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Vector and Cell Line Engineering Technologies Toward Recombinant Protein Expression in Mammalian Cell Lines

Seyedeh Hoda Jazayeri[1,2] · **Amir Amiri-Yekta**[2] · **Salahadin Bahrami**[2] · **Hamid Gourabi**[2] · **Mohammad Hossein Sanati**[3,2] · **Mohammad Reza Khorramizadeh**[4]

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Abstract The rapid growth of global biopharmaceutical market in the recent years has been a good indication of its significance in biotechnology industry. During a long period of time in recombinant protein production from 1980s, optimizations in both upstream and downstream processes were launched. In this regard, one of the most promising strategies is expression vector engineering technology based on incorporation of DNA opening elements found in the chromatin border regions of vectors as well as targeting gene integration. Along with these approaches, cell line engineering has revealed convenient outcomes in isolating high-producing clones. According to the fact that more than 50% of the approved therapeutic proteins is being manufactured in mammalian cell lines, in this review, we focus on several approaches and developments in vector and cell line engineering technologies in mammalian cell culture.

✉ Mohammad Hossein Sanati
m-sanati@nigeb.ac.ir

✉ Mohammad Reza Khorramizadeh
khoramza@tums.ac.ir

[1] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran

[2] Department of Genetics, Reproductive Biomedicine Research Center, ACECR, Royan Institute for Reproductive Biomedicine, P.O. Box: 14155-6343, Tehran, Iran

[3] Department of Medical Genetics, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

[4] Biosensor Research Center, Endocrinology and Metabolism Molecular-Cellular Sciences Institute, Tehran University of Medical Sciences, P.O. Box: 1411413137, Tehran, Iran

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Introduction

In 2016, nine of the top ten pharmaceutical products have being biologics [1]. According to the Intercontinental Marketing Services (IMS Health), biologics, biosimilars, and nonoriginal biologics (NOBs) are increasing their share of the global pharmaceutical market and dominating sales in 2017. As an example, in 2002, biologics specialized 11% ($46 billion) of global sales; however, it is estimated to reach ~ 20% ($212 billion) in 2017 [2]. This trend is getting started from recent years and projected to move faster according to numerous patent expirations in the coming years [2]. During 2012–2013, sales of biologics increased 18.2% in the USA, being extremely higher than the growth rate of the past 4–5 years. This rise is more than 7-fold in comparison with only 2.5% growth in the total pharmaceutical sector sales in 2012. Despite the high risk of biopharmaceutical development, potential of great reward causes the rising percentage share, which reflects the stabilization of product sales, success of many recently launched products, and the growth of existing products [3].

Meanwhile, a vast number of researches in therapeutic protein production are performed in prokaryotes and yeast systems [4–11]; however, mammalian cell lines are the most frequently used platforms for biopharmaceutical production on the market. This is due to the capability of mammalian cell lines to perform posttranslational modification, identical to the native human proteins [12–14]. Nevertheless, mammalian cell culture productivity is extremely lower than that of microbial cells and their cultivation is laborious, cost-intensive, and complicated process [15, 16]. It should be also noted that presenting the biopharmaceuticals to the market is a time-consuming process and may be a decade-long procedure; however, the biologics production industry have a growing trend and make opportunities for those who identify the gradual nature of this industry [17].

During more than three decades in recombinant protein production, vast optimizations of cell productivity are performed in mammalian cell systems. Whereas lots of these advancements are kept as commercial secrets, many reviews covering cell line development technologies are available [16, 18, 19]. This review focuses on strategies in vector and cell line engineering technologies in mammalian cell culture based on protein production and presents different approaches aimed at the increasing recombinant protein expression.

Vector Engineering Technologies

One of the most common bottlenecks in mammalian protein production is high variability between different clones [20, 21]. In addition, despite the initially high level of recombinant protein, expression reduction is frequently observed during prolonged culture [20, 22]. Accordingly, maintaining high gene expression level is a critical factor in heterologous mammalian gene expression [23]. Stability is determined by maintaining the 70% of initial productivity without using selection reagent, considering to the toxicity and high cost of these reagents [24, 25]; meanwhile, plasmid vector engineering technologies are suitable strategies for improving and preserving the efficiency of high-producing clones [18].

The vast variability and loss of stability in recombinant clones is emergent of cell line development procedure which can be made by deletion of transgene from host cell genome

which mostly undergoes gene amplification [26]. The use of mutagenic selection agent intended to amplify transgene copies can lead to chromosome breaks and transgene copy loss which frequently causes genetic instability and unstable expression. On the other hand, the selection pressure should be retained in culture medium despite its disadvantages as removal of selection agent may result in up to 80% decline in productivity [27, 28].

It should be noted that as expression constructs are often introduced into a host cell genome by random integration, the level of transgene expression depends on the integration site on the chromosome. With due attention to the fact that most genomic sites are transcriptionally repressive, it is not surprising that many clones are unable to express high levels of transgene [29]. The repressive effect can cause epigenetic silencing of adjacent genes by histone deacetylation and methylation of the transfected DNA's promoter [30, 31]. Two frequent strategies can be applied to preserve DNA from integration-dependent repression or negative position effects. One strategy is based on incorporation of DNA opening elements found in the chromatin border regions of an expression vector to protect the gene from surrounding chromatin influences. Another method relies on site-specific integration or recombination to conduct transgene into a previously specified locus which is transcriptionally active. In the following, these two effective approaches are discussed (Fig. 1).

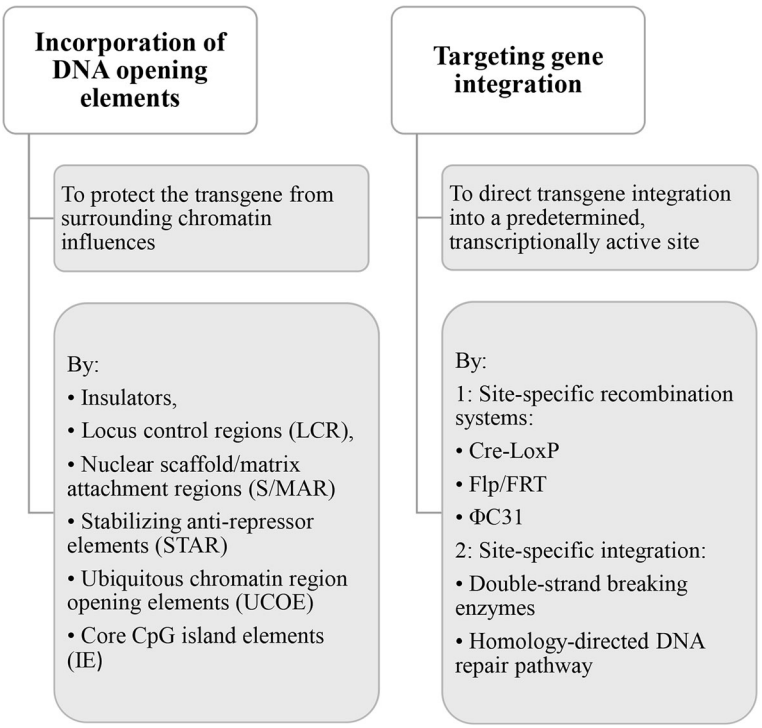

Fig. 1 Strategies toward DNA protection from negative position effects. Two approaches can be used to protect DNA from integration-dependent repression. One approach is based on incorporation of DNA opening elements found in the chromatin border regions of expression vector to protect the gene from surrounding chromatin influences. Another method relied on site-specific integration or recombination to conduct transgene integration into a predetermined site that is transcriptionally active

DNA Opening Elements

There are several commonly used DNA elements that tend cells to overcome silencing such as insulators, locus control regions (LCR), nuclear scaffold/matrix attachment regions (S/MAR), stabilizing anti-repressor elements (STAR), ubiquitous chromatin region opening elements (UCOE), and the core CpG island elements (IE) [23, 32]. Flanking a transgene with these elements can decrease the clonal expression variability and lead to easier identification of acceptable clone producer.

Among different chromatin open elements, S/MARs are one of the most practical ones. These elements are believed to facilitate the attachment of chromatin loops to the nuclear scaffold or matrix. S/MARs range from 300 base pairs to several kilo base pairs long and around 70% of their sequence is composed of AT. These elements may contain several nucleotide motifs covering base-unpairing regions (BURs), potential replication origins (ORI), triple-helical or H-DNA structure sequences, retro element insertion hot spots, etc. There are also certain proteins, bind to insulators and/or S/MAR elements such as 11–zinc-finger and CTCF, which is thought to exclude DNA methyl transferases from its binding region, so it can affect local genomic methylation and regulate gene expression. CTCF binding factor appears to be sensitive to methylation, so lack of CTCF can lead to methylation [33].

A simple method to increase the recombinant protein expression, takes advantage of insulators activity by incorporating them into an expression vector [34]. Some studies have shown the relation of protein expression with orientation and copy-number of insulators or S/MARs [35, 36]. These elements can be added *in cis* to a vector at the upstream of the promoter/enhancer or at the downstream of the polyadenylation (polyA) or both. There is also an additional benefit from using S/MARs at both borders of a reporter gene [29]. Furthermore, adding the MAR element on a distinct plasmid (*trans*), along with the expression vector, can increase recombinant gene expression up to 10-fold over control group [37]. Moreover, using constructs with two copies of S/MARs *in cis* can exceptionally increase the number of high-expressing clones. However, using three copies of S/MAR *in cis* did not exhibit higher expressing clones or better stability in comparison with using two copies [33]. Among different MAR elements, chicken lysozyme MAR (cMAR) and human interferon β MAR (iMAR) revealed more effective impact at increasing the GOI (gene of interest) expression in stable transfected cells.

UCOE is another frequently used chromosomal element with methylation-free CpG islands close to transcriptional start site and are endogenously localized next to the ubiquitously expressed genes [38, 39]. UCOEs provide a high expression level of the GOI in CHO and BHK21 and maintain the expression stability for more than 100 generations [40–42]. Betts et al. evaluated the effect of UCOEs in stability and productivity of recombinant protein expression in MTX amplification system within CHO DG44 cell line. They observed that cell lines containing UCOE have superior growth characteristics, greater transcriptional activity per gene copy, and lower clone variation in comparison with non-UCOE cell lines [22].

Another effective approach at preventing DNA methylation can be achieved by using the core CpG island elements (IEs), with the small size of 120 base pair, making its application easier in comparison with several thousand base pairs of other elements [43]. In general, it should be noted that several parameters need to be optimized for selection of proper sequences to make a sequence behavior as a chromatin border. Among different factors, expression vector components are one the most important parameters [44].

The Plasmid Vector Composition

One of the important factors in the productivity and stability of recombinant mammalian cells is the composition of plasmid vector [45]. Effective segments of vectors consist of promoters, enhancers, polyA signals, selection cassettes, expression augmenting elements, as well as the GOI 33334 [29, 46]. Three commonly applied promoters are isolated from the human cytomegalovirus (CMV) major immediate-early gene, the simian virus 40 (SV40), and the CHO elongation factor 1α (EF1α) gene [47, 48]. Researches have shown the susceptibility of CMV to gene silencing leading to declining production levels in long-term culture [49]. In addition, protein production instability using the SV40 promoter in CHO cells has been announced [50]. Studies suggested more resistance in silencing of endogenous mammalian promoters, such as EF1α, than viral promoters [51]. Applying EF1α promoter along with some regions upstream and/or downstream of the CHO EF1α gene has shown to be more dynamic than the only use of CMV and SV40 [48]. However, Ho et al. reported that despite the lower expression level of SV40 promoter, this promoter has higher expression stability in comparison with EF1α and CMV. This is due to more resistance to transcriptional silencing by DNA methylation according to the lower number of CGs dinucleotides in SV40 promoter region [52].

On the other hand, it is demonstrated that efficiency of DNA opening elements are associated with the cells and different vector components specially promoters [44]. Studies have shown that experimental testing is necessary for recognition of the best combinations between chromosomal elements and promoters to obtain higher expression level and stability [41]. Yeo et al. developed a collection of several hCMV promoters joined with one or two copies of IE elements in different positions of the hCMV enhancer and core promoter. They reported that the altered hCMV accompanied by one copy of IE and the enhancer and core promoter in reverse orientation has the best effect on promoting expression stability. It is also indicated that one third of IE containing clones preserved 70% of their initiating expression amount during 8 weeks of culture in the absence of selection pressure, whereas none of the wild-type clones had expression level above 50% [23]. However, Ho et al. demonstrated that using CPG-free promoters could decrease the early gene silencing but do not influence on long-term stability since silencing via histone modifications could still take place [53].

In another experiment a comparison of quantity and stability of recombinant gene expression among SV40, EF1α and CMV promoters were performed by using iMAR and cMAR [52]. It is reported that the addition of MAR would improve the stability of gene expression in SV40, although showed no impact on CMV and EF1α. On the other hand, there are conflicting results on EF1α and CMV promoters [15, 54] and there is a probability of cMAR or iMAR effects on stable gene expression for these promoters [52]; the similar result is achieved in a study conducted by Lonza in insertion of cMAR into glutamine synthetize expression vector representing no effect in monoclonal antibody expression level [54]. These studies may reveal that the activity of MAR elements is mostly related with the chromosomal sites when the expression is in low level but had minor influence in highly active chromosomal positions [29]. Therefore, MARs may be less effective on transgene expression by using strong promoters. In other words, insertion of MARs to a well-optimized vector would assign lower profit in comparison with a simpler vector [52].

On the other hand, a method for establishment of high recombinant antibody production cells using plasmids with two different elements containing a mammalian replication initiation region (IR) and MAR is reported. Plasmids bearing both IR and MAR enhance more

effectively their copy numbers in gene amplification. It is pointed out that the level of recombinant antibody expression in this method is strongly dependent on the cell type. Araki et al. reported clones expressing stable high level of the antibody with the specific protein production rate of 29.4 pg/cell/day in CHO DG44 cells [55].

Moreover, according to the time and labor intensiveness of DHFR/MTX procedure and instability of high-producing cells, the combination of IR/MAR and DHFR/MTX was proved in another study as these two gene amplification processes relies on various principles. The IR/MAR-DHFR fusion strategy was shown to yield more amplification stability and elevated recombinant protein over a longer span of time in comparison with performing each method separately. It is pointed out that efficient gene amplification cannot be achieved by using just IR or MAR or unrelated sequence with the same size [56].

Targeting Gene Integration

While random integration is widely applied to establish specific CHO cell lines in order to achieve stable and elevated expression of recombinant proteins, in most cases, expression levels are not desirable according to low activity of integration location in the target genome.

Two different strategies can be applied to integrate the GOI in a location with favorable expression particulars, site-specific recombination systems and site-specific integration by using double-strand breaking enzymes and homology-directed DNA repair pathway.

Site-Specific Recombination Systems

One of the site-specific recombination systems is Cre/loxP, which can be applied for targeting genes to special locations in the genome showing superior expression level [57, 58]. The important factor in this system is a 34 base pair recognition site called loxP site (ATAACTTC GTATA *ATGTATGC* TATACGAAGTTAT) which can be recognized by an enzyme named Cre recombinase that can accelerate the recombination among two LoxP sites [59]. The LoxP site includes two identical 13-bp inverted repeats flanking an 8-bp spacer region (Fig. 2a) [59]. Recombination among two LoxP sites can result in different outcomes relying on the position and the direction of these sites. If they have an identical direction and rest on one DNA molecule, the sequence between them will be excised. If they are on the same DNA molecule but with opposite orientation, the sequence between them will be inverted, and if these two sites presents on two different DNA molecules, the recombination event will lead to

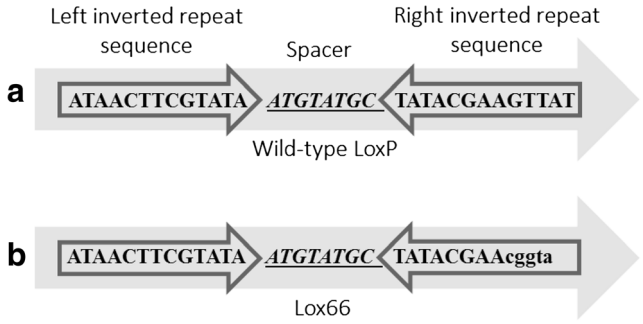

Fig. 2 Comparison of wild-type loxP (**a**) and mutant lox66 (**b**) sequences. The LoxP site is composed of an 8-bp spacer region flanked by two identical 13-bp inverted repeats

exchanging fragments of DNA between these molecules [59] (Fig. 3a–c). Since these reactions are reversible, it is possible to perform the opposite of the excision reaction. This reaction can be done if we have a DNA fragment flanked by two LoxP sites with the identical direction [57, 59]. In this case, the Cre recombinase can insert the DNA fragment inside the LoxP site in the target DNA molecule. There have been several LoxP mutants developed either in the spacer region or the 13-bp inverted repeats (e.g., lox71, lox66) (Fig. 2b). Because of the fact that some of these sites are not involved in the Cre-mediated recombination with one another, these new mutant LoxP sites have made it possible to exploit distinct irreversible recombination between these sites [59]. The remaining LoxP sites in these recombination products will be different from the original ones in a way that they are incompatible with each other (e.g., recombination between lox 71, lox 66 that yields a double mutant which cannot be recognized by Cre protein) [59]. Moreover, scientists constructed a vector carrying the green fluorescent protein (GFP) and DHFR while a loxP recognition site was inserted upstream part of them. This vector was transfected into DHFR-deficient CHO cells, and the highest fluorescence-producing clones were selected. These clones could be presumed highly active concerning transcription and gene amplification. In another study, a vector containing genes for both antibody subunits (light and heavy chains), along with a resistant marker and LoxP site, was generated and transfected together with Cre recombinase into the selected cells. The site-specific recombination facilitated by Cre protein led to the integration of the construct into the active locus. The generated clones produced human monoclonal antibody four times more than that of the previous one under similar conditions [57, 60]. By using this strategy, it is feasible to develop CHO cell lines that encompass a LoxP site in a position that boost the production of the genes integrated into this location. Once such a cell line is produced and isolated, different genes can be targeted to its active site to achieve the desirable protein expression. The Cre recombinase system can be applied as a transient expression system and be co-transfected with the vector containing the GOI to carry out the Cre/loxP recombination reaction. In 2011, Obayashi et al. developed a new integration system that can facilitate serial gene integration into the CHO cell genome starting with a single loxP site as the integration target site. In the next step, inserting multiple copy of the gene into the same location, called accumulative gene integration system (AGIS), simulated amplification in the target gene leading to the increase of recombinant

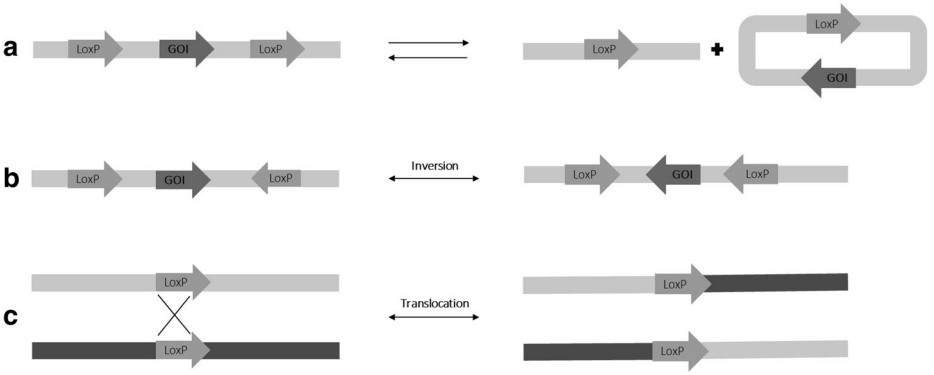

Fig. 3 Schematic of targeting gene integration. Site specific recombination between two LoxP sites can result in different outcomes. **a** If two loxPs are being in the same orientation and on the same DNA molecule, the sequence between them will be excised. **b** If two loxPs are being on the same DNA molecule with opposite orientation, the sequence between them will be inverted. **c** If two loxPs exist on two different DNA molecule, the recombination leads to exchanging fragments of DNA

protein [61, 62] (Fig. 4). A year later, another group of scientists used the same strategy to integrate multiple antibody genes into a pre-selected chromosomal locus of CHO cells [63]. Again in 2015, Kawabe et al. used AGIS to establish CHO cells that produce recombinant scFv-Fc in a high manner [64]. Matsuyama et al. applied Cre/LoxP system to achieve the conditional expression of mutant cell division cycle 25 homolog B (CDC25B) to obtain high and stable producing CHO DG44-derived cell line with the aim of improving the transgene amplification efficiently [65].

The second site-specific recombination system is Flp/FRT that is similar to Cre/LoxP, with the exception that recombination is facilitated by an enzyme called Flp recombinase performed between DNA sequences with a 34 base pair FRT sequence (5' GAAGTTCCTATTC *TCTA GAAA* GTATAGGAACTTC 3′). The Flp/FRT recombination reaction is also reversible, but its recombination specificity is low (< 10%) compared to Cre/loxP (80%) [66]. In 2007, Huang et al. generated a vector containing two weakened markers (β-galactosidase and DHFR) together with an FRT sequence. Three clones out of 20 showed promising activity and were

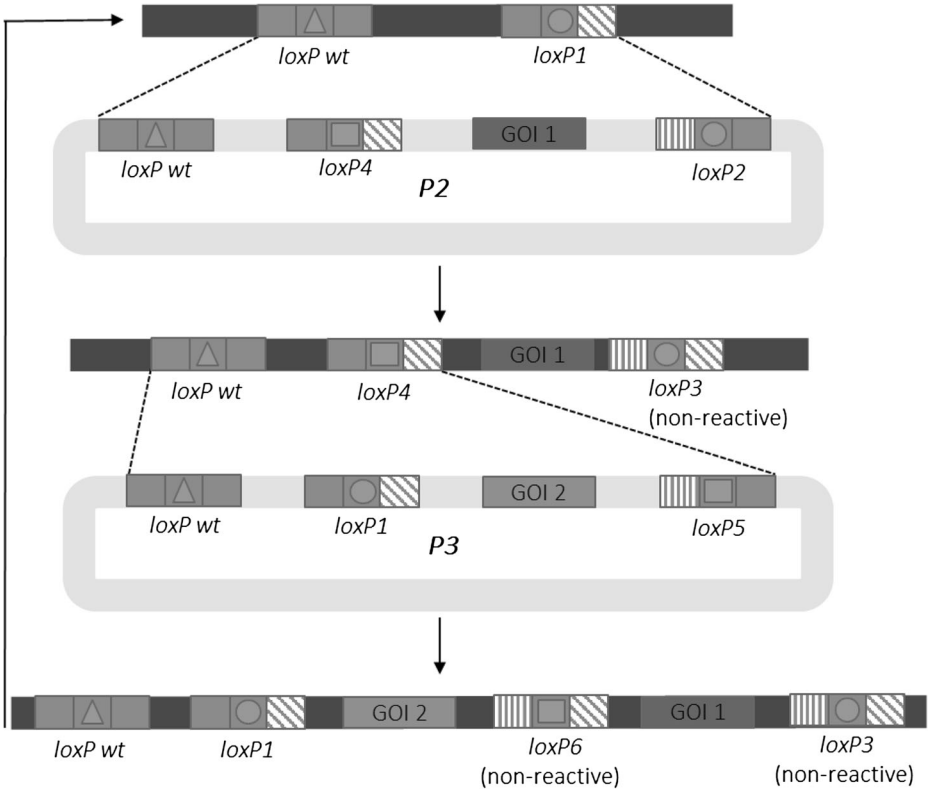

Fig. 4 A schematic representation of AGIS using Cre-mediated cassette exchange. Cre recombinase catalyzes a recombination reaction between two loxPs which is composed of an 8-bp asymmetric spacer region flanked by two 13-bp symmetric arms. Each LoxP site is depicted with three boxes. Wild-type spacer, mutated spacers 1 and 2 are represented by a triangle, circle, and square in the center box, respectively. Arm regions are depicted by two rectangle boxes at both ends of the LoxP site, the mutated arms are shown by the slanting-striped box. The Cre recombinase can facilitate recombination between those two sites which contain the same spacer and at least one wild-type arm. The loxPs with double-mutated arms that are generated after each recombination, can no longer be catalyzed by Cre- recombinase. A gene construct can be integrated into a target site multiple times in a successive and repeatable manner. This figure is adapted from Kameyama [62] with permission from authors

successfully used for inserting antibody genes into the desired locations. The best cell line was able to produce 200 mg/L of full-length anti-CD20 antibody in 6 days [67]. In another study, scientists used this recombination system with a vector entailing a neomycin resistance marker and an FRT site. They were able to isolate some clones producing tPA with high levels of 2.2 μg/10^6 cells/24 h protein production [68].

The third site-specific recombination system is ΦC31 system that is somewhat different from the other two mentioned systems [58]. First, the ΦC31 integrase enzyme accelerate recombination among two different sites, attP and attB, and second, the recombination reaction yields two new sites which cannot be recognized by integrase, so this type of recombination is not reversible (Fig. 5). The recombination specificity for this system is also below 10% similar to FLP recombinase. A mutant ΦC31 integrase enzyme was generated by using protein engineering, ΦC31o, the recombination specificity of which is as high as Cre recombinase. The ΦC31 integrase can catalyze recombination between an attB site and attP-like sequences in the target genome [66]. In this case, a vector entailing a luciferase coding gene fused with an attB site was transfected along with a ΦC31 enzyme carrying plasmid into CHO cells. Production of Luciferase expression using this procedure was 60 times higher than that of random transfection [69]. A cloning strategy, the gateway® cloning system, has been developed by using Bacteriophage lambda att site recombination that can also be applied to this target integration process. In this strategy, four various sites (attB, attP, attL, and attR) and two different integrase enzymes (BP Clonase™ II catalyzing recombination between attB and attP and LR Clonase™ II catalyzing recombination between attL and attR) are involved. Upon recombination between attB and attP by BP Clonase, the resulting sites will be attL and attR and a recombination between attL and attR will produce attB and attP sites [70, 71]. These two reverse reactions can be applied to facilitate recombinase-mediated cassette exchanges.

Site-Specific Integration Using Nucleases and DNA Repair Pathways

The site-specific recombination systems, referred above, are limited by the fact that a time-consuming process is needed for achieving clones within the GOI in a highly active location in its genome.

Genomic characteristics of several CHO cell lines are now available [72]. These data make it possible to design specific nucleases that can cause double-strand breaks in a specific location in the cell DNA [73]. Depending on the target location for integration of the transgene, different nucleases can be applied to induce the double-strand breaks (DSBs). These nucleases include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas9) RNA-guided nucleases [74]. Normally, the DSBs are repaired by one of these two major DNA damage repair pathways: non-homologous end-joining

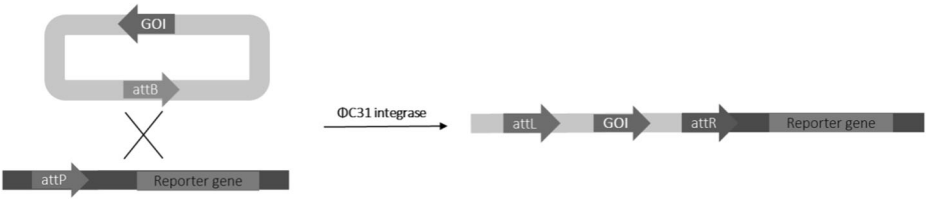

Fig. 5 Site-specific recombination system by ΦC31 system. Recombination between two different sites, attP and attB by ΦC31 integrase enzyme yields two new sites, attR and attL, which integrase cannot recognize them

(NHEJ) or homology-directed repair (HDR) [75, 76]. The HDR repair pathway can be induced with the concurrent introduction of homologous fragments of DNA and the nuclease carrying vectors [76]. By introducing another construct containing DNA fragments homologous to the flanking regions of the break site, along with the vector entailing the nuclease gene, it can be possible to induce targeted insertion/deletion (indel) mutations or accurate sequence modification in the target genome [74, 76].

In CRISPR/Cas9 system, the nuclease activity of the enzyme is guided by a short single RNA (single-guide RNA (sgRNA)) that can be engineered in a way inducing the DSB in specific target sites [77]. Ronda et al. investigated CRISPR/Cas9 system in order to apply it to CHO cells [78, 79]. They showed that a high efficient targeted mutation can be achieved in CHO cells by using this system (about 47.3%). Recently, researchers used the CRISPR/Cas9 genome editing system to insert a construct containing a fluorescent marker that was flanked by short-homology arms compatible with the DSB induced by this system. A 3.7-kb cassette was inserted at three different loci in CHO cells and led to the expression of GOI in CHO cells [73, 80]. Shin et al. demonstrated that balanced high-level expression of Cas9 nuclease and sgRNA increase the expression levels nearly three times more than that of typical methodology [81]. The CRISPR/Cas9 systems is also applied for production of specific knockout CHO cell lines [82, 83].

Cell Line Engineering Technologies

In spite of extensive characteristics gathered in mammalian cell lines, many limitations are still challenges being dealt with process control, media formulations, and cell engineering approaches [84–86]. One of the most common methods in cell line engineering is based on arresting cell cycle growth through overexpression of endogenous cyclin-dependent kinase inhibitors (CKIs), and using microRNAs (miRNAs) [87–89]. Along with these strategies, researchers have engineered cells in order to increase transcription rate, transgene expression, specific posttranslational modifications and decrease protein misfolding, aggregation, and degradation [90]. Overexpression of antiapoptotic proteins, like bcl-2 family members [91] and Bcl-x(L) [92, 93], expression of growth factors like acidic fibroblast growth factor (aFGF) or recombinant insulin-like growth factor (LR3-IGF) also can lead to increasing cell viability and augmentation of protein production [20].

Growth Arrest Strategies

Targeting regulators of cell cycle checkpoints by overexpression of CKIs leads to the increase of Q_P; however, the CKI overexpression has been observed no adequate amount of incorporation in production processes of some proteins [87, 94]. Du et al. developed an approach based on a small molecule compound that selectively inhibits cyclin-dependent kinases (CDKs) 4/6 and leads to the G1-checkpoint targeting. Using this inhibitor made the cell proliferation completely controlled in recombinant CHO cell lines during the production procedure, with up to 110 pg/cell/day specific productivity, and with regard no negatively changed of glycan profile [87].

In another study, the effects of a cell cycle checkpoint kinase, ataxia telangiectasia, and Rad3-related (ATR), downregulation on gene amplification and productivity of recombinant CHO cells were investigated. Using a small interfering RNA (siRNA) expression vector against ATR generated more high-producing cells after three different MTX concentrations of gene amplification as compared with mock cells [95].

Along these strategies, several approaches such as using chemical agents, decreasing cultivation temperature and changing medium PH may lead to growth arresting. These

strategies have focused on enhancing time integral of viable cell density (IVCD) and specific protein productivity (Q_p) [96]. Numerous chemical additives proved to have positive effects on the production of heterologous proteins, in spite of reduction or arresting cell growth by regulating CDK and CKI activity and inhibiting histone deacetylases. Sodium butyrate (NaBu), pentanoic acid (valeric acid), and dimethyl sulfoxide (DMSO) are routine compounds in present bioprocesses [97]. It has been demonstrated that low concentrations of NaBu (0.1–0.5 mM) can affect the production of heterologous proteins in CHO cells as much as 2–4-fold (98).

More specifically, reducing cultivation temperature is a routine strategy in growth control leading to decreasing in IVCD and increasing the recombinant protein synthesis. Due to the necessity of balancing between increasing Q_P and reducing IVCD, the hypothermic growth is achieved by two distinctive phases differentiated by temperature. After initial growth phase at physiological temperatures (37 °C), leading to proliferation of cells, the production phase at sub-physiological temperatures (30–33 °C) is performed to induce growth arrest during the S or G1 phase of the cell cycle and enhance protein expression. Decreasing cultivation temperature approach has been shown different effects based on variable conditions including the GOI, promoters, vectors, host cells, culture media, etc. [87]. Low manipulation in environmental pH (±0.2) has been found to have considerable effect on the Q_P of low temperature cells within the production phase of a biphasic culture [99]. Accurate pH adjustment enhances the proportion of viable cells remarkably. Simultaneous variations in these factors lead to reduction in nutrient use and product aggregation along with maximum protein yields in a complex culture system [97]. Growth control strategies induce G1-phase cell cycle growth arrest via CKI activation. These complex signaling pathways will lead to the generation of several cellular contexts influencing the product quality and could hinder the application of these techniques to the commercial production procedure [100].

MicroRNAs

Small regulatory miRNAs are identified as important tools in cell line engineering. This is due to their properties including association with important processes like apoptosis, proliferation, or protein biosynthesis, along with no loading bar in cell translation despite their capability in regulating entire physiological pathways [84, 101, 102].

According to the capability of miRNA to affect the expression of various genes, the employment of miRNAs could be more helpful than ordinary approaches for a single gene manipulation. In this case, the effect of several miRNAs in CHO cells was investigated. As an example, inhibition of Mmu-miR-466h-5p as a pro-apoptotic miRNA, led to larger amount of protein production in comparison with ordinary cells (103). In another study, engineered CHO cells expressing a pro-productive miRNA, miR-557, have shown remarkable increase in final product yields [104]. In two other surveys, stable CHO cells engineered with miR-30 and miR-17, exhibited enhanced maximum cell density and specific productivity of approximately 2-fold [84, 105]. On the other hand, it is demonstrated that cell line engineering using miRNA sponge transcripts could efficiently improve the cell density and longevity; therefore, it can increase product yield. Sanchez et al. have shown a superior increase in CHO protein production by disrupting the miR-7 activity through sponge decoy vectors. The miR-7 sponge changes the course of miR-7 from its targets and causes up to 40% improvement in cell density and about 2-fold increase in product yield in a fed-batch culture [106].

The aforementioned outcomes represent the miRNAs potential to manufacture more productive cell lines along with higher viable cell density and lengthened viability.

Conclusion

Nowadays, significant advances in recombinant protein expression lead to the increase of both quantity and quality of yields, decrease the process timescale and make the upstream procedures convenient toward downstream applications.

Plasmid vector engineering seems to have an important role on increasing the recombinant protein expression. The vector components, especially promoters and even the physical distances between different elements, appear to be important. Incorporating chromatin open elements into an expression cassette can help to improve gene expression significantly, but several parameters need to be optimized for achievement full benefits of this technology. The effectiveness of the final vector depends not only on any individual component but also on cross-talk and interplay among them [33]. In spite of considering above points, in the most cases, expression level is not desirable according to low activity of the integration location in the target genome. Targeting gene integration with the aim of conducting transgene to a transcriptionally active predetermined site has revealed encouraging outcomes in isolating very high-producing clones.

On the other hand, process control, media formulations, and cell engineering are approaches which have been addressed for solution of many problems related to low production in mammalian cells [84, 85]. Among these approaches, cell cycle growth arrest is one of the most common methods of inducing controlled proliferation, and is based on different approaches including using chemical agents, decreasing cultivation temperature and cell engineering. Some growth arrest strategies, including temperature shift or chemical additives induce several signaling pathways and will lead to the generation of different cellular contexts, influencing product quality attributes. An alternative approach can be achieved by cell engineering via targeting regulators of cell cycle checkpoints including CKIs. However, the impact of CKIs overexpression on expression of some proteins is insufficient; therefore, alternative cell cycle checkpoint kinases and miRNAs have been studied.

According to unique properties of the manufacturing process for each protein, it is important to direct the decision toward one approach and optimize the efficient paths for generation of proteins. Combination of mentioned sophisticated strategies readily provides the production of gram quantities of a specific therapeutic protein.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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