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The fickle CHO: a review of the causes, implications, and potential alleviation of the CHO cell line instability problem

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Chinese hamster ovary (CHO) cell-based bioproduction of recombinant proteins can now routinely achieve >5 g/L titers in fed-batches. This progress is partly due to the rapid adaptability of CHO cells to various genetic manipulations and changing process conditions. An inherently plastic genome allows for this adaptability; however, it also gives CHO cells the propensity for genomic rearrangements. In combination with the genomic and metabolic demand of high producer cells, CHO cell plasticity manifests itself in the bioproduction process as **cell line instability**, by way of a decline in productivity and product quality. In this review, we provide a definition for titer and quality stability and discuss the main causes of the CHO instability phenomenon and advances in clone selection and genetic manipulations. We also discuss advances in systems biology efforts that can provide new strategies for early prediction of CHO cell instability, which will help to identify multi-gram per liter titer cell lines that can maintain production stability and reproducible product quality over extended culture durations.

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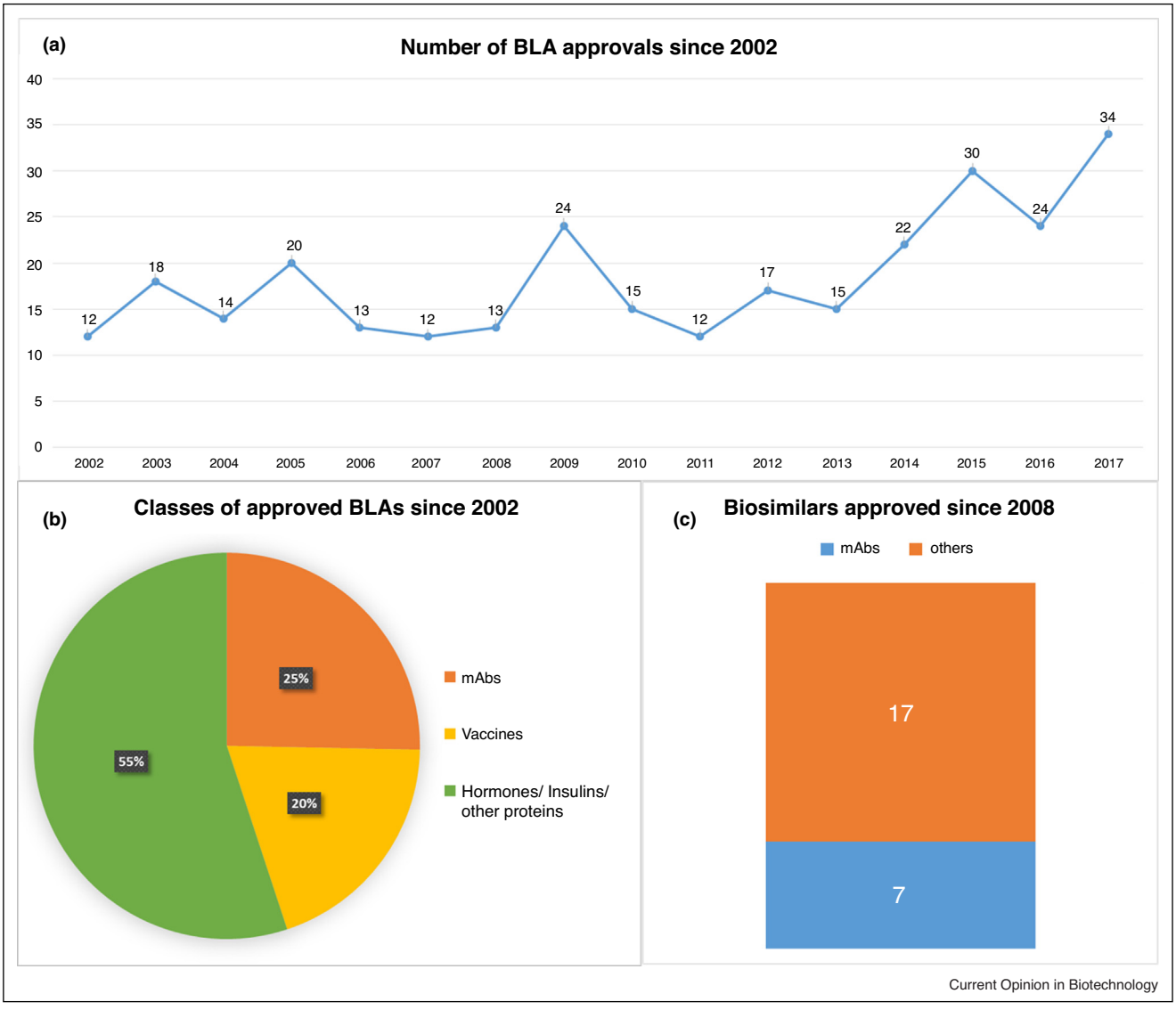
Introduction

Since 2002 there have been 316 biotherapeutic approvals (including biosimilar and combination use approvals) by the FDA. The number of new approvals per year has been consistent in the past four years and in the current year as of August 2018 there have been five new approvals: four monoclonal antibodies (mAbs) erenumab, tildrakizumab, ibalizumab, rastuzumab and one biosimilar protein drug epoetin alfa-epbx. In the new approvals category, from 2002 to present, mAbs (novel and new indications) make up 22% of all the New Drug Application (NDA) and Biologic License Application (BLA) approvals and 30% of all biosimilar approvals since 2008. (Figure 1: Source U.S. BIOPHARMACOPEIA Registry of Biopharmaceutical Products http://www.biopharma.com/approvals last accessed September 28, 2018). Chinese hamster ovary (CHO) cell lines have been preferentially used to produce recombinant therapeutics and are used for manufacturing approximately 70% of all recombinant biopharmaceutical proteins and for all mAbs (adalimumab, bezlotoxumab, avelumab, dupilumab, durvalumab ocrelizumab, and brodalumab) approved since 2016 [1,2]. It has been demonstrated that CHO cells have many process-amenable qualities such as tolerance to genetic manipulations, ease of adaptation to manufacturing process scales, rapid growth rates, and the inherent ability to perform human-compatible post-translational modifications. The sum of many incremental advances [3] over the past 30 years of biopharmaceutical development efforts have led to establishment of CHO cell culture processes demonstrating titers of 13 g/L in fed-batch production [4]. In the age of the biosimilars [5], and with better understanding of protein efficacy and the importance of analytics adherence for regulatory compliance [6], the new challenge in upstream process development is to manufacture large quantities of recombinant protein exhibiting a consistent charge, size, and efficacy profile.

It has been proposed by others [7••,8] that chromosomal rearrangements and other genomic variants observed in the CHO cell line karyotype have, in part, led to individual cell lines with performance amenable to industrial processes. However, because of this genome plasticity, one of the problems often encountered is that cells in culture can inadvertently show a decrease in recombinant protein production during long periods of culture and even during extended time at the final bioreactor scale [9•]. Various mechanisms have been identified as causes of the observed instability, such as gene loss [10], gene silencing [11•,12,13], and increased susceptibility to cellular stresses [10,14]. Production instability has also been known to arise from distal factors such as increasing apoptosis [15] and global gene changes [16••] as well as whole genome/epigenome changes [17••]. Unintended and unpredictable gene changes also come with a risk of changing the expression of cellular genes associated with glycosylation, protein

Figure 1

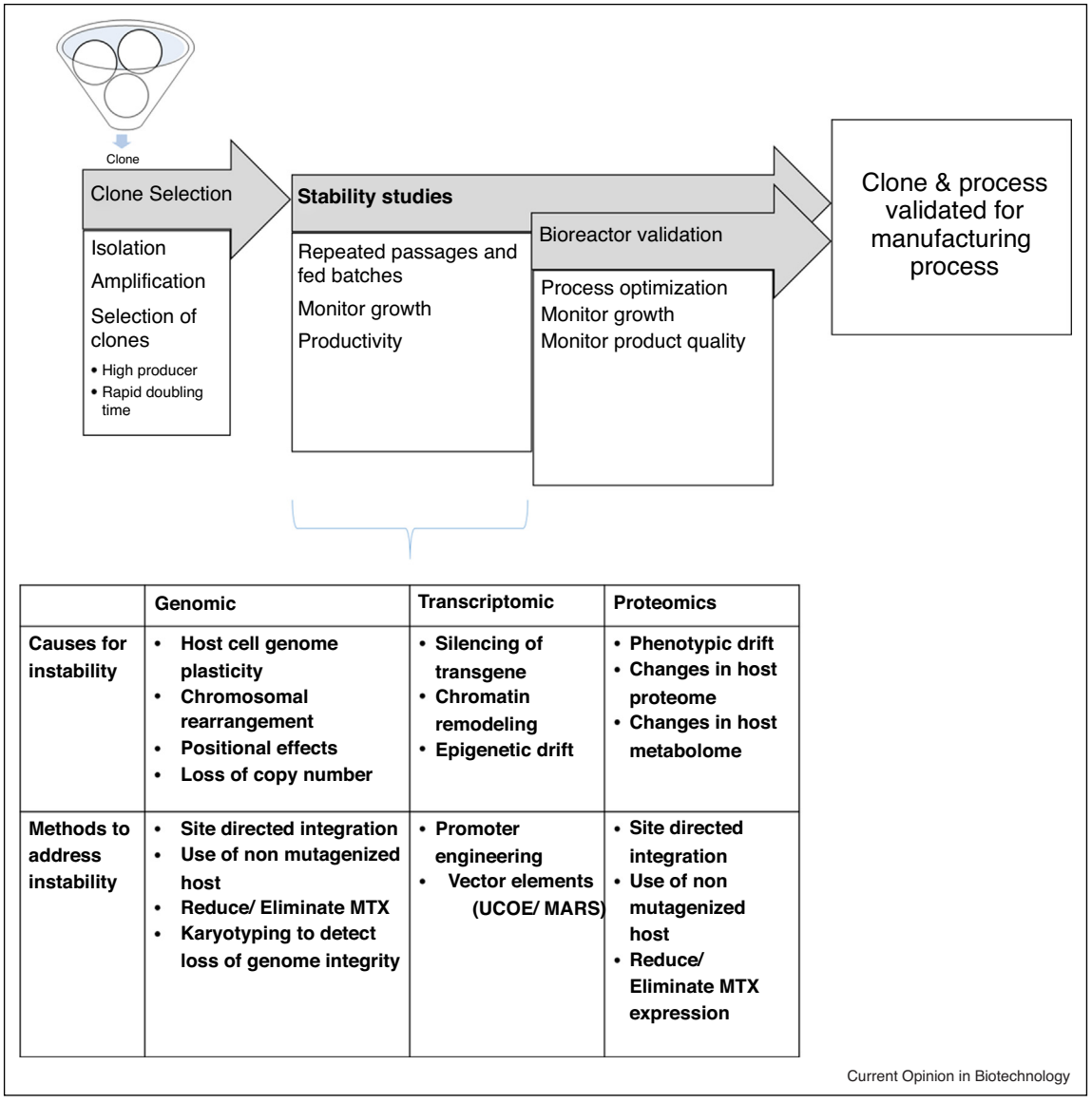


Growth of biological therapeutics. **(a)** The number of new biological license approvals granted since 2002. **(b)** Classes of biotherapeutics approved since 2002. **(c)** Total number of new approvals for biosimilar mAbs versus other biosimilar therapeutics.

folding, proteases and molecular chaperones. Additionally, biopharmaceutical manufacturing processes are susceptible to perturbations in temperature, pH, osmolality, and oxygen transfer. These changes can perturb dynamically the transcriptome [18]. Therefore, local transgene and global host cell genome deviations can inadvertently lead to decreases in process yield, impact timelines, increase costs, change protein quality, and cause regulatory compliance issues [10]. It has also been observed that properties of selected cells are frequently lost over time and properties within clones derived from the same cell population may vary significantly [19,20]. Overall, there has been a consensus among many labs that CHO cells display heterogeneity in growth and phenotype properties accompanied by genomic instability over time. Therefore, stability in a production process cannot be guaranteed over time [14,21].

In this review, we expand on the current definitions of cell line instability to include product quality instability (Figure 2). A list of all known causes of CHO cell instability from literature will be enumerated and current efforts to overcome instability by these mechanisms will be discussed. Finally, we discuss the need for new tools to increase our understanding of CHO cell instability. Continuous implementation of innovation and systems-based approaches to identify, monitor, and engineer solutions to eliminate instability altogether are

Figure 2



	Genomic	Transcriptomic	Proteomics
Causes for instability	• **Host cell genome plasticity** • **Chromosomal rearrangement** • **Positional effects** • **Loss of copy number**	• **Silencing of transgene** • **Chromatin remodeling** • **Epigenetic drift**	• **Phenotypic drift** • **Changes in host proteome** • **Changes in host metabolome**
Methods to address instability	• **Site directed integration** • **Use of non mutagenized host** • **Reduce/ Eliminate MTX** • **Karyotyping to detect loss of genome integrity**	• **Promoter engineering** • **Vector elements (UCOE/ MARS)**	• **Site directed integration** • **Use of non mutagenized host** • **Reduce/ Eliminate MTX expression**

Known causes of CHO instability and methods to alleviate product instability.

among the current needs in the field of CHO cell line development.

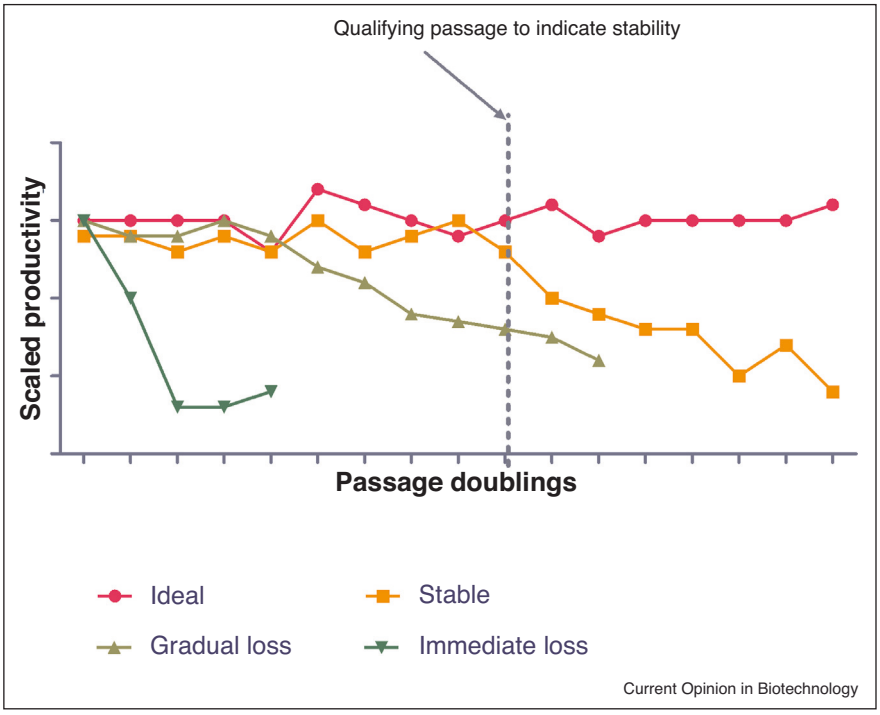
Definitions for cell line stability

Every industrial manufacturing process to generate product must follow strict criteria to ensure reproducibility, compliance and quality. Biotherapeutic production processes are unique as they utilize living cells in culture. Even though the engineering controls have been well established, for biological systems there are some expected deviations from replicates. In a robust, well-monitored process, a stable cell line can be defined as a homogenous cell population that has the following attributes:

1 Retention of 70% or more of volumetric productivity titer over 70 generations (60 generations is typically the period required to scale up from master cell bank to production volume).
2 Demonstrate 'no clinically meaningful differences' (FDA Orange Book https://www.fda.gov/drugs/informationondrugs/ucm129662.htm last accessed 28 September, 2018) from reference product as estimated by structure, function, purity, chemical identity and bioactivity.

Productivity profiles for long-term stability studies fall in the following categories (Figure 3):

Figure 3



Hypothetical examples of the types of production instability observed in a clonal generation platform.

- Ideal: cells show less than 15% deviation (within limits of measurement) in production titers at the end of 100 doubling cycles.
- Stable: cells may exhibit deviations in measurements within the tolerance of the assay for more than 60 doublings.
- Gradual loss: cells may show measurable and gradual reduction over many passages in fewer than 60 doublings.
- Immediate loss: cells may show abrupt and sudden decline in productivity within a few passages.

In a typical cell line selection protocol, transfected cells are cloned via limiting dilution seeding. The final selected clone needs to have high titer as well as maintain consistent and comparable product quality. In a well-defined production process, it is the cell line that has the most significant impact on quality attributes. Indeed, glycosylation, protease expression, ammonia, and lactate levels in culture are clonal attributes that significantly impact the product quality [22]. It is necessary to monitor product quality via high-throughput assays for attributes such as charge distribution analysis and molecular weight of the selected top clones to ensure a selection of desirable host cell properties and optimal and consistent product quality.

It is estimated that cell line **product titer** instability occurs in 8–63% of all recombinant CHO cell lines [23]. Early production instability may be attributed to loss of transgene expression due to epigenetic mechanisms and gradual loss of instability may be linked to non-producing subpopulations [24]. However, instability is ***independent*** of the choice of CHO host (DG44 versus CHO-K1), amplification procedure (glutamine synthase, GS, versus dihydrofolate reductase, DHFR, selection) and occurs in the presence and absence of selection pressure (e.g. methotrexate, MTX and methionine sulfoximine (MSX)); making this an extremely widespread phenomenon in current cell line development protocols. Currently, there are insufficient data on the influence of any of these parameters on **product quality** instability.

Mechanistic causes for the CHO cell instability phenotype

Although there have been reports of instability issues in various cell lines and products [25], loss of final titer and/or deviations from set quality parameters, are inherent and unpredictable. While the causes of CHO instability are not fully elucidated, some of the better understood molecular mechanisms leading to instability are reviewed here (Table 1).

Table 1

CHO cell instability causes and potential alleviation

Causes of CHO cell instability	Methods to overcome	Reference
Host cell propensity to chromosomal rearrangements	Employ non mutagenized hosts like CHOZN®	[41]
	Zinc finger nuclease (ZFN)-Modified CHO Cell Lines	
Loss of transgene	Site-specific integration	[50]
	Change in selection strategies	[24]
Loss of transcriptional activity	Promoter engineering	[44,45•]
	Vector elements to promote euchromatin state	[43•]
Phenotypic/genotypic drift of cells in culture	Karyotyping cells to monitor genome integrity	[47••]
	Transcriptome and proteome characterization to counter product quality impedance	[49••]

Inherent instability of the CHO genome

The underlying reason for instability is due, in part, to the genomic plasticity of CHO cells. CHO-ori cells were immortalized and established in the Puck lab [26] and faster growing cell populations were isolated. These cells consisted of 11 pairs of chromosomes. Thereafter many random mutagenesis and chemical treatments were employed to generate the CHO DG44 and CHO K1 hosts [7••]. Hundreds of such subcultures may have occurred before a CHO host was employed for biomanufacturing (Figure 4 provides a brief history of CHO cells). In addition to the chromosomal anomaly, DNA template mutations due to transcriptional errors have been observed at a higher background rate in CHO cells in long-term culture [27]. These observations indicate that the cell line may have a compromised DNA repair and transcription machinery. Sequencing has also revealed that the CHO chromosomes show a high occurrence of haploidy [28]. Because of this genomic instability, clones may appear with all the preferred qualities for manufacturing biotherapeutics, but these clones may exhibit genotypic and phenotypic drift that can cause changes to the product titer and quality. Cell line instability in CHO host cells has not been well understood compared to that in production cell lines. Since production cell lines are generated through transfection of host cells with transgenes, followed by isolation of clones (or pools) that stably express transgenes, the genomic/chromosomal instability of host cells is likely to be passed on to production cell lines.

Genomic influences on loss of productivity

Transfection of high copy number plasmids can result in recombinant DNA integration with the host genome. The transgene integration site is known to impact the expression of the transgene. For example, in high producer cells, gene inserts are frequently observed in large chromosomes [29], whereas in low producing or unstable cells, gene integration occurs more frequently at known fragile sites (which are sites along the genome especially susceptible to DNA breakage) [21].

The selection system used to generate clones can also influence the stability of transgene expression. The most commonly used selection systems are MTX-based selection for the CHO-DG44 host and the MSX-based system for the CHO-GS −/− host. MTX-based selection methods work on the principle of inhibiting the *dhfr* gene. During MTX treatment, the DNA synthesis pathway is inhibited, and CHO cells are susceptible to severe genetic rearrangement [14].

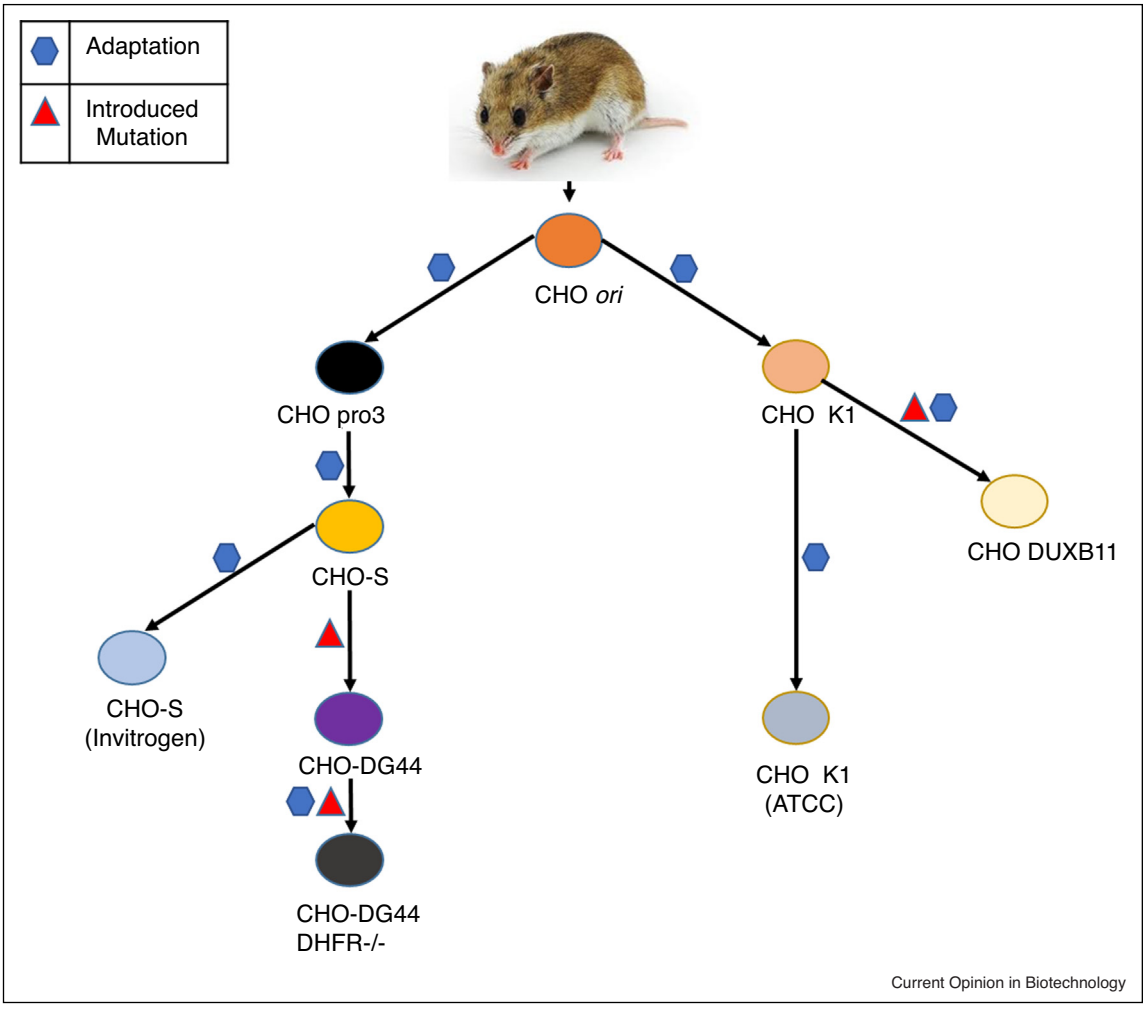
Transgene location and copy number also influence the expression of the recombinant gene. In MTX-mediated amplification, transgene integration is random and may result in many copies both locally and distally. In cases of multi-copy inserts and significant gene amplification, clones tend to lose these copies (and subsequently productivity) [12]. Indeed it is a large metabolic burden for cells to continuously express high levels of recombinant protein. A commonly observed mechanism by which cells break this metabolic commitment is through spontaneous elimination of the transgene from the host genome [30,31].

CHO cells can also lose copy number in the absence of selective pressure. Such a spontaneous loss of transgene presumably occurs as a result of DNA rearrangement [8]. Decreases in productivity may also occur in less-amplified systems such as (MSX)-selection where there have been reports of gene expression loss due to increased susceptibility to apoptosis in the cell clones [24].

Transcriptional silencing

Transcription is influenced by various factors such as DNA methylation, nucleosome positioning, histone modifications and variants, transcriptional complex binding, and by influence of non-coding RNAs. These factors can function independently, or in combination, to affect recombinant expression. Additionally, transcription-level modulation can be independent of genomic factors that reduce transgene copy number as there have been reports of loss of productivity in long-term culture without loss of transgene copies [29]. Typically, strong viral promoters

Figure 4



A simplified representation of historical CHO cell line development indicating various steps of adaptation and mutation performed to generate the widely used industrial hosts.

are used to drive expression of recombinant proteins; however, they contain CG-rich regions which are susceptible to silencing via methylation. Interestingly, it has been reported by Ho *et al.* that the use of CpG-free promoters reduced early transgene silencing but did not improve long-term stability [11•]. Indeed, histone modification patterns in producer cell lines have been known to continuously undergo modifications leading to gene silencing of the transgene as well as cellular proteins [10,17••,30]. In addition, a study of antibody-producing cells during long-term cultures revealed a reduction in global histone acetylation [13], indicating that production stability and product quality may be influenced by the epigenome.

Genomic/phenotypic drift

The time from the delivery of the recombinant DNA into the host cell nucleus for chromosomal integration, to clone selection, to commercial manufacturing can take many months. During this process, high-producing cell lines can manifest heterogeneous expression patterns or lose expression of the recombinant protein. It has been reported that after clone selection, the genomic homogeneity of clones that are derived from selected pools is not substantially different from selected clones. However, phenotypic drift has been observed in cell line development processes [17••]. Several reports indicate changes of the transcriptome occur during the changing nutrient and metabolite concentrations encountered by cells during batch or fed-batch culture. Up to 1400 mRNAs are differentially regulated in a culture during batch operations and during production cycles in response to the varying culture environment [31–33]. Other studies by Li *et al.* have demonstrated that changes in gene expression occur more frequently in long term cultures of unstable populations. It is interesting to note that the instability in

these studies was not due to transgene copy loss or localized epigenetic regulation of transcription but rather due to differential regulation of distal genes involved in RNA transport, mRNA translation and stability, and cell cycle regulation causing an overall change in the clonal phenotype [16••]. In addition, production instability has also been reported to occur due to increased susceptibility of cells to apoptosis following multiple passages due to overexpression of annexin V and caspase 3 beyond 50 passages [24]. It has been demonstrated that CHO cells show a loss of volumetric productivity due to overexpression of stress-related markers and changes in cellular metabolism after subculturing over many passages [10].

It is worth noting that protein folding and cell stress pathways frequently overlap. For a recombinant cell line producing multi-gram per liter titers, an increase in stress markers in cultures may indicate some level of product misfolding and aggregation [34]. Increased apoptosis leads to the release of proteolytic host cell proteins (HCP) into the culture medium. HCPs increase the risk of inactive, or even harmful, fragments and aggregates [35]. During extended production cycles, HCPs released from dead cells and secreted from viable cells accumulate extracellularly at a much higher level than they do in batch culture, thereby potentially impacting product quality. In particular, proteases and glycosidases that accumulate in culture medium negatively affect the quality of the expressed target [36••]. In addition to the global changes in CHO cells during their production life, it has been reported that specific pathways, such as glycosylation, may be silenced during long-term culture. For the *N*-glycosylation pathway, 21 out of 24 genes examined showed significant differential regulation over the course of a fed-batch culture and led to less sialylation of the recombinant IFN-γ [37].

It is well established that CHO cell lines show chromosomal rearrangements frequently during adaptation/selection and even during standard cultures. Because of the large number of single nucleotide variations (SNV) and point mutations that may occur in a typical cell line development process, the underlying causes and alleviations of subclonal variations as well as phenotypic drift-induced product titer and quality instability remains a challenge.

Strategies to overcome CHO cell line instability

Traditionally, cell line development protocols have relied on random integration of a transgene into the genome. Selection/amplification may lead to localized amplification or amplification of the transgene in distal spots. Unfortunately, the lack of control in this method may lead to unwanted positional effects. Further, the multiple copies created may be subjected to deletion or silencing, resulting in titer instability. In addition, the CHO cells' propensity for genomic rearrangements, epigenetic changes, transcriptional silencing, and proteomic changes can impact cell line stability. To overcome these issues, organizations select and test hundreds of clones to identify stable clones. Below are some successful strategies that have been employed to address cell line stability.

Overcoming genomic hindrances to CHO cell line stability

To circumvent random gene insertion, different approaches for site-directed integration of transgenes using site-specific nucleases are now available. Multiple groups have reported low levels of off-target impact, homogeneous expression, and sustained stable expression over many passages [38•,39•]. Now, with the availability of a detailed CHO genome [40•], it is possible to customize the site-specific integration into CHO cells with greater precision.

Given the mutagenic history of CHO cells and the prevalence of chromosomal haploidy in the prominent CHO-DG44 and CHO-K1 hosts, industrial platforms are moving towards the use of a CHO-GS knockout cell host. This cell line was generated by zinc finger nuclease gene editing methods and not by chemical mutagenesis. In addition, this cell line does not require multistep amplification and, therefore, it is subject to reduced transgene deletion and silencing in downstream cell selection methods [41].

In the future, there is value in the prediction of cell line stability. If any instability is a result of genomic rearrangement, such changes can be measured by karyotyping of the cells and may serve as markers and indicators for production instability [32].

Overcoming transcriptional barriers to CHO cell line stability

Loss of productivity due to transgene silencing has been widely reported [12] and there are efforts to increase the expression of the transgene. For example, various regulatory elements have been incorporated into expression vectors to enhance recombinant protein expression. Matrix attachment regions (MARs) are genomic DNA sequences that serve as attachment points within the DNA to anchor chromatin to the nuclear matrix during interphase. MARs have been shown to increase transgene expression levels as well as the proportion of positive colonies in CHO cell expression systems [42]. An increase in the number of successful transfectants, with a greater than 12-fold productivity increase, and higher retention of expression over cultures, has been reported by using MARS elements conjugated with strong vectors [43•]. A number of other chromosomal elements such as Locus Control Regions (LCRs) and Ubiquitous Chromatin Opening Elements (UCOEs) are utilized in recombinant protein production with improvements both in sustained transgene

transcription and stable production in fed-batch cultures [44]. In addition to vector engineering, the use of synthetic promoters, CpG free promoters, and cytomegalovirus (CMV) mutants have resulted in clones with a reduced propensity for transgene silencing [44,45•]. In addition to increasing productivity from the transgene, vector elements have also resulted in sustained expression of recombinant protein over several passages [44].

Overcoming phenotypic drift-induced barriers to CHO cell line stability

Some of the major reasons for the industrial prevalence of CHO cells are their phenotypic adaptability and plasticity. Indeed, CHO cells can be selected to achieve vastly different phenotypes such as resistance to virus infection and expression of a desired glycoform [46]. Despite these successes, constant phenotypic drift is an inherent problem. In a study by Baik and Lee, CHO-DUK cells and their subclones were investigated for chromosomal rearrangements. The results demonstrated that cell clones exhibit a karyotypically heterogeneous population in host cells that were under no metabolic burden or other types of selection pressures. This observation suggests that chromosomal rearrangements happen even during routine subculturing [47••]. Because instability is a result of multiple changes to cellular activities, systems biology approaches will be helpful to better understand and overcome stability problems. The availability of a significantly improved Chinese hamster reference genome [40•] will aid genome engineering efforts. Moreover, efforts have been made to determine optimal integration sites [38•,39•] leading to an increased likelihood of generating cell populations with consistent protein production. Similarly, host cell engineering efforts to remove proteases that impact product quality are aided with the improved genome. In a recent study, new information from an improved genome assembly was used to identify targets with similar predicted functions and characteristics of a difficult-to-remove host cell protein [48•]. Although there may be some benefits, it is unlikely that the community will move away from the use of CHO cells in the near future. Therefore, understanding the biological mechanisms for instability, identifying biomarkers, and incorporating bioinformatics-based genome mining at early stages of cell line development to identify stable clones are among current needs. As one early example, studies have revealed the importance of mitochondrial function in maintaining cellular biosynthetic capacity of high producing cells [49••]. The identification of these genes and pathways can tentatively serve as markers for identifying traits of stable producers in long-term cultures.

Discussion

Currently, CHO cells have reached a reasonably high productivity and the bottleneck for process development often lies with downstream processing steps. With the first wave of biotherapeutics coming off patent, biosimilar production will require more consistent product titer stability for both managing the costs of manufacturing as well as product quality stability. As new biomolecules such as viral antigens, vaccines, and bi-specific and tri-specific (monoclonal antibodies with multiple unique binding sites) enter the production pipeline, product quality maintenance may gain priority over further increases in productivity. Moreover, the total cost-of-goods analysis in the manufacturing cycle lists cell culture processes as having a low influence on total costs [4]. Therefore, there is a justification to incorporate screening steps in the cell line development process for selecting cell lines that deliver a stable and consistent product via identification of markers for stability without any impact on the cost of deliverables. We also believe that greater efforts to understand the problem of production instability will be valuable in understanding the all the routes leading to instability. Loss of recombinant transgene copies, transgene silencing and overall phenotypic drift are only a subset of relevant factors associated with this complex phenomenon. The possibility of predicting cell-line stability, and ultimately designing cell line engineering strategies to prevent it, is very exciting and may be necessary steps as the field moves towards integrated continuous bioprocessing methods.

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