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Review:

Improving the secretory capacity of Chinese hamster ovary cells by ectopic expression of effector genes: lessons learned and future directions

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Abbreviations: ATF6c, activating transcription factor 6; CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; effector gene, a transgene ectopically expressed with the purpose of obtaining a phenotypic change; IE, inducible expression; EPO, erythropoietin; ER, endoplasmic reticulum; HEK, human embryonic kidney; MAb, monoclonal antibody; pcd, pg per cell per day; PDI, protein disulphide isomerase; PTM, post-translational modification; q_p, specific protein productivity**;** r-protein, recombinant protein; SCS, single-cell sorting; SEAP, secreted embryonic alkaline phosphatase; SEE, stable episomal expression; SGE, stable gene expression; TGE, transient gene expression, YY1, transcription factor Yin Yang 1; VEGF, vascular endothelial growth factor; XBP-1S, spliced form of X-box-binding protein 1.

Abstract

Chinese hamster ovary (CHO) cells are the preferred cell factory for the production of therapeutic glycoproteins. Although efforts primarily within bioprocess optimization have led to increased product titers of recombinant proteins (r-proteins) expressed in CHO cells, post-transcriptional bottlenecks in the biosynthetic pathway of r-proteins remain to be solved. To this end, the ectopic expression of transgenes (effector genes) offers great engineering potential. However, studies on effector genes have in some cases led to inconsistent results. Whereas this can in part be attributed to product specificity, other experimental and cellular factors are likely important contributors to these conflicting results. Here, these factors are reviewed and discussed with the objective of guiding future studies on effector genes.

Keywords: Cell engineering, Chinese hamster ovary (CHO) cells, ectopic expression, endoplasmic reticulum, ER stress, gene dosage, product quality, recombinant protein production, secretion bottleneck, specific productivity

1. Introduction

Chinese hamster ovary (CHO) cells are the most frequently used cell host for biopharmaceutical production of glycoproteins (Walsh, 2014). Besides being the host cell used for the first approval of a recombinant biopharmaceutical produced in mammalian cells in 1986 (Wurm, 2004), CHO cells are the preferred choice for a number of reasons. First, CHO cells can easily be adapted for high-density suspension growth in a chemically defined, serum-free medium in large-volume cultures (Kim et al., 2012; Sinacore et al., 2000). Second, gene amplification methods have been established for CHO cells, leading to high specific productivity (q_p) of recombinant protein (r-protein) in stable cell lines (Durocher and Butler, 2009). Third, CHO cells are less prone to virus infection than other mammalian production cell lines and are therefore regarded as a safe host for the production of human therapeutics (Berting et al., 2010). Last, CHO cells and other mammalian cells are the platform of choice for the production of human recombinant glycoproteins because of their ability to correctly make human-like post-translational modifications (PTMs), in particular glycosylation (Butler and Spearman, 2014). Human-like PTMs turn r-protein products into functional drug molecules with reduced immunogenicity, prolonged serum half-life and high pharmacological efficacy in the human body (Walsh and Jefferis, 2006).

The production of r-proteins in CHO cells in optimized bioprocesses can reach q_p of 50–90 pg per cell per day (pcd) (Hacker et al., 2009). As previously pointed out by Khan and Schröder (2008), professional secretory plasma cells are capable of secreting IgM at a rate of 200–400 pcd (Fazekas et al., 1980; Randall et al., 1992). This clearly indicates that nature's physiological limit not yet has been reached and thus, intracellular rate-limiting steps in protein production remain to be resolved. Indeed, post-transcriptional rate-limiting steps in the biosynthetic pathway of r-proteins have been reported multiple times in CHO cells (Johari et al., 2015; Kallehauge et al., 2016; S. J. Kim et al., 1998; Ku et al., 2008; Schröder et al., 1999) as well as in other mammalian cells (Barnes et al., 2004; Fann et al., 1999). The presence of a post-transcriptional bottleneck suggests that there are many opportunities to improve the secretory pathway machinery in CHO cells. Moreover, artificial protein scaffolds such as fusion proteins are becoming more popular in the biopharmaceutical industry with increasing market shares (Aggarwal, 2014). These non-native scaffolds are in general more prone to misfolding (Lee et al., 2007). Thus, the cost-efficient production of these difficult-to-express fusion proteins will most likely require substantial engineering of the folding machinery in the secretory pathway.

Engineering CHO cells by the ectopic expression of transgenes (hereafter referred to as effector genes) is an attractive solution to improve the secretory capacity of CHO cells. In many cases, such engineering efforts have led to positive effects on q_p on a variety of r-proteins (see recent reviews (Fischer et al., 2015; Hussain et al., 2014; Nishimiya, 2013)). This multitude of studies showing positive effects clearly underpins the potential of modulating the expression of effector genes. However, as previously pointed out (Hussain et al., 2014; Kim et al., 2012; Mohan et al., 2008), some effector genes are flawed by inconsistent effects. To exemplify this, all published studies on r-protein productivity (volumetric productivity or q_p) in CHO cells with the ectopic expression of the widely studied protein disulphide isomerase (PDI) are listed in Table 1. PDI is an endoplasmic reticulum (ER)-resident enzyme conferring disulphide isomerase activity (Hatahet and Ruddock, 2009). Moreover, PDI forms and reduces disulphide bonds in nascent polypeptides in the lumen of the ER and in parallel inhibits the aggregation of folding intermediates through its function as a chaperone (Appenzeller-Herzog and Ellgaard, 2008). The reported effects of overexpressing PDI on volumetric productivity and q_p vary from a two-fold decrease through no effect to a 1.4-fold increase. This inconsistency, can to some extent, be explained by product specificity, as several

different r-proteins have been used as model proteins. In fact, PDI overexpression only increased q_p for one of four monoclonal antibody (MAb) variants in a parallel experimental setup (Pybus et al., 2014). However, many cellular and experimental factors are at play when examining how an effector gene affects volumetric productivity and q_p (Fig. 1). Thus, it is likely that factors other than product specificity are involved in the inconsistency of PDI's effect on volumetric productivity and q_p of r-proteins.

In contrast to PDI, the effect of many effector genes on volumetric productivity or q_p has only been reported once (Hussain et al., 2014; Nishimiya, 2013). Notwithstanding product specificity, it is likely that a considerable number of these effects are conditional – for example, specific to the monoclonal cell line or the expression platform being used. The applicability of such conditional effects is often limited to the research group in question and not to the CHO engineering field in general. Here, cellular and experimental factors that potentially affect the outcome when studying effector genes will be described and discussed. If these factors are appreciated, the risk of unintentionally investigating conditional effects can be minimized and the chance of finding true positive effects can be increased.

2. CHO host cell lines

In 1957, the immortalized, original CHO cell line (the common ancestor for all CHO cell lines) was established from the ovaries of an outbred female Chinese hamster (Puck, 1957; Wurm, 2013). This original cell line has led to a multitude of commercially available and proprietary CHO cell lines (Wurm, 2013). Being an immortalized cell line, the genome of CHO cells is inherently unstable (Frye et al., 2016). Moreover, dihydrofylate reductase deficiency (DHFR) in the widely used DXB11 and DG44 cell lines was achieved by subjecting cells to radiation- and chemical-mediated mutagenesis (Urlaub et al., 1983; Urlaub and Chasin, 1980). Thus, host CHO cell lines constitute a genomically diverse family in terms of single nucleotide polymorphisms (Lewis et al., 2013), copy-number variations (Kaas et al., 2015) and karyotypes (Wurm and Hacker, 2011). Moreover, it has recently been suggested to regard CHO host cell lines as 'quasispecies', emphasizing the extensive genetic heterogeneity residing in the CHO host cell family (Wurm, 2013).

When CHO host cell lines are compared, they are found to be not only genetically divergent but also phenotypically diverse. For example, it has been shown that the ER size in the CHO-K1 host cell line is larger compared to a DXB11-derived host cell line, and the mitochondrial mass was also found to be higher in CHO-K1 cells (Hu et al., 2013). These phenotypic differences could explain the approximately 10-fold lower q_p observed for the DXB11-derived host cell line compared to CHO-K1 cells, which was obtained for two different MAbs from stable gene-amplified clones (Hu et al., 2013). In a recent CHO bibliome study by Golabgir et al. (2016), a meta-analysis of bioprocess studies showed that the cell growth rate and q_p of DXB11- compared to DG44-derived cell lines were significantly higher and lower, respectively. Although the bibliome data consist of a range of process conditions and experimental setups, both DG44 and DXB11 are DHFR-deficient cell lines. Consequently, the gene-amplification process and clone selection are therefore comparable, warranting the comparison of q_p. Moreover, CHO-K1-derived cells were found to grow slower than DG44-derived cells in the bibliome study. The observed differences between CHO-K1, DXB11 and DG44 host cells clearly illustrate the phenotypic diversity that resides within the family of CHO host cells.

Being such a diverse family of cell lines, the following question arises: Are effects on q_p of an effector gene transferable across CHO host cell lines? In the PDI example, at least four CHO host cell lines (DXB11, DG44, CHO-K1 and CHO-S) have been used (Table 1). Because of product specificity and other factors, it is not possible to make any conclusion on the influence of CHO host cell lines on PDI as an effector gene. As for the spliced form of X-box-binding protein 1 (XBP-1S), it has been shown that ectopic XBP-1S expression was able to increase the volumetric productivity of erythropoietin (EPO) in CHO-K1 as well as in the murine NS0 myeloma cell line (Ku et al., 2008). Moreover, Tastanova et al. (2015) found that the CHO-derived transcription factor Yin Yang 1 (YY1) improved the volumetric productivity of a MAb (Rituximab) upon co-transfection in a transient expression setup in CHO-K1 and CHO-S cells. Moreover, YY1 expression also improved the volumetric productivity of MAb in DG44- and DXB11-derived stable cell lines. In a transient expression setup using the human orthologue, the positive effect of YY1 on volumetric productivity was also observed in human embryonic kidney (HEK) cells, human cervical cancer cells (HeLa) and human fibrosarcoma cells (HT-1080), which are immortalized cell lines with genomes vastly different from CHO cells. Future studies will show whether the generic effect of YY1 and XBP-1S between genomically distinct cell lines is an exception that proves the rule.

3. Clonal variation

Phenotypic heterogeneity is observed not only between CHO host cell lines but also within the cell population of CHO host lines. In fact, CHO cells are known for being able to adapt to changes in process conditions, which has been exploited in the industry to generate clonally derived cell lines with enhanced manufacturing capabilities (Frye et al., 2016). Moreover, the majority of cells in host cell lines seem to be intrinsically incapable of high production of MAb, and universally competent cells are likely relatively rare cases (O'Callaghan et al., 2010). Functional heterogeneity residing in the host cell population is typically referred to as clonal variation (Fig. 2). Although not CHO cells, how clonal variation is generated (phenotypic drift) has been elegantly demonstrated in the murine cell line NS0 (Barnes et al., 2006). Three rounds of limiting dilution were performed, and variation in cell growth rate was observed after each round of subcloning. This phenomenon has subsequently been observed in subclones of a CHO-K1-derived host cell line (Davies et al., 2013). The cell growth rate changed for approximately half of the clones during extended culture time, demonstrating that both static (inheritable) phenotypes and phenotypic drift were observed. Moreover, the initial cell growth rate of the subclones varied substantially, which demonstrates the presence of clonal variation within the host cell line. When analysing single cells in a monoclonal cell line culture, large variation in transgene expression has been observed, which could not be attributed to variables such as cell cycle and cell size (Pilbrough et al., 2009). Since this variation was shown to fluctuate within a relatively short period, phenotypic drift may partly originate from non-genetic diversity. However, a comprehensive genome and epigenome characterization of a CHO-K1 host cell line adapted to growth in three different media showed high variation in genome sequence both as a result of media adaptation and under constant culture conditions over time (Feichtinger et al., 2016). Based on these observations and as previously stressed by Frye et al. (2016), absolute genetic homogeneity in a cell culture does not seem achievable because of the genomic plasticity inherent in immortalized mammalian cell lines.

Clonal variation for recombinant CHO cell lines does not originate only from functional heterogeneity in the host cell line, as genetic heterogeneity is also introduced during the generation of recombinant cell lines (Fig. 2). For example, chromosomal aberrations were observed in 10 of 16 stable gene-amplified GFP-expressing cell lines not observed in the DG44 host cell line (Derouazi

et al., 2006). In addition, significant differences in specific growth rate and q_p of subclones from a gene-amplified MAb-producing clonally derived DG44 CHO cell line have been observed (N. S. Kim et al., 1998). Thus, recombinant monoclonal cell lines generated by gene amplification are genetically and phenotypically diverse.

Does an observed phenotypic difference between two recombinant cell lines originate from clonal variation or from stable expression of an effector gene? If only one control cell line and one effector gene-expressing cell line (both monoclonal) are being investigated, it is not possible to rule out that an observed phenotypic difference originates from clonal variation (Stockholm et al., 2007). Instead, this would require a number of monoclonal cell lines in both categories to demonstrate that the difference is a consequence of expressing the effector gene. In the PDI example, only single clonally derived producer cell lines have been used as host cell lines when analysing the effect of constitutively expressing PDI (Table 1). These monoclonal producer cell lines have then been used as hosts to generate either *quasi*-monoclonal or *mono*-monoclonal PDI-expressing lines (see Fig. 2 for definitions). Thus, the observed effects of PDI expression are likely biased by clonal variation, as *quasi*-polyclonal and *mono*-monoclonal cell lines in general do not represent the average phenotype in the host cell line. This bias can be addressed if employing polyclonal producer cell lines (see 'Clonality and clonal variation' in section 7).

In summary, caution must be exercised when investigating the effects of expressing effector genes in clonally derived producer cell lines. At a minimum, *quasi*-polyclonal or *mono*-monoclonal cell lines from two but preferably three different monoclonal producer cell lines should be examined. Alternatively, transient expression or stable episomal expression of the r-protein product circumvents the bias originating from clonal variation (see section 7).

4. Effector gene origin

The main application of CHO cells in the biopharmaceutical industry is to produce human-like glycoproteins. Although humanized, some MAb biopharmaceuticals are chimeric molecules of mouse and human amino acid sequences (Ahmadzadeh et al., 2014). Thus, human and, to some extent, murine-derived polypeptide sequences are expressed in a heterologous CHO-based context. In view of this, should the origin of an effector gene be human, CHO or mouse? In the PDI example (Table 1), all three origins have been reported, although human PDI is overrepresented (four of six studies). This observation is consistent with an overall preference for the human origin of effector genes (Fischer et al., 2015).

The argument for using a human effector gene would be that the effector gene protein is expected to directly interact with the r-protein. In other words, it is thought to facilitate a favourable interaction between two autologous (human) molecules that is less likely to take place between two heterologous molecules (direct effect; see Fig. 1). In contrast, the argument for using a CHO effector gene would be that an effector gene is expected to interact with host cell molecules (indirect effect through element X; see Fig. 1). For example, an effector gene-encoded transcription factor and a chaperone are expected to interact with host cell molecules (DNA) and the r-protein, respectively. Thus, the choice of effector gene origin depends on the function of the effector gene.

The influence of effector gene origin on CHO cell line engineering has not been systematically investigated. However, it has been shown that both CHO- and human-derived PDI can improve q_p of human MAb-related r-proteins in CHO cells (Johari et al., 2015; Mohan et al., 2007; Pybus et al., 2014) (Table 1). Moreover, XBP-1S has been shown to increase volumetric productivity and q_p in

CHO cells – both the human (Becker et al., 2008; Cain et al., 2013; Pybus et al., 2014) and murine (Hansen et al., 2015; Ku et al., 2008) orthologues. In contrast, only the CHO-derived and not the human orthologue of YY1 was able to increase volumetric productivity in CHO cells and vice versa in human cells (Tastanova et al., 2015). As pointed out by the authors, this clearly indicates that interaction with host-specific co-factors was required.

Although there might be a few cases where a human effector gene orthologue is needed, CHO orthologues are more likely to be functional because they will be present in a non-foreign (autologous) cellular context like YY1. Thus, using CHO-derived effector genes likely increases the chances of identifying true positive effects and at the same time minimizing the risk of false negative effects. Furthermore, applying CHO-derived effector genes would enable direct comparisons of the expression level between the recombinant effector gene and the endogenous gene. Accordingly, this would entail comparisons of effector gene doses across experiments and studies (see section 6).

Cloning CHO and Chinese hamster gene sequences has recently become a more straightforward task owing to the drafts of Chinese hamster and CHO cell line genomes (Brinkrolf et al., 2013; Lewis et al., 2013; Xu et al., 2011). Moreover, efforts to improve the quality of the Chinese hamster genome and to refine annotations are currently ongoing (Kremkow et al., 2015). The availability of correctly annotated Chinese hamster gene sequences is likely to facilitate the autologous, ectopic expression of effector genes in CHO cells in future CHO cell line engineering studies.

5. Secretion bottleneck and ER stress

A non-linear relationship between the transcript level and q_p in CHO cells shows that there is a post-transcriptional bottleneck in the biosynthetic pathway of r-proteins. Such a bottleneck has been reported in CHO cells upon transient expression of an Fc-fusion protein (Johari et al., 2015) and EPO (Ku et al., 2008), although only EPO titer and not q_p was reported for the latter example. In addition, a post-transcriptional bottleneck has been reported in stable MAb-producing CHO cells (S. J. Kim et al., 1998) and NS0 myeloma cells (Barnes et al., 2004). Moreover, a post-translational rate-limiting step (hereafter referred to as a 'secretion bottleneck'; Fig. 3A) has been demonstrated in stable CHO cell lines expressing antithrombin III (Schröder and Friedl, 1997) as well as in baby hamster kidney cells constitutively expressing activated protein C (Fann et al., 1999). In these two cases, a non-linear relationship between the intracellular level of r-proteins and q_p was observed, which demonstrates that the bottleneck was downstream of translation and translocation and therefore within the secretory pathway (ER, Golgi and secretory transport vesicles). Most intracellular whole MAb molecules in stable CHO cell lines have been found to be in the early part of the secretory pathway (between ER and *cis*-Golgi) for cell lines with and without a secretion bottleneck (O'Callaghan et al., 2010). The study by O'Callaghan et al. also demonstrated that bottlenecks in the biosynthetic pathway of the same MAb molecule are cell line-specific, irrespective of q_p. For example, in one cell line with q_p of 7 pcd, the folding and assembly rate of MAb was particularly slow, whereas in another cell line with q_p of 8 pcd, secretion was the rate-limiting step.

The unfolded protein response (UPR) is a homeostatic transcriptional program that is induced when the capacity of folding and processing incoming nascent polypeptides in the ER is exceeded (Moore and Hollien, 2012; Walter and Ron, 2011). This protein folding perturbation is called ER stress. If high levels of ER stress conditions persist, the UPR will eventually become pro-apoptotic (Jäger et al., 2012; Moore and Hollien, 2012; Sano and Reed, 2013). ER stress originating from r-

protein expression has been observed several times. For example, q_p of certain r-proteins has been shown to correlate with ER stress levels upon transient expression (Johari et al., 2015; Ku et al., 2010). Once post-transcriptional and/or secretion bottleneck conditions were established, ER stress levels increased abruptly upon a higher expression level of r-protein (Fig. 3B). Moreover, cell lines expressing seemingly difficult-to-express r-proteins have been shown to have increased levels of ER stress compared to cell lines expressing easy-to-express r-proteins (Johari et al., 2015; Le Fourn et al., 2014; Sommeregger et al., 2016). These examples show that ER stress and secretion bottlenecks are intimately linked.

In two studies by Ku et al., a post-transcriptional bottleneck was identified at high gene doses of transiently expressed EPO (Ku et al., 2010, 2008). Ectopic expression of XBP-1S was found to improve the volumetric productivity of EPO only at gene doses causing ER stress and with a post-transcriptional bottleneck phenotype (Ku et al., 2008). Consequently, some ER-related effector genes – such as XBP-1S – can improve volumetric productivity and/or q_p only in conditions where a post-transcriptional bottleneck is present (Fig. 3A). Being an ER-localized protein, the effects of PDI on q_p likely depend on whether such bottlenecks are present. However, the presence of a post-transcriptional bottleneck has been investigated for only two of eleven r-proteins (Table 1). Preferably, non-secretion bottleneck as well as secretion bottleneck conditions should be established when analysing effector genes, which would minimize the risk of obtaining false negative results. Indeed, an inadequate conclusion would have been drawn if only a low gene dosage of EPO had been used by Ku et al. (2008).

Obtaining positive effects on q_p of effector genes in conditions without post-transcriptional bottlenecks is an interesting supposition. To the knowledge of the authors, no studies have methodically investigated this topic. Although not investigated, it seems likely that a post-transcriptional bottleneck was not present when expressing Rituximab in CHO-S cells in the study by Tastanova et al. (2015), as we have obtained a >10-fold higher q_p in a comparable transient expression setup in shake flasks (Hansen et al., 2015). Nevertheless, a three-fold increase in Rituximab titer was obtained upon YY1 overexpression in CHO-S (Tastanova et al., 2015). In a stable CHO-DG44-derived clone expressing MAb, the positive effect of YY1 was also observed, and this effect could not be ascribed to an increase in the cell growth rate or transcript level of MAb heavy and light chains. The increase in q_p combined with an unchanged transcript level suggests that the secretion rate per transcript must be three-fold higher, indicating that the effect is post-transcriptional. Differences in the translation rate have been predicted between stable monoclonal CHO cell lines expressing the same MAb by mathematical modelling (O'Callaghan et al., 2010), suggesting that the effect of YY1 could be an increased translation rate. Alternatively, it could be a decreased degradation of folding intermediates mediated by the ER-associated degradation pathway (Hussain et al., 2014; Merulla et al., 2013) or autophagy- and lysosomal-mediated degradation (Kim et al., 2013). Nevertheless, the YY1 example implies that a secretion bottleneck phenotype does not seem to be a prerequisite for improving q_p.

6. Effector gene dosage

Whether an effector gene is able to improve q_p depends not only on the presence of the effector gene-encoded protein but also on the expression level. In other words, the outcome of expressing an effector gene on q_p depends on the effector gene dosage (Brown and James, 2015; Xiao et al., 2014). In a study by Davis et al. (2000), CHO clones stably expressing an Fc-fusion protein with different expression levels of PDI ('low PDI', 'medium PDI' and 'high PDI') were used (Table 1).

Whereas the low PDI clone had no apparent effect on product titer, the medium PDI had increased intracellular levels of the Fc-fusion protein but no effect on titer and the high PDI clone had increased intracellular levels of Fc-fusion protein and a two-fold decrease in titer. If leaving clonal variation out of consideration, this example shows that the folding machinery can be overwhelmed when an ER-localized enzyme exceeds an optimal expression level range. Like ER-resident enzymes, gene dosage titration is also important when investigating transcription factors as effector genes. Johari et al. (2015) observed increased volumetric productivity of two MAb variants when expressing either XBP-1S or cleaved activating transcription factor 6 (ATF6c) at lower gene doses and no effect at the highest gene dosage. Moreover, the gene dosage for both effector genes inversely correlated with cell growth, suggesting that the expression of XBP-1S and ATF6c generated a fitness cost. When Tastanova et al. (2015) investigated how the gene dosage of the transcription factor YY1 affected the volumetric productivity of a variety of MAb molecules and CHO cell lines, an optimal gene dosage range of YY1 was observed. These examples demonstrate the importance of gene dosage titration.

Two complementary strategies were employed to titrate the YY1 gene dosage by transient expression: the filler plasmid (empty vectors) principle and promoters with different strengths (Tastanova et al., 2015). Filler plasmids are used to titrate the effector gene-encoding plasmid (Estes et al., 2015; Rajendra et al., 2015, 2012), whereas promotors with different strengths drive transcription at different rates (Brown and James, 2015; Qin et al., 2010). The filler plasmid principle is a cost-efficient solution in transient expression-based setups, as no additional cloning work is needed besides a single plasmid preparation of the filler plasmid. Moreover, a linear relationship between the transfected plasmid load and mRNA can be expected at relatively low gene doses (Johari et al., 2015; Ku et al., 2008; Rajendra et al., 2015). When controlling the expression level of stably integrated effector genes, using a set of promotors with different strengths is the preferred choice (Brown and James, 2015).

Only a single gene dosage has been used when investigating the effect of PDI expression on nine of eleven r-proteins (Table 1). This means that, in nine of eleven cases, it is unknown whether an optimal or adverse gene dosage was used. This indicates that a substantial number of studies on ectopic expression of effector genes have only employed a single gene dosage, suggesting that the gene dosage space for many studied effector genes remains to be explored.

In summary, a wide range of effector gene doses should preferably be used in the attempt to find an optimal range of expression. Moreover, the expression level of the effector gene and the endogenous gene should be compared to facilitate comparisons across studies, which requires that CHO-derived effector genes be used (see section 4). These efforts would facilitate higher chances of drawing valid conclusions as well as higher chances of identifying conditions where effector genes improve q_p.

7. Expression platforms

There are several different platforms for the ectopic expression of r-proteins in CHO cells. r-proteins can be expressed either transiently from a non-integrated plasmid or from a gene stably integrated into the genome. Transient gene expression (TGE) is a widely used technology for the rapid production of r-proteins, usually during a 2-10 day batch or fed-batch process (Baldi et al., 2007). TGE is the preferred production method during the early stages of drug development for preclinical studies, as a sufficient quantity of r-protein can be obtained within a short period of time (Kim et al., 2012). However, the lower protein yield achieved with TGE in CHO cells has

historically been a major drawback compared to the substantially higher yields obtained with stable gene expression (SGE). Thus far, extensive efforts to improve TGE yields in CHO cells have been made by optimizing the culture environment (Galbraith et al., 2006; Ye et al., 2009), transfection efficiency (Mozley et al., 2014; Rajendra et al., 2015, 2012), vector systems (Cho et al., 2001; Mariati et al., 2010) and host cell line (Cain et al., 2013; Daramola et al., 2014; Macaraeg et al., 2013). Therefore, TGE has become a robust and flexible system, applicable to multiple r-proteins, expression volumes and bioprocesses with substantially increased yields of up to 3 g/L for MAb-producing CHO cells (Liu et al., 2015).

SGE is a prerequisite for the stable, large-scale manufacturing of r-proteins as biologics for clinical applications (Noh et al., 2013; Wurm, 2004). Stable cell lines are typically generated using selection based on a selective marker expressed on the same plasmid as the r-protein (Priola et al., 2016). If desired, the gene encoding the r-protein can be amplified to increase q_p, although gene-amplification in general reduces the stability of volumetric productivity and q_p in extended culture conditions (Chusainow et al., 2009). The two most widely used gene amplification systems are the DHFR system and the glutamine synthetase system (Noh et al., 2013). Nowadays, companies report titers of >10 g/L for MAb production (Gronemeyer et al., 2014). As an alternative to random transgene integration, *piggyBac* or *sleeping beauty*-mediated transposition can be used, where transgene integration into highly transcribed regions of the host genome is favoured. This leads to a generally higher rate of transgene transcription compared to the random integration of plasmids (Ding et al., 2005; Galvan et al., 2009; Wilson et al., 2007). Consequently, higher q_p and stability compared to stable producer clones obtained by random integration have been achieved with MAb titers of up to 7.6 g/L (Matasci et al., 2011; Rajendra et al., 2016).

Inducible expression (IE) platforms support the idea of a regulated, biphasic r-protein expression throughout the production phase, *e.g.*, being turned off during growth and turned on only during the late exponential and stationary phase. The inducible nature of the IE platform provides a powerful expression system for r-proteins that might confer toxicity when being expressed by constitutive promoters. Several systems with either repressor or activator configurations have been developed for mammalian cells to achieve tight control of gene expression. Among many, there are i) antibiotic-based regulation systems, such as the Tet-Off-On system regulated by tetracyclin (Gossen and Bujard, 1992; Mohan et al., 2007), the Pip system regulated by streptogramin (Fussenegger et al., 2000) and the E.REX system regulated by macrolides (Weber et al., 2002); ii) an aptamer-based regulation system (Werstuck and Green, 1998); and iii) the cumate gene-switch (Gaillet et al., 2010; Mullick et al., 2006). All these inducible systems have been successfully used in CHO cell lines, and a 0.24 g/L titer of an Fc-fusion protein has been reported for this system (Gaillet et al., 2010).

Expression from replicating episomes in CHO cells reduces the loss of plasmid and thereby prolongs the nuclear retention time of the plasmid after cell divisions (Van Craenenbroeck et al., 2000). Episomal replication can be achieved using, *e.g.*, the Polyomavirus large T gene (PyLT) and its origin of replication (PyOri) (Heffernan and Dennis, 1991), while plasmid maintenance and segregation can be accomplished using Epstein-Barr virus nuclear antigen-1 (EBNA-1) and its origin of replication (OriP) (Lupton and Levine, 1985; Yates et al., 1984). Using these two sets of complementing viral components, the episomal platform was reported to increase and prolong TGE yields of a growth hormone and MAb in CHO cells in comparison to non-replicating plasmid controls (Codamo et al., 2011; Kunaparaju et al., 2005). Without antibiotic-based selection, expression from replication-proficient episomes can be regarded only as a quasi-stable gene expression platform, as episomes eventually will be lost with a half-life of approximately 8-9 days

(Silla et al., 2006). Without selection, titers of up to 2 g/L MAb have been reported (Daramola et al., 2014). However, if antibiotic-based selection is applied, a stable pool of cells can be obtained that stably replicates and segregate episomes for more than two months (Silla et al., 2006). Thus, when combined with selection, the system can be regarded as stable episomal expression (SEE).

Although all the described expression systems have been used successfully to express r-proteins in CHO cells, each expression platform has advantages and disadvantages when used for effector gene expression. In Table 2, four selected expression systems are compared as platforms for analysing how effector genes affect q_p. It is important to note that other expression systems are available – such as stable pools and monoclonal cell lines obtained by lentiviral vector-mediated gene transfer (Oberbek et al., 2011) or stable pools obtained by *piggyBac* transposons (Matasci et al., 2011). In addition, the four expression systems can be combined in different ways – such as using IE (Chung et al., 2004) or TGE (Hayes et al., 2010) in a monoclonal stable producer cell line. Nevertheless, the selected expression platforms serve to highlight important aspects to consider when choosing a platform and interpreting the final data.

Duration – from transfection to answer: The preparation of plasmids carrying effector and r-protein genes takes approximately the same time for all four expression platforms. However, the time span from transfection to an answer varies substantially. TGE is clearly the fastest track, as experiments are typically done within two to three days (Hansen et al., 2015; Johari et al., 2015; Ku et al., 2008). SEE is also relatively fast, as antibiotic-based selected SEE cells can be obtained within two weeks (Silla et al., 2006). In contrast, both IE and SGE platforms require generation and characterization of monoclonal cell lines that take months to perform (Noh et al., 2013).

Gene dosage and combining genes: In terms of gene dosage titration (Rajendra et al., 2012) and combining genes (Nishimiya et al., 2013), TGE offers complete flexibility, as a variety of different plasmids (filler plasmid and/or plasmids carrying different effector genes) can be co-expressed. This is not the case for SEE, as the copy numbers of two plasmids with different genes expressed, in some cases, will drift towards selection for the plasmid/gene giving rise to the lowest fitness cost (personal communication with Dr Mikael Rørdam Andersen, Technical University of Denmark). To avoid this drift, the r-protein gene and effector gene must be co-expressed from the same plasmid. Relatively few cell lines are manageable to maintain in parallel, which decreases the throughput of gene combinations for the IE and SGE platforms. Because the effector gene is constitutively expressed in the described IE and SGE platforms (Table 2), the effector gene dosage is not titratable in these platforms. Notably, recent advances in promoter engineering in CHO cells make it possible to precisely control the gene dosage in TGE and possibly also in SEE, IE and SGE platforms spanning over two orders of magnitude (Brown et al., 2014).

Transfection stress and variability: A considerable drawback of TGE is the cytotoxic effect from transfection, here termed ‘transfection stress’ (Fig. 2). Different transfection reagents induce different levels of cytotoxicity in terms of impeded cell growth rate and a drop in viability (our own unpublished observations), as well as the inhibition of protein synthesis (Underhill et al., 2003). Notably, transfection stress can be reduced through process or cell engineering optimization (Johari et al., 2015; Macaraeg et al., 2013; Majors et al., 2008). In addition, variability in transfection efficiency is an inherent problem for TGE (Hansen et al., 2015; Liu et al., 2008); however, optimization and selection of the appropriate method can reduce variability substantially (Davies et al., 2013). For the SEE, IE and SGE platforms, transfection stress is most likely not an issue because of the lengthy (≥2 weeks) antibiotic-based selection processes.

ER stress: The expression of r-proteins is inducible for the TGE (transfection) and IE (addition of inducing agents) platforms. In contrast, r-proteins are constitutively expressed during the selection processes in the SEE and SGE platforms. Whereas the effector gene and the r-protein are co-expressed during the selection process in the SEE platform, the r-protein is typically first integrated into the genome (monoclonal cell line; Fig. 2) and subsequently the effector gene (*quasi/mono*-monoclonal cell line; Fig. 2) in the SGE platform. Thus, in contrast to the TGE, IE and SEE platforms, the SGE platform subjects cells to ER stress before investigating whether the effector gene of interest can alleviate the ER stress originating from r-protein expression. In other words, some cells with a high r-protein expression level and consequently a high level of ER stress will likely not survive during the selection processes (Hu et al., 2013) (Fig. 3B). These highly relevant, stressed cells will therefore not be part of the effector gene test case, in contrast to the TGE and IE platforms. This could introduce a bias towards cells with lower level of ER stress or cells inherently capable of coping with high levels of ER stress. It is important to note that stable producer cells adapted to a permanent increase in ER stress levels can be obtained (Sommeregger et al., 2016); however, the ER stress levels in these clones are not high enough to induce apoptosis. Instead, conditions with pro-apoptotic levels of ER stress are more likely to be established by TGE and IE because of the inducible nature of the two systems. Notably, the SEE platform does not allow for pro-apoptotic levels of ER stress to persist throughout the selection process. However, it does enable an effector gene of interest to alleviate pro-apoptotic levels of ER stress, because the effector gene and r-protein are co-expressed throughout the entire process.

Secretion bottleneck: Post-transcriptional and secretion bottlenecks have been reported in CHO cells for TGE (Johari et al., 2015; Ku et al., 2008) and SGE platforms (O'Callaghan et al., 2010). To the best of the authors' knowledge, a secretion bottleneck has not been reported in CHO cells for IE platforms, which likely is a result of a presumably low transcription rate of the r-protein gene compared to the TGE and SGE platforms. Naturally, the occurrence of a secretion bottleneck is protein-specific, and it is likely that, for some difficult-to-express proteins, a secretion bottleneck can be readily obtained. A post-transcriptional bottleneck in CHO cells has been reported upon expression from replicating episomes (Pybus et al., 2014); however, this was without antibiotic-based selection and is therefore not regarded as SEE (Table 2). A relatively high average q_p (10 pcd for MAbs) can be obtained using the SEE platform (personal communication with Dr Meelis Kadaja, Icosagen Cell Factory Ltd, Estonia), implying that post-transcriptional and/or secretion bottleneck conditions can be established using the SEE platform.

Clonality and clonal variation: When using TGE and SEE platforms, a representative pool of cells from the host cell line is being tested (polyclonality), whereas clonally derived cells are typically used in the IE and SGE platforms (monoclonality). Whilst polyclonal cells represent an average phenotype of all cells in the host cell line, monoclonal cell lines typically represent a favoured phenotype identified within the functionally heterogeneous pool of cells (Fig. 2). Thus, different questions are being put forward when expressing effector genes in polyclonal and monoclonal cells. If q_p is increased upon effector gene expression in polyclonal cells, this finding can likely be transferred to the majority of cells within the host cell line. This is not necessarily the case for clonally derived cell lines expressing effector genes (*mono*-monoclonal; see section 3). Thus, findings from a clonally derived cell line might be conditional; that is, the effect only applies to the cell line in question (O'Callaghan et al., 2010).

Validation: Since SGE is the preferred production platform for therapeutic proteins in the industry (Noh et al., 2013), any effector gene should preferably be validated in a SGE context – that is, in high-producer monoclonal cell lines. For example, effects obtained from TGE are sometimes

transferable to a SGE context (Tastanova et al., 2015) and sometimes not (Mohan and Lee, 2010). The need for validation is obviously a drawback of the TGE, SEE and IE platforms. However, if measures described here are taken – such as applying different effector gene doses and establishing conditions with and without post-transcriptional and/or secretion bottlenecks – the success rate of validating effector genes is likely to increase.

In summary, TGE is the preferred platform for screening effector genes because effector genes can readily be combined, gene dosage can easily be titrated and post-transcriptional and/or secretion bottlenecks can be obtained; in addition, TGE is not hampered by clonal variation and adaptation to ER stress (Table 2). A considerable drawback is the presence of transfection stress and transfection variability, which, however, can be addressed to some extent through process optimization. Once combinations of effector genes and gene doses have been identified, they need to be validated, preferably in multiple stable *mono*-monoclonal cell lines derived from different host cell lines.

8. Product quality

Although volumetric productivity and q_p are important measures, the ability to enhance product quality is equally important in the biopharmaceutical industry (Gramer, 2014). Volumetric productivity is the mass of produced r-protein per volume per culture time in cell-free supernatants. However, a significant fraction of the protein mass can be misfolded or incorrectly processed variants of the r-protein (Kunert and Reinhart, 2016). In such cases where product quality is impaired, an increase in product titer does not necessarily correlate with an increase in the yield of bioactive r-protein. Quality attributes defining the overall product quality are molecularly diverse features of the r-protein, such as misfolding/aggregation, incorrectly processed propeptides, enzymatic degradation and amino acid sequence variations (Gramer, 2014). Moreover, glycosylation is probably the most important quality attribute of therapeutic glycoproteins, because the pharmacokinetic effects of undesired glycosylation patterns can be decreased drug efficacy or increased antigenicity (Bertozzi et al., 2009; Butler and Spearman, 2014). Recent advances in glycoengineering and descriptions of how effector genes can be used to modulate glycosylation have been described in recent reviews (Bennun et al., 2016; Dicker and Strasser, 2015; Spahn and Lewis, 2014). Because of scope limitations, only misfolding/aggregation and propeptide processing in relation to effector genes will be described here.

Some secreted proteins are expressed as inactive proprotein precursors containing one or more inhibitory propeptides that need to be proteolytically cleaved off by propeptidases before full activity is achieved (Wiederanders et al., 2003). Since these propeptides in general are essential for protein folding (Chen and Inouye, 2008), propeptide-containing r-proteins need to be expressed as proproteins to prevent misfolding and degradation. However, there are multiple examples of insufficient cleavage of propeptides of r-proteins expressed CHO cells, leading to the secretion of a mixture of inactive proprotein and mature, correctly processed r-protein (Preininger et al., 1999; Sathyamurthy et al., 2015, 2012; Wasley et al., 1993). In these studies, the ectopic expression of effector genes encoding propeptidases increased the percentage of correctly processed r-proteins. This demonstrates that the propeptidase machinery within CHO cells can be the bottleneck for the production of propeptide-containing r-proteins. Solving this type of bottleneck is unique to propeptide-containing r-proteins and is therefore not generally applicable for enhancing q_p of other types of r-proteins.

Product quality in terms of misfolding and aggregation is intimately linked to the secretory pathway. Schröder et al. (2002) demonstrated that increasing the r-protein expression level of

antithrombin III through gene amplification in stable clonally derived CHO cells gave rise to the formation of disulphide-bonded aggregates. Similarly, lowering the gene dosage when transiently expressing an Fc-fusion protein in HEK cells has also been shown to reduce aggregate formation (Estes et al., 2015). In the PDI example (Table 1), the product quality has been investigated in only one of eleven examples. Here, a 1.2-fold increase in q_p of an Fc-fusion protein was observed upon PDI overexpression (Johari et al., 2015). However, PDI overexpression was found to impair product quality through a 1.4-fold increase in the formation of high molecular aggregates. Thus, the overall effect of PDI overexpression was a decrease in the number of correctly folded Fc-fusion protein molecules produced per cell per unit time. This study elegantly illustrates the importance of analysing quality attributes when studying how effector genes affect q_p.

9. Concluding remarks and future directions

A desirable goal of engineering the secretory capacity of CHO cells is to generate a universally competent cell line ('super-CHO') capable of manufacturing all r-proteins in demand. In general, this seems difficult to achieve because of product specificity (McLeod et al., 2011). For example, it has been reported that host cell line subclones being able to produce a MAb at relatively high q_p levels compared to the host cell pool were not able to produce high levels of an Fc-fusion protein (O'Callaghan et al., 2015). Moreover, two similar antibody fragments have been shown to differentially influence the cell's physiology in terms of different proteomic responses in a product-specific manner (Sommeregger et al., 2016). Similarly, effector genes have been shown to increase q_p in a MAb-variant-specific manner (Pybus et al., 2014). However, some effector genes can increase q_p for different types of r-proteins in CHO cells. For example, XBP-1S was able to increase q_p and/or product titer for secreted embryonic alkaline phosphatase (SEAP) and vascular endothelial growth factor (VEGF) (Tigges and Fussenegger, 2006), EPO (Ku et al., 2008), MAb (Becker et al., 2008; Cain et al., 2013; Pybus et al., 2014), an Fc-fusion protein (Johari et al., 2015) and α_1-antitrypsin and C1 esterase inhibitor (Hansen et al., 2015). Similarly, YY1 expression was able to increase product titer for SEAP, VEGF and MAb (Tastanova et al., 2015). This type of *general effector genes* seems to be able to traverse product specificity. It is important to note that general effector genes most likely also will fall short when it comes to r-proteins that require specialised modifications, such as the cleavage of propeptides (Sathyamurthy et al., 2015) and γ-carboxylation of clotting factors (Kumar, 2015). Thus, positive effects of general effector genes are likely not generally valid *per se*, but the effect may be retrieved once specialized post-translation modifications are no longer constituting a bottleneck.

The function of proteins encoded by general effector genes is probably conceptually different compared to effector gene-encoded proteins with more product-type specific effects (*i.e.* not general effector genes). In order to traverse product-specificity, the effect of general effector genes is probably multifaceted. Such multifaceted effects can be the result of simultaneously changing the expression level of several genes. Several cellular processes and/or molecules are able to confer such effects; for example phosphorylation (Rajesh et al., 2015), microRNAs (Barron et al., 2011; Hackl et al., 2012), transcription factors (Adachi et al., 2008; Harding et al., 2003) and histone marks (Dahodwala and Sharfstein, 2014). The molecules involved in these processes do not interact directly with the r-protein and the effects are therefore indirect (Fig. 1). An attractive advantage of using general effector genes is the possibility of modulating the expression level of more genes than is currently possible when co-expressing multiple single effector genes. However, some effects from general effector genes may also be adverse due to pleiotropic, undesired regulation of a subset

of genes (Stearns, 2010). Thus, investigating and eliminating these potential adverse effects of a general effector gene of interest may improve the desired phenotype.

Although single general effector genes seem to be part of the answer, multiple (general) effector genes are likely a more coherent and robust solution for increasing the secretory capacity (Harreither et al., 2015; Seth et al., 2007). In fact, multiple reactions within the biosynthetic pathway of MAb seem amenable for improvement (O'Callaghan et al., 2010). This notion is supported by non-limiting examples of studies demonstrating an increase in volumetric productivity or q_p in CHO cells upon the expression of effector genes related to translation and translocation (Le Fourn et al., 2014), ER folding processes (Borth et al., 2005; Chung et al., 2004; Hwang et al., 2003) and protein transport and secretion (Florin et al., 2009; Peng et al., 2011; Peng and Fussenegger, 2009) (Fig. 1). Several reports have demonstrated synergistic effects on the volumetric productivity or q_p of co-expressing multiple genes in CHO cells (Cain et al., 2013; Le Fourn et al., 2014; Mohan and Lee, 2010; Peng and Fussenegger, 2009), demonstrating the potential of combining effector genes. In addition to effector gene overexpression, downregulating endogenous genes might be equally important for increasing q_p (Feichtinger et al., 2016; Harreither et al., 2015). Finally, supplementing small-molecule chemical chaperones combined with effector gene overexpression has been shown to exceed the limits of functional heterogeneity present in the CHO-S host cell line (Johari et al., 2015).

When modulating the expression of multiple effector genes, the relative stoichiometry between gene transcripts (gene dosage) becomes an important aspect to consider to obtain an optimal effect on q_p (Xiao et al., 2014). However, q_p is not the only relevant factor: the time integral of viable cell density (accumulated viable cell number) and product quality attributes are important measures to control in production bioprocesses for increasing the product yield (Gramer, 2014; Kim et al., 2012). This is not a simple task, because process parameters (for example metabolite concentration and osmolality) in a bioreactor can change throughout a bioprocess (Justice et al., 2011). These dynamic changes affect the overall physiology and fitness of the cell and are likely to affect transcription rates in a promoter-specific manner (Brown and James, 2015). Such bioprocess-dependent variables can therefore interfere with the gene dosage of single and multiple effector genes. Thus, highly context-specific promoter designs are needed to simultaneously control the expression level of multiple effector genes during production bioprocesses (see recent review (Brown and James, 2015)). In combination with improved designs of vector elements, site-specific integration of expression cassettes into so-called safe harbour sites (Papapetrou et al., 2011) in the CHO genome has the potential of enabling predictable and stable expression levels of effector genes in clonally derived cell lines. Based on the game-changing CRISPR-Cas9 technology (Mali et al., 2013), site-specific integration into CHO cells is now possible at a relatively low cost with applicable efficiencies (Lee et al., 2016, 2015).

The global cellular view of systems biology and 'omics-based approaches holds a unique potential to identify pathways and gene networks comprising novel effector genes (Datta et al., 2013; Gutierrez and Lewis, 2015; Kildegaard et al., 2013). These networks would most likely be tedious to identify using a classical reductionist's approach where typically only a few genes are being tested. Indeed, as recently stated by Clarke and Lee (2014), the CHO community is currently generating 'omics data at an ever-increasing rate. This is supported by the following non-exhaustive examples on CHO 'omics studies: genomics (Lewis et al., 2013; Xu et al., 2011), transcriptomics (Becker et al., 2011; Doolan et al., 2008), translatomics (Courtes et al., 2013), proteomics (Baycin-Hizal et al., 2012; Carlage et al., 2009), metabolomics (Selvarasu et al., 2012; Zang et al., 2011) and integrative 'omics (Clarke et al., 2012). Moreover, a consensus genome-scale reconstruction of

CHO cell metabolism has now been established (Hefzi et al., 2016) which potentially opens up new designs of CHO cell lines (Kaas et al., 2014). In fact, using the genome-scale model to assess resource utilization suggests modulating expression of effector genes related to the secretory pathway is a more efficient way of improving q_p compared to typical bioprocess treatments such as sodium butyrate and hypothermia (Hefzi et al., 2016).

As mentioned by Hussain et al. (2014), some CHO 'omics studies were probably hampered by clonal variation (Fig. 2), because only a few monoclonal cell lines were compared. As previously described, CHO cells – even within a host cell line – are functionally and genomically diverse and only a fraction of the cells are universally competent in terms of r-protein production (O'Callaghan et al., 2010). Moreover, the 'omics profile of gene-amplified clonally derived high producer cells are probably vastly different compared to before transfection, due to, for example, adaption to ER stress. Thus, an 'omics profile of a high producer might comprise information on the end point of coping with a high secretion load. However, the profile does not necessarily contain any information on the cellular mechanism(s) involved in coping with the initial secretion load that the cell is subjected to right after transfection and/or gene amplification events. Instead, the intrinsic capability for coping with the initial secretion load is more likely to be found in non-transfected cells (non-adapted cells; Fig. 2). In fact, Harreither et al. (2015) were able to show that the capability to obtain high q_p from transient expression is reflected in the native transcriptome of host cell line subclones. Besides being a potential source of novel candidate effector genes, 'omics-based profiling of non-adapted monoclonal cells holds the potential to give fundamental insight into the functional heterogeneity of CHO cells.

Historically, the CHO lineage was not generated for r-protein production but for the investigation of molecular and classical cell genetics (Wurm, 2013). Moreover, CHO cells do not originate from dedicated secretory tissues like plasma cells and β-cells. By default, CHO cells therefore seem to be a suboptimal cell line for r-protein production, with many opportunities for improvement. The significant advances achieved through the ectopic expression of effector genes (Fischer et al., 2015) are likely only the tip of the iceberg, as relatively few of the approximately 20,000 genes in the Chinese hamster genome (Kremkow et al., 2015; Lewis et al., 2013) have been investigated. Furthermore, combining effector genes increases the number of possible test conditions dramatically. However, the number of possible test conditions can be reduced through systems biology- and 'omics-based approaches. Despite valuable guidance from systems biology, several novel effector genes (single genes and combinations) remain to be investigated. In conclusion, the cellular and experimental factors described here will likely aid future investigations of effector genes and thereby accelerate the development of CHO cell lines with optimized secretory capacity.

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Tables

Table 1. Studies on how ectopic expression of protein disulphide isomerase (PDI) affects volumetric productivity or q_p of r-proteins in CHO cells.

r-protein and reference	r-protein expression[a]; clonality[b]	Post-transcriptional bottleneck	Origin of PDI	PDI expression[a]; clonality[c]	Gene dosage	Host cell; cell culture	Effect on titer/q_p
Interleukin-15 (Davis et al., 2000)	SGE; 1 monoclonal cell line	Not investigated	Human	SGE; *quasi*-polyclonal	Single gene dose	CHO-DXB11; 2-3 days batch culture	No effect
Fc-fusion protein (Davis et al., 2000)	Stable expression; 1 monoclonal cell line	Not investigated	Human	SGE; *quasi*-polyclonal and 3 *mono*-monoclonal cell lines	3 clones with different levels of PDI expression	CHO-DXB11; 2-3 days batch culture	Polyclonal: No effect *Mono*-monoclonal: 2-fold decrease in titer for clone with highest PDI expression
MAb (Borth et al., 2005)	SGE; 1 monoclonal cell line	Not investigated	Not re-ported	SGE; *quasi*-polyclonal	Single gene dose	CHO dhfr$^-$; 3 days batch culture	1.37-fold increase in q_p
Thrombopoietin (Mohan et al., 2007)	SGE; 1 monoclonal cell line	Not investigated	CHO	IE; 2 *mono*-monoclonal cell lines	Single gene dose	CHO-DXB11; 3 days batch culture	No effect
MAb (Mohan et al., 2007)	SGE; 1 monoclonal cell line	Not investigated	CHO	IE; 2 *mono*-monoclonal lines	Single gene dose	DG44; 2 days batch culture	1.15–1.27-fold increase in q_p
MAb (Hayes et al., 2010)	SGE; 1 monoclonal cell line	Not investigated	Human	TGE; 1 *mono*-monoclonal line	Single gene dose	CHO-K1SV; 2 days batch culture	No effect
MAb (4 variants)	TGE (episomal-	Yes (predicted	Human	TGE; polyclonal	Single gene dose	CHO-K1 EB27; 10	No effect for 3 variants and 1.3-fold increase in

(Pybus et al., 2014)	replication); polyclonal	by modelling)				days fed-batch culture	q_p for 1 variant.
Fc-fusion protein (Johari et al., 2015)	TGE; polyclonal	Yes	Human	TGE; polyclonal	3 gene doses	CHO-S; 3 days batch culture	Gene dosage/effect on q_p: 10%/no effect, 20%/1.2-fold increase (1.4-fold increase in aggregate formation), 40%/1.3-fold increase.
α_1-antitrypsin (Hansen et al., 2015)	TGE; polyclonal	Not investigated	Mouse	TGE; polyclonal	Single gene dose	CHO-S; 3 days batch culture	1.2-fold decrease in q_p
C1 esterase inhibitor (Hansen et al., 2015)	TGE; polyclonal	Not investigated	Mouse	TGE; polyclonal	Single gene dose	CHO-S; 3 days batch culture	1.6-fold decrease in q_p
MAb (Our unpublished data)	TGE; polyclonal	Not investigated	Mouse	TGE; polyclonal	Single gene dose	CHO-S; 3 days batch culture	1.3-fold decrease in q_p

[a)] For definition and abbreviation of expression platforms, see Table 2.

[b)] Clonality of cells expressing the r-protein before transfection of the effector gene (SGE). r-proteins and effector genes are co-expressed in TGE platforms (polyclonal). For definition of clonality, see Figure 2.

[c)] Clonality of cells expressing PDI. For definition of clonality, see Figure 2.

Table 2. Selected expression platforms available for analysing how effector genes affect q_p in CHO cells.

	Transient gene expression (TGE)[a]	Stable episomal expression (SEE)[b]	Inducible expression (IE)[c]	Stable gene expression (SGE)[d]
Time from transfection to answer:	**2-3 days**[e]	**~2 weeks**	>2 months	>2 months
Gene dosage easily titrated:	**Yes**	No	**Yes**	No
Genes easily combined:	**Yes**	No	No	No
Transfection stress and variability:	*Yes*	**No**	**No**	**No**
Cells adapted to ER stress:	**No**	**(No)**[f]	**No**	Yes
Secretion bottleneck easily obtained:	**Yes**	**(Yes)**[g]	No	**Yes**
Clonality:	**Polyclonal**	**Polyclonal**	*Mono*-monoclonal[h]	*Mono*-monoclonal[h]
Clonal variation:	**No**	**No**	Yes	Yes
Validation by SGE needed:	Yes	Yes	Yes	**No**

a) Transient, plasmid-based co-expression of r-protein and effector genes. Examples: (Hansen et al., 2015; Johari et al., 2015; Ku et al., 2008; Tastanova et al., 2015).
b) Stable episomal co-expression of r-protein and effector genes after antibiotic-based selection (without selection resembles TGE). No published examples of co-expression are available, but the expression system is described here: (Kunaparaju et al., 2005; Silla et al., 2006). The r-protein gene and effector gene must be co-expressed from the same plasmid to prevent a drift in copy number between the two genes (see 'Gene dosage and combining genes' in section 7 for details).
c) Inducible expression of a stably integrated gene encoding the r-protein in a clonally derived cell line constitutively expressing an effector gene of interest. No reported examples of this specific setup are available to the best of the authors' knowledge, but examples of the expression system are described here: (Mohan et al., 2007; Mohan and Lee, 2010).
d) Constitutive (stable) co-expression of stably integrated genes encoding r-protein and effector genes in clonally derived cell lines. Examples: (Dreesen and Fussenegger, 2011; Haredy et al., 2013; Tastanova et al., 2015).
e) Features regarded as advantages are highlighted in boldface type.
f) See 'ER stress' in section 7 for details.
g) See 'Secretion bottleneck' in section 7 for details.
h) *Quasi*-polyclonal cell lines can also be used; however, *mono*-monoclonal cell lines are commonly used. For definition of *quasi*-polyclonal and *mono*-monoclonal cell lines, see Fig. 2.

Figure legends

Figure 1. Cellular and experimental factors affecting the biosynthetic pathway of r-proteins in CHO cells. Graphical representation of a CHO cell co-expressing an r-protein (gene, mRNA, polypeptide and protein in red letters) and an effector gene (gene and protein in blue letters). Effects from external factors are indicated with dashed arrows. Effector protein-mediated effects are shown with thin line arrows, and whether these effects are direct or indirect through interactions with an unknown DNA or protein element (element X) are illustrated. Nucleus, ER, Golgi and transport vesicles are depicted in the upper half of the cell. Since localization of effector proteins is gene-dependent, cytoplasmic organelles are not depicted in the lower half of the cell. PTM, post-translational modification.

Figure 2. Clonal variation in stable CHO cell lines. A typical process of generating a clonally derived stable CHO cell line co-expressing an r-protein and an effector gene is illustrated. CHO cells are depicted as circles, and the red circle is a high-producer cell clone selected for transfection of the effector gene. To depict the heterogeneity within the cell pools, the q_p level of each cell is illustrated as black and grey pie charts for transient and constitutive expression of the r-protein, respectively. Clonality of each phase is indicated. *Quasi*-polyclonal and *mono*-monoclonal mean that the polyclonal cell line and monoclonal cell line, respectively, both originate from a monoclonal r-protein producer cell line. Whether cells have adapted to expression of the r-protein is indicated (see 'ER stress' in Table 2). The extent of clonal variation throughout the process is depicted and is defined as the possible phenotypic difference from an average cell in the host cell line. Transfection-mediated stress is depicted (see 'Transfection stress' in Table 2). SCS: single-cell sorting.

Figure 3. Secretion bottleneck and ER stress. A) Schematic depiction of a secretion bottleneck upon co-expression of an r-protein and an effector gene. The black solid line shows the relationship between translation rate of an r-protein and q_p. The grey solid line illustrates that expression of certain effector genes gives rise to a positive effect on q_p only when a secretion bottleneck is present. The grey and black dashed lines indicate the onset translation rate for a secretion bottleneck with and without expression of a q_p-increasing effector gene, respectively. B) Schematic graph showing how secretion bottlenecks affect ER stress levels. Same colour code as in panel A is used except for the red line, which indicates the threshold between anti- and pro-apoptotic ER stress levels.

References

Adachi, Y., Yamamoto, K., Okada, T., Yoshida, H., Harada, A., Mori, K., 2008. ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. Cell Struct. Funct. 33, 75–89.

Aggarwal, R.S., 2014. What's fueling the biotech engine-2012 to 2013. Nat. Biotechnol. 32, 32–9.

Ahmadzadeh, V., Farajnia, S., Feizi, M.A.H., Nejad, R.A.K., 2014. Antibody humanization methods for development of therapeutic applications. Monoclon. Antib. Immunodiagn. Immunother. 33, 67–73.

Appenzeller-Herzog, C., Ellgaard, L., 2008. The human PDI family: Versatility packed into a single fold. Biochim. Biophys. Acta - Mol. Cell Res. 1783, 535–548.

Baldi, L., Hacker, D.L., Adam, M., Wurm, F.M., 2007. Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol. Lett. 29, 677–84.

Barnes, L.M., Bentley, C.M., Dickson, A.J., 2004. Molecular definition of predictive indicators of stable protein expression in recombinant NS0 myeloma cells. Biotechnol. Bioeng. 85, 115–21.

Barnes, L.M., Moy, N., Dickson, A.J., 2006. Phenotypic variation during cloning procedures: analysis of the growth behavior of clonal cell lines. Biotechnol. Bioeng. 94, 530–7.

Barron, N., Sanchez, N., Kelly, P., Clynes, M., 2011. MicroRNAs: Tiny targets for engineering CHO cell phenotypes? Biotechnol. Lett. 33, 11–21.

Baycin-Hizal, D., Tabb, D.L., Chaerkady, R., Chen, L., Lewis, N.E., Nagarajan, H., Sarkaria, V., Kumar, A., Wolozny, D., Colao, J., Jacobson, E., Tian, Y., O'Meally, R.N., Krag, S.S., Cole, R.N., Palsson, B.O., Zhang, H., Betenbaugh, M., 2012. Proteomic analysis of Chinese hamster ovary cells. J. Proteome Res. 11, 5265–76.

Becker, E., Florin, L., Pfizenmaier, K., Kaufmann, H., 2008. An XBP-1 dependent bottle-neck in production of IgG subtype antibodies in chemically defined serum-free Chinese hamster ovary (CHO) fed-batch processes. J. Biotechnol. 135, 217–23.

Becker, J., Hackl, M., Rupp, O., Jakobi, T., Schneider, J., Szczepanowski, R., Bekel, T., Borth, N., Goesmann, A., Grillari, J., Kaltschmidt, C., Noll, T., Pühler, A., Tauch, A., Brinkrolf, K., 2011. Unraveling the Chinese hamster ovary cell line transcriptome by next-generation sequencing. J. Biotechnol. 156, 227–235.

Bennun, S. V., Hizal, D.B., Heffner, K., Can, O., Zhang, H., Betenbaugh, M.J., 2016. Systems Glycobiology: Integrating Glycogenomics, Glycoproteomics, Glycomics, and Other 'Omics Data Sets to Characterize Cellular Glycosylation Processes. J. Mol. Biol. 428, 3337–52.

Berting, A., Farcet, M.R., Kreil, T.R., 2010. Virus susceptibility of Chinese hamster ovary (CHO) cells and detection of viral contaminations by adventitious agent testing. Biotechnol. Bioeng. 106, 598–607.

Bertozzi, C.R., Freeze, H.H., Varki, A., Esko, J.D., 2009. Glycans in Biotechnology and the Pharmaceutical Industry, in: Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E. (Eds.), Essentials of Glycobiology, 2nd Edition. Cold Spring Harbor Laboratory Press, NY.

Borth, N., Mattanovich, D., Kunert, R., Katinger, H., 2005. Effect of increased expression of protein disulfide isomerase and heavy chain binding protein on antibody secretion in a recombinant CHO cell line. Biotechnol. Prog. 21, 106–111.

Brinkrolf, K., Rupp, O., Laux, H., Kollin, F., Ernst, W., Linke, B., Kofler, R., Romand, S., Hesse, F., Budach, W.E., Galosy, S., Müller, D., Noll, T., Wienberg, J., Jostock, T., Leonard, M., Grillari, J., Tauch, A., Goesmann, A., Helk, B., Mott, J.E., Pühler, A., Borth, N., 2013. Chinese hamster genome sequenced from sorted chromosomes. Nat. Biotechnol. 31, 694–5.

Brown, A.J., James, D.C., 2015. Precision control of recombinant gene transcription for CHO cell synthetic biology. Biotechnol. Adv. 34, 492–503.

Brown, A.J., Sweeney, B., Mainwaring, D.O., James, D.C., 2014. Synthetic Promoters for CHO Cell Engineering 111, 1638–1647.

Butler, M., Spearman, M., 2014. The choice of mammalian cell host and possibilities for glycosylation engineering. Curr. Opin. Biotechnol. 30, 107–112.

Cain, K., Peters, S., Hailu, H., Sweeney, B., Stephens, P., Heads, J., Sarkar, K., Ventom, A., Page, C., Dickson, A., 2013. A CHO cell line engineered to express XBP1 and ERO1-Lα has increased levels of transient protein expression. Biotechnol. Prog. 29, 697–706.

Carlage, T., Hincapie, M., Zang, L., Lyubarskaya, Y., Madden, H., Mhatre, R., Hancock, W.S., 2009. Proteomic profiling of a high-producing Chinese hamster ovary cell culture. Anal. Chem. 81, 7357–62.

Chen, Y.-J., Inouye, M., 2008. The intramolecular chaperone-mediated protein folding. Curr. Opin. Struct. Biol. 18, 765–70.

Cho, M.S., Yee, H., Brown, C., Jeang, K.T., Chan, S., 2001. An oriP expression vector containing the HIV-1 Tat/TAR transactivation axis produces high levels of protein expression in mammalian cells. Cytotechnology 37, 23–30.

Chung, J.Y., Lim, S.W., Hong, Y.J., Hwang, S.O., Lee, G.M., 2004. Effect of doxycycline-regulated calnexin and calreticulin expression on specific thrombopoietin productivity of recombinant Chinese hamster ovary cells. Biotechnol. Bioeng. 85, 539–46.

Chusainow, J., Yang, Y.S., Yeo, J.H.M., Ton, P.C., Asvadi, P., Wong, N.S.C., Yap, M.G.S., 2009. A study of monoclonal antibody-producing CHO cell lines: What makes a stable high producer? Biotechnol. Bioeng. 102, 1182–1196.

Clarke, C., Henry, M., Doolan, P., Kelly, S., Aherne, S., Sanchez, N., Kelly, P., Kinsella, P., Breen, L., Madden, S.F., Zhang, L., Leonard, M., Clynes, M., Meleady, P., Barron, N., 2012. Integrated miRNA, mRNA and protein expression analysis reveals the role of post-transcriptional regulation in controlling CHO cell growth rate. BMC Genomics 13, 656.

Clarke, C., Lee, K.H., 2014. Special Focus: An ‘omics approach to Chinese hamster ovary based pharmaceutical bioprocessing. Pharm. Bioprocess. 2, 351–353.

Codamo, J., Munro, T.P., Hughes, B.S., Song, M., Gray, P.P., 2011. Enhanced CHO cell-based transient gene expression with the epi-CHO expression system. Mol. Biotechnol. 48, 109–15.

Courtes, F.C., Lin, J., Lim, H.L., Ng, S.W., Wong, N.S.C., Koh, G., Vardy, L., Yap, M.G.S., Loo, B., Lee, D.Y., 2013. Translatome analysis of CHO cells to identify key growth genes. J.

Biotechnol. 167, 215–224.

Dahodwala, H., Sharfstein, S.T., 2014. Role of epigenetics in expression of recombinant proteins from mammalian cells. Pharm. Bioprocess. 2, 403–419.

Daramola, O., Stevenson, J., Dean, G., Hatton, D., Pettman, G., Holmes, W., Field, R., 2014. A high-yielding CHO transient system: Coexpression of genes encoding EBNA-1 and GS enhances transient protein expression. Biotechnol. Prog. 30, 132–141.

Datta, P., Linhardt, R.J., Sharfstein, S.T., 2013. An 'omics approach towards CHO cell engineering. Biotechnol. Bioeng. 110, 1255–1271.

Davies, S.L., Lovelady, C.S., Grainger, R.K., Racher, A.J., Young, R.J., James, D.C., 2013. Functional heterogeneity and heritability in CHO cell populations. Biotechnol. Bioeng. 110, 260–274.

Davis, R., Schooley, K., Rasmussen, B., Thomas, J., Reddy, P., 2000. Effect of PDI overexpression on recombinant protein secretion in CHO cells. Biotechnol. Prog. 16, 736–743.

Derouazi, M., Martinet, D., Besuchet Schmutz, N., Flaction, R., Wicht, M., Bertschinger, M., Hacker, D.L., Beckmann, J.S., Wurm, F.M., 2006. Genetic characterization of CHO production host DG44 and derivative recombinant cell lines. Biochem. Biophys. Res. Commun. 340, 1069–1077.

Dicker, M., Strasser, R., 2015. Using glyco-engineering to produce therapeutic proteins. Expert Opin. Biol. Ther. 15, 1501–16.

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y., Xu, T., 2005. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell 122, 473–83.

Doolan, P., Melville, M., Gammell, P., Sinacore, M., Meleady, P., McCarthy, K., Francullo, L., Leonard, M., Charlebois, T., Clynes, M., 2008. Transcriptional profiling of gene expression changes in a PACE-transfected CHO DUKX cell line secreting high levels of rhBMP-2. Mol. Biotechnol. 39, 187–99.

Dreesen, I.A.J., Fussenegger, M., 2011. Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. Biotechnol. Bioeng. 108, 853–866.

Durocher, Y., Butler, M., 2009. Expression systems for therapeutic glycoprotein production. Curr. Opin. Biotechnol. 20, 700–7.

Estes, B., Hsu, Y.R., Tam, L.T., Sheng, J., Stevens, J., Haldankar, R., 2015. Uncovering methods for the prevention of protein aggregation and improvement of product quality in a transient expression system. Biotechnol. Prog. 31, 258–267.

Fann, C.H., Guarna, M.M., Kilburn, D.G., Piret, J.M., 1999. Relationship between recombinant activated protein C secretion rates and mRNA levels in baby hamster kidney cells. Biotechnol. Bioeng. 63, 464–472.

Fazekas, S., St. Groth, D.E., Scheidegger, D., 1980. Production of Monoclonal Antibodies: Strategy and Tactics. J. Immunol. Methods 35, 1–21.

Feichtinger, J., Hernández, I., Fischer, C., Hanscho, M., Auer, N., Hackl, M., Jadhav, V., Baumann,

M., Krempl, P.M., Schmidl, C., Farlik, M., Schuster, M., Merkel, A., Sommer, A., Heath, S., Rico, D., Bock, C., Thallinger, G.G., Borth, N., 2016. Comprehensive genome and epigenome characterization of CHO cells in response to evolutionary pressures and over time. Biotechnol. Bioeng. 9999, 1–14.

Fischer, S., Handrick, R., Otte, K., 2015. The art of CHO cell engineering: A comprehensive retrospect and future perspectives. Biotechnol. Adv. 33, 1878–1896.

Florin, L., Pegel, A., Becker, E., Hausser, A., Olayioye, M. a., Kaufmann, H., 2009. Heterologous expression of the lipid transfer protein CERT increases therapeutic protein productivity of mammalian cells. J. Biotechnol. 141, 84–90.

Frye, C., Deshpande, R., Estes, S., Francissen, K., Joly, J., Lubiniecki, A., Munro, T., Russell, R., Wang, T., Anderson, K., 2016. Industry view on the relative importance of "clonality" of biopharmaceutical-producing cell lines. Biologicals 6–11.

Fussenegger, M., Morris, R.P., Fux, C., Rimann, M., von Stockar, B., Thompson, C.J., Bailey, J.E., 2000. Streptogramin-based gene regulation systems for mammalian cells. Nat. Biotechnol. 18, 1203–1208.

Gaillet, B., Gilbert, R., Broussau, S., Pilotte, A., Malenfant, F., Mullick, A., Garnier, A., Massie, B., 2010. High-level recombinant protein production in CHO cells using lentiviral vectors and the cumate gene-switch. Biotechnol. Bioeng. 106, 203–15.

Galbraith, D.J., Tait, A.S., Racher, A.J., Birch, J.R., James, D.C., 2006. Control of culture environment for improved polyethylenimine-mediated transient production of recombinant monoclonal antibodies by CHO cells. Biotechnol. Prog. 22, 753–62.

Galvan, D.L., Nakazawa, Y., Kaja, A., Kettlun, C., Cooper, L.J.N., Rooney, C.M., Wilson, M.H., 2009. Genome-wide mapping of PiggyBac transposon integrations in primary human T cells. J. Immunother. 32, 837–44.

Golabgir, A., Gutierrez, J.M., Hefzi, H., Li, S., Palsson, B.O., Herwig, C., Lewis, N.E., 2016. Quantitative feature extraction from the Chinese hamster ovary bioprocess bibliome using a novel meta-analysis workflow. Biotechnol. Adv. 1–13.

Gossen, M., Bujard, H., 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U. S. A. 89, 5547–51.

Gramer, M.J., 2014. Product quality considerations for mammalian cell culture process development and manufacturing. Adv. Biochem. Eng. Biotechnol. 139, 123–66.

Gronemeyer, P., Ditz, R., Strube, J., 2014. Trends in Upstream and Downstream Process Development for Antibody Manufacturing. Bioengineering 1, 188–212.

Gutierrez, J.M., Lewis, N.E., 2015. Optimizing eukaryotic cell hosts for protein production through systems biotechnology and genome-scale modeling. Biotechnol. J. n/a-n/a.

Hacker, D.L., De Jesus, M., Wurm, F.M., 2009. 25 years of recombinant proteins from reactor-grown cells - where do we go from here? Biotechnol. Adv. 27, 1023–7.

Hackl, M., Borth, N., Grillari, J., 2012. miRNAs--pathway engineering of CHO cell factories that avoids translational burdening. Trends Biotechnol. 30, 405–6.

Hansen, H.G., Nilsson, C.N., Lund, A.M., Kol, S., Grav, L.M., Lundqvist, M., Rockberg, J., Lee, G.M., Andersen, M.R., Kildegaard, H.F., 2015. Versatile microscale screening platform for improving recombinant protein productivity in Chinese hamster ovary cells. Sci. Rep. 5, 18016.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., Bell, J.C., Hettmann, T., Leiden, J.M., Ron, D., 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol. Cell 11, 619–633.

Haredy, A.M., Nishizawa, A., Honda, K., Ohya, T., Ohtake, H., Omasa, T., 2013. Improved antibody production in Chinese hamster ovary cells by ATF4 overexpression. Cytotechnology 65, 993–1002.

Harreither, E., Hackl, M., Pichler, J., Shridhar, S., Auer, N., Łabaj, P.P., Scheideler, M., Karbiener, M., Grillari, J., Kreil, D.P., Borth, N., 2015. Microarray profiling of preselected CHO host cell subclones identifies gene expression patterns associated with increased production capacity. Biotechnol. J. 10, 1625–38.

Hatahet, F., Ruddock, L.W., 2009. Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. Antioxid. Redox Signal. 11, 2807–50.

Hayes, N.V.L., Smales, C.M., Klappa, P., 2010. Protein disulfide isomerase does not control recombinant IgG4 productivity in mammalian cell lines. Biotechnol. Bioeng. 105, 770–779.

Heffernan, M., Dennis, J.W., 1991. Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells. Nucleic Acids Res. 19, 85–92.

Hefzi, H., Ang, K.S., Hanscho, M., Bordbar, A., Ruckerbauer, D., Lakshmanan, M., Orellana, C.A., Baycin-Hizal, D., Huang, H., Ley, D., Martínez, V.S., Kyriakopoulos, S., Jiménez, N.E., Zielinski, D.C., Quek, L.E., Wulff, T., Arnsdorf, J., Li, S., Lee, J.S., Paglia, G., Loira, N., Spahn, P.N., Pedersen, L.E., Gutierrez, J.M., Lund, A.., Nagarajan, H., Thomas, A., Abdel-Haleem, A.M., Zanghellini, J., Kildegaard, H.F., Voldborg, B.G., Gerdtzen, Z.P., Betenbaugh, M.J., Palsson, B.O., Andersen, M.R., Nielsen, L.K., Borth, N., Lee, D.Y., Lewis, N.E., 2016. A consensus genome-scale reconstruction of CHO cell metabolism for improved biotherapeutic protein production. Cell Syst. In press.

Hu, Z., Guo, D., Yip, S.S.M., Zhan, D., Misaghi, S., Joly, J.C., Snedecor, B.R., Shen, A.Y., 2013. Chinese hamster ovary K1 host cell enables stable cell line development for antibody molecules which are difficult to express in DUXB11-derived dihydrofolate reductase deficient host cell. Biotechnol. Prog. 29, 980–985.

Hussain, H., Maldonado-Agurto, R., Dickson, A.J., 2014. The endoplasmic reticulum and unfolded protein response in the control of mammalian recombinant protein production. Biotechnol. Lett. 36, 1581–1593.

Hwang, S.O., Chung, J.Y., Lee, G.M., 2003. Effect of doxycycline-regulated ERp57 expression on specific thrombopoietin productivity of recombinant CHO cells. Biotechnol. Prog. 19, 179–84.

Johari, Y.B., Estes, S.D., Alves, C.S., Sinacore, M.S., James, D.C., 2015. Integrated cell and process engineering for improved transient production of a “difficult-to-express” fusion protein by CHO cells. Biotechnol. Bioeng. 112, 2527–42.

Justice, C., Brix, A., Freimark, D., Kraume, M., Pfromm, P., Eichenmueller, B., Czermak, P., 2011. Process control in cell culture technology using dielectric spectroscopy. Biotechnol. Adv. 29, 391–401.

Jäger, R., Bertrand, M.J.M., Gorman, A.M., Vandenabeele, P., Samali, A., 2012. The unfolded protein response at the crossroads of cellular life and death during endoplasmic reticulum stress. Biol. Cell 104, 259–270.

Kaas, C.S., Fan, Y., Weilguny, D., Kristensen, C., Kildegaard, H.F., Andersen, M.R., 2014. Toward genome-scale models of the Chinese hamster ovary cells: incentives, status and perspectives. Pharm. Bioprocess. 2, 437–448.

Kaas, C.S., Kristensen, C., Betenbaugh, M.J., Andersen, M.R., 2015. Sequencing the CHO DXB11 genome reveals regional variations in genomic stability and haploidy. BMC Genomics 16, 1–9.

Kallehauge, T.B., Kol, S., Rørdam Andersen, M., Kroun Damgaard, C., Lee, G.M., Faustrup Kildegaard, H., 2016. Endoplasmic reticulum-directed recombinant mRNA displays subcellular localization equal to endogenous mRNA during transient expression in CHO cells. Biotechnol. J. 11, 1362–1367.

Khan, S.U., Schröder, M., 2008. Engineering of chaperone systems and of the unfolded protein response. Cytotechnology 57, 207–31.

Kildegaard, H.F., Baycin-Hizal, D., Lewis, N.E., Betenbaugh, M.J., 2013. The emerging CHO systems biology era: harnessing the 'omics revolution for biotechnology. Curr. Opin. Biotechnol. 24, 1102–7.

Kim, J.Y., Kim, Y.-G., Lee, G.M., 2012. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. Appl. Microbiol. Biotechnol. 93, 917–30.

Kim, N.S., Kim, S.J., Lee, G.M., 1998. Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: Stability in the absence of selective pressure. Biotechnol. Bioeng. 60, 679–688.

Kim, S.J., Kim, N.S., Ryu, C.J., Hong, H.J., Lee, G.M., 1998. Characterization of chimeric antibody producing CHO cells in the course of dihydrofolate reductase-mediated gene amplification and their stability in the absence of selective pressure. Biotechnol. Bioeng. 58, 73–84.

Kim, Y.J., Baek, E., Lee, J.S., Lee, G.M., 2013. Autophagy and its implication in Chinese hamster ovary cell culture. Biotechnol. Lett. 35, 1753–63.

Kremkow, B.G., Baik, J.Y., Macdonald, M.L., Lee, K.H., 2015. CHOgenome.org 2.0: Genome resources and website updates. Biotechnol. J. 2011, 931–938.

Ku, S.C.Y., Ng, D.T.W., Yap, M.G.S., Chao, S., 2008. Effects of overexpression of X-box binding protein 1 on recombinant protein production in Chinese hamster ovary and NS0 myeloma cells. Biotechnol. Bioeng. 99, 155–64.

Ku, S.C.Y., Toh, P.C., Lee, Y.Y., Chusainow, J., Yap, M.G.S., Chao, S.H., 2010. Regulation of XBP-1 signaling during transient and stable recombinant protein production in CHO cells. Biotechnol. Prog. 26, 517–526.

Kumar, S.R., 2015. Industrial production of clotting factors: Challenges of expression, and choice of host cells. Biotechnol. J. 10, 995–1004.

Kunaparaju, R., Liao, M., Sunstrom, N.-A., 2005. Epi-CHO, an episomal expression system for recombinant protein production in CHO cells. Biotechnol. Bioeng. 91, 670–7.

Kunert, R., Reinhart, D., 2016. Advances in recombinant antibody manufacturing. Appl. Microbiol. Biotechnol. 100, 3451–3461.

Le Fourn, V., Girod, P.-A., Buceta, M., Regamey, A., Mermod, N., 2014. CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. Metab. Eng. 21, 91–102.

Lee, G.W., Fecko, J.K., Yen, A., Donaldson, D., Wood, C., Tobler, S., Vunuum, S., Luo, Y., Leonard, M., 2007. Improving the Expression of a Soluble Receptor:Fc fusion Protein in CHO Cells by Coexpression with the Receptor Ligand, in: Cell Technology for Cell Products. Springer Netherlands, Dordrecht, pp. 29–39.

Lee, J.S., Grav, L.M., Pedersen, L.E., Lee, G.M., Kildegaard, H.F., 2016. Accelerated homology-directed targeted integration of transgenes in Chinese hamster ovary cells via CRISPR/Cas9 and fluorescent enrichment. Biotechnol. Bioeng. 113, 2518–2523.

Lee, J.S., Kallehauge, T.B., Pedersen, L.E., Kildegaard, H.F., 2015. Site-specific integration in CHO cells mediated by CRISPR/Cas9 and homology-directed DNA repair pathway. Sci. Rep. 5, 8572.

Lewis, N.E., Liu, X., Li, Y., Nagarajan, H., Yerganian, G., O'Brien, E., Bordbar, A., Roth, A.M., Rosenbloom, J., Bian, C., Xie, M., Chen, W., Li, N., Baycin-Hizal, D., Latif, H., Forster, J., Betenbaugh, M.J., Famili, I., Xu, X., Wang, J., Palsson, B.O., 2013. Genomic landscapes of Chinese hamster ovary cell lines as revealed by the Cricetulus griseus draft genome. Nat. Biotechnol. 31, 759–65.

Liu, C., Dalby, B., Chen, W., Kilzer, J.M., Chiou, H.C., 2008. Transient transfection factors for high-level recombinant protein production in suspension cultured mammalian cells. Mol. Biotechnol. 39, 141–153.

Liu, C.Y., Spencer, V., Kumar, S., Liu, J., Chiou, H., Zmuda, J.F., 2015. Attaining High Transient Titers in CHO Cells. Genet. Eng. Biotechnol. News 35, 34–35.

Lupton, S., Levine, A.J., 1985. Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. Mol. Cell. Biol. 5, 2533–2542.

Macaraeg, N.F., Reilly, D.E., Wong, A.W., 2013. Use of an anti-apoptotic CHO cell line for transient gene expression. Biotechnol. Prog. 29, 1050–8.

Majors, B.S., Betenbaugh, M.J., Pederson, N.E., Chiang, G.G., 2008. Enhancement of transient gene expression and culture viability using Chinese hamster ovary cells overexpressing Bcl-x(L). Biotechnol. Bioeng. 101, 567–78.

Mali, P., Esvelt, K.M., Church, G.M., 2013. Cas9 as a versatile tool for engineering biology. Nat. Methods 10, 957–63.

Mariati, Ho, S.C.L., Yap, M.G.S., Yang, Y., 2010. Evaluating post-transcriptional regulatory elements for enhancing transient gene expression levels in CHO K1 and HEK293 cells. Protein Expr. Purif. 69, 9–15.

Matasci, M., Bachmann, V., Baldi, L., Hacker, D.L., De Jesus, M., Wurm, F.M., 2011. Rapid recombinant protein production from pools of transposon-generated CHO cells. BMC Proc. 5, P34.

McLeod, J., O'Callaghan, P.M., Pybus, L.P., Wilkinson, S.J., Root, T., Racher, A.J., James, D.C., 2011. An empirical modeling platform to evaluate the relative control discrete CHO cell synthetic processes exert over recombinant monoclonal antibody production process titer. Biotechnol. Bioeng. 108, 2193–204.

Merulla, J., Fasana, E., Soldà, T., Molinari, M., 2013. Specificity and regulation of the endoplasmic reticulum-associated degradation machinery. Traffic 14, 767–77.

Mohan, C., Kim, Y.-G., Koo, J., Lee, G.M., 2008. Assessment of cell engineering strategies for improved therapeutic protein production in CHO cells. Biotechnol. J. 3, 624–30.

Mohan, C., Lee, G.M., 2010. Effect of inducible co-overexpression of protein disulfide isomerase and endoplasmic reticulum oxidoreductase on the specific antibody productivity of recombinant Chinese hamster ovary cells. Biotechnol. Bioeng. 107, 337–46.

Mohan, C., Park, S.H., Chung, J.Y., Lee, G.M., 2007. Effect of doxycycline-regulated protein disulfide isomerase expression on the specific productivity of recombinant CHO cells: Thrombopoietin and antibody. Biotechnol. Bioeng. 98, 611–615.

Moore, K.A., Hollien, J., 2012. The Unfolded Protein Response in Secretory Cell Function. Annu. Rev. Genet. 46, 165–183.

Mozley, O.L., Thompson, B.C., Fernandez-Martell, A., James, D.C., 2014. A mechanistic dissection of polyethylenimine mediated transfection of CHO cells: to enhance the efficiency of recombinant DNA utilization. Biotechnol. Prog. 30, 1161–70.

Mullick, A., Xu, Y., Warren, R., Koutroumanis, M., Guilbault, C., Broussau, S., Malenfant, F., Bourget, L., Lamoureux, L., Lo, R., Caron, A.W., Pilotte, A., Massie, B., 2006. The cumate gene-switch: a system for regulated expression in mammalian cells. BMC Biotechnol. 6, 43.

Nishimiya, D., 2013. Proteins improving recombinant antibody production in mammalian cells. Appl. Microbiol. Biotechnol. 98, 1–12.

Nishimiya, D., Mano, T., Miyadai, K., Yoshida, H., Takahashi, T., 2013. Overexpression of CHOP alone and in combination with chaperones is effective in improving antibody production in mammalian cells. Appl. Microbiol. Biotechnol. 97, 2531–2539.

Noh, S.M., Sathyamurthy, M., Lee, G.M., 2013. Development of recombinant Chinese hamster ovary cell lines for therapeutic protein production. Curr. Opin. Chem. Eng. 1–7.

O'Callaghan, P.M., Berthelot, M.E., Young, R.J., Graham, J.W.A., Racher, A.J., Aldana, D., 2015. Diversity in host clone performance within a Chinese hamster ovary cell line. Biotechnol. Prog. 31, 1187–200.

O'Callaghan, P.M., McLeod, J., Pybus, L.P., Lovelady, C.S., Wilkinson, S.J., Racher, A.J., Porter, A., James, D.C., 2010. Cell line-specific control of recombinant monoclonal antibody production by CHO cells. Biotechnol. Bioeng. 106, 938–951.

Oberbek, A., Matasci, M., Hacker, D.L., Wurm, F.M., 2011. Generation of stable, high-producing CHO cell lines by lentiviral vector-mediated gene transfer in serum-free suspension culture.

Biotechnol. Bioeng. 108, 600–10.

Papapetrou, E.P., Lee, G., Malani, N., Setty, M., Riviere, I., Tirunagari, L.M.S., Kadota, K., Roth, S.L., Giardina, P., Viale, A., Leslie, C., Bushman, F.D., Studer, L., Sadelain, M., 2011. Genomic safe harbors permit high β-globin transgene expression in thalassemia induced pluripotent stem cells. Nat. Biotechnol. 29, 73–8.

Peng, R.-W., Fussenegger, M., 2009. Molecular engineering of exocytic vesicle traffic enhances the productivity of Chinese hamster ovary cells. Biotechnol. Bioeng. 102, 1170–81.

Peng, R.W., Abellan, E., Fussenegger, M., 2011. Differential effect of exocytic SNAREs on the production of recombinant proteins in mammalian cells. Biotechnol. Bioeng. 108, 611–620.

Pilbrough, W., Munro, T.P., Gray, P., 2009. Intraclonal protein expression heterogeneity in recombinant CHO cells. PLoS One 4, e8432.

Preininger, A., Schlokat, U., Mohr, G., Himmelspach, M., Stichler, V., Kyd-Rebenburg, A., Plaimauer, B., Turecek, P.L., Schwarz, H.P., Wernhart, W., Fischer, B.E., Dorner, F., 1999. Strategies for recombinant Furin employment in a biotechnological process: complete target protein precursor cleavage. Cytotechnology 30, 1–16.

Priola, J.J., Calzadilla, N., Baumann, M., Borth, N., Tate, C.G., Betenbaugh, M.J., 2016. High-throughput screening and selection of mammalian cells for enhanced protein production. Biotechnol. J. 1–13.

Puck, T.T., 1957. The genetics of somatic mammalian cells. Adv. Biol. Med. Phys. 5, 75–101.

Pybus, L.P., Dean, G., West, N.R., Smith, A., Daramola, O., Field, R., Wilkinson, S.J., James, D.C., 2014. Model-directed engineering of "difficult-to-express" monoclonal antibody production by Chinese hamster ovary cells. Biotechnol. Bioeng. 111, 372–85.

Qin, J.Y., Zhang, L., Clift, K.L., Hulur, I., Xiang, A.P., Ren, B.-Z., Lahn, B.T., 2010. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. PLoS One 5, e10611.

Rajendra, Y., Kiseljak, D., Baldi, L., Hacker, D.L., Wurm, F.M., 2015. Transcriptional and post-transcriptional limitations of high-yielding, PEI-mediated transient transfection with CHO and HEK-293E cells. Biotechnol. Prog. 31, 541–49.

Rajendra, Y., Kiseljak, D., Manoli, S., Baldi, L., Hacker, D.L., Wurm, F.M., 2012. Role of non-specific DNA in reducing coding DNA requirement for transient gene expression with CHO and HEK-293E cells. Biotechnol. Bioeng. 109, 2271–8.

Rajendra, Y., Peery, R.B., Barnard, G.C., 2016. Generation of stable Chinese hamster ovary pools yielding antibody titers of up to 7.6 g/L using the piggyBac transposon system. Biotechnol. Prog. 30, 429–42.

Rajesh, K., Krishnamoorthy, J., Kazimierczak, U., Tenkerian, C., Papadakis, a I., Wang, S., Huang, S., Koromilas, a E., 2015. Phosphorylation of the translation initiation factor eIF2α at serine 51 determines the cell fate decisions of Akt in response to oxidative stress. Cell Death Dis. 6, e1591.

Randall, T.D., Parkhouse, R.M., Corley, R.B., 1992. J chain synthesis and secretion of hexameric IgM is differentially regulated by lipopolysaccharide and interleukin 5. Proc Natl Acad Sci U S

A 89, 962–966.

Sano, R., Reed, J.C., 2013. ER stress-induced cell death mechanisms. Biochim. Biophys. Acta 1833, 3460–70.

Sathyamurthy, M., Kim, C.L., Bang, Y.L., Kim, Y.S., Jang, J.W., Lee, G.M., 2015. Characterization and expression of proprotein convertases in CHO cells: Efficient proteolytic maturation of human bone morphogenetic protein-7. Biotechnol. Bioeng. 112, 560–8.

Sathyamurthy, M., Lee, J.S., Park, J.H., Kim, Y.J., Jeong, J.Y., Jang, J.W., Lee, G.M., 2012. Overexpression of PACEsol improves BMP-7 processing in recombinant CHO cells. J. Biotechnol. 164, 336–9.

Schröder, M., Friedl, P., 1997. Overexpression of recombinant human antithrombin III in Chinese hamster ovary cells results in malformation and decreased secretion of recombinant protein. Biotechnol. Bioeng. 53, 547–559.

Schröder, M., Körner, C., Friedl, P., 1999. Quantitative analysis of transcription and translation in gene amplified Chinese hamster ovary cells on the basis of a kinetic model. Cytotechnology 29, 93–102.

Schröder, M., Schäfer, R., Friedl, P., 2002. Induction of protein aggregation in an early secretory compartment by elevation of expression level. Biotechnol. Bioeng. 78, 131–40.

Selvarasu, S., Ho, Y.S., Chong, W.P.K., Wong, N.S.C., Yusufi, F.N.K., Lee, Y.Y., Yap, M.G.S., Lee, D.Y., 2012. Combined in silico modeling and metabolomics analysis to characterize fed-batch CHO cell culture. Biotechnol. Bioeng. 109, 1415–1429.

Seth, G., Charaniya, S., Wlaschin, K.F., Hu, W.-S., 2007. In pursuit of a super producer-alternative paths to high producing recombinant mammalian cells. Curr. Opin. Biotechnol. 18, 557–64.

Silla, T., Tagen, I., Geimanen, J., Janikson, K., Abroi, A., Ustav, E., Ustav, M., Mandel, T., 2006. Vectors, cell lines and their use in obtaining extended episomal maintenance replication of hybrid plasmids and expression of gene products. WO2006084754 A1.

Sinacore, M.S., Drapeau, D., Adamson, S.R., 2000. Adaptation of mammalian cells to growth in serum-free media. Mol. Biotechnol. 15, 249–57.

Sommeregger, W., Mayrhofer, P., Steinfellner, W., Reinhart, D., Henry, M., Clynes, M., Meleady, P., Kunert, R., 2016. Proteomic differences in recombinant CHO cells producing two similar antibody fragments. Biotechnol. Bioeng. n/a-n/a.

Spahn, P.N., Lewis, N.E., 2014. Systems glycobiology for glycoengineering. Curr. Opin. Biotechnol. 30, 218–24.

Stearns, F.W., 2010. One hundred years of pleiotropy: a retrospective. Genetics 186, 767–73.

Stockholm, D., Benchaouir, R., Picot, J., Rameau, P., Neildez, T.M.A., Landini, G., Laplace-Builhé, C., Paldi, A., 2007. The origin of phenotypic heterogeneity in a clonal cell population in vitro. PLoS One 2, e394.

Tastanova, A., Schulz, A., Folcher, M., Tolstrup, A., Puklowski, A., Kaufmann, H., Fussenegger, M., 2015. Overexpression of YY1 increases the protein production in mammalian cells. J. Biotechnol. 219, 72–85.

Tigges, M., Fussenegger, M., 2006. Xbp1-based engineering of secretory capacity enhances the productivity of Chinese hamster ovary cells. Metab. Eng. 8, 264–72.

Underhill, M.F., Coley, C., Birch, J.R., Findlay, A., Kallmeier, R., Proud, C.G., James, D.C., 2003. Engineering mRNA translation initiation to enhance transient gene expression in chinese hamster ovary cells. Biotechnol Prog 19, 121–129.

Urlaub, G., Chasin, L.A., 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. U. S. A. 77, 4216–20.

Urlaub, G., Käs, E., Carothers, A.M., Chasin, L.A., 1983. Deletion of the diploid dihydrofolate reductase locus from cultured mammalian cells. Cell 33, 405–12.

Van Craenenbroeck, K., Vanhoenacker, P., Haegeman, G., 2000. Episomal vectors for gene expression in mammalian cells. Eur. J. Biochem. 267, 5665–78.

Walsh, G., 2014. Biopharmaceutical benchmarks 2014. Nat. Biotechnol. 32, 992–1000.

Walsh, G., Jefferis, R., 2006. Post-translational modifications in the context of therapeutic proteins. Nat. Biotechnol. 24, 1241–52.

Walter, P., Ron, D., 2011. The unfolded protein response: from stress pathway to homeostatic regulation. Science 334, 1081–6.

Wasley, L.C., Rehemtulla, A., Bristol, J.A., Kaufman, R.J., 1993. PACE/furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. J. Biol. Chem. 268, 8458–65.

Weber, W., Fux, C., Daoud-el Baba, M., Keller, B., Weber, C.C., Kramer, B.P., Heinzen, C., Aubel, D., Bailey, J.E., Fussenegger, M., 2002. Macrolide-based transgene control in mammalian cells and mice. Nat. Biotechnol. 20, 901–7.

Werstuck, G., Green, M.R., 1998. Controlling gene expression in living cells through small molecule-RNA interactions. Science 282, 296–8.

Wiederanders, B., Kaulmann, G., Schilling, K., 2003. Functions of propeptide parts in cysteine proteases. Curr. Protein Pept. Sci. 4, 309–26.

Wilson, M.H., Coates, C.J., George, A.L., 2007. PiggyBac transposon-mediated gene transfer in human cells. Mol. Ther. 15, 139–45.

Wurm, F., 2013. CHO Quasispecies—Implications for Manufacturing Processes. Processes 1, 296–311.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nat. Biotechnol. 22, 1393–8.

Wurm, F.M., Hacker, D., 2011. First CHO genome. Nat. Biotechnol. 29, 718–720.

Xiao, S., Shiloach, J., Betenbaugh, M.J., 2014. Engineering cells to improve protein expression. Curr. Opin. Struct. Biol. 26, 32–38.

Xu, X., Nagarajan, H., Lewis, N.E., Pan, S., Cai, Z., Liu, X., Chen, W., Xie, M., Wang, W., Hammond, S., Andersen, M.R., Neff, N., Passarelli, B., Koh, W., Fan, H.C., Wang, J., Gui, Y., Lee, K.H., Betenbaugh, M.J., Quake, S.R., Famili, I., Palsson, B.O., Wang, J., 2011. The

genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. Nat. Biotechnol. 29, 735–41.

Yates, J., Warren, N., Reisman, D., Sugden, B., 1984. A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. U. S. A. 81, 3806–10.

Ye, J., Kober, V., Tellers, M., Naji, Z., Salmon, P., Markusen, J.F., 2009. High-level protein expression in scalable CHO transient transfection. Biotechnol. Bioeng. 103, 542–51.

Zang, L., Frenkel, R., Simeone, J., Lanan, M., Byers, M., Lyubarskaya, Y., 2011. Metabolomics Profiling of Cell Culture Media Leading to the Identification of Riboflavin Photosensitized Degradation of Tryptophan Causing Slow Growth in Cell Culture. Anal. Chem. 83, 5422–5430.

Fig. 1

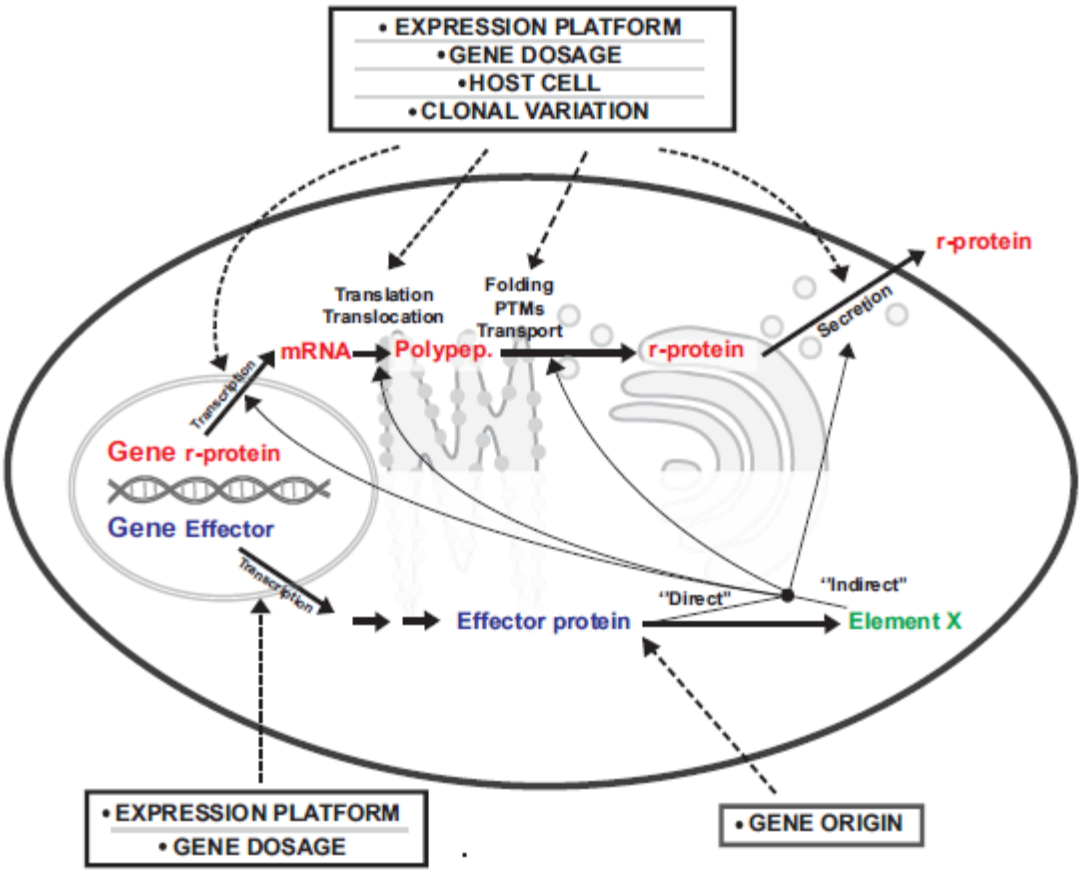

Fig. 2

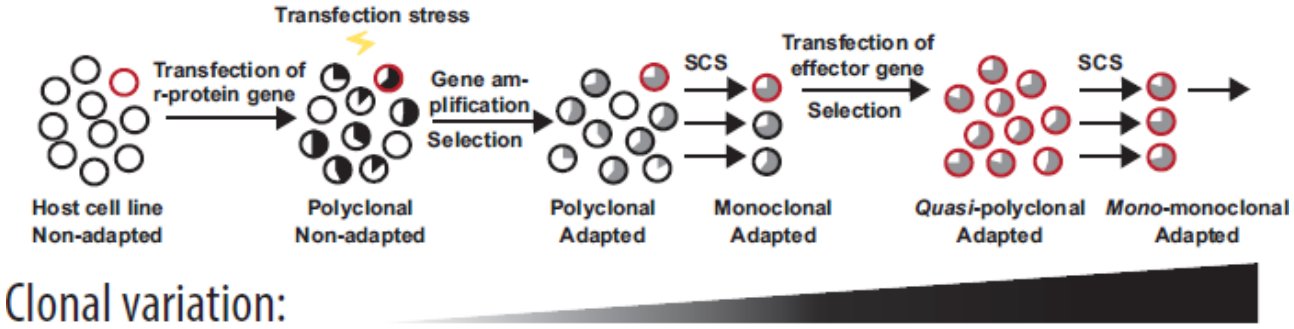

Fig. 3