Enhancing Production of Recombinant Proteins from Mammalian Cells

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Abstract—The bio-manufacturing of recombinant proteins from mammalian cell cultures requires robust processes that can maximize protein yield while ensuring the efficacy of these proteins as human therapeutics. Recognizing that the challenge of improving protein yield and quality can be met through various approaches, this paper presents three strategies currently being developed in our group. A method for rapidly selecting subpopulations of cells with high production characteristics is proposed. This method combines the efficiency of green fluorescent protein/fluorescence-activated cell sorting (GFP/FACS)–based screening with homologous recombination to generate and select high-producing subclones. Next, the development of chemically defined, protein-free media for enhancing monoclonal antibody production is described. Analysis of culture media effects on the genome-wide transcriptional program of the cell is presented as a means to optimize the culture media and identify potential targets for genetic manipulation. Finally, we propose a method for increasing the extent of intracellular sialylation by improving the transport of CMP-sialic acid into the trans-Golgi. This is hypothesized to increase the sialic acid availability, and may enhance the degree of sialylation in the glycoprotein product.

Index Terms—Clonal Selection, Glycosylation, Mammalian Cell Culture, Media Design

I. INTRODUCTION

RECOMBINANT protein production using mammalian cells offers several advantages over microbial systems. For example, mammalian cells are able to secrete the protein product, hence, obviating the need for cell homogenization. Furthermore, they are able to perform post-translational modifications, like glycosylation, which are necessary for the efficacy of protein drugs targeted as human therapeutics. However, mammalian cells have significantly slower growth rates compared to microbes and are much more complex in their nutritional requirements. To meet the demands of commercial production and regulatory requirements, bio-manufacturers are faced with the challenge of maximizing productivity whilst controlling product quality.

Recognizing that the challenge of improving protein yield and quality can be met through various approaches, this paper describes three strategies currently being developed in our group. Firstly, to generate cell lines that can produce high yields of recombinant proteins, a method for rapidly selecting high-producing mammalian cell clones following recombination with the gene of interest is proposed. Apart from the productivity of a specific cell line, another factor that has significant impact on recombinant protein yield and quality is the culture media. We describe our efforts to design a chemically defined, protein-free media for enhancing protein production. In addition, the effects of culture media on gene expression are being studied to gain insights into the impact of the media environment on cell physiology. Finally, as a potential method to improve the efficacy of recombinant proteins produced through cell culture, we put forth a proposal for enhancing the sialylation process in Chinese Hamster Ovary (CHO) cell lines.

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II. A GFP-BASED SCREEN FOR HIGH-PRODUCING CLONES

A key step in the generation of cell lines producing recombinant proteins is the selection of viable clones following the incorporation of the gene of interest into the host cell. For industrial-scale bioproduction, a clone in which the product gene is stably integrated and yields a high level of the protein product is highly desired.

During the engineering of recombinant cell lines, the product gene is integrated together with a selection marker, which enables successfully transfected clones to be isolated from the population. Resistance to antibiotics (e.g. neomycin gene encoding resistance to G418) or enzyme analogs (e.g. dihydrofolate reductase gene encoding methotrexate resistance) are two common types of selection markers. However, these selection markers have been shown to increase the metabolic burden on the cells, which leads to a reduction in cell growth [1, 2]. In a heterogeneous population, high-producing subclones are rare and would tend to be diluted out by the faster growing non- or low-producing cells. To overcome this problem, limited dilution methods have been used. Since the integration of a recombinant gene into

the host genome is a largely random event, the proportion of cells containing multiple copies of stably integrated genes would be small compared to those with low copy numbers. There is a significant probability that a random sample of the total number of transfected cells may not contain the highest, stable producer. Thus, to isolate a subclone with an increased production rate, many wells would need to be screened and tested. In general, limited dilution methods are tedious and time consuming.

To alleviate some of these problems, we propose a novel technique for selecting high-producing cell clones that uses the Green Fluorescent Protein (GFP) as a selection marker, coupled with sorting through a Fluorescence Activated Cell Sorter (FACS). Fig. 1 shows a schematic of the proposed method. The ease and efficiency of GFP/FACS-based screening methods had been demonstrated in previous studies [3, 4]. In these previous reports, GFP was incorporated as part of a fusion protein [5] or part of a bicistronic construct using either an internal ribosomal entry site (IRES) [6] or a two promoter system [3]. Cells with a high-level of GFP correlated with a high level of the protein product of interest. This can be attributed to the stable integration of a high number of copies of the recombinant gene or that the gene had been integrated into sites of very high transcriptional activity. Although these methods have been shown to be effective, there may be some concern regarding the use of GFP-containing cell lines for the production of human therapeutics. Furthermore, it appears unnecessary to burden the cell's metabolic machinery with GFP production after the subpopulation has been isolated. This cellular resource could potentially be diverted to increasing cell growth or recombinant protein production. With these in mind, we propose a modification of the GFP/FACS technique, as illustrated in Fig. 1.

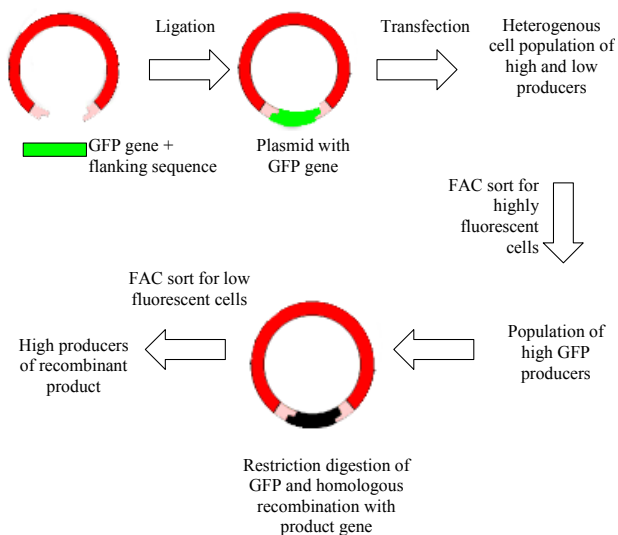Initially, plasmid vectors containing the cDNA encoding for

Fig. 1. Schematic of novel technique for rapid selection of high-producing cell clones.

GFP are constructed. In addition, known sequences flanking both sides of the GFP gene are included to facilitate subsequent homologous recombination and strand exchange. Cell transfected with the GFP-containing vector are then FACS sorted to screen for cells with high fluorescent intensity, which would correspond to high-producing subclones. These subclones are subsequently subjected to homologous recombination, exchanging the GFP gene with the desired protein gene. Cells that have successfully acquired the desired protein gene are then FACS selected by screening for cells with low fluorescent intensity. Finally, the selected clones are expanded and tested for production and stability.

As FACS enables a large number of cells to be easily screened, the chance of obtaining high producing clones would be greatly increased compared to limited dilution methods. Moreover, the procedure is less labor-intensive and may significantly reduce the time required to generate clones for bioproduction.

III. MEDIA DESIGN AND ITS EFFECTS ON GENE EXPRESSION

The culture media has an important impact on both the yield and quality of recombinant proteins from mammalian cells. Mammalian cells require a combination of both nutritive (e.g. sugars and amino acids) and non-nutritive (e.g. trace metals, vitamins and co-factors) components to support cell growth and protein production. In addition, the media environment has been shown to affect protein glycosylation, which is an important aspect of protein quality and influences its efficacy as a therapeutic [7, 8].

Traditionally, cell culture media were supplemented with serum, usually derived from fetal calves, to provide essential non-nutritive components as well as some nutritive compounds. Although some industrial cultivation processes still rely on serum supplementation, newer processes are directing towards serum-reduced or serum-free culture media for technical and economic reasons. For example, variability between serum lots can have a significant impact on culture performance. Serum is also quite costly (>US$200/L). These serum-free media usually consists of basal formulations fortified with insulin, transferrin, albumin and other protein materials, which are generally obtained from animal sources. However, there are concerns that these sources harbor potential adventitious contamination from animal-borne viruses or prions. This has spurred efforts to design media with greater biochemical definition, free from animal-derived components. In addition, the use of protein-free media may facilitate downstream purification, possibly simplifying the purification process or improving product recovery.

Our current research focuses on improving protein production through the design of chemically-defined, protein-free media. Insulin is one of the most common protein supplements for serum-free formulations. It is involved in a variety of cellular functions, including glycolysis, cell cycling and anti-apoptosis [9]. Previous work has shown that
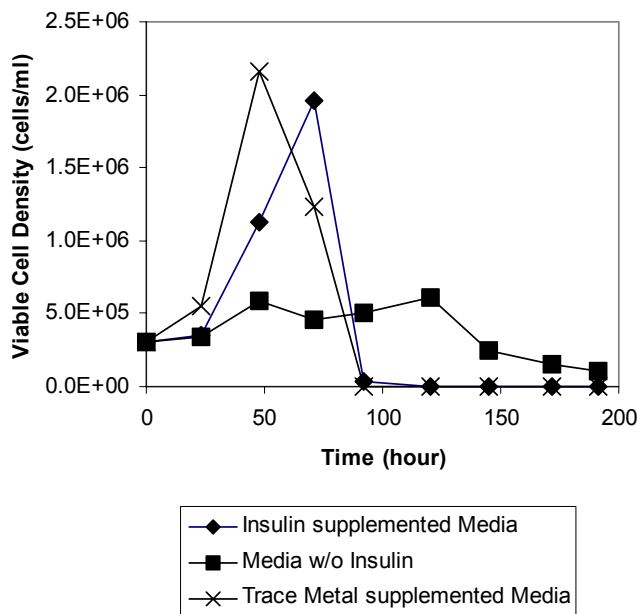
Fig. 2. Batch culture of the hybridomaCRL1606 in various media.

Fig. 3. Antibody production of CRL1606 in various media.

withdrawal of insulin leads to a reduction in viable cell density and viability [10]. As insulin is usually obtained from bovine pancreas, there is a potential risk of contamination from animal-borne viruses. Some researchers have substituted bovine insulin with recombinant insulin produced in *E. coli* [11]. However, this still carries the risk of foreign protein contamination and is a relatively costly solution. On the other hand, we have found that the insulin requirement can be effectively overcome by supplementing the media with the appropriate trace metals. Working with the murine hybridoma, CRL 1606, we have demonstrated that the cells could proliferate in insulin-free media in the presence of a trace metal supplement, without the need for adaptation (Fig. 2). Furthermore, monoclonal antibody production was not adversely affected by the substitution (Fig. 3). Studies to understand the insulin-mimetic qualities of these trace metals are currently in progress. Additionally, the media formulation is being optimized to further increase monoclonal antibody production.

Traditionally, most media formulations are generally derived through numerous empirical tests and is fairly time-consuming. A novel approach to tackle the problem of media design is to apply high-throughput analytical tools, such as the DNA microarray, to analyze the effects of various media conditions on a genome-wide level. By understanding the genetic profile of the cells grown in different media, we can potentially identify "deficiencies" in the formulation, leading to a more rational approach to media design. In addition, based on the transcriptional profile, we can also select candidate genes that may be genetically manipulated to modify the nutritional requirements of the cells. The potential for the latter application is illustrated by the work reported in [12], in which the authors obviated the requirement of a CHO cell line for basic fibroblast growth factor by overexpressing
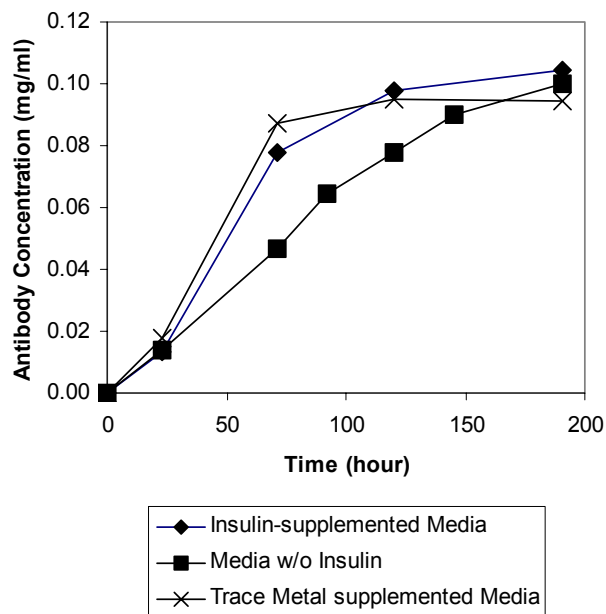
cyclin E.

IV. IMPROVING SIALYLATION OF GLYCOPROTEINS

The efficacy of recombinant glycoproteins as a human therapeutic is strongly dependent on their carbohydrate structures, or glycosylation [7]. Glycosylation has been implicated in bioactivity, receptor binding, susceptibility to proteolysis, immunogenicity and *in* vivo clearance rate [13]. As an example, Fig. 4 shows the full N-linked glycan structure of interferon-γ produced in recombinant CHO cells. Sialic acid (SA), the terminal sugar for N-linked glycosylation, plays a major role in a glycoprotein's circulatory lifetime. The presence of sialic acid can prevent recognition of the glycoprotein by asialoglycoprotein receptors and hence prolong its circulatory lifetime [14]. Therefore, for certain therapeutic functions, it is desirable to maximize the degree of sialylation of the glycoprotein to enhance its quality and efficacy. Furthermore, regulatory authorities are increasing requirements for the control of the glycosylation characteristics of glycoproteins produced from cell culture processes.

Sialylation is governed by two opposing processes: intracellular sialylation and extracellular desialylation. Extracellular desialylation is thought to be caused by sialadases released through cell lysis, especially during the later phase of cell culture. Harvesting the product before extensive cell lysis occurs can readily reduce the extent of extracellular desialylation. The use of sialidase antisense technology [15] or sialidase inhibitors [16] are other alternatives that have been reported. On the other hand, improving intracellular sialylation is significantly more challenging. Intracellular protein glycosylation is a tightly orchestrated chain of events; it requires the expression and
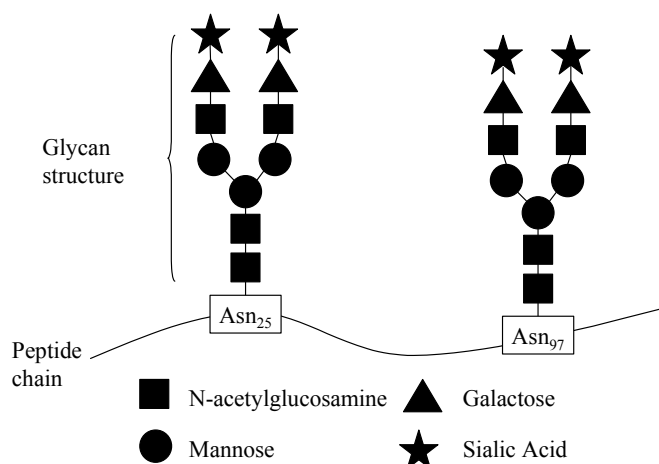
Fig. 4. Biantennary N-linked glycan structure of interferon-γ produced from CHO cells.

Fig. 5. The sialylation process in the Golgi.

subcellular localization of glycosyltransferases and glycosidases, the availability of intraluminal nucleotide sugar substrates, the streric accessibility of the glycosylation site for oligosaccharide processing and the transit time available for oligosaccharide formation [17]. There have been various attempts to improve intracellular sialylation through overexpression of sialytransferases [18, 19] or feeding of nucleotide sugars [2, 20, 21]. For example, Gu and Wang [17] fed up to 20 mM of N-acetylmannosamine (ManNAc), a sialic acid precursor, to CHO cells producing interferon-γ and observed an increase in percentage sialylation from 60 to 80%. However, no further improvement was observed with feeding of 40 mM ManNAc. Similarly, overexpressing sialytransferases had only limited success in augmenting the extent of sialylation [18, 19]. These results suggest that, apart from sialic acid and sialytransferase availability, other processes may be limiting intracellular sialylation. It is hypothesized that the transport of sialic acid into the trans-Golgi via the sialic acid transporter may be the limiting factor which governs the rate of intracellular sialylation. Fig. 5 illustrates the sialylation mechanism in the Golgi. Prior to sialylation, sialic acid is produced in the cytosol and subsequently activated with cytidine triphosphate to form cytidine monophosphate-sialic acid (CMP-sialic acid) in the nucleus. CMP-sialic acid is then transported from the nucleus through the cytosol and into the trans-Golgi. Once there, the sialic acid is added to the growing sugar chain by sialytransferases. It is proposed that the availability of CMP-sialic acid in the trans-Golgi is limited by its rate of transport through the sialic acid transporter (Fig. 5). Thus, overexpressing the CMP-sialic acid transporters in the cells may potentially relieve the sialic acid limitation within the Golgi, thus increasing the degree of glycoprotein sialylation. Overexpression of the sialic acid transporters will need to be coupled with other strategies, such as the feeding of nucleotide sugar precursors (e.g. ManNAc) or overexpressing sialytransferase to further improve intracellular sialylation in the cells.
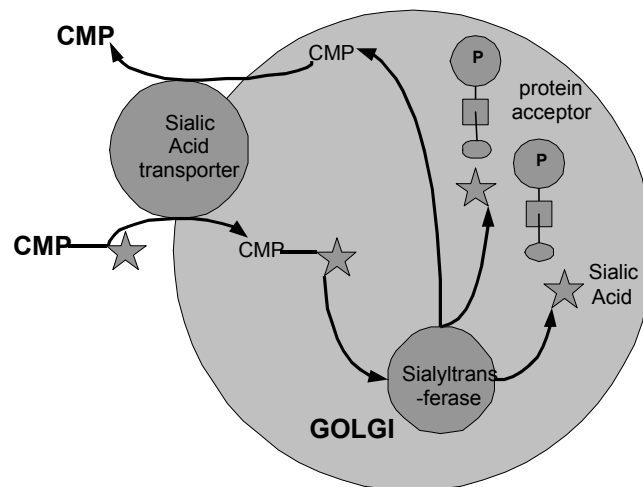
V. CONCLUSION

In this paper, we outlined some of the strategies for improving the production of recombinant proteins from mammalian cells currently being pursued in our group. Whilst each of these have been described separately, there is a potential for integrating these strategies to engineer a robust, high-producing cell line growing in a chemically-defined media that is able to maximize the production of recombinant proteins with the right glycosylation qualities. Such integrated strategies may become essential to satisfy the increasing demands for therapeutic proteins.

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