) uniform gene expression across the cell population at all induction levels. These attributes can be utilized to repress the expression of a protein and consequently of the responsible gene. At the same time, the increase at the concentration of the inducer results in synthesis of new proteins. Consequently, repressor level can be linked proportionally with the concentration of the inducer. What can also have to be considered is that the expression of all genes with identical promoters are also linearly depended on the concentration level of the inducer. Therefore, similar circuits can have potential use within mammalian cells, where controlled and uniform gene expression across the whole cell population is desired. That is a promising cell engineering strategy with which the regulation of gene expression in CHO cells could result in enhanced new antibody-producing cell lines (Nevozhay et al., 2013).

5. Conclusions

CHO cells are the main manufacturing platform to produce therapeutic proteins with global sales expected to reach up to US$200 billion by 2022. A dramatical increase in the production yields of different CHO cell lines has been documented during the last thirty years, as novel cellular engineering strategies are constantly deployed. Usually, CHO cell lines are being established after thorough cell screening of randomly mutants. Most recently, after mapping the whole CHO cell genome, it is easier to apply target genome engineering methodologies based on systematic approaches which all lead to higher product quality and yields.

Even though mammalian cells are the dominant platform for mAb production they face certain limitations the majority of which can be dealt with cell engineering approaches. These include regulating the cell cycle progression and the regulation of apoptosis which both can prolong the cell viability in the culture. Engineering of the chaperone molecules can lead to proper folded polypeptide chains, while metabolic engineering approaches can target to minimizing the accumulation of toxic by-products such as ammonia and lactate.

Cell line development has played a major role to the production of various products too. Host-cell engineering using micro-RNAs is an engineering strategy targeting multiple genes simultaneously hence it is preferred over single gene expression strategies. Gene editing tools have also been used for cell line development. Amongst them are the ZFNs, TALENs and lately in the spotlight CRISPR-Cas9 whose use has been linked with treating genetic diseases such as HIV and Leukemia. It is crucial though ethical boundaries to be established as CRISPR-Cas can be used to target specific sites of human genome as well. In CHO cells it can facilitate KI and KO of genes of interest for improving the quality and yields of mAbs production.