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RESEARCH

Enhanced CHO Cell-Based Transient Gene Expression with the *Epi*-CHO Expression System

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Abstract Transient gene expression systems in mammalian cells continue to grow in popularity due to their capacity to produce significant amounts of recombinant protein in a rapid and scalable manner, without the lengthy time periods and resources required for stable cell line development. Traditionally, production of recombinant monoclonal antibodies for pre-clinical assessment by transient expression in CHO cells has been hampered by low titers. In this report, we demonstrate transient monoclonal antibody titers of 140 mg/l with CHO cells using the episomal-based transient expression system, *Epi*-CHO. Such titers were achieved by implementing an optimized transfection protocol incorporating mild-hypothermia and through screening of a variety of chemically defined and serum-free media for their ability to support elevated and prolonged viable cell densities post-transfection, and in turn, improve recombinant protein yields. Further evidence supporting *Epi*-CHO's capacity to enhance transgene expression is provided, where we demonstrate higher transgene mRNA and protein levels of two monoclonal antibodies and a destabilized enhanced green fluorescent protein with *Epi*-CHO compared to cell lines deficient in plasmid DNA replication and/or retention post-transfection. The results demonstrate the *Epi*-CHO system's capacity for the rapid production of CHO cell-derived recombinant monoclonal antibodies in serum-free conditions.

Keywords Transient gene expression · CHO · Monoclonal antibodies · Episomal · Mammalian cell culture · Biopharmaceutical

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Introduction

In order to quickly evaluate large numbers of drug candidates, industry has moved toward producing material for early-stage product development via transient gene expression (TGE) systems. TGE yields have improved dramatically over the last decade due to research into a variety of parameters, including; the expression vector [1], the development of advanced chemically defined and serum-free media, their supplementation, and the feeding strategy implemented post-transfection [2, 3] and the direct addition of a variety of chemical agents such as epigenetic modulators [4], cell cycle regulators [5], growth factors [6], and agents thought to directly enhance transfection efficiency and promote plasmid maintenance [3]. Culturing under mild hypothermic conditions has also resulted in significant improvements in expression levels [6–8].

A significant challenge for TGE is rapid plasmid copy number dilution during cell division, which results in a significant reduction in recombinant protein titers at the

end stages of the process. To address this issue, episomal systems have been designed to maintain or increase plasmid copy number after transfection. TGE studies have frequently utilized HEK293 cells stably expressing Epstein-barr virus nuclear antigen 1 (EBNA-1) (HEK.EBNA) [9, 10]. EBNA-1 drives the replication and maintenance of plasmid DNA containing the EBV latent origin of replication, *OriP,* which in turn promotes elevated and prolonged expression of the recombinant protein of interest [10–13]. Recent reports using the HEK.EBNA system have generated mAb titers in excess of 1 g/l [14], whereas, the highest reported mAb titers generated from CHO cell-based TGE in the literature are considerably lower and range from 60 to 90 mg/l [3, 7, 8]. Stably transfected CHO cells remain the host of choice for the large-scale commercial production of therapeutic proteins and yields in these systems now routinely exceed 1 g/l. As a result, there is strong demand from industry for the development of efficient CHO cell-based, TGE systems with increased productivity [15].

Unlike human or simian-derived lines, rodent cell lines such as CHO are reported to be non-permissive for EBV-based plasmid replication [12, 16]. Interestingly, the capacity to maintain *OriP*-containing plasmid DNA over many generations and the potential for EBNA-1 to act as a transcriptional enhancer has been demonstrated in some murine cell lines [17]. For this reason, vectors containing the EBNA-1 gene and *OriP* sequence such as pCEP4 (Invitrogen), pEAK8, and pEAK12 (formerly available from Edge Biosystems, Gaithersberg, MD) have been used for transient expression with CHO cells [7, 13, 18].

Previously we reported on the development of *Epi*-CHO, an episomal transient expression system for CHO cells [18]. This system utilizes elements from the mouse polyomavirus (PyV)—Py virus origin of replication (*PyOri*) and the Py large-T antigen (PyLT)—to promote the replication of plasmid DNA in CHO cells after transfection. In addition, it also employs EBNA-1 and *OriP* from EBV to facilitate the retention and segregation of plasmid DNA during cell division. In practice, the *Epi*-CHO system has two components: a stable, suspension-adapted CHO cell line producing PyLT (CHO-T) and the expression plasmid pPyEBV, which contains the EBNA-1 gene, *PyOri*, and *OriP* sequences. Previously, we demonstrated functional plasmid replication with the *Epi*-CHO system. Additionally, with the incorporation of a media replacement strategy, increased production of human growth hormone was witnessed after 11 days compared to non-episomal controls.

In this report, we have detected EBNA-1 and PyLT protein in transfected cells as well as demonstrated enhanced mAb mRNA levels with the *Epi*-CHO system when compared to non-episomal controls. In addition, we show the effectiveness of the *Epi*-CHO system for the production of recombinant mAbs in batch culture, achieving a titer of 140 mg/l, which represents a 64% increase over the previously highest reported level of transient mAb production in CHO cells [8].

Materials and Methods

Plasmid Construction

pEGFP-C1 (Clontech, Palo Alto, CA), pPyEBV, and vectors expressing d2EGFP were previously described [18]. pPyEBV was modified to express the heavy chain (HC) and light chain (LC) of an IgG1 (Ab2) on separate plasmids (pPyEBV-Ab2HC and pPyEBV-Ab2LC) and an IgG4, (Ab1) on the same plasmid (pPyEBV-Ab1). Similar Ab1 variants were created for pPyOri, pBasic [18], and pCEP4 (Invitrogen, Carlsbad, CA). Plasmid DNA for transfection was purified using the Qiagen Plasmid MaxiPrep Kit (Qiagen, Valencia, CA).

Cell Culture

Suspension CHO-T [18] and CHO-K1 (ATCC CCL-61) cells were maintained in CHO-S-SFMII serum-free medium (Invitrogen) supplemented with 4 mM GlutaMax (Invitrogen) and 0.4% (v/v) anti-clumping agent (ACA) (Invitrogen). Unless otherwise stated, cells were maintained at 37°C in 7.5% CO_2 and 80% humidified air. Cell density and viability were determined using the Cedex HiRes Cell Counter (Innovatis AG, Bielefeld, Germany).

Liposome-Mediated Transfections

Cells in mid-logarithmic growth phase were diluted to 1.5×10^6 cells/ml in growth media (without ACA). For transfection, Lipofectamine 2000 (Invitrogen) was complexed with plasmid DNA at a ratio of 1:2.5 (w:v) using 2 μg DNA/ml culture. Each component was diluted separately in CHO-S-SFMII (12.5% of initial culture volume) and incubated for 10 min at room temperature (RT), mixed, then incubated for a further 25 min at RT and added to cells. Small-scale transfections (2–50 ml) were incubated at 37°C for 4–6 h post-transfection on an orbital shaker at 170 rpm. Cultures were then diluted with an equal volume of growth medium supplemented to achieve final concentrations of 0.4% (v/v) ACA, 100 μg/l IGF-1 (Novozymes, Adelaide, Australia) and 8 mM GlutaMax. Cultures were then incubated at 32°C (mAb expressing transfectants) or 37°C (d2EGFP expressing transfectants).

EGFP and mAb Quantification

EGFP and d2EGFP expression was quantified by flow cytometry using the FACSAria II (BD, San Jose, CA). Images of cells expressing d2EGFP were acquired using the Olympus CKX41 fluorescent inverted microscope (Olympus, Japan). mAb concentrations in culture supernatants were quantified by ELISA [19].

SDS-PAGE and Western Blotting

CHO cells ($\sim 1 \times 10^6$ cells) were lysed and analyzed with SDS-PAGE using the NuPAGE system (Invitrogen). Separated proteins were then transferred to a BioTrace PVDF membrane (Pall Corporation, Penascola, FL) and probed for the detection of PyLT and EBNA-1 using rat anti-polyoma virus large-T antigen and goat anti-EBV nuclear antigen polyclonal antibodies (Abcam, Cambridge, UK). Horseradish-peroxidase conjugated rabbit-anti rat IgG (for PyLT) and rabbit-anti goat IgG (EBNA-1) (Zymed, South San Francisco, CA) were used as secondary antibodies and detected by chemiluminescence using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

qRTPCR

Total RNA extracted from $\sim 1 \times 10^6$ cells using the RNeasy RNA Extraction Kit (Qiagen) was treated with DNaseI (Invitrogen) and converted to cDNA using the SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen) with oligo dT primers, all performed according to the manufacturer's protocol. cDNA samples for qPCR were diluted 1:5 in RNase free water, with qPCR reactions (in triplicate) performed using the Sybr-Green PCR Master Mix (Applied Biosystems, Foster City, CA) with the ABI 7900HT Real-Time PCR System (Applied Biosystems). Primers for the amplification of Ab1HC (forward 5′-TGG AGT GGG AGA GCA ATG G-3′, reverse 5′-CGG TTA GCC TGC TGT AGA GGA A-3′), Ab1LC (forward 5′-CTC TGT TGT GTG CCT GCT GAA-3′, reverse 5′-CTG GGA GTT ACC CGA TTG GA-3′), EBNA-1 (forward 5′-GGG AGA CGA CTC AAT GGT GTA AG-3′, reverse 5′-CGG TGT GTT CGT ATA TGG AGG TAG T), and glyceraldehyde-phosphate dehydrogenase (GAPDH) (forward 5′-AAG GCT GTG GGC AAA GTC AT-3′, reverse 5'-CAT ACT TGG CAG GTT TCT CC) were purchased from Geneworks (Adelaide, Australia). Results were analyzed using SDS software (v 2.2.2) (Applied Biosystems) with relative mRNA levels of Ab1HC and Ab1LC quantified by normalization to those of GAPDH.

Results and Discussion

Prolonged, Enhanced mAb and d2EGFP Expression Using the *Epi*-CHO System

To examine *Epi*-CHO's capacity to express mAbs, we monitored the expression of an IgG4 mAb (Ab1) using our optimized lipofection method over 14 days. CHO-T cells transfected with pPyEBV-Ab1 ("*Epi*-CHO") attained the highest yield of mAb compared to the remaining

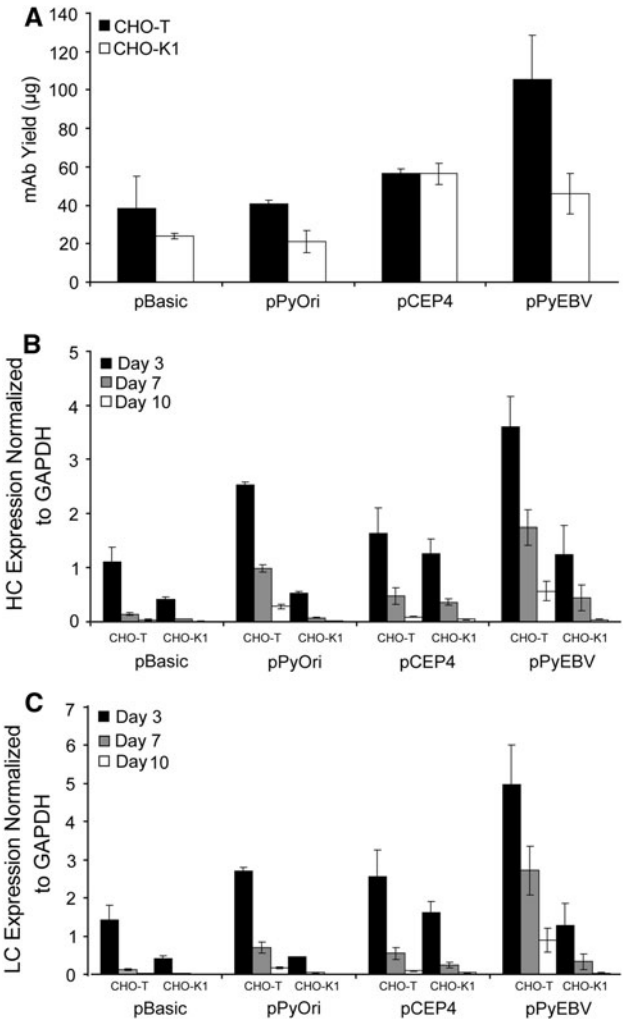

Fig. 1 Effects of plasmid replication and retention on mAb expression. CHO-T and CHO-K1 cells were transfected by lipofection in a final volume of 5 ml CHO-S-SFMII in 6-well plates with different plasmids expressing the mAb Ab1: pPyEBV-Ab1 (plasmid replication and retention competent), pCEP4-Ab1 (plasmid replication deficient), pPyOri-Ab1 (plasmid retention deficient), or pBasic-Ab1 (plasmid replication and retention deficient). **a** The final yield of Ab1 accumulated over 14 days from all transfectants was quantified by ELISA. **b**, **c** Total RNA extractions and cDNA conversion were performed on $\sim 1 \times 10^6$ transfected cells collected at days 3, 7, and 10 post-transfection. Using qRT-PCR, mRNA levels of Ab1HC and Ab1LC were quantified and normalized to GAPDH mRNA levels. $n = 3 \pm$ SD

transfectants, which were deficient for plasmid replication (pCEP4 transfectants), plasmid retention (pPyOri transfectants), or both (pBasic transfectants) (Fig. 1a). When compared to CHO-K1, pCEP4-Ab1 transfectants (for both cell lines) generated similar yields of mAb. This was expected since the effects of EBNA-1/*OriP* should be the same, regardless of the CHO cell line used. However, yields in CHO-T cells were higher when using pPyOri and pPyEBV compared to CHO-K1 transfectants (Fig. 1a). To further evaluate the ability for *Epi*-CHO to promote prolonged TGE, we monitored mRNA transcript levels post-transfection by qRT-PCR. mRNA levels for both Ab1HC and Ab1LC genes were higher with pPyEBV-transfected CHO-T cells compared to those observed with the control vectors and CHO-K1 transfectants (Fig. 1b, c). These results demonstrate that EBNA-1/*OriP* mediated plasmid retention combined with plasmid replication driven by PyLT and its cognate-binding elements promote prolonged expression when compared to each of the elements in isolation.

To directly examine the ability of the *Epi*-CHO system to prolong the period of TGE, we utilized vectors that expressed a destabilized variant of EGFP (d2EGFP), which has a half-life of approximately 2 h [20], allowing analysis of new protein synthesis without the bias of protein accumulation over time. The mean d2EGFP fluorescence intensity (Fig. 2a) and the number of fluorescent cells (Fig. 2b) were consistently higher in CHO-T cells transfected with pPyEBV-d2EGFP ("*Epi*-CHO") compared to the control vectors and all CHO-K1 transfectants. The benefits of the episomal elements comprising the *Epi*-CHO system were previously demonstrated by expression studies which incorporated selective pressure [18]. Here, we have demonstrated the maintenance of higher levels of GFP over prolonged periods in the absence of selective pressure, in accordance with the notion that TGE is principally based on non-selective conditions.

Together these results also indicate that, while the system does not provide an indefinite plasmid replicative or maintenance effect, crucially *Epi*-CHO provides substantial improvements in protein expression (compared to a non-episomal equivalent) over the timescale of a typical batch or fed batch culture. Similar trends can also be seen with reports using HEK.EBNA systems, whereby expression declines at a greater rate after 7 to 12 days post-transfection in the presence of EBNA-1 driven plasmid replication [14, 21].

Expression of PyLT and EBNA-1 in CHO Cells

Previously, a functional copy number assay was performed to demonstrate the presence of replicated plasmid DNA post-transfection with the *Epi*-CHO system; however, evidence for the expression of PyLT and EBNA-1 protein in CHO cells had not been shown [18]. Direct observation of EBNA-1 expression from pCEP4-derived vectors has also not been reported in detail [22]. We confirmed expression of PyLT protein in CHO-T cells (Fig. 3a) and in addition, EBNA-1 protein (Fig. 3b) and transcript (Fig. 3c) was detected in all transfected CHO cells. EBNA-1 mRNA transcript and protein levels are significantly higher with the *Epi*-CHO system compared to pCEP4-transfected

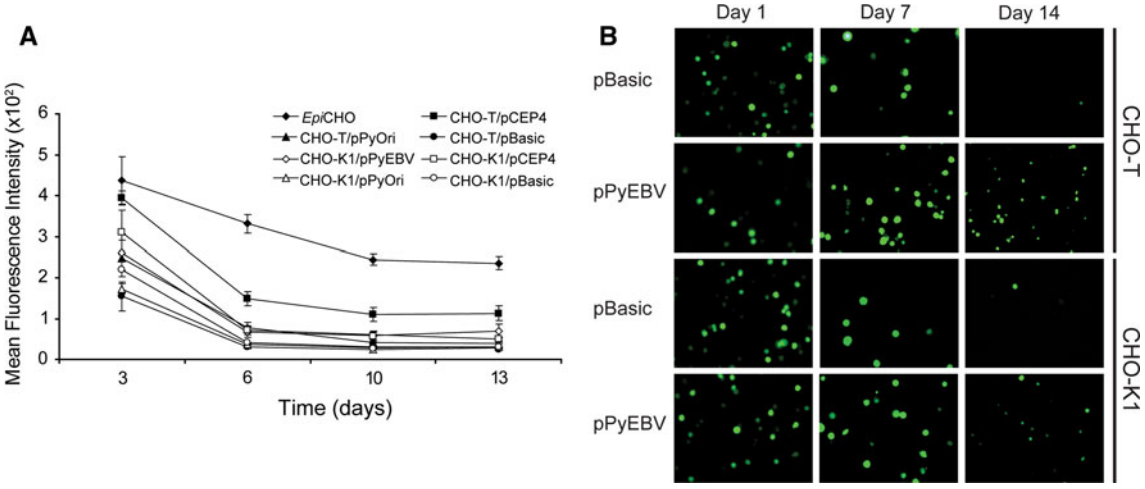

Fig. 2 Prolonged expression of a destabilized EGFP for 14 days. CHO-T and CHO-K1 cells were transfected by lipofection in a final volume of 5 ml CHO-S- SFMII in 6-well plates with pPyEBV-d2EGFP, pCEP4-d2EGFP, pPyOri-d2EGFP, or pBasic-d2EGFP and monitored for d2EGFP expression over 14 days post-transfection. Cultures were diluted with an equal volume of fresh medium at days 3, 6, 10, and 13 post-transfection. **a** The mean d2EGFP fluorescence intensity of viable transfected cells was quantified by flow cytometry at days 3, 6, 10, and 13 post-transfection. **b** Images of CHO-T and CHO-K1 cells transfected with pPyEBV-d2EGFP and pBasic-d2EGFP were obtained using fluorescent microscopy (10× objective used on days 1 and 7) (4× objective used on day 14). $n = 3 \pm$ SD

CHO-T cells and both CHO-K1 transfectants. With the latter, EBNA-1 expression is visible by day 5 but rapidly declines after this point, which is also the case for CHO-T cells transfected with pCEP4. Conversely, in CHO-T/pPyEBV transfectants, EBNA-1 expression is maintained over a longer period (Fig. 3b). Since EBNA-1 may mediate its effects through both plasmid maintenance and transcriptional enhancement [17], the increased expression could also be a contributing factor to the elevated recombinant protein yields seen with the *Epi*-CHO system. However, the qRT-PCR data suggest that expression of EBNA-1 is low in comparison with the transcript levels recorded for Ab1HC and Ab1LC (Fig. 1b, c). Further improvements in plasmid retention rates with *Epi*-CHO could be obtained through the examination of alternative, stronger promoters for EBNA-1 or through the provision of additional factors to enhance the EBNA-1/*OriP* interaction in rodent cells [9, 22].

Elevated mAb Yields Generated with Improved Growth Media

The removal of animal-derived serum from all mammalian cell bioprocesses is highly desired by regulatory authorities, driving significant research into the development of serum-free and chemically defined media for suspension cell growth. Such formulations have allowed significant improvements in viable cell growth and their application to TGE has enhanced recombinant protein yields [3, 23]. Furthermore, while CHO-S-SFMII allows for efficient transfection with Lipofectamine 2000; it is not suited to prolonged high-density cell growth. To harness *Epi*-CHO's capacity to maintain expression for greater than 7–10 days, various chemically defined and serum-free media were screened for their ability to facilitate increased cell densities, prolonged cell viability post-transfection, and in turn enhance recombinant protein yields. These media included CD CHO (Invitrogen), SFM4CHO and CDM4CHO (HyClone), and ProCHO5 and PowerCHO2 (Lonza). Growth studies at 32 and 37°C provided significant improvements in viable cell growth compared to CHO-S-SFMII, with ProCHO5 providing higher, sustained viable cell densities and prolonged cell viability over 15 days (data not shown). However, attempts to use our optimized lipofection protocol in any of the serum-free and chemically defined growth media above generated poor transfection efficiencies and low mAb yields (data not shown). These results reflect data in reports which have demonstrated that the presence of polyanionic molecules (such as dextran sulfate) in media at the time of transfection can inhibit transfection through neutralization of cationic transfection complexes [24, 25]. The alternate approach taken involved transfecting CHO-T cells in CHO-S-SFMII, diluting cultures with CHO-S-SFMII, CD CHO, CDM4CHO, ProCHO5, PowerCHO2, or SFM4CHO post-transfection and transferring cultures to mild-hypothermia 4 h post-transfection, with the latter change incorporated due to reports demonstrating improved TGE in transfected CHO cells when transferred to mild-hypothermia post-transfection [6, 7]. All media (with the exception of CDM4CHO) attained higher Ab2 concentrations than the CHO-S-SFMII control, with ProCHO5-fed cells obtaining the highest at ~140 mg/l (Fig. 4b). This medium also supported sustained viable cell densities above 2.5×10^6 cells/ml from days 7 to 15, with viability above 90% during this same period (Fig. 4c, d). The percentage of viable cells expressing EGFP was comparable between all transfectants (Fig. 4a). Similar

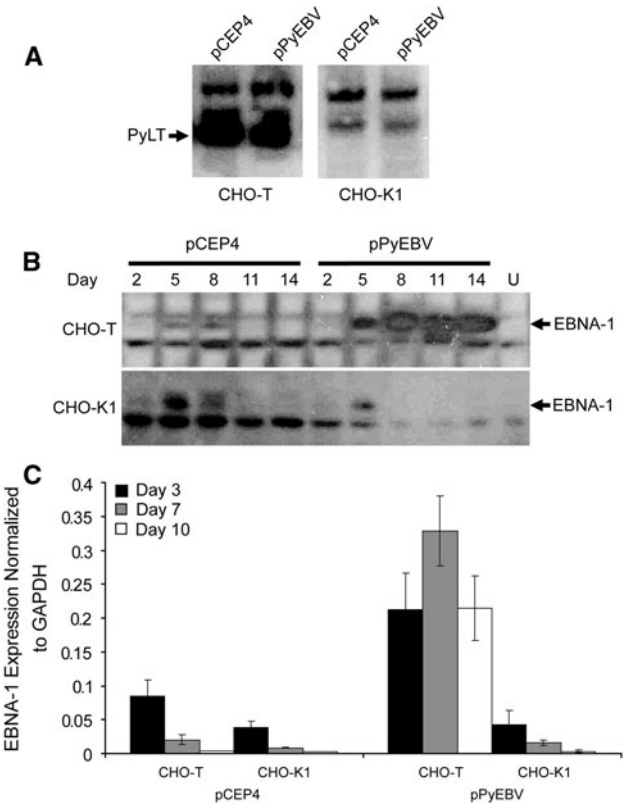

Fig. 3 PyLT and EBNA-1 expression detected in transfected CHO cells. CHO-T and CHO-K1 cells were transfected using lipofection in a final volume of 5 ml CHO-S-SFMII in 6-well plates with plasmids expressing the mAb Ab1: pPyEBV-Ab1 or pCEP4-Ab1. **a** Total protein extracted from ~5×10^4 cells collected at day 2 post-transfection were examined for PyLT expression by western blotting. **b** Similarly, EBNA-1 expression in CHO-T and CHO-K1 transfectants was examined at days 2, 5, 8, 11, and 14 post-transfection by western blotting. Untransfected CHO-T cells (represented by "U") were used as a negative control. **c** Total RNA extractions and cDNA conversions were performed on ~1×10^6 cells collected at days 3, 7, and 10 post-transfection. The mRNA levels of EBNA-1 were quantified and normalized to those of GAPDH using qRT-PCR. $n = 3 \pm$ SD

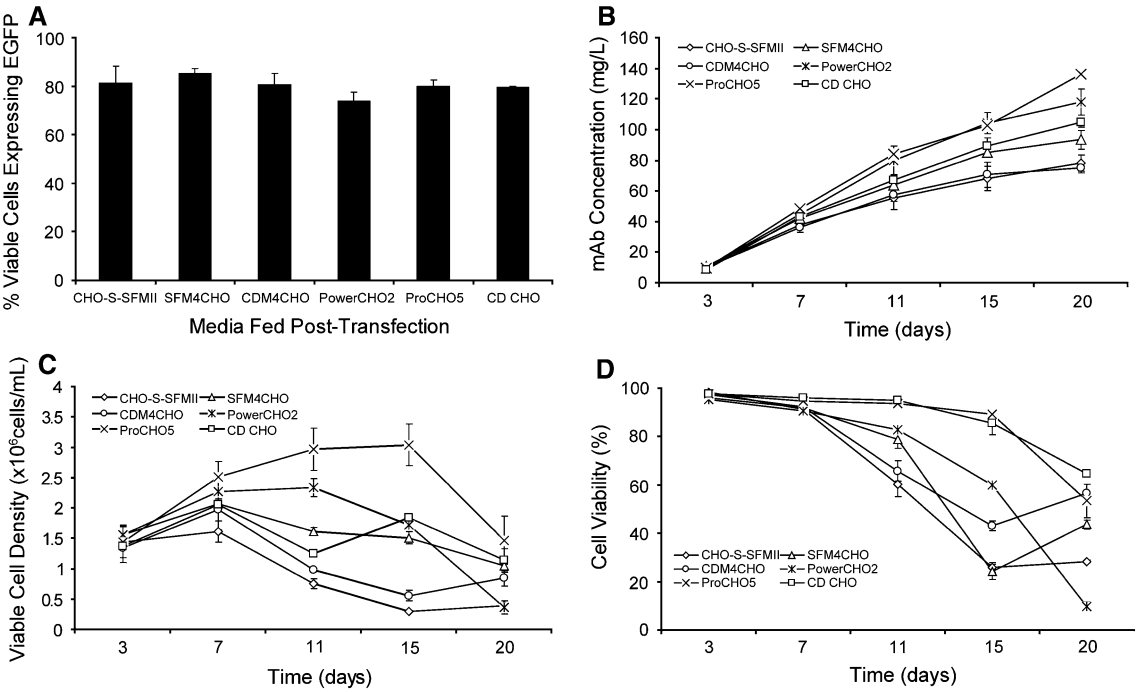

Fig. 4 High levels of mAb produced using the *Epi*-CHO system after 20 days. CHO-T cells were co-transfected using lipofection with pPyEBV-Ab2HC/pPyEBV-Ab2LC/pEGFP-C1 at a ratio of 47.5:47.5:5, respectively. Transfections were performed in 25 ml CHO-S-SFMII using 125-ml shake flasks. 4 h post-transfection, cultures were diluted (to $\sim 0.75 \times 10^6$ cells/ml) with an equal volume of CHO-S-SFMII, SFM4CHO, CDM4CHO, PowerCHO2, ProCHO5, or CD CHO supplemented with 8 mM GlutaMax, 0.4% (v/v) ACA and 100 μg/l IGF-1 to a final volume of 50 ml. Cultures were maintained for 20 days post-transfection without additional feeding. **a** The percentage of viable cells expressing EGFP was quantified by flow cytometry on day 3 post-transfection to ensure transfection efficiency was similar with all cultures. **b** Ab2 levels in the supernatant were quantified by ELISA at days 3, 7, 11, 15, and 20 post-transfection. **c**, **d** Viable cell density and cell viability was monitored over the 20 days, with samples collected at days 3, 7, 11, 15, and 20 post-transfection. $n = 2 \pm$ SD

studies using an optimized PEI transfection method with ProCHO5 and incorporating mild-hypothermia had previously achieved 60–80 mg/L [7]. While the studies presented here were essentially run in batch mode and at mild-hypothermia, the feed post-transfection allowed for sustained high viable cell densities over a longer time period. Providing such conditions was critical in harnessing *Epi*-CHO's capacity to enhance and prolong TGE post-transfection.

Conclusions

In this report, we have evaluated the performance of the *Epi*-CHO system using control vectors that were either deficient in plasmid replication, plasmid retention, or both to highlight the importance of plasmid amplification and maintenance in enhancing recombinant protein yields. Despite the growing popularity of TGE systems (including CHO cell-based systems), transfection efficiency, product yields, and cost of goods continue to be the key issues. The *Epi*-CHO system has been designed to overcome some key limitations associated with CHO cell-based TGE [18]. It provides the ability to replicate plasmid DNA after transfection and maintain extrachromosomal plasmid DNA over several cell generations in order to improve recombinant protein titers. When compared to a non-episomal system, we have demonstrated that elevated and prolonged recombinant protein expression can be achieved using the *Epi*-CHO system. We have confirmed the maintenance of recombinant mAb and EBNA-1 expression by qRT-PCR and western blotting versus control systems that were either deficient in plasmid replication and/or retention functions. Furthermore, through the screening of various media, we have developed a serum-free transient process incorporating mild-hypothermia with an optimized lipofection protocol that has generated up to 140 mg/l of mAb, with no additional feeding post-transfection. This represents a 64% increase on the highest previously reported titer in CHO cells [8]. The *Epi*-CHO system represents a high-producing CHO cell-based TGE system, providing substantial recombinant protein yields.

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