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RESEARCH

Assessment of UCOE on Recombinant EPO Production and Expression Stability in Amplified Chinese Hamster Ovary Cells

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Abstract CHO cells are the most frequently used host for commercial production of therapeutic proteins, and DHFR-mediated gene amplification is extensively applied to generate cell lines with increased protein production. However, decreased protein productivity is observed unpredictably during the time required for scale-up with consequences for yield, time, finance and regulatory approval. In this study, we have examined the interaction between Ubiquitous Chromatin Opening Elements (UCOE) and DHFR-linked amplification in relation to cell expression stability. In summary, the inclusion of UCOE elements generated cells that (1) achieved higher cell densities and exhibited increased production of recombinant mRNA per cell and protein yield, (2) allowed isolation of greater numbers of high-producing clones, (3) resulted in greater mRNA recovery per recombinant gene copy, (4) retained stable mRNA and protein expression after amplification provided Methotrexate (MTX) was present (but not in the absence of MTX when instability was observed) and (5) conferred copy number-dependent expression to linked transgene, suggesting that they are resistant to positional gene-silencing effects. It was concluded that the inclusion of UCOEs within expression constructs offers significant advantages for certainty of cell line generation (and the number of recovered clones for more detailed characterisation/optimisation) and that UCOEs are compatible with DHFR amplification protocols. The data suggested that enhanced cell line recovery by transcriptional enhancement of selection markers, such as DHFR, could be achieved.

Keywords UCOE · Chinese hamster ovary cells · Gene amplification · Mammalian cell culture · Instability · Methotrexate (MTX)

Abbreviations

MTX	Methotrexate
UCOE	Ubiquitous chromatin opening element

Introduction

Due to an increasing demand for increased production of clinical grade proteins, improvements in protein yields from mammalian cells and a reduced time for production are key objectives. Productivity has been improved over the last 20 years due to the identification and selection of specific host cells, vector developments, cell culture conditions and downstream processing [1, 2]. Among mammalian cells, CHO cells are the predominant host for commercial production of therapeutic proteins because of well-characterised platform technologies that allow for transfection, amplification and selection of high-producing clones [3, 4]. Over the past decades, extensive testing and safety data have been amassed which ultimately eases the regulatory approval process, which ensures that CHO cells are likely to remain as the major platform for the production of therapeutic protein in the near future [5, 6]. Gene amplification

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methods, in particular Methotrexate (MTX)-mediated amplification, are extensively applied to generate large amount of recombinant proteins [7]. Despite their widespread use and commercial significance, clonal heterogeneity and expression instability remain the key obstacles to "predictable" recombinant protein production in mammalian cell lines. In order to isolate a stable high-producing clone, a large number of clones need to be screened [8–10]. Consequently, it can be an arduous and costly process to identify suitable high-producing clones for commercial production. The location of integrated plasmid DNA into the CHO cell chromosome [11] and silencing of the transgene as a result of epigenetic gene regulation such as DNA methylation [12–14] and histone deacetylation [15] are suspected to play a role in instability. In order to overcome these challenges, recent studies have focused on various regulatory elements that can be incorporated into the expression vectors to maintain transgenes in a transcriptionally active state including Locus control regions (LCRs) [16], Scaffold/Matrix Attachment Regions (S/MARs) [17, 18] and Ubiquitous Chromatin Opening Elements (UCOEs) [14, 19–21]. UCOEs are derived from the heterogeneous nuclear ribonucleoproteins A2 B1/chromobox protein homologue 3 (HNRPA2B1/CBX3) housekeeping gene locus and are defined as being comprised of an extended methylation-free CpG-island and normally bi-directional promoters that are divergently transcribed [20, 22]. It has been used in combination with retroviral vectors in various cell lines including teratocarcinoma cell line P19 and hematopoietic stem cells and their differentiated progeny to improve and stabilise transgene expression [14, 23–26]. Anti-silencing activity was associated with strongly reduced DNA methylation of promoter, which strongly favours its application in PSC-based cell and gene therapy. In CHO and BHK21 cells, UCOE provided a higher level of transgene expression following stable transfection [19, 21, 27–29] and maintained the stability of protein expression over 100 generations [30]. In this study, a UCOE vector was used in combination with MTX amplification to improve the frequency of positive clones and to achieve a high volumetric recombinant protein production. In addition to this, its ability to maintain stability of recombinant EPO production in CHO-DG44 cells was evaluated.

Materials and Methods

Cell Culture and Media

The parental CHO-DG44 cells were originally supplied by British Biotech. All transfected cell lines were grown in RPMI medium supplemented with L-Glutamine (4 mM final, Lonza Group, Switzerland) and 10 % (v/v) Fetal Bovine Serum (FBS) (growth medium) either with or without MTX selection. When MTX selection was required, MTX was added to a final concentration of 250 nM. 1× Hypoxanthine and Thymidine (HT) solution (Gibco) was added to the growth medium for DHFR-deficient non-transfected parental CHO-DG44 cells. Cells were cultured routinely in T-75 flasks at 37 °C and 5 % CO_2 and sub-cultured every 48–72 h by rinsing with Dulbecco's phosphate-buffered saline (DPBS-Lonza), detaching with trypsin, and quenching by adding the growth medium. An appropriate volume of cells was diluted in fresh growth medium to give a cell density of 1×10^5 viable cells/ml. Long-term culture (LTC) was performed by continuous sub-culture for a total of 77 days in growth medium with or without MTX. Media, DNA and RNA samples for further experimental work (protein, gene copy number and mRNA expression analyses) were taken on days 0 and 77 of LTC. Growth characteristics were assessed by light microscopy using an improved Neubauer haemocytometer. Samples were appropriately diluted and mixed 1:1 with 0.5 % (w/v) Trypan Blue in PBS.

Vector Construction

pSKa (containing IRES-DHFR) and pCR2.1®EPO (Professor Alan Dickson, University of Manchester, UK) were digested with Nsi1 and EcoR1, and linearised fragments corresponding to EPO and pSKa were purified and ligated. The resulting EPO-IRES-DHFR cassette was then inserted into pCET901 (Cobra Biologics, UK) by digesting with Xba1 and EcoR1. This resulted in flanking EPO-IRES-DHFR with hCMV and pA sequences. The resulting hCMV-EPO-IRES-DHFR-pA cassette was isolated from p901-EPO and inserted into the 8 kb UCOE-containing pCET1010 vector (Cobra Biologics, UK) using Age1 restriction endonucleases (p1010-EPO, see Supporting Fig. 1 for detailed structure of the vectors used).

Transfection, Amplification and Cloning

CHO cells were transfected with the appropriate linearised plasmids, and limiting dilution was performed as described in [31]. Cell lines were maintained in 6-well plates, and EPO production was assessed by ELISA. The ten cell lines that showed the highest amount of EPO were counted, and an equal number of cells from each cell line were pooled and made up to a volume of 50 ml with RPMI + 10 % (v/v) FBS, to give a final concentration of 1.5×10^5 cells/ml. The pooled cells were then diluted in growth medium containing 250 nM MTX to give a range of dilutions (1.5×10^5, 3×10^4 and 6×10^3 cells/ml). Limiting dilution cloning was then continued as described previously until cell lines were in 6-well plates (in 25 days for UCOE and 33 days for non-UCOE clones) [31]. EPO

production was assessed by ELISA; the top three cell lines, in terms of EPO production, were scaled up to T-75 flasks, and frozen stocks were made.

Detection of EPO by Enzyme-linked Immunosorbent Assay (ELISA)

EPO accumulation was measured using a sandwich ELISA. Nunc 96-well immunoassay plates were coated with the capture antibody (R&D Systems, Minneapolis, USA), allowing the detection of captured EPO which was detected with goat anti-rabbit IgG antibody peroxidase conjugate (Sigma-Aldrich, UK). Reactions were visualised by TMB substrate solution, and OD of each well was measured at 450 nm.

Genomic DNA Extraction

An adapted version of the protocol detailed by Blin and Stafford (1976) was used to extract genomic DNA from CHO cells [32]. Approximately, 2×10^7 cells were harvested by centrifugation at 100 g for 10 min at room temperature. The cell pellet obtained was washed three times in 1× PBS with centrifugation between each wash as above. The final pellet was then resuspended in 100 µl of 1× PBS, and 3 ml of EDTA-Sarcosine solution [0.1 M EDTA, pH8.0, containing 0.5 % (w/v) N-Lauroyl-Sarcosine] was then added to the pellet in a dropwise fashion with continuous gentle mixing. 60 µl of Proteinase K (10 mg/ml) and 10 µl of RNase A (10 mg/ml) were then added. The mixture was incubated at 55 °C for 2 h and mixed by inversion every 15 min. The sample was purified by three rounds of phenol extraction and ethanol precipitation. DNA concentration and purity were measured by NanoDrop ® UV–Vis Spectrophotometer (Thermo Scientific via ABgene, Epsom, Surrey, UK).

RNA Extraction

RNA was extracted from cells in T-75 plates using TRIzol ® (Invitrogen, Paisley, UK) according to the manufacturer's instructions. RNA concentration was quantified using NanoDrop ® UV–Vis Spectrophotometer and extracts treated with DNaseI (Sigma) to remove any trace contamination of genomic DNA. The RNA was subsequently used as a template for cDNA. Reverse transcriptase production of cDNA was performed using a cDNA Synthesis Kit (Bioline, London, UK), according to the manufacturer's instructions.

qPCR and qRT-PCR Analysis

The genomic DNA samples, cDNA samples and negative control samples (parental genomic DNA or samples which had not been reverse transcriptase treated) were prepared for qPCR by diluting in ddH_2O- or DEPC-treated water. A series of standards (created from the recombinant plasmid vector or from a chosen cDNA sample) were also used during each essay. The target gene was quantified by normalising to β-actin. PCR primers are shown in Table 1. Primers were diluted in ddH_2O- or DEPC-treated water to give a final concentration of 10 µM and then mixed at a 1:2 ratio with 2× SYBR ® Green I qPCR MasterMix (Eurogentec Ltd.). qPCR reactions containing 5 µL sample/standard and 15 µL appropriate primers/reaction mix were set up as triplicates in 96-well plates. The qPCR reaction was performed using Chromo 4 Thermal Cycler and Opticon 4 software (Biorad) with following settings: 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 10 s, elongation at 72 °C for 20 s and denaturation of primer dimers at 76 °C for 1 s. A final elongation step at 72 °C for 10 min was performed.

Table 1 Primer sequences used in PCR reactions

Name	Sequence (5′–3′)
EPO forward (DNA)	GTA ACA ACT CCG CCC CAT T
EPO reverse (DNA)	ACA GCC AGG CAG GAC ATT C
EPO forward (RNA)	TGG GAG CCC AGA AGG AAG
EPO reverse (RNA)	CTC CCC TGT GTA CAG CTT CAG
β-Actin forward (DNA)	ACT GCT CTG GCT CCT AGC AC
β-Actin reverse (DNA)	CAT CGT ACT CCT GCT TGC TG
β-Actin forward (RNA)	TGT GAC GTT GAC ATC CGT AAA
β-Actin reverse (RNA)	GCA ATG ATC TTG ATC TTC ATG

Preparation of Standard Curves and DNA Samples

The p1010-EPO vectors were diluted to a final concentration of 1,000,000—457 copies per 5 µl reaction, in a background (10 ng/µl) of genomic DNA isolated from non-transfected CHO-DG44 cells. Background DNA was used to ensure that the efficiency of the PCR reaction was the same for all samples. One sample was dedicated as the 'check' sample that was run on all plates and used to normalise the total DNA content in each well.

Results

Analysis of Growth Characteristics and Productivity of Initial CHO-EPO Cell Lines During Long-Term Culture

The expression of EPO protein was determined by ELISA where 60 clones for UCOE and 48 clones for non-UCOE group were analysed. UCOE cell lines showed significantly

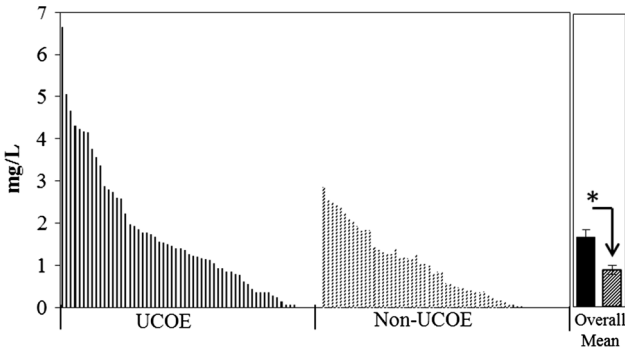

Fig. 1 Volumetric EPO production (mg/l) for initial CHO-EPO cell lines cloned by limiting dilution cloning and scaled up to 6-well plates. Cells were grown in RPMI medium plus 4 mM L-Glutamine and 10 % (v/v) FBS till approximately 90 % confluent. Medium samples were taken and analysed by ELISA to determine volumetric EPO production. Overall mean values are shown ± SEM for each group ($n \geq 60$). *Filled square* UCOE, ▨ non-UCOE. *Asterisk* indicates $p < 0.05$, using independent samples *t* test to compare UCOE and non-UCOE cell lines

higher EPO production than non-UCOE cell lines (Fig. 1). The three highest producing cell lines (in terms of volumetric EPO expression) were grown continuously for up to 77 days, and viable cell densities, volumetric EPO production and specific productivity were analysed over long-term cultivation.

The maximal viable cell densities for UCOE and non-UCOE cell lines were similar in early and late generations (Fig. 2a). A difference was observed in the growth profile of the cultures generated at the start and end of long-term culture with late generation cell lines exhibiting a more prolonged stationary phase and the viability of the late generation cultures declining more suddenly on day 9, presumably as a result of nutrient depletion (Fig. 2a).

Volumetric EPO production from UCOE cell lines was shown to be significantly higher than that of non-UCOE cell lines for all cells (Fig. 2b). Although non-UCOE cell lines showed a trend towards a loss in production (apart from N0E2 cell line where an increase in titer was observed), no significant difference was observed between the group mean values for volumetric production at day 0 and 77 for either UCOE or non-UCOE cell lines. Similarly, UCOE cell lines showed consistently higher specific productivity than the non-UCOE group, and this was reflected in the group mean values with specific productivity

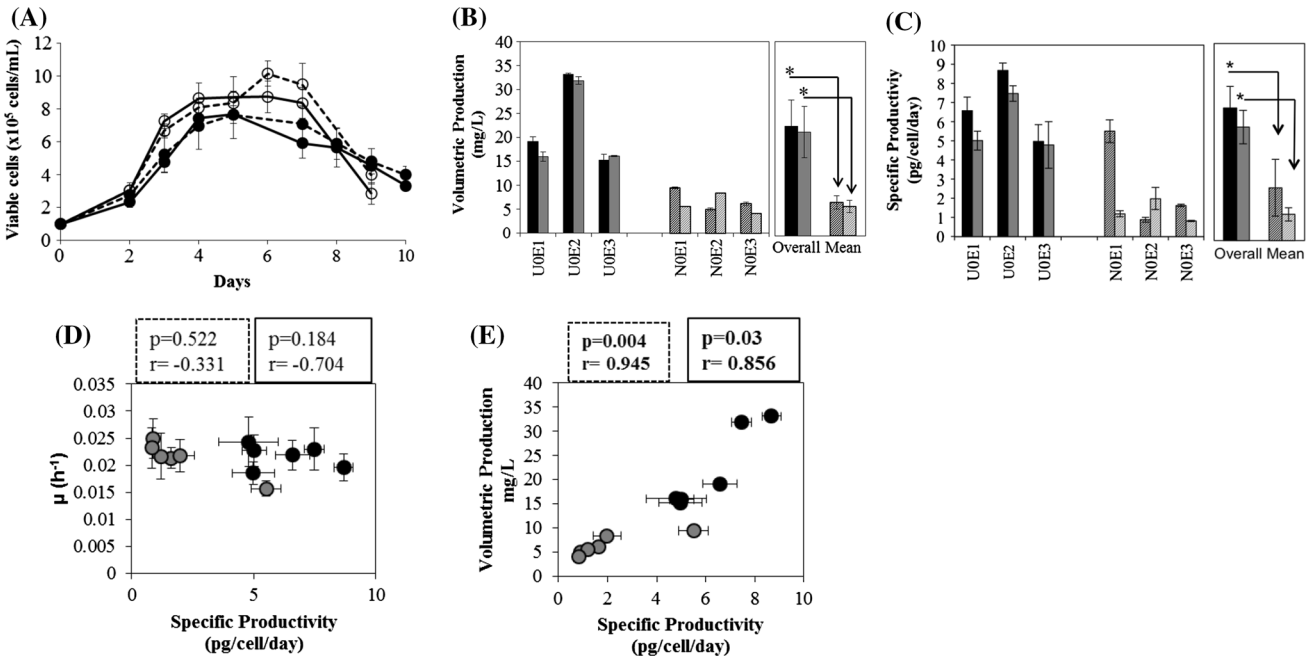

Fig. 2 Analysis of growth and productivity for three high-producing clones over long-term culture. **a** Viable cell densities were determined by light microscopy and trypan blue exclusion from samples taken during batch culture. Values are presented as average of each group ± SEM ($n = 3$). *Thick line* UCOE, *dotted line* non-UCOE, *filled circle* early generation, *open circle* late generation. **b** Recombinant EPO production was assessed by ELISA. Maximum recombinant EPO production, obtained at the end of batch cultures, is shown for each clone. **c** Specific productivity was calculated during the exponential phase, using samples and cell counts from 48 to 96 h. Two biological replicates were set up, and error bars represent the range. Overall mean values are presented as ± SEM ($n = 3$). *Asterisks* indicate $p < 0.05$, using independent samples *t* test to compare UCOE and non-UCOE cell lines (at the same time of long-term culture). *Filled square* Early generation UCOE, *grey square* late generation UCOE, ▨ early generation non-UCOE, ▨ late generation non-UCOE. **d** Cell growth rate and **e** volumetric production are compared with specific productivity for individual cell lines at all times. Correlation coefficients (*r*) and statistical significance (*p*) are noted above the scatter plots (*filled circle* UCOE; *grey circle* non-UCOE)

remaining constant over the long-term culture in UCOE cell lines (Fig. 2c). Conversely, a decrease was observed in non-UCOE, although this was not statistically significant. The behaviour of cell line N0E2 contrasts markedly from that of cell lines N0E1 and N0E3 (Fig. 2c). The relative change in volumetric production between early and late generation was 1.1-fold for both UCOE and non-UCOE group. On the other hand, the specific productivity decrease was twofold in non-UCOE cell lines, whereas this was again 1.1-fold in UCOE cell lines. Figure 2d, e shows comparisons of cell growth rate, total EPO production and specific productivity. No clear correlation was observed between cell growth and specific productivity in either UCOE or non-UCOE cell lines (if early generation, N0E1 cell line is omitted, Fig. 2d). Volumetric production was strongly correlated with specific productivity in both UCOE and non-UCOE cell lines (Fig. 2e).

Establishment of Amplified CHO-EPO Cell Lines

The top ten EPO-producing cell lines were selected from both UCOE and non-UCOE groups in order to address the effect of amplification on the stability of recombinant protein production. Each group of selected cell lines was pooled and treated with 250 nM MTX. More than 60 single colonies were observed for the UCOE cell lines after limiting dilution cloning, whereas only 38 single colonies were recovered from non-UCOE cell lines. All single colonies observed in non-UCOE cell lines, and a total of 40 single colonies from the UCOE group were scaled up to 6-well plates.

Volumetric production of EPO for the selected cell lines over a period of 72 h was determined by ELISA (Fig. 3). The group mean value of EPO expression for the UCOE cell lines was significantly higher than the non-UCOE group (84 ± 6.2 and 20.7 ± 1.9 mg/l, respectively, Fig. 3). The three highest producing cell lines (in terms of EPO production) from both UCOE and non-UCOE groups were selected for further long-term culture studies. Cells were sub-cultured every 2–3 days continuously for 77 days either with or without MTX selection with samples taken on day 0 and 77.

Effect of Amplification on Growth Characteristics and Productivity During Long-Term Culture

As shown in Figs. 2a and 4a, amplified UCOE cell lines at early generation showed similar maximum cell densities to the non-amplified UCOE cell lines suggesting that growth was not affected by MTX pressure. In contrast, the maximum cell densities were lower in the amplified non-UCOE group compared to the non-amplified cell lines in early generation. The UCOE cell lines were found to have higher

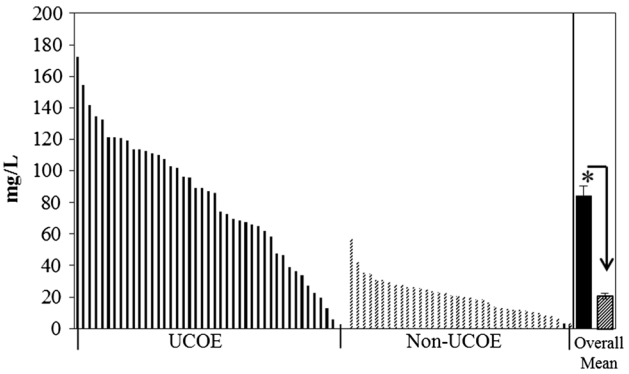

Fig. 3 Volumetric EPO production (mg/l) for amplified CHO-EPO cell lines. The top ten highest producing initial cell lines were pooled and treated with 250 nM MTX. Cells were cloned by limiting dilution cloning and scaled up to 6-well plates. Cells were grown in RPMI medium plus 4 mM L-Glutamine and 10 % (v/v) FBS with 250 nM MTX till approximately 90 % confluent. Medium samples were taken and analysed by ELISA to determine volumetric EPO production. Overall mean values are shown ± SEM for each group ($n \geq 38$). *Asterisk* indicates $p < 0.05$, using independent samples *t* test to compare UCOE and non-UCOE cell lines. *Filled square* UCOE, ▨ non-UCOE

maximum cell densities compared to the non-UCOE cell lines in the early and late generations in the presence and absence of MTX (Fig. 4a). Moreover, an increase in the maximum cell densities was observed in both the UCOE and non-UCOE cell lines over time in the presence and absence of MTX. As shown in Fig. 4b, the UCOE cell lines showed significantly higher specific cell growth rates compared to the non-UCOE cell lines at all times following MTX amplification. The cell growth rate increased over prolonged culture in both UCOE and non-UCOE cell lines in the presence and absence of MTX.

Consistent with the results obtained from the initial cell lines, total EPO production was lower in the non-UCOE cell lines both in the presence and absence of MTX (Fig. 5a, b). The volumetric EPO production was relatively stable in all UCOE cell lines in the presence of MTX for 77 days (Fig. 5a, also see Supplementary Table I). An increase, though not statistically significant, in total EPO production was observed in the non-UCOE cell lines in the presence of MTX over long-term culture. All cell lines showed a decrease in total EPO production over prolonged culture in the absence of MTX of 78 and 87 % in UCOE and non-UCOE cell lines, respectively (Fig. 5b). However, the UCOE cell lines still showed significantly higher EPO production compared to the non-UCOE cell lines (group mean values; 36.5 ± 3, 11 ± 3.7 mg/l, respectively).

The UCOE cell lines showed higher specific productivity compared to non-UCOE cell lines at early generation. However, this was not statistically significant. Additionally, no significant difference was observed between late generation UCOE and non-UCOE cell lines in

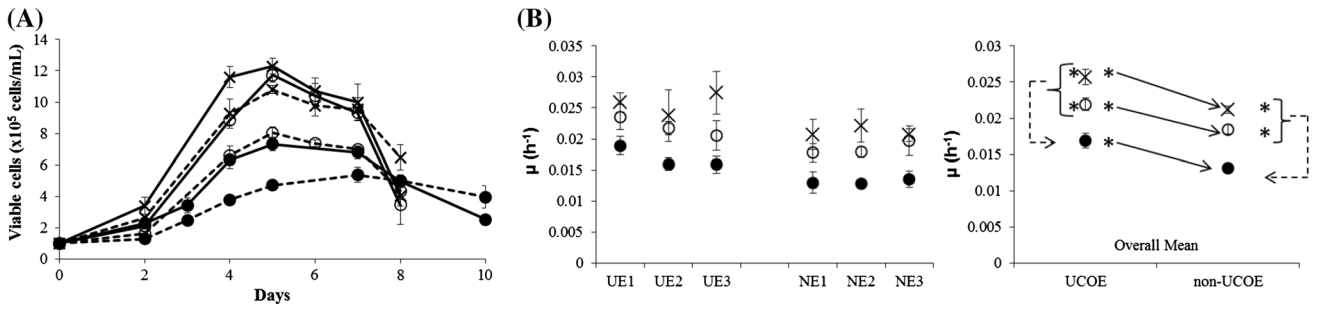

Fig. 4 Growth analysis of amplified CHO-EPO cell lines. Cells were sub-cultured every 2–3 days up to 80 days either with or without MTX selection. **a** The viable cell numbers are presented as the average of each group ± SEM ($n = 3$). **b** The cell growth rates were calculated during the exponential phase of batch culture. Prefixes UE- and NE- stand for EPO producing UCOE and non-UCOE cell lines, respectively. Two biological replicates were set up, and error bars represent the range. Overall mean values are presented as ± SEM ($n = 3$). *Asterisks* indicate $p < 0.05$, using paired samples *t* test to compare early and late generation cultures (for the same group of cell lines). *Thick line* UCOE, *dotted line* non-UCOE, *filled circle* early generation, *open circle* Late generation with MTX, *cross symbol* late generation without MTX

the presence of MTX (Fig. 5c). However, the specific productivity was significantly higher in the UCOE cell lines compared to the non-UCOE group at the end of long-term culture in the absence of MTX (Fig. 5d). A decrease in specific productivity was observed in all UCOE cell lines over long-term culture in the presence of MTX, as reflected in the mean value (Fig. 5c). A relatively strong negative correlation between growth rate and specific productivity was observed in UCOE cell lines, whereas there was no consistent correlation in non-UCOE cell lines (Fig. 5e). Furthermore, volumetric EPO production and specific productivity increased proportionally in both UCOE and non-UCOE cell lines (Fig. 5f).

Copy Number and mRNA Analysis Over Long-Term Culture in Amplified CHO-EPO Cell Lines

Since previous studies indicated that instability of recombinant protein production could result from a loss in the number of copies of recombinant vector integrated within the host cell genome [33], this was assessed by qPCR to determine if variations in recombinant EPO production were as a result of the change in gene copy number per cell (Fig. 6a, b). Samples were taken at the start and end of long-term culture from the cell lines growing in the presence and absence of MTX selection. All UCOE cell lines showed lower EPO gene copy numbers compared to non-UCOE cell lines at both early and late generations, as reflected by the group mean values (Fig. 6a). Furthermore, a decrease in the plasmid copy number was observed in all UCOE cell lines both in the presence and absence of MTX, with the latter more pronounced and also reflected in the group mean values (Fig. 6b). All non-UCOE cell lines displayed a decrease in gene copy numbers during prolonged culture in the absence of MTX, although this was not statistically significant, presumably due to a large standard deviation (Fig. 6b). Furthermore, a relatively strong positive correlation was observed between the gene copy number and specific productivity in the UCOE cell lines when early generation UE3 cell line is omitted, suggesting a copy number-dependent expression. However, there was still strong correlation between UE3 cell line early and late generations ± MTX ($p < 0.001$, $r = 0.960$). Conversely, there was no clear correlation observed between copy number and EPO expression from non-UCOE group (Fig. 6c, d).

EPO mRNA expression was assessed by qRT PCR to examine whether the differing levels of protein production were due to changes in EPO gene transcription (Fig. 7a, b). The UCOE and non-UCOE cell lines showed a similar amount of EPO mRNA expression in the presence of MTX (Fig. 7a). The relative mRNA expression was significantly higher in the UCOE cell lines compared to the non-UCOE group after 77 days in the absence of MTX (Fig. 7b). However, despite having similar or higher levels of mRNA expression, the UCOE cell lines had lower gene copies per cell compared to the non-UCOE cell lines, suggesting that UCOE provides higher transcriptional activity.

The UE1 and UE2 cell lines showed a decrease in EPO mRNA expression over prolonged culture in the presence of MTX, whereas mRNA expression remained constant in the UE3 and NE1 cell lines (Fig. 7a). In contrast, an increase in mRNA expression was observed in the NE2 and NE3 cell lines over long-term culture in the presence of MTX (Fig. 7a). All cell lines displayed a decrease in EPO mRNA expression when MTX selection was removed (Fig. 7b). However, the UCOE cell lines still had higher mRNA expression than the non-UCOE cell lines at the end of long-term culture. mRNA expression was strongly correlated with specific productivity in both UCOE and non-UCOE cell lines (Fig. 7c, d). A strong positive correlation was observed between the gene copy number and mRNA

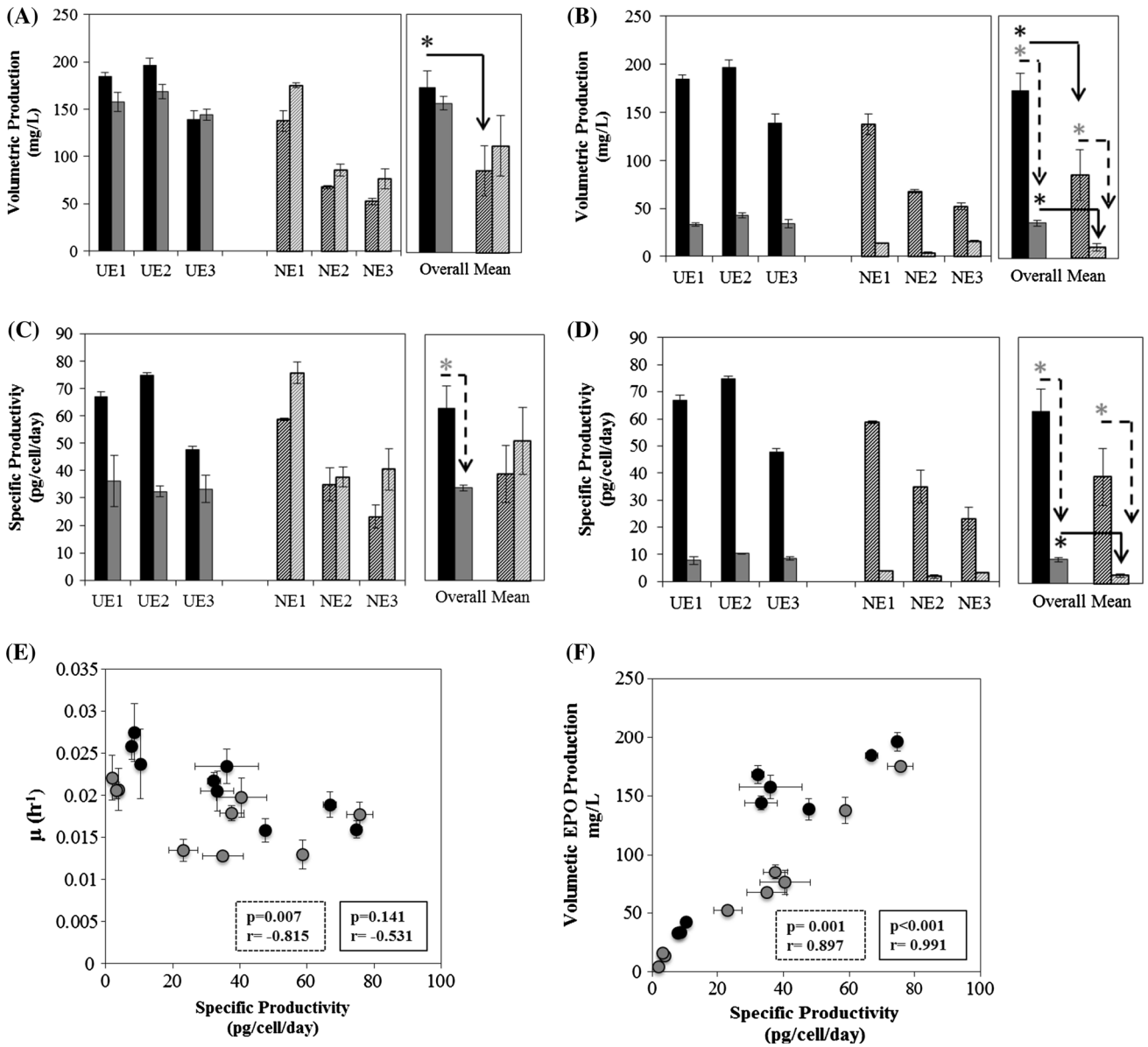

Fig. 5 Volumetric and specific productivity for three amplified high-producing clones over long-term culture. Recombinant EPO production was assessed by ELISA. **a** Volumetric EPO production in the presence and **b** absence of MTX. Specific productivity was calculated as described in Fig. 2. **c** Specific productivity for the cell lines in the presence and **d** absence of MTX. Two biological replicates were set up, and error bars represent the range. Overall mean values are presented as ± SEM ($n = 3$). *indicates $p < 0.05$, using independent samples t test to compare UCOE and non-UCOE cell lines (at same generation). *Asterisks* indicate $p < 0.05$, using paired samples *t* test to compare early and late generation cultures (for the same group of cell lines). *Filled square* Early generation UCOE, *grey square* late generation UCOE, ▨ early generation non-UCOE, ▨ Late generation non-UCOE. **e** Growth rate and **f** volumetric production are plotted against the specific productivity. Correlation coefficients (*r*) and statistical significance (*p*) are noted within the scatter plots (*filled circle* UCOE; *grey circle* non-UCOE)

expression in the UCOE cell lines (by omitting the early generation UE3 cell line, Fig. 8a). The non-UCOE cell lines showed a relatively strong correlation between the gene copy number and mRNA expression (Fig. 8b).

Table 2 summarises the effect of long-term culture on protein productivities and genetic parameters in both UCOE and non-UCOE cell lines. According to the results, the loss of specific productivity in the UE1 and UE2 cell lines in the presence of MTX was as a result of a decline in the EPO gene copies, which was also mirrored by a decrease in EPO mRNA expression. The UE3 cell line had a smaller decrease in cell-specific productivity in the presence of MTX. This cell line displayed similar amounts of EPO mRNA content at the beginning and end of long-term culture when MTX was present. The UE1 and UE2 cell lines showed more than an 86 % loss of productivity

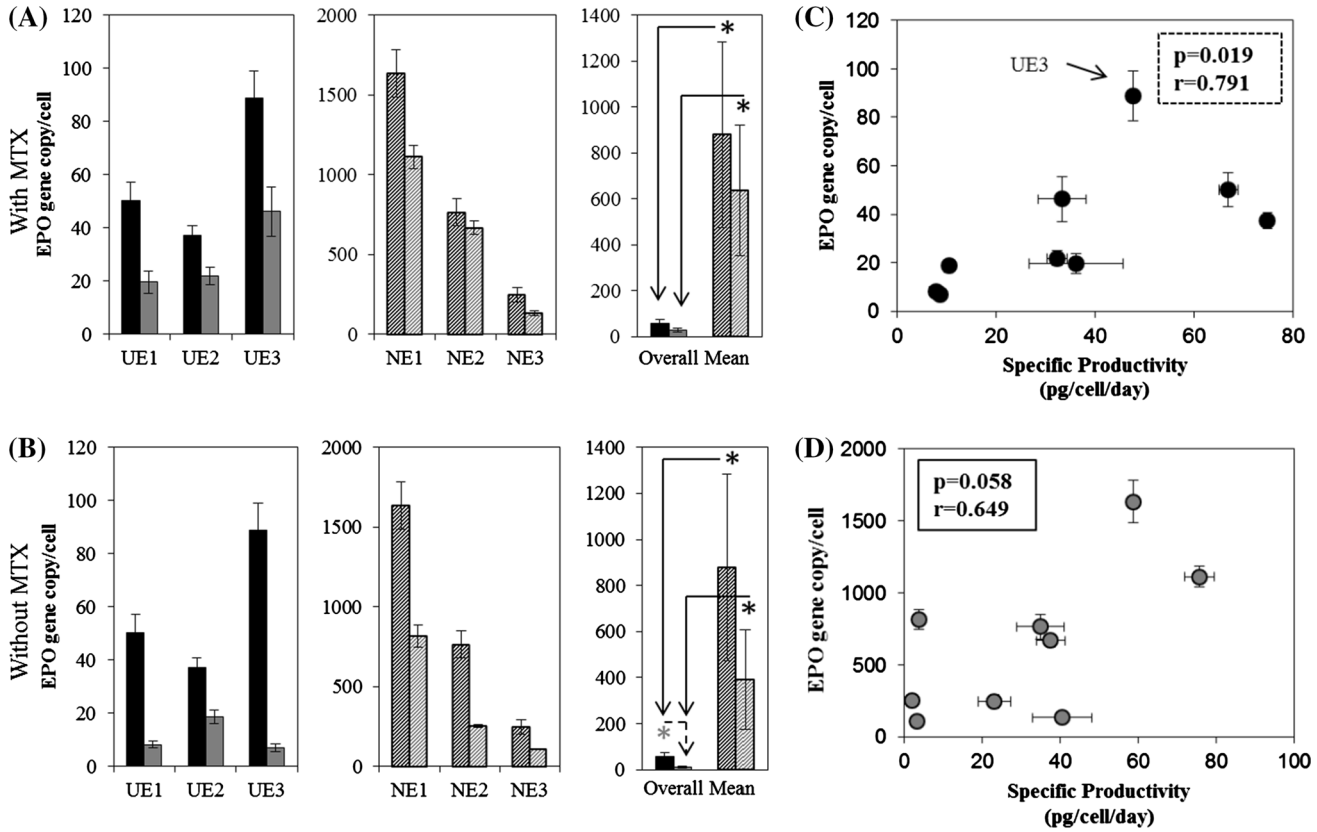

Fig. 6 Analysis of EPO gene copy number over long-term culture by qPCR. Genomic DNA content was normalised using β-actin primers specific for DNA. **a** EPO gene copy number in the presence and **b** absence of MTX. Error bars represent standard deviation (SD) of three replicates. *Asterisks* indicate $p < 0.05$, using independent samples *t* test to compare UCOE and non-UCOE cell lines (at same generation). *Asterisks* indicate $p < 0.05$, using paired samples *t* test to compare early and late generation cultures (for the same group of cell lines). *Filled square* early generation UCOE, *grey square* Late generation UCOE, ▨ Early generation non-UCOE, ▨ Late generation non-UCOE. EPO gene copy number and specific productivity are compared against each other in the scatter plots for **c** UCOE and **d** non-UCOE cell lines. Correlation coefficients (*r*) and statistical significance (*p*) are noted within the scatter plots (*filled circle* UCOE; *grey circle* non-UCOE)

after 77 days of culture in MTX-free medium. These cell lines also showed a decrease in EPO gene copies per cell in the absence of MTX (83 and 50 %, UE1 and UE2, respectively). However, both the UE1 and UE2 cell lines still had relatively high mRNA contents, suggesting that translation and secretion of a complicated product like EPO is a further impediment in the production of recombinant protein production [34–36]. The loss of productivity was as a result of gene copy loss in the UE3 cell line in the absence of MTX, as productivity, EPO gene copy number and mRNA content decreased concurrently. These results suggest that even though UCOE may provide resistance to gene-silencing mechanisms, UCOE-containing cell lines are still prone to instability due to gene copy loss or other mechanisms involved in protein production such as translation, folding or secretion of recombinant protein. Therefore, when using UCOE in combination with MTX amplification for the creation of recombinant cell lines, screening over long-term culture may still be required.

Among the non-UCOE cell lines, the NE1 cell line showed a decrease in gene copy number, while mRNA expression remained constant and specific productivity increased over time in the presence of MTX, which may suggest that the loss of gene copy occurred in transcriptionally passive sites (Table 2). Further decrease in protein production was observed in the absence of MTX, which was reflected in loss of mRNA expression. However, the gene copy number was still relatively high suggesting that some epigenetic silencing might occur in the absence of MTX. The NE2 cell line showed stable productivity and relatively constant gene copy numbers over long-term culture in the presence of MTX, whereas an increase in mRNA expression was observed, which was accompanied by a decrease in mRNA expression. A further loss of EPO gene copy was also observed in the absence of MTX, but gene copy loss was not proportional to the change in protein production and mRNA expression, suggesting that the NE2 cell line also might be experiencing some gene silencing in the

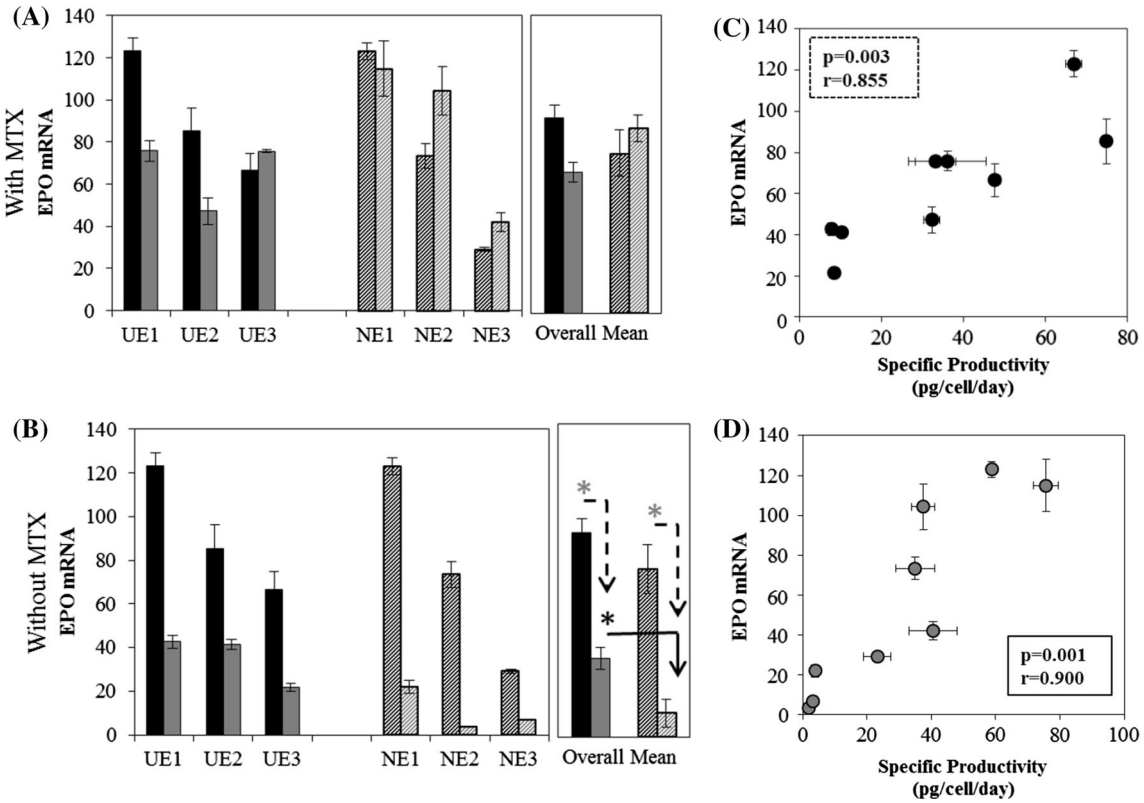

Fig. 7 Analysis of EPO mRNA expression over long-term culture by qRT PCR. Samples were normalised using mRNA β-actin primers. **a** EPO mRNA expression in the presence and **b** absence of MTX. *Error bars* represent standard deviation (SD) of three replicates. *Asterisks* indicate $p < 0.05$, using independent samples *t* test to compare UCOE and non-UCOE cell lines (at same generation). *Asterisks* indicate $p < 0.05$, using paired samples *t* test to compare early and late generation cultures (for the same group of cell lines). *Filled square* early generation UCOE, *grey square* late generation UCOE, ▨ Early generation non-UCOE, ▨ Late generation non-UCOE. EPO mRNA and specific productivity are compared against each other in the scatter plots for **c** UCOE and **d** non-UCOE cell lines. Correlation coefficients (*r*) and statistical significance (*p*) are noted with the scatter plots (*filled circle* UCOE; *grey circle* non-UCOE)

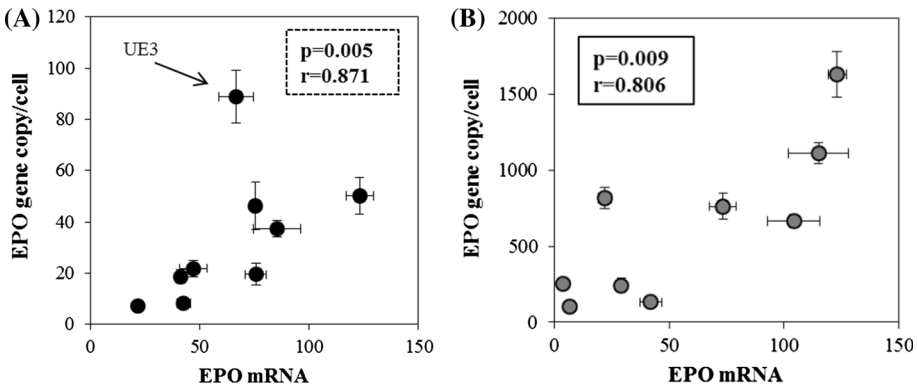

Fig. 8 EPO gene copy number and EPO mRNA are compared against each other in the scatter plots for **a** UCOE and **b** non-UCOE cell lines. Correlation coefficients (*r*) and statistical significance (*p*) are noted within the scatter plots (filled circle UCOE; *grey circle* non-UCOE)

absence of MTX. Although the NE3 cell line showed a decrease in gene copy number over time in the presence of MTX, mRNA expression and protein production increased over long-term culture, suggesting that the remaining EPO genes are in a more active state. This cell line showed significant loss of specific productivity during prolonged culture in the absence of MTX together with decreased EPO mRNA expression and gene copy numbers.

Discussion

The main priority in recombinant protein production is to obtain the maximum volumetric production in a short time period. To achieve high volumetric production, it is desirable to develop cells that can grow to high maximum cell densities and hopefully show high specific productivity. The results presented here demonstrate that high

Table 2 Comparison of specific productivity, gene copy number and RNA expression and their change during long-term culture

Cell Line	Generation	Specific productivity (pg/cell/day) ±SD	% Decrease in specific prod.	EPO gene copy/cell ±SD	% Decrease in gene copy	EPO mRNA ±SD	% Decrease in mRNA
UE1	Early	66.9 ± 1.9		50.1 ± 4.2		123.1 ± 6.3	
	Late + MTX	36.1 ± 9.4	46	19.5 ± 4.3	61	75.7 ± 4.7	38
	Late − MTX	7.8 ± 1.4	88.3	8.2 ± 1.2	83.6	42.7 ± 3	65
UE2	Early	74.8 ± 1		37.4 ± 3.3		85.4 ± 10.9	
	Late + MTX	32.3 ± 2	56.8	21.8 ± 3.2	41.5	47.2 ± 6.2	45
	Late − MTX	10.3 ± 0.1	86.2	18.7 ± 2.7	50	41.4 ± 2.3	51
UE3	Early	47.7 ± 1.3		88.9 ± 10.3		66.6 ± 8	
	Late + MTX	33.3 ± 4.9	30.2	46.2 ± 9.3	48	75.6 ± 0.6	−14
	Late − MTX	8.5 ± 0.6	82.1	7 ± 1.5	93	21.6 ± 1.8	68
NE1	Early	58.7 ± 0.6		1633.1 ± 148.6		123 ± 3.9	
	Late + MTX	75.7 ± 3.9	−28.9	1111.5 ± 71	31.9	114.9 ± 13	7
	Late − MTX	3.8 ± 0.01	93.5	817.4 ± 68.7	49.9	22 ± 3	82
NE2	Early	35 ± 6.1		763.2 ± 85.2		73.4 ± 5.8	
	Late + MTX	37.6 ± 3.6	−7.4	668.2 ± 39.2	12.4	104.3 ± 11	−42
	Late − MTX	1.9 ± 0.5	94.6	252.4 ± 9.7	66.9	3.5 ± 0.1	95
NE3	Early	23.2 ± 4.2		244.3 ± 45.3		29.1 ± 1.1	
	Late + MTX	40.5 ± 7.6	−74.6	131.6 ± 15.7	46	42 ± 4.6	−44
	Late − MTX	3.2 ± 0.01	86	106.2 ± 3.4	56.5	6.7 ± 0.3	77

frequency of positive clones with improved volumetric production can be achieved after one round of MTX amplification using UCOEs in vector construct.

The initial results showed that the non-amplified UCOE cell lines maintained a stable and high volumetric production over long-term culture, whereas two of the non-UCOE cell lines displayed a decrease in volumetric EPO production at the end of prolonged culture. Furthermore, cell lines containing the UCOE element showed significantly higher cell-specific productivity than non-UCOE cell lines. Specific productivity was stable for up to 77 days in the UCOE cell lines. In contrast, a decrease in the cell-specific productivity was observed in two of the non-UCOE cell lines. These results are consistent with previous studies, where inclusion of UCOE in the vector construct resulted in higher levels of recombinant protein production [19, 21, 27, 28, 30] and improved the transgene expression in hematopoietic stem cells [25, 26]. Otte et al. compared the effect of different DNA elements, including UCOEs, S/MARs, cHS4 insulator and anti-repressor elements, on protein expression levels [37]. They reported that the inclusion of UCOE in expression vectors did not result in an increase in reporter gene expression compared to the controls. In their study, CHO-K1 cells were transfected with vector constructs including shortened 2.6 kb UCOE upstream of hCMV-d2eGFP. The neomycin selection marker was located on a different plasmid that was co-transfected with the UCOE-d2eGFP expression construct. In the current study, an 8 kb UCOE element has been used with a DHFR selection marker on the same plasmid. Previous studies have shown that the truncated 1.5 kb UCOE, which is included within the 2.6 kb sequence used by Otte et al., was fully functional [21, 30]. Therefore, a reason for the difference in transgene expression may be attributed to the selectable markers being on the same or separate plasmids. It is likely that UCOE may improve the expression of linked transgene if the selectable marker is located on the same plasmid.

UCOE cell lines displayed significantly higher volumetric EPO production than the non-UCOE cell lines following MTX amplification. The growth analysis showed that the UCOE cell lines reached higher maximum cell densities than the non-UCOE cell lines when MTX is present, suggesting that UCOE cell lines have a higher tolerance to MTX than the non-UCOE group. Cell growth is an important parameter when screening for a suitable cell line for therapeutic protein production as maximum volumetric productivity can be achieved by a combination of a maximum amount of cells and a high specific productivity. There are conflicting reports as to the effect of over-expression of recombinant protein on the growth of CHO cells. Several studies reported that the level of recombinant protein production was inversely correlated with cell-specific growth rate [34, 38, 39], whereas others suggested

no clear correlation between recombinant protein production and cell growth [33, 40]. Recombinant CHO-EPO cell lines used in this study were created with a bi-cistronic vector, which contains an internal ribosomal entry site (IRES) to maintain a linkage between the EPO and DHFR genes. Therefore, providing that the UCOE cell lines displayed higher EPO production than non-UCOE cell lines and EPO and DHFR mRNAs are a part of bi-cistronic transcription unit, it is suggested that the enhanced cell density in these cell lines may be as a result of increase in DHFR protein production, although this was not tested. An increase in the growth rate and viable cell densities was observed over prolonged culture in both UCOE and non-UCOE groups in ± MTX conditions. This may be as a result of cell lines developing alternative resistance to MTX [41]. Despite clonal derivation using limited dilution cloning, cell populations can become quite heterogeneous in terms of growth rate and productivity following MTX amplification [41–43]. Therefore, it is likely that a cell population that was growing more quickly would become dominant during long-term culture, which may result in the higher growth rate and viable cell densities that were observed at the end of long-term culture [31].

UCOE cell lines displayed higher specific productivities than non-UCOE cell lines at early generation. Although UCOE cell lines showed a decrease in cell-specific productivity over prolonged culture in the presence of MTX, this did not outweigh its beneficial effect on overall productivity. The UCOE cell lines showed stable and high levels of volumetric EPO production for up to 77 days in the presence of MTX. The cell-specific and volumetric productivity decreased in both UCOE and non-UCOE cell lines in the absence of MTX. However, these were still significantly higher in UCOE cell lines compared to the non-UCOE group. Analysis of EPO gene copies and mRNA levels showed that the enhanced EPO production observed in UCOE cell lines was as a result of increase in transcription (Table 2).

After amplification, increases in protein production are not always proportional to copy number as the amplified transgene may be in an unfavourable location with varying expression characteristics. In the current study, a significant correlation between specific EPO productivity and EPO gene copy numbers in UCOE cell lines was observed, suggesting the expression of the transgene may be copy number dependent. This observation may also indicate potential mechanistic similarities between UCOE and the LCR and S/MAR elements [44, 45]. Similarly, Zhang et al. incorporated UCOE in lentiviral vectors and reported GFP expression in haematopoietic cells, which was strongly correlated with gene copy number [23]. However, Williams et al. found that UCOE was linked to the hCMV-eGFP expression vector and no significant correlation was indicated between plasmid copies per cell and protein expression [30]. In their study, the cell lines tested had two or three copies of the integrated plasmid per cell, whereas in this study a larger number of EPO gene copies per cells were observed after amplification (between 7 and 89) and a general trend of increased specific productivity with increased EPO gene copies per cells was displayed. The reason for not observing a perfect linear relationship between plasmid copies per cell and EPO production could be that the UCOE plasmid may not be totally resistant to the effects of surrounding chromatin, or it may be due to limitations in experimental technique that result in the variation from a perfectly linear relationship. Another reason may be because other post-translational effects are limiting the production of recombinant protein from mRNA transcripts.

Another observation was that the UCOE cell lines showed a loss of EPO gene copies over long-term culture both in the presence and absence of MTX selection, which resulted in the loss of specific productivity. Earlier studies showed that S/MAR elements may comprise targets of DNA recombination or rearrangement events. Examples may include the many deletions and translocations observed in leukaemia and breast cancer that were related with S/MAR elements [46, 47]. In addition, S/MAR elements may be targets for retroviral integration which often occurs within or close to S/MARs [48]. Taken together, these data could reflect an enhanced recombigenicity in these genomic loci. A more recent study proposed that S/MARs promote homologous recombination [49]. This mode of action might result from their ability to maintain chromatin in an accessible state therefore providing an access to DNA-binding proteins i.e. DNA topoisomerase II, an enzyme that catalyses double-strand breaks [49, 50]. Assuming that UCOEs and S/MARs have a similar overall effect on chromatin structure, it may be suggested that UCOEs may increase the recombigenic events; hence, the use of UCOE in the expression construct may result in the loss of the inserted plasmid copies.

The non-UCOE cell lines were more heterogeneous in terms of their response to long-term culture in the presence of MTX. All three cell lines showed different levels of productivity, gene copy number and mRNA content. Previous studies have also reported that despite being derived from a single clone, cell lines can become quite heterogeneous following MTX amplification [11, 41, 51]. All non-UCOE cell lines had reduced productivity over long-term culture in the absence of MTX, with a ~90 % decrease in productivity, which contrasted with a ~50 % loss of gene copy that was observed in the absence of MTX. This suggests that it is not possible to attribute gene copy loss to the very low level of productivity in these cell lines, as they still contained more than 100 gene copies per

cell. Therefore, it may be that these cell lines are experiencing some gene silencing after removal of MTX selection.

In summary, data presented in this study provide evidence that UCOEs within the expression construct provide a high level of cell-specific productivity and volumetric production of secreted recombinant protein following one round of MTX-mediated gene amplification, in which the time needed for cell line development can be significantly reduced. This study has also demonstrated that UCOE cell lines maintain favourable growth characteristics, a key goal in cell line development, as well as conferring higher transcriptional activity per gene copy. There are several lines of research arising from this project. Future work could be performed to address the question 'Is UCOE maintaining an open chromatin environment following MTX amplification?' Using ChIP technology to investigate potential histone modifications associated with the linked transgene, association with transcription factors and other DNA-binding proteins would provide further information on the effect of UCOE on the local chromatin structure when used in combination with MTX amplification.

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