Review

Biotechnology and Bioengineering

An 'Omics Approach Towards CHO Cell Engineering

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ABSTRACT: Chinese hamster ovarian cells (CHO) cells have been extensively utilized for industrial production of biopharmaceutical products, such as monoclonal antibodies, human growth hormones, cytokines, and blood-products. Recent advances in recombinant DNA technology have resulted in the bioengineering of CHO cells that have robust gene amplification systems and can also be adapted to grow in suspension cultures. In parallel, recent advances in techniques and tools for decoding the CHO cell genome, transcriptome, proteome, and glycome have led to new areas of study for better understanding the metabolic pathways in CHO cells with the long-term goal of developing new biologics. This review paper discusses the recent advances in bioengineering strategies in CHO cell lines and the impact of the knowledge gained by CHO cell genomics, transcriptomics, and glycomics on the future of CHO-cell engineering.

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KEYWORDS: Chinese hamster ovary cells; bioengineering; glycomics; metabolic engineering

Introduction

Chinese hamster ovary cells (CHO) cells have been extensively utilized for industrial production of biopharmaceutical products such as monoclonal antibodies, hormones, cytokines, and blood-products (Table I). Many of these proteins are glycoproteins, post-translationally modified through glycosylation. Among the mammalian cells used in preparing biopharmaceuticals, including mouse myeloma cells, mouse fibroblast cells, human embryonic kidney 293 cells, baby hamster kidney cells, and human retina-derived PerC6 cells (Baldi et al., 2005; Barnes et al., 2001; Bebbington et al., 1992; Griffin et al., 2007; Jones et al., 2003), CHO cells are the most widely employed mammalian cell line (Birch and Racher, 2006; Jayapal et al., 2007; Omasa et al., 2010; Walsh, 2010; Zhu, 2012). The first clinically approved recombinant biologic produced in CHO cells was tissue plasminogen activator (Kaufman et al., 1985). Since then, it has been estimated that CHO cells produce over 70% of the therapeutic proteins in a global market valued at US $30 billion in annual sales (Jayapal et al., 2007; Walsh, 2010).

Over more than two decades, CHO cells have been routinely engineered to produce recombinant therapeutic proteins, in particular glycoproteins, that are non-immunogenic to humans; moreover, they have the potential to produce other non-protein pharmaceuticals, such as heparin (Baik et al., 2012; Butler, 2005; Jenkins et al., 1996; Walsh and Jefferis, 2006). As cloning techniques, expression vector design, and clonal selection methods have improved, combined with bioprocess optimization (e.g., media composition, feeds, etc.), volumetric productivity has steadily increased from 0.05 to 2–10 g/L (Huang et al., 2010; Wurm, 2004). While these approaches are effective, they are labor intensive, and more importantly, must be performed for every new bioproduct. In addition to the productivity challenges, biologics often require complex post-translational modifications, particularly glycosylation. Incorrect glycosylation can create a product that is non-functional, has an accelerated clearance rate, or is immunogenic.

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Table I. Selected biologics produced in CHO cells.

Tradename	Generic name	Product category	Biological Importance	Developer/ Manufacturer	FDA Approval Year
Zaltrap	Ziv-aflibercept	Recombinant fusion protein	Colon cancer drug	Sanofi Aventis US and Regeneron Pharmaceuticals	2012
Eylea	Aflibercept	Recombinant fusion protein	Wet (neovascular) age-related macular degeneration (AMD)	Regeneron Pharmaceuticals	2011
Actemra	Tocilizumab	Antibodies	Treatment of rheumatoid arthritis (RA)	Genentech	2010
Prolia	Denosumab	Antibodies	Osteoporosis in post menstrual women	Amgen	2010
Recothrom	Thrombin alpha	Blood factors, anticoagulants and thrombolytics	Coagulation Factor	ZymoGenetics, Bayer	2008
Arcalyst	Rilonacept	Recombinant fusion protein	Cryopyrin-Associated Periodic Syndromes	Regeneron Pharmaceuticals	2008
Xyntha	Factor VIII	Blood factors, anticoagulants and thrombolytics	Hemophilia A	Wyeth	2008
Herceptin	Trastuzumab	Antibodies	A single agent for treatment of HER2-overexpressing node-negative and node-positive breast cancer	Genetech	2008
VectibixTM	Panitumumab	Antibodies	Antineoplastic, metastatic colorectal cancer	Amgen	2006
MYOZYME®	Glucosidase alfa	Enzymes	Enzyme Replacement Therapy, Pompe disease	Genzyme	2006
Orencia	Abatacept	Others	Treatment of adults with moderate to severe rheumatoid arthritis	Bristol-Meyers Squibb	2005
Naglazyme	Galsulfase	Enzymes	Mucopolysaccharidosis VI	BioMarin Pharmaceuticals	2005
Luveris	Lutropin alpha	Hormones	Luteinizing hormone for treatment of infertility	Merck Serono	2004
Avastin	Bevacizumab	Antibodies	Treatment of first-line metastatic colon or rectum cancer	Genentech	2004
Aldurazyme	Laronidase	Enzymes	Mucopolysaccharidosis I	Genzyme	2003
Amevive	Alefacept	Immunosuppressive dimeric fusion protein	Chronic plaque psoriasis	Biogen Idec	2003
Advate	Factor VIII	Blood factors, anticoagulants and thrombolytics	Hemophilia A	Baxter	2003
Xolair	Omalizumab	Antibodies	Asthma treatment	Genetech	2003
Raptiva	Efalizumab	Antibodies	Treatment of plaque psoriasis	Serono, Genentech	2003
Fabrazyme	Agalsidase bet	Enzymes	Recombinant human alpha galactosidase A for treatment of Fabry disease	Genzyme	2003
Rebif	Interferon beta-1a	Interferons	Glycosylated interferon beta-1a for treatment of multiple sclerosis	Serono	2002
Humira	Adalimumab	Antibodies	Human IgG1 monoclonal antibody	Abbott	2002
Zevalin	Ibritumomab tiuxetan	Antibodies	Therapeutic radiopharmaceutical for treatment of non-Hodgkin's lymphoma	IDEC Pharmaceuticals (part of Biogen Idec)	2002
Aranesp	Darbepoetin Alfa	EPO and colony-stimulating factors	2nd generation recombinant form of erythropoetin for treatment of anemia	Amgen	2001
MabCampath	Alemtuzumab	Anribodies	Treatment of chronic lymphocytic leukaemia	Genzyme Corporation	2001
Cathlo Activase	Alteplase	Blood factors, anticoagulants and thrombolytics	Tissue-plasminogen activator (t-PA) for treatment of acute myocardial infarction	Genentech	2001
Ovidrel	Choriogonadotropin alfa		Recombinant human chorionic gonadotropin, r-hCG	Serono	2000
ReFacto	Factor VIII	Blood factors, anticoagulants and thrombolytics	Hemophilia A	Wyeth	2000
TNKase	Tenecteplase	Blood factors, anticoagulants and thrombolytics	Tissue plasminogen activator for treatment of myocardial infarction	Genentech	2000
Thyrogen	Thyrotropin alfa	Hormones	Thyroid cancer	Genzyme	1998

Table I. (*Continued*)

Tradename	Generic name	Product category	Biological Importance	Developer/ Manufacturer	FDA Approval Year
Enbrel	Etanercep	Antibodies	A tumor necrosis factor antagonist	Immunex, now Amgen	1998
Follistim	Follitropin beta	Hormones	Follicle stimulating hormone for treatment of infertility	NV Organon	1997
Benefix	Factor IX	Blood factors, anticoagulants and thrombolytics	Hemophilia B	Wyeth, Genetics Institute	1997
Gonal-F	Follitropin alfa	Hormones	Follicle stimulating hormone for treatment of anovulation and superovulation	Merck Serono	1997
Rituxan	Rituximab	Antibodies	Treatment of patients suffering from B-cell non-Hodgkins lymphoma	Genentech and IDEC Pharmaceuticals (now Bioegen Idec)	1997
Avonex	Interferon beta-1a	Interferons	Glycosylated interferon beta-1 for treatment of multiple sclerosis	Biogen Idec	1996
Cerezyme	Imiglucerase	Enzymes	Beta-glucocerebrosidase	Genzyme	1994
Bioclate	rh Factor VIII	Blood factors, anticoagulants and thrombolytics	Hemophilia A	Aventis Behring	1993
Pulmozyme	Dornase alfa	recombinant human deoxyribonuclease I (rhDNase)	Cystic fibrosis	Genentech	1993
Recombinate	Factor VIII	Blood factors, anticoagulants and thrombolytics	Hemophilia A	Baxter	1992
Procrit	Epoetin alfa	EPO and colony-stimulating factors	Erythropoetin	Orthobiotech	1990
Epogen	Epoetin alfa	EPO and colony-stimulating factors	Erythropoietin	Amgen	1989
Activase	Alteplase	Blood factors, anticoagulants and thrombolytics	Tissue-plasminogen activator (t-PA) for treatment of acute myocardial infarction	Genentech	1987

References:
http://www.gene.com/gene/about/corporate/history/timeline.html, accessed on April 2nd–3rd 2012;
www.FDA.gov, accessed on April 2nd–3rd 2012 to August 22nd–23rd 2012;
Walsh (2010);
Jayapal et al. (2007);
http://www.centerwatch.com/drug-information/fda-approvals/default.aspx?DrugYear=2012, accessed on August 22nd 2012.

Beyond simple relationships (e.g., feeding galactose increases galactosylation), there is little understanding of how process conditions affect product quality, again requiring empirical optimization (Andersen et al., 2009).

Recent advances in techniques and tools for rapid, low-cost genome and transcriptome sequencing, as well as advances in proteomics, metabolomics, and glycomics have permitted an unprecedented characterization of organisms and cultured cell lines. The recent sequencing of the CHO genome (Xu et al., 2011), combined with efforts to characterize the proteome, metabolome, and glycome under various conditions will permit a similar understanding of these industrially relevant cell lines (Dietmair et al., 2012; Kim et al., 2012b; Meleady et al., 2012a,b; North et al., 2010; Selvarasu et al., 2012; Tep et al., 2012). This understanding may then translate into more rapid bioprocess optimization for novel therapeutic proteins and improved development of biosimilars, ''generic'' versions of existing therapeutic bioproducts. In particular, using 'omics to develop an understanding the relationship between process conditions and glycosylation may be very important for development of biosimilars, as it will be very difficult to reproduce the glycosylation patterns of a commercial bioproduct with a new cell line and no knowledge of original bioprocess conditions. Hence, the current FDA guidance indicates that clinical testing will likely be required for approval of biosimilars. 'Omics may also play an important role in CHO-cell engineering by providing an understanding of how to optimize transgene expression as well as how engineering the host CHO cell (e.g., for reduced apoptosis) affects its growth and the productivity of the bioproduct. This review paper discusses the different strategies that are employed in CHO-cell engineering, the approaches that have been taken to optimize CHO-cell engineering, and the scope of 'omics to further optimize CHO cell engineering.

Strategies for CHO Cell Engineering

CHO cells have been engineered for the production of biopharmaceuticals and non-protein pharmaceuticals using three broad strategies, genetic engineering to introduce the genes for heterologous protein production, cellular engineering to alter phenotypes for improved productivity and growth, and metabolic engineering to produce novel products, including altered glycoforms on recombinant proteins. These approaches are discussed in greater detail below.

CHO Cell Genetic Engineering

The first, most conventional, and most widely used method of CHO cell engineering for the past two decades has been genetic engineering, wherein the CHO cells are genetically engineered for the production of recombinant proteins (Jayapal et al., 2007; Walsh, 2010). The principle strategies for production of recombinant proteins in CHO cells are: (1) identification of the gene-of-interest that needs to be expressed in the host cell; (2) expression-vector design and optimization of the coding sequence for expression in CHO cells; (3) cloning the gene-of-interest (i.e., transgene) into a suitable expression vector; (4) transfection and optimization of transgene integration into the host genome; (5) clonal selection; followed by (6) optimization of expression levels by gene amplification as required (Fig. 1).

CHO Cell Cellular Engineering

The second most widely used strategy for CHO cell bioengineering is cellular engineering. Cellular engineering of CHO cells involves optimizing the cellular processes in the cell line with the long-term goal of creating more robust bioprocesses and higher production. These approaches include engineering the cells to resist apoptosis (Dorai et al., 2009, 2010; Majors et al., 2009) to reduce lactate production (Jeon et al., 2011; Zhou et al., 2011), and to improve glycosylation patterns (Jeon et al., 2011; Majors et al., 2009; Mohan et al., 2008, 2009; Son et al., 2011; Zhou et al., 2011). A variety of mechanisms can be employed to alter these cellular processes, including silencing or over-expressing individual genes in a cellular pathway and modifying the expression of entire groups of genes using microRNAs.

Gene silencing is an important approach to cellular engineering. Strategies for gene silencing include interfering RNA (RNAi) and gene targeting, often employing a variety of nucleases. RNA interference technology has the potential to silence multiple genes in different cellular pathways for optimum productivity and quality of bioengineered proteins (Wu, 2009). RNA interference has been employed to target: (1) apoptosis [e.g., caspase-3 and 7 (Sung et al., 2005, 2007), Bak & Bax (Lim et al., 2006), and requiem (Wong et al., 2006a)]; (2) glycosylation [e.g., 1,6 fucosyl-transferase (Mori et al., 2004; Yamane-Ohnuki et al., 2004) and sialidase (Ngantung et al., 2006)]; and (3) enzymes such as dihydrofolate reductase (Hong and Wu, 2007; Wu et al.,

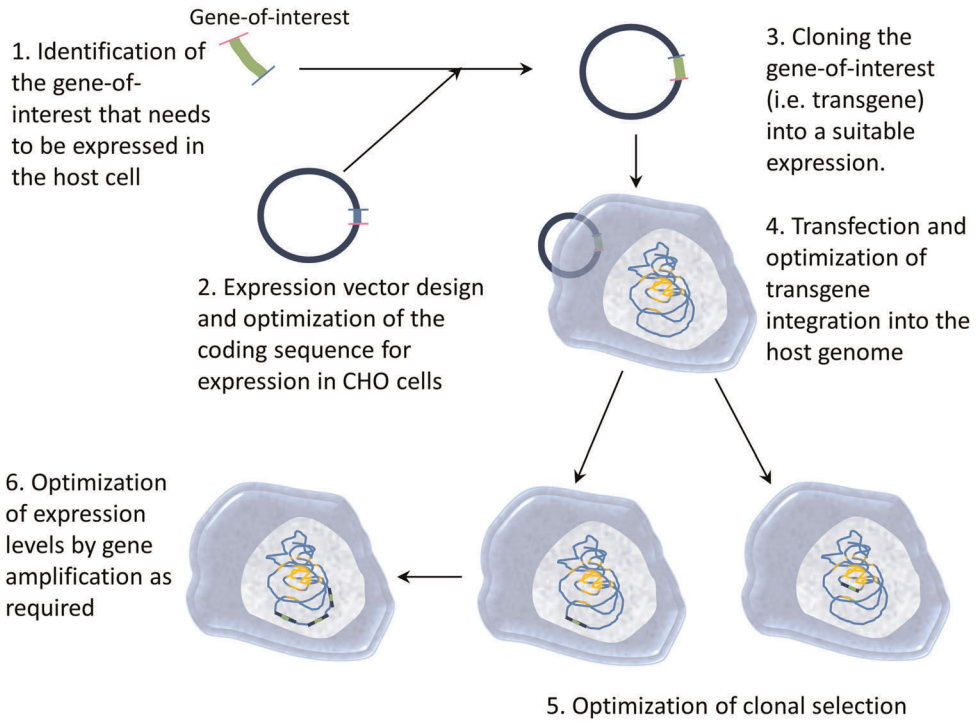

Figure 1. Recombinant DNA Technology in CHO cells.

2008). Alternate emerging strategies for targeting gene expression include a variety of nucleases, such as zinc finger nucleases, homing endonucleases (or meganucleases), and transcription-activator-like effector nucleases (Gaj et al., 2012; Gustafsson et al., 2012; Kim et al., 2012a; Silva et al., 2011). For example, zinc-finger nucleases have been used to silence expression of Bak and Bax proteins to produce apoptosis-resistant CHO cells (Cost et al., 2010). Meganucleases have been associated with homologous gene targeting, and in recent years, meganucleases have been used to develop high-throughput gene targeting techniques for efficient and cost effective cell line development (Cabaniols et al., 2010).

Before a CHO genome sequence was available, gene silencing in CHO cells could be achieved either by sequencing the gene of interest or by designing silencing sequences against highly conserved regions using mouse and human genomes. With the availability of complete genome information, gene-silencing sequences can more readily be designed.

The sequencing of the CHO genome has provided an additional opportunity for regulating gene expression, the use of microRNAs. MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression, and hence, cell physiology. These miRNAs work by down-regulating gene expression of large groups of mRNAs, generally by binding to the 3′UTR of the mRNA and inhibiting translation. MicroRNAs may affect cell growth, apoptosis, metabolism, secretion, and specific productivity of recombinant proteins with no additional translational burden to the host cell (Barron et al., 2011, 2012; Bort et al., 2012; Gammell et al., 2007; Hackl et al., 2011; Hammond et al., 2012; Jadhav et al., 2012; Johnson et al., 2011; Lin et al., 2011; Meleady et al., 2012a; Muller et al., 2008). Having a complete genome sequence permits identification of miRNAs in the genome sequence, generally by homology with known miRNAs from other organisms. Once the miRNA sequences are identified, they can either be overexpressed or silenced to achieve the desired regulation of gene expression (Hackl et al., 2012).

CHO Cell Metabolic Engineering

Metabolic engineering of a CHO cell line requires over-expressing and/or down-regulating specific proteins in a metabolic pathway, such that the cells produce a novel product. This approach has been used extensively in prokaryotic cells and lower eukaryotes for production of novel antibiotics, biofuels, and specialty chemicals (Curran and Alper, 2012; Lee et al., 2012). However, the use of metabolic engineering in CHO cells and other mammalian cell systems is less common. Metabolic engineering has been employed (as described above) to modify fucosylation and sialylation of therapeutic glycoproteins, including antibodies and non-antibody proteins (Jeong et al., 2009; Prati et al., 1998; Wong et al., 2006b; Yamane-Ohnuki et al., 2004). However, the next step is to use metabolic engineering to produce non-protein bioproducts. For example, CHO cells have a specific pathway for the biosynthesis of heparan sulfate proteoglycans; metabolic engineering of this heparan sulfate pathway might permit the production of the biopharmaceutical drug, heparin (Baik et al., 2012). While introducing a novel functionality into a cell is a rather straightforward application of genetic engineering technology, and even knocking out a gene can be done relatively easily with modern nuclease editing tools (e.g., zinc-finger nucleases), predicting how overexpressing or down-regulating certain genes affects the physiology of the cell and whether the modification will achieve the desired outcome is a much more difficult problem. Here the tools of 'omics may play an important role in both identifying perturbations in the overall physiology and in identifying bottlenecks, regulatory factors, and missing activities that prevent achieving the desired outcome. [Figs. 2 and 3].

Current Technologies for Optimization of CHO Cell Bioprocesses

Maximization of expression from the ''straightforward'' genetic engineering of CHO cells can be achieved by a variety of approaches: optimization of the expression vector, including the promoter and flanking sequences; clonal screening and selection, and optimization of extrinsic factors such as media and other bioprocess conditions (e.g., temperature, pH; Becerra et al., 2012; Freimark et al., 2012; Jardon et al., 2012; Jing et al., 2012a; Li et al., 2012; Taschwer et al., 2012). These approaches often have a combinatorial effect on stability and productivity of transgene expression. Approaches for optimization and the impact of 'omics technologies on these approaches are discussed in detail below.

Vector Design and Targeting

An efficient transfection method, optimized vector design, and robust clonal selection methods are required to effectively obtain stable clones. Transfection is well established, using a variety of methods including calcium phosphate precipitation, cationic lipid transfection, electroporation, and nucleofection (Graham and van der Eb, 1973; Seth et al., 2007). In a stable transfection, once the cells have been transfected, they are subjected to the selective pressure that is governed by the co-expressed selection gene. The daughter cell that has stably integrated the expression vector into its chromosome survives selection pressure and will hopefully also express the transgene. The integration of the expression vector in the daughter cell's chromosome is a random event that may, or may not, result optimal expression of the transgene due to silencing or other epigenetic effects (Wurm, 2004). Integration of the expression vector in the heterochromatin may result in

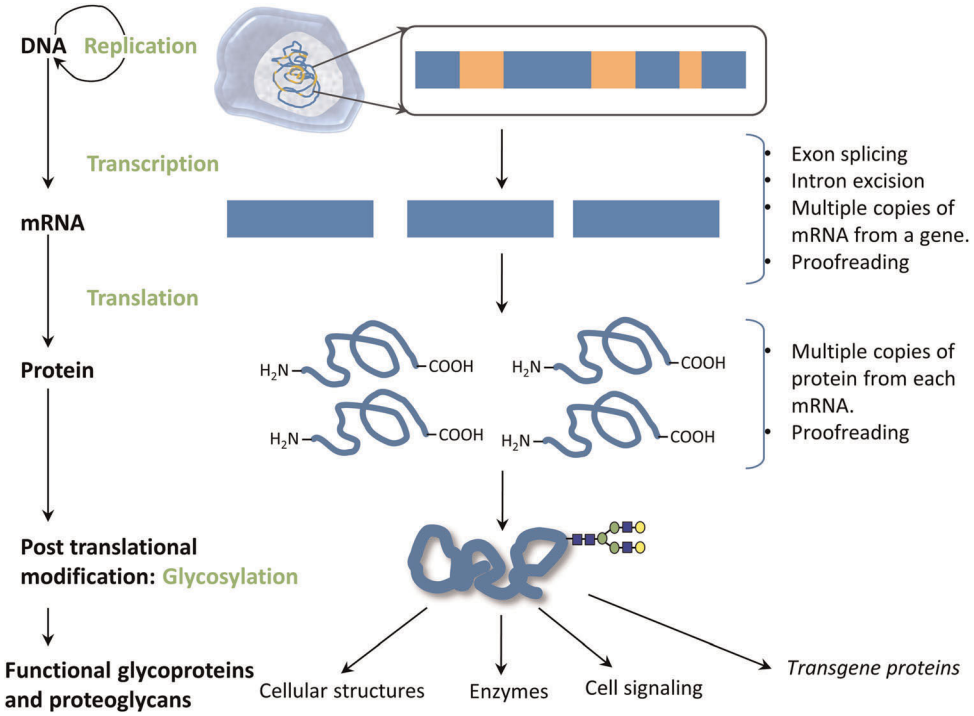

Figure 2. Flow of information in the cell.

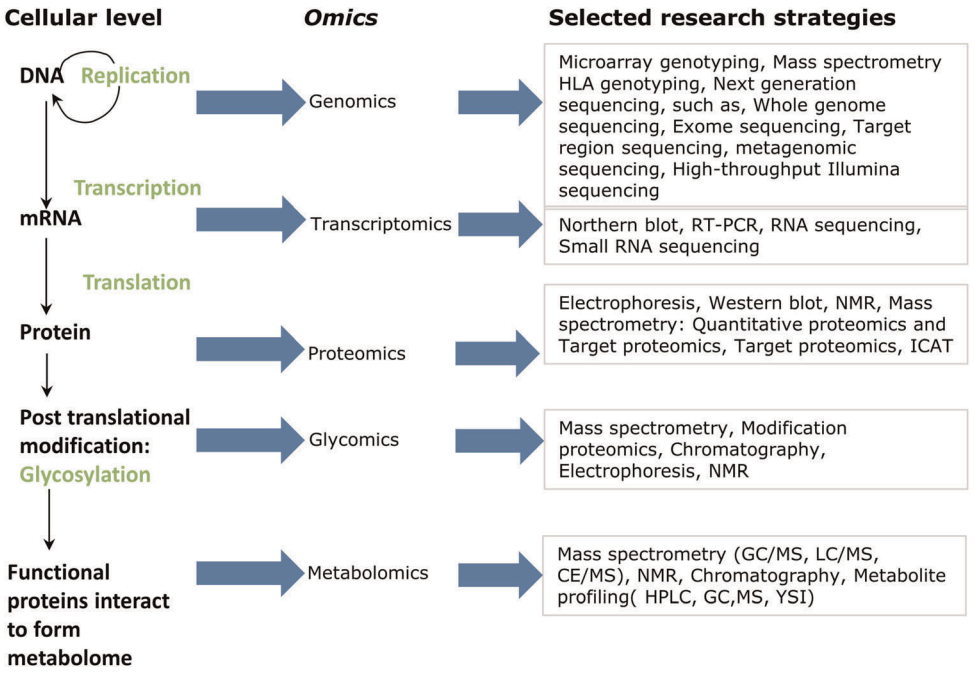

Figure 3. Research strategies in 'omics.

silencing or low level expression of the transgene (Kwaks and Otte, 2006). However, integration of the expression vector into the more transcriptionally active euchromatin still may not result in optimal transgene expression due to other local factors (Kwaks and Otte, 2006). Moreover, high expression of transgenes may be silenced by histone deacetylation or DNA methylation. These caveats in mammalian-cell transgene expression have led to novel research focusing on expression vector optimization, including promoter design, to establish highly efficient, stable transgene expression with high frequency.

A simplified expression vector design for transfection purposes consists of the cDNA of the transgene driven by a strong viral or cellular promoter/enhancer (Gopalkrishnan et al., 1999; Ringold et al., 1981; Wurm, 2004). The expression vector may also contain a selection marker, such as an antibiotic resistance gene that is driven by a weak promoter. Alternatively, the selection marker can be provided by co-transfecting the selection marker on a separate vector. However, beyond this minimal vector, there have been numerous approaches to optimize the expression vector, including gene targeting and promoter design.

When a transgene integrates into certain chromosomal regions of the host cell line, clones are produced that are more productive and stable (Kuystermans et al., 2007; Wilson et al., 1990). This observation has driven the development of novel approaches to maximize gene expression by gene targeting or expression-vector modification. Improvements in expression vector design for achieving high level, stable expression of the transgene include flanking the transgene with: (1) cis-regulatory sequence for transcription control [e.g., CHO elongation factor-1 alpha (Running Deer and Allison, 2004)]; (2) chromatin opening sequences [e.g., matrix attachment regions (Girod et al., 2005, 2007; Kim et al., 2004; Zahn-Zabal et al., 2001), ubiquitous chromatin opening elements (UCOE) (Benton et al., 2002), and antirepressor elements (Kwaks et al., 2003)]; and (3) gene targeting to transcriptionally active sites such as the Cre/Lox site-specific recombination system (Kito et al., 2002) and the Flp/FRT site-specific recombination system (Huang et al., 2007).

Promoter regions are essential for gene transcription. For genetic engineering in CHO cells, viral promoters and more recently, cellular promoters have been used. In addition, synthetic promoters have also been recently proposed, building on common motifs seen in cellular and viral promoter regions (Grabherr et al., 2011). Examples of strong viral promoters include the CMV promoter (human and mouse cytomegalovirus), SV40 promoter, and Rous Sarcoma Virus (RSV) long-terminal-repeat (LTR) promoter. Cellular promoters may be housekeeping genes, such as CHO-derived elongation factor-1 (CHEF-1), Chinese hamster Cofilin (CHCF) and the Chinese hamster 14-3-3 epsilon promoter (Kwok-Keung Chan et al., 2008). Knowledge of the CHO cell genome will add promoter sequences of more housekeeping genes, accelerating cellular promoter designs.

Clonal Selection

Once daughter cells have stably integrated a transgene, stable clones are selected based on cell health, cell viability, and quantity of transgene protein expression. The clonal selection technique that is employed is usually based on cell type (adherent or suspension) and whether the exogenous protein is secreted outside the cell, localized inside the cell, or present on the cell surface. Regardless of the protein localization, clonal screening generally requires laborious analysis of hundreds or even thousands of clones to find an optimal clone (Bailey et al., 2002; Chung et al., 2003; Freimark et al., 2010; Kumar and Borth, 2012). Moreover, CHO cells exhibit heterogeneity within a single clone, making it more important to select clones based on both productivity and quality of the bioengineered protein formed by a given clone (Kim et al., 1998; Pilbrough et al., 2009). A review by Browne and Al-Rubeai describes recent developments in the cloning methods (Browne and Al-Rubeai, 2007). Techniques employed for clonal selection have been traditional limiting dilution cloning (Puck and Marcus, 1955), flow cytometry and cell sorting (Borth et al., 2000; Klapperstuck et al., 2009; Kumar and Borth, 2012; Lee and Lee, 2012; Nicolette et al., 2011; Pichler et al., 2011; Vanderbyl et al., 2001), and gel microdrop technology (Hammill et al., 2000). More recently, these techniques have been combined with automation to improve throughput and reduce the labor involved (Kacmar and Srienc, 2005; Shi et al., 2011). Recent advances in clonal selection based on quality of the bioengineered protein include high-throughput analysis based on sialic acid content in the bioengineered glycoprotein (Markely et al., 2010; Park et al., 2010). The number of molecules of sialic acid attached to a bioengineered glycoprotein often impacts its biological activity (Kaneko et al., 2006), protease degradation (Goldwasser et al., 1974; Tsuda et al., 1990), serum half-life (Ngantung et al., 2006), solubility (Sinclair and Elliott, 2005), and thermal denaturation (Goldwasser et al., 1974; Tsuda et al., 1990).

Optimization of Extrinsic Factors

For the past 25 years, much of bioprocess optimization has been performed by screening process conditions using design of experiment (DOE) approaches to optimize for higher cell growth and/or for higher levels of recombinant protein productivity (Bollin et al., 2011; Kim and Lee, 2009; Legmann et al., 2009; Rouiller et al., 2012). Extrinsic factors such as media components, pH, temperature, and bioreactor design influence cell growth, cell productivity, and transgenic protein structure, and by implication, function as well. An example is glucose, which is the main carbon source in almost all mammalian cell culture media. Very low concentrations of glucose in the media adversely impact cell viability and decrease glycosylation-site occupancy in the transgenic glycoprotein product (Hayter et al., 1992). Media components such as copper sulfate, manganese sulfate, and

zinc sulfate and bioreactor temperature, pH, and shear stress can also impact cell growth and viability as well as quantity and quality of the transgenic protein produced (Andersen et al., 2009; Dahodwala et al., 2012; Nam et al., 2008). Additional additives such as sodium butyrate can inactivate histone deacetylases, increasing histone acetylation, and increasing expression of the transgene (Jiang and Sharfstein, 2008). However, all of these approaches have global effects on cell physiology, rather than targeted effects on the protein of interest, suggesting that an integrated understanding of transcriptional, translational, regulatory, and metabolic responses would aid in a more directed approach to improving productivity by altering bioprocess conditions.

'Omics Approaches Towards CHO Cell Engineering

Deciphering the CHO Cell Genome

A recent study has determined the genome sequence of the parental CHO-K1 cell line (Xu et al., 2011), representing a major milestone for cellular and metabolic engineering of CHO cells. The CHO-K1 genome was established using a de novo sequencing technique and subsequently assembled by short oligonucleotide analysis package (SOAP). This CHO-K1 cell line is an ancestral cell line, and current clones used for research and industrial production of biologics are expected to have additional genetic variations. The ancestral CHO-K1 cell line has a 2.45 Gb genome, and analysis of the transcriptome sequence predicts more than 24,000 genes (Xu et al., 2011).

Among these 24,000 predicted genes, special emphasis was given to the genes that are involved in glycosylation and viral-susceptibility genes. The homologs of 99% of the human genes involved in glycosylation are present in CHO-K1. However, expression of approximately 141 of these glycosylation homologs, or nearly half of the glycosylation-related genes, was not observed during the exponential phase in the CHO-K1 cell growth (Xu et al., 2011). Having this information provides guidance in strategies for cellular engineering to obtain desired glycans on glycoproteins and proteoglycans for metabolically engineering CHO cells to produce these biopharmaceuticals.

CHO Cell Transcriptome

Almost all cells in a multicellular organism will have the same genome sequence. However, depending upon the function and type of the cell, genes will be differentially expressed, resulting in a cell-specific pattern of gene expression. CHO cells are derived from Chinese hamster ovaries and thus, will have their own set of genes that are turned on or turned off. Moreover, CHO cells, like other eukaryotic cells have chromosomes that are composed of both exons and introns. Message processing from the primary transcript to mRNA requires exon-intron splicing often with multiple alternative splice sites, yielding different proteins from the same gene. In addition, CHO cells used for production of biologics have been subcloned from parental CHO-K1 or CHO-DG44, and thus there is heterogeneity among these subclones caused by aneuploidy and chromosomal rearrangements (Cao et al., 2012). Thus, genome sequencing provides only the first snapshot of what constitutes a CHO cell; transcriptional analysis can provide a better (though still incomplete) picture of the physiology.

The most powerful tools for transcriptome studies have been DNA microarrays, quantitative real-time PCR (q-RT-PCR), and RNA interference. Many of these techniques rely on the availability of known DNA sequences. Until the recent the genome sequencing of CHO cells, research was limited to incomplete CHO cell microarrays (Doolan et al., 2012; Melville et al., 2011; Wlaschin et al., 2005) and studies relying on murine DNA sequences (Yee et al., 2008b). Despite these limitations, a number of studies have been published profiling the transcriptional responses of CHO cells to different cell culture conditions (Baik et al., 2006; Clarke et al., 2011; Jing et al., 2012b; Kantardjieff et al., 2010; Kim et al., 2012a; Klausing et al., 2011; Nissom et al., 2006; Shen et al., 2010; Szperalski et al., 2011; Yee et al., 2008a). With the availability of the complete genome sequence, it is expected that commercially produced CHO-cell DNA microarrays will be available within the next year or two (personal communication).

Recent studies on unraveling the CHO cell transcriptome by Becker et al. (2011) have identified more than 29,000 transcripts from several CHO cell lines cultured under different growth conditions (adherent, serum-free, serum-dependent, etc.), using pyrosequencing technology followed by assembly of the transcriptome sequence data with Newbler Assembler software. This study resulted in 1.84 million reads that were assembled into 32,801 contiguous sequences, 29,184 isotigs, and 24,576 isogroups. The results showed that more than 70% of the assembled data of the CHO transcriptome was similar to mouse (*Mus musculus*) and also closely related to rat (*Rattus norvegicus*) transcriptomes (Becker et al., 2011). Using this transcriptome information, the metabolic pathways for glycolysis, citrate cycle, pentose phosphate pathway, and other related carbohydrate metabolism pathways have been reconstructed. The CHO transcriptome also showed the presence of all genes that code for enzymes involved in the major steps in the *N*-glycosylation pathway in CHO cells (Becker et al., 2011).

CHO Cell Proteomics

The transcription of genomic DNA to mRNA, followed by the translation to finally form proteins is a well-controlled process inside the cell. However, translation rates of

individual proteins vary, as do mRNA stabilities (Valencia-Sanchez et al., 2006; Valleriani et al., 2011). Moreover, defective mRNAs and misfolded proteins are deleted due to the robust proofreading system of the cell (Chakrabarti et al., 2011; Eisele and Wolf, 2008; Valencia-Sanchez et al., 2006). Thus, examining the transcriptome can provide insight into regulation of cellular processes, but mRNA levels are not necessarily directly correlated with the protein expression levels for their protein products (Baycin-Hizal et al., 2012; Guo et al., 2008). In addition, proteins are subject to a variety of post-translational modifications that affect biological activity, particularly phosphorylation and protein–protein interactions (Flott et al., 2011; Lu et al., 2011). Thus proteomic analysis, particularly quantitative assays of proteins and their modifications can provide addition insight into changes in cell physiology upon expression of an exogenous recombinant protein or changes in culture conditions. A variety of techniques including electrophoresis (2D-PAGE), traditional Western blotting, two-dimensional difference in gel electrophoresis (2D-DIGE) and MS-based techniques have been employed for proteomic profiling in CHO cells (Baik and Lee, 2010; Carlage et al., 2009; Doolan et al., 2010; Kumar et al., 2008; Meleady, 2007; Meleady et al., 2008, 2011, 2012b). With the availability of the CHO genome sequence, proteomic studies will also be facilitated, as less de novo sequencing will be required to identify differentially expressed proteins under various conditions.

A recent study by Betenbaugh and co-workers analyzed the proteome of CHO-K1 cells, building on the information available in the newly sequenced CHO genome (Baycin-Hizal et al., 2012). They separated and analyzed secreted proteins, total protein from cell lysates, and glycoproteins from cell lysates. By comparing their mass-spectral data with genomic and transcriptomic data from CHO cells (rather than mouse, rat, or human), they were able to sequence and identify an order of magnitude more CHO proteins than are currently available in the databases. In addition, by comparing protein and nucleic acid sequences, they were able to identify the codon bias of CHO cells (i.e., the preferred codon(s) for each amino acid) more accurately than ever before. They found a substantial difference between CHO and human codon biases, suggesting strategies for codon optimization for production of human proteins in CHO cells. In addition, by comparing the relative abundance of individual transcripts and their associated proteins, they were able to establish that in general, transcript levels are well correlated with protein expression levels, but there are a number of genes that were significantly over or under represented when transcript and protein levels were compared. Finally, they were able to examine individual genes in important biological pathways (e.g., apoptosis) and demonstrate that while in most cases both mRNA and protein were present at detectable levels in the cells, in some cases only mRNA was observed and in others only protein, highlighting the importance of integrating genomic, transcriptomic, and proteomic studies.

CHO Cell Metabolomics

While genomics, transcriptomics, and proteomics tell which genes are present, expressed, and working in a cell, they cannot identify the biochemical reactions that are proceeding in a cell or determine the concentrations of small molecules and macromolecules involved in those reactions. These metabolites include sugar molecules, amino acids, nucleosides, amines, and fatty acids. Metabolomics is a qualitative and quantitative analysis of these cellular metabolites. From the concentrations of these metabolites, known biochemical pathways, and the presence of genes for the appropriate enzymes in the genome, a metabolic reconstruction can be obtained. Recently a number of studies have optimized techniques for studying intracellular and extracellular metabolites in CHO and other cultured mammalian cells (Chong et al., 2009; Dietmair et al., 2010; Sellick et al., 2009). The most widely used techniques have been nuclear magnetic resonance spectroscopy and mass spectrometry (Ma et al., 2009). Understanding cellular metabolism will aid in rapid development of optimal media formulations that permit extended growth with limited buildup of toxic metabolic waste products (e.g., lactate, ammonia) or elevated osmolarity. In addition to media optimization, metabolic engineering of CHO cells can be performed to reduce detrimental metabolites. Several previous studies reported decreased lactate production using a variety of engineering strategies including siRNA-mediated lactate dehydrogenase-A (LDH-A) knockdown or expression of the fructose transporter (GLUT5) in CHO cells (Kim and Lee, 2007; Wlaschin and Hu, 2007; Zhou et al., 2011). To successfully knockdown expression of LDH, it was necessary for the investigators to clone and sequence the CHO LDH gene, an approach that will no longer be necessary with the complete genome sequence. Interestingly, when LDH activity was reduced, rather than channeling more glucose into the TCA cycle, glucose consumption was reduced to avoid buildup of pyruvate, highlighting the importance of understanding metabolic interactions.

CHO Cell Glycomics

CHO cell glycomics may be broadly categorized into two main aspects of glycomics that impact the transgene expression and function, (1) the importance of post-translation modifications (PTMs) of proteins, and (2) the structure of the CHO cell glycocalyx. The cell's glycocalyx contains abundant cell-specific glycans attached to proteins (i.e., glycoproteins and proteoglycans) and lipids (i.e., glycolipids). These cell-surface glycoconjugates can trigger and enhance binding to growth factors and chemokines, activating cell signaling pathways and resulting in cell–cell communications (Evans and Roger MacKenzie, 1999). For example, the glycosaminoglycan chains of heparan sulfate proteoglycans that are differentially sulfated in a cell-specific manner contain multiple cell-specific binding sites for growth factors, blood coagulation factors, and chemokines.

Proteins produced in CHO cells often undergo post-transcriptional modifications, the most common of which is glycosylation. Glycosylation PTMs dictate the stability and functionality of the resulting glycoconjugates. Glycosylation of lipids also takes place, resulting in glycolipids. In glycoproteins and proteoglycans, glycosylation involves the addition of N- and O-linked glycans on proteins. Glycosylation confers functional diversity to a protein, and defective glycosylation of proteins often leads to inactive or abnormal proteins that may result in defects in cellular processes, including those in development, immune reactions, and cell signaling pathways (Dwek, 1995, 1998; Haltiwanger and Lowe, 2004; Sharon and Lis, 1993; Varki, 1993). CHO cells produce proteins with biologically similar glycosylation patterns to those found in humans, leading to stability, low immunogenicity, and in vivo clearance rates similar to their human counterparts, resulting in their in vivo therapeutic efficiency. However, there are distinct differences between the CHO cell glycome and the human glycome. For example, non-human animals often terminate their *N*-glycans with *N*-glycolylneuraminic acid. In contrast, humans, incapable of synthesizing this form of sialic acid, may be immunologically sensitive to therapeutic proteins generated in CHO cells, which may occasionally contain *N*-glycolylneuraminic acid (Noguchi et al., 1995). Thus, control of this modification and other steps in the CHO cells glycan biosynthetic pathways is critical in the preparation of safe therapeutic glycoproteins.

Unlike DNA, RNA, and proteins that are encoded by template driven biosynthesis, glycans are formed in a non-template driven biosynthetic pathway and also lack any known proof-reading mechanisms (Paulson and Colley, 1989). Glycan biosynthesis involves the synchronized action of glycosyltransferases and other glycan modifying enzymes (i.e., epimerases, sulfotransferases, etc.) that orchestrate the site-specific attachment and modifications of glycans to proteins in the endoplasmic reticulum and Golgi (de Graffenried and Bertozzi, 2004). Most of these glycotransferases and glycan modifying enzymes have multiple isoforms that are specific to the cell. Thus, each type of cell has its own diverse and heterogeneous set of glycans attached to proteins and lipids, producing cell-specific glycoproteins, proteoglycans, and glycolipids.

As the number of glycoproteins that are being produced in CHO cells increase, there has been an increase in research on the biology and chemistry of protein glycosylation. The technology used for the characterization of glycans isolated from CHO cells has also improved, with a focus on sensitivity, precision, and ease and rapidity of analysis. Techniques that are currently used for analyzing glycoproteins, proteoglycans, and glycolipids and their attached glycans include high-performance anionic exchange chromatography with pulsed amperometric detection, micellar electrokinetic capillary chromatography, capillary isoelectric focusing, capillary zone electrophoresis, matrix-assisted laser desorption ionization mass spectrometry, capillary electrophoresis with laser-induced fluorescence, hydrophilic interaction liquid chromatography, weak anionic exchange, reverse phase, electrospray ionization tandem mass spectrometry, matrix-assisted laser adsorption–desorption ionization time of flight, collision-induced dissociation, electron transfer dissociation, and electron capture dissociation. A detailed review of the techniques for analyzing protein glycosylation has recently been published (Andersen et al., 2009). Newly developed techniques for analyzing glycans may help to decipher the structure of the glycans in CHO cells.

The CHO cell glycome is composed of polysaccharides (i.e., hyaluronan, glycogen, etc.), glycolipids, N- and O-linked glycans in glycoproteins, and the acidic polysaccharides of proteoglycans (i.e., heparan sulfate, chondroitin/dermatan sulfate; Fig. 4). However, since glycan biosynthesis is a non-template driven process, simply knowing the desired structure for desired biological function does not provide the information required to produce these glycans. Moreover, although the glycosyltransferases are encoded by the genome, their activity does not have a linear relationship to their transcriptome level in the cell at any given time point (Varki, 1998). There are two approaches for quantifying and understanding the enzymes involved in shaping the glycomes of CHO cells, namely, (1) quantifying enzyme activity and, (2) functional genetics approaches that analyze the function of metabolic enzymes through the gain-of-function and loss-of-function mutants (Esko et al., 1985). The knowledge gained by studying the enzymology of glycomics has opened potential applications in developing new therapeutics by metabolic engineering of the CHO cells, such as metabolic engineering of the biopharmaceutical drug heparin (Baik et al., 2012).

Heparin is highly sulfated version heparan sulfate that is utilized as an anticoagulant drug. It is currently derived from mucosal tissues of slaughtered animals such as pig intestine or cow lungs. The annual sales of pharmaceutical heparin are over $3 billion and it is prepared in 100 metric ton amounts annually (Liu et al., 2009). Animal-sourced heparin is more likely to contain impurities such as viruses and prions than are bioengineered or metabolically engineered drugs. Moreover, as the recent heparin contamination crisis suggests, while impurities/contaminants/adulterants could be present in either animal-sourced or bioengineered or metabolically engineered heparin, contaminants are much more likely to be present in animal-sourced heparin because these are prepared in part, at a slaughterhouse and are subject to food-related regulations while a bioengineered or metabolically engineered heparin would be prepared in a cGMP facility operating under stringent drug-related regulations (DeAngelis, 2012; Guerrini et al., 2009).

Both the potential for viral and prion contamination, as well as the difficulty in monitoring and controlling processing in a non-cGMP facility have led to an interest in the development of pharmaceutical heparins from non-animal sources. Heparin and heparan sulfate share a similar biosynthetic pathway in the Golgi. Biosynthesis initiates

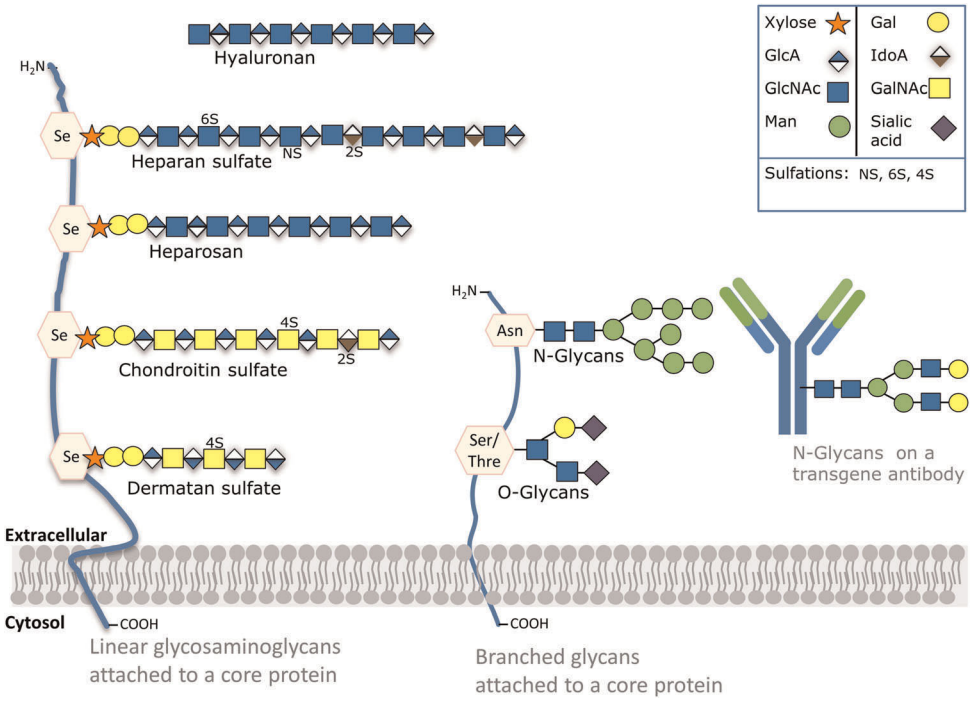

Figure 4. The CHO glycome.

with formation of a tetrasaccharide linker that is attached to serine residues of a core proteins followed by polysaccharide chain elongation and modification (deacetylation, epimerization, and sulfation; Esko and Selleck, 2002). Chain elongation and modification involves multiple isoforms of different enzymes resulting in a heterogeneous population of heparan sulfate, having differential functional protein binding sites. Complete modification including the action of the critical 3-O-sulfotransferase-1 enzyme yields heparin with an antithrombin-binding site, conferring anticoagulant activity. In animals, heparin biosynthesis is also tissue-specific; heparin is found attached to the serglycin core protein and stored in the intracellular vesicles in mast cells. In contrast, heparan sulfate is present in almost all cells and is attached to transmembrane core proteins or secreted into the extracellular matrix. CHO cells produce heparan sulfate on their cell surface. Genomic analysis by Xu et al. (2011) confirms that the CHO-K1 genome has genes for some of the sulfotransferases that are involved in the sulfation of heparan sulfate and heparin. These genomic data are in accordance with the analysis of protein expression of heparan sulfate and heparin sulfotransferases in adherent and suspension CHO cells, suggesting that certain of these enzymes that are critical for the synthesis of anticoagulant heparin are absent in CHO cells (Baik et al., 2012; Esko and Stanley, 2009; Zhang et al., 2006). Specifically, NDST2 and HS3st1 are critical for synthesis of anticoagulant heparin; though stably transfecting suspension-adapted CHO cells with these enzymes produced heparin with low activity (Baik et al., 2012). Thus, the preparation of CHO-cell heparin represents an interesting and important challenge of 'omics approaches towards CHO-cell bioengineering.

Future Directions: Challenges and Opportunities

Decoding the genome and transcriptome of the CHO-K1 cell represents a milestone in the field of CHO bioengineering, with detailed proteomic analyses soon to follow. A future challenge in CHO bioengineering is to address CHO cell line heterogeneity. Currently, genomic data is publically available for only CHO-K1 cells; however, active sequencing projects are expected to make the sequence of a number of cell lines, including CHO DG44 and CHO-S, as well as the sequence of the Chinese hamster available soon (data presented at Cell Culture Engineering XIII, April 2012). Currently, most biopharmaceutical therapeutics are produced in different clones of suspension-adapted CHO cells. Since most of these cell lines have emerged from either CHO-K1 or DG44 parents, they will likely have high degree of similarity in their genome sequences. However, due to culturing conditions, adaptations, epigenetic modifications, and random mutations, there will be a diverse set of transcriptomes, proteomes, and glycomes, even if the genome sequences are similar. It remains to be seen how well cellular engineering strategies will transfer from one cell line to another.

Despite these challenges, having a "sequenced organism" opens up a wealth of opportunities and possibilities for

strategies to improve productivity and cell line robustness, as well as reducing both cell line-selection and process-development times, hastening products into the clinic and to market. In addition, knowledge of the genome, transcriptome, proteome, and glycome aids in developing strategies for metabolic engineering of CHO cells, permitting them to serve as hosts for novel bioproducts, thus creating a new paradigm of metabolic engineering of mammalian cells.

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