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Review

Recent advances in the application of genetic and epigenetic modalities in the improvement of antibody-producing cell lines

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ABSTRACT

There are numerous applications for recombinant antibodies (rAbs) in biological and toxicological research. Monoclonal antibodies are synthesized using genetic engineering and other related processes involved in the generation of rAbs. Because they can identify specific antigenic sites on practically any molecule, including medicines, hormones, microbial antigens, and cell receptors, rAbs are particularly useful in scientific research. The key benefits of rAbs are improved repeatability, control, and consistency, shorter manufacturing times than with hybridoma technology, an easier transition from one format of antibody to another, and an animal-free process. The engineering of the host cell has recently been developed method for enhancing the production efficiency and improving the quality of antibodies from mammalian cell lines. In this light, genetic engineering is mostly utilized to manage cellular chaperones, decrease cell death, increase cell viability, change the microRNAs (miRNAs) pattern in mammalian cells, and glycoengineered cell lines. Here, we shed light on how genetic engineering can be used therapeutically to produce antibodies at higher levels with greater potency and effectiveness.

1. Introduction

Recombinant antibodies (rAbs) are extremely specific detection probes in research and diagnostics. They have attracted increasing attention as the fastest-developing class of therapeutic proteins. Antibody production has been intensely hastened by *in vitro* selection systems, in particular phage display [1,2]. A growing type of recombinant production system, such as gram-negative and -positive bacteria, yeasts, filamentous fungi, insect and mammalian cells to transgenic plants and animals, has progressed [3,4]. Almost all therapeutic antibodies are now manufactured in mammalian cell lines to reduce immunogenicity risk due to altered non-human glycosylation patterns and decreased levels of endotoxin contamination [5]. The strategy to generate antibodies in mammalian cells with higher yields is either transient transfection or the establishment of stable cell lines. Antibodies could be manufactured on a large scale in mammalian cells, like human embryonic kidney (HEK) and Chinese hamster ovary (CHO) cells [6,7].

Genetic engineering includes various modalities for the intentional manipulation of genetic material to modify, repair, or promote form or activity [8]. Recombinant DNA technologies comprise the chemical splicing (recombination) of strands of DNA from different organisms to each other. The chimeric DNAs are used to produce wealthy proteins through either transfer into prokaryotic (such as *E.coli*) or transfer into eukaryotes (like mammalian cells) host cells [9,10]. Meanwhile, host

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cells are further manipulated at the genetic or epigenetic level to boost the yield of recombinant proteins. The Nobel Prize in Physiology and Medicine in 2006 was awarded for the discovery of RNA interference (RNAi), a gene silencing technique by double-stranded RNA [11]. Synthetic biology approaches also are in progress for harnessing the power of nature to solve problems in medicine, manufacturing as well as agriculture [12]. Besides, genome editing tools such as zinc finger endonucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the Clustered regularly interspaced short palindromic repeats/CRISPR associated nuclease (CRISPR/Cas) system have improved our competence in a diversity of arenas, ranging from basic research to applied biotechnology and biomedical studies [13,14]. Engineering is mainly used for reducing cell death and increasing its viability, control of cellular chaperones, altering the miRNA pattern of mammalian cells, and finally glycoengineering of cell lines [15,16]. Herein, we will focus on the therapeutic utility of genetic engineering in the context of antibody generation. Also, a glimpse into its application in antibody validation and CHO cell line generation has been delivered.

2. Cell culture processes for antibody production

Cell culture expertise has advanced drastically over the past decades and is now largely recognized as a dependable, robust, and relatively mature technology [17]. A spectrum of biotherapeutics is presently manufactured utilizing cell culture plans in large-scale producing facilities. These products are established for both commercial utility and clinical investigations. The efficient operation of cell culture technology needs the optimization of several variables [18,19]. First, creating cell lines to produce target molecules at high efficiencies to reduce operating costs is urgently required. Second, optimizing culture conditions and supplements as well as bioreactor condition is important to facilitate high productivity and provide product quality qualifications. Besides, a suitable online and offline sensor is needed for offering information that eventually promotes process control [20]. Finally, we require determining culture performance at several scales to guarantee smooth scale-up. Efficient operations required appropriate plans for expansion, scale-up, specification, and validation of processes up to facilitate successful operation and meet compliance with existing regulations.

The cell culture policy for antibody generation includes common host cells, expression vector, transfection and selection of approaches throughout cell line generation, standard cell culture media, procedure control, and scale-up methodologies throughout procedure optimization [21]. This method eases fast procedure progress and also ensures anticipated efficiency in scale-up, facility fit, and downstream procedure integration. Therapeutic antibodies are largely manufactured in mammalian host cell lines comprising CHO cells, NS0 murine myeloma cells, and also PER.C6® human cells [22,23].

The CHO cells include a diversity of lineages comprising CHO-DXB11 (or DUKX), CHO-K1, CHO-DG44, and CHO-S with a common ancestor. In 1956, Theodore Puck generated the original CHO cell line by isolation of spontaneously immortalized fibroblasts from a culture of ovarian cells of a Chinese hamster [24]. One year late, CHO-K1 was established from a subclone of the original cell line, and then CHO-DXB11 was established by chemical mutagenesis of CHO-K1 in 1980 [25]. The dihydrofolate reductase (dhfr) deficiency facilitated the steady insertion of transgenes once co-transfected with a functional copy of the dhfr gene as a selection marker. In 1983, the establishment of the CHO-DG44 lineage was managed by the depletion of both dhfr alleles by mutagenesis of a different CHO cell starting population. CHO-S cell line was derived from another CHO cell starting population in 1991 [25]. Now, all of them are broadly applied to the manufacture of biopharmaceuticals.

The chief ingredients of the cell culture media are similar for all CHO cell lines and NS0 cells. However, particular growth media have been modified based on discrete cellular necessities like cholesterol for NS0 cells [26]. The choice of expression method is defined by its capacity to provide great productivity with satisfactory product quality competencies along with the inclinations of individual corporations, usually affected by their historical involvements. In terms of producibility and scalability, mammalian cells are difficult to work with. This difficulty is mainly a result of low harvest, moderate complexity, serum requisite, and shear sensitivity [27]. Recent developments in this technology now ensure specific efficiency of over 20 pg/cell/day for production cell lines. Additionally, high titers of up to ~10 g/L and cell densities of over 20×10^6 cells/mL in fed-batch procedures have been reported by some producers [28]. The improvement of efficiency per cell is earned by choosing greatly productive clones accompanied by the use of suitable medium components and bioreactor operation circumstances. Now, the incorporation of high titers and large capability has progressively transformed the emphasis of cell culture technology from acquiring even higher titers to adjusting antibody quality and process reliability at all progress steps and manufacturing scales [29].

The establishment of a cell line, selection of the clones with an appropriate yield of the protein of interest, and optimization of the culture medium are procedures that are initiated in small-scale systems. A such system comprises 96-well plates, shaker flasks, and bench-scale bioreactors [30]. When proper conditions are determined, the procedure is commonly moved to a pilot scale to examine scalability and generate material for preliminary toxicology investigation. After that, the procedure must be performed at a larger scale for the generation of a clinical ingredient under current good manufacturing practices (cGMP) regulations. Upon the progress of a commercial cell culture procedure for manufacturing a biological yield in pilot scales, the commercialization procedure is started with process characterization, scale-up, technology transfer, and validation of the production procedure [31].

3. The genome editing tools

Concerning the engineered bacterial nucleases, the progress of genome editing tools has facilitated the possibility of directly affecting genomic sequences in eukaryotic cells [32]. Current development in evolving programmable nucleases has critically accelerated the development of gene editing from theory to clinical practices [33].

3.1. CRISPR-based systems

In 1987, CRISPRs were originally discovered in *E. coli* and later in numerous other bacteria species [34]. In 2005, scientists found their similarities to phage DNA and suggested their contribution to bacterial and archaea adaptive immune defense versus insulting foreign DNA by stimulating RNA-guided DNA cleavage [35]. CRISPR-Cas systems classically are categorized into two classes according to the structural differences of Cas genes accompanied by their organization shape. In this light, class 1 CRISPR-Cas systems include multiprotein effector complexes, while class 2 systems contain only a single effector protein [36]. Overall, six CRISPR-Cas types and about 29 subtypes have been discovered. The type II CRISPR/Cas9 system, the most well-known type of CRISPR, relies on a single Cas protein from *Streptococcus pyogenes* (SpCas9) [37,38]. The CRISPR/Cas9 system consists of two constituents, including a single-stranded guide RNA (sgRNA), a particular 20 base-pair (bp) sequence, along with a Cas9 endonuclease. The sgRNA is designed to complement the target DNA region in a sequence-specific mode [39]. The target regions essentially are followed by an upstream short DNA sequence required for the compatibility with the Cas9 protein exploited, termed protospacer adjacent motif (PAM) [40]. Upon linking between designed sgRNA and target DNA by Watson-Crick base pairing, Cas9 specifically slices the DNA to shape double-stranded DNA breaks (DSBs) [41]. Then, DNA-DSB repairing systems start the DNA repairing process via non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms (Fig. 1). The Cas9 variants have been offered in Table 1.

CRISPR/Cas9 has some superiority over endonuclease-based ZFN or TALEN. For instance, reengineering of the particular enzyme to fit each

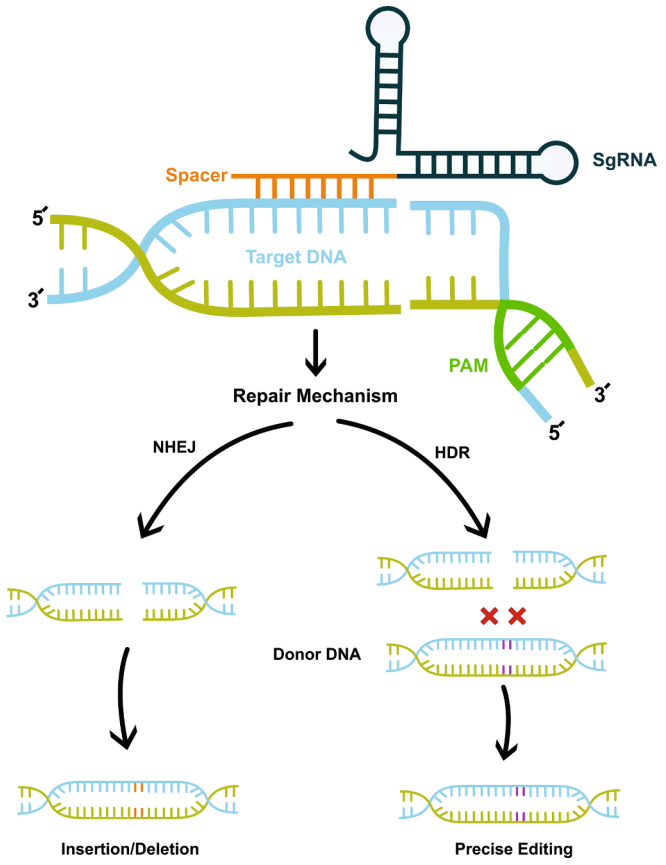

Fig. 1. CRISPR-Cas9 genome editing tool. Recognition, cleavage, and repair are the three steps that make up the CRISPR/Cas-9 mechanism for genome editing. The target sequence in the gene of interest is recognized by the designed sgRNA through a complementary base pair. The double-stranded break is caused by the Cas-9 nuclease at a site three base pairs upstream from the protospacer adjacent motif, and it is then fixed by cellular mechanisms using either non-homologous end joining (NHEJ) or homology-directed repair (HDR).

Table 1
Cas9 variants.

Variants	PAM sequence (5′ to 3′)	Utilization	Ref
SpCas9	NGG	Multiplex genome editing in mammalian cells	[154]
SpCas9-VRER	NGCG	Editing the previously inaccessible sites in zebrafish embryos as well as human cells	[155]
SaCas9	NNGRRT (R represents A or G)	More efficient genome edition by the AAV-SaCas9-gRNA vector system	[156]
evoCas9	NGG	Restricting unspecific cleavage of a difficult-to-discriminate off-target region and fully perturbing the cleavage of two additional off-targets	[157]
SpCas9-NG	NG	More efficient and accurate genome edition in mouse zygotes and also somatic culture cells	[158]
SpRY	NA	Exact editing to prolong almost to the whole genome	[159]
CjCas9	GAAAGCAC	In vivo genome edition within muscles of dystrophin KO mice	[160]
xCas9–3.7	NG, GAA, GAT	Base replacement of C.G → T.A and A.T → G.C for the pathogenic mutation sites	[161]

Note: Streptococcus Pyogenes Cas9 (SpCas9), Small Cas9 ortholog from Staphylococcus aureus (SaCas9), Campylobacter jejuni Cas9 (CjCas9), Adeno-associated viral (AAV) vectors, Protospacer adjacent motif (PAM), Not applicable (NA).

target sequence is required for ZFN or TALEN means, while Cas9 is equal in all cases [42]. ZFNs and TALENs request much more labor and are less cost-effective compared to the CRISPR/Cas9 system. In addition, CRISPR/Cas9 can trigger multiple loci editing, thereby making it easier, more efficient, and more scalable than other gene editing tools [43]. The most desired delivery method is the direct delivery of CRISPR ribonucleoprotein (RNP), including a complex of Cas9 protein and *in vitro* transcribed sgRNA. This has several advantages, such as speedy action, unlimited stability, and reduced antigenicity.

Since the first in-body clinical trial with CRISPR-Cas9 is managed, the risks related to injecting the genome editing means to patients become gradually relevant [44]. Anti-Cas9 antibodies should be insignificant because Cas9 is an intracellular protein and the majority of therapeutic interventions aim to temporarily express or deliver the recombinant Cas9 directly to target cells [45]. Wang and colleagues found SpCas9-specific antibodies two weeks after the delivery of the adenoviral Cas9, defying this presumption in their seminal study [46]. Specific clinical applications of Cas9 may not be significantly hampered by pre-existing anti-Cas9 antibodies. However, their presence (especially at high titers) suggests that people likely have memory T cells and B cells that can mount an adaptive immune response to Cas9 or to cells presenting Cas9 antigenic epitopes, which could pose a potential efficacy or safety concern [47]. It has been demonstrated that the use of bacterial proteins in therapeutic interventions, such as pseudomonas toxin for targeted cancer therapies, causes strong immune reactions that render the interventions ineffective [48]. The immunogenicity of all CRISPR/Cas9-based therapeutic products should therefore be evaluated. Risk assessment is predicated on two questions: (1) does the therapeutic induce anti-drug antibodies (ADAs), and (2) what, if any, are the clinical results of these ADAs? The questions can be answered by using a well-established standard assay development and statistical methodology for identifying positive ADA in clinical samples [49].

In order to create a mouse model for human non-alcoholic steatohepatitis (NASH), they sought to interfere with phosphatase and tensin homolog (PTEN) expression in hepatocytes [46]. While they were able to edit the genome, they noticed antibodies with the IgG1, IgG2a, and IgG2b subtypes, marking a host immune response to adenoviral Cas9. In addition, Chew et al. showed that Cas9 expression in mice induced an immune response [50] (Fig. 2), regardless of the method of delivery. Muscles that expressed Cas9 displayed a resulting enrichment in CD45 + leukocytes, particularly myeloid cells (CD11b + Gr1- monocytes, macrophages, and/or dendritic cell subsets), as well as T cells (CD3 + CD4 + and CD3 + CD8 +). Intriguingly, they discovered four T cell receptor ß-chain (TCR-ß) clonotypes that were shared by all Cas9-exposed animals, one of which was recognized as truly Cas9-responsive [50](Fig. 2) [51].

The primary immunity versus therapeutic Cas9 delivery attenuates its effectiveness post-transplantation and brings about substantial safety concerns. Thus, monitoring for anti-Cas9 antibodies during clinical progress by robust and reliable assays is strongly suggested. For instance, ELISA-based assays could detect antibodies against SaCas9 and SpCas9 in human sera. This method revealed the prevalence of anti-SaCas9 and anti-SpCas9 antibodies, respectively, to be 10% and 2.5% [49].

Cas9 nickases have been engineered to produce new variants due to the wild-type (WT) SpCas9′s lax requirements for DNA complementarity. Single-strand breaks (SSBs) rather than double-strand breaks (DSBs) are produced by these variants. The D10A point mutation in SpCas9 nickase results in RuvC nuclease domain inactivation; as a result, this form of nickase only cleaves the target DNA [52]. Two adjacent gRNAs and paired nickases are used to produce DSBs. It has been announced that in human cells, eSpCas9 (1.1) and SpCas9-HF1 variants demonstrate significantly decreased off-target cleavage. The off-target effects happen once Cas9 performs on untargeted genomic region and brings about cleavages that may induce unwanted consequences. In contrast, on-target effect is favored and refers to modification inside of

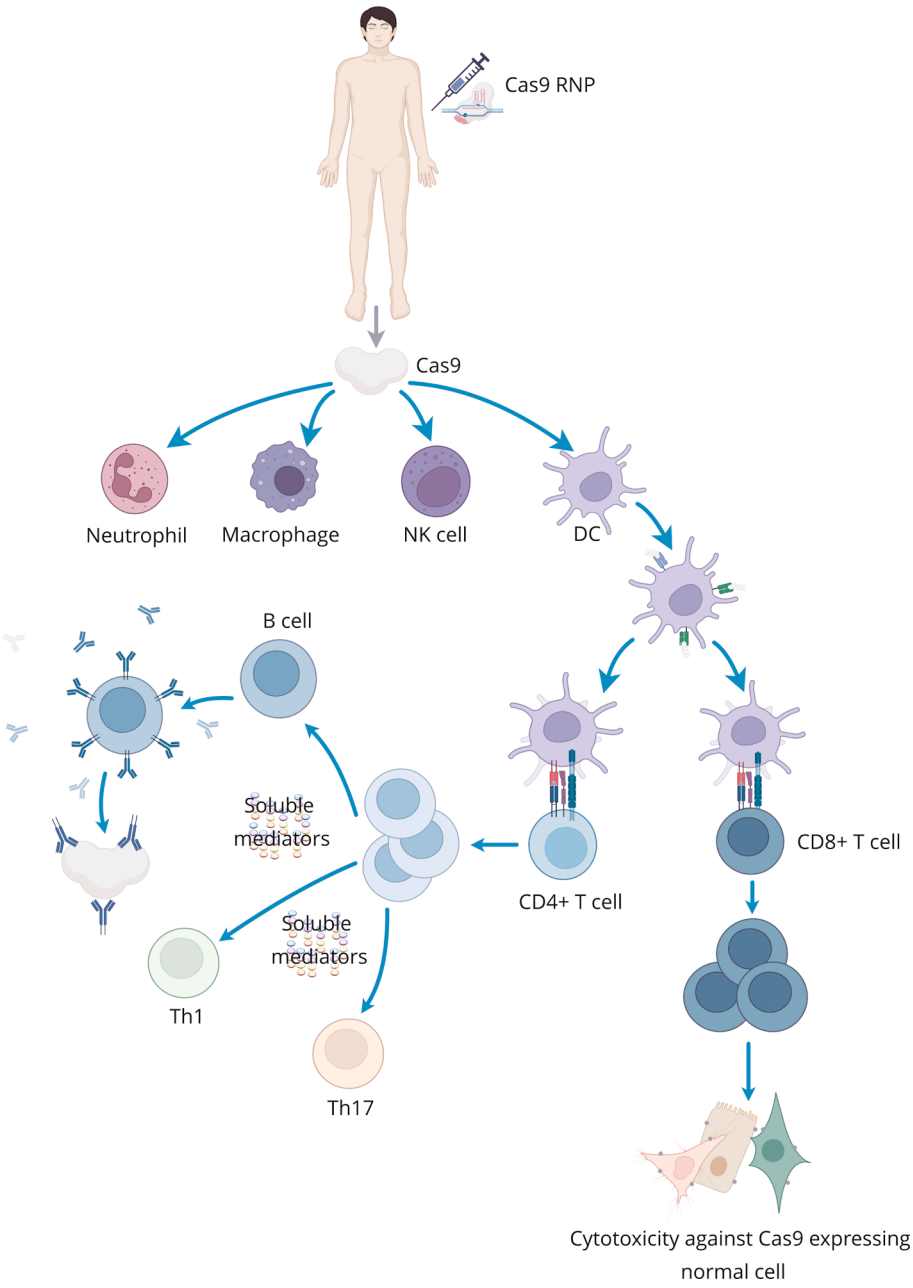

Fig. 2. The innate and adaptive immune response to Cas9. Cas9 may trigger innate and adaptive (acquired) immune responses including humoral immunity and cell-mediated immunity in mouse models, as well as the existence of anti-Cas9 antibodies and T cells in human plasma. Pre-existing immunity to therapeutic Cas9 delivery could reduce its effectiveness in vivo and raise serious safety concerns (Created with BioRender).

target gene. The off-target sites are usually sgRNA-dependent, as Cas9 is showed to tolerate up to 3 mismatches between sgRNA and genomic DNA [53]. SpCas9-HF1 effectively rules out the possibility of off-target effects since both Cas9 nickases must nick their targets for the generation of a DSB dot. There is no information available about the targets of discrimination or fidelity advance [54]. As a result, Doudna et al. demonstrated that both eSpCas9 (1.1) and SpCas9-HF1 are trapped in an inactive state when bound to mismatched targets using a single-molecule Forster resonance energy transfer (FRET) experiment [55]. Furthermore, it was found that REC3 and Cas9′s non-catalytic domain recognize target complementarity and regulate the HNH nuclease to regulate overall catalytic competence. The same research team made use of this phenomenon to create a new, hyper-accurate Cas9 variant (HypaCas9) that exhibits high genome-wide specificity in human cells without sacrificing on-target action [55,56].

3.2. *TALEN and ZFN*

The boundaries of biological research are being redefined by a potent class of tools called zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) [57]. These chimeric nucleases are made up of sequence-specific DNA binding modules that can be programmed and a non-specific DNA cleavage domain. By causing DNA double-strand breaks that encourage error-prone NHEJ or HDR at particular genomic locations, ZFNs and TALENs enable a wide range of genetic alterations [58]. The first edited crop that led to the treatment of cancer was achieved using TALEN. As described, CRISPR is partially easier to construct and much easier to multiplex [59]. However, given the specific setting of TALEN systems, it has been developed to manage new functionalities. The generally applied TALEN system consists of two components, including TALE proteins bound to FOK1 endonuclease

[60]. TALEs proteins, which were primarily discovered in 2009 from the phytopathogenic bacterial genus Xanthomonas, are a sort of DNA binding proteins containing central DNA binding domain (DBD) with 12–28 repeats, a nuclear localization signal (NLS) and an acidic domain [61]. Besides, ZFN is an extensively applied programmable DNA binding protein mainly found in the African clawed frog Xenopus laevis [62]. ZFNs are a member of zinc finger (ZF) transcription factors merged with FokI endonuclease [63]. Again, FokI catalytic domains must heterodimerize for DNA cleavage and then recall of cellular DNA repair system and gene editing [64].For customizable nucleases to bring relevance for genetic analysis and clinical utility, such nucleases is required to manifest severe specificity toward their intended DNA targets. Nonetheless, complex genomes frequently comprise manifold copies of sequences that are equal or highly homologous to the intended DNA target, causing off-target effect as well as cellular toxicity. To circumvent this concern, structure [65] and selection-based [66] strategies are exploited to establish promoted ZFN and TALEN heterodimers with better cleavage specificity and decreased toxicity. Sood and colleagues recently applied directed evolution to establish a hyperactivated variant of the FokI cleavage domain, Sharkey, which demonstrate a $>$ 15-fold enhancement in cleavage performance compared to traditional ZFNs [67] and is directly compatible with several ZFN architectures [66]. Also, increasing proofs show that 4 to 6 zinc-finger domains for each ZFN half enzyme considerably improves action and specificity [68,69]. Other approaches for increasing ZFN activity comprise the employment of transient hypothermic culture conditions to raise the expression levels of nuclease [70], and the applying fluorescent surrogate reporter vectors that could permit for the enrichment of ZFN and TALEN-affected cells [70]. The specificity of ZFN-mediated genome editing also could be promoted by the progress of zinc-finger nickases (ZFNickases) [71,72].

Site-specific nucleases have enabled the introduction of targeted modifications in a number of model organisms common to biological research. In addition to valuable animal models like zebrafish [73], rats and mice [74], both ZFNs and TALENs are applied to introduce targeted modifications in plants, such as Arabidopsis [75] and several crop species [76], facilitating the incorporation of valuable traits, such as disease and herbicide-resistance.

4. Genome engineering in antibody production

4.1. Promoting cell survival/growth

Mammalian cells were shown to experience apoptosis upon insult, glucose deprivation, and extension of batch cell culture. Aside from that, bypassing cell apoptosis and increasing its growth is broadly applied to substantially boost the yield of therapeutic proteins. Programmed cell death could be postponed by the generation of modified cell lines to greatly express the anti-apoptotic genes or negatively regulate pro-apoptotic genes causing enriched product yields.

Apoptosis negatively adjusts cell survival during cell culture. Zhang et al. (2018) suggested a cost-effective process to increase cell viability and generation of an antibody through the transient co-transfection with anti-apoptotic genes Bcl-xL and Mcl-1 in CHO cells [77]. They showed that genetically modified CHO cells experienced a decreased level of apoptosis and enhanced cell survival with an improved anti-PD1 antibody production yield of about 82% and 34% in cells co-transfected with Bcl-xL and Mcl-1, respectively [77]. When anti-apoptotic genes Bcl-xL and Mcl-1 were compared in relevant prospects, Bcl-xL gene showed more efficient in anti-apoptotic impact and productivity was substantially increased [77]. Different effects of two apoptotic proteins on apoptosis [78], autophagy[79,80], and cell cycle [81]may account for the variations in promoting productivity. These results were consistent with earlier reports showing that the engineered cell lines with Bcl-xL stably expressed led to an enhance in yield of between 70 and 270 percent after 14 days in fed-batch [82].

Besides, Bcl-xL in combination with rapamycin resulted in deterred cell apoptosis, extended cell lifetime, and increased anti-PD1 monoclonal antibody expression levels by about 100% in CHO cells [83]. Rapamycin, an mTOR signaling inhibitor, precedes its positive effects by increasing cell viability- not by affecting cell growth [83]. Growth inhibition and cell death without cell cycle arrest in G0/G1 point to a more widespread mechanism for controlling cell growth and death in fed-batch cultures [83]. It's possible that another mechanism, rather than the functioning of the cell cycle, is more responsible for the growth arrest and cell death observed in fed batches. Based on the delays seen in all phases of hybridisms, such mechanism appears to be general for all cell cycle phases. In this sense, growth arrest and cell death caused by cell cycle arrest in a cell line that is normally governed may be symptoms rather than causes, with the symptom masking the underlying cause [83].

In addition, the genetically modified CHO cell line to highly express anti-apoptotic proteins E1B-19 K and/or Aven experienced better survival compared to the parent cell line following glucose deprivation [84]. In co-immunoprecipitation and yeast two-hybrid screens, it was discovered that both E1B-19 K and Bcl-2 bound to Bax and Bak, providing biochemical evidence for the relationship between E1B-19 K and the cellular Bcl-2 family members [85,86]. At least in the case of the proapoptosis protein Bax, binding to the BH3 domain mediates the inhibition of E1B-19 K [87]. E1B-19 K also binds to the proapoptosis BH3 domain proteins Nbk/Bik and BNIP3, which were discovered using E1B-19 K as bait in yeast two-hybrid studies. This suggests that E1B-19 K may inhibit apoptosis through additional mechanisms [87,88]. Interestingly, E1B-19 K and/or Aven expressing CHO cells also displayed a longer operating lifetime and greater final antibody titer (increased by about 40–55%) in fed-batch bioreactors [84].

Lee et al. (2013) also revealed that Bcl-2 overexpression as an anti-apoptosis agent along with Beclin-1 for autophagy induction could protect the cells from cell death under stressful culture conditions like sodium butyrate exposure [89].Under stressful culture conditions brought on by sodium butyrate treatment or hyperosmolality, Bcl-2 overexpression has also been shown to increase the production of foreign proteins by rCHO cells [90]. Despite being depleted of nutrients, sodium butyrate treatment or hyperosmolality can result in stressful culture conditions, and the co-overexpression of Bcl-2 and Beclin-1 has been shown to prevent cell death under other stressful cultures [89].

These studies deliver proof that pro-autophagy engineering in combination with anti-apoptosis engineering results in a synergistic impact and positively increases the lifetime of engineered CHO cells. Finally, Safari and coworkers (2020) showed that CRSISPR-mediated ablation of caspase-7 gene could increase the expression of recombinant protein in the CHO cell line largely by inducing the cell cycle arrest in the G2/M phase [91]. Likewise, co-down-regulation of both caspase-3 and caspase-7 by using siRNA in CHO cells could promote the cell viability and increase culture longevity [92].

4.2. Targeting genes involved in protein synthesis, folding, and secretion

In the cell, the interaction of a various of protein cofactors, which termed as molecular chaperones, is needed for folding the most newly synthesized proteins [93]. These molecules identify and interact with nascent polypeptide chains and partially folded intermediates of proteins, thus deterring their both aggregation and misfolding. The 40-kDa heat shock protein (HSP40; DnaJ), 60-kDa heat shock protein (HSP60; GroEL), and 70-kDa heat shock protein (HSP70; DnaK) families, as the most critical chaperons, play key roles in this regard [94] (Fig. 3). The accessibility of high-resolution structures has enabled a more comprehensive elucidation of the complex chaperone machinery and mechanisms, comprising the ATP-dependent reaction cycles of the GroEL and HSP70 chaperones. For both of these chaperones, the binding of ATP induces a crucial conformational change causing the release of the bound substrate protein [95]. Although the central role of the HSP70/HSP40 chaperone system is to decrease the accumulation of newly

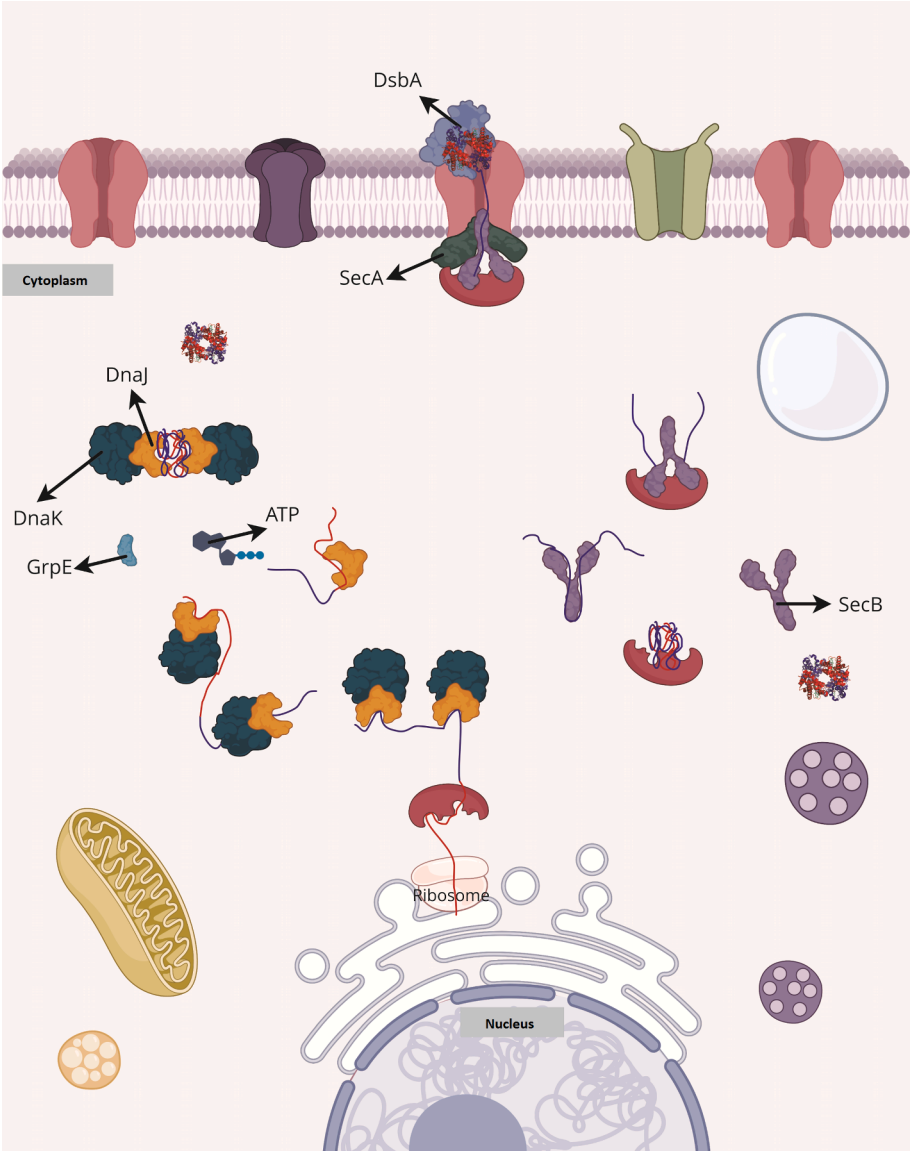

Fig. 3. Chaperone mediated folding pathway. The interaction of a variety of protein cofactors known as molecular chaperones is necessary for the folding of the majority of newly synthesized proteins in the cell. These molecules identify and bind to developing polypeptide chains and intermediate stages of partially folded proteins to stop them from aggregating and misfolding (Created with BioRender).

synthesized proteins, the HSP60 chaperones also ease the actual folding process by delivering a secluded environment for individual folding molecules, thereby improving the unfolding and refolding of misfolded intermediates [96].

The aptitude to establish recombinant proteins in a secreted form by cultivated mammalian cells is extremely appropriate because they can release proteins with human-like post-translational alterations. Though, efforts to express a particular antibody at high levels in CHO cells may lead to low yield. The CHO cells engineering to overexpress signal receptor protein 14 (SRP14) could enhance proper processing and secretion of recombinant proteins. SRP14 directs secretory proteins toward the rough endoplasmic reticulum (ER) membrane, and its deficiency results in an inefficient targeting of preproteins and brings about defects in secretion [97]. Thus, SRP14 up regulation enables the expression of difficult-to-express proteins at high levels, and also enhanced the generation of an easy-to-express protein.

It has been previously found that intracellular assembly of antibodies' light and heavy chains are a main restricting step for overall cell-specific productivity because secretion levels boost with higher light chain expression rates and heavy chain accumulates intracellularly in the existence of too little light chain [97]. PDI is a multifunctional redox chaperone of ER facilitating protein folding. Also, BIP or glucose-regulated protein 78 (Grp78) is a vital ubiquitous resident of ER. Previous work has shown that a human-antibody-producing recombinant CHO cell line did not increase its intracellular content according to the increased expression of antibodies. Due to the low level of intracellular protein disulfide isomerase (PDI) and heavy chain binding protein (BIP) in CHO cells in comparison to that of hybridoma cells, scientists have sought various modalities to up-regulate their expression [98].

There have been previous studies on the impact of PDI overexpression on the rates of monoclonal antibody production (qAb), but the results are still ambiguous. In CHO cells, PDI overexpression reduced TNFR:Fc secretion but had no effect on IL-15 secretion [99]. The authors of a different study that examined how dexamethasone-induced PDI overexpression affected antibody secretion in a murine hybridoma noted that while PDI overexpression had no effect on secretion rates, it had a positive impact on the cells' ability to maintain their culture for a longer period of time [100]. In this light, Borth et al. revealed that BIP

overexpression, both alone and plus PDI, decreased the specific secretion levels, while PDI overexpression alone increased product secretion levels in CHO cells [98]. A moderately increased expression of PDI is required to permit the secretion of large amounts of antibody, despite the fact that overexpression of PDI per se does not increase productivity. The presence of an autologously increased intracellular PDI content in high secreting cell lines supports this conclusion. In another study, PDI and ER oxidoreductase (ERO1L) were overexpressed in CHO cells to assess their effects on protein secretion levels. ERO1L preserves PDI in an oxidized state so that disulfide bond creation happens. Transient expression of ERO1L individually or along with PDI improved antibody secretion levels by 37% and 55%, respectively [101]. At the transcriptional level of Ab synthesis, it was determined that co-overexpression of PDI and ERO1L had no discernible impact. Under temporary gene expression, the bottlenecks in the folding process, translation, and post-translational processing appeared to have been relieved [101]. In contrast, intracellular product accumulation and impaired secretion were also seen in stable clones that co-expressed PDI and ERO1L [101].

Besides, a recent report also indicated that transient overexpression of X-box binding protein 1 (XBP-1) could enhance protein titers by up to 2.5-fold in CHO and NS0 cell lines [102]. XBP-1 is a central regulator of the cellular secretory axis and unfolded protein response (UPR) [103]. It acts as a transcription activator and boosts the expression of various genes related to protein secretion as well as ER biosynthesis. Contrary to previous report [104], the effects of XBP-1S overexpression on protein productivity were found to depend on the expression levels of recombinant proteins [102]. Given that XBP-1 proteins from various species were used in the two studies, the differences between the two studies may be explained by differences between species. The earlier research used human XBP-1, whereas more recent studies used the mouse homolog. Also, in contrast to transient expression, the stable expression of XBP-1S was not positively affect the antibody productivity of stable cell lines [102]. Also, Berger et al. (2020) found that overexpression of Foxa1 and Erp27, which binds unfolded proteins in ER, could improve cell survival, and increase the secretion of difficult-to-express proteins [105]. Foxa1 overexpression improved cell viability, yields of easy- and difficult-to-express proteins, and cell density in fed-batch cultures while reducing reactive oxygen species. Two additional candidate genes, Ca3, which helps protect cells from oxidative stress, and Tagap, which is involved in signaling and cytoskeleton remodeling, were also upregulated by Foxa1 overexpression and produced more difficult- and/or easy-to-express protein [105]. The actin-mediated adaptation of cells in a suspension environment may be improved by increased expression of Tagap. As the actin cytoskeleton is involved in the regulation of the secretory pathway, Tagap upregulation could also help to improve therapeutic protein secretion [106].

4.3. Regulating microRNAs expression

MicroRNAs (miRNAs) post-transcriptionally adjust gene expression in most eukaryotic cells such as CHO cells. In light of this, miRNA engineering of CHO cells enables the production of difficult-to-express proteins and enhances achievement in cell line development. Based on the literature, various types of miRNAs like miR-744 are complicated in reducing productivity in CHO cells expressing therapeutic antibodies [107]. Raab et al. (2019) have revealed CRISPR/Cas9-mediated knockout of the miR-744 locus improved the antibody titers to 190–311 mg/L compared to 156 mg/L in non-modified cells. It seems that negative regulation of miR-744 results in the up-regulation of its target genes, such as cyclin B1 (ccnb1) and secreted frizzled-related protein 1 (sfrp1), leading to enhanced antibody generation and secretion [107].

Likewise, CRISPR/Cas9-mediated knockout of miR-27b, which typically performed as an apoptosis inducer, could ultimately increase CHO cell viability in the late stages of batch and fed-batch cultures [108]. By adversely affecting the PI3K/AKT pathway, miR-27b can inhibit cell proliferation and trigger apoptosis in human cells [109]. By partially activating TNFα-induced pathways, miR-27b mimics also result in the suppression of proliferation and the promotion of apoptosis [110] Thus inhibiting its activity could ease cell viability.

Likewise, repressing endogenous miR-378-3p function by miRNA-sponge-decoy could improve cell density by 59% mainly as a result of up-regulating ubiquitin carboxyl-terminal hydrolase 14 (USP14) protein levels [111]. UCH37 and USP14 can work independently to disassemble or trim but not degrade Ub chains, thus impeding the tagged protein's commitment to proteasome degradation. Usp14 is largely contributed to enhancing cell proliferation, while itsoverexpression in CHO cells improves cell growth. The possible mechanism behind the supportive effect of USP14 on cell growth might be activating NF-kB [112] and MAPK/JNK pathway [113].

Other line of studies have shown that overexpression of various miRNAs could increase cell viability and antibody production [114]. For instance, the study of the effects of miR-17 on CHO growth and protein productivity signified that miR-17 overexpression could result in a 2-fold increase in specific productivity. But stable miR-17 overexpression significantly boosts productivity while only slightly increasing growth. This unusual parallel induction of cell-specific growth rate and productivity necessitates a more thorough investigation of the precise interactions and effects brought on by miR-17 [115]. Based on previous reports, observed effects resulting from miR-17 overexpression might result from activating Wnt, JAK/STAT signaling and TGF-β pathway [116].In addition to miR-17, the potential of miRs-19b, and −92a as cell-engineering targets to promote rAbs generation has been validated in mammalian cells [117]. Stably transfected pools demonstrated 24–34% increment in specific productivity (qP) and 21–31% enhancment in titer relative to the parental clone, without substantial change in proliferation rates [117]. Likewise, Further, miR-92a improves rAbs productivity in mammalian cells by up-regulating intracellular cholesterol levels. The extracellular cholesterol concentrations for the miR-92a clones were likewise about two to three times higher than those of the control group [118]. MiR-92a overexpression increases cholesterol biosynthesis/secretion, whereas miR-92a knockout decreases systemic cholesterol levels in mice [119]. Cholesterol is significant because it makes up about 20 percent of the membrane lipid in the Golgi compartment and is thus important for vesicular transport from the ER and Golgi [120–122]. The relative Golgi surface area was found to be 18–26% higher in the miR-92a clones compared to the non–transfected clone, highlighting the significance of cholesterol levels [118]. Molecular analysis revealed that over-expression of miR-92a could reduce insig1 expression levels, a regulator of cholesterol biosynthesis [118]. Thus, miR-92a promotes cholesterol synthesis by inhibiting insig1, leading to enhanced intracellular cholesterol levels and Golgi volume and thus promoting protein secretion [118].

4.4. Glycoengineering of cell lines

Throughout antibody-dependent cell cytotoxicity (ADCC) the target cells are eliminated by monocytes as well as natural killer cells (NKCs) [123]. Lacking the fucose molecule from the antibody heavy chain's core N-linked glycan results in increased ADCC [124]. Although there is a direct relationship between afucosylated antibody levels and Fc-RIII binding or percentADCC activity, evidence indicates that nearly maximum Fc-RIII binding or percentADCC can be attained with only 50–60% afucosylated antibody species [124]. Thereby, numerous plans have been conducted to establish fully afucosylated antibodies. A usually applied and efficient method is ablating the fucosyltransferase 8 (FUT8) gene of the mammalian host cells (e.g., CHO) resulting in the creation of a fully afucosylated glycans antibody [125]. It is possible to choose the level of fucosylation for g/L levels of IgGs with strongly enhanced ADCC effector function when cells are designed to inhibit alpha-(1,6)-fucosyltransferase activity directly and specifically. For the development of next-generation therapeutic antibodies with improved

effector functions, the quick and effective method presented here should have a wide range of practical applications [125]. The main disadvantage of the FUT8-KO host is the necessity for conducting two separate cell line development (CLD) efforts for acquiring both primarily fucosylated and fully afucosylated antibody types for comparative investigations [124]. Also, the provision of primarily fucosylated and FUT8-KO clones with comparable sufficient product quality is an asset to certify that any detected ADCC benefit can be severely attributed to afucosylation [126]. The KO of UDP-galactose 4′ -epimerase (Gale) decreased peak cell density in CHO cells without affecting viability, according to a recent study [126]. Cell growth was negatively impacted at and above 200 M galactose supplementation; it is important to investigate whether these effects are brought on by a reduction in the amount of uridine available or by changes in the relative levels of nucleotide sugar levels [126]. The different formulations of the serum-free culture medium may be the cause of the slower cell growth observed with KO of the Gale gene here as compared to earlier studies. A supplementation of GalNAc, but not galactose, was shown to restore O-glycans [127]. Gale is also responsible for the interconversion of UDP-GlcNAc and UDP-GalNAc. However, Gale KO HeLa cells, but not Gale KO HEK cells, have shown a partial increase in intracellular UDP-GalNAc with galactose supplementation [128]. More research is required to determine how Gale KO affects O-glycosylation. O-linked glycans with O-GalNAc residue are frequently found in therapeutic glycoproteins like EPO [129] and thus this host system could also facilitate manipulation of such O-GalNAc glycans. Meanwhile, Zong et al. (2017) have shown that CRISPR/Cas9 mediated ablation of α-1, 6 fucosyltransferase (FUT8) gene in CHO cells led to generating defucosylated IgG1 antibodies with promoted binding affinities to human FcγRIIIa and higher ADCC [130]. They evaluated the ADCC activities in a liver cancer cell line (HepG2), and found that anti-HER2 IgG1 expressed by FUT8-/- cell lines had more evident impact in ADCC compared with wild type fucosylated IgG1 in liver tumor [130]. Meanwhile, Lin et al. (2021) also have developed a CRISPR-Cas13d system to efficiently inhibit the endogenous genes governing lactate production (LDHA), fucosylation (GFT), and cell death (DDIT3), hence blocking lactate accretion and core fucosylation in CHO cells [131]. This strategy resulted in reduced lactate accumulation and produced 59 ± 6% more IgG with less fucosylation and stronger ADCC efficacy [131]. Furthermore, the ablation of GDP-fucose transporter (SLC35C1) has demonstrated an alternative plan to establish CHO cells for the generation of fucose-free anti-HER2 antibodies [132]. Albeit, this intervention has no positive effect on antibody production and cell proliferation levels [132]. Besides the removal of core fucose from the N-glycans, recent studies demonstrated that eradication of sialic acid from antibodies also improves ADCC [133]. As a result, Haryadi et al. (2013) created a pair of zinc-finger nucleases and inactivated the GDP-fucose transporter gene (Slc35c1). The resulting mutant cell line, CHO-gmt5, lacks GDP-fucose and CMP-sialic acid transporters that are functional [133]. As a result, these cells can only generate asialylated and afucosylated glycoproteins. The removal of the core fucose from the N-glycans attached to Asn297 of human IgG1 is now widely acknowledged to significantly improve ADCC by increasing the antibody's ability to bind to FcγRIIIa [133]. Except for the gene editing tools, negative regulation of FUT8, GDP-mannose 4,6-dehydratase (GMD), and GDP-fucose transporter (GFT) using specific siRNA can be used to bring about stable manufacture of fully non-fucosylated antibodies with enhanced ADCC [134].

5. CHO cell engineering using glutamine synthetase (GS) gene knockout

Regarding the exclusive properties, CHO cells have been developed as the main workhorse for the production of therapeutic rAbs. However, detecting the high-producing clones among great numbers of low- or even non-productive clones can bargain CHO cell line generation [135]. Typically, numerous individual clones must be screened for the detection of a commercial clonal cell line with the appropriate yield and growth profile to ultimately create a suitable cell line for commercial use. This inefficient procedure for generating a CHO cell line is both time-consuming and laborious [25]. Two main CHO expression systems, including dihydrofolate reductase (DHFR)-based methotrexate (MTX) selection and glutamine synthetase (GS)-based methionine sulfoximine (MSX) selection, have been managed for the generation of CHO cell line for industrial application [136]. Choosing the recombinant cell lines in the GS-CHO system is according to the equilibrium between the GS gene expression introduced by the expression plasmid and the addition of GS inhibitor, methionine sulfoximine (MSX). The MSX is used to hinder excess GS. Thereby, GS expression from the endogenous GS gene in parental CHOK1SV cells may disturb the selection procedure [137]. Interestingly, GS-negative CHOK1SV cell lines were recently established by employing the genome editing tools like ZFN to impact the endogenous CHO GS gene specifically [138]. Development of GS-knockout CHO host cell line enables the fast production of high manufacturing clones with a decreased generation of lactate and ammonia in the lack of MSX [139,140]. In another study, the substantial productivity enhancements in both bulk culture and individual clonal cell line was enabled with the combined application of GS-knockout CHOK1SV cells and decreased SV40E promoters driving GS expression [141]. Moreover, lactate dehydrogenase-A (LDH-A) negative regulation in the mAb-producing cell line produced with the GS-based system could attenuate both ammonia and lactate rates, thus enhancing mAb galactosylation [142]. In addition to the CHO cells, the GS-knockout HEK293E cell line also could be applied as host cells to establish stable rHEK293E cells manufacturing antibodies by GS-mediated gene selection in the lack and presence of MSX [143]. A summary of recent studies concerning the utilizing genetic engineering in Ab generation has been provided in Table 2.

6. Genome editing tools for antibody validation

Mouse monoclonal hybridoma screening is the best approach applied to define new antigens accompanied by their biological relevance. Because the immunization of mice is done usually with a multifaceted antigenic material like whole cells or cellular fractions, tissue homogenates, cell extracts, or protein mixtures, this plan leads to the establishment of a panel of mAb with unidentified specificities [144]. Firstly, the initial screening and subsequent selection in functional tests are done. Secondly, the mAb must be identified to determine the antigen it binds to [145].

An important stage in mAb progress is the characterization and ultimate documentation of the specific target antigen and its responding epitope [144]. Although antibodies have been used extensively, there are no set standards that specify how they should be validated before use. Due to incomplete validation prior to use and/or lack of additional confirmation, many commercial antibodies have led to failed or unreplicable outcome, even projects being abandoned, leading to substantial time, money and sample loss [146]. As a result, there is a pressing need for antibody verification standards to be established. Based on the literature, various strategies have progressed for mAb identification and validation. Among them, enzyme-linked immunosorbent assay (ELISA), flow cytometry, and immunohistochemistry (IHC) are the most common. Besides, mass spectrometry (MS) analysis, immunoprecipitation (IP), Western blotting (WB), and immunofluorescence (IF) microscopy is applied for this purpose [147]. Since WB is the best assay for precisely validating antibodies against denatured proteins, it is generally regarded as the first step in the evaluation of new antibodies [148]. It is not the best standard method for antibody binding, though, in experimental assays where the antigen is in its natural conformation. The process used to fix tissue makes the issue more complicated because antibodies may recognize a specific epitope in fresh tissue but recognize different epitopes in the fixed tissue. This occurs as a result of the fact that after fixed

Table 2
Genetic engineering of cells aimed with production of higher concentration of Ab with better efficacy.

Target gene	Cell line	Expression pattern	Result	Ref
Bcl-xL Mcl-1	CHO	Up-regulation	Increased cell viability and production of anti-PD1 antibody	[77]
Bcl-xL	CHO-DG44	Up-regulation	Enhanced cell viability about 30%	[82]
E1B-19 K	CHO	Up-regulation	Increased cell viability and production of higher antibody concentration	[84]
Bcl-2 Beclin-1	CHO	Up-regulation	Protection of the cells from apoptosis	[89]
caspase-7	CHO	Down regulation	Stimulation of cell cycle arrest in the G2/M phase	[91]
SRP14	CHO-K1	Up-regulation	Increased the processing and secretion of IgG	[97]
PDI BIP	CHO	Up-regulation	BIP up-regulation reduced the specific secretion rate PDI up-regulation caused an increase of product secretion rate	[98]
PDI ERO1L	CHO	Up-regulation	Transient expression of ERO1L alone and with PDI enhanced q(Ab) by 37% and 55%, respectively	[101]
X-box binding protein 1	NS0 CHO	Up-regulation	Improved protein titers up to 2.5-fold	[102]
Erp27	CHO	Up-regulation	Promoted cell density, cell viability, and easy- and difficult-to-express protein yields	[105]
MicroRNA-744	CHO	Down-regulation	Enhnaced antibody titers to 190–311 mg/L versus a nontargeting (NT) sgRNA transfected clonal control with 156 mg/L	[107]
MicroRNA −27	CHO	Down-regulation	Increased the cell viability in late stages of batch and fed-batch cultures	[108]
MicroRNA-378-3p	CHO DP12	Down-regulation	Increased proliferation	[111]
Usp14	CHO DP12	Up-regulation	Increased proliferation	[111]
MicroRNA-7	CHO-K1	Up-regulation	Increased proliferation	[115]
MicroRNA-92a	CHO	Up-regulation	Promoted intracellular cholesterol levels and Golgi volume and thus improved protein secretion	[81]
FUT8	CHO-S	Down-regulation	Increased antibody generation (about 37%), and better ADCC (about 14-fold)	[130]
FUT8	CHO	Down-regulation	Increased ADCC	[162]
Gale	CHO	Down-regulation	Rapid synthesis of variably glycoengineered proteins	[126]
LDHA GFT DDIT3	CHO-K	Down-regulation	Prolonged longevity and production of IgG with less fucosylation and stronger ADCC	[131]
Slc35c1	CHO-K1	Down-regulation	Generation of fucose-free anti-Her2 antibody	[132]
GS	CHO	Down-regulation	Improved bulk culture productivity about 2–3 fold	[138]
LDH-A	CHO	Down-regulation	Reduced both ammonia and lactate levels, and increased Ab galactosylation	[142]
GS	HEK293E	Down-regulation	Selection of HEK293E cells in the absence and the presence of MSX	[143]

Note: Not applicable (NA).

processing, uncovered epitopes in natural proteins become accessible, and vice versa [149].Antibody validation is moderately simple once immunization is conducted with peptide or purified protein, mRNA, or DNA. However, it is problematic once immunization is conducted by whole cells or other complex antigens [150]. Defining the antigen specificity of a mAb is more difficult, once antibody reactivity is not sensed in Western blotting and/or immunoprecipitation assay. Regardless of protein-based means exploited for antibody characterization, a spectrum of gene-based methods, including cDNA expression or siRNA knockdown are applied for the validation of antibodies with limited reactivities. In the last years, CRISPR/Cas9 system also has become an ideal method for antibody-specificity validation. KO validation applies CRISPR's gRNA to guide the Cas9 endonuclease to the interest gene by sequence-specific targeting, creating DSBs neighboring targeted regions [151]. DSBs inspire repair mechanisms to generate a frameshift mutation, insertion, or deletion recently applied for KO cell line generation [152]. After that, antibody specificity is determined by the lack of off-target binding in the KO control lysate or cell lines. Recently, Zotova et al. have established the CEM T- and the Raji B cell lines with pooled libraries utilizing the genome-scale CRISPR-Cas9 knockout (GeCKO) library vectors [151]. They recognized BF4 as an anti-CD82 mAb. A deep sequence analysis of the GeCKO library transferred to the cell lines finally exhibited the successful selection of antibody-negative cells, leading to the efficient identification of mAb respecting the quality of cell library preparation [151]. Accordingly, it seems that this method is appropriate for the recognition of numerous other hybridomas and signifies an excellent alternative to the present protein- and gene-based strategies employed for mAb validation [153].

7. Conclusion

Though mammalian cells are employed for the manufacture of more effective mAbs for diagnostics and treatments of various human disorders, multiple boundaries hinder their dominance. Recent evolvement in cell engineering strategies at various steps facilitates the chance of cell utility for the generation of complex natured mAbs. Cell manipulation includes but is not restricted the adjusting cell apoptosis and growth, altering protein folding and secretion, glycoengineering, and also targeting miRNA expression. These modalities ease longer cell viability and higher cellular productivity and generate antibodies with higher affinities. In addition, genetic engineering can be used for decreasing metabolic by-product accumulation, applying favored changes in the target protein product as well as attenuation of impure host cell proteins. Meanwhile, Off-target effects are the major downside when we used CRISPR/Cas9 system for genome edition purposes. This effect may underlie unwanted editing or mutation in the host cell genome, conferring the importance of focusing on sgRNA designing. In addition to the sgRNA, befalling any mutation in the PAM sequence throughout the *in vitro* processing may limit the cleavage of the target DNA. Moreover, CRISPR-mediated modifications are largely permanent. Another drawback is the chance of triggering a deleterious immune response against the CRISPR/Cas system. In sum, we guess that genetic engineering can efficiently bring about a parading shift in antibody generation and can improve protein yields and product quality. This event in turn decreases drug production costs and enhances affordability for a large population.

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Data availability

No data was used for the research described in the article.

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