Mohamed Al-Rubeai
Editor

CELL ENGINEERING 6

Cell Line Development

Springer

Cell Line Development

Cell Engineering

Volume 6

Series Editor

Professor Mohamed Al-Rubeai
UCD School of Chemical and Bioprocess Engineering
University College Dublin
Dublin, Ireland

Editorial Board

Dr Hansjorg Hauser
Helmholtz Centre for Infection Research
Braunschweig
Germany

Professor Michael Betenbaugh
Johns Hopkins University
Baltimore, USA

Professor Martin Fussenegger
Swiss Federal Institute of Technology
Zurich, Switzerland

Professor. Nigel Jenkins
National Institute for Bioprocessing Research and Training
Dublin, Ireland

Dr Otto-Wilhelm Merten
A.F.M.-Genethon 11
Gene Therapy Program
Evry, France

For other titles published in this series, go to
www.springer.com/series/5728

CELL ENGINEERING

Vol. 6: Cell Line Development

Edited by

Mohamed Al-Rubeai

University College Dublin, Ireland

Springer

Editor
Prof. Dr. Mohamed Al-Rubeai
School of Chemical and Bioprocess Engineering
University College Dublin
Belfield, Dublin 4
Ireland

ISBN 978-90-481-2244-8 e-ISBN 978-90-481-2245-5
DOI 10.1007/978-90-481-2245-5
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009929356

© Springer Science+Business Media B.V. 2009
No part of this work may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission from the Publisher, with the exception of any material supplied specifically for the purpose of being entered and executed on a computer system, for exclusive use by the purchaser of the work.

Printed on acid-free paper

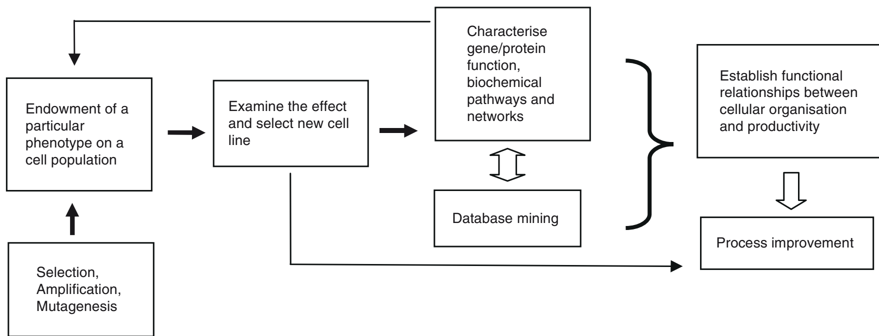
Springer is part of Springer Science+Business Media (www.springer.com)

Cell Engineering – Overview

The development of cell lines has undergone several advances over the years, essentially to meet the requirement to cut the time and costs associated with using such complex hosts as production platforms. This book reviews the aspects involved in the development of cell lines and the cell engineering approach that can be employed to enhance productivity, improve cellular metabolism, control proliferation and apoptosis, and reduce instability.

Cell engineering is a new research approach which began in the early 1990s, coinciding with an increasing interest in apoptosis. This approach of manipulation of apoptotic regulatory functions in cells was highlighted in an article appearing in 1995 in "Trends in Biotechnology", in which we suggested that through the manipulation of cell lines by transfecting them with anti-apoptotic genes, one may be able to enhance the robustness and survival of those cells in culture. Since then several papers have been published which demonstrate the effectiveness of this approach; this has since evolved to embrace methodologies offered by molecular biology for the development of cell lines, which could provide platforms to improve recombinant protein production and the efficiency of industrial culture processes. The underlying principles of cell engineering are very simple, essentially involving the identification of a gene of interest, expression of it into a cell, or knocking down its function and development of a new cell line. Today, cell engineering covers several topics, including expression engineering, and involves the use of technologies such as functional genomics, proteomics and metabolomics which have become an integral part of cell engineering. Its scope has also become broader but the underlying principle remains the same. It involves various strategies of adding and deleting genetic elements such as single and multiple gene insertion, gene disruption (genetic deletions), gene silencing, mutagenesis and directed evolution. The figure below is a diagrammatic representation, which is useful in explaining the interaction between cell engineering and "omics" technologies.

It is undeniable that integrating genomics and proteomics data with vast amounts of bioprocess data will improve significantly the analysis of the biological pathways involved, with a consequent discovery of lead genes that may serve as candidates for cell engineering. Ultimately, new cell engineering strategies should provide greater insight into regulatory networks within cells in a bioprocess environment, thus greatly advancing our understanding of cellular mechanics in conjunction with



improvements in overall productivity in the manufacturing environment; this will lead ultimately to efficient and safe processing of protein products.

Cellular engineering approaches can be used to integrate bioprocess improvements like high cell density cultures, operational strategies like fed-batch and perfusion cultures and high productivity (Qp) at a cellular/molecular level. However, this approach has only met with limited success, as very little is known about the cellular dynamics related to productivity. At a cellular level, Qp has been suggested to be dependent on events downstream of transcription, thus modifying the translational and secretory pathways that would overcome limitations of protein folding and assembly reactions.

In the pursuit of increasing productivity, molecular analysis of recombinant protein production at different organisational levels within the cells (transcript, polypeptide, assembly and secretion), together with metabolite data, in effective combination with gene expression data, should piece together a more comprehensive picture about the adaptive state of productivity. The increase in productivity will not only require important transcription and mRNA stability, but also a simultaneous increase in the post-translational capacity of the cell, including N-glycan biosynthesis and secretory functions. Energy metabolism appears to become a limiting factor, with high AMP levels and high oxidative stress taking place in highly productive cells. These changes are likely to be controlled at the gene transcription level, suggesting that a cell engineering approach can alter cellular organisation to support substantial increases in yield.

In conclusion, “direct” cell engineering approaches of manipulating apoptosis, proliferation, metabolism, glycosylation and secretion have resulted in several current and potential improvements in cell lines of biopharmaceutical importance. With the advent of genomic and proteomic tools, “indirect” cell engineering is becoming a useful strategic approach for the improvement of biopharmaceutical cell lines. In combination with advances in expression engineering, clone selection and media development, it is hoped to decrease development times and dramatically increase cell line productivity, thus reducing overall costs for the next generation of approved products.

Mohamed Al-Rubeai
19 March 2009

Contents

Use of MAR Elements to Increase the Production of Recombinant Proteins 1
Cori Gorman, Salina Arope, Mélanie Grandjean, Pierre-Alain Girod, and Nicolas Mermod

Expression Engineering – The IE2 Promoter/Enhancer from Mouse CMV 33
Markus O. Imhof, Philippe Chatellard, Michel Kobr, Renata Pankiewicz, Valérie Duverger, Léonard Bagnoud, Christophe Sauvage, and Christine Mossu

Defeating Randomness – Targeted Integration as a Boost for Biotechnology 53
L. Gama-Norton, P. Riemer, U. Sandhu, K. Nehlsen, R. Schucht, H. Hauser, and D. Wirth

Importance of Genetic Environment for Recombinant Gene Expression 83
Alan J. Dickson

Expression Vector Engineering for Recombinant Protein Production 97
Helen Kim, John Laudemann, Jennitte Stevens, and Michelle Wu

Cell Xpress ™ Applications in Development and Characterization of Biopharmaceutical Recombinant Protein Producing Cell Lines 109
Jennifer R. Cresswell, Nan Lin, Genova A. Richardson, and Kevin J. Kayser

Selection Methods for High-Producing Mammalian Cell Lines 127
S.M. Browne and M. AL-Rubeai

Engineering Mammalian Cells for Recombinant Monoclonal Antibody Production 153
Sarah L. Davies and David C. James

Engineering Cell Function by RNA Interference 175
Joseph A. Gredell, Hemant K. Kini, and S. Patrick Walton

Apoptosis and Autophagy Cell Engineering 195
Chaya Mohan, Yeon-Gu Kim, and Gyun Min Lee

Glycoengineering and Modeling of Protein N-Glycosylation 217
Sandra V. Bennun, Frederick J. Krambeck, and Michael J. Betenbaugh

Engineering the Secretory Pathway in Mammalian Cells 233
Ren-Wang Peng and Martin Fussenegger

Index 249

Use of MAR Elements to Increase the Production of Recombinant Proteins

Cori Gorman, Salina Arope, Mélanie Grandjean, Pierre-Alain Girod, and Nicolas Mermod

Abstract The biopharmaceutical industry continues to face the challenge of producing large amount of recombinant proteins for use as therapeutics, and eighty percent of protein therapeutics in clinical development are produced in mammalian cell systems. Approaches to increase production addressing growth conditions, such as the improvement of media composition and process control, or transcription of the recombinant gene via the use of strong promoters/enhancers and amplification of gene copy number, have increased the yields obtained from mammalian cells considerably over the past decades. However these processes remain laborious, and extensive screening of clones is often required, as stable cell line and/or protein production is not always obtained. Unstable or variable expression is linked to the location of transgene integration site, the regulation of gene expression, the silencing of genes, and the loss of gene copies. Genetic elements that may remodel chromatin to maintain the transgene in an active configuration are now being employed increasingly to improve protein production using mammalian cells. Here we will review how one type of such elements, the MARs, may increase transgene integration into the cell genome and decrease silencing effects to reduce expression variability. We also illustrate how inclusion of these elements in expression vectors leads to increased specific productivities ranging from 20 to 100 picograms per cell and per day (p/c/d), resulting in protein titers above 5 g/l.

C. Gorman and P.-A. Girod
Selexis SA, 18 chemin des Aulx, 1228 Plan-les-Ouates, Switzerland

C. Gorman
DNA Gateway International, Inc. 55 New Montgomery St. Ste 605
San Francisco CA, 94105, USA

S. Arope, M. Grandjean, and N. Mermod (✉)
Laboratory of Molecular Biotechnology, University of Lausanne, 1015, Lausanne, Switzerland
e.mail: nicolas. mermod@unil.ch

M. Al-Rubeai (ed.), *Cell Line Development*, Cell Engineering 6,
DOI 10.1007/978-90-481-2245-5_1, © Springer Science+Business Media B.V. 2009

1 Introduction to Epigenetic Silencing Issues in the Generation of Production Cell Lines

During the past two decades, cultured mammalian cells have become the most widely used platform for producing recombinant therapeutic proteins. Improving yield and stability of protein expression are, therefore, of considerable value to the industry. Previously, such improvements have mainly originated from optimizing downstream production processes and media development. Despite recent advances in the field of cell line generation, expression levels in mammalian cells are relatively low and often unstable over time; these events result in high development and production costs for therapeutic proteins. The average reported yields in mammalian cells (usually 0.5–2 g/l) are several fold lower than the yields from bacteria and yeast systems, while even the highest reproducible yields, 5 g/l, remain at least three fold lower than obtained in more simple production systems (Kwaks and Otte, 2006).

Despite significant effort, the current schemes for cell line development remain, to a large extent, empirical. There is a considerable degree of variability, and our understanding of the sources of variability in the mammalian cell line development process remains limited. This process is laborious, and extensive screening of clones, often spanning over several months, is still widely practiced in industry. Obtaining cell lines that maintain stable protein production is of utmost importance, particularly for industrial use where the goal is to commercialize the protein being produced. Problems with stability can impact the time and effort required to generate working and master cell banks. The loss of productivity between the initial cell isolates and the end-of-production cells can compromise regulatory approval and, in the worst-case scenario, may result in rejection of a particular cell line after months of development efforts. Unfortunately, the method of selecting cell lines relies heavily on a degree of chance. In part, this high degree of variability is due to the effect that random transgene integration into host cell chromosome exerts on transgene transcription often resulting in silencing. Another cause of variability results from the integration of a varying number of transgene copies from one clone to the next. Finally, a last source of variability is variegation, a phenomenon that results in the cycling of cells between productive and non-productive phases, which again may affect differently distinct cell clones.

The insertion of genes into certain areas of chromatin can lead to the so-called "position effect" (Wilson et al., 1990). Silencing of transfected genes in mammalian cells is a fundamental problem that probably involves the relatively inaccessible status of the DNA when it is imbedded in chromatin. Transgene integration, when it occurs through a random process, can either occur in highly condensed, silenced region of the chromatin, heterochromatin, or in more open and active chromatin, euchromatin (Eissenberg, 1989; Eissenberg et al., 1992; Zahn-Zabal et al., 2001). Integration into heterochromatin may result in minimal or no transgene expression. Because a large proportion of the genome is in the form of heterochromatin, the chance that a transgene integrates in, or close to, heterochromatin, and consequently is silenced or repressed, is high.

Other regions of the chromosome may be subjected to slow silencing effects (Pankiewicz et al., 2005). Because this slow silencing may not be readily apparent,

it can lead to a gradual loss of productivity that suddenly appears after an initial phase of seemingly stable expression during clone isolation and characterization (see chapter by Alan Dickson in this book). A related process, known as position effect variegation (PEV), is thought to result from the stochastic spread or retreat of heterochromatin towards or away from the gene location (Eissenberg et al., 1992; Volfson et al., 2006). PEV typically leads to clones that possess heterogeneous levels of expression when comparing distinct cells in the monoclonal population. Often, this heterogeneous expression is not apparent when determining the titer of secreted proteins such immunoglobulins in the cell supernatant, but nevertheless may limit the yield. The combined effects of random transgene incorporation with these chromatin-mediated epigenetic effects collectively result in only a small percentage (less than 1%) of the initially isolated cells being capable of producing high amounts of the desired protein (Girod and Mermod, 2003). Therefore, lengthy selection and screening procedures are often required to select and identify those cells with the proper growth, transgene expression, long-term stability, and protein quality properties required for large-scale production.

For several years, Barnes et al. (2001, 2003, 2004, 2006, 2007; Barnes and Dickson, 2006) have examined the process of loss of protein production in mammalian cell lines. Despite repeated rounds of cloning, the cell lines they derived showed a wide variation in terms of maximum obtainable cell densities, rates of growth, and accumulation of secreted recombinant protein (Barnes et al., 2001). Several lines of data suggest that rapid phenotypic drift may be occurring during culture and, therefore, the cells derived from a single cell, as a result of cloning, soon diverge to become a mixed population. In this context, the term ‘stable cell lines’ refers to cell populations that retain stability of expression during prolonged culture (Barnes et al., 2001).

Though a variety of mammalian cells have been used for recombinant protein production, including mouse myeloma derived (NS0), human embryonic kidney (HEK-293), baby hamster kidney (BHK) and more recently, the human retina derived (PerC6) cells, the most commonly used host cell lines remain the CHO cells. The popularity of CHO based expression is largely due to the ability to use DNA amplification techniques in these cells to increase transgene copy number. However, both the DHFR and GS methods of amplifying transgenes can result in genetic instability. CHO cells typically undergo genomic rearrangements and amplifications of the locus of DNA integration resulting in increased copy numbers for both DHFR and the protein of interest. Often, clones containing several hundred copies of the vector construct can be found following amplification. This high copy number does not however lead to uniform high expression or to stable production. It is commonly reported that recombinant protein production can drop significantly within the 2 months following high-producing clone selection, particularly when the selection pressure is removed (Fann et al., 2000; Jun et al., 2006; Kim et al., 1998a; Strutzenberger et al., 1999). For instance, Strutzenberger et al. (1999) have shown that when dhfr amplification with methotrexate was used, over 75% of the integrated transgenes were lost once the drug was removed. In the absence of selective pressure, expression is lost just weeks following selection. In one study, the relative decrease in specific productivity varied among subclones, ranging from 30% to 80% (Kim et al., 1998b; Kim et al.,

2001). Southern and Northern blot analyses showed that this decreased productivity resulted mainly from the loss of amplified antibody gene copies and their respective cytoplasmic mRNAs (Barnes et al., 2006).

Overall, it is clear from the examples presented above that there is a commercial need within the biotechnology industry to understand the problem of instability of protein production associated with recombinant mammalian cell lines. Loss of recombinant gene copy number and the overgrowth of non-producing populations of cells may result in low production; however, there are several other factors that may affect expression levels and stability of production. The possibility of improving cell-line stability and decreasing the variability associated with the generation of a cell line is now possible through the use of techniques that assure that the transgenes are actively transcribed following integration and that the chromatin surrounding the transgene remains capable of active transcription. The potential of these techniques for saving time as well as human and financial resources is extremely promising. This will also open up avenues for more rapid and effective use of additional types of mammalian cells, beyond CHO cells, as expression hosts (Barnes et al., 2004, 2007).

2 Causes of Instability During Mammalian Cell Production

The consistency of growth, productivity, or product characteristics with each successive generation of the cell line defines cell line stability and these factors contribute to the overall process consistency. Some of the issues that lead to instability of protein production in mammalian cells include gene amplification, loss of genetic material, methylation and the location of integration. Gene amplification occurs through the mechanism of chromosomal rearrangement, which involves chromosomal breaks (Andrulis et al., 1983; Melton et al., 1982). Amplification can result in decreased stability of transgene expression due to such breaks and rearrangements (Flintoff et al., 1984; Yoshikawa et al., 2000). CHO cells are known to have an unstable karyotype, with chromosome rearrangements arising from translocations and recombination events, as in the amplification procedures (Yoshikawa et al., 2000). As discussed above, the predominant use of CHO cells has been paralleled with gene amplification selection methods; however, loss of protein production following amplification has been reported for several proteins including interferon, tissue plasminogen activator, and antibodies. In some cases, production levels can reach a stable value after an initial decrease during the first 30 to 50 days of culture (Kim et al., 1998a). Even in the presence of selective pressure, Jun et al. (2006) found that the stability of antibody producing subclones was very poor. Furthermore, the specific secretion rate decreased by 50% after 100 passages even with selective pressure. This might be explained by the selective silencing of the transgenes at chromosomal sites that are prone to epigenetic regulation (position effect), as the amplified gene arrays are often scattered at multiple loci in the host genome. Thus instability may be a concern in the development of CHO cell

lines with DHFR and GS-mediated gene amplification (Kim et al., 1998a; Kim and Lee, 1999; Fann et al., 2000; Jun et al., 2006).

In addition to the potential loss of transgenes following amplification, the large number of repetitive gene sequences that results from this process may induce methylation of DNA sequences, thus preventing transcription (Fouremana et al., 1998). More recently, RNAi-mediated chromatin remodeling linked to the occurrence of repetitive sequences, such as the hundreds of copies that result from amplification, has been shown to contribute to gene silencing (Almeida and Robin, 2005; Morris, 2008). These observations provide a likely explanation for the fact that expression following amplification does not increase proportionally with the transgene copy number. Mechanisms by which repeated sequences such as inverse repeat transgene arrays and RNAi may trigger silent chromatin assembly include physical pairing of homologous sequences and/or DNA–RNA or RNA–RNA interactions (Selker, 1999; Matzke et al., 2001). The connection between RNAi and heterochromatin assembly has suggested a model for the RNA-mediated epigenetic structuring of the eukaryotic genomes. Double-stranded RNA is processed into small RNAs, which in turn provide specificity for targeting histone-modifying activities and epigenetic modification of the genome through homology recognition (Fig. 1; Grewal and Moazed, 2003).

Instability of expression can be due to the regulation of gene expression, the silencing of genes, and the loss of gene copies. However, it must be stressed that these mechanisms are not mutually exclusive, and often the regulation of gene expression and the occurrence of instability of expression involve interplay between different mechanisms. DNA is highly condensed into the chromatin structures, and this condensation often hinders the accessibility of DNA to transcription complexes (Felsenfeld, 1992, 1996; Woodcock and Dimitrov, 2001). Activation of transcription

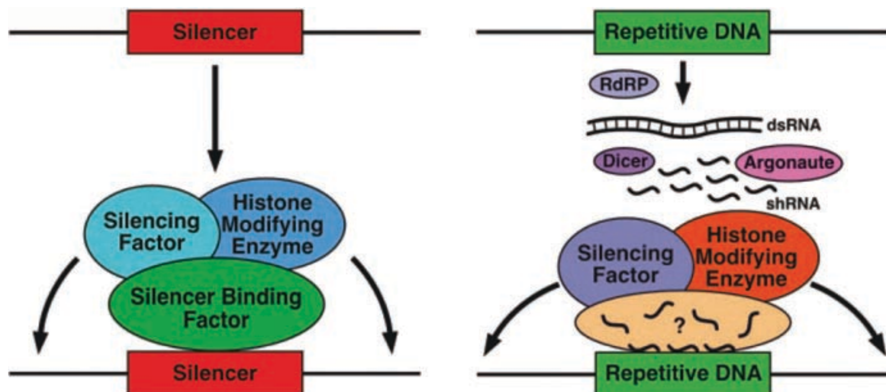


Fig. 1 Mechanisms for the initiation of heterochromatin. Heterochromatic structures can be nucleated by specific cis-acting sequences, called silencers, which are recognized by DNA binding proteins (*left*). Transcripts generated by repetitive DNA are processed into siRNAs by a mechanism requiring components of the RNAi machinery (from Grewal and Moazed, 2003, reprinted with permission from AAAS)

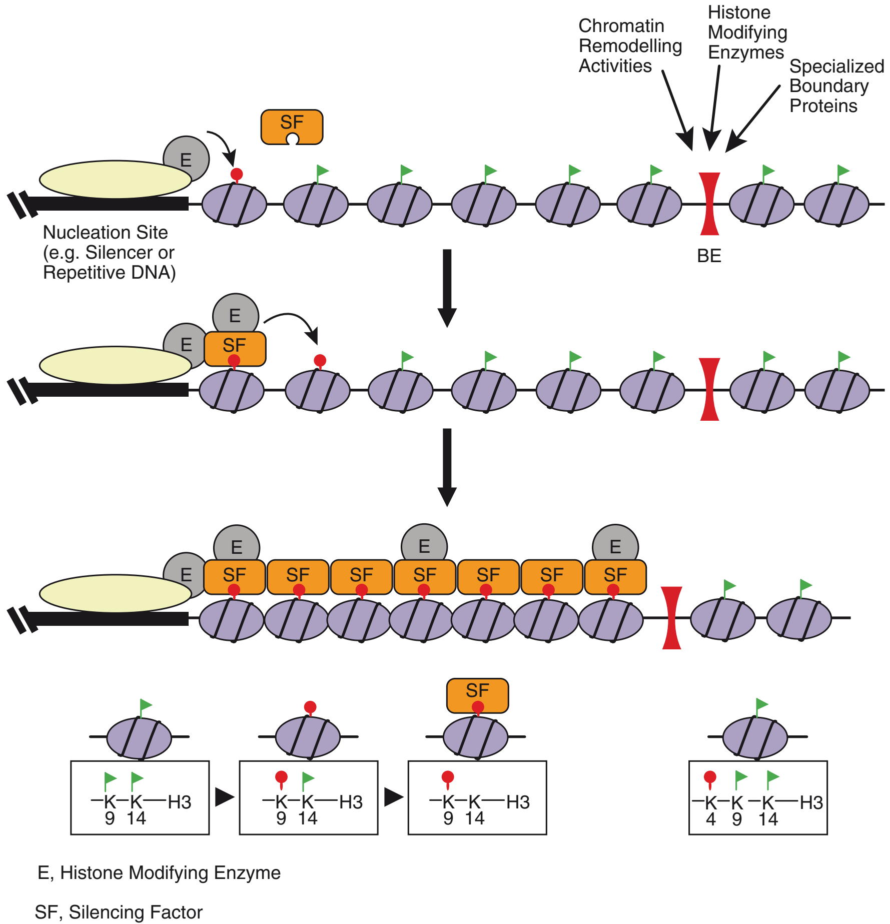


Fig. 2 Model for formation of silenced chromatin domains. After the recruitment to a specific heterochromatin nucleation site by proteins that directly bind DNA or are targeted by way of RNAs, histone modifying enzymes (E) such as deacetylases and methyltransferases modify histone tails to create a binding site for silencing factors (SF). Spreading of silencing complexes is blocked by the presence of boundary elements (BE). The modifications associated with the amino terminus of histone H3 in fission yeast heterochromatin (*bottom left*) and euchromatin (*bottom right*) are illustrated as an example (from Grewal and Moazed, 2003, reprinted with permission from AAAS)

requires the rearrangement of chromatin structure or chromatin remodeling. Chromatin remodeling, which is performed by a range of remodeling complexes, is a loose term used to define any event that alters the nuclease sensitivity of a DNA region (West et al., 2002; Grewal and Moazed, 2003) (Fig. 2).

The highly condensed heterochromatin domains are interspersed along with relatively decondensed euchromatic regions (Fig. 2; Grewal and Moazed, 2003). Given that heterochromatin structures, once nucleated, can spread in *cis*, resulting