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Mammalian cells genetic manipulations for enhanced monoclonal antibody production: from traditional to novel trends, a review

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Abstract

Novel therapeutics and monoclonal antibodies (mAbs) specifically, are under massive demands for the treatment of various illnesses. Research in cell engineering has been able to develop mammalian cell lines able to produce higher yields of potent recombinant proteins using different genetic manipulations. However, the public demands for these potent drugs keep increasing and as such, it is needed for the field of biopharmaceuticals to create highly efficient producers to increase volumetric productivity and lower overall costs. Several ways have been investigated up to date, from impacting on cells' natural growth pattern, by apoptosis regulation and cell cycle control, enhancement of its production pathways, by stimulating secretary factors, and production of enzymes to use growth-related metabolites. These approaches, most of the time, involve a considerable amount of resources and time, with an impact mostly on a couple of natural traits of mammalian cells. The ideal scenario is to use other vectors to impact entire pathways and the global cellular functioning to provide better workhorses. Novel approaches, namely micro RNAs (miRNAs), RNA interferences (RNAi) and CRISPR/Cas9, have been used in the last few years and have demonstrated a broader impact on cellular pathways, and also more efficiency in recombinant genes transfection or complete knock-out of specific sequences. In this review, traditional and novel aspects are described, discussed while expressing what approach to be taken next would be of interest, from a productivity standpoint.

Keywords: Apoptosis • Cell Cycle Regulation • Corticosteroids • Secretion system • Metabolic Engineering • miRNA • RNAi • CRISPR/Cas9 • CHO cells • Monoclonal Antibody

Introduction

The area of medicine is a subject in constant motion. Many drugs are being designed, clinically tried and approved. Monoclonal antibodies (mAbs) have been established as realistic solutions in the treatment of various diseases such as cancer, autoimmune conditions, metabolic and infectious diseases. The last few years have enabled mAbs to prove themselves as therapeutics, and as such, many drugs called "blockbusters" are in fact antibodies. There are currently around eighty mAbs approved by the EMA and the FDA and many more are undergoing clinical trials. In the last decade, there has been a tremendous advance in the genetic engineering of mammalian cells, which helped to develop many cell lines producing recombinant therapeutics including bispecific antibodies, antibody-drug conjugates, and novel antibody-live scaffolds. It is estimated that among the top-selling oncologic drugs in 2020, three will be mAbs (EvaluatePharma. World Preview 2015, Outlook to 2020, 2015). The commercial success of mAbs led to a significant increase in the demands at the production scale. To respond to such needs, mAbs-producing cell lines have been subject to various molecular manipulations over the years to overcome difficulties with human immune response, to improve their specificity, but mainly to optimize the production of such valuable therapeutic products. Molecular biology helps to provide answers to industrial, medical complications and can be of interest for further studies down the line.

Chinese ovary (CHO) cells, along with mouse myeloma derived NS0 and Sp2/0 cells, HEK293 and PER.C6 cells are the mostly used cells in biotherapeutics production, especially mAbs. Among those, the CHO cell remains the most widely used cell line for large-scale industrial production. Indeed, around 70% of all recombinant proteins produced are from CHO cells [1], the reasons being the safety of that cell line, the likeliness of standards compliance set up by competent authorities like the FDA, the glycosylation pattern of secreted proteins, high-level amplification capacity and ability to adapt to suspension culture which is preferred in industrial-scale productions. Hence why most of the studies on therapeutics production are based on CHO cells. Various aspects of CHO cells, and other cell lines, have been studied and manipulated to enhance desirable traits and/or inhibit undesirable ones. The purpose of these genetic modifications is to make the cell line as efficient as possible in its production profile and the quality delivered of the protein. In this review, many different aspects of productivity improvements are reported and examined.

Apoptosis regulation

Apoptosis corresponds to the programmed death of mammalian cells and is the main form of death in high-scale productions [2]. This natural phenomenon demonstrates a specific phenotype: the cellular volume decreases, the membrane is altered followed by nuclear fragmentation and total disintegration of the cell. Apoptosis affects cell concentration, the recombinant protein production and quality [3]. The idea behind the alteration of apoptosis is to increase the cultivation period and increase ultimately therapeutics production. As apoptosis belongs to the genetic information of

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the cell, cell engineering became the natural way to interfere with such a mechanism.

The most common approach is the use of the bcl-2 protein family reportedly involved in both aspects of cellular death as inducers and as inhibitors. This family is divided into 3 different groups according to their involvement in apoptosis: anti-apoptotic Bcl-2-like (bcl-2, bcl-xL, bcl-w, mcl-1), pro-apoptosis BH3-only (bim, bad, bid, PUMA, noxa) and pro-apoptotic Bax-like (bax, bak, bok) [4]. It is suggested that the numerous interactions between pro- and anti-apoptotic genes are major factors in the cell death regulation. Indeed, these interactions coupled with the ratio of each protein type will determine if the cell stays viable or goes into apoptosis [4]. Apoptosis signal is transmitted by a caspase-cascade phenomenon and Bcl-2 type proteins have an impact on caspases, principal effectors of cells apoptosis. Caspases are enzymes made of two subunits and are either initiators (caspase-2, -8, -9 and -10) or effectors (caspase-3, -6 and -7)[5]. The first class goes under autocatalytic activation which enables the induction of cell death, the second one is activated by an initiator and manages cell destruction through cleavage of different substrates [6]. Multiple cases have demonstrated the effects of Bcl-2-like proteins on caspases resulting in cellular lifespan extension (Table 1).

Table 1: Apoptosis regulation through the usage of anti- and pro-apoptosis genes from the Bcl-2 family.

Gene	Beneficial effect(s)	Reference
bcl-2	- caspase activation inhibition	Yang et al. [7]
	- inhibition of sodium butyrate effects	Kim and Lee, []
	- increase cell viability (2 days)	
	- increased antibody final titer (2-fold)	
bcl-xL	- increased cell viability	Chiang and Sisk. [8]
	- increased integrin production (2-fold)	
	- delay of autophagy	
mcl-1	- increased antibody production (20-35%)	Majors et al.[9]
	- increased cell viability (25% after 14 days)	
bax + bak (knockout)	- caspase activation failure	Cost et al.[10]
	- increased antibody production (2 to 5-fold)	
	- increased antibody production (82% with bcl-xL, 34% with mcl-1)	Zustiak et al. [11]
		Zhang et al., 2017 [12]

However, most studies involved in gene regulation of apoptosis gave a great variance in the genes' efficiency in either the down-regulation of pro-apoptotic genes or stimulation of anti-apoptosis. Even in the event of clonal variability, a global comparison of each separate gene would determine the genes with the highest efficiency within the same conditions. These genes may be subjected to genetic modifications to enhance even further the attribute of interest. The co-transfection of most proficient pro- and anti-apoptotic genes within mammalian cells would provide results of high interest for optimization at large-scale therapeutics production. The same principle would apply to the Bcl-2-like protein's effect on protein production.

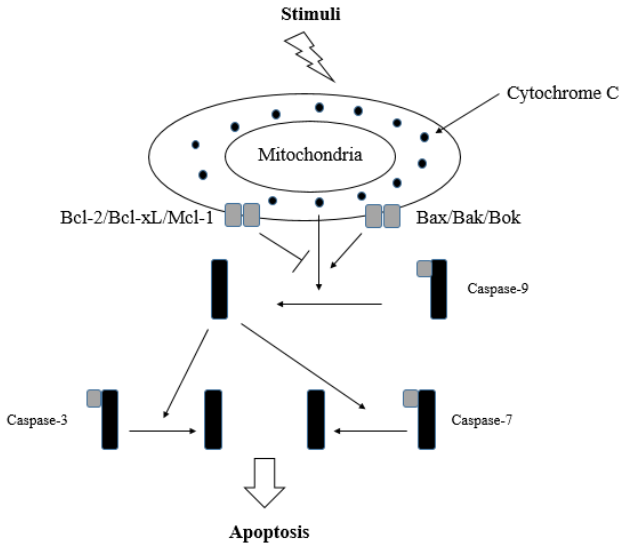

Figure 1: Illustration of caspase-cascade reaction of apoptosis and the implication of Bcl-2 family genes [13,14].

RNA interference interacts with mRNA translation and has a clear impact on gene expression and became a valuable tool in cell engineering because of the impact on the regulation of gene expression [15]. It has been used against pro-apoptotic gene expression, namely Bax, Bak, Alg-2 and a transcriptional factor, resulting in a noticeable delay of apoptosis, increased cell viability and protein production [16,17]. Druz et al. have used stable expression of short hairpin RNA fragment to specifically inhibit pro-apoptotic miRNA mmu-miR-466h-5p, which in turn delayed caspases effectors activation (caspases-3 and -7) by more than 35 hours [18]. It helped the increased expression of five different pro-apoptotic genes and finally an 11% increase in specific productivity while increasing cell viability. The authors have highlighted the snowball effect of the mechanism alteration from a single miRNA inhibition. Members of the miR 297-669 cluster also have anti-apoptotic features and it would be interesting to evaluate the pro-apoptotic potential of other cluster members and compare them while inhibited along with mmu-miR-466h-5p. Multiple miRNAs showed involvement in apoptosis regulation [19-23]. Another mammalian cell's natural trait, i.e. cell cycle has been the prime focus of several studies where the growth pattern is altered to provide positive outcomes.

Cell cycle regulation

Cell cycle regulation, similarly to apoptosis regulation, aims to increase cell viability/density and cultivation period to increase volumetric productivity [24, 25]. The cell cycle is made of four different phases: G0/G1, S, G2, and M, and each of these steps is controlled by checkpoints (Figure 1). Cycle regulation can induce a stoppage in cellular growth at the G2/M checkpoint [26]. However, stopping cell growth at the G1 phase is preferable as mammalian cells were observed to be metabolically more active, larger in size, and greatly capable of expressing genes involved in ribosome biosynthesis [27,28].

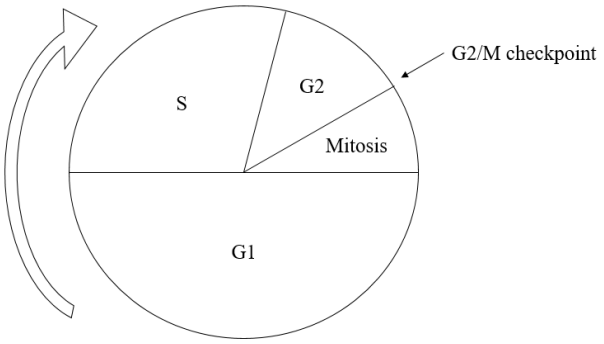

Figure 2: Diagram of cell cycle phases.

Ibarra and colleagues have managed to increase IgG4 antibody production through growth stoppage of NS0 cells at the G1 phase by inducible expression of p21CIP1 gene [29]. However, the combination of inducible expression of cell cycle regulation factor with Bcl-2 protein expression has not promoted higher resistance to apoptosis. The combination of both G1 stoppage and anti-apoptotic Bcl-2, in theory, capable of extending cell lifespan, suggests that the cell machinery at the G1 stage, being pro-efficient in cellular development, might reach a limitation in its translation capacity. The silencing or knockout of pro-apoptotic genes might be a preferable substitute, as it would relieve the cell from over-expressing another gene. The manipulation of miRNA/RNAi might also be of interest in that case.

mAb production has been improved up to 110pg/cell/day, equivalent to a 2 to 3-fold increase, using a cell cycle inhibitor, specific of the G1 cyclin CDK4/6, completely arresting cell progress [30]. Caspases inhibiting genes or the use of miRNAs, either or both co-transfected with cell cycle arrest inducible expression system or a cell cycle inhibitor, would logically be another way to improve the lifespan of the cells.

The cell cycle can be regulated through the temperature impact on metabolic mechanisms. CHO cells lower growth rate at mild temperatures have been proven to result in reduced volumetric productivity, even if specific cellular protein productions are improved [31-33]. Further investigations were focusing on downregulating cold-inducible RNA-binding protein (CIRP), formerly believed to be actively inhibitor of cell growth at low-temperature culture and enhancing cell adaptation to the cooler environment. The growth of CHO cells in these conditions was improved but without any clear impact on volumetric productivity [31,34]. To remedy this matter, CHO cells were first grown in normal conditions at 37^{o}C and were subjected to a temperature shift at the production phase resulting in increased volumetric productivity [35,36] . A fully comprehensive understanding of the mechanism of hypothermic cellular adaptation is required to impact protein productivity through cell engineering. The possibility to stop cell proliferation at the G1 stage in hypothermic conditions should help in increasing the volumetric productivity considering the metabolic enhancement but without impact on growth rate. An improvement in specific productivity requires higher secretion capacity for the protein to be available in the medium. The enhancement of that pathway has been of major importance to increase overall production and for downstream processing and quality/purity aspects.

Secretion system.

Mammalian cells have a key characteristic over microbial strains for therapeutics production with their capacity to secrete protein extracellularly, facilitating downstream processing. The protein secretion pathway in mammalian cells is quite complex and remains to be fully exploited. However, key secretion regulators have been well studied and demonstrated themselves as valuable tools for enhancing protein production. All proteins are naturally co-translationally targeted to the endoplasmic reticulum (ER), a major contributor in post-translational protein processing, and then transported to the Golgi apparatus. The protein is packed into secretory vesicles that will fuse with the membrane to release the component outside of the cell. In some cases, proteins might fail to be folded correctly and can result in the accumulation of unfolded protein in the ER, which provokes an acute stress state called unfolded protein response (UPR).

The X-box-binding-protein 1 (XBP-1) is a human transcription factor and has been used in secretion engineering in mammalian cells to improve specific protein productivity. Tigges and Fussenegger have shown the efficiency of the gene xbp-1 in monitoring secretion bottlenecks, responsible for UPR [37]. The resulting transformed CHO-K1 cells demonstrated that increasing levels of XBP-1 resulted in enlargement of endoplasmic reticulum and Golgi apparatus allowing a steadier flow of protein to be transported through the secretion pathway, avoiding UPR occurrence. The spliced form of Xbp-1, namely Xbp-1S, is a transcription activator and activates genes involved in the protein secretion pathway and the biosynthesis of ER [38]. In cases where bottlenecks are achieved, the overexpression of XBP-1S increased the protein titer by 2.5-fold. Gulis et al utilized the Xbp-1 gene ligated into the doxycycline-inducible system, which was transfected effectively into IgG-producing CHO cells [39]. The IgG production increased along with increasing doxycycline concentration and Xbp-1 expression. Other UPR transcription factors, GADD34 and ATF4, were studied with similar positive outcomes (Table 2).

Table 2: Secretory pathway factors.

Gene	Impact	Reference
Xbp-1	- prevent UPR	Tigges and Fussenegger [37]
	- increased protein production	Becker et al. [40]
		Gulis et al. [39]
Xbp-1S	- increased protein production (2.5-fold)	Ku et al. [38]
ATF4	- increased specific productivity	Ohya et al. [41]
		Haredy et al. [42,43]
GADD34	- increased protein production	Omasa et al. [44]
Sly1, Munc18c	- increased secretory capacity	Peng et al. [45]
Munc18b	- increased secretory capacity	Peng et al. [46]
S132A	- increased protein production (with Xbp-1)	Florin et al. [47]
		Rahimpour et al. [48]
SNAP-23, VAMP8	- increased secretory capacity	Peng et al. [49]
SRP14	- improved difficult-to-express antibodies production	Le Fourn et al. [50]

The Sec1/Munc18 (SM) proteins family are key components in plasma membrane fusion regulation. Soluble N-ethymaleimide-sensitive factor attachment receptors, or SNAREs, are proteins anchored to both transport vesicles and target membranes. They ensure proper vesicle-membrane specificity and trigger membrane fusion by assembling into 4- α -helix-bundle complex catalyzed by proteins from the SM family. In this family, genes Sly1 and Munc18c, in conjunction with Xbp-1 engineering, showed a positive impact on vesicle synthesis and increased secretory specific capacity up to 40pg per cell per day of anti-CD20 antibody [45]. Expression of Munc18b enhances the secretory capacity of HEK-293, HeLa and HT-1080 cells [46]. As mentioned above, SNAREs are involved in the fusion of vesicles with respective targets in the plasma membrane. SNAP-23 and VAMP8 were stably expressed in different mammalian cell lines and showed to improve SEAP production [49].

Another area that has been investigated is the central part of the pathway consisting of the recruitment of members from the protein kinase D (PKD). These enzymes interact with CERT proteins which are responsible for transport between the ER and the Golgi apparatus. This interaction regulates the secretion capacity of the Golgi organelles [51]. A mutant version of the S132 gene, CERT-S132A, was co-transfected with Xbp-1 in CHO with a reported protein production increase of 35% [48].

Signal peptides modifications are regularly used to help in the secretion of recombinant protein. They are comprised of 15 to 30 amino-acids and bound to the antibody N terminus of the antibody. Signal peptides help in the mediation and of protein translocation from cytoplasm to the periplasmic region and finally get cleaved during the process [52]. Young and Rance demonstrated that it was possible to express a specific gene with a signal peptide from other species with higher protein production [53]. Also, it has been observed that secreted antibodies with signal peptides on both chains resulted in high secretion, whereas the removal of these from both chains gave low or no secretion of antibody [54]. Hence, the choice of signal peptide remains a considerable challenge because of the variable region within antibodies [55]. Le Fourn et al have been able to use the expression of signal recognition particle, namely SRP14, in Trastuzumab- and Infiximab-producing CHO cells and enabled the transformed cells to produce difficult-to-express respective mAbs [50]. Haryadi et al. manipulated the original Rituxan signal peptide and the outcome resulted in a two-fold increase of the mAb titer [56]. The high complexity of the mammalian secretory pathway can be enhanced in many different ways but to provide the best theoretical candidate, genetic modifications would have to occur at all levels of the pathway involving UPR transcription factors, mediators between ER and Golgi apparatus, SNAREs and signal peptides (native or modified). In that sense, secretion optimization relies more on anticipation of bottlenecks formation, UPR prevention and enhancement of protein transport within the cell. Each case where protein production increases are notified through cell engineering, secretion factors would have to be involved totally or partially to enable cells to reach their secretory potential. Also, an increased protein production activity would indirectly involve a higher metabolite production, which can become detrimental to both product and cell culture. The genetic modifications to be brought to resolve such issues would have to be made on the metabolic aspect.

Metabolic engineering

Ammonia and lactate are both derived from mammalian cell cultivation and their accumulation in the media can alter cell growth and damage product quality. The accumulation of both compounds is due to the presence of glutamine and glucose within the culture medium. The engineering of cellular metabolism enables cells to use these metabolites efficiently. To reduce ammonia produced from glutamine, a less ammoniagenic substrate, glutamate, was used. Recombinant CHO cells expressing glutamine synthetase (GS) were able to convert glutamate with ammonia into glutamine and showed less ammonia production in the glutamate-based culture [57].

Several studies have worked with the expression of cytosolic yeast pyruvate carboxylase 2 (PYC2) gene in different mammalian cell lines and resulted in lower lactate production [58,59]. PYC2 was transfected into CHO cells using pMPYC-CHO, a dual selection vector with antibiotics, and the rCHO cells had a higher mAbs production and a noticeable improvement in its glycosylation profile [60]. The use of PYC2 combined with dual selection in CHO cells provided the highest productivity, with an improved N-glycan

profile. Several genetic manipulations are required at the genomic level of the host cell line for cell growth, proliferation, and survival, as the functions of mAbs are dependent on several other proteins. An increase in protein yield has been observed, as the host cell is not able to cope up with the production of heterologous protein, probably due to increased stress and toxicity [13]. Hence, gene manipulation at the vector level, for creating favorable metabolic patterns, facilitates the reduction of cellular processes that affect the quality profile of mAbs i.e. folding, waste reduction. It is now clear that traditional modifications made within mammalian cells genetic information only act on one or a couple of factors. The key strategy for novel techniques is to be more efficient and to be able to act on several elements involved. Micro RNAs and CRISPR/Cas9 techniques are bringing answers from that standpoint.

Micro RNAs (miRNAs) and CRISPR/Cas9 cell engineering

miRNAs are endogenous small non-coding RNA fragments that impact gene expression in mammalian cells highly conserved across species [61]. miRNAs have a proven ability to control entire physiological pathways by targeting a consequent number of genes [62], and individual miRNAs can regulate several cellular phenotypes simultaneously [63]. Table 3 shows that miRNAs have been widely studied and provide improved protein productivity.

Table 3: List of miRNAs with a direct impact on mammalian cell production capacities.

miRNA(s) studied	Over or stable expression	Impact	Reference
miR-7	Overexpressed	- increased specific productivity	Barron et al. [64]
miR-557, miR-1287	Stable	- increased cell proliferation	Strotbek et al.[65]
		-increased productivity	
miR-17, miR-19b, miR-92a, miR-20a	Stable	- increased specific productivity	Loh et al. [66]
miR-17, miR-92a	Stable	- increase growth and productivity (miR-17, 3-fold)	Jadhav et al. [67]
		- increased productivity (miR-92a)	
miR-2861	Stable	- increased productivity	Fischer et al.[68]
miR-92a	Over-expressed	- increased specific productivity	Loh et al.[66]
miR-483	-	- increased productivity	Emmerling et al.[23]
miR-557	Stable	- increased productivity	Fischer et al. [69]
miR-15a, miR-16-1 (silencing)	-	- increased specific productivity	Pairawan et al. [70]

Considering their ability to regulate entire molecular networks, the use of miRNAs for improving the efficiency of recombinant protein production by CHO cells is gaining considerable interest. Several other miRNAs have been studied with clear beneficial aspects in cell proliferation and protein productivity [65-67]. The enzyme histone deacetylase (HDAC) is an inhibitor of protein secretion, miR-2861 was stably expressed in CHO cells and was shown to regulate HDAC5 after translation, promoting protein expression [68]. A challenge for industries is the stable expression of difficult-to-express therapeutics as their secretion might not be as effective as desired. The pro-productive miRNA miR-557 increased by 2-fold difficult-to-express mAb titer when stably expressed [69].

The alternative approach in miRNA studies is to inhibit the function of a specific miRNA. miR-15a and miR-16-1, both apoptosis enhancers, were abolished through stable expression of their specific decoy transcript, or miRNA sponges, in anti-CD52-producing CHO-K1 cells [70]. The recombinant mAb production yield increased by 3.37-fold after four days of cultivation. The field of miRNAs is focusing considerable interest, but similarly to the Bcl-2 protein family, there is a degree of variance in terms of impact on the secretory pathway of mammalian cells. The difference might be explained by the nature and the role of the miRNA target in the protein production process. An in-depth research of miRNAs efficiency in impacting cellular pathways would be a breakthrough in cell engineering. Indeed, it would enable direct impact and/or inhibition of multiple entire pathways to reach a highly suitable recombinant cell profile. Even though different co-transfection of several miRNAs in expression vectors have already been used (see Table 3), different miRNAs transfection combinations would be interesting to evaluate. CRISPR-Cas9 is a novel molecular tool that comprises a guide RNA fragment directing a Cas9 endonuclease, capable of cleaving specific DNA sequences [71]. The usage of Cas9 has allowed accurate gene integration with 34% more efficiency than transformation methods previously studied [72]. The proven capacity of integration of large fragments incorporating regulation factors for signal peptides, translation, and glycosylation, while producing stable cell lines and without cell machinery obstruction would be interesting to explore further.

Conclusion

mAbs are produced by mammalian cells and are used in different medical cases such as the treatment of severe diseases. The capacity of post-translational modifications from eukaryotic producers makes them a suitable candidate for biotherapeutics with high potency. Indeed, glycosylation, a post-translational modification step, has been proven many times to convey correct, and even enhanced at times, cytotoxic characteristics. The improvements in the field of cell engineering during the last decade have helped to elaborate mammalian cell lines with increased specific productivity. Many studies have focused on mammalian cell lines, CHO cells especially, natural traits to improve production yields, such as both apoptosis and cell cycle regulations, metabolic engineering and secretory pathway.

The first approach for apoptosis and cell cycle regulation is to extend the life span of producing cells within the culture at the stationary phase or G1 growth phase. From the cell cycle regulation aspect, blocking cellular growth at the G1 phase and using a member of the Bcl-2 protein family has not been showed to promote apoptosis resistance to date. However, the addition of

increased cellular metabolic capacities and a longer lifespan would have attractive attributes. Also, to our knowledge, there has not been a study yet on the Xbp-1 impact on CHO cells stopped at the G1 growth level, as the secretion ability of the cell would be exploited in that regard. One challenge when greater cell proliferation is achieved is the management of metabolites accumulation and metabolic engineering has been focused to provide viable solutions. Recombinant cells produced the enzymes capable of using or to lower their metabolite production. mAb productions were significantly improved and in some cases, an improvement of the protein N-glycan profile was noticed. This strategy would help in protein isolation/purification and further downstream processing. The enhancement of the cellular secretion pathway is also a valuable tool. Although quite complex, several ways to provide a positive impact have been discussed above. Xbp-1 secretion factor is one of the most studied proteins with multiple interactions within secretory pathways of mammalian cells and act directly on protein secretion rather than an intermediate step in the process. Several papers have used the influence of Xbp-1S in conjugation with SM and CERT proteins and both aspects have produced increased protein production. A lot of these techniques used have shown a direct or indirect impact on cell protein production. Nevertheless, from a large-scale producing pharmaceutical standpoint, the elaboration of suitable cell lines needs to be achieved in a short period avoiding possible mutations. The optimization of the vectors used for transfection, the timing required for the transformed cells pools screening, the scale-up, and the final productivity are all factors to take into account while choosing the modifications to be brought to the original cells. In that regard, novel molecular techniques involving mRNA-RNAi (no translation requirement), and CRISPR/Cas9 are the ones that have the smallest bearing in cellular metabolic machinery and the highest efficiency without altering protein folding/glycan structure [73]. However, certain undesirable traits might be incorporated through miRNA because of the capacity of a wide range of changes within the cells' genome. The fact that these methods are not fully mastered today explains why the more traditional approaches are still highly valued.

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