


RESEARCH ARTICLE

WILEY

Serum-free suspension cultured human cells can produce a high-level of recombinant human erythropoietin

Luciano Costa e Silva¹ | Matheus Henrique dos Santos¹ | Tarik Reis Heluy¹ |
 Rafael Tagé Biaggio¹ | Alexander Rodrigo Ferreira² | Jonathan Milhomens² |
 Simone Kashima² | Wassim El Nemer^{3,4,5} | Sara El Hoss^{3,4,5} | Virgínia Picanço-Castro² |
 Dimas Tadeu Covas² | Kamilla Swiech^{1,2} 

¹School of Pharmaceutical Sciences of
 Ribeirao Preto, University of Sao Paulo,
 São Paulo, Brazil

²Center for Cell-based Therapy/Blood
 Center of Ribeirão Preto, Ribeirão Preto
 Medical School, University of São Paulo,
 São Paulo, Brazil

³Université de Paris, Paris, France

⁴Institut National de la Transfusion
 Sanguine, Paris, France

⁵Laboratoire d'Excellence GR-Ex, Paris,
 France

Correspondence

Kamilla Swiech, School of Pharmaceutical
 Sciences of Ribeirao Preto, University of
 Sao Paulo, Ribeirao Preto, Sao Paulo,
 Brazil.
 Email: kamilla@fcfrp.usp.br

Funding information

Conselho Nacional de Desenvolvimento
 Científico e Tecnológico, Grant/Award
 Number: 573754-2008-0; Coordenação de
 Aperfeiçoamento de Pessoal de Nível
 Superior, Grant/Award Number: 1544865;
 Fundação de Amparo à Pesquisa do Estado
 de São Paulo, Grant/Award Numbers:
 2008/578773, 2012/04629-8, 2013/08135-2;
 Coordination for the Improvement of
 Higher Education Personnel

Abstract

Currently, the majority of commercial recombinant therapeutic proteins are produced in nonhuman expression systems. Human cells are capable of producing recombinant proteins with post-translational modifications that are more similar to native proteins which reduce immunogenicity. This article describes the potential of the human cell lines Sk-Hep-1, HKB-11, and Huh-7, cultured in serum-free suspension conditions, to produce a complex recombinant glycoprotein, human erythropoietin (EPO). Recombinant cell lines were generated by lentiviral transduction and sorted by flow cytometry. All the recombinant cells presented high-specific cell growth rates and a high level of rhEPO production. Maximum rhEPO concentrations achieved by Sk-Hep-1, HKB-11, and Huh-7 cells were 112.5 µg/mL, 112.7 µg/mL, and 571 µg/mL, respectively. The levels of rhEPO production by Huh-7 cells were higher than the levels commonly reported in the literature for serum-free suspension cultures. The rhEPO produced demonstrated biological activity measured through in vitro differentiation of CD34+ cells. In view of this, we demonstrate that Sk-Hep-1, HKB-11, and Huh-7 cell lines have good characteristics to be used as host cells for the production of complex recombinant glycoproteins, with special emphasis on Huh-7 due to enhanced EPO production.

KEYWORDS

erythropoietin, human cell lines, recombinant glycoproteins, serum-free suspension culture

1 | INTRODUCTION

Mammalian cells are the preferred expression system for recombinant therapeutic protein production because they can synthesize proteins with molecular structures and biochemical properties similar to those naturally occurring in

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2020 The Authors. *Engineering Reports* published by John Wiley & Sons, Ltd.

humans.^{1,2} Chinese hamster ovary (CHO) cells are the most commonly used mammalian cell-based expression system,³ being responsible for 60%-70% of all recombinant products produced. This can be explained by the fact that CHO cells are capable to produce recombinant proteins at high productivity with titers up to grams per liter of protein, to synthesize human-like glycosylation patterns, are well adapted to serum-free suspension cultures, are refractory to a number of human viruses, and have established regulatory track record.^{4,5} Other cell lines, such as baby hamster kidney cells (BHK21), murine myeloma cells (NS0 and Sp2/0), human embryonic kidney cells 293 (HEK293), and human fibrosarcoma cell (HT-1080) are used for therapeutic protein production as well.^{6,7} Nonhuman mammalian cells have proven reliable for recombinant protein production, synthesizing proteins that are similar to those naturally occurring in humans. However, there are some differences in glycosylation patterns among mammalian species, which have had a significant impact on the quality of the recombinant protein produced.^{4,8} CHO cells, for instance, may not adequately perform glutamic acid γ -carboxylation, post-translational modification required for activity of some therapeutic recombinant proteins such as coagulation factors VII and IX.³

The main structural differences between recombinant proteins produced in nonhuman vs human mammalian cells include the presence of the nonhuman sialic acid *N*-glycolylneuraminic acid (Neu5Gc) and the absence of $\alpha(2,6)$ linkages of sialic acid to galactose.^{3,9} The presence of these *N*-glycans can contribute to immunogenicity and neutralization of the corresponding recombinant therapeutic proteins since humans have demonstrated the ability to produce circulating antibodies against them.¹⁰⁻¹⁴ Neu5Gc uptake either via consumption of animal-sourced food or of contaminated biopharmaceuticals may lead to the onset of chronic inflammation and is suspected to increase the risk of cancer and heart attack.¹⁵

Using human cells for recombinant protein production is a viable way to overcome issues related to nonhuman cell-based expression systems. Human cells can generate more natural, human-like recombinant proteins with higher quality characteristics including lower immunogenicity, greater biological activity, and increased half-life.¹⁶ There are human cell-based expression systems that have demonstrated promising outcomes for recombinant protein production⁶: AGE1.HN (Probigen AG; a human cell line developed from primary neuronal precursor cells), CAP cells (CEVEC's Amniocyte Production; an immortalized cell line based on primary human amniocytes), HEK293, PER.C6 (Crucell; a human embryonic retina cell line), and HT1080 (human fibrosarcoma cell).¹⁷⁻²¹ However, variations in glycan structure persist even among human cells, for example between differentiated normal human cells and tumor cells or when comparing erythropoietin (EPO) produced recombinantly to EPO obtained from natural sources.²²⁻²⁵

The human cell lines used in this study, Sk-Hep-1, HKB-11, and Huh-7, were already successfully used for the production of recombinant coagulation factors VII, VIII, and IX²⁶⁻³⁰ and adapted to serum-free suspension cultivation.³¹ In this research, we selected human EPO as a model protein to further evaluate the potential of these cells to produce complex recombinant glycoproteins.

2 | MATERIAL AND METHODS

2.1 | Cell Lines, culture conditions, and vectors

The HKB-11 (ATCC CRL-12568), Sk-Hep-1 (ATCC HTB-52), and Huh-7 (cordially provided by Prof. Dr. Carlos Augusto Pereira from Butantan Institute) cells were previously adapted to serum-free suspension culture.³¹ HKB-11 and Sk-Hep-1 cells were cultured in HyClone CDM4CHO medium (GE, lifesciences), supplemented with 6 mM Glutamax (ThermoFischer Scientific) and 0.1% of Pluronic F-68 (ThermoFischer Scientific) and the Huh-7 cells were cultured in CD 293 AGT medium (ThermoFischer Scientific) supplemented with 2 mM Glutamax (ThermoFischer Scientific). Cells were maintained in 125 mL Erlenmeyer flasks containing 20 mL of culture medium at 150 rpm orbital stirring, in a 5% CO₂ environment at 37°C. Subcultures were performed every 2-3 days, at a seeding concentration of 3×10^5 cells/mL. The lentiviral vectors p1054, pCMV Δ R 8.91³² containing the HIV-1 gag, pol, rev and tat genes, and pMD2.VSVG³³ encoding the VSV-G envelope were kindly provided by Dra. Virginia Picanço-Castro of the Hemocentro of Ribeirão Preto—University of São Paulo, Brazil. The p1054 vector has the synthetic sequence of the gene encoding human EPO, an ampicillin resistance gene and a reporter gene encoding the eGFP protein.

2.2 | Lentiviral production and titration

For lentiviral production, HEK293T cell line was cultured in 75-cm² T-flasks containing 10 mL of DMEM culture medium supplemented with 10% fetal bovine serum and transfected using a mixture of DNA and the cationic polymer polyethyleneimine. For transfection, the three plasmids were used in the following proportions: 20 µg of vector p1054, 13 µg of vector pCMVΔR8.91, and 7 µg of vector pMD2 VSVG. After 48 hours of transfection, the supernatant was collected, centrifuged at 450×g for 5 minutes at 4°C, filtered (0.45 µm filter), and stored at −80°C. A gene transfer assay was performed in HEK293 to titer viral particles produced in HEK293T. Briefly, HEK 293 cells were plated at 3×10^5 cells/well in six-well plates and aliquots of 10, 20, 50, 100, and 500 µL of the lentivirus-containing supernatant were added to the cells. Cells were maintained in culture for 4 days and then trypsinized for flow cytometry analysis (GFP measure) for viral titer quantification.

2.3 | Generation of stable rhEPO producing-cell lines

Aliquots containing 1 MOI (Multiplicity of infection) of the produced lentiviral particles were added to the cultures of SK-Hep-1, HKB11, and Huh-7 cell lines. At the time of transduction, 1 mL of the cell suspensions were seeded at a concentration of 3×10^5 cells/well in a 6-well plate. After 72 hours, the cells were taken for analysis of green fluorescent protein (GFP) expression as well as cell viability by flow cytometry. All assays were performed in duplicate. To obtain a homogeneous recombinant cell population, recombinant cells were sorted by flow cytometry using the BD FACSAria cytometer. The selected cells were recultured, and after the third passage, they were reanalyzed for the percentage of postsorting GFP positive cells. The cells were then expanded and frozen to create the recombinant cell banks.

2.4 | Selection of the best media for rhEPO production

With the objective to select an appropriate culture medium for the production of rhEPO, two culture media were evaluated for each cell: the media where cells presented better growth rates and viability during adaptation process³¹ and the Freestyle 293 Expression media (Thermo Fischer Scientific). For this purpose, 1×10^6 cells/mL of each cell line was inoculated into 20 mL of each media in 125 mL Erlenmeyer flask and cultured under the aforementioned culture conditions. The production of rhEPO was monitored for 72 hours. Every 24 hours, 1 mL aliquots were removed, and after centrifugation at 1200 rpm the supernatants were frozen at −20°C for further analysis. Cell densities and viability were measured by the dye exclusion method using Trypan blue (0.4%) in a hemocytometer. The level of rhEPO was determined by Human Erythropoietin ELISA Kit (ab119522) according to the manufacturer's instructions. EPO mass concentration (µg/mL) was estimated from the IU/mL concentration obtained from ELISA using the conversion factor of 150 U/µg, based on the third WHO International Standard for Erythropoietin (National Institute for Biological Standards and Control (NIBSC code: 11/170)).³

2.5 | Recombinant cell pool characterization

2.5.1 | Gene copy number determination

The number of copies of the 1054-EPO lentiviral vector inserted into the cell genome was calculated by the quantitative PCR (Polymerase Chain Reaction) technique. Genomic DNA was extracted from cells using a DNeasy Tissue kit (Qiagen, Valencia, California), according to the manufacturer's instructions. For amplification of the 1054-EPO lentiviral vector, primers, and TaqMan hydrolysis fluorescence probes (Thermo Fisher Scientific) specific for LTR (Long Terminal Repeat) viral region were used. The total reaction volume was 20 µL per well, 10 µL of TaqMan Master Mix, 1 µL containing the LTR 5' primer (5'-TGCTTTTGGCTTGTTACTGTGGG-3'), 1 µL LTR 3' (5'-CTAGTTACCAGAGTCACACAA-3') 0.5 µL of hydrolysis fluorescence probes, and genomic DNA samples diluted in 5 µL of pure nuclease-free water. The reaction conditions were 50°C for 2 minutes; 95°C for 10 minutes, 95 for 15 seconds and 60°C for 1 minute (40 cycles), using a 7500 Real-Time PCR System. Nontransduced cells were used as a negative control (NC). Data were processed with the

SDS 2.1 software package (PerkinElmer, Waltham, Massachusetts; Applied Biosystems, Carlsbad, California). For absolute quantification, a 1054-EPO standard curve was constructed using a 10-fold serial dilution ranging from 10^1 to 10^6 plasmid copies per well. With the knowledge of the molecular weight of the plasmid and the insert, the corresponding plasmid copy number was determined.³⁴ The standard curve was generated by linear regression of the Ct values plotted against the logarithm of their template copy numbers. The absolute gene copy number in samples was quantified by analyzing 100 ng of genomic DNA of each cell line. The absolute number of copies was divided by the corresponding number of genome molecules related with 100 ng of genomic DNA of each cell line. Each qPCR run was performed in triplicate.

2.5.2 | Relative mRNA expression

The transduced cell lines were analyzed for the level of rhEPO gene expression by RT-qPCR (Real Time- Quantitative PCR). For this, the total RNA was extracted from 1×10^6 cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. Quantification and purity of total RNA were evaluated by spectrophotometer reading at wavelengths of 260 and 280 nm. Only 260/280 ratios between 1.8 and 2.0 were considered acceptable. The cDNA was produced using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). An amount of 2 μ g of total RNA, 5 μ L of 10X RT buffer, 10 mmol/L dNTPs, 5 μ L of random primer, 0.15 μ L of ribonuclease inhibitor, and 2.5 μ L of the MultiScribe Transcriptase enzyme were added to a final volume of the reaction of 50 μ L. The GeneAmp PCR Cyclor System 9700 (Applied Biosystems) was set for a cycle of 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. The methodology of quantitative mRNA expression analysis used primers, and FAM/MGB-NFQ conjugated hydrolysis fluorescence Taqman probes specific for a cDNA region of the rhEPO and the reference genes. The final volume of the reaction was 20 μ L per well, consisting of 1 μ L of 20X TaqMan gene expression assay containing the primers and the hydrolysis probe, 10 μ L of 2X TaqMan Master Mix, and 100 ng of cDNA diluted in 4 μ L of ultra-pure water. The volumes of all reactions were achieved using pure nuclease-free water. Data analysis was performed according to the relative expression of the target gene to its control, through equation $2^{-\Delta\Delta Ct}$.³⁵ Quantitative PCR was carried out in 7500 real-time PCR System (Applied Biosystems), 40 cycles were performed consisting of 50°C for 2 minutes, 95°C for 10 minutes, 60°C for 1 minute. Normalization was performed using the geometric mean of the Ct (Cycle threshold) of the reference genes. The reference genes were the endogenous $\beta 2$ microglobulin and β -actin genes, and each qPCR run was performed in triplicate.

2.6 | Characterization of growth, metabolism, and rhEPO production by recombinant cells

After determining the best media for each cell, a kinetic characterization was performed regarding cell growth, metabolism, and rhEPO production. For this, 5×10^5 cells/mL were inoculated into a 125-mL Erlenmeyer flask containing 20 mL of each media. Cultures were maintained for 312 hours. Every 24 hours, 2 mL aliquots were collected, and after centrifugation at 1200 rpm the supernatants were frozen at -20°C for the subsequent analysis. The culture volume was adjusted to 20 mL by adding fresh media for each 2 mL aliquot taken. Glucose, lactate, glutamine, and glutamate concentration in the culture supernatants was carried out using the YSI 2700 biochemical analyzer (Yellow Springs Instruments). Ammonia concentration was determined using an ion-selective electrode (Orion 720A⁺, Thermo Scientific, South Logan, Utah) according to the manufacturer's instructions.

The maximum specific growth rate (μ_{\max}) was estimated from the slope of the best-fitting line of the logarithm of viable cell density vs time during the exponential growth phase. Specific EPO productivity (qEPO), specific consumption, or production rates of glucose (qGlc), lactate (qLac), and ammonium (qNH₄) were calculated by plotting the products or substrate concentrations against the values of the integral of viable cells (IVC) during exponential growth phase, except for qEPO in which IVC was estimated from 48 hours to 216 hours, a time-period related to the rhEPO production phase. A positive signal for specific rate indicates production and negative consumption. The IVC is the area under the viable

cell growth curve and was estimated by the software Origin 8.5. The glucose-lactate yield coefficient was calculated as shown in the equation below:

$$Y_{\text{lac}}^{\text{glc}} = \frac{\Delta \text{lac}}{\Delta \text{glc}}$$

where, Δlac is the variation of lactate concentration in the beginning and end of the exponential phase and Δglc is the variation of glucose concentration in the same interval.

2.7 | In vitro terminal erythroid differentiation assay

The biological activity of the rhEPO produced by the recombinant cells was evaluated by the ability to induce erythroid differentiation. For this, peripheral blood mononuclear cells (PBMC) were obtained from the leukoreduction system chambers of apheresis platelet donors, with informed consent, according to the institutional ethics committee (CAAE 98950818700005440). CD34⁺ cells were isolated from PBMC by magnetic—positive selection—cell sorting (Miltenyi Biotec).

The culture method to enrich the erythroblast population was adapted from Freyssinier et al.³⁶ and Hu et al.³⁷ This differentiation protocol was set into two phases. In phase 1, CD34⁺ cells were cultured for 7 days in Iscove's Modified Dulbecco's Medium (ThermoFisher) supplemented with 15% (v/v) of BIT9500 serum substitute (Stem Cell Technologies), 100 U/mL Penicillin-Streptomycin (ThermoFisher), 2 mM GlutaMax (ThermoFisher), 100 ng/mL Stem Cell Factor, 100 ng/mL interleukin IL-6, and 10 ng/mL interleukin IL-3 (Miltenyi Biotec). In phase 2, cells were cultured for 12 days in the previously described media with the replacement of IL-6 for 2 U/mL EPO either in-house recombinantly expressed in human cell lines SK-Hep-1, HKB-11, and Huh7 or commercially available CHO-expressed EPO (R&D Systems). The NC was cultured in EPO-deprived phase 2 media for the same period. Cells were cultivated at 37°C, 5% CO₂, and 85% humidity.

Cultured cells were immunophenotyped at days 0, 4, 8, and 12 of phase 2. Briefly, 1×10^5 cultured cells were washed and suspended in 2%FBS-PBS and then labeled with FITC-conjugated anti-CD235a, also known as Glycophorin A (GPA) (BD Biosciences). Labeled cells were then analyzed in a FACS Aria I cytometer (BD Biosciences). Flow cytometry data analysis was conducted in FCS Express 6.06.0014 (DeNovo Software).

Cell morphology was analyzed by May-Grünwald-Giemsa (MGG) staining of cytospin samples. At days 0, 4, 8, and 12 of phase 2, 1×10^5 cells were washed and suspended in 200 μL of 1 \times PBS and then centrifuged in microscopy glass slides at 100 \times g for 5 minutes. After drying, slides were stained in May-Grunwald (Sigma MG500) for 5 minutes, washed in PBS for 1 minute, stained in Giemsa solution (Sigma GS500) for 15 minutes, and rewashed in PBS for 1 minute. The cells were imaged using an inverted microscope (Carl Zeis Axiovert A1).

2.8 | Statistical analysis

All statistical analyses were conducted in GraphPad Prism version 6.05 for Windows (GraphPad Software).

3 | RESULTS AND DISCUSSION

The host cell line chosen to produce the glycoprotein has a strong influence on the glycosylation since different host systems may express different glycosylation enzymes and transporters that influence the specificity and heterogeneity in glycosylation patterns.⁴ Consequently, the selection of the appropriate host system is extremely crucial for the production of therapeutic glycoproteins.¹ To study the potential of human cell lines for industrial production of recombinant glycoproteins, we have generated recombinant human cell lines under suspension serum-free conditions by a lentiviral transduction approach. We have chosen EPO as our model for this study based on its molecular complexity, mainly due to its glycan profile. EPO is a glycoprotein that contains approximately 40% carbohydrate in its structure.³⁸ Carbohydrate structures are critical for many biological properties of recombinant proteins, these include pharmacokinetics, secretion, stability, receptor recognition and antigenicity, protein conformation, and biological activity.³⁹

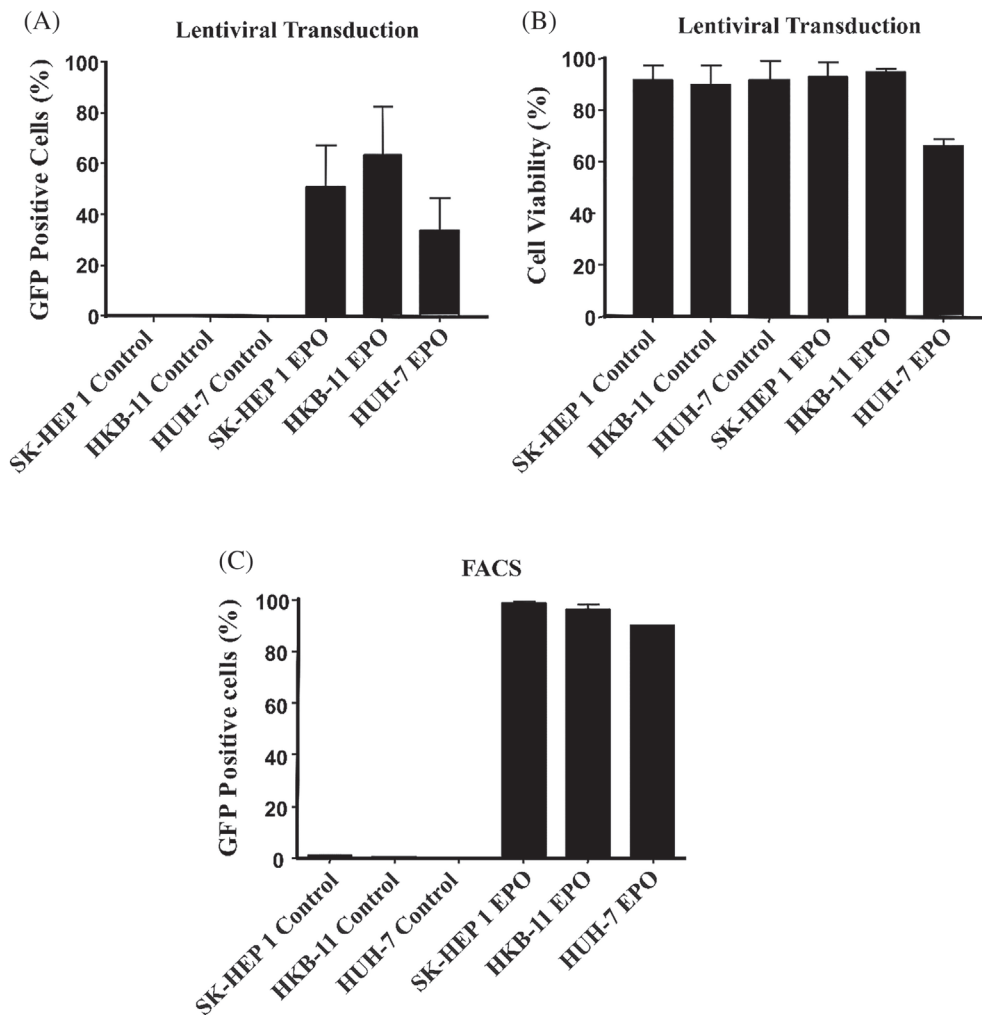


FIGURE 1 Transduction efficiency (A), viability (B), and percentage of GFP-expressing cells enrichment after sorting (C) of the Sk-Hep-1, HKB-11, and Huh-7 recombinant cell lines. Cell lines were transduced at a density of 3×10^5 /mL with lentiviral vectors carrying the rhEPO gene using an MOI of 1. The data presented show the average of three independent experiments ($n = 3$)

3.1 | Recombinant rhEPO producing-cells were successfully generated

Lentiviral-based gene transfer enables the generation of recombinant cell lines with higher and stable levels of transgene expression when compared to classical DNA delivery methods.⁴⁰ A lentivirus-derived vector carrying the coding region of a rhEPO gene, whose expression is linked to the GFP through an internal ribosome entry site, was used for the generation of stable recombinant Sk-Hep-1, HKB-11, and Huh-7 cells. We transduced the cells at a MOI of 1. Figure 1 shows the percentage of GFP positive cells (A) as well as the cell viability (B) after transduction. HKB-11 cells presented a higher percentage of positive GFP cells, 63%, followed by Sk-Hep-1 and Huh-7 with 50% and 34%, respectively. Regarding viability, only Huh-7 presented a decline, showing that this cell line can be more sensitive to the transduction processes. To enrich the population of rhEPO producing cells, GFP-positive cells were sorted by flow cytometry reaching levels >90% for all cell lines (Figure 1C).

3.2 | rhEPO production and levels of mRNA expression are not related to the gene copy number

After the generation of the pool of recombinant cells, we evaluated different culture media to select an appropriate for the production of rhEPO. We compared the culture medium previously selected for each cell line during the adaptation process (CDM4CHO for Sk-Hep-1 and HKB-11 and CD293 AGT cells for Huh-7 cells)³¹ with Freestyle 293 medium, a culture medium widely used for recombinant protein production. To evaluate which media had the highest rhEPO productivity, each culture was monitored for 72 hours.

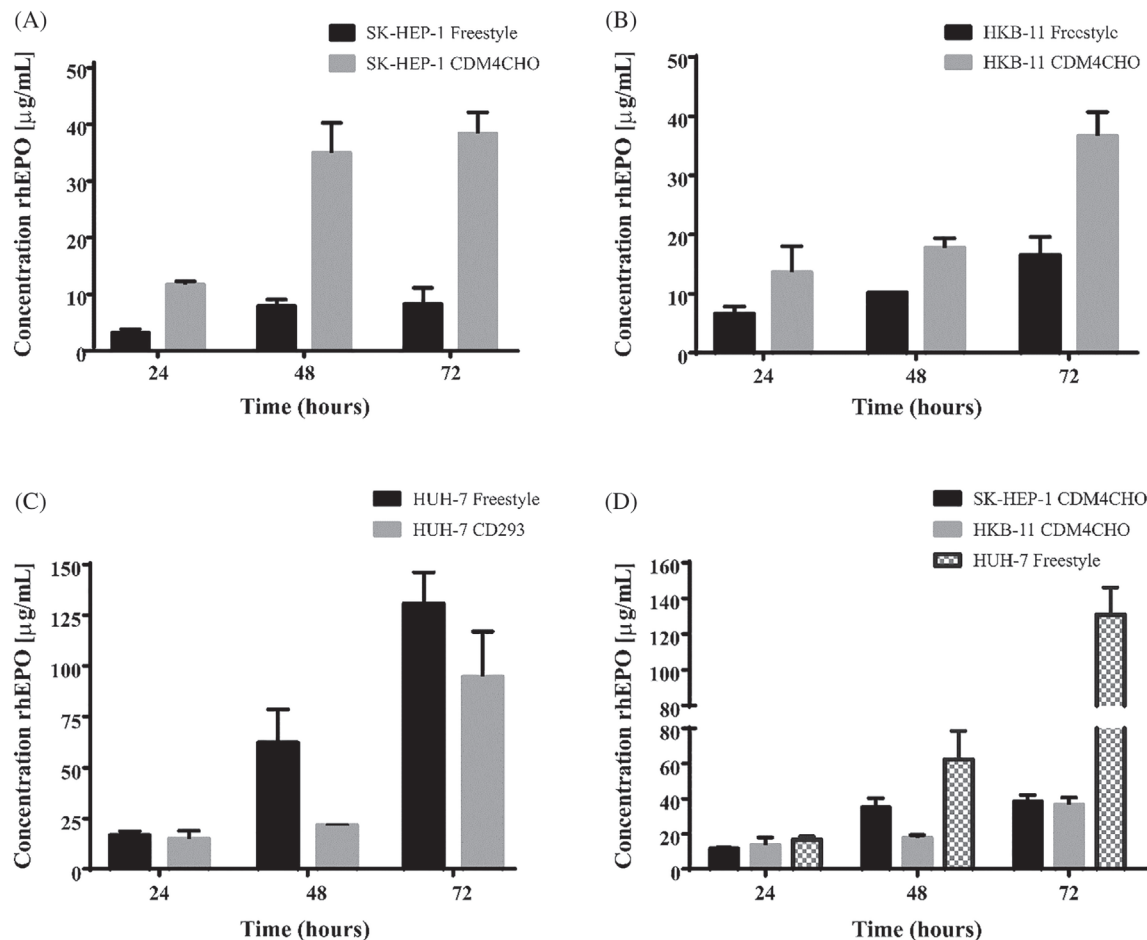


FIGURE 2 Volumetric production of rhEPO for Sk-Hep-1 (A), HKB-11 (B), and Huh-7 (C) using different culture media. The yield of rhEPO was compared using CDM4CHO, CD293 AGT, and Freestyle 293 media. (D) Volumetric production using the best culture media for each cell. The production of rhEPO was monitored for 72 hours ($n = 2$)

As shown in Figure 2, Sk-Hep-1 (A) and HKB-11 (B) cell lines achieved a maximum rhEPO concentration of 38.35 μg/mL and 36.65 μg/mL when cultured in CDM4CHO medium, and 8.3 μg/mL and 16.55 μg/mL when cultured in Freestyle 293, respectively, confirming CDM4CHO as the best medium for cell growth as well as for rhEPO production. For the Huh-7 cells (C), the best rhEPO production was achieved with the Freestyle 293 medium when compared to CD293 AGT, reaching a maximum concentration of 130 μg/mL and 94.96 μg/mL, respectively, demonstrating that the medium elected for cell growth was not the one that presented the best volumetric protein production. rhEPO specific production of each cell line was also evaluated. As shown in Figure 3, Huh-7 cells had higher specific production than Sk-Hep-1 and HKB-11 cell lines.

We also compared the gene expression of the cells to the nontransduced controls and the cell line that presented the highest relative expression was the Huh-7, with the rhEPO gene 122 294-fold higher than its nontransduced control, followed by the Sk-Hep-1 line with expression 2352-fold and the HKB-11 1097-fold higher than its nontransduced control (Figure 4). At this point, these results corroborate with the higher rhEPO production by Huh-7.

Detrimental effects on karyotypic stability are related to the high gene copy number integrated into the genome of mammalian cells, especially after long periods of cultivation. High gene copy number might impair cell growth, cell survival and proliferation of surviving cells, outbalancing any beneficial effect of enhanced specific productivity.^{41,42} On the other hand, high levels of integrated copies into the genome can improve the productivity of the cell.⁴³ In this research, we used an MOI of 1 to favor a low number of integrations per cell, targeting the stability of the recombinant cell populations. As shown in Table 1, HKB-11 cells had the higher number of inserted copies, with a total of 10.1 copies/cell followed by the Sk-Hep-1 with 8.3 copies/cell, whereas Huh-7 cells had a significantly lower value, with 1.97 copies/cell. Huh-7 cells had a lower gene copy number compared to Sk-Hep-1 and HKB-11, suggesting that

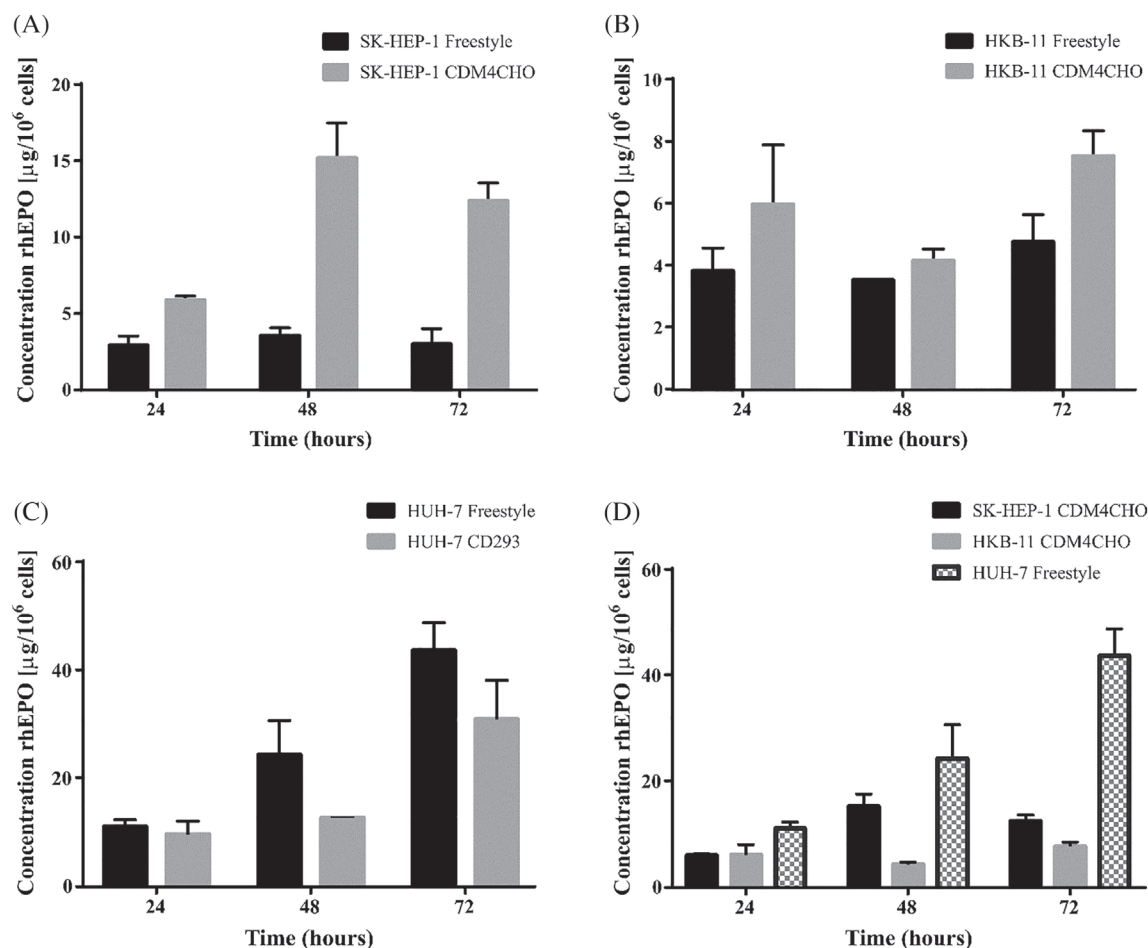


FIGURE 3 Specific production of rhEPO for Sk-Hep-1 (A), HKB-11 (B), and Huh-7 (C) using different culture media. For Sk-Hep-1 and HKB-11 the comparison was made using CDM4-CHO and Freestyle 293 media and for Huh-7 CD293 AGT and Freestyle media. Figure D compares the specific productivity using the best culture media. The production of rhEPO was monitored for 72 hours ($n = 2$)

Cell line	Copy number/cell
SK-Hep-1	8.3
Hkb-11	10.1
Huh-7	1.97

TABLE 1 Number 1054-EPO lentiviral vector copies per cell

The analysis was carried out by qPCR using 100 ng of genomic DNA extracted from SK-Hep-1, HKB11, and Huh-7 cell lines.

the high level of expression achieved by this cell line was not related to the number of copies integrated into the cell genome, but to the transcriptional activity of the integration site. Putting these results together it is possible to note that there was no correlation between the number of copies integrated into the genome of the cells and the rhEPO production level.

3.3 | Growth and metabolic characterization

As shown in Figure 5 (A), Sk-Hep-1 cells showed cell growth up to 96 hours postinoculum (5×10^5 cells/mL), reaching a maximum cell concentration of 2.14×10^6 /mL. After 96 hours the cells remained in a stationary phase of growth until the

FIGURE 4 The fold change in expression of the EPO mRNA in transduced cells relative to nontransduced cells. The samples were analyzed using real-time quantitative PCR. The expression fold change of the target gene was calculated using the $\Delta\Delta C_t$ method. The reference gene is the geometric mean of the ΔC_t values of the β Actin and $\beta 2$ microglobulin gene. Three replicates of each reaction were performed

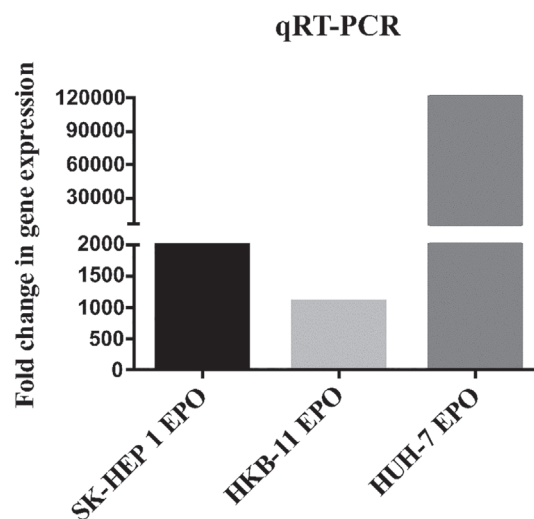


TABLE 2 Parameters for Sk-Hep-1, HKB-11, and Huh-7 cultured under suspension serum-free conditions

	Sk-Hep-1	HKB-11	Huh-7
μ_{\max} (h^{-1})	0.0237 ± 0.0006	0.0218 ± 0.0008	0.0312 ± 0.0012
Xv max (10^6 cells/mL)	2.14 ± 0.07	5.74 ± 0.08	4.36 ± 0.19
Dt (h)	29.31 ± 0.78	31.88 ± 1.14	22.23 ± 0.90
IVC (10^6 cells*h/mL)	535.9 ± 7.9	1129.3 ± 1.5	771.6 ± 0.6
qEPO (pg/cell/day)	5.904 ± 1.848	2.496 ± 0.432	22.272 ± 5.568
qGlc (pmol/cell/day)	2.376 ± 1.390	3.828 ± 0.424	4.718 ± 1.286
qLac (pmol/cell/day)	7.752 ± 4.800	1.128 ± 0.033	8.100 ± 0.288
qNH ₄ (pmol/cell/day)	0.245 ± 0.074	0.110 ± 0.000	0.379 ± 0.077
Ylac/Glc (mmol/mmol)	1.127 ± 0.037	0.444 ± 0.004	1.426 ± 0.552
[EPO]max ($\mu\text{g/mL}$)	112.75 ± 26.94	112.50 ± 10.60	571.54 ± 129.09
P ($\mu\text{g/h}$)	0.67 ± 0.16	0.67 ± 0.06	3.40 ± 0.76

The maximum specific growth rate (μ_{\max}), Xv, maximum density of viable cells, Dt, doubling time, IVC, integral of viable cells during the whole culture period, qEPO, specific rate of rhEPO production, qGlc, specific rate of glucose consumption, qLac, specific rate of lactate production, qNH₄, specific rate of ammonia production, Ylac/Glc, glucose-lactate yield coefficient, [EPO]max, maximum concentration of rhEPO, P rhEPO productivity per h.

end of the culture. Cell viability remained above 90% throughout the culture. The maximum specific growth rate during the exponential growth phase was 0.0237 hour^{-1} (Table 2). HKB-11 cells (Figure 5B) showed strong cell growth up to 120 hours, reaching a maximum cell density of 5.74×10^6 cells/mL, with a maximum specific growth rate of 0.0218 hour^{-1} (Table 2). These cells had no stationary phase as with the Sk-Hep-1 cell, with a decrease in cell density and viability in the final days of culture. At the end of 312 hours, both cell density and viability fell by approximately 50% in comparison to their respective maximum points. Huh-7 cells (Figure 5C) showed a cell growth pattern similar to HKB-11 cells for up to 96 hours of culture; reaching a maximum cell density of 4.36×10^6 cells/mL, after that the cell density began dropping, resulting in a final cell concentration of 1.7×10^6 cells with a viability of 30% and a maximum specific growth rate of 0.0312 hour^{-1} (Table 2). The cell density values achieved by all the cell lines are compatible to values obtained for the culture of the HKB-11 and SK-Hep-1 cells³¹ and other human cells such as HEK-293 and PER.C6 under suspension serum-free conditions.⁴⁴⁻⁴⁸

Understanding the metabolism of cell lines is an essential step in improving the performance of mammalian cell culture processes. Knowing the metabolism of the lineages allows for the characterization of substrates and metabolites that should be monitored and controlled in the culture medium to increase cellular growth and productivity. For Sk-Hep-1

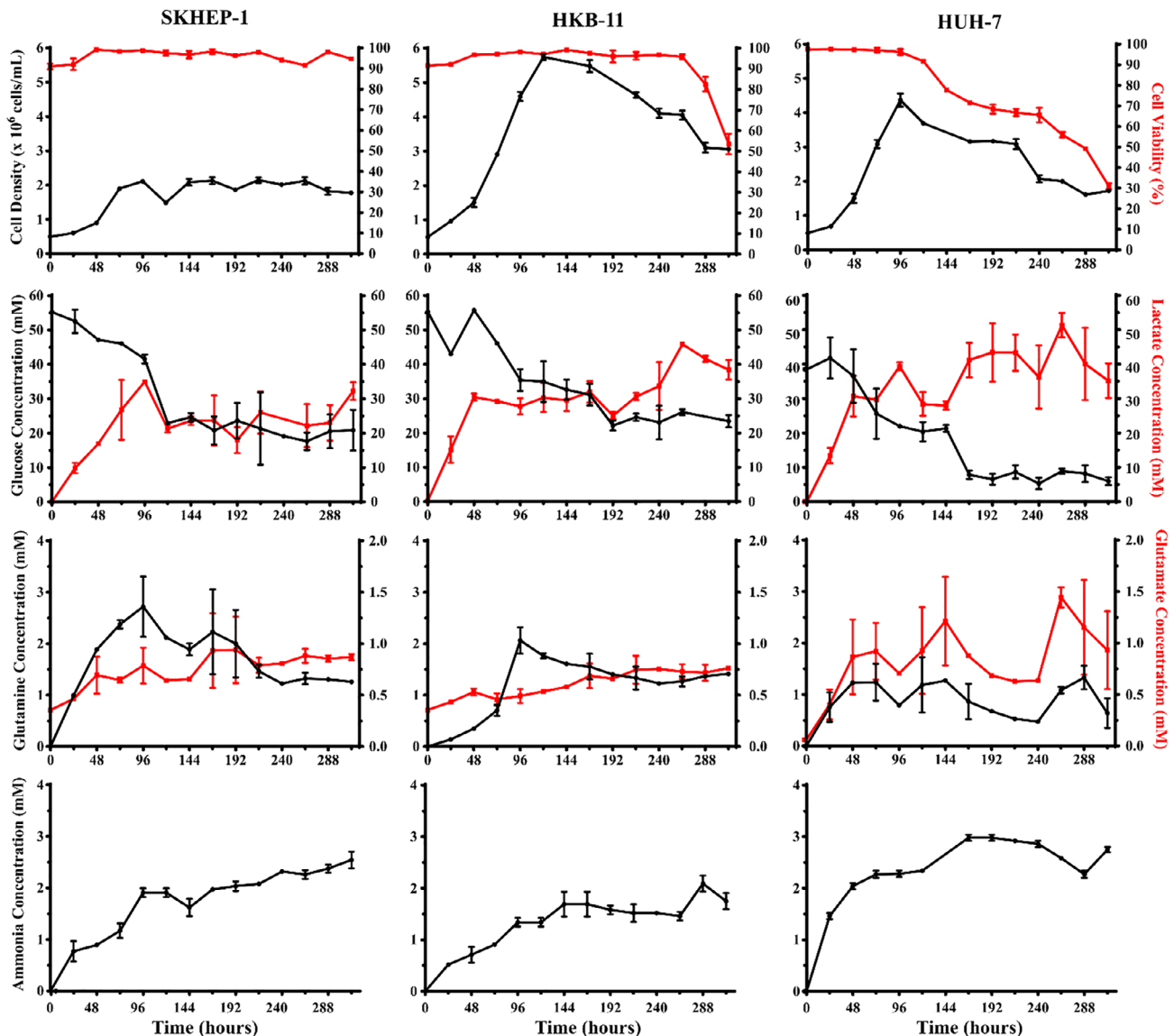


FIGURE 5 Growth kinetic and metabolism of recombinant Sk-Hep-1, HKB-11, and Huh-7 cell lines cultured under serum-free suspension conditions. Growth, glucose consumption, lactate production, glutamine consumption, glutamate production, and ammonia production by the Sk-Hep-1 (A), HKB-11 (B), and Huh-7 (C) cells ($n = 2$)

cells it is possible to observe a decrease in glucose concentration for up to 120 hours of cultivation. Lactate, which is a product of glucose metabolism, showed an increase in the concentration at the start of cultivation, reaching 35 mM at 96 hours and remaining in the range of 18–32 mM until the end of cultivation. There was no total consumption of glutamine during cultivation, it reaches a minimum concentration of 1.25 mM in 240 hours, and remains in this level until the end of the culture. Regarding ammonia, which is a byproduct of glutamine and glutamate metabolism, Sk-Hep-1 cells had a rapid increase in ammonia concentration, reaching a maximum concentration of 2.5 mM at the end of the culture.

For the HKB-11 cell line it was not possible to observe a drop in glucose concentration in the first 2 days, but from then on it began to fall, stabilizing after 192 hours and maintaining a concentration of approximately 30 mM. Lactate increased significantly in the first 48 hours, subsequently reaching its maximum concentration in 264 hours (46 mM). The levels of glutamine consumption and glutamate production in the HKB-11 cells were lower than in Sk-Hep-1 cells. The concentration of ammonia increases more slowly than the Sk-Hep-1 cells, which can be correlated with the lower production of glutamate and consumption of glutamine. The maximum concentration of ammonia reached was 2 mM in 288 hours.

During the Huh-7 cultivation the glucose concentration began to fall after 24 hours, reaching its minimum concentration in 312 hours. The cells were more sensitive with the glucose drop and the increase of the concentration of the

metabolites produced in comparison with the other cell lines tested, showing more significant and pronounced decreases in the number of total viable cells. The Huh-7 line reached a lactate concentration of 40 mM in 96 hours and a maximum ammonium concentration of 3 mM in 168 hours. All metabolites increased more markedly than in other lines and at higher levels. It is possible to infer from this that the Huh-7 cell had a considerably higher metabolic flux than those of the previous lines.

One of the main consequences of the metabolic behavior of *in vitro* cultured animal cells is the exacerbated formation of the lactate and ammonium ions. When these products exceed specific critical concentrations, they inhibit cell growth and may decrease the production of recombinant protein.^{49,50} Lactate can cause a significant reduction in the pH of the medium, which may inhibit cell growth, but this effect can be avoided with a control system that keeps the pH within an optimal range. However, the primary mechanism by which lactate appears to affect cultures negatively is increasing osmolarity of culture media.

In all cultures, glucose exhaustion was not observed. All cell lines showed a rapid accumulation of lactate in the first 72 hours, which may be related to rapid growth. In general, concentrations less than 20 mM do not affect the cell, whereas 20–40 mM disrupt productivity, and above this value cell growth is inhibited.^{51,52} As can be seen in Figure 5B and C, HKB-11 and Huh-7 cells reach lactate inhibitory concentration for mammalian cells in 264 and 96 hours respectively, which may be related to the decrease of viable cells and with cell growth limitation for Huh-7 cells (96 hours); Sk-Hep-1 cells did not reach the lactate concentration value considered inhibitory. Changes in extracellular pH and osmolarity as a result of increased lactate production are thought to be responsible for cell growth inhibition.⁵³ Also significant is the influence of this metabolite on the quality of the recombinant product, especially on glycosylation. Lactate has been reported to inhibit cell growth and cell metabolism and decrease productivity in CHO, BHK cells, and hybridomas, in part due to changes in osmolarity of culture environment.^{50,54,55}

The ammonium ion has a more significant impact on the culture of mammalian cells *in vitro* than lactate because concentrations of between 2 and 5 mM of ammonium ion can inhibit growth and impair productivity.⁵¹ Furthermore, a high concentration of ammonium has been shown to impair the glycosylation pattern of rhEPO by decreasing the terminal sialylation, the content of the O-linked glycan, and the tetra-antennary *N*-glycan.⁵⁶ Sk-Hep-1 cells reached the ammonium concentration considered to be inhibitory in approximately 96 hours of culture when cells enter in the stationary growth phase. At this point lactate is also near inhibitory concentration. From then on the culture entered a stationary phase where it remains until the end of the culture, without presenting decay of the total viable cells. The HKB-11 cells showed a decrease in the number of viable cells shortly after reaching their maximum concentration in 120 hours, and the ammonium and lactate levels in this period are not considered as inhibitory.⁵¹ The Huh-7 cell line reached both ammonium and lactate inhibitory concentration in the first 96 hours, which correlates with cell growth limitation and decay of viable cells. The intensity of Huh-7 metabolism could be a burden associated with higher mRNA expression of rhEPO as well as higher protein yield as shown in the previous results. The higher level of rhEPO expression could have led to the accumulation of unfolded or misfolded protein in the endoplasmic reticulum (ER) lumen of the Huh-7 cells inducing ER stress that can be detrimental and affect cell viability.⁵⁷

Regarding glucose specific consumption (qGlc) the values obtained for each cell were very similar to those found in another human cell line, HEK293, with a range from 1.75 to 7.92 PMol/cell/day. For qLac, HKB-11 showed a low value (1.128 PMol/cell/day) compared to those found in literature for HEK293 (3.05 to 16 PMol/cell/day).^{58–64} For glucose-lactate yield coefficient (Y_{lac/Glc}), HKB-11 also presented a low value (0.444 mmol/mmol) compared to other human cell lines (0.5–2.3 mol/mol),^{59,61–64} which can be explained by a lactate consumption in exponential phase, a common phenomenon in HEK293,^{60–62} one of the cell employed to establish HKB-11 cell line.⁶⁵

3.4 | Huh-7 cells achieved higher levels of rhEPO production than Sk-Hep-1 and HKB-11 cells

rhEPO production was monitored throughout the 216-hours culture period using the ELISA assay. The volumetric rhEPO production of Sk-Hep-1, HKB-11, and Huh-7 achieved a maximum concentration of 112.5 µg/mL, 112.7 µg/mL, and 571 µg/mL (Table 2, Figure 6), respectively, and the production of all cells was sustained during the whole process, achieving the higher concentration at the end of the culture (Figure 6). Corroborating with the previous results, Huh-7 cells produced higher rhEPO compared to HKB-11 and Sk-Hep-1 cells, which achieved virtually the same volumetric production. This yield is comparable to some results obtained using well-established production platforms for recombinant glycoproteins, like CHO, BHK, and HEK293. In 2002, Yoon and colleagues, analyzing the effects of temperature on the

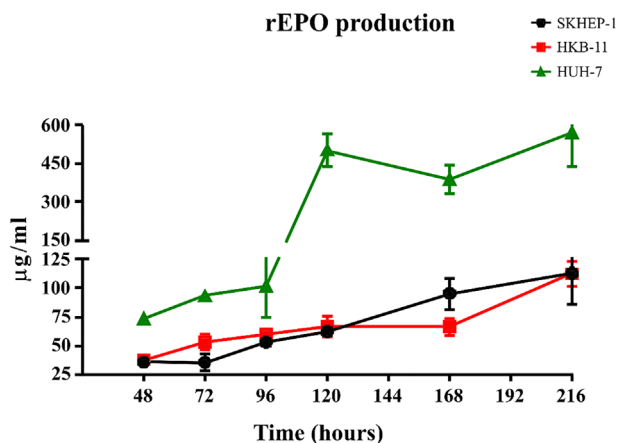


FIGURE 6 Kinetic of rhEPO production of Sk-Hep-1, HKB-11, and Huh-7 cells cultured under serum-free suspension conditions. SK-Hep and HKB-11 cells were cultured in CDM4-CHO medium and Huh-7 cells in Freestyle 293 medium ($n = 2$)

production of rhEPO by CHO cells achieved a maximum concentration of 109.2 µg/mL of rhEPO in cultures maintained at 33°C and 42.8 µg/mL at 37°C.⁶⁶ The qEPO values obtained by CHO cells (2.16 pg/cell/day at 37°C and 8.4 pg/cell/day at 33°C) were also comparable to the ones obtained in our present work, except for Huh-7 where the value was considerable higher. In a similar study using CHO cells, Ahn and colleagues reached a maximum concentration below 20 µg/mL in cultures maintained at 37°C and approximately 140 µg/mL in cultures maintained at 32°C. The highest qEPO obtained was 9.2 ± 0.9 pg/cell/day at 30°C culture,⁶⁷ which is lower than the 22.272 ± 5.568 pg/cell/day by the Huh-7 cells used in this study.

After 13 days of culture, Gaillet and colleagues achieved a maximum rhEPO concentration of 206 µg/mL using stable CHO cells generated by lentiviral transduction and cultured in serum-free conditions. In that study, an MOI of 200 was used for the transduction of the cells with an average of 19 gene copies integrated per cell.⁴³ Also using lentiviral vectors for generation of stable CHO cells, Baranyi and colleagues⁶⁸ generated clones producing on average 50–250 µg/mL of rhEPO. In a recent work Chin and colleagues generated HEK293 cells producing EPO, adapted then to serum-free suspension conditions and obtained cells pools expressing 3680 to 21 730 U/mL (24.5–144.9 µg/mL) in batch mode. To further optimize rhEPO production the authors cultured the cells in a bioreactor operated under feed-batch conditions, where the cells were able to produce 92 700 U/mL (696 µg/mL) with qEPO of 18.1 pg/cell/day, values lower than obtained in this work for Huh-7 cells cultured in batch in Erlenmeyer flasks.

Despite the high expression of rhEPO by Huh-7 cells, its culture showed an inhibitory concentration of both ammonium and lactate, which can inhibit growth and decrease cell viability and may interfere with the glycosylation of this protein. However, there are some strategies to decrease the concentration of these metabolites during cultivation, such as substitution of glutamine by some ammoniagenic substrates such as glutamate, pyruvate, alpha-ketoglutarate, or a few dipeptides, fed-batch for glutamine replacement or limitation of the initial glutamine concentration⁶⁹; and in lactate case, a fed-batch strategy using galactose/glutamate.^{51,70}

The maximum rhEPO concentration obtained in the cultures of Sk-Hep-1, HKB-11, and Huh-7 cells are comparable and even superior in the case of Huh-7 cells to those found under similar conditions of serum-free or nonserum-free suspension cultures. Sk-Hep-1, HKB-11, and Huh-7 cells have also proven to be able to produce high amounts of other complex recombinant glycoproteins. In 2017, a study using the Sk-Hep and HKB-11 cells showed that these cells were able to secrete 2.6–5.0-fold more recombinant human coagulation factor VII (rhFVII) (585.4–1176.6 ng/mL) than BHK-21 cells (222.6 ng/mL) under static serum-supplemented culture.²⁷ Bomfim and colleagues showed that the amount of recombinant human coagulation factor IX (rhFIX) secreted by SK-Hep-1 cells was 1.6-fold higher than by 293T cells and the biological activity of rhFIX secreted by SK-Hep-1 cells was 4-fold higher (186 mUI/10⁶ cells) than by 293T (47 mUI/10⁶ cells).²⁶

3.5 | Produced rhEPO was able to sustain differentiation of CD34+ cells into erythroid cells in vitro

To evaluate the biological activity of the produced rhEPOs, we performed an erythroid terminal differentiation assay with peripheral blood-derived hematopoietic progenitor cells.³⁷ To achieve this, we measured the expression of GPA, a major

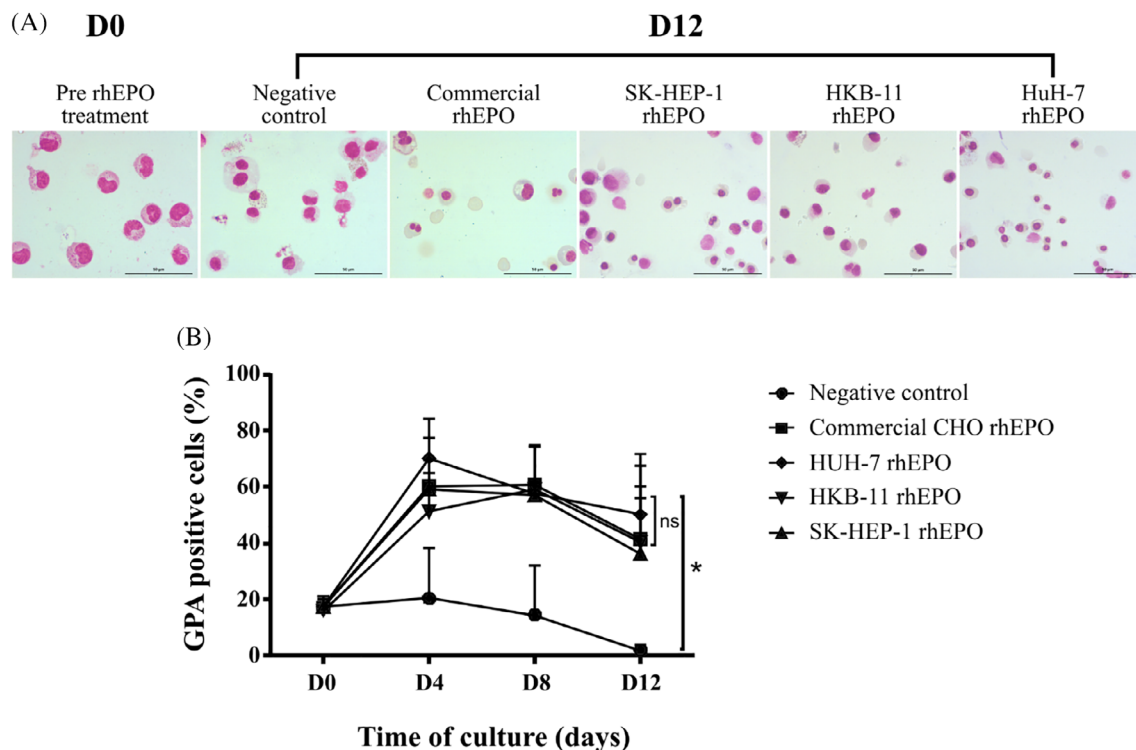


FIGURE 7 Functional analysis of the produced rhEPO. (A) Mean percentage of GPA positive cells throughout the culture. (B) Representative images of the morphology of cytopinned MGG stained cells from day 0 and day 12 from each group (63× magnification). The negative control represents CD34⁺ cells cultured in EPO-deprived phase 2 media. *Two-way ANOVA and Turkey's multiple comparison correction test computed with 95% Confidence Interval

erythroid surface marker⁷¹ during a 2-phase culture system. On Day 0 of Phase 2 of each independent experiment, samples presented similar levels of GPA positive cells (Figure 7B). Except for the NC (Phase 2 culture deprived of rhEPO), all samples displayed an important increase of GPA positive cells throughout the experiment, with the peak on Day 4. Of note, cells treated with rhEPOs showed a comparable performance to the cells treated with the commercial EPO regarding the percentage of GPA positive cells. Since erythropoiesis is an EPO-dependent process, it is expected that samples deprived of EPO should not terminally differentiate.⁷² This fact was supported by the morphologic characteristics of the samples among the different groups (Figure 7A). NC cells preserved a morphology similar to the early erythroid progenitors, maintaining a Day 0-like aspect. In contrast, cells cultured either with the commercial or rhEPOs presented a higher number of terminally differentiated erythroid cells and even enucleated reticulocyte-like cells. These results showed that the rhEPO produced by Sk-Hep-1, HKB-11, and Huh-7 cells under serum-free conditions was functional and biologically active, with no significant difference when compared to a commercially available EPO produced in CHO cells.

4 | CONCLUSION

In the present study, we show that the Sk-Hep-1, HKB-11, and Huh-7 cell lines were easily genetically modified to produce a recombinant protein in serum-free suspension conditions. Besides, the cells presented good cell growth characteristics and were able to produce high levels of a recombinant complex glycoprotein, EPO. HKB-11 cells stood out for their ability to grow at high cell densities in batch culture. The Huh-7 cell line showed superior results when compared to Sk-Hep-1 and HKB-11 cells regarding the level of EPO produced, which makes it a promising alternative for the production of recombinant proteins. This cell line however produced the higher amount of ammonia. The effect of this by-product on recombinant protein quality attributes must be evaluated. Further studies are also needed in order to develop specific serum-free culture media for these cells as well as to establish optimized culture conditions to enable an enhanced cell growth and recombinant protein production profile. The scalability and compatibility of these cells with a bioreactor are

also essential parameters that need to be evaluated. The glycosylation profile of the recombinant proteins produced by the cells is also a relevant feature to be analyzed, since this feature can demonstrate the quality and heterogeneity of the protein produced, as well as influence the selection of the later production strategies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge funding from CNPq (573754-2008-0), FAPESP (2008/578773, 2012/04629-8, and 2013/08135-2), and Coordination for the Improvement of Higher Education Personnel (CAPES) (financial code 001) (1544865).

PEER REVIEW INFORMATION

Engineering Reports thanks the anonymous reviewers for their contribution to the peer review of this work.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Kamilla Swiech  <https://orcid.org/0000-0002-4811-9751>

REFERENCES

1. Durocher Y, Butler M. Expression systems for therapeutic glycoprotein production. *Curr Opin Biotechnol*. 2009;20:700-707.
2. Walsh G. Biopharmaceutical benchmarks 2018. *Nat Biotechnol*. 2018;36:1136-1145.
3. Chin CL, Goh JB, Srinivasan H, et al. A human expression system based on HEK293 for the stable production of recombinant erythropoietin. *Sci Rep*. 2019;9:16768.
4. Goh JB, Ng SK. Impact of host cell line choice on glycan profile. *Crit Rev Biotechnol*. 2018;38:851-867.
5. Lalonde ME, Durocher Y. Therapeutic glycoprotein production in mammalian cells. *J Biotechnol*. 2017;251:128-140.
6. Dumont J, Euwart D, Mei B, Estes S, Kshirsagar R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. *Crit Rev Biotechnol*. 2016;36:1110-1122.
7. Ghaderi D, Zhang M, Hurtado-Ziola N, Varki A. Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. *Biotechnol Genet Eng Rev*. 2012;28:147-175.
8. Zhu J. Mammalian cell protein expression for biopharmaceutical production. *Biotechnol Adv*. 2012;30:1158-1170.
9. Lee JS, Ha TK, Lee SJ, Lee GM. Current state and perspectives on erythropoietin production. *Appl Microbiol Biotechnol*. 2012;95:1405-1416.
10. Fliedl L, Grillari J, Grillari-Voglauer R. Human cell lines for the production of recombinant proteins: on the horizon. *Nature Biotech*. 2015;32:673-679.
11. Ghaderi D, Taylor RE, Padler-Karavani V, Diaz S, Varki A. Implications of the presence of N-glycolylneuraminic acid in recombinant therapeutic glycoproteins. *Nature Biotech*. 2010;28(8):863-869.
12. Tangvoranuntakul P, Gagneux P, Diaz S, et al. Human uptake and incorporation of an immunogenic nonhuman dietary sialic acid. *Proc Natl Acad Sci USA*. 2003;100(21):12045-12050.
13. Galili U, Rachmilewitz EA, Peleg A, Flechner I. A unique natural human IgG antibody with antialpha-galactosyl specificity. *J Exp Med*. 1984;160:1519-1531.
14. Noguchi A, Mukuria CJ, Suzuki E, Naiki M. Immunogenicity of N-glycolylneuraminic acid-containing carbohydrate chains of recombinant human erythropoietin expressed in Chinese hamster ovary cells. *J Biochem*. 1995;117:59-62.
15. Varki A. Uniquely human evolution of sialic acid genetics and biology. *Proc Natl Acad Sci USA*. 2010;107:8939-8946.
16. Swiech K, de Freitas MC, Covas DT, Picanço-Castro V 2015. Recombinant glycoprotein production in human cell lines. *Methods Mol Biol*, 1258: 223-240.
17. Graham FL, Smiley J, Russel W, Nairn CR. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36:59-67.
18. Pau MG, Ophorst C, Koldijk MH, Schouten G, Mehtali M, Uytdehaag F. The human cell line PER.C6 provides a new manufacturing system for the production of influenza vaccines. *Vaccine*. 2001;19:2716-2721.
19. Schiedner G, Hertel S, Bialek C, Kewes H, Waschütza G, Volpers C. Efficient and reproducible generation of high-expressing, stable human cell lines without need for antibiotic selection. *BMC Biotechnol*. 2008;8:1-11.
20. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer*. 1974;33:1027-1033.
21. Niklas J, Schröder E, Sandig V, Noll T, Heinzle E. Quantitative characterization of metabolism and metabolic shifts during growth of the new human cell line AGE1.HN using time resolved metabolic flux analysis. *Bioprocess Biosyst Eng*. 2011;34:533-545.
22. Reichel C. Differences in sialic acid O-acetylation between human urinary and recombinant erythropoietins: a possible mass spectrometric marker for doping control. *Drug Test Anal*. 2013;5(11-12):877-889.
23. Skibeli V, Nissen-Lie G, Torjesen P. Sugar profiling proves that human serum erythropoietin differs from recombinant human erythropoietin. *Blood*. 2001;98:3626-3634.

24. Varki A, Freeze HH, Manzi AE. Preparation and analysis of glycoconjugates. *Curr Protoc Mol Biol*. 2009;88:1-11.
25. Varki A, Cummings RD, Aebi M, et al. Symbol nomenclature for graphical representations of glycans. *Glycobiology*. 2015;25:1323-1324.
26. Bomfim AS, Corrêa de Freitas MC, Picanço-Castro V, et al. Human cell lines: a promising alternative for recombinant FIX production. *Protein Expr Purif*. 2016;121:149-156.
27. Freitas MCC, Bomfim AS, Mizukami A, Picanço-Castro V, Swiech K, Covas DT. Production of coagulation factor VII in human cell lines Sk-Hep-1 and HKB-11. *Protein Expr Purif*. 2017;137:26-33.
28. Picanço-Castro V, Biaggio R, Cova D, Swiech K. Production of recombinant therapeutic proteins in human cells: current achievements and future perspectives. *Protein Pept Lett*. 2013;20:1373-1381.
29. Amaral RLF, Bomfim A de S, Soares Neto MA, et al. Approaches for recombinant human factor IX production in serum-free suspension cultures. *Biotechnol Lett*. 2016;38:385-394.
30. Rosa NG, Swiech K, Picanço-Castro V, Russo EMS, Soares Neto MA, De Castilho-Fernandes A.; Faça VM, Fontes AM, Covas DT. 2012. SK-HEP cells and lentiviral vector for production of human recombinant factor VIII. *Biotechnol Lett* 34 (8):1435-43.
31. Biaggio RT, Abreu-Neto MS, Covas DT, Swiech K. Serum-free suspension culturing of human cells: adaptation, growth, and cryopreservation. *Bioprocess Biosyst Eng*. 2015;38(8):1495-1507.
32. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol*. 1997;15(9):871-875.
33. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol*. 1998;72(11):8463-8471.
34. Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods*. 2003;278:261-269.
35. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem*. 2009;55(4):611-622.
36. Freyssinier J, Lecoq-Lafon C, Amsellem S, et al. Purification, amplification and characterization of a population of human erythroid progenitors. *Br J Haematol*. 1999;106(4):912-922.
37. Hu JP, Liu J, Xue FM, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. *Blood*. 2013;121(16):3246-3253.
38. Jelkmann W. Erythropoietin: structure, control of production, and function. *Physiol Rev*. 1992;72(2):449-489.
39. Butler M, Spearman M. The choice of mammalian cell host and possibilities for glycosylation engineering. *Curr Opin Biotechnol*. 2014;30:107-112.
40. Oberbek A, Matasci M, Hacker DL, Wurm FM. Generation of stable, high-producing CHO cell lines by lentiviral vector-mediated gene transfer in serum-free suspension culture. *Biotechnol Bioeng*. 2011;108(3):600-610.
41. Batard P, Jordan M, Wurm F. Transfer of high copy number plasmid into mammalian cells by calcium phosphate transfection. *Gene*. 2001;270:61-68.
42. Lattenmayer C, Trummer E, Schriebl K, et al. Characterization of recombinant CHO cell lines by investigation of protein productivities and genetic parameters. *J Biotechnol*. 2007;128(4):716-725.
43. Gailliet B, Gilbert R, Broussau S, et al. High-level recombinant protein production in CHO cells using lentiviral vectors and the Cumate gene-switch. *Biotechnol Bioeng*. 2010;106(2):203-215.
44. Havenga MJ, Holterman L, Melis I, et al. Serum-free transient protein production system based on adenoviral vector and PER.C6 technology: high yield and preserved bioactivity. *Biotechnol Bioeng*. 2008;100(2):273-283.
45. Loignon M, Perret S, Kelly J, et al. Stable high volumetric production of glycosylated human recombinant IFN α 2b in HEK293 cells. *BMC Biotechnol*. 2008;8:65.
46. Maranga L, Aunins JG, Zhou W. Characterization of changes in PER.C6 cellular metabolism during growth and propagation of a replication-deficient adenovirus vector. *Biotechnol Bioeng*. 2005;90(5):645-655.
47. Sun X, Goh PE, Wong KT, Mori T, Yap MG. Enhancement of transient gene expression by fed-batch culture of HEK 293 EBNA1 cells in suspension. *Biotechnol Lett*. 2006;28(11):843-848.
48. Xie L, Pilbrough W, Metallo C, et al. Serum-free suspension cultivation of PER.C6(R) cells and recombinant adenovirus production under different pH conditions. *Biotechnol Bioeng*. 2002;80(5):569-579.
49. Gambhir A, Korke R, Lee JC, Fu PC, Europa A, Hu WS. Analysis of cellular metabolism of hybridoma cells at distinct physiological states. *J Biosci Bioeng*. 2003;95(4):317-327.
50. Lao MS, Toth D. Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. *Biotechnol Prog*. 1997;13(5):688-691.
51. Altamirano C, Berrios J, Vergara M, Becerra S. Advances in improving mammalian cells metabolism for recombinant protein production. *Electron J Biotechnol*. 2013;16(3).
52. Wagner A, Marc A, Engasser JM, Einsele A. Growth and metabolism of human tumor kidney cells on galactose and glucose. *Cytotechnology*. 1991;7(1):7-13.
53. Slivac I, Blajic V, Radosevic K, Kniewald Z, Gaurina SV. Influence of different ammonium, lactate and glutamine concentrations on CCO cell growth. *Cytotechnology*. 2010;62(6):585-594.
54. Cruz HJ, Freitas CM, Alves PM, Moreira JL, Carrondo MJ. Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells. *Enzyme Microb Technol*. 2000;27(1-2):43-52.
55. Ozturk SS, Riley MR, Palsson BO. Effects of ammonia and lactate on hybridoma growth, metabolism, and antibody production. *Biotechnol Bioeng*. 1992;39(4):418-431.

56. Yang M, Butler M. Effects of ammonia on CHO cell growth, erythropoietin production, and glycosylation. *Biotechnol Bioeng*. 2000;68(4):370-380.
57. Hussain H, Maldonado-Agurto R, Dickson AJ. The endoplasmic reticulum and unfolded protein response in the control of mammalian recombinant protein production. *Biotechnol Lett*. 2014;36:1581-1593.
58. Henry O, Durocher Y. Enhanced glycoprotein production in HEK-293 cells expressing pyruvate carboxylase. *Metab Eng*. 2011;13:499-507.
59. Karengera E, Robotham A, Kelly J, Durocher Y, Crescenzo G, Henry O. Altering the central carbon metabolism of HEK293 cells: impact on recombinant glycoprotein quality. *J Biotechnol*. 2017;242:73-82.
60. Liste-Calleja L, Lecina M, Lopez-Repullo J, Albiol J, Solà C, Cairó J. Lactate and glucose concomitant consumption as a self-regulated pH detoxification mechanism in HEK293 cell cultures. *Appl Microbiol Biotechnol*. 2015;99:9951-9960.
61. Martínez-Monge I, Comas P, Triquell J, Lecina M, Casablancas A, Cairó J. A new strategy for fed-batch process control of HEK293 cell cultures based on alkali buffer addition monitoring: comparison with O.U.R. dynamic method. *Appl Microbiol Biotechnol*. 2018;102:10469-10483.
62. Martínez-Monge I, Albiol J, Lecina M, et al. Metabolic flux balance analysis during lactate and glucose concomitant consumption in HEK293 cell cultures. *Biotechnol Bioeng*. 2018a;116:388-404.
63. Petiot E, Jacob D, Lanthier S, Lohr V, Ansoorge S, Kamen A. Metabolic and kinetic analyses of influenza production in perfusion HEK293 cell culture. *BMC Biotechnol*. 2011;84(1):12.
64. Siegwart P, Côté J, Male M, Luong J, Perrier M, Kamen A. Adaptive control at low glucose concentration of HEK-293 cell serum-free cultures. *Biotechnol Prog*. 1999;15(4):608-616.
65. Cho MS, Yee H, Chan S. Establishment of a human somatic hybrid cell line for recombinant protein production. *J Biomed Sci*. 2002;9(6):631-638.
66. Yoon SK, Song JY, Lee GM. Effect of low culture temperature on specific productivity, transcription level, and heterogeneity of erythropoietin in Chinese hamster ovary cells. *Biotechnol Bioeng*. 2003;82(3):289-298.
67. Ahn WS, Jeon JJ, Jeong YR, Lee SJ, Yoon SK. Effect of culture temperature on erythropoietin production and glycosylation in a perfusion culture of recombinant CHO cells. *Biotechnol Bioeng*. 2008;101(6):1234-1244.
68. Baranyi L, Doering CB, Denning G, et al. Rapid generation of stable cell lines expressing high levels of erythropoietin, factor VIII, and an antihuman CD20 antibody using lentiviral vectors. *Hum Gene Ther Methods*. 2013;24(4):214-227.
69. Ha TK, Lee GM. Glutamine substitution: the role it can play to enhance therapeutic protein production. *Pharm Bioprocess*. 2015;3(3):249-261.
70. Altamirano C, Paredes C, Illanes A, Cairó JJ, Gòdia F. Strategies for fed-batch cultivation of t-PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium. *J Biotechnol*. 2004;110:171-179.
71. Robinson J, Sieff C, Delia D, Edwards PA, Greaves M. Expression of cell-surface HLA-DR, HLA-ABC and glycophorin during erythroid differentiation. *Nature*. 1981;289(5793):68-71.
72. Krantz S. Erythropoietin. *Blood*. 1991;77(3):419-434.

How to cite this article: Costa e Silva L, dos Santos MH, Heluy TR, et al. Serum-free suspension cultured human cells can produce a high-level of recombinant human erythropoietin. *Engineering Reports*. 2020;2:e12172. <https://doi.org/10.1002/eng2.12172>