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Synthetic biology approaches: the next tools for improved protein production from CHO cells

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Since the approval of the first biotherapeutic protein produced from CHO cells in 1987, researchers have been studying how to improve protein titer and product quality, mainly through cell line development and bioprocess optimization. With recent advances in genetic editing methods (CRISPR/Cas systems) together with large scale systems biology data, further improvements have been made. Here we outline recent progress in protein production from CHO cells through genetic editing and look to the future of improvements through synthetic biology approaches. We describe new work in the expansion of the genetic parts toolkit, including novel promoters, terminators, transcription factors, and genetic circuits, and how these synthetic parts will be used synergistically to continue improvements to protein production.

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Introduction

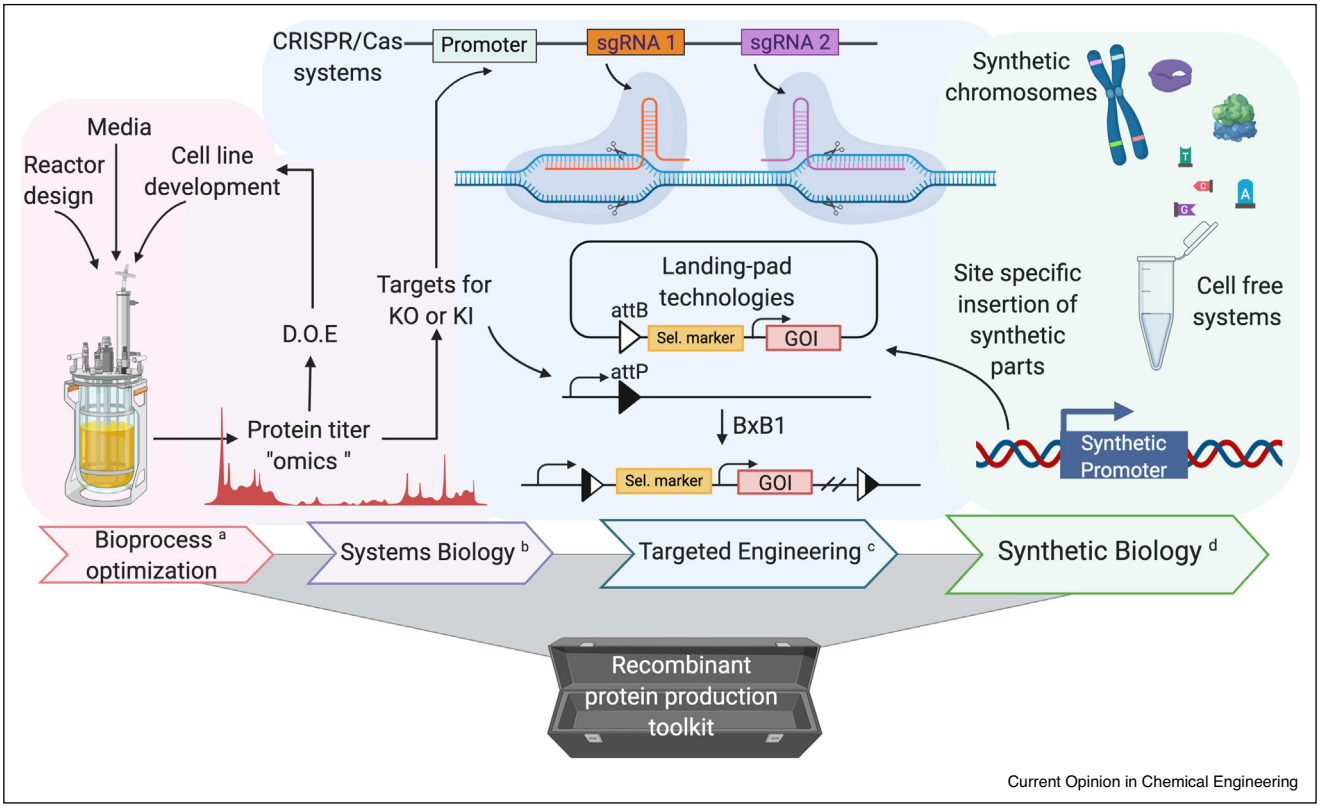
Chinese hamster ovary (CHO) cells are the standard mammalian cell line used for biotherapeutic protein production, commonly monoclonal antibody (mAb) glycoproteins. They are responsible for producing over 70% of recombinant therapeutic proteins [1]. Production via CHO cells has many advantages, most notably the ability to produce human-like post-translational modifications. However, one major drawback is genomic instability, where unfavorable changes to productivity and product quality can occur as population doubling level (PDL) increases [2]. This is commonly attributed to gene loss due to recombination or epigenetic silencing. While bioprocess optimization has improved product quality and productivity, the recent emergence of systems biology has given researchers additional tools to improve their understanding of CHO cell physiology. With increasingly available 'omics' data, predictive models can reveal cellular mechanisms that impact recombinant protein productivity [3]. This understanding, together with simple targeted gene editing methods have greatly improved well-established methods for increased productivity and improved product quality. The next 'tool' to enable progress will come from incorporating synthetic parts into the CHO cell genome using advances in genetic engineering (knockouts and targeted insertions), enabling greater control over the cellular environment, and building new capabilities on the cellular level that are not existent in classically derived cell lines (Figure 1). Here, we will review recent progress in improving recombinant protein production from CHO cells, as well as outline how synthetic biology approaches will play a key role in future progress.

Systems biology analysis informs bioprocess optimization and targeted engineering

Bioprocess optimization has improved protein titer to as high as 10 g L^{-1} for some proteins [4].This has been achieved through improvements to media (chemically defined), feeding strategies, and other culturing methods [5] (Figure 1a). Recently, bioprocesses have not only been optimized to improve titer and specific productivity of recombinant proteins, but also product quality, mainly glycosylation. [6]. Changes to N-linked glycosylation of monoclonal antibodies, especially at the conserved Asn297 residue can significantly impact antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity, anti-inflammatory properties and antibody clearance rate. Given a therapeutic glycoprotein, an ideal glycosylation pattern could be achieved via media additives (e.g. galactose, fucose, mannose, ammonia, amino acids, minerals). However, the use of media additives is not always economically viable in large-scale bioreactors for biopharmaceutical production and some additives can have negative effects on cell growth or protein titer.

The collection of 'omics' data, spurred by the publication of the CHO-K1 genome [7], give insight into further optimization of bioprocesses as well as pinpoint targets for genome editing (Figure 1b). An early success demonstrated metabolomics' insight into uptake of nutrients and accumulation of unwanted by-products to modify culture media [8]. Together transcriptomic and glycomic data recently identified the B4GALT gene family as the major contributor to galactosylation and MAN1/2 and MGAT1/2 genes as major contributors to mannosylation

Figure 1



The expanding toolkit for improved protein production from CHO cells.
Different tools have emerged overtime including bioprocess optimization, systems biology and targeted engineering. The next wave of progress will come from synthetic biology approaches.

in two host cell clones derived from CHO-DG44 and one derived from CHO-K1 [9]. This type of data is key for future studies' ability to identify genetic targets to manipulate for higher productivity or a tailored glycan profile.

Genome engineering efforts

High specific productivity of recombinant proteins has classically been achieved by gene amplification using either a dihydrofolate reductase/methotrexate (DHFR/MTX) or glutathione synthase/methionine sulfoximine (GS/MSX) selection system. These methods are limited by quasi-random integration of the gene of interest (GOI) followed by labor intensive screening. Site-specific cell line engineering methods such as protein-based (zinc finger nucleases, ZFNs [10]; transcription activator-like effector nucleases, TALENs [11]) and ribonucleoprotein-based (CRISPR-Cas) methods offer the ability to insert or knockout genes in desired loci, with CRISPR systems as the most currently used and facile. Thus, CRISPR/Cas and its numerous variations (Table 1) enable high level control to study CHO cell lines for increased stable expression, reduced apoptosis, tailored glycosylation profiles, and other applications.

Permanent gene disruptions and deletions using CRISPR/Cas9 enable the study of gene functions in CHO cell lines (Figure 1c). The Class 2 Type II Cas9 effector protein targets genomic loci via a protein–DNA interaction (GC-rich protospacer adjacent motif, PAM) and a gRNA-DNA complementarity resulting in a blunt dsDNA break. The first example of gene disruption using the Cas9 system in CHO cells disrupted the glycosylation genes FUT8 and COSMC where an indel frequency of 99.7% was achieved with lectin selection for FUT8 but without selection was 47.3% for COSMC [22]. FACS sorting of Cas9 linked to GFP can increase Cas9 indel frequencies significantly [12,13••,14–17,18•,19]. Disruption of multiple genes (multiplexing) has been successfully demonstrated through a triple gene knock-out by co-transfection of multiple guide RNA expression plasmids to disrupt FUT8, and pro-apoptotic genes BAK and BAX, resulting in 59% of the isolated clones having multiple knockouts and 35% having triple knockouts

Table 1

Applications of CRISPR-Cas methods demonstrated in CHO cells

Application	Protein/guide RNA source	Type/size of edit	Genes targeted/insertion sites	Maximum efficiency	Ref.
Knockout	Cas9-sgRNA plasmids	Indels	FUT8 COSMC [FUT8	99.7% (with lectin selection) 47.3% 66.3%	[22]
	3 GFP-2A-Cas9-sgRNA plasmids	Indels	BAK BAX]	66.3% 75.8%	[12]
	Cas9-2A-GFP-sgRNA-tRNA-sgRNA plasmid Cas12a-2A-GFP-sgRNA array plasmid	Gene deletion	[FUT8]	d.n.r.	[13••]
	2 Cas9 + sgRNA plasmids	Gene deletion	[Dnmt3a]	46.2%	[21]
	2 Cas9-2A-GFP-sgRNA plasmids	Gene deletion	[B4GALT1] [B4GALT2] [B4GALT3] [B4GALT4]	d.n.r.	[14]
	4 Cas9-2A-GFP-sgRNA plasmids	Indels	[B4GALT1 B4GALT2 B4GALT3 B4GALT4]	d.n.r.	[15]
	Cas9-2A-GFP-sgRNA plasmids	Gene deletion	[Repressor element] (500 bp + SV40 polyA signal)	20–25%	[17]
	10 GFP-2A-Cas9-sgRNA plasmids	Indels	[MGAT4A MGAT4B MGAT5 ST3GAL3 ST3GAL4 ST3GAL6 ST3GNT2 FUT8 SPPL3 GLUL]	d.n.r.	[16]
	Cas9-2A-GFP-sgRNA-tRNA-sgRNA plasmids	Gene deletion	[MicroRNA-744]	d.n.r.	[18•]
	Cas9-2A-GFP plasmid with 2 U6-gRNA	Gene deletion	[FUT8] [B4GALT1]	d.n.r.	[57••]
	GFP-2A-Cas9-sgRNA plasmids	Indels	AASS AFMID DDC GAD1 GAD2 HPD LOC100759874 PRODH PRODH2	d.n.r.	[19]
	crRNA + ATTO-550 labeled tracrRNA + gRNAs + Cas9 protein	Indels	GS	Up to 75%	[59]
	GFP-2A-Cas9 + 2 sgRNA plasmids each step	Gene deletion	[NEU1] [NEU2] [NEU3] [BAK] [BAX]	d.n.r.	[20]
Insertion	Cas9 + sgRNA plasmids + 750 bp-donor DNA-750 bp plasmid	3.7 kb	COSMC Mgat1 LdhA	27.8% 0.6% 7.4%	[38]
	Cas9 + sgRNA plasmids + 750 bp-donor DNA-750 bp linear DNA	5.6kb	C12orf35 HPRT GRIK1	48.4–66% for mCherry 25.4–41.7% for anti-PD1 mAb	[60]
	Cas9 + sgRNA plasmid + circular LP donor plasmid	~5kb	Novel genomic sites in supported long-term transgene expression	Integrate landing pad 5% for sLP1-1 50% for sLP20 Loci dependent	[41•]

Table 1 (*Continued*)

Application	Protein/guide RNA source	Type/size of edit	Genes targeted/insertion sites	Maximum efficiency	Ref.
Interference	dCas9-KRAB-sgRNA plasmid	N/A	DHFR	Enhanced EGFP expression ~3.8 fold Enhanced G-CSF ~2.3 fold	[28]
	dCas9-KRAB-Mecp2 plasmids	N/A	BAK	0.25 fold	[29]
			BAX	0.4 fold	
			CASP3	0.5 fold Decreased mRNA expression	
Activation	VPR-dCas9 + sgRNA plasmid	N/A	MGAT3 ST6GAL1	Up to 100 fold Up to 1500 fold	[30]

Bracketed gene names signify multiplexed editing. A single bracketed gene name signifies the deletion of that gene by multiplexed editing.

[12]. Other examples of multiplexing used monocistronic expression of multiple gRNAs on a single plasmid or the use of tRNA or IRES strategies for polycistronic expression [13••] (Table 1).

Targeted disruptions can help overcome bioprocess limitations; recombinant human erythropoietin (rhEPO) is normally unable to be produced in fed-batch culture due to low sialylation in late phase. Sialylation was improved threefold by deleting NEU1, NEU2, and NEU3, and cell viability was increased from 47% to 82% by deleting BAK and BAX, allowing production of rhEPO [20]. Similarly, transgenic expression and long-term stability have been enhanced by knock-out of the methyltransferase DNMT3A, resulting in reduced epigenetic silencing though histone modification and DNA methylation [21]. Knockouts are not relegated to coding regions only; the microRNA-744 was deleted via Cas9, reducing miRNA-induced silencing of genes critical to antibody production and thus improved antibody titer up to twofold [18•] (Table 1). To minimize off-target activity of CRISPR-based techniques, multiple bioinformatic tools have been developed to aid in design of gRNAs (notably CRISPy, CCTop, and Benchling all have configurations specific to CHO cells) [22,23].

In addition to making gene disruptions and deletions, CRISPR/Cas systems, alongside native DNA repair machinery, can be used for precise and efficient integration of transgenes into the CHO genome by homology directed repair (HDR). However, HDR occurs at a low frequency (less than 5% [24]) compared to non-homologous end joining (NHEJ), leading to a time-intensive and labor-intensive screening process. HDR frequency can be improved up to 75% in CHO cells by counteracting HDR-limiting steps (knockdown of MRE11, PARI and overexpression of RAD51) [24]. In K562 cells HDR frequency reached 86% by inhibiting NHEJ through expression of a mutant 53BP1 fused to Cas9, which acts as a competitor to the native NHEJ-promoting 53BP1 [25]. Similarly, expressing an engineered variant of the NHEJ-inhibiting protein RAD18 increased HDR threefold in HeLa cells [26] (Table 1).

The CRISPR Cas12a (formerly Cpf1), a Class 2 Type V, differs from Cas9 in 1) a 5′ T-rich PAM, 2) a staggered dsDNA break, 3) targeting mediated via a single and short (~43 nt) crRNA, and, importantly, 4) the ability to process pre-crRNA into mature crRNAs without an additionally expressed protein [27]. This all-in-one crRNA maturation ability obviates the need for multiple plasmids or an IRES site to multiplex edits, allowing the deletion of entire genes without using a tRNA-based polycistronic expression system and maintaining high knockout efficiency [13••].

CRISPR/Cas systems can also be used to alter gene expression, without making a dsDNA break. CRISPR interference (CRISPRi) is used to inhibit gene expression in a targeted manner by co-expression of a modified Cas effector protein and a compatible guide RNA. The method, using a nuclease deficient Cas9 (dCas9) fused to a transcriptional repressor (typically KRAB) has been used to repress transcription of the *dhfr* selection marker to further amplify the GOI in the presence of MTX selection pressure, enhancing the productivity of granulocyte colony stimulating factor (G-CSF) ~2.3-fold [28]. To reduce apoptosis, endogenous apoptotic genes BAX, BAK and CASP3 were downregulated by CRISPRi, improving CHO cell viability by 50% [29] (Table 1).

Similarly, CRISPR activation (CRISPRa) can activate expression of desired genes in CHO cell lines to alter the transcriptome without transgene insertion. CRISPRa uses a dCas9 fused with a transcriptional activator (VPR) to activate transcription including endogenous silenced genes [30], which can make up nearly half of all genes on the CHO genome [31]. The transcription of two silenced glycosyltransferases was increased up to 100-fold for MGAT3 and 1500-fold for ST6GAL1 using CRISPRa,

resulting in desired glycan structures (bisecting *N*-acetylglucosamine and α2,6 sialic acid) not commonly produced in CHO cells (Table 1) [30].

In contrast to the defined genetic modifications of CRISPR, transposon-mediated technologies are used to generate heterogeneous cell populations with stable recombinant protein production through quasi-random integration. Type *2* transposons are selfish, 'cut and paste', mobile genetic elements that move via transposases. Biotechnologically, these naturally occurring elements have been repurposed to insert a GOI into the CHO cell genome (e.g. PiggyBac, Tol2, Sleeping Beauty, Leap-In) [32,33]. GOIs flanked by transposase recognized inverted terminal repeats are quasi-randomly inserted through transient expression of a cognate transposase [34]. These heterogeneous populations can stably produce antibodies at productivities and titers higher than conventional plasmid transfection but typically less than clonally derived populations [35]. However, the transposon method is faster (less than 1 month compared to ~6 months for clonal cell line generation) [33,36].

Recombinase Mediated Cassette Exchange (RMCE), uses site-specific recombinases such as Cre/*lox*, Flp/*FRT*, φC31/*att* P/B systems and BxB1 systems [37–39], to insert transgenes at defined loci (Figure 1c). First a 'landing pad' (LP) with a recombination site is inserted into a desirable location by HDR. Once this cell line has been developed, a GOI with a selectable marker is inserted into the landing-pad by a matching recombinase. This technique requires a well-defined insertion site amenable to high expression [40]; which have been identified via lentiviral integration screens [41•,42]. This RMCE method is multiplexable and robust, as genes have been incorporated into three different loci with one transfection and multiple copies of a gene can be integrated into the CHO genome [41•].

The next step: building synthetic biology parts for maximal control

The next generation of improvements towards a CHO cell system with sustained high productivity and tunable product quality parameters will come from the incorporation of synthetic parts with the goal of designing, not screening for a desired phenotype (Figure 1d). Utilization of synthetic biology by promoter, terminator and transcription factor engineering has been studied to improve protein production in mammalian systems, and synthetic genetic circuits offer a method of dynamic and tunable control of gene expression. Promoter engineering has been the most frequently reported, as increasing and maintaining transcriptional activity is a straightforward way to increase protein expression. The available library of promoters for heterologous gene expression in mammalian cells is small, and the primary selection criterion appears to be often based on precedent rather than properties. Mammalian expression promoters frequently have varying levels of protein expression in different cell types [43] and are prone to silencing [44].

The cytomegalovirus (CMV) promoter, the most frequently used in CHO cell studies, contains ~40 transcription factor regulatory elements (TRFEs) identifiable through bioinformatic analysis, yet the sequence-function relationship is not well understood [45]. The CRE and NF-κB TRFEs have been postulated to be the primary drivers of expression based on work that first systematically blocked TF binding to TRFEs and observed the resulting decrease in reporter gene expression. Destruction of all the CRE or all the NF-κB sites on the genetic level consistently resulted in ~50% reduction in expression while destroying all CRE and NF-κB sites simultaneously reduced expression by ~80% [45].

With a better understanding of the mechanism of CMV-driven gene expression in CHO cells, medium-sized libraries of rationally designed promoters have been synthesized to improve protein production. While rational library synthesis limits library size to the order of 10's to 100's, this mechanistic knowledge enabled identification of improved promoters with a 25% smaller sequence footprint than the wild type CMV and increased recombinant protein production by up to 2.5-fold [46,47••]. Using transcriptomic data, promoters can now be designed to sustain increased protein production for particular cell lines [46] or under specified culturing conditions [47••]; TFREs identified upstream of genes upregulated during mild hypothermic conditions (32°C) were used to create improved chimeric promoters comprising multiple TFREs upstream of the minimum core CMV [47••].

Non-rational design methods have been used to create larger libraries (thousands to tens of thousands) of promoters to identify novel regulatory elements. A CHO-K1 genomic library of 1−4 kb fragments inserted upstream of the CMV promoter and screened via fluorescence activated cell sorting (FACS) to isolate high expressing cells led to the discovery of a novel regulatory element (E77) that improved stable gene expression [48]. This 2894 bp E77 fragment had not previously been used as a regulatory element, however it does contain known TFREs including AP-1, E-box, and GATA-1. The elucidation of novel regulatory elements increases the number of tools for the future design of engineered or synthetic promoters.

Synthetic promoters have also been designed in an attempt to reduce gene silencing due to methylation at CpG sites. Introduction of single point mutations removing commonly methylated cytosines in the CMV promoter showed the removal of a single cytosine located 179 bp upstream of the transcriptional start site stabilized

recombinant IgG-IL2 production in stably transfected CHO cells [49]. However, removal of CpG sites is not a simple panacea for promoter engineering. Another study compared a CpG-free and CpG-rich promoter (a combination of mouse CMV, human elongation factor 1 alpha, and a synthetic intron at the 5′ untranslated region) and found that the CpG-free promoter did not improve long-term expression compared to the CpG rich promoter, and gene silencing was perhaps linked to histone deacetylation [50]. Synthetic chimera promoters have shown promise in increasing stable expression compared to the wild type CMV by combining the CMV promoter and CpG island elements. CpG islands from housekeeping genes (e.g. hamster adenine phosphoribosyltransferase gene [51], Chinese hamster B-actin gene [44]) can avoid silencing by preventing DNA methylation, as these genes are constitutively expressed in a cell.

While an emphasis has been put on creating synthetic promoters for improved protein production, terminators (3′ UTR region) are also a transgene element that should be designed and synthesized. Terminators typically have a smaller sequence footprint than promoters but can also tune gene expression, albeit to a lesser extent. Synthetic terminators that used the generic mammalian structure as a scaffold but modulated the upstream element, spacers, polyA, and downstream elements with sequences derived from the SV40, GAPDH, ACTB, EF1a, and consensus elements resulted in increasing the dynamic range up to 11-fold of reporter protein output [52•]. These terminators were comparable or outperformed the SV40 viral terminator. Interestingly, some codependency exists between the terminator, promoter and GOI, further highlighting the future of designing a cooperative set of synthetic genetic elements to produce specific proteins.

Transcription factor (TF) engineering presents an interesting area of research to increase and control protein expression in mammalian systems. A randomized library (~2000 in size) of artificial zinc finger protein transcription factors (ZFP-TF) was successfully screened for increased production of a mAb (max improvement of 10-fold) [53]. However, recent TF studies in CHO cells have focused on the overexpression of transcription factors for increased productivity. By overexpressing Xbp1s and cYY1, specific productivity was increased by up to fourfold [54,55]. and between 4 and 13-fold (depending on the cell line), respectively [56].

Synthetic genetic circuits using TF-TRFE pairs have been introduced into CHO cells to control the *N*-glycosylation pattern of a mAb [57••]. Here, genes associated with fucosylation (FUT8) and galactosylation (B4GALT1) were placed under control of small molecule-inducible promoters and inserted into the genome via landing pad technology. Through this method, the glycosylation could be tightly controlled (fucosylation and galactosylation ranging 0–97% and 0–87%). Synthetic genetic circuits have proven to achieve a much higher level of control versus media supplementation which was previously the gold standard in modulating glycosylation profiles. Future progress will include the creation of synthetic genetic circuits comprising engineered TF-TFRE pairs for increased specificity and tunability of protein production [46].

Future directions

The next wave of progress in the production of recombinant proteins by CHO cells will come from a greater control of cellular physiology. Tools enabling hyper-precise genetic editing (e.g. 'search and replace' [58]) and epigenetic edits, dynamic control of gene expression through 'parts' development [49,52•,57••], coupled with systems biology insight [3] will allow rapid and robust control over physiological processes. Developments in expansion of the genetic code through incorporation of non-canonical amino acids, use of artificial chromosomes to deliver large amounts of DNA, and cell free expression systems will continue to expand the capabilities of the CHO platform.

Conflict of interest statement

Nothing declared.

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