Recombinant proteins therapeutic use | essay

Recombinant therapeutic proteins produced in Mammalian cells represent a major class of the Biopharmaceutical industry. The Biopharmaceuticals produced by these proteins are mainly used for medical applications. Most recombinant proteins in this category are thus complex molecules that need to be post translationally modified for their full biological activity. Since post translational modifications are being effectively done in mammalian cells they have become the preferred hosts for the recombinant protein expression. The selection of High producing cell lines is a very important step in the process development for the production of Biopharmaceuticals. Traditional methods of selecting cell lines are often time consuming and they significantly limit the number of clones that can be screened. In order to meet the growing demand for therapeutic proteins produced from Chinese Hamster Ovary cells new methods of screening Mammalian cells have to be developed. Efficient selection methods of the cell line are very useful for selecting high yielding cell clones for production. The new methods thus developed must have high levels of expression and must be cost effective, efficient and in a high-throughput manner.

Introduction:

The importance of recombinant proteins for therapeutic applications has been increasing tremendously. Currently almost 60% of all the recombinant therapeutic proteins are being produced in mammalian cells. Mammalian cells are the preferred hosts when compared to other expression systems because mammalian cells have the ability to generate high quality proteins that are similar in their biochemical properties to the naturally occurring human forms. The advantage of the mammalian cells as well as the growing

demand for high quality recombinant therapeutics is driving researchers to develop new methods for selecting high producing cell lines. The new methods must thus be time-consuming, simple, efficient and high-throughput. The following sections will clearly outline the different expression systems and the advantages of Mammalian cells (CHO cells) that are being used for the production of therapeutic proteins. It will also give a detailed description about the different selection methods for producing a high yielding cell line for industrial production of the proteins.

Recombinant protein Expression:

Recombinant protein expression is the foundation of today's biomolecular research and the developing Biotechnology industry (Baldi, L et al 2006). Recombinant proteins are basically manipulated forms of proteins which are generated in various ways to produce large amounts of the protein. Recombinant proteins represent a major class of Biopharmaceuticals. The pathway for the development of stable cell lines for the production of recombinant proteins should be carefully regulated at each step so as to get high yields of the purified protein (Kito, M et al 2002).

- Selection
- Transfection
- MTX/MSX
- Screening
- Gene Amplification
- Expansion
- Cell Line Characterisation & Process Development

Figure 1: Development of stable cell lines for the production of recombinant proteins

Expression Systems:

The expression system can be broadly classified into two categories namely the Prokaryotic systems and the Eukaryotic systems. The advantages of the prokaryotic and the eukaryotic systems have to be clearly understood before selecting a cell line for the production of Biopharmaceuticals. The prokaryotic systems are advantageous because they can be easily grown to high cell densities and also of their ease of cloning. The disadvantages of this system are it is normally not used in industrial processes because of the plasmid instability, hyperglycosylation problems and it also does not perform all post-translational modifications. Whereas the eukaryotic systems are mostly preferred because they can be easily grown to high cell densities, high cloning capacity, high and stable expression of the heterologous gene and also has the ability of performing post-translational modifications. (Wurm, F. M. 2009)

- Prokaryotic Systems
- Bacterial [E. coli and Bacillus subtilis]
- Expression Systems
- Eukaryotic Systems
- Yeast
- Baculovirus
- Mammalian cell lines
- Filamentous Fungi

Figure 2: Various expression systems

Mammalian cell lines

The Mammalian Cells have become the preferred hosts for the expression of complex proteins with authentic post-translational modifications that are seen in the natural host (Wurm, F. M. 2004). The big boost to mammalian cell culture came with the development of hybridoma technology (Kohler and Milstein, 1975) who showed that mAbs can be expressed in large quantities in in vitro cell culture. There are various mammalian expression systems namely

- Chinese Hamster Ovary (CHO) Cells
- NSO Hybridoma Cells
- Baby Hamster Kidney Cells (BHK)
- PER. C6 Human Embryonic Retinal Cell Line

The CHO cell lines and the NSO cell lines are more commonly used (Dickson, A. J. et al, 2002). The Chinese Hamster Ovary (CHO) cells are the most important cell lines for the production of recombinant proteins (Hacker, L. D 2009). They are more commonly used due to their high levels of protein expression, post translational modifications, easily genetically engineered, stable glycosylation and can excrete high titers of recombinant proteins in solution. CHO cells are used in studies of genetics, toxicity screening, nutrition and gene expression particularly expression of recombinant proteins. CHO cells have become the mammalian equivalent of E. Coli in research and development (Puck 1985). CHO cell lines are the cell line of choice when long-term (stable) gene expression and high yields of proteins are required (Kaufman, 1982).

4. 1 CHO Cells:

The CHO cells used in the manufacturing of proteins originated in 1957 from a primary culture of ovarian cells of Chinese Hamster (Scientific name: Cricetulus griseus) (Puck, et al., 1958). Puck obtained a female Chinese hamster from Dr. George Yerganian's laboratory at the Boston Cancer Research Foundation and used it to derive the original Chinese Hamster Ovary (CHO) cell line. Having a very low chromosome number (2n= 22) for a mammal, the Chinese Hamster is an ideal model for radiation cytogenetics and tissue culture. CHO cells are widely used in studies of genetics, toxicity screening, nutrition and gene expression particularly expression of recombinant proteins. CHO cells are widely used to produce antibodies using both Dihydrofolate Reductase (DHFR) and Glutamine Synthetase (GS) expression systems. DUKX-B11 (Urlaub and Chasin, 1980) and DG44 (both lacking dhfr) are the most commonly used CHO strains with DHFR expression vectors. The GS system uses CHO-K1 and CHOK1SV.

Mammalian Selectable Markers:

A wide range of mammalian selectable markers have been developed to isolate stable cell lines. Dihydrofolate reductase (DHFR) and Glutamine synthetase (GS) (Lonza Biologics) are two such markers used in the industrial production of commercially valuable proteins. These expression vectors usually have strong promoters to drive expression of the antibody genes. The various steps involved in the insertion of plasmid into the cells for expression can be explained with the help of a diagram.

5. 1 DHFR Expression System

DHFR is the oldest and the most well-studied dominant selection marker routinely used to isolate stable cell lines. DHFR is a small monomeric enzyme that catalyses the conversion of folic acid to tetrahydrofolate (THF). The DHFR system uses the folate analogue Methotrexate (MTX) to inhibit the function of DHFR (Fann et al., 2000; Yoshikawa et al., 2000). MTX binds and inhibits DHFR stiochiometrically, forcing the cells to undergo gene rearrangements and amplification for survival. The main function of the DHFR gene is to facilitate vector amplification. The DHFR gene is usually under the control of a weak promoter thus reducing the promoter interference and thus facilitates in increasing the expression of the immunoglobulin genes.

5. 2 GS Expression Systems

GS catalysis provides the pathway for the synthesis of glutamine. GS expression vector contains the GS gene downstream of a SV40 promoter (Bebbington et al 1992, Barnes et al 1990)). The GS system does not rely upon amplification to achieve high productivities. Instead, these rely on insertion of the antibody construct into a transcriptionally active region to achieve high productivities. In the absence of glutamine in the growth medium, the GS enzyme plays an essential role in the survival of the mammalian cells. This GS system uses Methionine Sulphoximine (MSX) as an inhibitor (Kaufman and Schinke, 1981; Bebbington et al 1992). The GS system allows for the rapid selection of high producing cell lines.

Methods for selecting High-producing Mammalian Cell lines

The selection of high producing mammalian cell lines is a very important process in the production of Biopharmaceuticals. The market is said to increase rapidly and hence there is an increasing need for developing new methods for selecting cell lines. These methods must be efficient, cost-effective and high-throughput. The traditional methods are time consuming and they are also limited by the number of clones that can be feasibly screened. Thus alternatives are being developed for the selection of high-producing cell lines.

6. 1 Traditional Methods:

The cloning method is mainly dependent on the individual properties of a particular cell line. At low cell density the individual cells can be discerned and cloning is usually carried out in petri dishes, multiwell plates or flasks (Davis, J. M., 2002). The various traditional methods include spotting technique, cloning rings and subsequent excision of colonies (Davis, J. M., 2002). The most commonly used method is Limiting Dilution Cloning (LDC) (Puck, T. T., and Marcus, P. I., 1955) because of the low cost and relative simplicity of the method. The disadvantage of this method is that it takes more than eight months for the selection of the clone and subsequent product analysis. Protein secretion cannot be measured individually and thus outgrowth of sub clones is required. The high level of downstream analysis means these methods are constrained by the number of cells that can be feasibly analyzed. Thus in order to find a cell line with required characteristics, tens of thousands of clones need to be evaluated (Drake, R., 2005) which is a time consuming process.

6. 2 Flow cytometry and cell sorting:

The flow cytometry and cell sorting methods considerably increase the number of cells that can be screened. In these cells single cells can be isolated from mixed populations even when they are present at very low frequencies. Another advantage is that several million cells can be screened in a short time (Rubeai, M. et al, 1991). This method has been facilitated by the availability of antibody and ligand-conjugated fluorochromes enabling isolation of cells based on cell surface protein expression.

6. 2. 1 Surface protein expression

In certain cell lines there exists a relation between the surface expression of recombinant proteins and levels of the secreted proteins. (Marder, P et al, 1990). Recent development in this selection method (De Maria et al, 2007), is done in CHO cells. CHO cells were transfected with a recombinant protein linked to CD20 (non-fluorescent reporter molecule). The proteins are linked by an internal ribosome entry site (IRES). Cells are screened by flow cytometry and are subsequently cloned. The high levels of expression can be predicted accurately at an early stage and it also enables monitoring of instability in protein expression over time easily. Instability can either be detected by a large degree of heterogeneity in fluorescence intensity or by the presence of two separate populations.

6. 2. 2 Green Fluorescent Protein

In this the cells can be isolated based on the levels of the intracellular proteins using reporter molecules such as Green Fluorescent Protein (GFP) (Gubin, A. N et al, 1997). In mammalian cells GFP has been used for the selection of high producing clones by co-expression with recombinant

proteins and selection based on fluorescent intensity (Meng, Y. G et al 2000). A correlation between Human Embryonic Kidney cells co-expressing a recombinant rat serotonin receptor protein and GFP were studied. (Mancia, F et al 2004). The cultures were then subjected to repeated rounds of sorting and expansion of cells. It has been observed that the highest GFP fluorescence intensity resulted in significant increase in recombinant protein production.

6. 3 Methods based on cell secretion rate

The above methods are not applicable for all cell types. Secreted proteins have the property of dissociating from the cells and the flow cytometry is not used to detect proteins that are not cell associated. Thus the following methods retain the recombinant protein in the vicinity of the cell that produces it.

6. 3. 1 Gel microdrop technology

Gel microdrop technology facilitates the measurement of protein secreted by a single cell (Browne, M. S., 2007). In mammalian systems it has been used to separate secreting from non-secreting hybridomas (Gray, F et al 1994). It is also used to detect low-producing populations (Hammill et al, 2000) and is applied for the isolation of high producing cells. This method can be used to measure any product to which a complementary fluorochrome-conjugated antibody is available.

6. 3. 2 Matrix based secretion assays

In this the protein is immobilized on an artificial matrix on then cell surface. The cells are labeled with biotin and are tagged directly with an avidin

antibody (Manz, R et al 1995) or via an avidin bridge using affinity capture surface display (ACSD) technique (Holmes, P., and Rubeai, M. 1999). The avidin bridge maximized the binding capacity of the matrix. Cells are incubated in high viscosity medium to minimize diffusion and the bound protein is labeled with fluorescent tag (Broth, N et al 2000). This method has shown to reduce the duration of the selection process by 25% and the clones generated were 5 times more than the clones obtained with LDC.

6. 4 Automated systems

6. 4. 1 Laser enabled analysis and processing (LEAP)

The LEAP system employs a process in which the unwanted cells are eliminated by laser. First the cells are cultured on a matrix and then the specific protein is stained with a fluorescent labeled antibody. Image analysis is carried out and once the cells of interest have been identified, the unwanted cells are eliminated by a laser beam and this allows the cell to proliferate. A five to twenty fold increase in productivity has been observed and also high levels of the secreted protein (Hanania, E. G et al 2005).

6. 4. 2 Automated Colony Pickers:

The automated colony pickers were initially used for bacterial or fungal colonies but recently these have been applied to mammalian cells. The cells are immobilized on a semi-solid medium and incubated to form colonies. After addition of the fluorescently labeled capture antibody, the secreted protein can be viewed as a halo around the colony (Lee, C et al 2006). When measuring the secretion by fluorescence, data from the white light and the fluorescent images are merged and the system excludes fluorescent colonies

that are close to non-fluorescent colonies thus preventing contamination with non-producing cells.

Conclusion

Mammalian cell culture is an expensive process with low yields and long development times. Due to the growing demand of therapeutic protein in mammalian cells we have to develop methods for the selection of these cell lines so that there is high yield of the protein in less time.