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Using cell engineering and omic tools for the improvement of cell culture processes

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Abstract Significant strides have been made in mammalian cell based biopharmaceutical process and cell line development over the past years. With several established mammalian host cell lines and expression systems, optimization of selection systems to reduce development times and improvement of glycosylation patterns are only some of the advances being made to improve cell culture processes. In this article, the advances pertaining to cell line development and cell engineering strategies are discussed. An overview of the cell engineering strategies to enhance cellular characteristics by genetic manipulation are illustrated, focusing on the use of genomics and proteomics tools and their application in such endeavors. Included in this review are some of the early studies using the 'omic' technique to understand cellular mechanisms of product synthesis and secretion, apoptosis, cell proliferation and the influence of the physicochemical environment. The article highlights the significance of integrating genomics and proteomics data with the vast amounts of bioprocess data for improved analysis of the biological pathways involved. Further improvements of the techniques and methodologies used are needed but ultimately, the new cell engineering strategies should provide great insight into the regulatory networks within the cell in a bioprocess environment and how to manipulate them to increase overall productivity.

Keywords Cell engineering · Proteomics · Genomics · Bioinformatics · Biopharmaceuticals

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Introduction

It has been over two decades since the first FDA-approved biopharmaceutical process was established and since then significant strides have been made in the development of process strategies and technological platform for optimal production of biopharmaceuticals. One such technological platform is based on the use of mammalian cell lines for the production of complex biomolecular products. In 2006 it was reported that over 165 new biopharmaceutical entities received FDA approval (Walsh 2006). Mammalian cell lines command an effective monopoly towards production of complex therapeutic proteins that require post-translational modifications (Walsh 2006; Walsh and Jefferis 2006). This unique advantage outweighs the costs associated with mammalian cell culture, which are far greater in terms of development time and manufacturing

when compared to bacterial culture. Moreover, in comparison to bacteria the scale up and optimization of mammalian cell cultures to meet production demands has been problematic due to the low productivity and instability of the cell lines used.

The development of cell lines has undergone several advances over the years, essentially to meet the requirement to cut the time and costs associated with using such a complex hosts as production platforms. This paper will review the aspects involved in the development of cell lines and the cell engineering approach that can be employed to enhance productivity, improve cellular metabolism, control proliferation and apoptosis, and reduce instability.

Direct cell engineering has been applied for years to enhance cell lines through the manipulation of single genes that play important roles in key metabolic and regulatory pathways but recently consideration has been given to the use of indirect cell engineering approach, utilizing genomic and proteomic techniques and tools, to aid the discovery process of novel targets for metabolic manipulation within the host cell line. It is hoped that the use of these new techniques and tools will greatly advance our understanding of cellular mechanics in conjunction with improvement of overall productivity in the manufacturing environment and lead to efficient and safe processing of protein products.

Cell line development

Host cell lines

The most common host cell line used for the production of therapeutics is Chinese Hamster Ovary (CHO) (Cockett et al. 1990, Milbrandt et al. 1983) but apart from this cell line, others that are commonly used for the production of recombinant proteins are: mouse myeloma derived (NS0) (Barnes et al. 2001, Bebbington et al. 1992), human embryonic kidney (HEK-293) (Baldi et al. 2005), baby hamster kidney (BHK) (Wurm 2004) and more recently, the human retina derived (PerC6) (Jones et al. 2003) cell lines. For the purpose of process homogeneity, reproducibility and safety, chemically defined protein free medium has become a necessity. However, some media formulations still contain one or two recombinant proteins (such as recombinant human insulin) depending on the cell requirement. The NS0 cell line is the only exception to anchorage dependency and will only need to undergo adaptation to serum free or chemically defined protein free media while the other cell lines maintain their anchorage dependent characteristics unless adapted to suspension culture.

The use of these common cell lines, has led to several sub-cell lines utilizing specific expression technology that have become an industry standard such as CHO DHFR (dihydrofolate reductase system) (Lucas et al. 1996), where a mutant CHO DHFR- cell line is transfected with a vector containing the target gene along with the DHFR marker gene. Selection occurs in the absence of the metabolites hypoxanthine and thymidine and gene amplification is accomplished by exposing the cell line to MTX concentrations, which inhibit DHFR enzyme activity. CHO GS (Glutamine Synthetase) (Cockett et al. 1990) and NS0 GS (Bebbington et al. 1992) are another standardized selection system, where cells are transfected with a vector containing the gene of interest in addition to the glutamine synthetase (GS) gene. GS is an enzyme that allows the cell to synthesize its own intracellular glutamine from glutamate and the ammonia group provided from asparagines (Barnes et al. 2000). NS0 cells are preferred for this system because they have no endogenous GS activity unlike its CHO counterpart. GS cells are selected in glutamine free media and gene amplification can occur by the addition of the GS inhibitor methionine sulphoximine (MSX). The GS cells require lower gene copy numbers, when compared to the DHFR system, for a similar expression level and thus give the added advantage of a shorter selection period. Another advantage of the GS selection system is that the cultures produce less of the toxic metabolite, ammonia, which can negatively affect the glycosylation of the produced recombinant protein (Yang and Butler 2000a; b).

Expression engineering

Most of the expression systems used are inserted into the cellular chromosome by means of random transgene integration events (Wurm 2004). In this process a positional effect can occur where transgene expression is dependent on the position of integration. With standard vector systems, if integration occurs in the heterochromatin, no or minimal transgene expression occurs while integration into the euchromatin will not guarantee maximal transgene expression (Kwaks and Otte 2006). This is supported by evidence that CHO cell line clones with amplified genes located on specific chromosomal regions were found to be more productive and stable than other type of clones (Yano et al. 2006). Thus the copy number of an integrated gene may not indicate a stable expression since deterioration over time may occur due to repeat induced silencing. Targeting transgene integration by the use of the Cre/loxP recombination system and Flp/FRT (Flp-recognition target) in combination with recombinase mediated cassette exchange (RCME) (Baer and Bode 2001) means it is possible to create cell lines which can be selected for high expression of a specific tag, such as Green Fluorescent Protein (GFP). The selected clones can be assessed for stability and are transfected with the gene of interest so that RCME takes place and a high producing cell line with the gene of interest is obtained (Baer and Bode 2001, Feng et al. 1999).

Over the years the use of epigenetics to augment gene expression has been gaining momentum. Several new technologies have been introduced to overcome positional dependent inactivation. For example, using chemicals such as sodium butyrate and sodium propionate, which inhibit histone deacteylases since histone actylation is generally related to enhanced expression, but these compounds tend to have cytotoxic effects as well (Chun et al. 2003, Davie 2003). An alternative method to overcome positional dependent inactivation is the use of vectors with a matrix attachment region (MAR) allowing for the repression of some of the silencing. This allows an open chromatin structure to be maintained, although it doesn't directly reflect the copy number, and its molecular basis is still not fully understood (Barnes and Dickson 2006, Kwaks and Otte 2006). The discovery of gene regulatory elements which give dominant chromatin opening and resist silencing have started to be used in an industrial setting (Trish et al. 2002). These DNA fragments composed of methylation free-CpG islands are known as ubiquitous chromatin opening elements (UCOE) which have been isolated from ubiquitously expressed housekeeping genes (Antoniou et al. 2003), which can be resistant to heterochromatin mediated silencing. When linked to the human cytomegalovirus (*h*CMV) promoter, UCOE's can give stable high level expression with resistance to silencing of the transgene associated with these elements (Williams et al. 2005).

The use of licensed compounds for the induction/repression of gene transcription, such as antibiotic resistance operons including macrolide inducible resistance operons and pharmacological agents that can be used to regulate gene expression, have been exploited for biopharmaceutical production (Weber et al. 2002). An example of such an approved compound is tetracycline in the common tetracycline regulatory system (Gossen and Bujard 1992). The use of vectors that use these compounds, for multiregulated multigene metabolic engineering (Fussenegger et al. 1998a; Fux et al. 2004) will be useful with our increased greater understanding of the complex metabolic and gene regulation networks within the host cell, giving opportunities for application of these technologies to increase product yields.

Optimization of selection procedures

Traditionally, selection of a high producing cell line is a costly process due to the time and labor-intensive procedures involved. It has been referred to as one of the main bottlenecks of getting a producer cell line out of development and into the manufacturing environment (Borth et al. 2000; Carroll and Al-Rubeai 2004). A heterogeneous cell population that undergoes selection for high producers have the disadvantage that the high producing clones mainly have a reduced proliferation rate due to the energy being diverted towards production (Borth et al. 2000;

Kim and Lee 1999; Kromenaker and Srienc 1994). However, recently we found from genomics and metabolomics data that the increased specific recombinant protein productivity in arrested or slow growing cells is the result from overall increase in cell growth rather than from a diversion of cellular energy (unpublished data). As a consequence, cell selection using the limited dilution method is usually employed which results in multiple screens of hundreds of wells to select out the appropriate high producing subclone.

Several high throughput techniques for selection of high producing subclones have been conceived in the past few years, some have utilized fluorescence activated cell sorting (FACS) technique (Böhm et al. 2004; Borth et al. 2000; Carroll and Al-Rubeai 2004, 2005; Holmes and Al-Rubeai 1999). A strategy for selection of high producers described by Holmes et al. (1999) using an affinity surface display matrix consisting of avidin and neutravidin, allowed the capture antibody and cell to become irreversibly linked. This complex forms the primary means of capturing the secreted target antibody product which can then be fluorescently labeled for sorting by flow cytometry. This method significantly speeds up the selection process (Carroll and Al-Rubeai 2004), where the critical path activity of commercial cell lines can be minimized to 31 weeks compared to a conventional 40 (Racher 2006).

Optimisation of glycosylation patterns

Mammalian cells have the unique ability to produce therapeutic proteins with oligosaccharides attached to their serine/threonine (*O*-linked glycosylation) or asparagines (*N*-linked glycosylation) residues (Butler 2005; Seth et al. 2006a). The functional biological activity of the glycoprotein is directly affected by glycosylation, including its trafficking and folding within the host cell (Scallon et al. 2006; Walsh and Jefferis 2006; Willey 1999). Once secreted, solubility, aggregation, stability and immunogenicity of the protein may be affected by its glycosylation pattern (Kobata 1992; Sinclair and Elliott 2005; Willey 1999; Wyss and Wagner 1996).

N-glycosylation is initiated in the ER, and O-glycosylation can be initiated in either the ER or Golgi apparatus, but due to processing inconstancies glycans can have frequent structural heterogeneities (Patel et al. 1992; Seth et al. 2006a; Varki 1998). The most common therapeutic monoclonal antibody approved biopharmaceutical is of the immunoglobulin G (IgG) class (Walsh and Jefferis 2006). This glycoprotein has several isoforms which are cell line and clone dependent, plus the structure is influenced by the culture environment (Chee Furng et al. 2005; Patel et al. 1992; Raju et al. 2000). This structure or the pattern of glycosylation is dependent on the glycosyltransferases present in the Golgi apparatus. Mouse cells have different glycosylation patterns to humans because they generate Galα1,3-Galβ1,4-GlcNAc residues due to the presence of α1,3 galactosyltransferase. These residues are highly immunogenic in humans and can have an impact on the decision of which cell line to use when this type of glycosylation is present in the end product (Butler 2005; Jenkins et al. 1996). In addition, most host mammalian cell lines (other than the human derived) may not possess the same set of glycosylation enzymes present in humans which may pose an immunogenic threat. It is known that human glycans predominately contain terminal *N*-acetylneuraminic (NANA) sialylation of the glycoproteins, found to vary in other species (Raju et al. 2000). In fact, most species predominantly have *N*-glycolyl-neuraminic acid (NGNA) rather than NANA. CHO cells have the advantage of the presence of a high percentage of NANA sialylation, but still include some NGNA sialylation (Baker et al. 2001).

Stability

The consistency of growth, productivity, or product characteristics with increasing generation number defines cell line stability and contributed to overall process consistency. With this in mind, the stability of a genetic, hence physiological, heterogeneic biopharmaceutical cell line is reflected by no deviation of the recombinant product quality and quantity across its manufacturing window (Racher 2004). Therefore, the recombinant product undergoes extensive quality control by assessment of product physiochemical,

biochemical, and immunochemical properties under stringent analytical tests since it is the key feature for regulatory approval of a bioprocess (Crommelin et al. 2003; Katterle et al. 2006). In terms of cell line productivity, studies have reported that copy number may not be a viable indicator for productivity and cell line stability rather that mRNA level can be more of a predictive indicator (Barnes et al. 2003, 2004). The location of the amplified genes also determines the stability since studies on CHO cells during prolonged culture showed that in the absence of selective agent such as Methotrexate (MTX), genes have higher stability when located in the telomeric regions of chromosomes (Kim and Lee 1999; Yoshikawa et al. 2000). Recently research has shown that over-expression of human telomerase reverse transcriptase (hTERT), which adds hexameric repeats to the chromosome ends preventing telomeric loss, enhances chromosomal stability in CHO cell lines and possibly production stability (Crea and Al-Rubeai 2006).

Cell engineering

Direct approach to cell engineering

Cell engineering has benefited greatly from recombinant DNA technology and the ability to design complex expression systems. Apart from epigenetic strategies; inducible vectors can be used to regulate gene transcription for over-expression or silencing of key regulatory genes. The direct approach to cell engineering is to introduce to or omit from the cell a gene or genes by genetic engineering methods to endow a particular phenotype in order to improve the cellular processes. Table 1, illustrates some of the cell engineering strategies attempted to control apoptosis, proliferation and productivity within cell lines.

Apoptosis engineering

Some of the first direct approach strategies for cellular engineering looked at prolonging the viability of cells in culture by delaying the onset of apoptosis (Al-Rubeai 1998; Al-Rubeai and Singh 1998; Cotter and Al-Rubeai 1995). One anti-apoptotic protein known to suppress the mitochondrial apoptotic pathway is *bcl-2* (Gupta 2003). The over-expression of the *bcl-2* gene in plasmocytoma (myeloma) (Rabinder 1996), NS0 (Tey et al. 2000b), CHO (Tey et al. 2000a), and hybridoma (Simpson et al. 1998) cells has imparted improved robustness to nutrient deprivation and toxin exposure, including longer survival in intensified culture systems (Tey and Al-Rubeai 2004). Some of the first studies showed that when hybridoma cells over-expressed the Bcl-2 protein it significantly increases cell viability and productivity (Simpson et al. 1997). When myeloma cells, in perfusion culture, were engineered to express E1B-19K (the adenovirus Bcl-2 homologue) the resulted 2-fold increase in viable cell densities allowed for a 40% increase in monoclonal antibody yield (Mercille and Massie 1999). Another approach utilized small interfering RNA (siRNA) (Brummelkamp et al. 2002; Elbashir et al. 2001) constructs directed at mRNA sequences of two pro-apoptotic proteins, Bax and Bak (Lim et al. 2006). Although, silencing was not optimal it was enough to give extended survival in culture and increase IFNγ-product yield in CHO cells by 35% when compared to the control.

Cell cycle engineering

This direct approach has also been used to manipulate the cell cycle genes to increase or decrease proliferation rate and thereby to enhance productivity. When *c-myc* gene transfected into CHO cells a significant increase in proliferative rate of CHO cell line is demonstrated (Ifandi and Al-Rubeai 2005). Over-expression of both *c-myc* and *bcl-2* resulted in a cell line with increased proliferation and maximum cell densities while a decrease in apoptosis was achieved (Ifandi and Al-Rubeai 2005). Another cell cycle associated gene, *p21*, has had its potential for inducing cytostasis exploited. Research has shown that by using an inducible $p21^{CIP1}$ vector a 4 fold increase in antibody productivity resulted in a suspension NS0 culture (Watanabe et al. 2002). Further work showed that this engineered cell line had an increased cell volume, ribosomal protein S6, mitochondrial activity and mitochondrial mass when induced suggesting that $p21^{CIP1}$

Table 1 Summary of some of the successful cell engineering strategies carried out over the past years

Reference	Year	Modification	Cell type
Anti-Apoptotic Engineering			
Itoh et al.	1995	*bcl -2*	hybridoma
Singh et al.	1996	*bcl -2*	myeloma
Tey et al.	2000a	*bcl -2*	NSO
Tey et al.	2000b	*bcl -2*	CHO
Simpson et al.	1997	*bcl -2*	hybridoma
Perani et al.	1998	*bcl -2*	hybridoma
Ifandi and Al-Rubeai	2005	*bcl -2*	CHO
Meents et al.	2002	*bcl -2, bcl -xL*	CHO
Mastrangelo et al.	2000b	*bcl -2, bcl -xL*	CHO, BHK
Figueroa et al.	2001	*bcl -2, bcl -2* deletion mutant	CHO
Figueroa et al.	2004	*aven, bcl -xL*	CHO
Figueroa et al.	2006	*aven, e1B-19K*	CHO
Chung et al.	1998	*bcl -2*	hybridoma
Jung et al.	2002	*bcl -xL*	hybridoma
Gauthier et al.	1996	*bcl -xL*	hybridoma
Charbonneau et al.	2003	*bcl -xL*	hybridoma
Sauerwald et al.	2002	*XIAP*	CHO, HEK292
Sauerwald et al.	2003	*XIAP, CrmA*	CHO, HEK293
Lee and Lee	2003	*bcl -2*	CHO
Mercille and Massie	1999	*elB-19K*	NSO,BHK
Lasunskaia et al.	2003	*hsp70*	NSO
Terada et al.	1997	*bag-1, bcl -2*	hybridoma
Crea et al.	2006	*htert*	CHO
Veraitch and Al-Rubeai	2005	*neo, bcl -2*	NSO
Lim et al.	2006	*RNAi to Bak, RNAi to Bak*	CHO
Wong et al.	(2006b)	*siRNA to Alg2, siRNA to Requim*	CHO
Cell-Cycle Engineering			
Koester et al.	1995	IRF-1	BHK
Kirchhoff et al.	1996	IRF-1	BHK
Geserick et al.	2000	IRF-1	BHK
Mazur et al.	1998	$p21^{CIPI}$, $p27^{KIPI}$,$P53^{275p}$	CHO
Fussenegger et al.	1998b	$p21^{CIPI}$, $p27^{KIPI}$, bcl_{XL}	CHO
Watanabe et al.	2002	$p21^{CIPI}$	NSO
Bi et al.	2004	$p21^{CIPI}$	CHO
Ifandi and Al-Rubeai	2003	c-myc	CHO
Glycosylation Engineering			
Mori et al.	2004	siRNA to α2,3-siayltransferase	CHO
Weikert et al.	1999	siRNA to α1,6-fucosyltransferase	CHO
Umaña et al.	1999	β1-4-N-acteylglucosaminyltransferase III	CHO
Davies et al.	2001	β1-4-N-acteylglucosaminyltransferase III	CHO

induced cell cycle arrest uncouples cell growth from cell cycle progression (Bi et al. 2004). This in-turn was directly related with productivity. Early work with inducing cell arrest was done utilising the interferon regulatory factor 1 (IRF-1) to regulate cell growth (Kirchhoff et al. 1993; Koester et al. 1995) and increase production in BHK-21 cells via estradiol induction (Kirchhoff et al. 1996). However, this work demonstrated that when using the IRF-1 cytostatic gene the main task is keeping the viability high as this has a tendency to decrease with time. By controlling the addition and removal of estradiol, hence modulate IRF-1 activity, it is possible to overcome the loss in viability (Carvalhal et al. 2001). Other studies have also investigated the use of cytostatic genes on CHO cells secreting alkaline phosphatase (SEAP) with similar effects of higher productivity when cell are growth arrested (Carvalhal et al. 2003; Mazur et al. 1998). An example is the use of the $p27^{KIP1}$ gene induced by acetylaldehyde gas which allowed

increased SEAP production in HEK.EBNA cells (Werner et al. 2006). Apart from the control of cell cycle by the $p27^{KIP1}$ gene, the use of acetylaldehyde as an inducing agent has several advantages in terms of low cost, precise fine tuning of transgene expression (Weber et al. 2004), the use of concentrations below observable toxicities (Hartenbach and Fussenegger 2005; Weber et al. 2005b), and ease of removal prior to downstream processing (Weber et al. 2005a).

Glycosylation engineering

Cell engineering of glycosylation patterns (Davies et al. 2001; Natsume et al. 2006; Shields et al. 2002; Shinkawa et al. 2003; Umaña et al. 1999; Warner 1999; Weikert et al. 1999) and in vitro glycosylation (Butler 2005; Raju et al. 2001) has provided new possibilities for biopharmaceutical design and optimization thus lowering immunogenicity effects, increasing stability and enhancing functional activity. Weikert et al. (1999) engineered CHO cells that secreted either tissue necrosis factor receptor–IgG1 fusion protein (TNFR-IgG) or tissue plasminogen activator (TNK-tPA) to over-express α2,3-sialyltransferase and the resultant glycoproteins had a considerable longer pharmacokinetic mean residence time in rabbit models. Bisected oligosaccharides have been implicated in enhanced antibody dependant cellular cytotoxicity (ADCC) activity. When CHO cells were engineered to over express β1,4 N-acetyl glucosaminoyltransferase (GnTIII), the bisected oligosaccharide glycoforms, ADCC activity was increased 20–100 fold (Davies et al. 2001; Umaña et al. 1999). A silencing approach has also been used in CHO cells to increase the antibody effector function of ADCC by introducing siRNA targeting α1,6 fucosyltransferase (FUT8) mRNA (Mori et al. 2004). Recently, one of the first biopharmaceuticals was approved for the market that included engineered modifications of glycosylation which extent its half life in human patients (Walsh and Jefferis 2006).

Engineering of metabolic pathways

Apart from manipulation of the apoptosis, cell cycle and glycosylation pathways the focus has been on glycolysis to enhance energy use and reduce cytotoxic by-product formation. For instance, by enhancing pyruvate carboxylase expression, which converts pyruvate to oxaloacetate (OAA). The increase in oxalacetate conversion to malate via the malate-aspartate shuttle for entry into the tricarboxylic acid cycle decreased pyruvate concentration which in turn reduced lactate formation (Irani et al. 2002, Seth et al. 2006a); however, no data have been presented on the effect of oxygen uptake rate which should give a more conclusive picture. In another study, antisense technology has been employed to knockdown the enzyme LDH in CHO cells. LDH is involved in the reversible reaction of lactate acid from pyruvate and interestingly, this strategy reduced the overall glycolysis rate, likely due to reduced NADH to NAD^+ formation, which is coupled to the pyruvate-lactate reaction (Seth et al. 2006a). This work illustrates the complexity of metabolic pathway engineering and how multiple gene engineering in a single host might be a possible future strategy to overcome some of these problems. The drawback of the antisense and RNA interference (RNAi) technologies is that they lack the precision in controlling expression levels with the consequence that using this technology for metabolic flux control may prove to be difficult.

Other engineering routes

Cell engineering research has also started to focus on other regulatory elements of the cell such as mRNA processing pathways and microRNA (miRNA). Deciphering of the mRNA processing pathways is still in its infancy. It is known that some RNA processing events can affect cell productivity. Recently, it has been discovered that if X-Box binding protein 1 spliced (S) (XBP-1(S)) was obtained from the transcript XBP-1 by mRNA processing, it had the possibility to lead to increased secretion, cell size, membrane synthesis, and energy production (Brewer and Hendershot 2005, Yoshida et al. 2006). Tigges et al. (2006) have transfected a CHO cell line with XBP-1(S) and demonstrated a 6-fold increase in SEAP production compared to the control cell line (Tigges and Fussenegger 2006). On the

contrary, this may not pertain to all cases as observed when CHO cells expressing antithrombin III (AT-III) had decreased productivity by 0.7 fold when over-expressing XBP-1(S) (Hayashi et al. 2006). When it comes to the use of miRNA, for now, it is limited since many miRNA and the pathways they affect are still being identified (Ford 2006). These small, 17–24 nucleotide (debatable), non-coding, regulatory RNAs, may comprise a network that regulates 30% of genes within the cell, thus controlling a substantial amount of protein production (Bentwich et al. 2005; Cheng et al. 2005; Ford 2006; Hwang and Mendell 2006). Genes may have several target sites for either one miRNA or a few different miRNAs which makes it possible for combinatorial regulation to fine tune activities (Plasterk 2006). Recent work by Cheng et al. (2005) found that two miRNA; miR-21 and miR-24, profoundly increased cellular proliferation while miR-214 resulted in decreasing apoptosis in HeLa cells. However, the effect of these miRNA seems to be cell specific. Since the complexity of miRNA targeting also gives it the power to regulate several genes at the same time, it might prove to be very useful in engineering cell lines which can bypass apoptosis, have optimal productivity and proliferation by using only 1 or 2 miRNA constructs. Non-transcription events, such as miRNA and mRNA processing pathways, will still require further study but they will nevertheless represent another tool in the cell engineers' arsenal.

Indirect approach to cell engineering

The indirect approach takes a more global view of the cellular pathways and its regulation. By identifying or constructing of the desired phenotype into the host, the factors conferring the phenotype can be discovered and used to engineer improved cell lines (Bailey et al. 2002). A cell line with a particular phenotype either produced by selection, silencing or over-expression can be employed for characterisation according to global gene and protein expression profiles. Thanks to the growth of information from bioinformatics, data mining and pathway analysis of resultant profiles, important genetic and protein expression information pertaining to the phenotype can now be obtained with relative ease. Once the factors that establish the conferred phenotype are elucidated, a direct cell engineering approach may be applied to the pathway in question, an approach that may eventually contribute to overall process optimisation (see Fig. 1). Using this method a greater understanding of the cellular biochemical pathway interactions is gained and it is hoped that the discovery of novel targets can contribute to an overall increase in cell

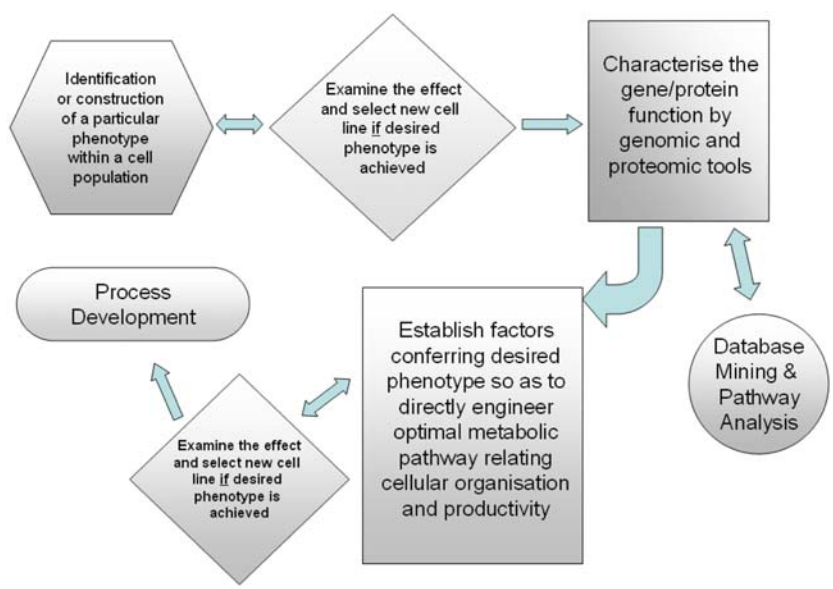

Fig. 1 Example of workflow for an indirect engineering strategy employing the use of omic tools for development of cell lines

productivity. Even if the target cell line does not respond well to the first attempt of this approach the valuable knowledge gained should aid future engineering strategies.

Genomics and proteomics-tools for indirect cell engineering

In order to apply the indirect approach, new tools have emerged to aid this endeavor. In fact, great efforts have been made in the last ten years to identify genes and pathways relevant to cells enhancing productivity, to identify the metabolic bottlenecks, to understand the mechanism of protein synthesis, to develop better nutrients and media formulation, to reduce apoptosis and increase growth rate, and to design processes with the aim to increase overall productivity. Two new technologies have recently become available which allow for a thorough high throughput assessment of changes at gene (genomics) and protein (proteomics) levels involved in determining productivity in different environmental conditions and to establish functional relationships between cellular organisation and productivity. By using these two new methodologies, genomics and proteomic analysis, together in combination with cell engineering strategies we are one step closer towards a better understanding of cellular behavior during bioprocessing.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE or 2DE) is the most common proteomic technique and with the recent advent of 2D-differential gel electrophoresis (DIGE) (Lilley and Friedman 2004) combined with advances in quantitative analysis, in image warping and spot detection algorithms (Miller et al. 2006), followed by MALDI-TOF (matrix-assisted laser-desorption/ionization time-of-flight) mass spectrometry (MS) or LC-MS/MS (liquid chromatography) for protein identification (Aebersold and Mann 2003; Gorg et al. 2004), a powerful proteomic tool has been generated. In genomics the use of high-density oligonucleotide GeneChip microarrays for transcriptomics analysis has become a common method to analyse gene expression. These technologies allow for deeper insights into cell metabolism and physiology under process and manufacturing conditions. In addition, these two approaches seem to be a more precise analytical tools for monitoring hundreds to thousands expression changes at gene and protein levels and could significantly contribute to cell culture and cell line development efforts which may, in the near future, achieve specific productivities of over 90 pg $cell^{-1}$ day^{-1} (Wurm 2004).

Before discussing some of the applications of this technology and reviewing the progress made, some of the alternative underlying technologies available for such development should be mentioned. When looking at the platforms that can be used for expression analysis in the genomics field or gene transcriptome analysis, the area has undergone significant development from early cDNA microarrays (Schena et al. 1995). The available platforms today are varied (reviewed in Kuo et al. 2006) and the general conclusion is that, overall, most platforms today show good agreement on expression profile data; however, commercial platforms consistently out-performed in-house platforms for internal and external consistency with the best platforms being one-dye, such as Applied Biosystems (ABI), Affymetrix, and Amersham (now GE Healthcare) systems (Kuo et al. 2006).

In proteomics, apart from 2D-gel techniques, the use of MS (as the primary quantitative step) has become a powerful and alternative tool over the last years, with the ability to analyse large numbers of proteins in complex biological samples through development of methods as MudPIT (multi-dimensional protein identification technology), SILAC (stable isotope labeling by amino acids), ICAT (isotope-coded affinity tags), iTRAQ, or ^{16}O-to-^{18}O for functional and quantitative proteomics (Ong and Mann 2005; Roe and Griffin 2006). For example, more than 2000 proteins could be analyzed by SILAC labeling (de Godoy et al. 2006). However, the problem is that in applying these new methods adequate high performance MS machines and properly skilled scientists to operate these systems are necessary. Therefore, 2D-electrophoresis will probably be the method of choice, for the moment, in most of the academic research laboratories in order to carry out quantitative proteome profiling.

Recent studies have shown the potential of applying genomic and proteomic techniques to gain insights into intracellular changes when environmental conditions are altered (e.g. changes in pH, osmolarity and temperature). Further on, expression changes of genes and proteins directly or indirectly involved in the metabolic processes have been identified in different cell clones and cell lines. However, the field of studying the genome and proteome of mammalian cell culture are still in its infancy (Table 2) in comparison with microbial culture; nevertheless, it has a clear objective in identifying new targets for cell line engineering strategies to improve productivity, proliferation, and viability.

The first application of a large-scale protein analysis approach for mammalian cell culture was reported by (Passini and Goochee 1989). The cellular response of hybridoma cell lines grown in a bioreactor system experienced different environmental stresses (heat shock, agitation and sparging) were analysed by 2DE. Later, work done by Lee and co-workers (Lee et al. 1996a) took the initial steps towards the use of large-scale quantitative proteome analysis as part of an indirect cell engineering strategy for the improvement of specific recombinant protein production and proliferation rates in mammalian cell lines. Detailed 2DE profile maps were established for CHO cells cultivated in media supplemented with foetal calf serum, insulin, or basic fibroblast growth factors. Twenty-four gene products involved in growth factor signaling were identified as differentially regulated. Additionally, the regulation and interaction of transcription factor E2f-1 with cyclin A and E, as well as involvement of cyclin D1 in cell cycle regulation has been found to have an impact on controlling cell growth stimulation. Subsequently studies indicated that over-expression of transcription factor E2f-1 resulted in an increased proliferation in serum free and protein free media (Lee et al. 1996b).

Elucidating the biological pathways regulated by different environmental conditions may contribute to a further understanding of the mechanisms that control cell survival and productivity. Studying the role of these pathways may identify putative targets to interfere with these events, allowing the development of more robust and productive cell lines. Temperature is a significant parameter which can be manipulated to alter the production of recombinant proteins. Kaufmann et al. (1999) have characterized the synthesis of specific cold-induced proteins and molecular changes at posttranslational level resulted from cultivation of recombinant CHO cells at 30°C. Under this hypothermic condition accumulation of cells in G1 phase occurred, with an increased specific productivity and delayed onset of apoptosis. A preliminary analysis of the CHO transcriptome and proteome induced by low temperature have recently been published (Baik et al. 2005), where commercially available rat and mouse cDNA microarrays were used. Overall, transcriptome analysis revealed that low culture temperature could lead to changes in gene expression in various cellular processes such as metabolism, transport and signaling pathways. Complementary to the earlier findings (Kaufmann et al. 1999) 26 different kinds of proteins induced by low-culture temperature were identified by the work of Baik et al. (2006).

Recently, the transcription profile of a NS0 6A1 cell line cultivated at 37°C, 34°C and 22°C has been compared (Swiderek and Al-Rubeai 2006). Microarray analysis revealed that the altered transcription at different temperatures was associated with a broad spectrum of functions within a wide range of regulated genes that were implicated in metabolic pathways such as the urea cycle and metabolism of amino groups. Also affected were the signaling pathways: apoptosis, death receptor, endoplasmatic reticulum stress pathway, and cell cycle checkpoints. Major changes occurred at the transcription level when NS0 6A1 cells were grown at 22°C leading to repression of genes involved in metabolic pathways in contrast from cells grown at 37°C and 34°C where the latter condition had the highest product yield. These observations indicate that the beneficial effect of reduced temperature can only be obtained if the cells are cultured at a condition that does not reduce the catabolic and anabolic needs of cells.

Transcriptional profiling of apoptotic pathways in batch and fed-batch CHO cultures (Wong et al. 2006a) revealed that during periods of high

Table 2 Published papers dealing with genomic and proteomic analysis in mammalian cell culture

Cell line	Comparison	Culture Condition	Analysis		Source
			Genomic	Proteomic	
Hybridoma Cell line	Temperature, sparging, agitation	Bioreactor/suspension	–	+	Passini et al. (1989)
CHO-K1	10% FCS, insulin, basic fibroblast growth factors	adherent/T-flask/batch	–	+	Lee et al. (1996)
NS0/NS0_r (cholesterol independent)	Cholesterol-dependent/-independent	Serum-free/suspension/spinner flask/batch	+	+	Seth et al. (2005)
CHO	Exponential growth phase—reference map	Serum-free/suspension/2L bioreactor/fed-batch	–	+	Champion et al. (1999)
CHO-K1	70–80% confluence—reference map	Adherent/T-flask/batch	–	+	Hayduk et al. (2004)
CHO-K1	0.5 mM n-butyrate acid, 80 μM zinc sulphate	Adherent/T-flask/batch	–	+	van Dyk et al. 2003)
Murine hybridoma cells	Hyperosmotic—390 mOsm/kg	Suspension/T-flask/batch	+	–	Shen et al. (2005)
CHO	Hyperosmotic—300, 350, 400, 450 mOsm/kg	Adherent/6-well plates/batch	–	+	Lee et al. (2003)
MAK mouse-mouse hybridoma cell line	Nutrient feeding—metabolic shift	Serum-free/suspension/fed-batch to continuous culture	–	+	Seow et al. (2001)
CHO	0–2.5% DMSO	Adherent/T-flask/batch	–	+	Li et al. (2006)
CHO-K1	Temperature shift—37°C to 31°C	Adherent/T-flask/batch	–	+	Kaufmann et al. (1999)
CHO	Temperature shift—37°C to 33°C	Adherent/T-flask/batch	+	+	Baik et al. (2006)
GS-NS0	Temperature—37°C, 34°C and 22°C	Suspension/T-flask/batch	+	–	Swiderek et al. (2006)
CHO-SEAP	High vs low producer	Adherent/T-flask/batch	–	+	Hayduk et al. (2005)
GS-NS0/wild type NS0	Producer vs non-producer	Suspension/spinner flask/batch	+	–	Khoo et al. (2007)
GS-NS0	High vs low producer	Suspension/T-flask/batch	–	+	Alete et al. (2005)
GS-NS0	High vs low producer	Suspension/T-flask/batch	–	+	Smales et al. (2004)
GS-NS0	Mid-exponential phase—reference map	Serum-free/shaker flask/batch	–	+	Smales et al. (2003)
GS-NS0	High vs low producer	Suspension/T-flask/batch	–	+	Dinnis et al. (2006)
Murine hybridoma cells	Zinc as insulin replacement	Serum-free/shaker flask/batch	+	–	Wong et al. (2006c)
CHO	Batch and fed-batch culture	Serum-free/suspension/5L bioreactor/batch&fed-batch	+	–	Wong et al. (2006a)

viability, most pro-apoptotic signaling genes were down-regulated but upon loss in viability, several early pro-apoptotic signaling genes were up-regulated. At later stages of viability loss, late pro-apoptotic effector genes such as *caspases* and *DNases* were up-regulated. These findings enabled the development of cell lines which are apoptosis resistant (Wong et al. 2006b).

Van Dyk et al. (2003) have presented the protein expression profiles of CHO-K1 cells under normal growth conditions and when exposed to media additives such as zinc sulphate and n-butyrate acid, which are known to have a positive effect on recombinant protein production. Comparison of the expression patterns has revealed a number of cellular proteins induced by thioredoxin, GRP75 and enolase. Surprisingly, there was no increased intracellular response of stress proteins (chaperones) to the addition of zinc sulphate and n-butyrate. Beyond, 2DE analysis has been contributed to gain insight into the cellular changes of recombinant antibody-expressing CHO cells to hyperosmotic stress (Lee et al. 2003). Change of osmolarity from 300 to 450 mOsm/kg in culture media through addition of salts and sugars resulted in a decrease in cell proliferation but increase in specific productivity. Only a few significant proteins such as pyruvate kinase and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were differentially regulated, as a consequence of increased glucose consumption and subsequently energy metabolism. Down-regulation of tubulin reflected the reduced cell growth. Further, a genome-wide analysis of a murine hybridoma systems (Shen and Sharfstein 2005) subjected to hyperosmotic stress revealed changes on gene transcription levels of several functional groups, such as transcription regulation, cell cycle regulation and apoptosis. In this study, the immunoglobulin heavy chain gene showed no significant change in its mRNA level; however, the immunoglobulin kappa chain gene (light chain) expression was significantly up-regulated, which may contribute to the enhanced antibody production. Many of differentially deregulated genes under osmotic shock might be suggested as candidates for cellular engineering to improve culture viability and longevity.

By maintaining mammalian cultures at low lactate to glucose ratios high cell concentration with high viability were achieved (Europa et al. 2000). It is suggested that the identification of specific proteins or genes involved in the manipulated metabolism could contribute to cell engineering of improved cell lines. Therefore, 2DE has been utilized to analyze the proteome of mouse hybridomas during high consumption of glucose and amino-acids which resulted in a large formation of toxic metabolites (lactate and ammonia) in comparison to a shift in metabolism by sustaining low nutrient feeding in a continuous mode. The cellular response to a metabolic shift showed at least eight proteins to be differentially expressed including metabolic enzymes (GAG, NDUS8) and proteins involved in protein degradation (L1) (Seow et al. 2001). Another investigation focused on the effect of 0–2.5% DMSO presence in culture medium by protein expression profiling to view intracellular changes in cell physiology. DMSO as a medium additive was shown to prolong culture duration, enhance protein production and simultaneously decrease nutrient consumption of CHO cells. Four enzymes related to glycolysis were down-regulated suggesting the enhancement of redistribution of substrate metabolism flux (Li et al. 2006). Limiting steps for further improvements of recombinant protein expression were attributed towards protein post-translational modification and secretion machineries. This was assumed due to the lack of over-expressed chaperon proteins in the presence of DMSO which support protein folding and secretion.

The genetic basis for differentiating a high producer clone from low producer is widely still unclear whether expression is limited by energy metabolism, metabolic regulation or protein folding and secretion capacity (Khoo et al. 2007). Unexpectedly, differences in gene expression profiling of producer and non-producer NS0 cell lines suggested that the selection process actually resulted in a producer clone that did not have higher number of expressed genes in relation to protein synthesis. In mammalian cells, specific productivity seems to be clone dependent as a consequence of random integration sites of foreign genes in the host chromosome (Yoon et al. 2004).

Thus it may not be plausible to expect to find a set of genes that have their expression levels always associated with high productivity across the different clones and cell lines. The complexity of this relationship was demonstrated in several studies on the proteome maps of NS0 clones varying in their specific recombinant antibody production rates where the identification of a total of 79 proteins and their categorization into groups suggested that high producer cells are more functionally equipped in some cellular areas, together facilitating improved recombinant protein production instead of only single genes (Alete et al. 2005; Dinnis et al. 2006; Smales et al. 2004). In a study that looked at the proteomic comparison of different productivities of recombinant CHO clones selected under high MTX concentrations, it has been suggested that actin capping protein, one of 21 differentially expressed proteins, might play an important role in heterologous protein secretion as well (Hayduk and Lee 2005). High producer clones expressing SEAP showed as much as a 4-fold increase in actin capping protein expression. Subsequently, studies of CHO clones treated with the small drug molecule cytochalasin D, mimicking the actin cytoskeleton protein, showed an increase of 2- to 3-fold of SEAP productivity within these cells postulated due to modifications in the secretion pathways.

A transcriptomic and proteomic analysis of NS0 cells grown at different densities in perfusion culture have identified a total of 47 genes and 53 proteins that were deregulated at high cell density (Krampe and Al-Rubeai 2006). Specifically, it was found, that up-regulation of gene and protein expression involved in energy metabolism, anti-apoptosis, and cell cycle checkpoints ensured cell survival in high cell density populations. Although no change in expression of protein folding and assembly was observed, expression of chaperone proteins was significantly increased. Another proteomic study looked at differences between an over-expressing c-myc CHO cell line and its control (Kuystermans and Al-Rubeai 2006). This proteomic approach was used to discover factor(s) involved in c-myc-CHO cells that promote the increased proliferative capacity and to gain insights into the mechanism by which c-myc modulates cell growth and apoptosis. Unique changes in protein expression level for 23 proteins were identified including proteins associated with proliferation such as Hsp70 and proteins implicated in exocytosis and cell-to-cell adhesion such as Annexin A2. A large number of proteins identified were involved in the cytoskeletal network, such as actin like protein, β-actin, and F-actin (also involved in the secretory pathway (Hayduk and Lee 2005)) and others in energy metabolism such as ATP synthetase, phosphoglycerate mutase-1 and triophosphate isomerase. It was postulated that a balance exists between proliferation and protein production most likely integrated into the predicted extensive c-myc sub network. The increase in F-actin (which is known to hold up the secretory pathway) and the decreased expression in ER proteins have resulted in reduced secretion of heterologous protein as was subsequently demonstrated by the transfection of the cell line with the hSEAP-hFc construct.

The first combined transcriptomic and proteomic analysis was accomplished on a NS0 cell line by Seth et al. (2005). In this study, the gene and protein expression profile of NS0 cholesterol-dependent phenotype, cultivated under standard cholesterol-dependent growth conditions (NS0) was compared to cells adapted to cholesterol-independent conditions (NS0 revertant) (Seth et al. 2005). Overall, a large number of genes were expressed differentially and mostly down-regulated in the cholesterol biosynthesis, lipid metabolism and central energy metabolism. It was deduced that the deficient expression of a single gene, Hsd17b7, allowed for cholesterol auxotrophy. Subsequent work confirmed, through the use of a cell-engineering route, the role of Hsd17b7 in cholesterol dependency of NS0 cells (Seth et al. 2006b).

Integration of proteomic and genomics analysis

We are continually finding ways to optimise the use of these tools effectively. Further improvements of the techniques and methodologies which address the variation in biological samples and lack of standard procedures (number of replicates

and statistical methods) are being addressed including the merging of the ''omic'' disciplines to give a more complete global understanding of a biological system. With a well established transcriptomic technology and a well advancing proteomic technology, the next few years will most likely see the further integration of these two technological platforms to describe complex biological systems. Since intracellular expression information does not flow in a unidirectional path from transcriptome to proteome it is more likely that a better view would be obtained by examining an integrated view of transcriptomic and proteomic profiles of the same system. At the mRNA level, observations in gene expression give only information on some of the effectors of biological functions while proteins are usually seen as the effectors of biological functions (Chan 2006), although specific time point snapshots of both these systems (transcriptome and proteome) might not be enough to fully understand the regulatory complexity of the biological systems.

Proteins and mRNA transcripts have varied half-life, where mRNA is known to have a half-life from minutes to hours, the half-life of proteins can be from minutes to days. This can give discrepancies in the data, which must be accounted for when analyzing profiles and integrating the two data sets. Temporal integrative profiling experiments might shed some light on these discrepancies and give us a better understanding of the regulatory pathways that interact within the cell (Ideker et al. 2001). At the same time bioinformatics analyses that allow for the interpretation of both data sets on one platform will be advantageous (Tian et al. 2004).

Conclusions

Direct cell engineering approaches have resulted in several current and potential improvements in cell lines of biopharmaceutical importance. With the advent of genomic and proteomic tools, indirect cell engineering for the improvement of biopharmaceutical cell lines is becoming a useful strategic approach. In combination with advances in expression engineering, selection and media development it is hoped to decrease development time and dramatically increase cell line productivity reducing overall costs for the next generation of approved products.

The analyses of gene and protein expression profiles, alone, in cells undergoing the physiological changes provide great insight into the regulatory systems. The examples discussed, showed clearly that by interaction of cells with their extracellular environment some specific pathways are activated. The comparisons of genes and metabolic pathways whose changes in expression profiles influence the phenotype lead to new targets for bioprocess engineering. Therefore, the tools of functional genomics and proteomics could be applied directly in cell and metabolic engineering to identify new targets for improved phenotypes. An additional benefit from this is the knowledge gained to have a fundamental understanding of genetic mechanisms involved in recombinant protein production.

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