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Research review paper

Improving recombinant protein production in CHO cells using the CRISPR-Cas system

Ali Kerem Kalkan [a,b], Fahreddin Palaz [c], Semeniuk Sofija [d,e], Nada Elmousa [f], Yuri Ledezma [f,h], Elise Cachat [d,e,g], Leonardo Rios-Solis [d,f,i,*]

[a] Department of Bioengineering and Imperial College Centre for Synthetic Biology, Imperial College London, London, UK
[b] Environmental Engineering Department, Gebze Technical University, Turkey
[c] Faculty of Medicine, Hacettepe University, Ankara 06100, Turkey
[d] Centre for Engineering Biology, University of Edinburgh, Edinburgh EH9 3BF, UK
[e] Institute of Quantitative Biology, Biochemistry and Biotechnology, School of Biological Sciences University of Edinburgh, Edinburgh EH9 3BF, UK
[f] Institute for Bioengineering, School of Engineering, University of Edinburgh, Edinburgh EH9 3DW, UK
[g] UK Centre for Mammalian Synthetic Biology, University of Edinburgh, Edinburgh EH8 9YL, UK
[h] Biology Department, Faculty of Pure and Natural Sciences, Universidad Mayor de San Andrés, Bolivia
[i] School of Natural and Environmental Sciences, Molecular Biology and Biotechnology Division, Newcastle University, Newcastle Upon Tyne, NE1 7RU, UK

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ABSTRACT

Chinese hamster ovary (CHO) cells are among the most widely used mammalian cell lines in the biopharmaceutical industry. Therefore, it is not surprising that significant efforts have been made around the engineering of CHO cells using genetic engineering methods such as the CRISPR-Cas system. In this review, we summarize key recent studies that have used different CRISPR-Cas systems such as Cas9, Cas13 or dCas9 fused with effector domains to improve recombinant protein (r-protein) production in CHO cells. Here, every relevant stage of production was considered, underscoring the advantages and limitations of these systems, as well as discussing their bottlenecks and probable solutions. A special emphasis was given on how these systems could disrupt and/or regulate genes related to glycan composition, which has relevant effects over r-protein properties and in vivo activity. Furthermore, the related promising future applications of CRISPR to achieve a tunable, reversible, or highly stable editing of CHO cells are discussed.

Overall, the studies covered in this review show that despite the complexity of mammalian cells, the synthetic biology community has developed many mature strategies to improve r-protein production using CHO cells. In this regard, CRISPR-Cas technology clearly provides efficient and flexible genetic manipulation and allows for the generation of more productive CHO cell lines, leading to more cost-efficient production of biopharmaceuticals, however, there is still a need for many emerging techniques in CRISPR to be reported in CHO cells; therefore, more research in these cells is needed to realize the full potential of this technology.

1. Introduction

Biopharmaceuticals are products derived from biological sources that can be manufactured in various cell lines through biotechnological methods (He et al., 2020; O'Flaherty et al., 2020). Although antibodies are predominant among the biopharmaceuticals approved by FDA, these products encompass a wide variety of therapeutics including cytokines, hormones, growth factors, vaccines and peptides, among others (O'Flaherty et al., 2020). A comprehensive market analysis has shown that the global biopharmaceutical market was valued at $237.2 billion in 2018, and it has been estimated that it will grow to $389.0 billion by 2024 (O'Flaherty et al., 2020). To this respect, the growing importance of biopharmaceutical biotechnology has led industry and academic stakeholders to improve the production of biopharmaceuticals by increasing the efficiency of transgene integration, enhancing the recombinant protein (r-protein) production, and modulating the glycan structures of r-proteins, all of which will help to accelerate the entire bioprocess and reduce the cost of production.

* Corresponding author at: School of Natural and Environmental Sciences, Molecular Biology and Biotechnology Division, Newcastle University, Newcastle Upon Tyne, NE1 7RU, UK
E-mail address: leo.rios@ncl.ac.uk (L. Rios-Solis).

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Chinese hamster ovary (CHO) cells are among the most widely used mammalian cell lines in the biopharmaceutical industry (Walsh, 2014) owing to a variety of reasons:

(1) They can produce proteins with complex bioactive post-translational modifications (PTMs) similar to human cells (Chin et al., 2019; Dumont et al., 2016).
(2) In contrast to most other mammalian-based expression systems, CHO cells allow the reaching of recombinant protein yield as high as gram per litre. Moreover, these cells are not easily affected by changes in oxygen levels, temperature, pressure, or pH during production (Dumont et al., 2016; Ghaderi et al., 2012).
(3) They allow reproducible large-scale manufacturing since they can be cultivated in suspension as well as in chemically defined serum-free media (Dumont et al., 2016).
(4) Moreover, human viruses are mostly unable to infect these cells, hence reducing biosafety concerns during production (Lalonde and Durocher, 2017).
(5) CHO cells have a well-established genetic toolbox (Kim et al., 2012).
(6) CHO cells have been utilised for the production of r-protein for decades to the point where both regulatory agencies and pharmaceutical companies have become well familiarised with the process of therapeutic discovery and development research based on CHO cells, enabling these pharmaceuticals to be available for patients in a shorter time compared to studies based on novel mammalian expression systems (Dumont et al., 2016).

Therefore, it is not surprising that significant efforts have been made around the engineering of CHO cells using genetic engineering methods. CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR associated) is a bacterial adaptive immune system against viruses and plasmids, which was later redeveloped as a programmable genome-editing tool (Hsu et al., 2014). CRISPR-Cas system provides easy, efficient, programmable and low-cost opportunities to edit eukaryotic cells for generating improved cell factories compared to conventional genome editing tools like zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) (Khan et al., 2020; Lee et al., 2015a). CRISPR-Cas9, the most widely used CRISPR-Cas system, is an RNA-guided DNA editing tool and has two key players: an effector protein (Cas9) and a guide RNA (gRNA) (Chen and Doudna, 2017). When gRNA binds Cas9, the ribonucleoprotein complex will search its target, and upon binding in a sequence-specific manner to its complementary region, which requires to be flanked with a protospacer adjacent motif (PAM), it will introduce a double-strand break (DSB) at a specific locus in DNA (Chen and Doudna, 2017). Using this system, it is possible to effectively delete endogenous genes and/or integrate transgenes (Moon et al., 2019; Shalem et al., 2014). The Cas9 from *Streptococcus pyogenes* cleaves DNA strands using two nuclease domains: RuvC and HNH. To expand the potential of the CRISPR-Cas9 system, researchers mutated either one or both domains (Mitsunobu et al., 2017). To this respect, an engineered version of Cas9, namely Cas9 nickase (nCas9), which carries only one functional nuclease domain (either RuvC or HNH), has been used with a pair of gRNAs to increase the specificity of CRISPR-based gene integration, reducing the rate of off-target editing (Mitsunobu et al., 2017; Ran et al., 2013). Moreover, to directly block the transcription or recruit transcriptional regulators to a desired genomic region without causing DSB, nuclease deficient Cas9 (dCas9) was generated and fused with different transcriptional regulator domains such as Kruppel-associated box (KRAB), VP64, and p65 and used to transiently activate or repress genes (Mitsunobu et al., 2017; Xu and Qi, 2019). Furthermore, as the CRISPR technology evolves, new, orthogonal CRISPR-Cas systems have been discovered and used instead of CRISPR-Cas9 in certain applications (Chen and Doudna, 2017; Garcia-Doval and Jinek, 2017). For instance, type V CRISPR-Cas12a system, an RNA-guided DNA editing tool, has a different PAM requirement (TTTN) than that of Cas9 (NGG), which can be used with Cas9 in an orthogonal way and expands the possible targets and within the genome (Shaw et al., 2022). On the other hand, unlike Cas9 and Cas12a, type VI CRISPR-Cas13 system targets RNA instead of DNA, which can be used to repress gene expression without changing the genome sequence (Chen and Doudna, 2017). For a comprehensive overview detailing engineered variants of Cas9, Cas orthologs and/or different types of CRISPR systems, we refer the readers to recently published reviews (Bharathkumar et al., 2022; Mitsunobu et al., 2017; Moon et al., 2019; Perčulija et al., 2021a).

Although most CRISPR-based studies employed the CRISPR-Cas system to knock out a single desired gene in CHO cells (Chan et al., 2016; Lu et al., 2018; McVey et al., 2016; Sun et al., 2015), the discovery of new types of CRISPR systems is expanding eukaryotic editing to multiplex gene editing and regulation (Blomeier et al., 2021; Bryson et al., 2021; Schweickert et al., 2021; Shen et al., 2020; Teng et al., 2019). Similarly, dCas nucleases fused to functional domains are used for transcriptional modulation (Shen et al., 2017; Xiong et al., 2019), base editing (Nishida et al., 2016), transcriptome engineering (Konermann et al., 2018), epigenome editing (Marx et al., 2021; Marx et al., 2018) and building genetic circuits (Lebar and Jerala, 2016; Minami and Shah, 2021): this created novel approaches for genetic engineering of CHO cells, thereby accelerating the development of enhanced CHO cell factories that can efficiently produce biopharmaceuticals.

In this review, we summarize the studies that have used the CRISPR-Cas system to improve r-protein production in CHO cells. Here, every relevant stage of production was considered, besides underscoring the advantages and limitations of these systems, as well as discussing their bottlenecks and probable solutions. Lastly, by putting forward novel CRISPR-Cas based systems established in CHO cells, possible applications to improve r-protein production in CHO cells are discussed for the near future.

2. CRISPR-mediated transgene integration methodologies

Traditional recombinant CHO cell-line development is done based on random integration of transgenes into the genome (Kim et al., 2012). However, uncontrolled insertion can cause inconsistent expression of transgenes and requires labour-intensive selection processes to obtain stable and high-expressing cell lines (Kim et al., 2012; Lee et al., 2019). Alternatively, transgenes can be integrated into designated genomic sites using site-specific integration (SSI) methods. SSI approaches have been developed using site-specific recombinases such as Flp/FRT and Cre/loxP, which however require previously established landing-pad cell lines, and the integration of transgenes is limited to the genomic regions that contain these systems (Baser et al., 2016; Kito et al., 2002). Another approach is by using programmable endonucleases such as ZFNs, TALENs and CRISPR-associated nucleases including Cas9. The CRISPR-Cas9 is a highly efficient method that is relatively simpler and more flexible to use compared to other tools such as ZFNs and TALENs owing to its RNA-mediated programmability (Adli, 2018). This section highlights how targeted gene integration is achieved in CHO cells by triggering DNA repair mechanisms via the CRISPR-Cas9 system.

Programmable nucleases create DSBs at the desired genomic sites. DSBs are then repaired by cellular DNA repair mechanisms, which can be harnessed to knock out a gene by creating insertions, deletions or causing frameshift mutations, or to insert transgenes into the genome by introducing donor DNA templates (Gaj et al., 2013). The use of programmable nucleases alleviates the need for an established cell line for gene integration, while transgenes can be integrated into any desired locus in the genome, including newly discovered transcriptionally active genomic hot-spots.

The two main DSB repair mechanisms in CHO cells are non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Mansour et al., 2008), both of which have been exploited for the integration of transgenes into the genome of CHO cell lines.

Furthermore, alternative knock-in strategies have been developed in CHO cells (Sakuma et al., 2015) using microhomology-mediated end-joining (MMEJ) (Bae et al., 2014) (Fig. 1A). Taking advantage of these DNA repair mechanisms in combination with using pre-validated genomic integration sites with high and stable transcriptional activity has led to the achievement of consistent productivity and cell line stability, as discussed in the next sections.

2.1. Repair mechanism based on non-homologous end-joining

Although NHEJ is an error-prone pathway that can create insertions and deletions when free DNA ends are ligated, it is the predominant DSB repair pathway in CHO cells (Mansour et al., 2008). Therefore, the NHEJ can be used for targeted transgene integration if precise editing at the target site becomes unnecessary. Targeted gene integration using CRISPR-Cas9 via NHEJ pathway requires a linear donor DNA or a plasmid DNA, in which the transgene is flanked by gRNA target sites, enabling the production of a linear transgene cassette in cells (Fig. 1A). In a preliminary study, a cytomegalovirus (CMV) promoter was inserted upstream of a genomic promoterless *Puro* gene in CHO cells via the NHEJ pathway using Cas9 (Bachu et al., 2015). Successful integration events were observed in 0.45% of the colonies obtained after puromycin selection. Based on this work, Wang et al. used the CRISPR-Cas9 system to insert a linear donor DNA encoding *eGFP-HsQSOX1b* and *hSurvivin* genes into a specific locus in the CHO-K1 genome (Wang et al., 2018). With the selection of eGFP positive cells using FACS, 3.85% of integration efficiency was achieved. In another proof-of-concept study, an NHEJ-based method called homology-independent target integration (HITI) (Suzuki et al., 2016) was used for eGFP gene integration into the *caspase-7* gene for knockout purpose in CHO cells (Safari et al., 2020). These studies suggest that NHEJ-mediated site-specific gene integration using CRISPR-Cas9 system is an efficient strategy for CHO cell line engineering.

2.2. Repair mechanism based on homology-directed repair

HDR uses a template donor DNA with long homology arms that flank the transgene, enabling precise integration of gene cassettes to minimize any random insertions and deletions (Fig. 1A). Many groups have focused on CRISPR-mediated targeted gene integration in CHO cells using the HDR pathway. In a proof-of-concept study, Lee et al. (2015b) performed HDR-mediated gene integration at *COSMC*, *Mgat1* and *LdhA* loci using the CRISPR-Cas9 system (Lee et al., 2015b). They used a donor plasmid, in which mCherry and neomycin resistance genes were flanked by the homology arms while ZsGreen1-DR was outside, enabling the selection of mCherry-positive and neomycin-resistant clones while avoiding the selection of random integrants by excluding ZsGreen1-DR-positive cells. The assay design yielded a high rate of editing efficiency, ranging from 7% to 28% depending on the target loci. Although the study was promising, the method used included the integration of the mCherry gene into the CHO genome and required antibiotic selection, which is not preferred in industrial settings. Therefore, in a different study, the authors modified the method by expressing GFP and Cas9 from the same promoter, and used a separate donor plasmid in which erythropoietin (EPO) or rituximab (anti-CD20 monoclonal antibody) gene cassettes were flanked by the homology arms while mCherry was outside (Lee et al., 2016a). After mCherry and GFP positive cells likely to be exposed to genome editing events were enriched using FACS, non-fluorescent clonal cells were isolated to exclude random integrants. From six 96-well plates, 81 EPO and 76 rituximab clonal cells were obtained, where one EPO and one rituximab clone were precisely edited. In addition to these studies, Cas9-mediated HDR efficiency has been increased up to 1.75-fold in CHO cells with *Rad51* gene overexpression and simultaneous knockdown of *Mre11* and *Pari* genes, as these were identified as rate-limiting genes for HDR in CHO cells (Bosshard et al., 2019). Moreover, incubating CHO cells with a low concentration (0.125 mM) of hydroxyurea increased the proportion of CHO cells in the S phase of the cell cycle, thereby increasing the HDR-mediated transgene integration by 1.2–1.5-fold (Kwak et al., 2021).

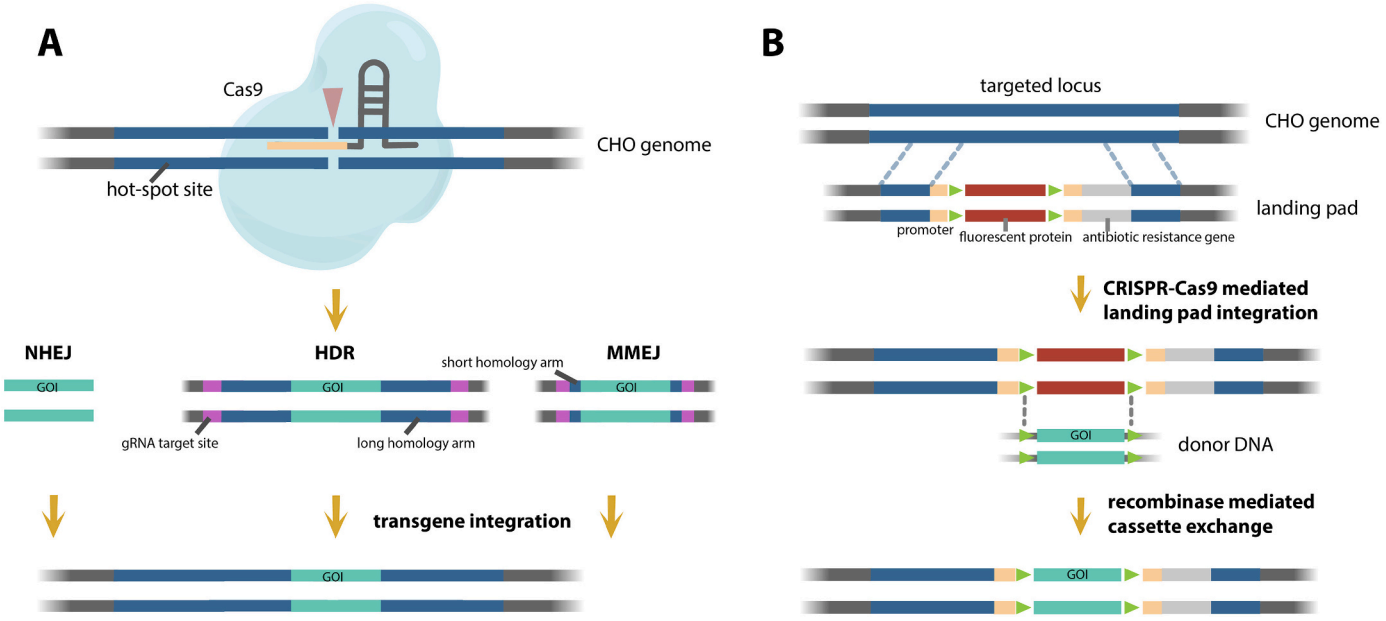

Fig. 1. Methods for site-specific gene integration using the CRISPR-Cas system in CHO cells. (A) Cas9 is targeted to a hot spot locus, creating a DSB. Then, one of the DSB repair pathways such as NHEJ, HDR, and MMEJ is harnessed for the integration of the gene of interest (GOI). These three pathways use donor DNAs with no homology arms, long homology arms of 0.5–1 kb, and short microhomologies of 20–40 bp, respectively. Double-cut donor (DCD), in which the integration cassette is flanked by gRNA target sites, is preferred for HDR and also may be used when performing MMEJ-based strategy. (B) Using CRISPR-Cas9, the landing pad carrying fluorescent protein encoding gene, antibiotic resistance gene, and recombinase sites is integrated into the targeted locus. Following the selection of antibiotic-resistant and fluorescent-positive cells, RMCE is performed to integrate GOI; and then fluorescent-negative cells are further selected.

2.3. Repair mechanism based on HDR and double-cut donors

Recently, it was found that the use of double-cut donors (DCDs), in which the homology arms are flanked by gRNA target sites, significantly increased HDR efficiency in mammalian cells (Fig. 1A) (Zhang et al., 2017). To alleviate the need for enrichment of precisely edited CHO cells, Shin and Lee (2020b) increased HDR-mediated integration efficiency using DCDs, which achieved Cas9-mediated simultaneous knock-in of CMV and EF1α promoters in a CHO-K1 cell line harbouring promoterless *EGFP* and *TagRFP657* genes. The rate of correctly edited clones was 1–2% for the adherent cell line and 2–4% for serum-free suspension-adapted cells. Furthermore, DCDs allowed up to a 36-fold increase in HDR-mediated integrants compared to that of conventional circular donors. In another study, the authors discovered that a modified vector system consisting of a gRNA/DCD vector and a Cas9 expression vector with the weight ratio of 8:2 enabled a 1.5-fold increase in HDR-mediated targeted gene integration via DCDs (Shin et al., 2021). These studies indicated that the use of DCDs substantially increases the efficiency of HDR-mediated transgene integration using Cas9 in CHO cells. Therefore, DCDs facilitate enrichment-free and multiplexed gene integration besides reducing the time and effort required for obtaining desired integrants.

2.4. Repair mechanism based on microhomology-mediated end-joining

MMEJ pathway can be used for site-specific integration via short microhomologies (20–40 bp), which simplifies the construction of donor DNA compared to HDR (Fig. 1A). With the help of programmable nucleases, MMEJ has been harnessed by several studies for site-specific gene insertion in mammalian cell lines, including CHO cells (Nakade et al., 2014; Sakuma et al., 2015). Among those studies, Kawabe et al. integrated puromycin resistance gene and DsRed or an anti-prion single-chain Fv fused with Fc (scFv-Fc) antibody gene into *HPRT* locus using an MMEJ-based method known as the CRISPR-mediated precise integration into target chromosome (CRIS-PITCh) (Kawabe et al., 2018). When the optimised CRIS-PITCh-v2 method was used for DsRed integration, 28 colonies were obtained, of which 8 were selected and 4 were found to be correctly edited. However, HDR was more efficient than MMEJ for DsRed integration. Out of 21 colonies obtained by HDR-mediated DsRed integration, 5 were selected and found to be correctly edited. For scFv-Fc integration, the authors utilised CRIS-PITCh-v2 and obtained 4 clones that showed high and stable expression of scFv-Fc. The study suggests that the use of MMEJ is a promising way for Cas9-mediated gene integration in CHO cells.

Recently, Hamaker and Lee (2020) developed a reporter system for evaluating the activity of CRISPR-mediated knockout or knock-in events in CHO cells. They compared the efficiency of Cas9-mediated site-specific integration of mCherry open reading frame using four different methods: NHEJ, MMEJ with 20 bp microhomologies, HDR with 250 bp homology arms and HDR with 700 bp homology arms, which yielded 0.18%, 0.23%, 0.65% and 1.90% of integration efficiencies, respectively. In these assays, linear donor DNAs were used. Compared to circular donors with 850 bp homology arms, the linear HDR donor with 700 bp homology arms showed a 2.3-fold increase in integration efficiency.

The use of DCDs (Shin et al., 2021; Shin and Lee, 2020b) or linearised DNA donors (Hamaker and Lee, 2020) in HDR provides Cas9-mediated site-specific integration with relatively high efficiency, such that multiplexed knock-in can be achieved even without additional enrichment processes (Shin et al., 2021; Shin and Lee, 2020b). Therefore, future studies may focus on increasing the efficiency of NHEJ- and MMEJ-mediated site-specific integration methods and further optimising the HDR-mediated knock-in method using DCDs.

2.5. Choosing a suitable repair pathway

It is hard to suggest a method that is suitable for every type of gene editing in mammalian cells (Banan, 2020). For instance, HDR is often the preferred repair pathway when precise gene integration is desired due to its higher accuracy and specificity (Zhang, M. et al., 2021). On the other hand, the insufficient efficiency of HDR in non-dividing cells, especially with large inserts, directs researchers to use NHEJ- and MMEJ-mediated SSI strategies (Zhang, M. et al., 2021). Nevertheless, these methods are not precise as HDR and often end up with mutations in the vicinity of 5′ and 3′ ends of the inserts. Moreover, NHEJ-based repair may give rise to incorrect insert orientation (Banan, 2020). MMEJ-mediated SSI, however, is still a useful strategy, especially when working with relatively larger DNA fragments and it provides a more controlled repair compared to NHEJ (Hamaker and Lee, 2018; Lau et al., 2020). Overall, HDR remains a leading strategy in the case of precise integration in dividing CHO cells (Shin and Lee, 2020a). Moreover, apart from what have been discussed above, with the small molecules that increase HDR:NHEJ ratio such as Scr7, as well as arresting cell cycle at S/G2 phase, HDR efficiency can be further improved, and such a strategy, to our knowledge, has yet to be explored in CHO cells (Banan, 2020; Zhang, M. et al., 2021). For a comprehensive overview of using these DNA repair mechanisms for site-specific integration in CHO cells, we refer readers to recent review articles (Hamaker and Lee, 2018; Shin and Lee, 2020a).

2.6. Hot-spots genomic regions for CRISPR-mediated transgene integration

Targeted transgene integration into pre-validated genomic sites with high and stable transcriptional activity is preferred to achieve consistent productivity and cell line stability (Fig. 1B). Such regions are called 'hot-spots' and there are many characterised hot-spot loci in the genome of CHO cells (Hamaker and Lee, 2018). To ensure high and stable transgene expression, Zhao et al. (2018) integrated mCherry and anti-PD-1 antibody genes (anticancer drug) into three potential hot-spot sites in CHO cells using CRISPR-Cas9 and DCDs. Compared to *HPRT* and *GRIK1* loci, the *C12orf35* was the most favourable locus in terms of productivity and stability, such that the *C12orf35*-integrated cell pool achieved 18- and 9.5-fold higher mCherry and anti-PD-1 expression than the random integration pool. After puromycin selection, stably transfected clones were obtained, among which 60% and 42% were confirmed to have successfully integrated mCherry and anti-PD-1 antibody genes, respectively. In another study, Zhou et al. (2019) integrated bevacizumab light chain and heavy chain genes with puromycin resistance gene into a transcriptional hot-spot site using Cas9. The donor plasmid comprised copGFP (from copepod *Pontellina plumata*) outside the homology arms, enabling the exclusion of random integrants by the selection of cop-GFP negative clones. After puromycin selection, copGFP negative cells were sorted by FACS and seeded. Among 74 clones, 1 was correctly edited. The stable clone yielded 0.006 pg/cell/day (pcd) of bevacizumab for up to 50 passages. Additionally, recent studies revealed a new potential hot-spot by successfully integrating reporter genes into a PhiC31 pseudo attP site in the CHO genome (Pourtabatabaei et al., 2021). These studies indicate that CRISPR-Cas9 mediated transgene integration into transcriptional hot-spots is a potential approach for producing recombinant CHO cell lines that can be used in biopharmaceutical research.

2.7. Creating hot-spots using CRISPR for recombinase-mediated site-specific integration

Recombinases are proteins derived from bacteriophages and fungi that catalyse DNA exchange reactions. As previously mentioned, with the help of these site-specific recombinases, synthetically engineered gene cassettes can be integrated into the recipient cell genome to correctly integrate recombinant genes in CHO cells without clonal

variation. However, targeted integration of transgenes through recombinases is limited to the genomic regions that contain specific recognition sequences. A synthetically designed landing pad can provide a safe harbour locus for Recombinase-mediated cassette exchange (RMCE) and therefore help overcome this limitation. Using CRISPR-Cas9 to integrate RMCE landing pads into a defined locus can further increase the efficiency of gene integration, enabling stable r-protein expression in CHO cells as described in Fig. 1B (Chi et al., 2019; Gaidukov et al., 2018; Grav et al., 2018; Sergeeva et al., 2020). A key advantage of using recombinase-based transgene integration is the reduced clonal variation due to target site-specificity. In this regard, Gaidukov et al. (2018) integrated 21 well-characterised landing pad sites into the genome of CHO-K1 cells using the CRISPR-Cas9 system, achieving efficiencies of ~5 to 50% for correctly integrating recombinant genes in CHO cells without clonal variation (Gaidukov et al., 2018). Consequently, the multi-Landing Pad platform enabled a stable and predictable insertion of large gene cassettes into the CHO-K1 genome using Bxb1 integrase that served as DNA recombinase (Gaidukov et al., 2018). In another study, researchers integrated a landing pad carrying a reporter gene (mCherry), lox sites for Cre-mediated recombination and a selection marker into the CHO genome using a similar CRISPR-mediated approach (Grav et al., 2018). Taking advantage of the gene-trap, they avoided the random integration of the gene of interest (GOI) and eliminated the need for drug selection, thus enabling stable site-specific integration of genes encoding four different r-proteins. Following the selection of r-protein-expressing cells, the authors generated clones that were able to grow consistently with minimal clonal variation for the extended duration of cultivation. In a further study, a similar RMCE landing pad design was utilised to systematically evaluate the effect of every component in the expression cassette on r-protein production by varying the promoter, GOI, integration site and 5′-flanking regulatory sequences (Pristovšek et al., 2019). It showed the generation of robust patterns of gene expression in different 5′ proximal regulatory elements occurring in defined CHO integration sites, yielding a wide range of transcriptional outputs. More recently, Cre recombinase-mediated site-specific integration was further engineered to improve the specific production of therapeutic proteins by increasing the gene copy number in the CHO genome (Sergeeva et al., 2020). Researchers expressed the hormone EPO and the Fc-fusion protein etanercept (ETN) through multicopy targeted integration and investigated the relationship between gene dosage and protein expression. Consequently, they successfully introduced four copies of the r-protein genes and reached a titer of 1 g/L and specific production of 12–14 pg/cell/day (pcd) in shake flask culture. This was a significant improvement for industrial use, compared to the 1–10 pcd production achieved through random integration (Ley et al., 2015; Pristovšek et al., 2018). A similar principle was also successfully utilised with PhiC31 integrase, enabling precise gene insertion in the CHO-S genome. Using CRISPR-Cas9, the authors generated a landing pad carrying two PhiC31 attP sites into the H11 locus, where they subsequently integrated a GFP encoding gene, achieving efficiencies of 97.7% and 13.1% with and without a selection step, respectively (Chi et al., 2019).

Overall, these studies indicate that RMCE is a powerful tool for site-specific integration of transgenes and that it can be improved by optimising the system design such as altering each component of the system or increasing the copy number of transgenes. CRISPR-Cas9-mediated homologous recombination, on the other hand, allowed for the insertion of a specially designed landing pad into the desired locus, which expanded the application profile of this method.

3. CRISPR-Cas based strategies to increase r-protein production

3.1. CRISPR-Cas9 mediated gene knockout studies

R-protein production in CHO cells can be influenced by many metabolic and signalling pathways associated with the regulation of the cell cycle and apoptosis (Chong et al., 2012; Courtes et al., 2014; Fan et al., 2015; Misaghi et al., 2013). Therefore, such pathways can be engineered to improve r-protein production using gene-editing tools and CRISPR-Cas. Hence, this section reviews recent studies employing CRISPR-Cas systems to knock in/out or regulate single or multiple genes related to r-production using Cas9, Cas13 or dCas9 fused with effector domains. In addition, the summary of these studies is presented in Table 1 and Fig. 2.

3.1.1. Targeting apoptosis genes

One of the most common ways to enhance r-protein production yield is the disruption of pro-apoptotic genes, which extends the lifespan of the CHO cells (Grav et al., 2015; Ha et al., 2020; Miao et al., 2018). For instance, CRISPR-Cas9 has been used for simultaneous knockout of three genes, namely fucosyltransferase 8 (*FUT8*), Bcl-2 homologous antagonist killer (*BAK*) and Bcl-2-associated X (*BAX*), two of which (*BAK* and *BAX*) encode pro-apoptotic proteins involved in a wide range of cellular functions (Grav et al., 2015; Ha et al., 2020). As a result, apoptotic caspase activation was significantly decreased (~90%) compared to control CHO-S cells. Moreover, in line with apoptosis inhibition, double and triple KO cells were generated, demonstrating improved culture longevity, while two clones showed increased production of rituximab (~1,87 fold) among three different clones (Grav et al., 2015). Successful apoptosis inhibition has also been achieved in another study when the *BAK* gene was targeted, where the coding gene for infliximab, a monoclonal antibody used in the therapy of certain autoimmune diseases, was integrated into the *BAK1* locus via CRISPR-Cas9 mediated homology-directed repair (HDR) (Miao et al., 2018). An alternative target for the enhancement of bioproduction is deubiquitinase cylindromatosis (*CYLD*), which participates in tumour suppression through NF-κB and Wnt/β-catenin signalling pathways[20]. Inhibition of *CYLD* using CRISPR-Cas9 technology resulted in improved cell viability, cell density and increased production of four different antibodies: trastuzumab (63%), rituximab (105%), IgG (50%) and one bispecific antibody (228%) (Lu et al., 2018).

Additionally, microRNAs are involved in the modulation of many signalling pathways including apoptosis in CHO cells (Jadhav et al., 2013). For example, targeting miR-744 precursor sequence, which was reported to be associated with the induction of apoptosis, led to increased r-protein production in CHO cells (Chen and Liu, 2016; Fischer et al., 2014) (Fig. 2A). Although the viable cell density of two of the generated clones was reduced by ~50% compared to a non-targeting sgRNA control, the authors observed up to 2-fold increase in IgG titer (Raab et al., 2019).

3.1.2. Targeting genes that inhibit r-protein production

A decrease in r-protein production is one of the major challenges in CHO cell-based systems, particularly over long-term culture. PI3K/AKT/mTOR is another important cellular pathway, which is involved in the cell's ability to sense and adapt to its environment (Fig. 2B). It has been shown that CHO cells produce lower amounts of r-proteins when mTOR is inhibited, while r-protein production increases upon mTOR upregulation (Edros et al., 2014). McVey et al. used CRISPR-Cas9 to knock out *TSC2* that inhibits mTORC1 activity in CHO cells, resulting in a 2-fold increase in protein production with comparable quality to that of control under fed-batch conditions (McVey et al., 2016).

Decrease in r-protein can also be due to transgene silencing and promoter hypermethylation during long term cultures (Bailey et al., 2012; Chusainow et al., 2009; Kim et al., 2011; Osterlehner et al., 2011; Yang et al., 2010). To address these challenges, Jia et al. (2018) used CRISPR-Cas9 to disrupt the CMV-driven DNA methyltransferase *(Dnmt3a)* without compromising cell growth and viability. Following 50 passages (~5 months), Dnmt3a-deficient cells exhibited distinctively greater (approximately 2-fold) eGFP protein levels than those of Dnmt3a expressing cells (Fig. 2C) (Jia et al., 2018). Similarly, in another study, the disruption of *Dnmt3b* yielded at least 1,6-fold higher r-protein

Table 1
Summary of the selected CRISPR-Cas studies that focus on increasing r-protein production in CHO cells.

Cell type	Genome Editing strategy	Target Gene/s	Goal	Effect	R-protein	Cell viability and growth	Reference
CHO-S	Multiplexed CRISPR-Cas9-Mediated Knockout	*BAK, BAX FUT8*	inhibit apoptosis	~1,87-fold increase in protein productivity	Rituximab	Improved culture longevity	(Grav et al., 2015)
CHO-K1	CRISPR-Cas9-Mediated Knockout	*BAK1*	inhibit apoptosis	Enhanced cell resistance to the serum starvation Stable expression of r-protein up to 25 passages	Infliximab	–	(Miao et al., 2018)
CHO-K1	CRISPR-Cas9-Mediated Knockout	*TSC2*	enhance cell adaptation to its environment	2-fold increase in protein production	GFP	Decreased cell growth and viability	(McVey et al., 2016)
CHO-K1	CRISPR-Cas9-Mediated Knockout	*DNMT3A*	To increase the stability of transgene expression	~2-fold increase in protein production Stable expression of r-protein up to 50 passages	EGFP	Comparable cell growth and viability	(Jia et al., 2018)
CHO-K1	CRISPR-Cas9-Mediated Knockout	*CYLD*	inhibit apoptosis	Increased r-protein production (63%, Trastuzumab; 105%, Rituximab; 50%, IgG; – 228%, A bispecific antibody)	Trastuzumab Rituximab IgG A bispecific antibody	Improved cell growth and viability	(Lu et al., 2018)
CHO	CRISPR-Cas9-Mediated Knockout	*LDHA*	prevent lactate accumulation (To inhibit apoptosis)	2.8-fold increase in r-protein production Enhanced energy and redox conditions for protein synthesis	Fc-fusion protein	Decreased cell density	(Wilkens et al., 2019)
CHO-S	CRISPR-Cas9-Mediated Knockout	*HPD, GAD2*	prevent lactate and ammonium accumulation (To inhibit apoptosis)	26% decrease in specific ammonium production 22% decrease in lactate production	–	Increased integral of viable cell density	(Ley et al., 2019)
CHO-DG44	CRISPR-Cas9-Mediated Knockout (Excising the entire gene)	miR-744 precursor sequence	inhibit apoptosis	~1,2–2-fold increase in monoclonal antibody (mAB) titer	IgG	Decreased viable cell density	(Raab et al., 2019)
CHO-DG44	CRISPR-Cas9-Mediated Knockout	*BMPRIA or BMPRII*	remove autocrine BMP signalling (To inhibit apoptosis)	~2.4-fold increase in the mean maximum concentration	BMP-4	Improved cell growth and viability	(Kim and Lee, 2019)
CHO-DG44	CRISPR-Cas9-Mediated Sequential Gene Knockout	*NEU1, 2, 3 BAK, BAX*	increase the content of sialylation of hEPO To inhibit apoptosis	1.4-fold increase in protein concentration	hEPO	Comparable cell growth and viability	(Ha et al., 2020)
CHO-K1	CRISPR-Cas9-Mediated Gene Knockout (indel mutations)	*CYP1A2, ATP5S, DGKI, or P3H2*	confirm genes affecting r-protein production based on the siRNA screen	1.9- fold increase in the specific productivity	Three different antibodies	Modest decrease in Cell growth (CYP1A2)	(Lin et al., 2021b)
CHO-K1	NHEJ-mediated knock-in strategy using CRISPR-Cas9	*hQSOX1, hSurvivin*	increase the metabolic stress, thereby inducing unfolded protein response in ER prevent cells from prolonged UPR-mediated apoptosis	6,40-fold increase in the antiapoptotic viability 5,55-fold increase in the yield of r-protein	GLuc	Improved cell viability	(Wang et al., 2018)
CHO-DUXB11	CRISPR interference (dCas9-KRAB)	*DHFR*	increase the copy number of gene coding for r-proteins	~3,8-fold increase in the EGFP expression ~2,35-fold increase in granulocyte colony stimulating factor	EGFP and granulocyte colony stimulating factor	Comparable cell growth	(Shen et al., 2017)
CHO-S	CRISPR interference (KRAB-dCas9, dCas9-KRAB, dCas9-KRAB-Mecp2)	*BAK, BAX, and CASP3*	inhibit apoptosis	~10% decrease in caspase activity (KRAB-dCas9) Significant decrease in relative mRNA levels (KRAB-dCas9, dCas9-KRAB, dCas9-KRAB-Mecp2)	EGFP	Significantly increased viable cell density (dCas9-KRAB-Mecp2, dCas9-KRAB)	(Xiong et al., 2019)
CHO-K1 CHO-DUXB11 CHO-DG44	Multiplexed CRISPR-Cas13d Mediated Gene Knockdown	*BAK, BAX PDK1*	inhibit apoptosis To prevent lactate accumulation	90,91-99,74% decrease in mRNA levels ~1,42-fold increase in antibody titer	IgG	Slightly slowed cell growth, comparable cell viability	(Shen et al., 2020)
CHO-K1	Multiplexed CRISPR-Cas13d Mediated Gene Knockdown	*DDIT3 LDHA GFT*	inhibit cell death To prevent lactate accumulation To inhibit fucosylation	38–83% decrease in mRNA levels ~1,48-fold increase in antibody titer	IgG	Improved culture longevity, modestly decreased viable cell density	(Lin et al., 2021a)

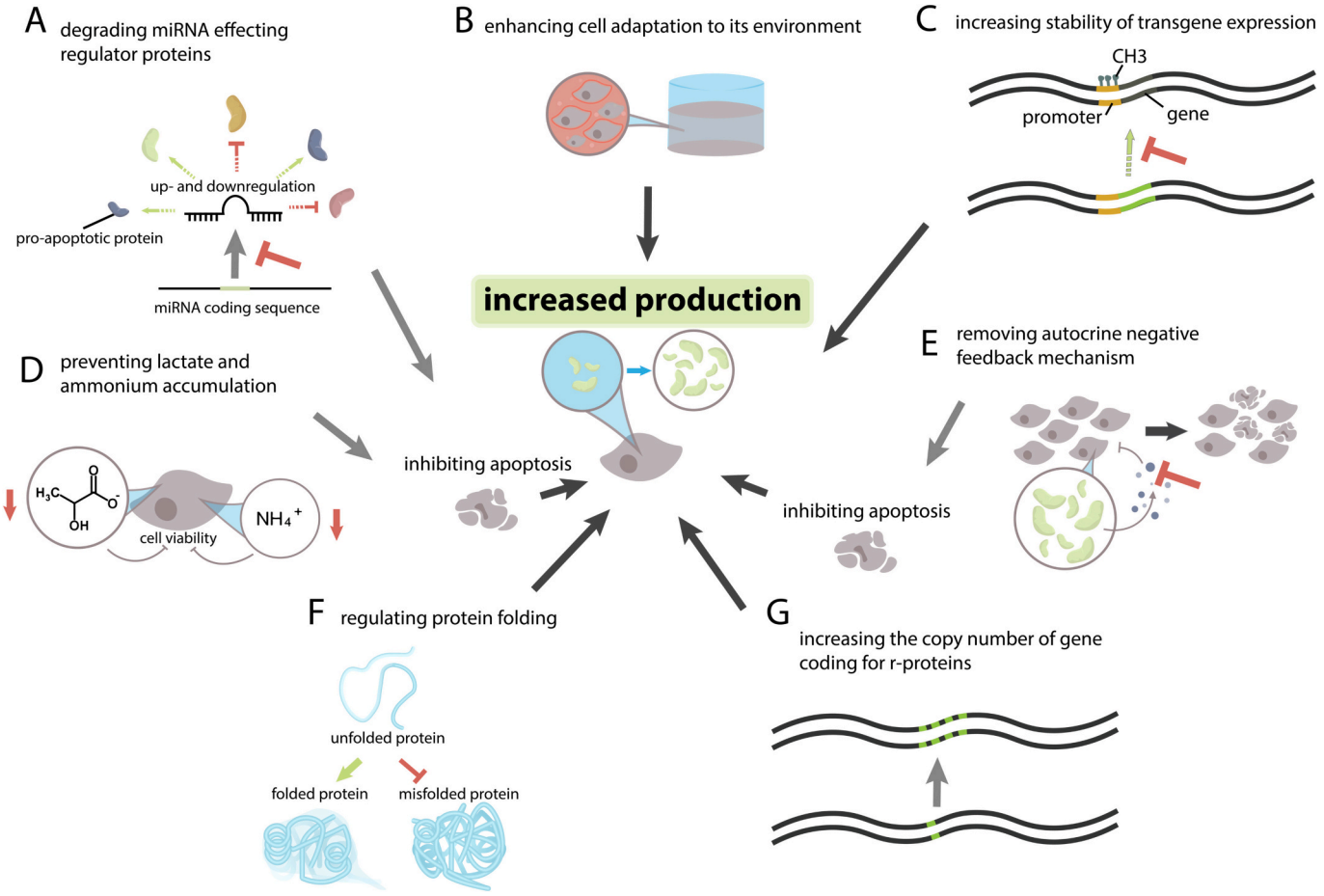

Fig. 2. Approaches for increasing r-protein production in CHO cells using CRISPR-Cas system.

production (Feng et al., 2021).

Another common reason for decreased long-term r-protein production in CHO cells is the accumulation of metabolic side products such as ammonium and lactate, which negatively affect cell growth (Fig. 2D). Therefore, recent studies have concentrated on reducing these growth-inhibiting compounds through media optimisation (Wilkens et al., 2011) and metabolic engineering strategies (Kim and Lee, 2007; Wlaschin and Hu, 2007). CRISPR-Cas9-based studies were also aimed at decreasing the accumulation of lactate and/or ammonium in CHO cells by targeting genes involved in amino acid catabolism (Ley et al., 2019) or lactate production directly (Wilkens et al., 2019). In a study that employed this strategy, a 2.8-fold increase in Fc-fusion Protein production was achieved (Wilkens et al., 2019).

Methods to improve the production of certain r-proteins might require a more bespoke approach since a particular r-protein can require specific alterations of a host cell line. For example, the recombinant production of human bone morphogenetic protein-4 (rhBMP-4) that plays a substantial role in promoting autocrine BMP signalling pathway is decreased when autocrine BMP signalling is upregulated (Kim and Lee, 2019) (Fig. 2E). Kim and Lee (2019) used CRISPR-Cas9 to disrupt *BMPRIA* or *BMPRII* genes to inhibit this signalling pathway in CHO-DG44 cells, thus achieving a ~ 2.4-fold greater mean maximum rhBMP-4 concentration than that of control. The study clearly indicated that protein-specific gene modifications in CHO cells may sometimes be required to improve the production yield and overcome problems associated with difficult-to-express proteins.

To date, many genes have been disrupted or overexpressed to increase the production of r-proteins in CHO cells. Nevertheless, one study took a step further through a comprehensive small interfering RNA (siRNA) screen to investigate the possible genes associated with r-protein production (Lin et al., 2021b). The authors targeted a comprehensive part of the CHO genome involving 2234 CHO genes for siRNA screening and identified genes that significantly enhanced antibody production upon downregulation. CRISPR-Cas9 was then used to disrupt selected genes. Although production dynamics of CRISPR-Cas9 mediated knockouts did not entirely correlate with siRNA screening results, three clones that exhibited increased r-protein production were isolated. Since gene knockdown and knockout may have different effects on cell growth and r-protein production, stable gene knockdown could be an alternative way to further evaluate genes identified through siRNA screening. On the other hand, CRISPR-dCas9-, CRISPR-dCas12a- or CRISPR-Cas13-mediated gene knockdown could be also used (Schweickert et al., 2021; Shen et al., 2020; Xiong et al., 2019).

3.2. *CRISPR-Cas9 mediated gene knock-in studies*

Different from the aforementioned studies, r-protein production in CHO cells can be increased through CRISPR-Cas-mediated knock-ins. For example, Wang et al. used NHEJ-mediated knock-in strategy to reduce apoptosis while increasing the production yield using CRISPR-Cas9 (Wang et al., 2018). In this regard, one of its two objectives was to increase the metabolic stress, thereby inducing unfolded protein response (UPR) in the endoplasmic reticulum (ER). UPR is a cellular reaction triggered in response to the disruption of ER homeostasis and decreases the unfolded protein load on the ER. Therefore, inducing UPR promotes protein folding and secretion (Fig. 2F). The other objective was to prevent cells from prolonged UPR-mediated apoptosis (Hetz, 2012; Hetz et al., 2015; Wang et al., 2018). So, the authors concentrated

on improving ER microenvironment and strengthening antiapoptotic ability by integrating *hSurvivin* and *hQSOX1b* genes into the CHO-K1 genome, respectively. The antiapoptotic viability, as well as the yield of r-protein (GLuc) production, was increased by 6,4-fold and 5,55-fold, respectively (Wang et al., 2018).

3.3. CRISPR-Cas mediated gene regulation studies

Another CRISPR-Cas strategy for increasing r-protein production in CHO cells is CRISPR interference (CRISPRi). For instance, Shen et al. (2017) employed an additional selection pressure using CRISPR-dCas9-assisted suppression of dihydrofolate reductase (*dhfr)* gene transcription (~85%) alongside methotrexate (MTX) selection. The stimulated CHO-DUXB11 cells amplify more copies of *dhfr* and adjacent *egfp* gene as MTX inhibits DHFR, a key enzyme for biosynthesis of glycines, purines and thymidylic acid. Therefore, following MTX-based selection and in response to increased levels of MTX, the surviving cells carry more *dhfr* gene copies (to compensate for its inhibition) and more copies of the GOI that is cloned adjacent to it (Fig. 2D). Compared to a selection method based on MTX alone, with the help of the extra selective pressure generated by CRISPRi, the authors achieved a ~3-fold increase in *egfp* copy number and ~3,8-fold increase in EGFP expression. Furthermore, using the CRISPRi approach, they successfully enhanced the production of granulocyte colony-stimulating factor (G-CSF) ~2,3-fold by swapping *egfp* for *gcsf* as the GOI adjacent to *dhfr*. Importantly, the growth was not affected by the extra pressure exerted by the CRISPRi system (Shen et al., 2017). Another study utilised a similar CRISPRi system to target endogenous apoptotic genes (*Bax*, *Casp3*) (Xiong et al., 2019). The authors constructed different repressor domains namely KRAB-dCas9, dCas9-KRAB and dCas9-KRAB-MeCP2 to find the most efficient fusion construct. Consequently, using CHO codon-optimised dCas9-KRAB-MeCP2, they successfully achieved a significant decrease (~10%) in apoptosis and an increase (~57%) in viable cell density (Xiong et al., 2019).

Another way for regulating gene expression in CHO cells is using the type VI CRISPR system, which includes several effectors such as Cas13a, Cas13b and Cas13d (Abudayyeh et al., 2017; Burmistrz et al., 2020). Unlike Cas9, Cas13 specifically binds and cleaves RNA recognised by a short CRISPR RNA (crRNA) (Abudayyeh et al., 2017); therefore, these systems can be repurposed for transcript knockdown or RNA editing in mammalian cells (Lin et al., 2020). Shen et al. harnessed this system to simultaneously suppress the transcription of multiple genes involved in lactate metabolism (*PDK1*) and apoptosis (*BAK*, and *BAX*). Consequently, mRNA levels were decreased by 96% on average, whereas cell viability was modestly decreased compared to that of non-targeting control; IgG titer, the r-protein of interest, was increased by ~1,6 fold (Lin et al., 2021a). In a similar study, CRISPR-Cas13d-based multiplex gene repression resulted in extended cell survival and increased production of IgG (~1,6 fold), following simultaneous knockdown of *LDHA*, *GFT* and *DDIT3* (Shen et al., 2020).

Overall, these studies show that despite the complexity of mammalian cells, there are many strategies to improve r-protein production, as described in Fig. 2 and summarised in Table 1. In this regard, CRISPR-Cas technology provides efficient and flexible genetic manipulation and allows for the generation of more productive CHO cell lines, leading to more cost-efficient production of biopharmaceuticals.

4. CRISPR-Cas strategies to modulate glycan structures of glycoproteins

Glycan composition is one of the main factors that contribute to the quality of recombinant proteins. It affects protein folding, solubility and intracellular trafficking, consequently influencing half-life, biodistribution and in vivo activity of recombinant biopharmaceuticals (Gupta and Shukla, 2018; Li et al., 2019; Tian et al., 2019). Therefore, efforts have focused on engineering glycosylation to achieve more efficient humanised r-proteins by targeting genes involved in glycosylation in CHO cells. To this day, programmable nucleases such as ZFNs and TALENs have been used (Haryadi et al., 2013; Heffner et al., 2018). Nonetheless, CRISPR-Cas based methods are simpler to engineer besides being more reliable, efficient and cost-effective (Javed et al., 2018; Li et al., 2013). Hence, this section examines recent studies that employed CRISPR-Cas systems to disrupt and/or regulate single or multiple genes related to glycan composition using Cas9, Cas13 or dCas9 fused with effector domains. In addition, the summary of these studies is presented in Table 2.

4.1. CRISPR-Cas9 mediated gene knockout studies to modulate glycan structures of glycoproteins

Recombinant monoclonal antibodies can exert their therapeutic effect through an immune mechanism known as antibody-dependent cellular cytotoxicity (ADCC). De/afucosylated antibodies have been shown to exhibit higher in vivo ADCC than their fucosylated counterparts, since fucosylation can hinder antibodies from binding to their target (Zong et al., 2017). Antibody defucosylation is a very common r-proteins glycan optimisation approach to improve antibody efficiency. Defucosylation can be achieved by inactivating the transfer of fucose residues to glycoproteins. Several studies have focused on the disruption of the *FUT8* gene to produce fully defucosylated antibodies using CRISPR-Cas9 (Chung et al., 2017a; Ronda et al., 2014; Sun et al., 2015; Zong et al., 2017). The proteins derived from FUT8 KO CHO cells can exhibit up to 100-fold higher antibody-dependent cellular cytotoxicity (ADCC) than that produced in wild type cells. In this aspect, defucosylated antibodies showed superiority in ADCC activity for destroying breast cancer cells (Zong et al., 2017). Other methods for inhibiting fucosylation of antibodies rely on reducing the levels of GDP-fucose in the Golgi, which can be achieved by de novo engineering or modifying GDP-fucose biosynthesis pathways, or impeding the transport of GDP-fucose into the Golgi by disrupting *FX* and *Slc35c1* genes (Chan et al., 2016; Louie et al., 2017). For instance, *Slc35c1* has been knocked out using three different genome editing methods, namely CRISPR-Cas9, TALENs and ZFNs. ZFNs was proven as the least efficient technique in terms of genome modification, while TALENs and CRISPR-Cas9 have managed to induce mutations with similar efficiencies. Interestingly, the mutant clones generated by TALENs exhibited comparable antibody production levels to parental cell lines, in contrast to the genetic manipulation with ZFNs and CRISPR-Cas9, where production levels were decreased by ~50%. However, none of these compromised cell growth or viability (Chan et al., 2016). To understand the effects of fucosylation on antibody efficacy, multiple cell lines capable of producing either fucosylated or defucosylated antibodies may need to be developed. This generally involves extensive screening of single clones. To shorten this process, Louie et al. used CRISPR-Cas9 to delete the *FX* gene involved in the de novo pathway of GDP-fucose synthesis in the absence of fucose (Louie et al., 2017). This resulted in a cell line capable of expressing either fully defucosylated antibodies or primarily fucosylated antibodies in the presence of fucose (via a salvage pathway leading to GDP-fucose production). Consequently, this system allowed a direct comparison for the effect of fucosylation on antibody efficacy using only one cell line, and allowed for the optimisation of fucosylation amount on glycoproteins by varying fucose titer in culture medium (Louie et al., 2017).

Another approach to increase ADCC of antibodies (IgGs) is by enhancing the ratio of IgG with α-2,6 sialylation via knockout of two α-2,3 sialylatransferases, along with the overexpression of α-2,6 sialyltransferase. α-2,3 sialyation results in decreased antibody binding to the therapeutic target, thus reducing their ADCC activity. To achieve this, CRISPR-Cas9 was used to delete *ST3GAL4* and *ST3GAL6* genes in CHO-K1 cells, which displayed no α-2,3 linkages on the expressed IgG according to sialidase treatment (Chung et al., 2017b). On the other hand, Ha et al. (2020) attempted to increase α-2,6 sialylation levels of

Table 2
The summary of CRISPR-Cas studies that rely on modulating glycan structures of glycoproteins.

Cell Type	CRISPR Based Genome Editing strategy	Goal	Important Result/s	R-protein	Cell viability and growth	Target Gene/s	Reference
CHO-K1	CRISPR-Cas9-Mediated Knockout	To inhibit fucosylation	11–42,5% indel frequencies 85% of indels induced frameshift mutations	–	–	FUT8	(Ronda et al., 2014)
CHO-K1	CRISPR-Cas9-Mediated Knockout	To inhibit fucosylation	9–25% indel frequencies	IgG	Comparable	FUT8	(Sun et al., 2015)
CHO-S	CRISPR-Cas9-Mediated Knockout	To inhibit fucosylation	16% indel frequency 25-fold higher ADCC activity	IgG1	Comparable	FUT8	(Zong et al., 2017)
CHO-K1	CRISPR-Cas9-Mediated Knockout	To inhibit fucosylation	6.4% indel frequency	ANTI-HER2	Comparable	SLC35C1	(Chan et al., 2016)
CHO-K1	CRISPR-Cas9-Mediated Knockout	To inhibit fucosylation and activate fucosylation in a fucose-dependent manner	Maximum %ADCC can be induced by 50–60% afucosylated antibodies	IgG1	Comparable (Cell growth)	FX	(Louie et al., 2017)
CHO-S	CRISPR-Cas9-Mediated Knockout	To limit glycosylation to early intermediates	Hybrid or complex glycan species decreased from 75% to 0,53%	HIV-1 envelope protein (Env) gp120	Comparable	MGAT1	(Byrne et al., 2018)
CHO-K1	CRISPR-Cas9-Mediated Knockout	To produce an IgG with an increased galactosylation content and inhibit fucosylation	Production of IgG with ~80% bigalactosylated and afucoyslated glycoforms	IgG	–	FUT8, ST3GAL4 AND ST3GAL6	(Chung et al., 2017a)
CHO-K1	CRISPR-Cas9-Mediated Knockout	To produce an IgG with an increased α-2,6 sialylation content and minimize α-2,3 sialylation	Production of >77% sialylated glycans and > 62% biantennary disialylated glycans	IgG	–	ST3GAL4 and ST3GAL6	(Chung et al., 2017b)
CHO-S	CRISPR-Cas9-Mediated Knockout	To mitigate immunogenic epitopes (N-glycolylneuraminic acids) fused to r-proteins	4.9–11% indel frequencies ~68% and ~ 53% decrease in mRNA expression of CMAH-62 and CMAH-1	EPO	Increased	CMAH	(Chai et al., 2020)
CHO-DG44	Sequentially CRISPR-Cas9-Mediated Knockout	To maintain sialylation	2–8% knockout efficiency 1.4-fold more EPO concentration 3.0-fold more sialic acid content	EPO	Comparable	NEU1, 2, and 3 BAK and BAX	(Ha et al., 2020)
CHO-S	CRISPR-dCas9-VPR-mediated gene activation	To express therapeutic glycoproteins with humanised glycan structures	Achieved ~87-fold MGAT3, ~880-fold St6gal1 gene activation and no activation of ALDH18A1	–	–	ALDH18A1, MGAT3 AND ST6GAL1	(la Karottki et al., 2020)
CHO-DuxB11	CRISPR-dCas9-TET1 mediated gene activation (de-methylation) CRISPR-dCas9-DNMT3A mediated gene silencing (methylation)	To stably activate and subsequently repress an endogenous gene	Stable gene activation in ~60% of transfected cells (>80 days) 5.4-fold decrease in ST6GAL1 mRNA expression	EPO-Fc	Comparable (growth)	ST6GAL1	(Marx et al., 2018)
CHO-DUXB11	CRISPR-dCas9-mediated gene silencing (methylation)	To stably repress an endogenous gene by different effector domains (DNMT3A, DNMT3A3L, KRAB and LSD1)	78% lower FUT8 mRNA expression (dCas9-DNMT3A3L) 3,1-fold FUT8 gene repression (dCas9-DNMT3A3L)	BFP	–	ST6GAL1 FUT8	(Marx et al., 2021)
CHO-S	Multiplexed CRISPR-Cas9 Mediated Gene Knockout	To generate agalactosylated r-proteins	~94% agalactosylated N-glycans (EPO) ~97% agalactosylated N-glycans (Rituximab)	EPO Rituximab	Decreased (growth)	B4GAL-T1, 2, 3	(Amann et al., 2018)
CHO-S	Multiplexed CRISPR-Cas9 Mediated Gene Knockout	To produce recombinant human plasma proteins with a humanised glycosylation pattern	Increased the A2G2 glycan structure on r-proteins from 3.5% to ~80%	α-1-antitrypsin and C1 esterase inhibitor	Comparable	MGAT4A, MGAT4B, MGAT5, ST3GAL3, ST3GAL4, ST3GAL6, B3GNT2, FUT8, SPPL3 AND GLUL	(Amann et al., 2019)
CHO	CRISPR-Cas9 mediated Knockout Screen	To screen all the substantial glycan variations that influence the biodistribution of lysosomal enzymes	3-fold extended circulation time (N-glycans capped with α2–3SA on R-proteins)	α-galactosidase A	Comparable	43 genes were targeted (alone or in combinations)	(Tian et al., 2019)
CHO-K1	Cas13d-mediated gene knockdown	To inhibit fucosylation	~53% GFT knockdown	IgG	–	GFT	(Lin et al., 2021a)

ADCC: Antibody-dependent cellular cytotoxicity.

recombinant human erythropoietin (rhEPO) by sequentially disrupting three sialidase genes using CRISPR-Cas9 and creating a large deletion at the target locus. Additionally, they knocked out two pro-apoptotic genes, namely *BAK* and *BAX*, which helped to further increase the sialic acid content and the proportion of highly sialylated glycans. Consequently, after 10 days of culture, the sialic acid content of rhEPO was 3-fold higher than that of WT control without compromising cell growth and viability (Ha et al., 2020).

Beyond increasing ADCC of antibodies, some studies used CRISPR-Cas9 to control glycosylation heterogeneity for improving the binding of glycan–dependent antibodies to antigens. This can be achieved through the disruption of genes involved in N-linked glycosylation. For instance, Byrne et al. inactivated the *MGAT1* gene in CHO cells, which led to the expression of gp120, an HIV envelope glycoprotein, with early oligomannose glycans. This way, the heterogeneity of N-glycosylation on gp120 was reduced, leading to improved binding of antibodies to the target epitope(Byrne et al., 2018). In addition to disruption of MGAT1, another group used CRISPR-Cas9 system to delete the *C1s* gene, which encodes a serine protease responsible for proteolysis of gp120 (Li et al., 2019). Gene disruption by CRISPR-Cas9 has been also used to mitigate immunogenic epitopes fused to r-proteins, resulting in CHO cell lines expressing more human-like therapeutic glycoproteins (Chai et al., 2020).

4.2. Multiplex genome editing studies in CHO cells to modulate and screen glycan structures of glycoproteins

CRISPR-Cas based methods allow for easy manipulation of multiple genes at once by concurrently expressing multiple gRNAs. Therefore, recent studies have taken advantage of this system and increased the number of target genes to further optimise the glycosylation of r-proteins expressed in CHO cells. In one study, the authors introduced indel mutations in 4 *B4Gal-T* gene isoforms responsible for galactosylation of N-glycans. IgG with agalactosylated N-glycans can be helpful in the therapy of lupus, since patients treated with agalactosylated antibodies showed improved symptoms (Amann et al., 2018). The use of different indel combinations allowed the authors to detect the effect of 4 genes separately and simultaneously. In contrast to previous studies, they found that *B4Gal-T4* does not have a substantial effect on galactosylation of N-glycans, since they achieved ~1% galactosylation by disrupting genes encoding only for the other 3 isoforms (Amann et al., 2018). Another study used CRISPR-Cas9 to target 10 genes in a multiplex manner to produce recombinant alpha-1-antitrypsin (A1AT) and plasma protease C1 inhibitor (C1INH) to produce r-proteins with humanised N-glycan structures (Amann et al., 2019). This was an important effort since the current production of these proteins relies on their isolation from human donor blood based on glycosylation differences between proteins expressed in human and CHO cells. However, the method limits the production scale and poses risks due to infectious particles, particularly non-enveloped viruses (Soucie et al., 2013). Consequently, N-glycan analysis of recombinant lA1AT and C1INH showed that their N-glycosylation profiles were fully humanised and exhibited comparable in vitro activity to native human plasma A1AT and C1INH. Moreover, SDS-PAGE indicated higher purity of recombinant human C1INH than that of human plasma lC1INH, demonstrating that engineered CHO cells may express higher quality proteins with comparable activities. However, it was also reported that multiplex CRISPR-Cas9-based gene disruption can impair cell growth due to off-target effects or deletions of genes that can significantly impact cell culture.

Since the glycan composition affects tissue targeting, biological activity and pharmacokinetics of glycoproteins, therapeutic proteins with distinct N-glycans can be used for certain applications (Sethuraman and Stadheim, 2006). CRISPR screening is a promising approach to evaluate thousands of genes that influence the glycosylation of r-proteins, thereafter expressing proteins with varying glycan structures. In this regard, a CRISPR-Cas9 based screen provided an alternative to select optimised N-glycan moieties for better delivery of lysosomal enzymes to hard-to-reach organs (Tian et al., 2019). It allowed extensive comparison between several substantial glycoforms of r-proteins expressed in CHO cell lines. Based on the CRISPR-Cas9-based comprehensive screen in the study, it was found that α-galactosidase A with a glycan structure capped with α2–3-linked sialic acid could increase circulation time and improve biodistribution in a mouse model of Fabry disease (a condition due to deficient α-galactosidase A) (Tian et al., 2019). Hence, it is safe to suggest that such large-scale screens can allow researchers to explore the key glycan structures that influence cellular uptake and biodistribution of r-proteins, thus facilitating the design of improved biotherapeutics.

4.3. CRISPR-Cas mediated gene regulation studies related to glycosylation of r-proteins

Targeted gene disruption is one of the leading strategies to optimise the protein production process in CHO cells. However, gene knockouts may not be sufficient to provide elaborate controls for glycosylation levels, which is important for manufacturing biopharmaceuticals (Hansen et al., 2017). CRISPR-Cas9 system can be repurposed to regulate transcription or for epigenome editing by fusing a dCas9 with an enzymatic domain such as epigenetic effectors or transcriptional activator/repressor (Sung and Yim, 2020; Xu and Qi, 2019). For instance, if dCas9 is fused to a transcriptional activator like the VPR, it can be used to increase the expression of endogenous genes by targeting the gRNA to a region upstream of the transcription start site. This approach was harnessed to activate three silenced glycosyltransferases and express therapeutic glycoproteins with humanised glycan structures (la Karottki et al., 2020). la Karottki et al. (2020) designed three gRNAs for each gene; interestingly, the most potent activation was obtained when all three gRNAs were transfected. Consequently, they increased the transcription of two genes, namely *Mgat3* (~87-fold) and *St6gal1 (~880-fold),* engineering CHO cells to synthesise glycoproteins with correct glycan structures. Surprisingly, all of the designed gRNAs failed to activate *Aldh18a1,* indicating the critical importance of gRNA design and identification of suitable endogenous target genes (la Karottki et al., 2020). A similar approach has been designed for targeted demethylation and subsequent methylation of a given gene promoter using TET1 and DNMT3A as catalytic domains, respectively. To increase the number of catalytic domains recruited at a specific region of the genome, the authors used the SUperNova tagging system, which enables multiple copies of TET1 and DNMT3a to be recruited at the target site (Fig. 3A) (Marx et al., 2018). Using this strategy, the authors first activated the *St6gal1* gene and observed sustained upregulation for over 80 days in nearly 60% of transfected CHO cells, while no negative effect on cell growth or r-protein productivity was observed. Following the targeted methylation, mRNA expression levels of *St6gal1* were decreased up to 5.4-fold. In a further study, a similar DNA methylation strategy was used to target a recombinant viral promoter, CMV, and endogenous *FUT8* promoter, in addition to the promoter of *St6gal1* (Marx et al., 2021). Chromatin immunoprecipitation assays verified that the targeted methylation using dCas9 fusions with DNMT3A and DNMT3A3L promoted repressive heterochromatin formation on these promoters. On the other hand, demethylating dCas9 fusions with TET1 caused heterochromatin repressive marks to disappear and promoted active promoter histone marks. This underscores that epigenetic engineering using dCas9-effectors might be an alternative to engineer CHO cells in a stable, tunable and reversible manner.

CRISPR-Cas13 system is another powerful tool for multiplex gene regulation in CHO cells (Blomeier et al., 2021; Lin et al., 2021a; Shen et al., 2020). A recent study demonstrated that Cas13d could be used for the knockdown of three genes in a multiplex manner with average efficiencies of ~60%. Although the cell growth rate was slower than that of the control, the stable cell line expressing Cas13 produced 59 ± 6% more IgG with less fucosylation, resulting in enhanced ADCC as a result of gene knockdown (Lin et al., 2021a).

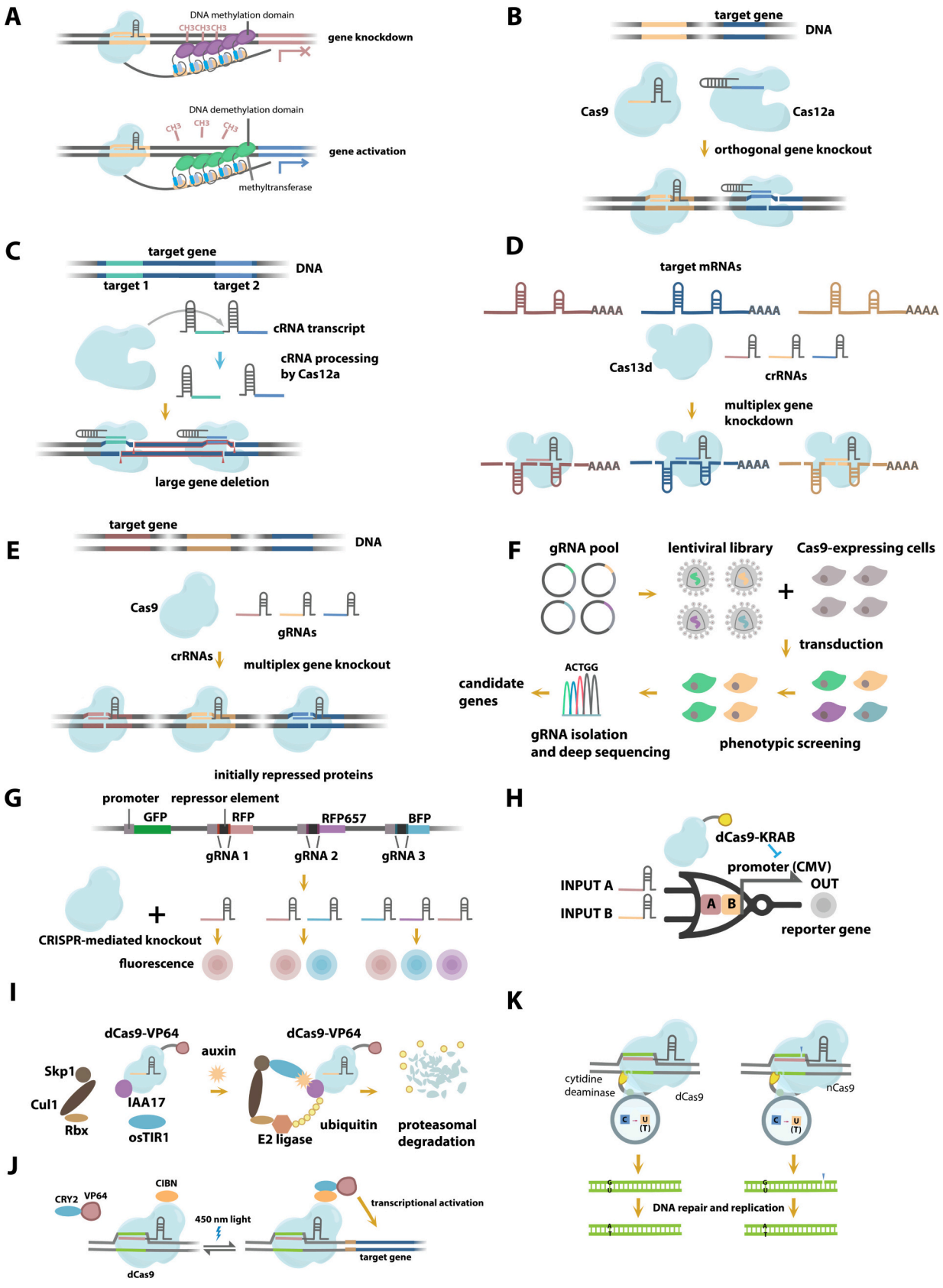

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Fig. 3. CRISPR-based gene editing and regulation methods in CHO cells. (A) Multiplexed orthogonal gene knockout using both Cas9 and Cas12a. Because each gRNA are activated only in the presence of corresponding enzyme, it will allow the editing of multiple genes without crosstalk. (B) CRISPR-Cas12a mediated large gene deletion. Cas12a can process a single transcript containing two crRNAs that target two distinct sites in a single gene, which generates a large gene deletion. (C) Cas13d-mediated multiplex gene knockdown. mRNAs that encode different proteins can be degraded by RNA-guided RNA cleavage. (D) Cas9-mediated multiplex gene knockout. Genes that encode different proteins can be degraded by RNA-guided DNA cleavage. (E) CRISPR-Cas9 mediated loss-of-function screening. gRNA plasmids are transfected into HEK293 cells, lentiviruses each of which contains a single gRNA are harvested and used for transduction in Cas9-expressing CHO cells. Following phenotypic screening, enriched gRNAs isolated and sequenced; candidate genes are then identified. (F) CRISPR-Cas9 mediated gain-of-function screening. A plasmid carrying initially repressed fluorescent proteins are stably integrated into CHO cells. By targeting different combinations of gRNAs to the repressor elements, it is achievable to express proteins in every combinations using only one cell line. (G) dCas9-KRAB based NOR gate. The NOR gate is inducible by two different gRNAs, the reporter protein is only expressed in the absence of two gRNAs. (H) CRISPR-Based site-specific epigenetic editing. Using DNA methylation and demethylation domains fused to dCas9, gene knockdown and activation can be performed, respectively. (I) Drug-induced dCas9-VPR mediated gene repression. In the presence of auxin, IAA17-dCas9-VPR are polyubiquitinated and degraded by the proteasome; the gene activation by dCas9-VPR are then inhibited. (J) Base editing. Cytidine deaminase fused with dCas9/nCas9 is used for programmable C to U editing. After DNA repair and replication cytosine is replaced with thymine. (K) Light-activated gene upregulation. Following light activation, dCas9-CIBN recruits CRY2-VP64, and generated fusion protein upregulate the target gene.

Taken together, the CRISPR-Cas based gene regulation system can be used in applications where endogenous gene activation/inactivation is required without changing the CHO genome, which may induce random disruption of genes and cause undesired effects. Moreover, multiple targets can be regulated at once by designing multiple gRNAs/crRNAs in CHO cells to generate glycan structures for all relevant objectives in a single procedure, such as increasing in vivo half-life and activity of glycoproteins, producing human-like structures as well as delivering r-proteins to desired organs.

5. Future trends/novel developments of CRISPR-Cas tools/ applications in CHO cells

In the following paragraphs, the recent methods to optimise the CRISPR-Cas system in CHO cell lines are discussed (see also Table 3), and possible future applications of CRISPR to achieve a tuneable, reversible, or highly stable editing of CHO cells.

5.1. Novel Cas nucleases for CHO cells genome editing and gene regulation

CRISPR technology can gain new functions as scientists discover new Cas nucleases with various properties, including alternative protospacer adjacent motif (PAM) recognition sequences and the ability to target different nucleic acids (DNA or RNA). For this reason, researchers have tested and used new Cas enzymes as an alternative to Cas9, to improve and facilitate genome editing or create more complex systems, achieving parallel engineering without crosstalk (Fig. 3B). To this end, Schmieder et al. (2018) utilised either Cas9 or Cas12a (Cpf1) nucleases to induce a large deletion using two different gRNAs, which enabled not only the disruption of coding sequences in the CHO genome but also the modification of regulatory regions (as frame-shift mutations are not effective to regulate these sites). In addition, inducing large deletion mutations by introducing two gRNAs provides easy detection of genomic alteration, which eliminates the need for other time-consuming methods. Moreover, since Cas12a is capable of pre-crRNA processing, it does not need any additional sequence (tracrRNA) to process the transcript, thereby allowing more crRNA to be placed into a single plasmid and facilitating multiplex gene editing (Schmieder et al., 2018) (Fig. 3C). Such a design would be helpful to generate a genome-wide knockout library that includes non-coding regions in the CHO genome, which have yet to be studied. On the other hand, by using Cas13, an RNA-guided RNA ribonuclease, researchers can knock down numerous genes by depleting specific mRNA transcripts in a multiplex manner (Fig. 3D), which can be an alternative to dCas9-based gene repression or knockout (Fig. 3E). Accordingly, recent studies reported that Cas13d (from *Ruminococcus flavefaciens*) can successfully repress three genes simultaneously in CHO cells (Lin et al., 2021a; Shen et al., 2020). It is highly possible to increase the number of genes targeted when using Cas13d since it can process the associated pre-crRNA into mature crRNAs like Cas12a, thereby allowing the integration of many crRNAs into a single transcript (Perčulija et al., 2021b). Among the drawbacks of this method is that Cas13 has collateral cleavage activity. In other words, it transforms into a nonspecific RNase upon activation by ssRNA sequence bearing complementarity to its crRNA spacer, which then degrades surrounding RNA non-specifically (Konermann et al., 2018). Although the collateral cleavage activity has not yet been observed in mammalian cells, no study directly investigates this activity in CHO cells and therefore further rational design and optimization of this method is recommended (Malci et al., 2022). Given the decreased cell density in the studies that used Cas13d for gene knockdown, such activity needs to be investigated regarding its negative effects on CHO cells (Konermann et al., 2018; Shen et al., 2020).

5.2. Gain or loss-of-function pooled CRISPR screening

CRISPR screening is a promising approach to evaluate thousands of genes that influence r-protein production in CHO cells. These studies can rely on either gain- (Eisenhut et al., 2018) or loss-of-function strategies (Fig. 3F and G) (la Karottki et al., 2021; Xiong et al., 2021). Furthermore, in different mammalian cells including HEK293T, researchers have used dCas9-based transcriptional regulation elements to screen genes involved in a relevant physiological activity (Gasperini et al., 2019). Similar studies based on dCas9-mediated repression or activation can be designed in CHO cells to screen genes using knockdown strategy and investigate the effects of a particular gene or the combination of genes on viability, cell density and productivity. Moreover, instead of RNAi screening, Cas13d-based screen can also be used to identify genes involved in protein production in CHO cells, as relevant studies in human cell lines indicated that Cas13d-based screen outperformed siRNA screening in terms of specificity (Cao et al., 2021; Xu et al., 2020; Zhang, Y. et al., 2021). Following this approach, researchers mapped the repressed genes during the adaptation process of CHO cells to a suspension culture by taking advantage of transcriptome sequencing (Lee et al., 2016b).

Recently, a genome-wide CRISPR-Cas9 knockout screen was performed to uncover the genes related to the unfolded protein response (UPR) (Xiong et al., 2021). Instead of lentiviral delivery, the authors used recombinase-mediated cassette exchange (RMCE) to generate gRNA expressing transgenic CHO cells. Among 18,353 genes, 76 genes were identified and validated as potential target genes to reduce ER stress. They investigated these genes in further experiments and, following the deletion of Zfx (Zinc-Finger Protein X-Linked) that encodes a protein involved in cell proliferation and apoptosis, viable cell density increased ~5-fold compared to two controls after 4 days of ER stress inducer treatment (tunicamycin).

Therefore, in this regard, CRISPR-dCas9- or Cas13d based screening platforms seem to offer suitable alternatives to gene knockdown and gene knockout to investigate the effects of a repressed gene or the combination of genes in CHO cells.

Gain-of-function screening is helpful to investigate the effects of genes on cellular phenotype; however, due to high variation between

Table 3
The summary of CRISPR-Cas studies in CHO cells.

Cell Type	CRISPR Based Genome Editing strategy	Major Advantage/s	Major Limitation/s	Possible application/s	Delivery method	Reference
CHO-K1	nCas9- and dCas9-PmCDA1 mediated Base Editing	Less cell toxicity than Cas9-mediated gene knockout	Restricted targetable sites	Gene knockout	Plasmid transfection	(Nishida et al., 2016)
CHO-K1	Cas9-mediated multiplex genome editing by iterative transfection	Better site-specific mutation (~1,14–2 fold)	Only two genes were targeted in the multiplex context	Multiplex gene knockout	Plasmid transfection	(Shin et al., 2015)
CHO-K1 HEK293T	Cas12a- and Cas9-mediated gene knockout	Ability to use CRISPR-Cas system for two different intentions in the same cell without any crosstalk. Ability to target regulatory regions of the CHO genome Easy-readout by conventional PCR/qPCR	One plasmid contains two gRNAs for a single target, limiting the number of possible targets to be integrated into a single transcript due to the maximum size limit	A genome-wide deletion library including non-coding regions	Plasmid transfection	(Schmieder et al., 2018)
CHO-K1 CHO-DUXB11 CHO-DG44	Cas13d-mediated multiplex gene knockdown	Rapid generation of stable pools with ~80%–90% knockdown Highly efficient and specific compared to RNA interference	Reducing cell density	Multiplex gene knockdown	Plasmid transfection Recombinase aided genomic integration (Sleeping Beauty-mediated)	(Shen et al., 2020)
CHO-K1	Cas13d-mediated multiplex gene knockdown	Rapid generation of stable pools with ~80%–90% knockdown Highly efficient and specific compared to RNA interference	Reducing viable cell density	Multiplex gene knockdown	Plasmid transfection Recombinase aided genomic integration (Sleeping Beauty-mediated)	(Lin et al., 2021a)
CHO-K1 HEK293T	dCas9-KRAB based genetic circuit (NOR gate)	Controlling single gene expression using two different gRNAs	The repression efficiency is ~%63 lower in CHO cells than that of HEK293T cells	Dynamic control of metabolic pathways	Plasmid transfection	(Lebar and Jerala, 2016)
CHO-K1 HEK293FT	Drug-induced repression of dCas9-mediated gene activation	Tunable gene repression (By using different drug-concentrations achieving dose-dependent repression of gene expression)	Irreversible effect	To assess the difference of a given gene up- and downregulation in a dosage-controlled manner	Recombinase aided genomic integration (Flp-mediated)	(Kleinjan et al., 2017)
CHO-K1 CHO-DG44	Light-induced dCas9-mediated gene activation	Tunable and reversible gene activation	Short-term activation (48 h)	To assess the difference of a given gene up- and downregulation within a short period in a light-dependent and reversible manner	Plasmid transfection Lentiviral transfection	(Minami and Shah, 2021)
CHO-K1	Cas9-mediated gene knockout (using preassembled Cas9 protein and gRNA complex)	The reduced risk for the unwanted integration into the CHO genome Achieving increased indel frequencies with less off-target effects	–	Multiplex gene knockout with less off-target effects	Ribonucleoprotein	(Lee et al., 2016b)
CHO-K1	Cas9-mediated multiplexable activation of artificially repressed genes	Eliminating time-consuming subcloning and screening experiments	It is tested only for a limited number of genes (up to 3)	Screening of the overexpression of single gene or multiple industrially relevant (up to 3) genes in various combination/s	Plasmid transfection Random genome integration	(Eisenhut et al., 2018)
CHO-S	Cas9-mediated large-scale loss-of-function screening	The ability to select relevant genes among thousands of genes	The lack of multiple knockout mutants	Finding new candidate genes in the CHO genome that are involved in r-protein production	Lentiviral transduction	(la Karottki et al., 2021)

subclones of CHO cells, a thorough investigation of the effects is difficult. To address this, a screening method was developed, which allowed overexpression of three genes with all different combinations (Eisenhut et al., 2018). In this system, all genes were first repressed and then transfected into CHO cells to be activated by CRISPR-Cas9 mediated deletion of the repressor element. Thus, by adding the corresponding gRNA(s) to the system, it is possible to achieve the activation of the selected gene(s) (Fig. 3G). A further step would be increasing the number of transfected genes in the system, thereby assessing the effects of numerous genes within the same setting. More recently, a large-scale pooled CRISPR loss-of-function screening (Fig. 3F) using approximately 16,000 gRNAs was employed to identify key genes that affected CHO cell metabolism upon glutamine deprivation, which is a substantial selection pressure for industrial applications (la Karottki et al., 2021) Consequently, among numerous genes, the authors found that the disruption of *Abhd11* enhanced the growth of CHO cells in a glutamine-free culture medium. These data clearly indicate that, as the number of genome-scale CRISPR screening studies increases, more unstudied genetic targets will be discovered, and with the help of new CRISPR techniques (Eisenhut et al., 2018; Lian et al., 2019), it will be possible to

assess their synergistic effect on the growth of CHO cells and r-protein production.

5.3. Engineering biological circuits in CHO cells

CRISPR-Cas system can be also used to construct biological synthetic circuits, which are designed to produce desired outputs in response to multiple user-defined inputs (Mukherji and van Oudenaarden, 2009; Xia et al., 2019). More specifically, genetic circuits would allow us to program gene expression using various inputs such as drugs, light, or other small molecules, thereby creating a biological machine that is tunable and controllable (Xia et al., 2019). Since it is programmable and easy to design, the CRISPR-Cas system has been used for several synthetic biology applications in bacteria, yeast and mammalian cells (Brooks and Alper, 2021; Xie and Fussenegger, 2018). However, there are limited studies using CRISPR-Cas based synthetic circuits in CHO cells (Kleinjan et al., 2017; Lebar and Jerala, 2016; Minami and Shah, 2021). for example, designed a NOR gate using dCas9-KRAB targeting a reporter gene in CHO cells. The NOR gate was inducible by two different gRNAs, where gRNAs had 7 recognition sites upstream of the reporter gene. In this system, the reporter protein was only expressed in the absence of two gRNAs (Fig. 3H). Importantly, the repression efficiency was lower in CHO cells (~11-fold) than that in HEK293T cells (~30-fold). Therefore, future studies should optimise the systems in CHO cells to achieve efficient dynamic control of gene expression. Further research should also be conducted to express these gRNAs via inducible promoters, thus dynamically controlling possible relevant metabolic pathways or cell growth and viability, which are crucial to r-protein production in CHO cells. Another study related to the field of synthetic biology showed that auxin (a plant hormone) could be used to induce degradation of dCas9-VPR in a dose-dependent manner by forming a degradable complex with auxiliary proteins, one of which was heterologously expressed (Fig. 3I) (Kleinjan et al., 2017). Such a system would allow tunable gene activation in mammalian cells. Although these studies were mainly based on HEK293FT cells, they also proved that the system can efficiently work in CHO cells. Furthermore, by using different condition-dependent promoters driving different components of this system including dCas9, degron (auxin) and auxiliary proteins (osTIR1), as well as different Cas orthologs simultaneously, it should be possible to engineer CHO cells in a specific, dosage- and timing-controlled manner.

5.4. Tools for reversible gene expression in CHO cells

Reversible gene expression can be useful for CHO cell engineering. However, popular drug-inducible systems do not offer rapid or cheap reversible platforms as they rely on the replacement of the culture medium. Due to the dynamic nature of r-protein production, light-based control could offer a more effective way to control genes that affect cell culture differently at different time points throughout the culture period. Recently, Minami and Shah (2021) investigated the use of optogenetics in CHO cells. By taking advantage of the light-activated CRISPR-dCas9 effector (LACE) system, they aimed to achieve temporal regulation of a model protein (eGFP) (Fig. 3J). Consequently, compared with the control, eGFP expression was increased up to 4-fold in response to light. However, an important problem regarding this method was the low stability of the system due to transient transfection, which efficiently worked for only 48 h. To solve this problem, the authors tried to stably express all components of the system (apart from eGFP) using lentiviral transfection but resulted in decreased signal-to-noise ratio. Stable expression of LACE components would be an important development to enhance light-induced CRISPR-based gene regulation. In this regard, following transient gene expression, it could be possible to generate stable cell lines or use different gene integration strategies such as recombinase assisted methods (Gaidukov et al., 2018). Recently, another light-based reversible system, a CRISPR-Cas13b-based optogenetic tool (PspCas13b), was designed to mediate mRNA knockdown in response to blue-light in HEK-293 T cells and was tested in CHO-K1 cells, achieving a comparable decrease in relative Firefly luciferase (FLUC) luminescence to that in HEK-293 T cells (~80%) (Blomeier et al., 2021). In the presence of blue light, PspCas13b was expressed and cleaved the mRNA of interest upon recognition, hence inducing downregulation of the given gene. The authors further enhanced the repression efficiency by integrating another light-induced tool into the system involving transcriptional repression and protein degradation, which reached up to 99% decrease in luminescence levels under blue light in HEK-293 T cells. Such systems remains to be explored in CHO cells in future studies (Blomeier et al., 2021).

Currently, although numerous reports have used CRISPR-Cas9 mediated single-gene knockout to obtain the desired phenotype in CHO cells (Chai et al., 2020; Fukuda et al., 2019; Ha et al., 2020; McVey et al., 2016; Raab et al., 2019; Sun et al., 2015), a substantial number of these engineered cell lines exhibited decreased cell viability, growth or cell density for which Cas nucleases mediated off-target effects might be responsible (Lin et al., 2021b; McVey et al., 2016; Raab et al., 2019; Wilkens et al., 2019). To overcome this problem, recent methods such as base editing (Fig. 3K) and prime editing could be a better alternative to CRISPR-Cas9 mediated KO in CHO cells, since they induce much lower off-target editing than Cas9 nuclease (Anzalone et al., 2019; Gaudelli et al., 2017; Nishida et al., 2016). Even though these methods were mainly studied in HEK293T cells, it is highly possible that soon, these techniques might be used in CHO cells in a standardized way (Beal et al., 2020), and provide gene knockout with reduced cytotoxicity, thereby resulting in comparable cell growth and viability besides promoting r-protein production.

6. Conclusion and future remarks

With the rapid expansion of the CRISPR toolbox, elaborate control of gene manipulation has become easier, which is important for generating cell factories that are more suitable for the production of biopharmaceuticals. However, there is still a need for many CRISPR emerging techniques to be reported in CHO cells; therefore, more research in these cells is needed to realize the full potential of this technology. For instance, preliminary studies in CHO cells mostly used natural CRISPR-Cas variants (Grav et al., 2015; Lin et al., 2021b; Lu et al., 2018; Schweickert et al., 2021; Shen et al., 2020); however, engineered CRISPR-Cas variants with higher DNA specificity (Hu et al., 2018; Kleinstiver et al., 2016; Lee et al., 2018; Vakulskas et al., 2018) and expanded targeting range (Gao et al., 2017; Hu et al., 2018; Kleinstiver et al., 2015; Kleinstiver et al., 2019; Kocak et al., 2019; Nishimasu et al., 2018; Strecker et al., 2019) could enhance the efficiency of gene manipulation and facilitate designing highly active gRNAs, thereby making it possible to increase the number of gRNAs per gene. In addition, engineered variants may reduce the off-target effects linked with decreased cell growth in CHO cells owing to their high specificity (Lin et al., 2021b; McVey et al., 2016; Raab et al., 2019; Wilkens et al., 2019). Furthermore, increasing the number of targets in a single procedure would be of interest in CHO cells, since there are many target mechanisms to improve r-protein production, such as inhibiting apoptosis, promoting protein folding and modulating glycan composition. Given the fact that with Cas12a up to 25 crRNAs can be expressed in a single transcript in mammalian cells (Campa et al., 2019), further studies in CHO cells could use a similar design to achieve multiple edits in a short amount of time. Nevertheless, expressing numerous crRNAs and targeting many genes simultaneously may increase the frequency of off-target effects and compromise cell growth (Amann et al., 2018), which are risks that should be considered for future research. Using a further strategy involving the generation of crRNA array libraries for CRISPR screening in CHO cells (Liao et al., 2019), researchers can explore the synergistic effect of combining numerous genes on cell growth, production and glycan composition of a specific r-protein. Moreover, using Cas9, Cas12, dCas9 and dCas12 fused with activator,

repressor domains, or epigenetic regulators (Breinig et al., 2019; Campa et al., 2019; Marx et al., 2021; Takasugi et al., 2020) and/or using truncated and full-length gRNAs in the same setting (Campa et al., 2019), multiplexed orthogonal genome editing and transcriptional regulation can be achieved in CHO cells, thus providing elaborate control of multiple targets simultaneously. Lastly, advances in genome editing and sensing technology with CRISPR systems (Anzalone et al., 2020; Xie et al., 2021) as well as developments in CRISPR-based synthetic circuits (Dominguez-Monedero and Davies, 2018; Ferry et al., 2017; Nakamura et al., 2019) may, in the future, help researchers construct smart systems that sense specific chemicals in the cell and respond by modulating recombinant production during cultivation of CHO cells, thereby improving the production process of r-proteins. Taken together, the CRISPR system provides more opportunities to generate optimised CHO cell factories for r-proteins and has the potential to decrease the time and cost of production, all of which are of interest to the pharmaceutical industry.

CRediT authorship contribution statement

Ali Kerem Kalkan: Conceptualization, Writing – original draft. **Fahreddin Palaz:** Writing – original draft. **Semeniuk Sofija:** Writing – review & editing. **Nada Elmousa:** Writing – review & editing. **Yuri Ledezma:** Writing – review & editing. **Elise Cachat:** Writing – review & editing. **Leonardo Rios-Solis:** Conceptualization, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest.

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