Biotechnology Journal

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Growth Rate Changes in CHO Host Cells Are Associated with Karyotypic Heterogeneity

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Chinese hamster ovary (CHO) cell line instability and clonality issues can affect cell culture phenotypes such as cell growth, productivity, or product quality and remain challenges for biopharmaceutical manufacturing. While there have been efforts for characterizing cell line instability in CHO production cell lines, a pre-existing level of cell line instability in CHO host cells has not been determined. In this study, cell line instability and chromosomal heterogeneity of the host, CHO-DUK cell line, is reported by using a karyotyping approach. Long-term cultures and karyotype analysis of CHO-DUK cells revealed that the growth rate was higher in later passage cultures, correlating with an increase in the population ratio containing the mar3 chromosome. To further investigate a correlation between growth rate and karyotype, CHO-DUK cells are subcloned by limiting dilution and the growth rate and karyotype of each subclone are determined. Subclones containing the mar3 chromosome exhibit higher cell growth rates than subclones without the mar3 chromosome. Finally, karyotype analysis indicate that CHO-DUK cells, as well as limiting-diluted subclones, exhibit a karyotypically heterogeneous population, suggesting that chromosomal rearrangements occur spontaneously and frequently even in non-engineered host cells. These results demonstrate CHO host cell line instability and suggest that chromosomal instability and karyotypic changes are associated with compromised clonality (heterogeneity), affecting cell line (in)stability in CHO host cells.

1. Introduction

Chinese hamster ovary (CHO) cells have been widely used for biopharmaceutical manufacturing. While many efforts have been made to achieve improved productivity and product quality of recombinant proteins produced in CHO cells,[1–3] a number of studies have described cell line instability, such as a decrease in productivity, reduced culture longevity, or varied host cell protein expression during extended cell cultures.[4–6] Cell line instability results in variability in the biomanufacturing process that can affect cost or regulatory compliance, yet the causes and mechanisms of cell line instability remain elusive. Given the hypothesis that cell line instability is associated with chromosomal rearrangements, we previously developed and applied a karyotype-based framework to quantify chromosomal rearrangements in secreted alkaline phosphatase (SEAP)-producing CHO cells that exhibited production instability.[4] Karyotyping and fluorescence in situ hybridization analysis demonstrated that the SEAP production instability was associated with a particular chromosomal rearrangement, suggesting a mutation-and-selection mechanism.[4,7] It has also recently been shown that a substantial number of genomic rearrangements occur after transgene integration.[8]

Cell line instability in CHO host cells has not been well understood compared to that in production cell lines. As production cell lines are generated through transfection of host cells with transgenes followed by isolation of clones (or pools) that stably express the transgenes, the genomic/chromosomal instability of host cells are likely to be passed on to production cell lines. Moreover, despite the importance and expectation of clonality assurance as part of regulatory filings, the pre-existing level of genetic heterogeneity (clonality) in host cell lines has not been studied.[9] In this study, we examined cell line instability of CHO-DUK cells (also known as DXB11 and DUKX), a commercially available CHO host cell line, through a long-term culture. We then applied a karyotype-based framework to evaluate the correlation between cell line instability and karyotypic changes. Finally, we report heterogeneous karyotypic populations of CHO-DUK cells and subclones that have been originated from single cells.

2. Experimental Section

2.1. Cell Line Maintenance and Long-Term Culture

CHO-DUK cells (ATCC, Manassas, VA) were adherently maintained in T-25 culture flasks (Corning Inc., Corning, NY) containing 5 ml Iscove's Modified Dulbecco's Medium (Hyclone Laboratories Inc., Logan, UT) supplemented with 10% dialyzed fetal bovine serum (Gibco, Grand Island, NY) and 1% hypoxanthine/thymidine solution (Gibco) and incubated at

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DOI: 10.1002/biot.201700230

37 °C and 5% CO_2. To examine cell line instability, CHO-DUK cells were subcultured for 75 days (25 passages) in the growth media described above.

2.2. Limiting Dilution and Batch Culture

Thirty six subclones were isolated by one round of limiting dilution cloning. CHO-DUK cells at passage 5 (P5) were plated out into 96-well plates at 0.2–1.0 cells per well in the growth media. The clonality was determined by visual inspection of single cell-derived colony formation using an EVOS XL microscope (Life Technologies, Carlsbad, CA). At 40–60% confluency, clonal cells were sequentially transferred to 24-well plates (Corning Inc.), 6-well plates (Corning Inc.), and T-25 culture flasks. For batch culture experiments, exponentially growing parental CHO-DUK cells (at P5, P15, and P25) and CHO-DUK subclones were seeded at 5×10^4 cells/ml in 6-well plates containing 3 ml growth media. Biological duplicates were sacrificed daily and viable cell density was determined using a hemocytometer and the Trypan blue (Gibco) exclusion method.

2.3. Karyotype Analysis

The karyotype analysis method was developed in our laboratory previously.[4] Briefly, parental CHO-DUK cells (at P5, P15, and P25) and CHO-DUK subclones in the exponential growth phase were exposed to colchicine (Sigma–Aldrich, St. Louis, MO), harvested, and spread onto glass slides. The cell spreads were trypsinized, washed, and then stained with Giemsa solution (Gibco). The chromosomes of metaphase cells imaged using a 100× oil immersion objective on an EVOS XL microscope were sorted and aligned using Adobe Photoshop software (CS5, Adobe Systems Incorporated, San Jose, CA) to create karyotypes and to classify chromosomal variants and rearrangements.

3. Results and Discussion

3.1. Increase in the Specific Growth Rate of Late Passages Is Associated with Karyotypic Changes

CHO-DUK cells were subcultured for 25 passages and batch cultures at the early (P5), mid (P15), and late (P25) passages were used to generate growth profiles. Because non-producing host cells were used, cell growth data are the most relevant measurable culture attributes. The viable cell densities of eight biological replicates in the exponential growth phase (days 1–4) per condition were used to calculate specific growth rate. The P25 cells exhibited a higher specific growth rate, maximum viable cell density, and integrated viable cell density by 18%, 30%, and 24% when compared to the P5 cells, respectively (**Figure 1**), demonstrating that CHO-DUK cells display unexpected phenotypic changes at late passages. Moreover, the higher growth rate of CHO-DUK cells in the late passage is consistent with a previous observation in CHO-SEAP cells[4]; when the only selection pressure is the growth rate (no selective drug), faster growing cells are more likely to be selected and enriched in late passages.

To examine if these same features are associated with a particular karyotype, the karyotypes of P5, P15, and P25 cells were compared. Fifty G-banded metaphase images per passage were analyzed by using a karyotype comparison framework previously developed in our laboratory[4] and the results are summarized in Figures 2 and S1, Supporting Information. It was observed that the ratios of the mar2 and mar3 chromosomes substantially changed over time. The ratio of mar2-containing cells decreased and the ratio of mar3-containing cells increased, implying that the presence of mar3 or the absence of mar2 might give the CHO-DUK cells a growth advantage.

3.2. mar3-Containing Subclones Exhibit Higher Specific Growth Rates

To verify whether the increase in the growth rate was a result of a specific karyotype, single clones of the P5 CHO-DUK cells were isolated by limiting dilution. Thirty-six subclones were established and subjected to batch cultures to determine specific growth rates as well as to determine karyotypes (10–27 metaphase cells per clone). Five subclones (15, 17, 22, 28, and 35) contained tetraploid chromosomes (≈40 chromosomes per cell) and were excluded from further analysis to rule out any potential effects of polyploidy on growth rate. The remaining subclones were classified into three groups based on which chromosomes each subclone contained – seven subclones contained only the mar2 chromosome (mar2 only), twenty subclones contained only the mar3 chromosome (mar3 only), and four subclones contained neither the mar2 nor mar3 chromosome (none) (Table S1, Supporting Information). A growth rate comparison revealed that the "mar3 only" group had subclones with higher growth rates, suggesting that this specific karyotype may be associated with increased growth rate (**Figure 3**). Isolation of a greater number of "mar3 only" subclones compared to the other two groups ("mar2 only" and "none") also implies that mar3-containing subclones with a growth advantage had a higher chance to be selected during the cloning steps. While a Student's *t*-test (2-tailed with unequal variances) indicates a marginal significance between the "mar2 only" and "mar3 only" groups ($p = 0.111$, possibly due to smaller sample number in the "mar2 only" group), it is noteworthy that all of the top 20% fastest growing subclones contain only the mar3 chromosome. Therefore, a possible scenario is that, as faster growing cells take over the population in a mixed cell culture, "mar3 only" cells became dominant in the CHO-DUK cultures at P15 and P25. While studies are needed to confirm a direct relationship between the growth rate and either the presence of mar3 or the absence of mar2, there have been other observations that a particular karyotype (or chromosomal rearrangement) can affect a cell's phenotype. For example, chromosomal translocation or inversion can lead to chimeric genes or gene disruption; and duplication or deletion of a chromosome can affect gene copy number, which is known to interrupt endogenous gene expression.[10] Indeed, Ritter et al. reported that the deletion of a telomeric region of chromosome 8 in CHO cells was associated with high productivity and stability.[11] Moreover, even structural variations within intergenic regions of the genome can affect an organism's phenotype

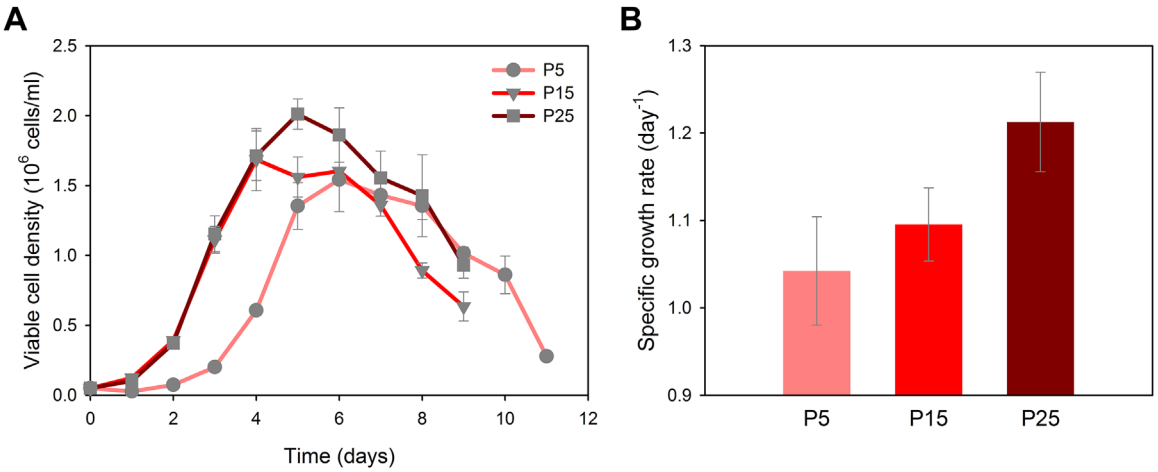

Figure 1. A) Viable cell density and (B) growth rate of CHO-DUK cells at P5, P15, and P25. The growth rate was calculated using the viable cell density in the exponential growth phase (1–4 days). Error bars represent the standard deviation of biological replicates.

during development.[12] Still, given the various growth rates within the same group clones (Figure 3), a karyotype change does not appear to be the sole factor that determines the growth rate.

3.3. CHO-DUK Cells and Limiting-Diluted Subclones Are Karyotypically Heterogeneous

Another observation from the karyotype analysis is that the CHO-DUK cells, at all passages as well as the majority of the limiting-diluted subclones, were karyotypically heterogeneous (Figure S1 and Table S1, Supporting Information). The clonality of CHO-DUK cells is unknown because the history of the cells, such as passage number before cell banking and whether they were re-cloned, is unavailable. Moreover, karyotypic and functional heterogeneity of other CHO host cells, presumably due to genomic instability, has been reported.[13–15] Therefore, the karyotypic heterogeneity of CHO-DUK cells is not surprising. However, considering the relatively short time (19–27 days) required to isolate and expand the single clones, the heterogeneous karyotypes within the subclones suggest a clonality issue for the cell line development process; even if a clonal cell line is developed from an individual cell, it appears to become heterogeneous quickly relative to the time scale of cell line development processes (i.e., CHO-Quasispecies).[16] The limiting dilution cloning process itself may exacerbate chromosomal instability as it can create a stressful environment to single cells,[17] resulting in a heterogeneous population.[15]

Finally, we observed that 10–15% of CHO-DUK cells are polyploid and were able to isolate five tetraploid subclones. In general, tetraploid cells can be generated by various mechanisms, including cytokinesis failure, mitotic slippage, and DNA replication or repair defects.[18] Once generated, polyploid cells are usually subject to cell-cycle arrest, followed by apoptosis.[18] However, the tetraploid subclones isolated in this study suggest that the tetraploid checkpoint, signal transduction, or apoptosis pathway may be impaired in these CHO cells, thereby providing opportunities for the polyploid cells to survive and to form cell lines.

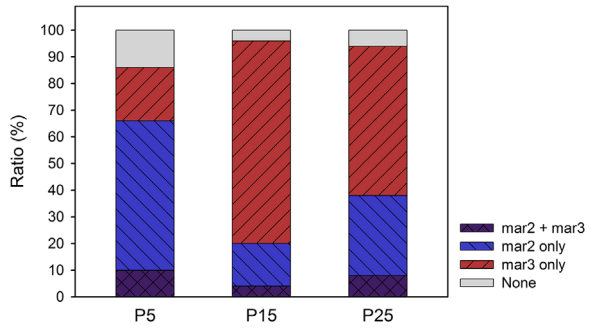

Figure 2. Ratio of the CHO-DUK metaphase population containing mar2 and/or mar3 chromosomes. Fifty metaphase cells per sample were used to calculate the ratios.

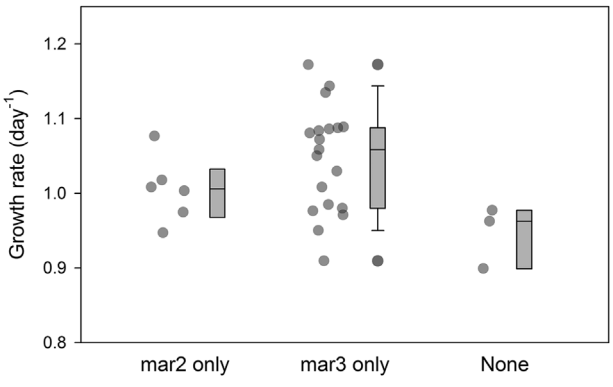

Figure 3. Growth rate of CHO-DUK subclones in the exponential growth phase. The growth rates of each subclone are shown in the dot and box plots. Median, upper and lower quartiles, and upper and lower 95% ranges are presented in the box plot.

4. Conclusions

Cell line instability and clonality assurance are increasingly recognized as a challenge in the biomanufacturing of therapeutic proteins in CHO cells. In this study, host cell line instability, and an increase in growth rates and maximum cell densities, was observed over long-term cultures of CHO-DUK cells. A karyotype-based framework was employed to identify karyotypic changes that may be associated with the cell line instability. Moreover, karyotype analysis of CHO-DUK subclones revealed that these subclones rapidly lost chromosomal clonality, resulting in karyotypic heterogeneity. These results suggest that inherent, spontaneous genomic changes occur regardless of genetic manipulation or amplification in CHO cells, potentially affecting both cell line (in)stability and clonality.

Abbreviations

CHO, Chinese hamster ovary; SEAP, secreted alkaline phosphatase.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

We are grateful for financial support from the National Science Foundation (1412365, 1539359, 1624698, and 1736123).

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

bioprocess engineering, cell line stability, CHO cells, clonality, continuous manufacturing, regulatory affairs

Received: May 17, 2017
Revised: August 26, 2017
Published online: September 22, 2017

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