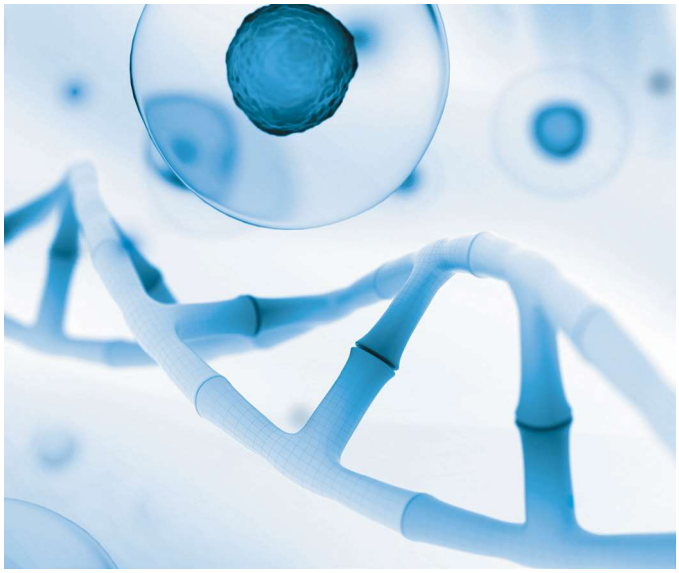
Re-programming CHO by Gene Editing, the New Frontier in Bioprocessing

For many years, Chinese hamster ovary (CHO) cells have been the cornerstone of the success of biopharmaceutical proteins. Their flexibility and adaptability to bioreactor culture conditions, compared to other mammalian systems, make them the system of choice for one of the most successful protein therapeutic classes, monoclonal antibodies. However, very little has changed since the approval of the first commercial product ever manufactured in mammalian cells in 1987. The advent of new gene editing technologies is revolutionising how the industry relates to CHO cell hosts. Gene editing, particularly CRISPR-related platforms and functional genomic screening are opening the door to new tailor-made cell hosts able to deliver desired product characteristics and show optimal culture performance in bioreactors. Long gone are the days where product and processes were subservient to the whims and behaviour of expressing cell lines.



Why the Cells from a Humble Rodent Became the Industry Standard

Since the approval of the first recombinant biotherapeutic, insulin, in 1982, the pharmaceutical industry has experienced an explosion in the development and commercialisation of protein therapeutics. The development and improvement of industrial manufacturing platforms has been a key enabler for this. Today, monoclonal antibodies and architectures derived from them constitute more than half of the protein therapeutics on the market and are, by far, the largest group of biopharmaceuticals currently in clinical development. At the heart of this success are the CHO cells that, since their establishment as a cell line, have become 'the' de-facto manufacturing platform for the large majority of protein-based therapeutics on the market and in development[1].

Chinese hamsters had been used as a laboratory model since the 1910s, but it was in 1957 when Theodore Puck managed to establish stable cultures of what would later turn out to be the "mother of all CHO cells", the strain CHO-K1 from which all existing bioprocessing CHO cell lines in use today derive[2]. In those early days, CHO cells already showed several interesting properties that made them attractive as a cellular model:

- They remain in uninterrupted culture for many generations without immortalisation or transformation.
- They have short doubling time (16–22 h).
- They are genetically simpler than other mammalian cells (Table 1).

Cell	Organism	Number Chromosomes	DNA size (Mb)	Doubling Time
HEK293	Human (kidney)	64	~9000?	34h
CHO-K1	Chinese Hamster (ovary)	21	2450	16–22h
Saccharomyces cerevisiae	Yeast	16	12.2	1.2–2h
Pichia pastoris	Yeast	4	9.4	2–5h
Bacillus subtilis	Bacteria	1	4.2	27 min
Escherichia coli	Bacteria	1	4	20 min

Table 1. Common cells used in the production of biotherapeutics

These features have led to CHO cells becoming an ideal model for research and biotechnology applications. On one hand, they present significant advantages for bioprocessing by allowing bioreactor cycle times to be substantially shorter. Also, their genetic simplicity makes them a favourable target for gene editing today.

Why Has CHO Not Been Displaced by Other Expression Platforms?

Many other platforms, particularly microbial organisms, are considerably easier to maintain in culture and are often able to produce large amounts of recombinant proteins but have not managed to displace the predominance of CHO. This is due to a combination of several factors:

- **Post-translational modifications (PTMs).** Microbial systems (even yeasts) are not very effective at replicating desired PTMs in proteins, particularly complex glycosylation patterns. By contrast, CHO in most cases (chiefly monoclonal antibodies) manages to do a reasonably good job.
- **Manufacturing costs are still a minor fraction of the price of a drug.** Typical manufacturing costs of monoclonal antibodies expressed in CHO cells can be as little as 1–5% of the final drug price[3].
- **Microbial or plant-based platforms are not as 'cheap' or 'fast' as one might expect.** Although microbial and plant-based systems can occasionally bring advantages in terms of bioprocessing costs and timelines, they have not yet managed to dramatically outperform CHO in terms of cost or overall development timelines.
- **Regulatory** (traceability and safety-related) and **infrastructure hurdles** for developing new therapeutic products can dissuade from switching platforms. Also, once a company has invested in developing the manufacturing structure for a given product class, they will not move away if it does not bring substantial benefits (reducing time, risks, and costs, or improving process performance).
- **CHO is still the industry 'standard'.** CHO is the leading platform for producing biotherapeutics in large quantities and at acceptable costs. One can transfer a CHO-based

process anywhere in the world and almost all existing CDMOs have experience and infrastructure to use CHO cells successfully.

Are New Gene Editing Platforms Marking the Onset of a New Age for CHO?

Very little changed with CHO during its first 50 or so years as a cell line. Initial efforts concentrated in moving away from adherent cultures requiring complex roller-bottles or multi-stack infrastructure that were poorly scalable. Probably, the most important event in CHO's history as a protein expression host was its adaptation to suspension culture. This transition allowed significant improvements in cell culture process control and consequently substantial increases in productivity and product quality. Further to this, the discovery of metabolic inhibitors; methotrexate (MTX) and methionine sulfoximine (MSX) for two particular enzymes; di-hydro folate reductase (DHFR) and glutamine synthetase (GS) respectively, provided selection markers to facilitate the selection of cells expressing a given gene of interest. Aside from this, the only substantial manipulation worthy of mention was the generation of the DHFR (double negative) mutant in the 1980s, giving rise to the CHO DG44 strain. Since then, nothing much happened to the CHO hosts cells used in bioproduction.

The arrival of the new millennium with the publication of the first CHO genome and emerging genetic technologies, reignited more systematic gene modification efforts[4,5]. Early projects included the generation of GS knockouts in CHO-K1 derivatives to improve selection pressure, whilst eliminating the need for MSX (potentially neurotoxic), and the generation of mutants with reduced fucosylation to enhance the effector-function activity of monoclonal antibodies *in vivo*[6,7].

New gene editing platforms have been appearing ever since[4,8]:

- Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), were the first genomic-editing technologies to be used in CHO. These platforms were successfully used to generate selection marker knockouts as well as other variants. However, these platforms present some limitations. They require considerable expertise in the design of the DNA-recognition domains and are not as 'high-throughput' and flexible when compared to more modern platforms, like CRISPR-based methods. This makes them unsuitable for high-throughput screening approaches and restricts their use to pre-validated targets. Still, they remain powerful, and efficient approaches for gene editing remain actively in use in the industry.
- Systems based in recombinant adeno-associated virus (rAAV) were next used for gene editing in CHO cells. Recombinant AAVs do not integrate in the host genome and are unable to generate replication-competent viruses in CHO. The platform constitutes a reliable method for gene editing, and the design of the edits is simple and does not require sophisticated knowledge or technologies. Also, the intellectual property situation around the use of rAAV is straightforward. However, the technique has a relatively low efficiency, and is slow in comparison to CRISPR-based methods.
- CRISPR-Cas9 is one of the latest platforms to arrive and from which many different variants have emerged. The CRISPR-Cas9 system does not require sophisticated design constraints, making it easier to implement, but also faster in performing edits. The modularity of the system enables multiplexing of gene edits. Also, high-throughput screening with large guide RNA libraries can be used to interrogate the impact of different genes in cellular function. CRISPR is extremely efficient compared to other gene editing platforms and this, combined with its speed, makes it the method of choice for many researchers. However, the IP behind CRISPR technologies is complicated, which could discourage the industry from fully exploring its commercial applications in the short term.

Gene-edited CHO Cell Lines for Biotherapeutic Production

The generally risk-averse bioproduction industry has been relatively slow in embracing new technologies to modify the genome of expression cell hosts. This may have been compounded by the complexity of technology access (including IP landscape), restrictive commercial licensing terms, perceived technical difficulty or even doubts about its benefits altogether. However, the increasing complexity of biotherapeutic molecular architectures (i.e. multi-specific scaffolds and complex fusion molecules), combined with an increased urgency for taking products to the clinic and streamlining development are forcing the industry to seek alternative technologies and processes.

In recent years gene editing technologies have been employed to solve various problems:

- **Incorporating desirable PTMs in the product.** CHO cells are known for not being able to produce the PTMs required for some therapeutic molecules. This has been addressed recently by several groups by incorporating enzymatic activities that were missing from CHO; for example, sialidases that could extend the product half-life.

- **Pharmacology and efficacy of product though glyco-engineering.** The glycoform attached to the Fc fragment of antibodies is known to play an important role in the pharmacology and effector-function activity of monoclonal antibodies and Fc fusion proteins. Equally, as mentioned above, the presence of specific human-like sialylation patterns can increase the half-life of protein therapeutics[9].

- **Safety of product.** The safety of biopharmaceuticals can be affected by the presence of potential pathogens in the preparation (virus) or the immunogenicity of the product. Strategies to address these risks include reducing viral permissivity of CHO cells and/or eliminating host cell proteins (HCPs) that could increase the risk of immune responses in patients[10,11].

- **Simpler, cheaper processes.** Downstream processing (purification) of protein biotherapeutics is a key bottleneck in bioproduction and probably the single most expensive unit operation, largely due to the costs of resins and time required to perform. Reduction or, whenever possible, elimination of difficult-to-remove contaminants could potentially have a significant impact in the economics of bioproduction but also in the quality and safety of the therapeutic product itself[11,12].

- **Process robustness and productivity.** CHO cultures require large amounts of energy to grow and express products. In bioprocessing, CHO cells are coached to produce as much protein as possible whilst reducing the expenditure of the cells in 'unnecessary' activities (like synthesising DNA, or non-desired host cell proteins, including proteases). Several lines of research are approaching this in different ways: from manipulating the metabolic circuitry in CHO cells, to eliminating HCPs, to promoting anti-apoptotic behaviour[12,13].

- **Consistency and speed in cell line generation.** The introduction of landing pads in CHO to direct the integration of the desired transgene into a specific location in the genome has been proposed as advantageous in increasing consistency of expression across different cell lines and also potentially accelerating the generation of expressing cell lines[14]. Such landing pads can also be used in combination with mammalian display technologies[15].

- **Streamline antibody discovery and development.** Mammalian display technologies allow the incorporation of additional selection criteria beyond simple ligand binding affinity. This 'cell-based developability' at such an early stage of development facilitates the identification and design of good binders that also are able to fold, assemble and express more favourably, reducing manufacturing and product stability risks that might derail product development later on, often at a very high cost[16].

The Future of Gene Editing in Bioproduction

The current revolution in gene editing is shifting the bioprocessing landscape and opening possibilities to manipulation[4]. Early gene editing platforms (ZFNs, TALENs, rAAVs, etc.) require a good understanding of the desired edit to be performed and the expected phenotypic result, which can often be a question of trial-and-error. In contrast, the simplicity of design afforded by CRISPR-derived platforms has enabled the generation of large screening libraries that make the complexity of whole mammalian genomes a manageable problem. This is where CRISPR screening, combined with comprehensive computational models that integrate different cellular pathways, can become a powerful tool in the identification of novel targets suitable for gene editing.

Genome-wide functional genomics CRISPR screening (Figure 1) is becoming a powerful tool in the identification of genome-phenotype functional relationships, primarily due to its simple design and 'programmability' compared to other platforms[17]. The availability of multiple CRISPR variants allows very sophisticated analysis combining knockout generation with gene modulation via CRISPRi (interference) or CRISPRa (activation) approaches, which can be particularly useful for genes that are either essential or can play different roles depending on relative abundance.

Multiple gene edits can be obtained simultaneously in a single cell, allowing targeting of complex interactions to achieve significant phenotypic effects as a result. This has recently been demonstrated by the simultaneous knocking out of a variety of HCP genes that synergistically contributed to a reduction in general HCP load and favouring the gene of interest productivity in CHO[12].

As mentioned above, CRISPR-based mammalian display has been proposed as an alternative to landing-pad recombinase-mediated display systems. This approach can potentially increase the size of libraries available for screening but also help merge the interface between antibody discovery and engineering with bioprocess development and manufacturing[16]. On the other hand, base editing and prime editing technologies are opening the door to simpler gene editing to fine-tune the activity of specific effectors relevant to a given product or process. Finally, the emergence of new CRISPR systems is opening alternative commercialisation routes to gene editing that might be currently blocked due to complex intellectual property landscapes. In addition, new smaller CRISPR systems might create opportunities to integrate nucleases in more sophisticated multi-functional architectures[18].

What is Next for CHO?

The industry is just peering out into a brand-new landscape where drug developers will have access to novel, even tailor-

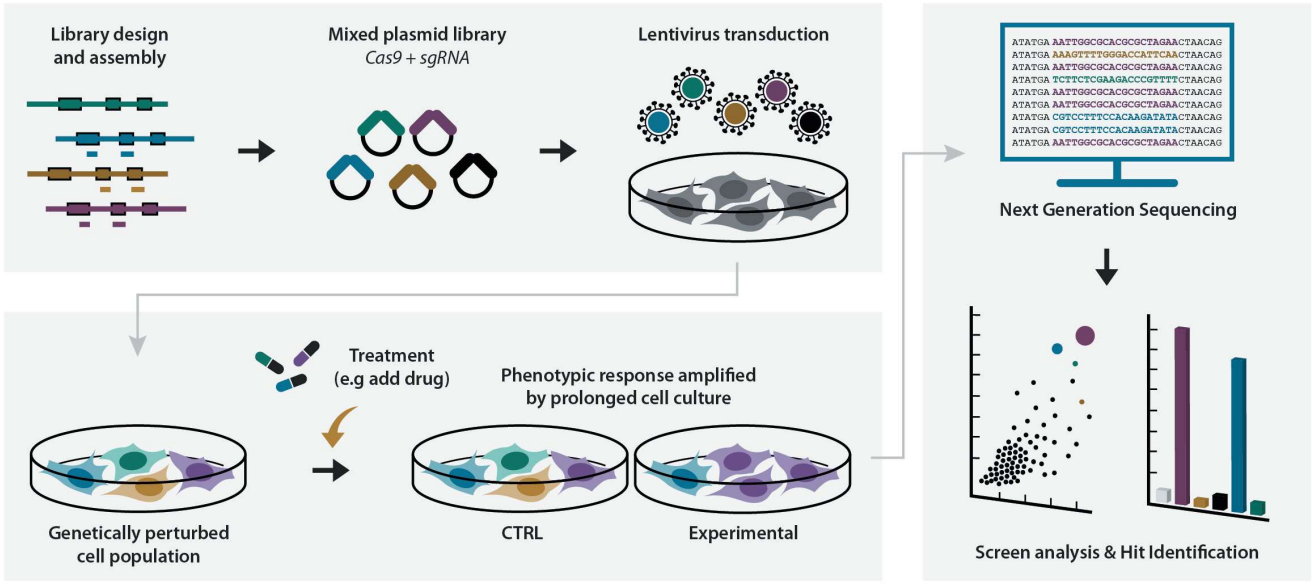


Figure 1: An example of a genome-wide functional genomic CRISPR screening workflow

made, expression platforms to accommodate their needs and enable them to produce protein biotherapeutics in entirely new ways. One cannot help but be surprised at how the industry has historically adapted its processes to the whims and biological designs of CHO cells, with substantial investments in infrastructure and technology over the years. For example, the market for media, cell culture and bioreactor technology is estimated to be in excess of $1 billion per year, whereas the market for downstream processing, including resins, filters and purification technologies is about ten times as large. However, the investment made by the industry in 'taming' CHO cells by re-programming their genomes, pales in comparison. Now the door is open to adapting the host design to 'ideal' or 'optimal' bioprocessing conditions and development needs. These may vary broadly depending on requirements introduced by disease condition, or specific commercialisation strategies, which could impact the required production scale, development timeline, on-demand manufacturing, or non-standard chemical composition. These new paradigms will shape future manufacturing practices and will have at their core more diverse, robust, and flexible cell hosts, which are still likely to include those derived from CHO cells.

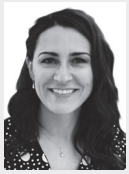
REFERENCES

1. Walsh, G. Biopharmaceutical benchmarks 2018. Nat. Biotechnol. 36, 1136–1145 (2018).
2. Vital tools, a brief history of CHO cells. LSF Magazine. 38–47 (2015) http://biomanufacturing.org/uploads/files/547998065159985597-cho-history.pdf
3. Kelley, B. Industrialization of mAb production technology: The bioprocessing industry at a crossroads. MABS 1. 443–452 (2009).
4. Gaj, T. et al. ZFN, TALEN and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 31, 397–405 (2013).
5. Lewis, N. et al. Genomic landscapes of Chinese hamster ovary cell lines as revealed by the Cricetulus griseus draft genome. Nat. Biotechnol. 31, 759–765 (2013).
6. Fan, L. et al. Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. Biotechnol. Bioeng. 109, 007–1015 (2012).
7. Yamane-Ohnuki, N. et al. Establishment of FUT8 knockout Chinese hamster ovary cells. Biotechnol. Bioeng. 87, 614–22 (2004).
8. Gaj, T. et al. Genome Engineering Using Adeno-associated Virus: Basic and Clinical Research Applications. Mol. Ther. 24, 458–464 (2016).
9. Wang, Q. & Betenbaugh, M.J. Metabolic engineering of CHO cells to prepare glycoproteins. Emerg. Top Life Sci. 2, 433–442 (2018).
10. Chiang, A.W.T. et al. Combating viral contaminants in CHO cells by engineering innate immunity. Sci. Rep. 9, 8827 (2019).
11. Gutierrez, A.H. et al. Of [hamsters] and men A new perspective on host cell proteins. Hum. Vaccin. Immunother. 8, 1172–1174 (2012).
12. Kol, S. et al. Multiplex secretome engineering enhances recombinant protein production and purity. Nature Comms. 11, 1908 (2020).
13. Baek, E. et al. Anti-Apoptosis Engineering for Improved Protein Production from CHO Cells. In: Meleady P. (eds) Heterologous Protein Production in CHO Cells. Methods in Molecular Biology (1603). Humana Press, New York, NY. (2017)
14. Hamakera, N.K. & Lee, K.H. Site-specific Integration Ushers in a New Era of Precise CHO Cell Line Engineering. Curr. Opin. Chem. Eng. 22, 152–160 (2018).
15. Zhou, C. et al. Development of a novel mammalian cell surface antibody display platform. MABS 2, 508–518 (2010).
16. Parthiban, K. et al. A comprehensive search of functional sequence space using large mammalian display libraries created by gene editing. MABS 11, 884–898 (2019).
17. Shalem, O. et al. High-throughput functional genomics using CRISPR–Cas9. Nature Rev. Genet. 16, 299–311 (2015).
18. Pausch, P. et al. CRISPR-CasΦ from huge phages is a hypercompact genome editor. Science. 369, 333–337 (2020).

Dr. Jesús Zurdo

Jesús is Global Head Bioproduction at Horizon Discovery. He co-founded Zyentia and Zapaloid, developing disease-modifying therapies against neurodegenerative disorders. He subsequently worked at Lonza and Rentschler Biopharma where he introduced new development and biomanufacturing technologies for protein biologics, cell and gene therapy and microbiome-modulating therapeutics. Jesús held research positions at the Universities of Oxford, Cambridge and at CBM-CSIC in Spain and has a PhD in molecular biology from the Autonomous University of Madrid.

Email: jesus.zurdo@horizondiscovery.com



Andrea Gough

Andrea is Global Product Manager of Bioproduction at Horizon Discovery. She has 10 years' experience in the biologic drug development space; focused in the field of cell line development (CLD). This experience developed working as an Application Specialist at Molecular Devices providing CLD advice to users of the ClonePix technology and onwards as a Product Manager at Solentim Ltd, supporting that product range. Andrea's position at Horizon is focused on supporting the future path of its Bioproduction Products and services.

Email: andrea.gough@horizondiscovery.com

Dr. Delphine Cougot

Delphine is Manager Bioproduction R&D at Horizon Discovery and has 15 years of R&D experience in academic and industry settings. Delphine holds a PhD in oncogenesis from the University of Paris. Following a postdoctoral position at the Gurdon Institute/University of Cambridge, she joined Horizon to lead the standardisation of immunohistochemistry assays across testing laboratories. Now in the Bioproduction group, she leads several R&D programmes which apply gene editing technologies to the improvement of bioproduction products and services.

Email: delphine.cougot@horizondiscovery.com