Biotechnol Lett
DOI 10.1007/s10529-013-1168-8

ORIGINAL RESEARCH PAPER

Unstable expression of recombinant antibody during long-term culture of CHO cells is accompanied by histone H3 hypoacetylation

Verenice Paredes · Jeong Soo Park · Yongsu Jeong · Jaeseung Yoon · Kwanghee Baek

Received: 9 December 2012 / Accepted: 18 February 2013

Abstract The gradual loss of recombinant protein expression in CHO cell lines during prolonged subculture is a common issue, referred to as instability, which seriously affects the industrial production processes of therapeutic proteins. Loss of recombinant gene copies, due to the genetic instability of CHO cells, and epigenetic silencing of transgene sequences, are the main reported causes of production instability. To increase our understanding on the molecular mechanisms inherent to CHO cells involved in production instability, we explored the molecular features of stable and unstable antibody producing cell lines obtained without gene amplification, to exclude the genetic instability induced by the gene amplification process. The instability of recombinant antibody production during long-term culture was caused by a 48–53 % decrease in recombinant mRNA levels without significant loss of recombinant gene copies, but accompanied by a ~45 % decrease in histone H3 acetylation (H3ac). Thus, our results suggest a critical role of H3ac in the stability of recombinant protein production.

Keywords CHO cell · Histone acetylation · Long-term culture · Production instability · Recombinant protein expression · Transgene silencing

V. Paredes · Y. Jeong · J. Yoon · K. Baek (✉)
Department of Genetic Engineering and Graduate School of Biotechnology, Kyung Hee University, Yongin-si 446-701, Republic of Korea
e-mail: khbaek@khu.ac.kr

V. Paredes
e-mail: vereniceph@khu.ac.kr

Y. Jeong
e-mail: yongsu@khu.ac.kr

J. Yoon
e-mail: jsyoon@khu.ac.kr

J. S. Park
PanGen Biotech Inc., Suwon, Republic of Korea
e-mail: jeongsoo_park@pangen.com

Introduction

A common and serious problem faced during the development of recombinant cell lines is the loss of productivity upon the prolonged subculture of cells. This production instability has been attributed to different causes and one of the main ones is the loss of recombinant genes over passaging of cells, which seems to be the result of chromosomal disruption/rearrangements inherent to the specific genetic instability background of cells (Kim et al. 2011) and/or induced by gene-amplification processes (Kaufman et al. 1983). The decline in the transcription of recombinant genes is another major cause of productivity drop, presumably due to the epigenetic silencing of recombinant sequences through promoter methylation (Yang et al. 2010) and histone modifications, such as deacetylation and methylation (Mutskov and Felsenfeld 2004). The nature of recombinant sequences, their tandem arrangement acquired during the amplification process, as well

Published online: 07 March 2013

as the insertion locus in the host genome, have all been proposed as key factors for gene silencing (Kaufman et al. 2008).

Histone modifications play a critical role in gene transcription regulation since they determine the transcriptional state of genes (Berger 2007). For instance, hyperacetylation of histones 3 and 4 tails is associated to an open chromatin structure (euchromatin) with an active gene transcription; conversely hypoacetylation is linked to transcriptional silencing and a condensed chromatin structure (heterochromatin). The involvement of histones modifications on transgene silencing has been studied in vitro, in chicken erythroid cells (Mutskov and Felsenfeld 2004) and in vivo, in mice tissues (Riu et al. 2007), where results clearly showed a correlation between loss of transgene expression and histone deacetylation at transgene sequences. Furthermore, a study in human fibrosarcoma HT1080 cells (Yan and Boyd 2006) revealed that histone modification patterns of genomic sequences flanking the transgene are determinant for its persistence or silencing. Indeed the influence of neighboring chromatin over the transgene expression, termed "position effect", has been extensively studied and pointed out as a key factor determining the stability of recombinant gene maintenance and expression (Lattenmayer et al. 2006; Yin et al. 2012).

Decreased efficiency of the post-transcriptional processes, such as translation, protein folding and secretion may also account for the instability of recombinant cell lines (Chusainow et al. 2009). Additionally, phenotypic alterations of cells arising during long-term culture, in terms of metabolism, growth behavior and sensitivity to stress, can also significantly affect the stability of recombinant protein production (Bailey et al. 2012).

To understand better the molecular mechanisms inherent to CHO cells inducing the instability of recombinant protein expression during prolonged culture, excluding the effect of gene amplification by selective pressure, we generated antibody producing CHO cell lines without gene amplification and studied their stability up to 30 passages. Then, the molecular features of stable and unstable cell lines, in terms of recombinant mRNA levels, recombinant gene copy numbers and histone acetylation levels at recombinant sequences, were analyzed at early and late stages of the long-term culture. We found that instability of recombinant antibody expression was caused by decreased levels of heavy chain (HC) and light chain (LC) mRNAs during late stages of culture, and that these were accompanied by decreased levels of H3 acetylation (H3ac) on the transgenes.

Materials and methods

Transfection vectors

The cDNAs encoding the recombinant antiCD20 HC and LC were obtained by artificial synthesis and then amplified by PCR using primers including sequences for specific restriction enzymes. The expression vectors, pMSG–HC and pMSG–LC (Fig. 1a), were generated by insertion of the amplified HC or LC cDNA downstream from the SV40 promoter into the previously obtained pMSG vector (Kim et al. 2004).

Cell culture and transfections

Dihydrofolate reductase (DHFR)-defective CHO (DG44) cells adapted to suspension culture in a CDM4CHO serum-free medium (HyClone) supplemented with 100 μM sodium hypoxanthine and 16 μM thymidine (HT supplement, Gibco, Invitrogen), were co-transfected by electroporation (Neon Transfection System, Invitrogen) with 2 μg DNA, consisting of a 50:50:1 molar ratio of HC and LC expression vectors (pMSG–HC/pMSG–LC) and the selection marker vector (pDCH1P) containing the DHFR gene (Ciudad et al. 1988). Transfected cells were then cultivated in a humidifier incubator at 37 °C, 5 % CO_2, in the CDM4CHO serum-free medium lacking HT supplement to obtain positive transfectants. Pools of transfectants with the highest productivities were subjected to single clonal selection by limiting dilution and the highest producer clones were subcultured every 3 days in the CDM4CHO serum-free medium lacking HT supplement for the long-term expression stability studies. A clone showing a stable antibody expression (AJ-68) and a clone showing a decreased expression (AE-40) over 30 passages were used for the molecular studies. These clones were never exposed to agents like methotrexate (MTX) for gene amplification.

Assays for antibody expression

Antibody titers of supernatant samples were determined by a human IgG ELISA kit (PanGen Biotech

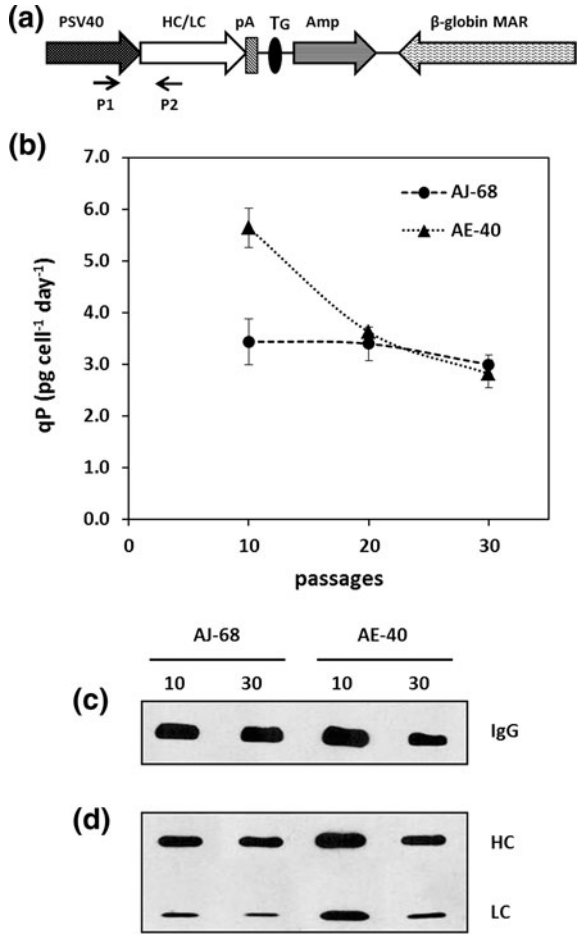

Fig. 1 Analysis of antibody productivity during long-term culture of CHO cell lines. **a** Schematic representation of pMSG–HC and pMSG–LC expression vectors (*Psv40* SV40 promoter, *pA* SV40 polyA, *TG* human gastrin terminator). The location of primers, P1 and P2, used for PCR analysis of ChIP enriched samples is shown below. **b** Cell-specific antibody productivities (qP) of CHO cell lines during the long-term culture. *Error bars* represent the standard deviations from three independent ELISA analysis. Western blotting at non-reducing (**c**) and reducing (**d**) conditions of supernatants taken at passages 10 and 30 of the long-term cultures

Inc.) according to the manufacturer's instructions. The cell specific productivity (qP; pg cell^{-1} day^{-1}) was calculated from a linear curve of antibody concentration versus integrated viable cell density.

Western blot

Supernatant samples were run in a 10 % SDS-PAGE under reducing and non-reducing conditions, and then analyzed by western blotting using an HRP-conjugated goat anti-human IgG (ZYMED) and the WEST-one detection system (Intron Biotechnology).

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from frozen cell pellets using the RiboEX total RNA isolation solution (GeneAll Biotechnology) according to the manufacturer's instructions. The mRNA levels were determined by RT-PCR, using 20 ng extracted total RNA as template, the Maxime RT-PCR premix kit (Intron Biotechnology) and specific primers to amplify the HC, LC and β-actin cDNAs. RT-PCR products were run in 1 % agarose gel, and mRNA levels were estimated by densitometric analysis of the observed bands using the ImageJ software.

Assays for gene copy number

Genomic DNA (gDNA) was extracted from frozen cell pellets using the DNeasy Blood & Tissue kit (Qiagen) following the instructions of the manufacturer. The HC and LC recombinant genes were then amplified by PCR using 10 ng of gDNA as template, the Maxime i-Taq premix kit (Intron Biotechnology) and specific primers to amplify the whole sequences of genes. The β-actin gene was also amplified for normalization purposes, as well as serial amounts of pMSG–HC and pMSG–LC expression vectors to be used as standards for quantification. PCR products were run in 1 % agarose gel, and the HC/LC gene copy numbers were estimated by densitometric analysis of the observed bands using the ImageJ software.

Assays for histone acetylation

Histone-DNA crosslinking

Cultivated cells at different passages were fixed with 1 % (v/v) formaldehyde at 37 °C for 10 min, then washed twice with PBS, pelleted by centrifugation and stored at −70 °C for the chromatin immunoprecipitation (CHIP) assays.

ChIP assays

ChIP assays were performed using the ChIP assay kit (Millipore), following the manufacturer's instructions. Briefly, fixed cells at different passages were lysed and

DNA was sheared by sonication with 10 sets of 4-second pulses at 30 % output. A 10 % volume of each lysate was saved to serve as input DNA. Lysates were incubated at 4 °C overnight with specific antibodies against acetylated histone 3, acetylated histone H4 (H4ac) (Millipore) or without antibodies for negative control, then immune-complexes were precipitated with salmon sperm DNA/protein A-agarose. Eluted histone–DNA complexes and input aliquots were adjusted to 0.2 M NaCl and incubated at 65 °C overnight to reverse crosslinks, followed by treatment with proteinase K (45 °C, 1 h) and RNAse A (37 °C, 30 min). DNAs were recovered by phenol/chloroform extraction and ethanol precipitation.

PCR analysis

Amounts of DNA associated to H3ac or H4ac in immunoprecipitated (IP), negative control (NC) and input (In) fractions were determined by PCR, using the Maxime i-Taq premix kit (Intron Biotechnology) and specific primers to amplify a 400 bp segment spanning the SV40 promoter and HC or LC coding sequences surrounding the transcription initiation site in expression vectors (Fig. 1a). A 400 bp segment of the endogenous β-actin gene was also amplified as an internal control for normalization purposes. IP-fractions, NC-fractions and 1:10 diluted In-fractions were used at 3 µl for PCR amplification. PCR products were then run in 1 % agarose gel, and a densitometric analysis of the obtained bands was carried out using the ImageJ software. The levels (% Input) of H3ac or H4ac at targeted sequences represent the ratio of the IP-fraction signal to that of In-fraction in each sample. The percentage of H3ac or H4ac reduction at passage 30 was calculated by comparison of the IP-fraction/In-fraction values obtained at this passage to those obtained at passage 10, which were set as 100 %.

Results and discussion

Analysis of antibody productivity during long-term culture in CHO cells

The recombinant antibody producing cell lines AJ-68 and AE-40 were obtained by co-transfection of expression vectors containing the antiCD20 HC and LC genes along with the plasmid containing the selection marker, as previously described in the materials and methods. The stability of antibody expression in these cell lines during long-term culture was then assessed by ELISA at passages 10, 20 and 30. As shown in Fig. 1b, the AE-40 cell line showed a higher initial antibody productivity than AJ-68, however it lost about 50 % of its initial productivity at 30 passages, whereas the AJ-68 cell line maintained its productivity above 90 % of initial along the long-term culture. To confirm the data obtained by ELISA and also to compare the expression levels of HC and LC, western blot analysis were performed under reducing and non-reducing conditions. Western blot analysis under non-reducing conditions also showed that the antibody expression in the AE-40 cell line substantially decreased at 30 passages (Fig. 1c), as the results obtained from ELISA. The relative expression of HC and LC was compared by western blot under reducing conditions and the results showed that the expression of both HC and LC in AE-40 cells similarly decreased at passage 30 (Fig. 1d) as that of whole antibodies. Since the cell line AJ-68 was able to highly sustain its antibody expression up to 30 passages, we considered this as a stable cell line; conversely the cell line AE-40, which lost its expression at a significant extent, was considered unstable.

Transcription levels and stability of transgenes during long-term culture

To understand the molecular basis for the unstable expression of the transgenes during long-term culture, the mRNA levels and the genetic stability of HC and LC genes were analyzed in the two cell lines. Decreased levels of HC and LC mRNAs, 48 and 53 % respectively, were detected by RT-PCR in AE-40 cells at passage 30, (Fig. 2a), which correlates well with the reduced levels of HC and LC detected by western blot, indicating that the decline of antibody expression in this cell line was caused by either an inefficient transcription of recombinant genes, their deletion from the host genome or a combination of both.

In order to determine whether the reduced levels of transcripts in AE-40 cells resulted from the instability of transgenes, the copy numbers of recombinant genes at early and late passages in the two cell lines were assessed by PCR. As shown in Fig. 2b, the numbers of HC and LC gene copies were 3.8- to 5-fold higher in

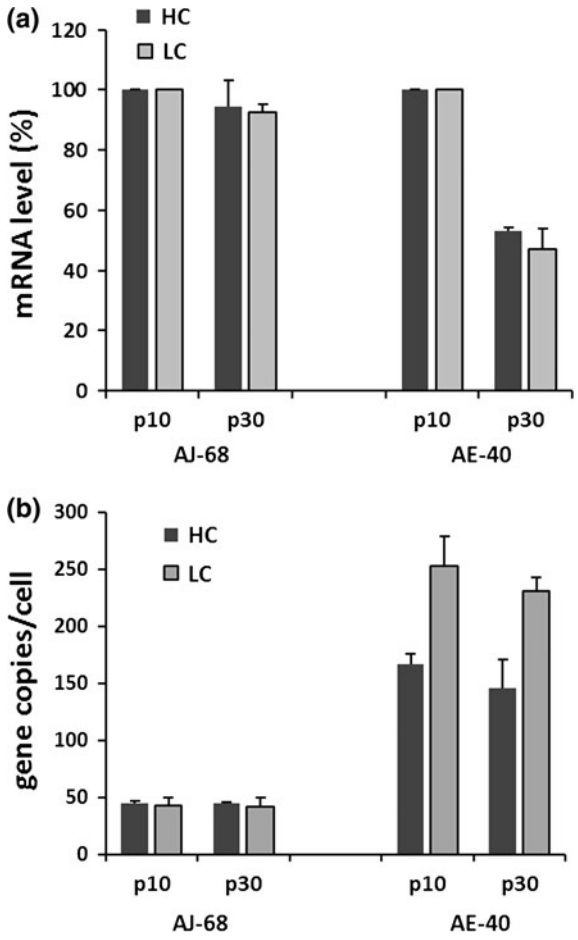

Fig. 2 The mRNA levels of antibody HC and LC and their gene stability during long-term culture. **a** HC and LC mRNA levels in the two cell lines at passages 10 and 30 were determined by semiquantitative RT-PCR. The signal densities of HC and LC amplicons were normalized to that of β-actin amplicons in each sample, and the percentage level of mRNA at passage 30 was estimated from the ratio of its signal to that of passage 10, which was set as 100 %. **b** HC and LC gene copy numbers in the two cell lines at passages 10 and 30 were determined by semiquantitative PCR. The signal densities of HC and LC amplicons were normalized to that of β-actin amplicons in each sample, and used to interpolate the gene copy number values from linear standard curves generated with the amplicons signals of serial amounts of expression vectors. In graphs **a** and **b** the *error bars* represent the standard deviations of triplicated samples' analysis

the AE-40 cell line, which could explain its higher initial productivity compared to that of AJ-68. Regardless of their differences in recombinant gene copy numbers, the two cell lines demonstrated a strong genetic stability as they largely retained the transgene sequences over the long-term subculture. Since decreased levels of HC/LC transcripts were detected in the unstable cell line with no significant loss of recombinant genes, the decline on antibody expression observed in this cell line seems to be caused by a deficient transcription of recombinant genes. Similar results were obtained by Chusainow et al. (2009), who suggested that the decline of the transgene expression was mainly caused by the silencing of transgene sequences. Although the precise molecular mechanisms by which host cells silence transgene sequences are still not fully clarified, an interesting model proposed by Mutskov and Felsenfeld (2004) postulates that transgene silencing involves a sequence of events, of which loss of histone H3/H4 acetylation and H3 lysine 4 methylation (H3K4me) are the early events accompanying the transcription inactivation of transgenes, while methylation of H3 lysine 9 and CpG sites on promoter sequences are the later events that assure a stable silenced chromatin state.

The acetylation levels of histones H3 and H4 during long-term culture

Since our results indicate that the reduction of HC and LC transcripts in the unstable cell line is likely caused by silencing of recombinant genes, we therefore explored the acetylation status of histones H3 and H4 at the vicinity of the transcription initiation site in the stable (AJ-68) and unstable (AE-40) cell lines at early and late passages through ChIP assays followed by PCR. Results showed that histones H3 and H4 at the transgenes were acetylated in both the stable and unstable cell line, and that the levels of acetylation were changed over time, especially in the unstable cells (Figs. 3a, b). As shown in Fig. 3c, the levels of H3ac associated to promoter-HC (p-HC) or promoter-LC (p-LC) target sequences were slightly reduced, 16 and 15 %, respectively, during 30 passages in the stable cells, while a substantial reduction, 45 and 46 %, was detected in the unstable cells at passage 30. Meanwhile the levels of H4ac associated to the same target sequences similarly decreased after 30 passages in both the stable cell line (p-HC 12 %, p-LC 15 %), and the unstable cell line (p-HC 20 %, p-LC 21 %) (Fig. 3d). This significant reduction of H3ac at the recombinant sequences in the unstable cell line correlates well with the reduced levels of HC and LC transcripts detected by RT-PCR at passage 30, suggesting that the transcriptional reduction of HC and

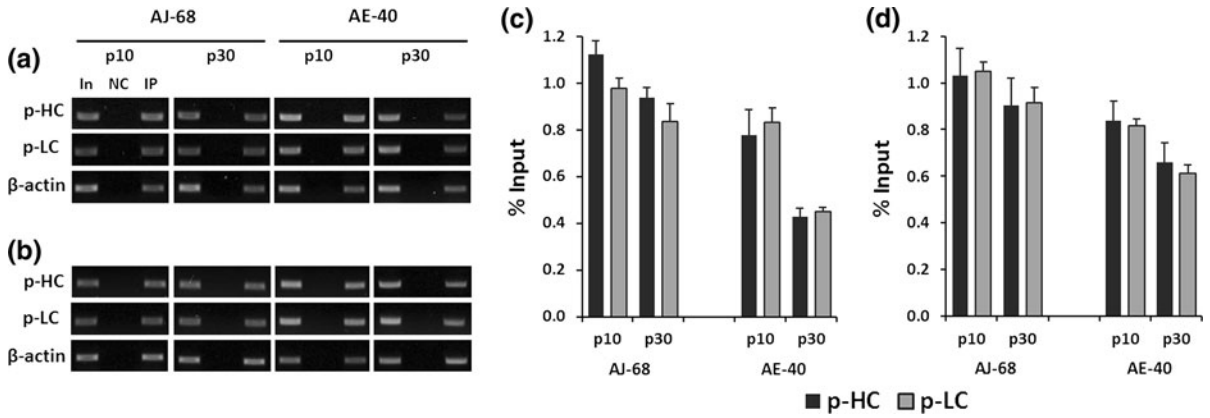

Fig. 3 The levels of histone acetylation at transgene sequences during long-term culture. Representative images of PCR products from immunoprecipitated DNAs with anti-H3ac (**a**) or anti-H4ac (**b**) antibodies in ChIP assays performed in stable (AJ-68) and unstable (AE-40) cells at passages 10 and 30. The signal of amplified target sequences (p-HC/p-LC) in IP-fractions is compared to that of 1 % In-fractions; and a segment of the β-actin gene was also amplified in each sample as an internal control. Fractions obtained without antibody were used as negative controls (NC). Quantitative levels (% Input) of H3ac (**c**) and H4ac (**d**) at targeted sequences in the two cell lines. *Error bars* represent the standard deviations of three independent ChIP-PCR assays

LC recombinant genes by histone H3 hypoacetylation was the major cause of the drop in the antibody productivity during the long-term culture. We could not find any significant differences of H4ac in both stable and unstable clones, suggesting that the reduced level of H4ac was not the major cause of the drop in the antibody productivity of the unstable cell line. Therefore, the level of H3ac at recombinant sequences seems to be important for the stability of recombinant protein expression during prolonged culture.

The specific factor(s) that induced the silencing of recombinant sequences in the unstable cell line remains unknown. However, as the results indicate, an important change in the chromatin structure, in terms of H3ac level, occurred on the recombinant sequences over the long-term culture. The chromatin environment at the transgene insertion site dictates its transcriptional state; moreover, chromatin regions permissive for stable transgene expression were enriched in H3ac and H3K4me, while non-permissive regions were poor in or depleted of these histone modifications and progressively silenced transgene expression, which was accompanied by a reduction of H3ac and H3K4me at the transgenic promoter (Yan and Boyd 2006). H3ac and H4ac have different roles in transcription regulation, chromatin folding and other cellular processes, therefore their regulation is carried out by different histone acetyltransferase (HAT) complexes (Shahbazian and Grunstein 2007). Accordingly, it may be possible that stable cells have the transgenes located in a permissive chromatin region where HATs are constantly recruited to maintain a histone hyperacetylation status favorable for transcription. On the other hand, transgenes sequences in unstable cells may be located in chromatin regions with a low recruitment of HATs, which results in a gradual reduction of H3ac and consequently in transgene silencing over cell generations. Another possibility is that the unstable cells have a great number of transgene copies arranged in tandem, which is known to induce silencing. Further analysis addressing the location and integration patterns of transgene sequences are needed to identify the primary causes that induced deacetylation of histone H3 at transgene sequences in unstable cells.

Several researchers have reported the use of histone deacetylase inhibitors (iHDACs), such as sodium butyrate, trichostatin A (TSA) and, more recently, valproic acid, to improve the expression of transgenes in different host cell types. Furthermore, a partial reactivation of silenced transgenes by treatment of recombinant CHO cells with butyrate and TSA was reported by Choi et al. (2005). Therefore, iHDACs may induce a global histone hyperacetylation, including at transgene sequences, which activates their transcription and prevents or partially reverts their silencing.

The results presented here provide evidence of the involvement of histone modifications in transgene

silencing in CHO cells, and therefore might be helpful in clarifying the mechanism by which recombinant gene expression is repressed in this widely used host cell, and to design efficient strategies to overcome this problem.

Acknowledgments This work was supported by the R&D Program (10035201) of MKE/KEIT, Korea.

References

Bailey LA, Hatton D, Field R, Dickson AJ (2012) Determination of Chinese hamster ovary cell line stability and recombinant antibody expression during long-term culture. Biotechnol Bioeng 109(8):2093–2103

Berger SL (2007) The complex language of chromatin regulation during transcription. Nature 447:407–412

Choi KH, Basma H, Singh J, Cheng PW (2005) Activation of CMV promoter-controlled glycosyltransferase and beta-galactosidase glycogenes by butyrate, tricostatin A, and 5-aza-2′-deoxycytidine. Glycoconj J 22(1–2):63–69

Chusainow J, Yang YS, Yeo JH, Toh PC, Asvadi P, Wong NS, Yap MG (2009) A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng 102(4):1182–1196

Ciudad CJ, Urlaub G, Chasin LA (1988) Deletion analysis of the Chinese hamster dihydrofolate reductase gene promoter. J Biol Chem 263:16274–16282

Kaufman RJ, Sharp PA, Latt SA (1983) Evolution of chromosomal regions containing transfected and amplified dihydrofolate reductase sequences. Mol Cell Biol 3(4):699–711

Kaufman WL, Kocman I, Agrawal V, Rahn HP, Besser D, Gossen M (2008) Homogeneity and persistence of transgene expression by omitting antibiotic selection in cell line isolation. Nucleic Acids Res 36(17):e111

Kim JM, Kim JS, Park DH, Kang HS, Yoon J, Baek K, Yoon Y (2004) Improved recombinant gene expression in CHO cells using matrix attachment regions. J Biotechnol 107: 95–105

Kim M, O'Callaghan PM, Droms KA, James DC (2011) A Mechanistic understanding of production instability in CHO cell lines expressing recombinant monoclonal antibodies. Biotechnol Bioeng 108:2434–2446

Lattenmayer C, Loeschel M, Steinfellner W, Trummer E, Mueller D, Schriebl K, Vorauer-Uhl K, Katinger H, Kunert R (2006) Identification of transgene integration loci of different highly expressing recombinant CHO cell lines by FISH. Cytotechnology 51(3):171–182

Mutskov V, Felsenfeld G (2004) Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. EMBO J 23(1):138–149

Riu E, Chen ZY, Xu H, He CY, Kay MA (2007) Histone modifications are associated with the persistence or silencing of vector-mediated transgene expression in vivo. Mol Ther 15(7):1348–1355

Shahbazian MD, Grunstein M (2007) Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem 76:75–100

Yan C, Boyd DD (2006) Histone H3 Acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression. Mol Cell Biol 26(17):6357–6371

Yang Y, Mariati, Chusainow J, Yap MG (2010) DNA methylation contributes to loss in productivity of monoclonal antibody-producing CHO cell lines. J Biotechnol 147:180–185

Yin Z, Kong QR, Zhao ZP, Wu ML, Mu YS, Hu K, Liu ZH (2012) Position effect variegation and epigenetic modification of a transgene in a pig model. Genet Mol Res 11(1): 355–369