Investigating Media Supplements to Enhance Chinese Hamster Ovary Cell Culture

A dissertation submitted for the degree of Ph.D.

By

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December 2019

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed:  (Candidate) ID No.: 15212896 Date: 25-11-19

To my lovely grandpa Pere

Who taught me the meaning of passion for your work.

Thank you for being one of the most kind, sweet and good person I have ever met.

I will never forget you, I miss you,

T'estimo moltíssim avi

Acknowledgements

Overall, I am very grateful to all the people I have met, which have made these last years an exceptional experience.

Firstly, I would like to thank Professor Martin Clynes for this intense and rewarding research experience over the last 6 years. From an original 6 months of working experience in NICB, to a year of Research Assistant and finally a 4.5 years of PhD; I can just be deeply grateful for giving me this opportunity and for all your enthusiasm along the way. I would also like to thank my first supervisor, Dr. Erika Hennessy. Even though having worked together for a short time, you were a great support during my firsts days, helping me to adapt to a different country with all your enthusiasm, fun and kindness. Thank you for encouraging me to stay in NICB, which has been an unforgettable experience at both personal and professional level.

To Dr. Padraig Doolan for getting on board as my supervisor. You have always had the door open for me and I really appreciated it. Thank you for all your support and enormous patience, especially with writing; I think we beat a record on the amount of drafts of the same paper! But also thank you for all the friendly talks, football chats and travel tips (Kerry is on my list before leaving Ireland), it has been a real pleasure to work with you.

To Gillian Smith and Anita White, thank you for all your work behind the scenes, essential for keeping NICB rolling, but also for your friendly chats and Friday beers in Nubar. I would also like to thank Mairead Callan, for the uncountable times you have saved my POs and orders, but also for the many chats in the kitchen.

Thank you to all the friends and colleagues in NICB that I have met during all these years, which have been there to give advice and help with any question I had. I would like to specially thank all the people from the first floor John, Neil, Nicola, Charles, Shannon and Ali for making me feel part of a group friends far from home and never stop inviting me to all the night games, dinners and all the group activities we have done, it has been real fun. I would like to specially thank my running therapist – favourite cook Ali. Thank you for all the laughing, crying, advice and support, you are one of a kind and I am really grateful we have met. Also, thank you to Shannon, for knowing when to take me out for a walk/coffee and encouraging me to keep going, thank you for being such a good friend. Finally, to my short time gym buddy Charles for all the laughs and chats.

I would also like to thank the guys from the second floor who have become my second family here in Ireland. This time here wouldn't have been the same without you guys. Specially, I would like to thank Antonio for being there at the good and bad times, for becoming a real friend; I am really glad we have met. Also, Ricardo for making me feel like home. Thank you for all the uncountable chats and advice at scientific but also personal level. Finally, to my flatmate and colleague, Giuseppe.

And now it is your turn. There are few things I will be taking home from Ireland, and one of them is you. When I decided to move abroad I wasn´t expecting to meet someone like you, and even less to

get married. Thank you Victor for being besides me, for all your patience (we both know it can get tough sometimes), for knowing how to calm me down and giving me the strength to keep going. For being my best friend, making me laugh and living this and future adventures with me.

Finally, I can't finish without thanking my family and friends from home. To my lovely parents and siblings, for all your support since way before the PhD, for always believing in me and making me feel I could achieve anything I wanted. To my lovely grandma, *iaia* Assumpció. You have finally got a doctor in the family, although not exactly the type you expected! I love you. Lastly, to Laura, Laureta and Ingrid, my lifelong friends, with whom I have shared laughs and cries, and that have been besides me even from the distance. Thank you for all your support.

Thesis structure

Section	Title	Status of publication	Authors	Contribution
-	Background on biopharmaceutical production	-	-	-
-	Preface to the Chapters – in-house serum-free media development	-	-	-
Chapter 1	Zinc supplementation increases protein titer of recombinant CHO cells	Accepted and published in Cytotechnology (doi: 10.1007/s10616-019-00334-1)	Capella Roca, B. Alarcon Miguez, A. Keenan, J. Suda, S. Barron, N. O'Gorman, D. Doolan, P.* Clynes, M.*	Main author. Primary contributor to the experimental design and execution.
Chapter 2	An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals	Accepted and published in Journal of Biological Chemistry (doi: 10.1074/jbc.RA119.011162) Work protected by intellectual property (patent application number 1911023.8)	Capella Roca, B. Lao, N. Barron, N. Doolan, P. Clynes, M.	Main author. Primary contributor to the experimental design and execution.
Chapter 3	Altered gene expression in CHO cells following polyamine starvation	Accepted (subject to reviewer's comments) in Biotechnology Letters (ID: BILE-D-19-00932)	Capella Roca, B. Doolan, P. Barron, N. O'Neill, F.* Clynes, M.*	Main author. Primary contributor to the experimental design and execution.
Chapter 4	Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO cells	Accepted in Biotechnology Progress (*doi: 10.1002/btpr.2954)*	Capella Roca, B. Lao, N. Clynes, M.* Doolan, P.*	Main author. Primary contributor to the experimental design and execution.

Research outputs

Publications

- Capella Roca, B., Alarcón Miguez, A., Keenan, J., Suda, S., Barron, N., O'Gorman, D., Doolan, P. and Clynes, M. (2019) Zinc supplementation increases protein titer of recombinant CHO cells. Cytotechnology. 71(5):915-924. DOI: 10.1007/s10616-019-00334-1
- Capella Roca, B., Lao, N., Barron, N., Doolan, P and Clynes, M. (2019) An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals. J. Biol. Chem. DOI:10.1074/jbc.RA119.011162
- Capella Roca, B., Lao T., N., Clynes, M. and Doolan, P. (2019) Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO. Biotechnology Progress. DOI: 10.1002/btpr.2954
- Capella Roca, B., Doolan, P., Barron, N., O'Neill, N. and Clynes, M. Altered gene expression in CHO cells following polyamine starvation. Biotechnol. Letters (manuscript ID:BILE-D-19-00932). Manuscript accepted (subject to reviewer's comments)

Poster presentations

- Capella Roca B., Joyce H., Meiller J., Doolan P., Clynes M. Development of Single-Cell Cloning Media for CHO Cell Lines. Poster presented at the 25th European Society for Animal Cell Technology (ESACT) conference; 2017 May 14th - 17th; Lausanne, Switzerland
- Capella Roca B., Doolan P., Clynes M. Copper and Zinc Supplementation Improve CHO-K1 VCD Levels in Suspension Culture. Poster presented at the 28th European Society for Animal Cell Technology -UK (ESACT-UK) annual meeting; 2018 Jan 10th - 11th; Leeds, UK
- Capella Roca B., Alarcon Miguez A., Keenan J., Suda S., Doolan P., Clynes M. Supplementation with high zinc concentrations increases final mAb and recombinant protein titer in DP12 and CHO-K1 cells. Poster presented at the 29th European Society for Animal Cell Technology -UK (ESACT-UK) annual meeting; 2019 Jan 9th - 10th; Tamworth, UK
- Capella Roca B., Alarcon Miguez A., Keenan J., Suda S., Doolan P., Clynes M. Supplementation with high zinc concentrations increases final mAb and recombinant protein titer in DP12 and CHO-K1 cells. Poster presented at the 26th European Society for Animal Cell Technology (ESACT) conference; 2019 May 5th – 8th; Copenhagen, Denmark

Scientific talks

- Capella Roca B., Doolan P., Clynes M. Copper and Zinc Supplementation Improve CHO-K1 VCD Levels in Suspension Culture. Poster flash presentation at the 28th European Society for Animal Cell Technology -UK (ESACT-UK) annual meeting; 2018 Jan 10th -11th; Leeds, UK

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Abbreviations

AC – Anticlumping agents

ATA – Aurintricarboxilyc Acid

ATCC – American Tissue Culture Collection

BA – Basic Additives

CDM – Chemically-Defined Media

CDS – Coding Sequence

CHO – Chinese Hamster Ovary

CMV – Cytomegalovirus

Co – Cobalt

Cu – Copper

DE – Differential Expression, Differentially-expressed

DHFR – Dihydrofolate Reductase

DMEM – Dulbecco's Modified Eagle's Medium

DMEM-F10 – Dulbecco's Modified Eagle Medium: Nutrient Mixture F-10

DMEM-F12 – Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DNA – Deoxyribonucleic acid

ELISA – Enzyme-Linked Immunosorbent Assay

EPO – Erythropoietin

ER – Endoplasmic Reticulum

FAC – Ferric Ammonium Citrate

FAS – Ferric Ammonium Sulphate

FBS – Fetal Bovine Serum

FC – Ferric Citrate

GFP – Green Fluorescent Protein

GS – Glutamine synthetase

hEPO – human EPO

IgG – Immunoglobulin G

IRES – Internal Ribosome Entry Site

LP – Lipids/Polyamines

mAb – Monoclonal Antibody

MARS – Matrix Attachment Regions

MEM – Eagle's Minimal Essential Medium

MEM – Minimum Essential Medium

MFI – Mean Fluorescence Intensity

miRNA – microRNA

Mn – Manganese

mRNA – messenger RNA

MSX – Methionine Sulfoximine

MTX – Methotrexate

NEAA – Non-Essential Aminoacids

PFM – Protein-Free Media

Pn – Passage number

PVA – Poly vynil Alcohol

RNA – Ribonucleic Acid

RQ – Relative Quantification

RT-qPCR – Quantitative reverse transcription polymerase chain reaction

SFM – Serum-Free Media

TGE – Transient Gene Expression

t-PA – tissue plasminogen activator

UCOE – Ubiquitous Chromatin Opening Elements

VCD – Viable Cell Density

Zn – Zinc

Abstract

Enhancement of CHO bioreactor performance has typically been derived from optimization of media formulations and feeding strategies, with advances in clone selection systems and cell engineering also playing an essential role. However, these breakthroughs in media development are usually not disclosed by the biopharmaceutical industry or media vendors due to commercial considerations. As a result, optimisation of CHO culture performance from the research sector is thus limited and time-consuming with undesired and/or unexpected effects in essential steps (e. g. transfection) also observed. To address this deficit in information, in-house serum-free and chemically-defined media (SFM and CDM) were developed as working tools to study the effects of media additives in culture performance. Investigating the titer-enhancing effects of zinc, the specific productivity of DP12 and rCHO-K1 cell lines could be significantly increased. A correlated effect was also observed at transcriptional level, with increased oxidative respiration metabolism also associated with the zinc-supplemented, higher-producing cultures. Building on from the knowledge gained, further investigation on essential additives for CHO survival was then performed, identifying putrescine as a vital supplement. Based on this phenotype, a novel auxotrophic-based selection system was designed. The method offers a drug-free, easy-to-apply and cost-effective system for cell line development, observed to successfully isolate hEPO- and GFP-expressing clones with stable production profiles for at least 42 generations. Further characterisation of the polyamine-dependent phenotype of CHO by gene expression microarray (Affymetrix) was then performed, suggesting an association between cessation of growth and increased G1/S transition but arrest at M/G1 checkpoint. Finally, to highlight the essential implications of media additives in other key steps for bioprocess optimisation, the effect of media additives in transfection was investigated. Assessing the efficiencies of liposome-, lipopolyplexes- and polymer-mediated transfections, an inhibitory role of ferric ammonium citrate was identified and a novel strategy to circumvent this inhibition was recommended.

Introduction

Background on biopharmaceutical production

In 1982, the first recombinant human DNA therapeutic, insulin (Humulin, Eli Lilly & Co.) was released to the market (Quianzon et al. 2012). Since then, over 316 biopharmaceuticals have been licensed in U.S. and Europe, with monoclonal antibodies (mAbs) the most predominantly approved drug (Fig. 1a) (Walsh 2018). Over the past five years, the rate of biopharmaceuticals approval has been observed to increase for the first time since 1995, with a total of 112 products licensed since 2014 – a 2-fold increase (Fig. 1b) (Walsh 2018). In 2017, total sales of up to $188 billion were recorded (Walsh 2018). The emergence of the biopharmaceutical patent cliff, with many "blockbuster" drugs coming off patent, is a main driver of this effect over the past number of years, resulting in a surge of biosimilar approvals and subsequent increase in competitiveness in the market. Several production platforms have been investigated and modified to generate therapeutics on an industrial scale. While some smaller proteins not requiring complex folding and post-translational modification - such as insulin and Hepatitis B vaccine - can be produced in *E. coli* or yeast, most complex therapeutics, such as monoclonal antibodies, are most efficiently produced in an active form by using mammalian platforms (Walsh, 2014) and for this reason mammalian cells are the dominant production platform (Walsh 2018) (Fig. 2). Bacterial expression systems lack glycosylation machinery and recombinant proteins produced by yeast display altered mannose patterns, so these platforms are thus limited to the production of non-glycosylated proteins (Lalonde and Durocher, 2017a). Similarly, other non-mammalian production platforms, including plant-derived systems (such as root carrot-derived cell lines), insect cells (such as Sf9) and transgenic animals (such as goat and rabbit) also include altered glycosylation patterns, resulting in only a few biopharmaceuticals and vaccines successfully approved for commercialisation with these platforms (see list in Dumont *et al.*, 2016).

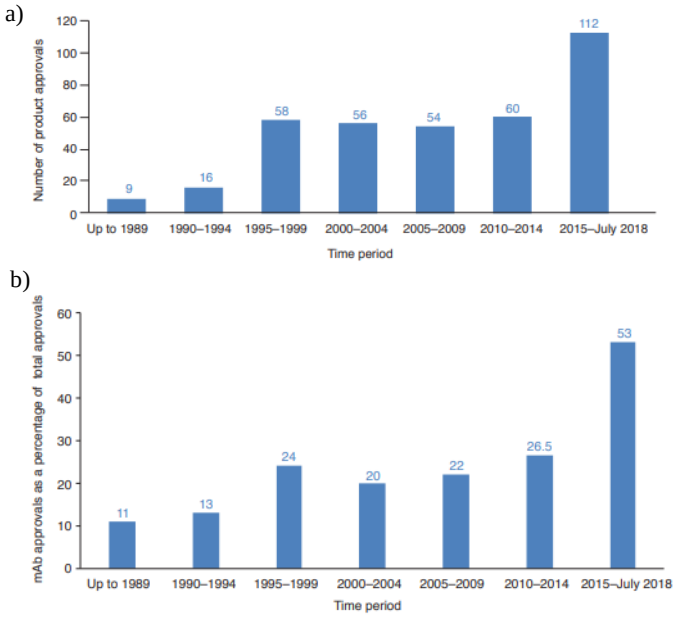

Fig. 1 a) Annual product approval over the indicated period. b) monoclonal antibody approval as a percentage of total approvals. Figure obtained from Walsh 2018.

Among mammalian-based expression platforms, Chinese Hamster Ovary (CHO) cells have remained the leading cellular system, involved in the production of nearly 70% of biopharmaceutical proteins (Butler and Spearman, 2014; Lalonde and Durocher, 2017b). As the first mammalian cell line to be used for the production of a commercial therapeutic back in 1986 (tissue plasminogen activator; t-PA), CHO cells have an extensive safety record and widespread regulatory acceptance (Kim, Kim and Lee, 2012). Several advantageous features include lack of human virus entry machinery, which increases the biosafety of therapeutic production, and high cell densities in serum-free and chemically-defined formulations at large-scale culture. Over the last few years, the development of amplification systems for this cell line have resulted in high specific productivities, with yields at the gram per litre levels reported (Butler and Spearman, 2014; Dumont *et al.*, 2016; Lalonde and Durocher, 2017b). Importantly, recombinant proteins produced in CHO cells include post-translational modifications resulting in bioactive, stable and compatible human therapeutics. Differing glycosylation patterns are sometimes observed between human and CHO products, although more humanised biopharmaceuticals can be obtained from several glycoengineering approaches, alteration of culture conditions and medium formulation strategies (Zhu, 2012; Butler and Spearman, 2014). Human-derived cell lines such as HEK293, PER.C6, HKB-11 and CAP have also been utilised over the past few years, with few therapeutic proteins developed from these platforms approved for the market in 2015 (e. g. rFVIIIFc, Dulaglutide and Velaglucerase alfa) (Zhu, 2012). Although human cell lines can facilitate acceptable glycosylation patterns for therapeutic activity, clear disadvantages are observed with these lines, such as the risk of pathogen contamination and the reported ability of the cells to produce Sialyl-Lewis^{x} modifications that could perturb bioavailability of the therapeutic (Kim, Kim and Lee, 2012). As a consequence of all the above, CHO cell lines are still the main workhorse for biopharmaceutical production.

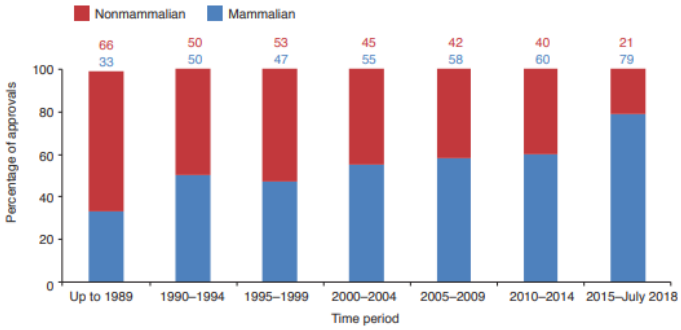

Fig. 2 Relative use of mammalian compared to nonmammalian-production system for the manufacture of biopharmaceuticals approved over the period of times indicated. Figure obtained from Walsh 2018.

The production of therapeutic proteins is time-consuming and laborious, consisting of the following stages: drug discovery, pre-clinical and clinical testing and approval, development and isolation of the producer clonal cell line, bioprocess culture and downstream purification. Targeted improvement of any of the stages will result in more efficient processes for the pharmaceutical industry. Given the vital role CHO cells play in the industry, enhancement of the culture performance profiles of this cell line (growth rate, viability, specific productivity, product quality, metabolite production, etc.) is a key goal of the biopharmaceutical sector. Three common strategies employed to improve the production process are: (i) media development and feeding strategies, (ii) bioprocess optimisation through bioreactor design, continuous bioreactor monitoring tools (e.g. pH, oxygen, temperature, glucose, lactate levels) and/or bioreactor culturing strategies (fed-batch, batch, perfusion) and (ii) generation of more productive cell lines by genetic engineering, clonal selection and amplification (Butler and Meneses-Acosta, 2012; Fischer, Handrick and Otte, 2015). These are briefly discussed below.

1. Media optimisation for improved growth and productivity

When CHO were first used for production of therapeutics (1986), yields of up to 100mg/L were reported (De Jesus and Wurm, 2011). Over the past three decades, this has increased almost 50-fold, with reported titers now between 3 - 10g/L - this has been achieved mainly from optimisation of feeding composition and bioprocess culture systems (Butler and Meneses-Acosta, 2012; Kelley, Kiss and Laird, 2018). Media formulations for biopharmaceutical cell culture have been developed to ensure sufficient nutrient availability to support healthy cell proliferation and high production profiles, while decreasing accumulation of toxic by-products derived from cell metabolism. Formulations are also expected to have minimal negative effects on end-product quality, such as increased aggregation of proteins or improper glycosylation patterns. Optimisation and research on media formulations and feeding compositions are continuously reviewed and updated in order to achieve enhanced bioprocessing. Metabolomics studies have become powerful tools in this area, identifying key parameters for efficient cell proliferation and product yield to be used for the improvement of bioprocesses (Dickson, 2014; Pereira, Kildegaard and Andersen, 2018).

1.1. Removal of serum: Serum-free and chemically-defined media development

Producer CHO cell lines used during biopharmaceutical production are clonally-expanded colonies isolated from a mixed population by using single-cell cloning assays to increase product reproducibility. However, these cells grow slowly or often fail to grow when cultured at low cell densities, resulting in poor culture performance (Zhu *et al.*, 2012). For this reason, further supplements are required in order to sustain cell growth. Fetal bovine serum (FBS) is a rich supplement obtained from foetal calves and contains a mixture of all the essential compounds required to promote cell proliferation *in vitro* (van der Valk *et al.*, 2018). Consequently, it was a widely-used

additive for CHO culture, consisting of a complex mixture of biomolecules which participate in the stimulation of cell proliferation due to the high content of growth factors and hormones (e.g. insulin, insulin-like growth factor 1 and 2, fibroblast growth factor, epidermal growth factor). It also acts as a source of large amounts of binding proteins which can act as carriers of trace elements, vitamins and lipids (e.g. transferrin, albumin). Moreover, these proteins also play a role in the detoxification of accumulated by-products, thereby reducing cell death. Proteins, peptides, minerals and cell adherence proteins, such as laminin or fibronectin are found in varying concentrations in FBS (van der Valk *et al.*, 2004, 2018).

Despite the advantages provided by this supplement, industrial production of therapeutics for human consumption demands the use of serum-free media formulations, mainly due to safety concerns arising from serum's animal-derived origin; these risks include possible viral, mycoplasma and/or prion contamination. Additionally, seasonal and continental variability leads to differences between batch-to-batch productions that may cause inconsistencies and impact batch productions of the eventual therapeutics (Gstraunthaler, 2003; van der Valk *et al.*, 2004). Moreover, serum's ill-defined composition may lead to the inclusion of additional downstream purification steps in order to remove contaminating animal proteins (David W Jayme and Smith, 2000). There are also ethical considerations with the use of FBS, as blood is extracted directly from the foetal bovine heart and processed to produce the serum (van der Valk *et al.*, 2004, 2018). As a consequence of the above, several approaches to reduce and replace serum supplementation have been pursued over the years, resulting in the development of a range of formulations that can be classified in four types: 1) *Serum-reduced media,* the result of initial attempts of serum-free media development which still requires low levels of FBS, along with additional supplements to ensure healthy cell growth; 2) *Serum-free media* (SFM), in which serum supplementation is replaced with growth factors and proteins (derived or not from animals); 3) *Protein-free media* (PFM), characterized by the complete removal of high molecular weight proteins. However, it may contain small peptides and/or protein hydrolysates; 4) *Chemically-defined media* (CDM), composed by well-defined supplements, which may or may not contain recombinant proteins. Additionally, media can be further defined by the term "*animal-derived component-free media*", indicating an absolute lack of animal-derived supplements (Fig. 3) (David W. Jayme and Smith, 2000; Van Der Valk *et al.*, 2010).

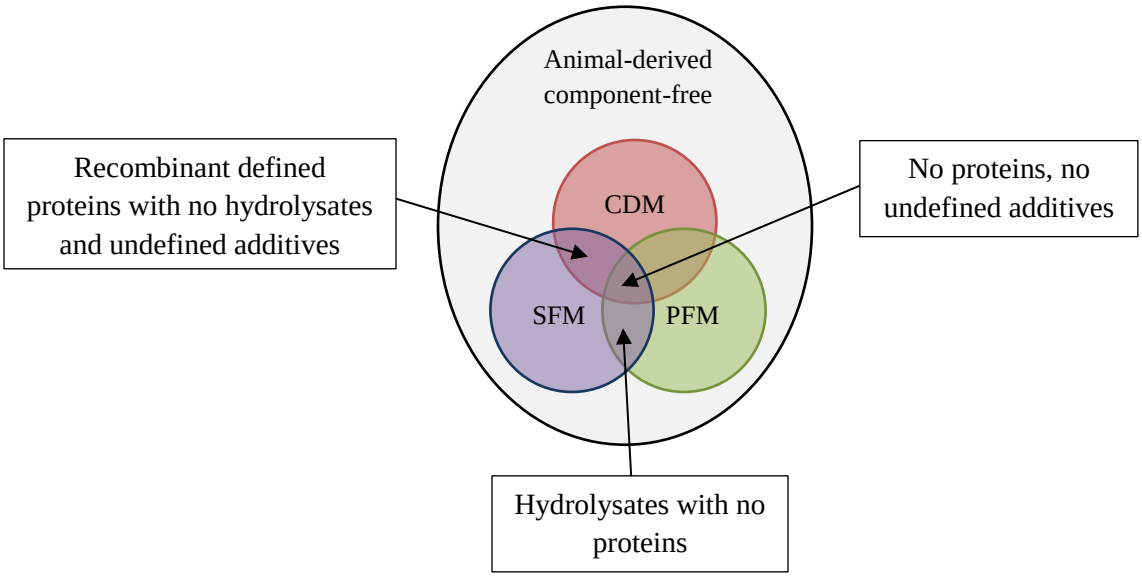

Fig. 3 Schematic of serum-removed media classification. A serum-free media might be also described as chemically-defined media if there is absence of undefined additives (such as hydrolysates) and contains recombinant proteins. A medium can be also defined as protein-free and serum-free if the formulation has no proteins. Finally, a third media definition includes lack of proteins and undefined peptides being then described as serum-free, chemically-defined and protein-free. Moreover, each of the formulations used for biopharmaceutical industry tend to avoid the use of animal-derived components. Thus, all three can be further classified as animal-derived component-free media.

1.1.1. Basal media

Media formulations lacking serum supplementation typically differ considerably depending on the nutritional requirements of the cell line, the recombinant product generated and the culture system. However, the development of a new formulation usually follows the same workflow: selection of the most suitable basal media and enrichment of the composition with essential nutritional supplements. Historically, the first successful attempt of *in vitro* tissue culture was based on a mixture of salts developed by Ringer and Buxton (named Ringer's solution), who successfully maintained functional frogs heart tissue for short periods of time (Yao and Asayama, 2017). Nonetheless, for the culture of cells and tissues for longer periods of time, additional supplementation with nutrients and pH buffering components was required to complete the formulation (Yao and Asayama, 2017). In 1955, Henry Eagle modified the saline solutions being used up until then by adding changing concentrations of L-aminoacids, vitamins, cofactors, carbohydrates and salts and tested them on two mammalian cell lines, HeLa and L-fibroblasts (Eagle, 1955). With this approach, essential compounds required for prolonged cell maintenance were identified, leading to the generation of the Eagle's Minimal Essential Medium (MEM), developed as a base media still requiring low percentages of serum

addition (Eagle, 1955; Van Der Valk *et al.*, 2010)**.** MEM was well-defined and the removal of any of the components resulted in cell death. From this basic approach, a chemically-defined formulation was sought. In 1963, serum supplemented to media was replaced by a combination of albumin and fetuin serum proteins, resulting in the development of Ham's F10 formulation. This medium was described as one of the first to successfully support isolation of single CHO cells in serum-free conditions (Yao and Asayama, 2017; Evans et al. 1956). Further investigations led to the development of a completely defined medium by replacing albumin and fetuin for linoleic acid and putrescine, resulting in (the still currently used) Ham's F12 medium (Ham, 1963b, 1963a; Ham, 1965). Variations of these formulations have since been designed, with DMEM (Dulbecco's Modified Eagle's Medium) being one of the most widely used formulas for cell culture. DMEM has a 4-fold increase in the quantity of vitamins and amino acid which supports the culture of a broad range of mammalian cell lines. Nutrient enrichment was achieved by combinations of these media, resulting in the development of the currently commercially-available DMEM-F12. This new formulation has been extensively used as a baseline medium for the development of serum-free formulations for several CHO cell lines (Van Der Valk *et al.*, 2010; van der Valk *et al.*, 2018). For instance, Huang *et al.* (2017) reported the development and optimisation of a chemically-defined and protein-free DMEM-F12-based media for the growth of an autocrine CHO cell line (Super-CHO), reaching up to $2.6x10^6$ cells/ml (Huang, Marquis and Gray, 2007). Similarly, DMEM-F12 was used as a basal SFM for the study of growth promoting agents, identifying insulin-like growth factor 1 (IGF-1) as an important growth-stimulating factor for DG44 cells (Chun *et al.*, 2003). Other basal media have been studied less extensively, such as alpha-MEM and IMDM. In 1998, a study to develop a producing medium for DG44 cells was performed using alpha-MEM. The final product reached maximal viable cell numbers of $5.6x10^5$ cells/ml, similar to the densities observed with 5 % FBS-supplemented media (Eun Jung Kim, Kim and Lee, 1998). IMDM (Iscove's Modified Dulbecco's Medium) is another popular baseline medium, which was derived from MEM (minimal essential medium) by replacing iron with potassium nitrate. For this reason, transferrin or protein-free iron chelator, like ferric citrate, must be added to support cell proliferation (Lee *et al.*, 1999; Kim *et al.*, 2006).

In order to generate further enriched formulations, combinations of two or more basal media have been also reported in the literature. For instance, Parampalli *et al.* published a SFM formulation based on a mixture of IMDM:Ham's F12 (1:1) where the concentration of the supplements was optimized, resulting in a final 1.4-fold increased viable cell density compared to the control ($1.45x10^6$cells/ml) (Parampalli *et al.*, 2007). In 2012, a combination of DMEM-F12:RPMI1640 (2:1:1) supplemented with amino acids, insulin, putrescine, ferric ammonium citrate and other components was developed for GS-CHO cells, reaching a maxim viable cell density of $3.7x10^6$cells/ml in a 5-day culture; similar to that achieved in control EX-CELL 302 commercial medium. Moreover, scaling-up the cultures to

2L bioreactors displayed reproducible viable cell density profiles when compared to 125ml shake flasks (Zhang *et al.*, 2013).

1.1.2. Additives

Basal media such as DMEM-F12 provides a mixture of organic and inorganic salts, amino acids, trace elements, vitamins, carbohydrates, putrescine, lipids and buffering agents essential for basic growth, but fails to support cell proliferation at high cell densities. In 1982, Murakami *et al.* identified insulin, ethanolamine, transferrin and selenium (ITES) as four vital additives to support mouse hybridoma cell lines in serum-free conditions (Murakami *et al.*, 1982). During early stages of serum-free and chemically-defined media, ITES became extensively popular for the growth of mammalian cell lines, resulting in a commercially available supplement for SFM development. Further investigations have led to the discovery of more chemically-defined additives that promote cell proliferation for long periods of time. In the following section, the most widely used supplements for CHO serum-deprived media are outlined.

1.1.2.1. Hormones and growth factors

Serum is rich in growth factors and hormones which participate in the activation of antiapoptotic and cell proliferation signals (Gospodarowicz and Moran, 1976). For this reason, initial attempts to sustain cell cultures in serum-free conditions focused on the supplementation of these proteins (Hayashi and Sato, 1976). Insulin has been defined as essential to sustain serum-deprived CHO cultures when supplemented at supraphysiological levels (1-20 μg/ml), inducing mitogenic signalling pathways with the activation of insulin like growth factor 1 (IGF-1) receptor (Keenan, Pearson and Clynes, 2006; Van Der Valk *et al.*, 2010; Ritacco, Wu and Khetan, 2018a). Supplementation of IGF-1 and its analogue LongR3 IGF-1 (Repligen) have been also explored, reporting similar or even increased viability profiles when compared to insulin, at lower supplemented concentrations than insulin (Morris and Schmid, 2000; Sunstrom *et al.*, 2000). Additional growth factors have been identified from a study of conditioned media samples, identifying fibroblast growth factor 8 (FGF8), hepatocyte growth factor (HGF) and vascular endothelial growth factor-c (Vegf-c) as growth promoting agents (Lim *et al.*, 2013). However, compared to serum and insulin, their growth promoting effects are less effective than those reported for insulin.

To obtain protein-free formulations, the mitogenic effects of zinc and aurintricarboxylic acid (ATA) supplementation have been exploited. Insulin-mediated growth-stimulatory effects mimicked by zinc-supplementation have been described in several cell lines (Tang and Shay, 2001; V. V. T. Wong *et al.*, 2006) including CHO (at 1.5mg/L Zn), although growth in insulin-replaced media was only observed with one of the two CHO cell lines tested (Wong, Ho and Yap, 2004). The role of zinc as an insulin-mimic has been associated with the activation of phosphoinositide 3-kinase/Akt pathway,

with no interaction with the insulin receptor (Ezaki, 1989; Tang and Shay, 2001). Alternatively, the antiapoptotic factor ATA has been previously reported as a growth-stimulation factor in PFM for CHO cells (Liu, Chu and Hwang, 2001a; Miki and Takagi, 2015). ATA has been described to induce its mitogenic activity through the activation of IGF-1 receptor response in a similar manner as insulin (Beery *et al.*, 2001).

1.1.2.2. Carbon sources

In most serum-free media, glucose is the main source of carbon and energy supplement for CHO cells. Industrial media compositions contain amounts at a range from 25 - 35mM. Variations of these glucose concentrations over or below the typical limits described have been shown to have an impact on the specific growth rate, productivity, nutrient consumption and product quality of CHO cells (Altamirano *et al.*, 2000; Wong *et al.*, 2005). From metabolic studies, it is widely known that CHO cultures in exponential phase consume glucose in large quantities which is converted to lactate through glycolysis, even in the presence of oxygen (Warburg effect) (Dickson, 2014). Limiting-glucose feeding approaches have been described to elongate the viabilities of the cultures though the reduction of lactate accumulation and without changing the transcriptomic expression of the cells (Wong *et al.*, 2005; Gowtham, Saski and Harcum, 2017). However, low specific growth and productivities are usually displayed as a result. Alternatively, glucose supplementation above 40 mM has been reported to increase specific productivity but to cause an accumulation of lactate that results in decreased specific cell growth (Lee *et al.*, 2015). For this reason, levels of glucose supplementation are usually maintained at 30 mM in growing cultures (Vergara *et al.*, 2018).

In order to reduce lactate accumulation, "slow metabolised" sugars, such as galactose, mannose or fructose, have been also tested. Complete replacement of glucose for either galactose or fructose has been reported to reduce cell growth and/or productivity, while mannose-containing cultures achieved similar specific growth rates compared to cultures in glucose-supplemented medium (Altamirano *et al.*, 2000).

L-glutamine is an important nutritional component of culture media, playing an essential role as a protein precursor as well as an important alternative energy source to glucose (Van Der Valk *et al.*, 2010). However, L-glutamine represents the main source of ammonia by-product, released for either its spontaneous breakdown or metabolism (Jagušić *et al.*, 2016). In order to overcome the lack of stability of this essential amino acid, dipeptides of L-alanine-glutamine (commercially available as Glutamax) or glycyl-glutamine have been developed, increasing the stability of the amino acid (Imamoto *et al.*, 2013; Ha and Lee, 2015). Alternative sources such as glutamate or pyruvate have been also used as replacements for glutamine, reporting substantial reduction in ammonium secretion

(Genzel *et al.*, 2008) and increased productivity profiles compared to non-supplemented cultures (Hong, Cho and Yoon, 2010).

1.1.2.3. Iron sources

Iron is essential for cell cycle progression, growth and division due to its involvement with many proteins and enzymes that participate in cell replication. Lack of iron supplementation results in G1/S phase arrest, reduction of cell growth and eventual cell death (Yu, Kovacevic and Richardson, 2007). However, excessive supplementation also may have detrimental effects. In media, free iron ions are highly reactive and may produce ROS species by Fenton redox-cycle reaction, damaging the cells (Graham, Bhatia and Yoon, 2019). For this reason, transferrin, the natural iron-chelating protein is supplemented in serum-free media formulations (Butler M. et al. 2015). Alternatively, in chemically-defined and protein-free media, iron has been delivered in the form of ferric citrate, ferric ammonium citrate, ferric sulphate, ferric ammonium sulphate or ferric gluconate (Zhang, Robinson and Salmon, 2006; Bai *et al.*, 2011; Zhang *et al.*, 2013). Sodium selenite has been also described as an effective iron-carrier, reporting final cell densities of $10x10^6$ cells/ml and mAb yields of 3 g/L in a 14-day fed-batch (Zhang, Robinson and Salmon, 2006). Another example is the iron chelating agent tropolone, which has not been extensively used in CHO due to patent protection (Zhang, Robinson and Salmon, 2006). It is noteworthy that, due to the essential nature of this micronutrient, basal media are routinely supplemented with traces of iron sources, such as DMEM-F12. However, as described above, additional supplementation to PFM and CDM has been observed necessary in order to improve cellular proliferation.

1.1.2.4. Trace elements

Most trace metals are already part of basal media formulations, such as DMEM-F12. It is noteworthy that, even though supplemented at very low levels, metals are essential to sustain healthy cell growth *in vitro*, being involved in many roles: regulatory factors, cellular respiration control, protection against oxidative stress and anti-apoptotic agents, among many others (Tan *et al.*, 1984; Gong *et al.*, 2001; Piret *et al.*, 2004; Chaderjian *et al.*, 2008a; Yuk *et al.*, 2014; Kim and Park, 2016).

Importantly, analysis of the composition of trace metals between two lots of SFM, revealed significant variations in copper, zinc, selenium and cobalt levels (Keenan *et al.*, 2018). These fluctuations have been associated with variations in the source and batch of raw materials used for the development of SFM formulations, such as hydrolysates (presence of iron salts) and insulin (zinc sometimes used as stabiliser). Metals leaching from glass and stainless steel vessels have also been reported, causing variability in media composition (reviewed in Graham, Bhatia and Yoon, 2019). Due to the vital involvement of metals in key cellular processes, alterations in culture performance may be observed in SFM as a consequence of small variations on the levels of these trace elements.

The most widely used trace metals for SFM development are copper, zinc and selenium, present as traces in basal media formulations. Supplementation of manganese, cobalt and other trace metals such as molybdenum and vanadium have been also reported with lower impact. Details on the role of these micronutrients are given below. It is also worth noting that further information and strategies for media optimisation based on trace metals addition are described in the next section.

1.1.2.4.1. Copper

Copper is an essential micronutrient for cell growth, being related to oxidative respiration and shift to lactate consumption during the stationary phase, thus elongating cell culture (Chaderjian *et al.*, 2008b; Luo and Pierce, 2012; Yuk *et al.*, 2014; Yuk, Russell, Tang, W. T. Hsu, *et al.*, 2015; Xu, Hoshan and Chen, 2016). Copper plays an essential role in the electron transport chain, being a cofactor of the cytochrome c oxidase subunit of complex IV (Kang *et al.*, 2014a). Low levels of this metal in media have been associated with a reduced number of complex IV (Kang *et al.*, 2014a) and increased lactate dehydrogenase function, thus accumulating lactate in the medium (Zagari *et al.*, 2013a; Kang *et al.*, 2014a; Nargund, Qiu and Goudar, 2015a). Following supplementation of copper concentrations above a defined threshold (13nM), induction of a metabolic shift to lactate consumption has been reported (Yuk, Russell, Tang, W.-T. Hsu, *et al.*, 2015), resulting in improved viable cell density, viability and productivity profiles (Luo *et al.*, 2012). However, reduced viabilities (82-90 %) have been observed in DUX-B11 cells cultured in serum-free conditions with concentrations of 50-100 µM Cu (Chaderjian *et al.*, 2005). In fact, increased production of reactive oxygen species (ROS) are associated with high copper concentrations in culture (Camakaris *et al.*, 1995).

1.1.2.4.2. Zinc

Being involved on the activation and function of more than 300 enzymes and transcription factors, supplementation with sufficient zinc concentrations is required for proper cell proliferation. The importance of this micronutrient in CHO cell growth was reported by Xu *et al*. Using design of experiments (DoE), $ZnS0_4$ (at 1mg/L) was identified as one of three most significant supplements inducing growth-promoting effects in CHO-K1 cultures in a CDM (Xu *et al.*, 2014). Zinc has been described as a powerful antioxidant in culture (Marreiro *et al.*, 2017), acting as a co-factor of superoxide dismutase, inducing the expression of glutathione, activating the metal transcription factor 1 – which plays an important role in regulating oxidative stress – and protecting from lipid peroxidation (Marreiro *et al.*, 2017). Zinc has been also observed to display anti-apoptotic properties in bioreactor-relevant conditions (Cotter and Al-Rubeai, 1995) and to stimulate glucose uptake (Ezaki, 1989). Zinc involvement in mRNA stability has been also reported, mediated by the interaction of zinc-finger proteins with nucleic acids (Taylor and Blackshear, 1995; Buchner *et al.*,

2015), while a beneficial effect on product titer in CHO has been also described following supplementation of zinc at high concentrations (Kim and Park, 2016).

1.1.2.4.3. Selenium

Selenium has been identified as an essential supplement for mammalian cell culture as well as in serum-deprived conditions, enhancing the healthy growth of cells (Mckeehan, Hamilton and Ham, 1976; Lebkowski, Schain and Okarma, 1995). It is extensively used for serum-free media development, with concentrations as low as 20 µg/L commonly supplemented (E J Kim, Kim and Lee, 1998; Kim, Kim and Lee, 1999; Lee *et al.*, 1999; Schröder, Matischak and Friedl, 2004). Selenium has been described to participate in oxidative-stress protection, constituting selenocysteine-containing proteins such as glutathione peroxidase and glutathione synthetase (Köhrle *et al.*, 2000; Schomburg, Schweizer and Köhrle, 2004). Detoxification of peroxide oxygen radicals by the activation of anti-apoptotic pathways has been also observed, promoting longevity of the cultures and improved growth profiles (Yoon *et al.*, 2002). Moreover, selenium has been described to play an essential role in MAPK pathway and therefore to induce mitogenic growth effects (Zeng and Combs, 2008).

1.1.2.4.4. Cobalt

Supplementation of cobalt to culture media has been shown to induce hypoxia-like conditions, resulting in the stimulation of glucose uptake and glycolytic pathways. Moreover, antiapoptotic protection following serum-deprivation has been also observed in HepG2 cells (Gong *et al.*, 2001; Piret *et al.*, 2002, 2004; Torii *et al.*, 2011). In 2014, cobalt was reported to modify the galactosylation patterns of a mAb for two CHO cell lines. However, this effect was substantially lower compared to manganese supplementation and each cell line presented different sensitivities to the metal, with decreased product titer observed at concentrations above 50 µM for cell line 1, but a similar phenotypic threshold concentration for cell line 2 was observed at just 5 µM (Hossler and Racicot, 2015).

1.1.2.4.5. Manganese

Manganese has been described as an antioxidant, acting as a cofactor of manganese superoxide dismutase (Mn-SOD) – a mitochondrial enzyme that eliminates reactive oxygen species by conversion into oxygen and hydrogen peroxide (Kaewpila *et al.*, 2008). Manganese plays an important role in the regulation of galactosylation and glycosylation processes and for this reason, this metal is usually supplemented in serum-free media formulations to enhance product glycosylation patterns (Kaufman, Swaroop and Murtha-Riel, 1994; Crowell *et al.*, 2007; Hossler,

Khattak and Li, 2009; Graham, Bhatia and Yoon, 2019). However, no effects on cell growth were observed in a separate study with supplementations up to 100 µM (Gawlitzek *et al.*, 2009a).

Other trace metals, such as vanadium has also been described to increase cell growth (Ritacco, Wu and Khetan, 2018b).

1.1.2.5. Amino acids

As the starting building blocks for the construction of proteins, amino acids are required for cell proliferation and productivity. In response to amino acid-limiting conditions, activation of several pathways is observed to decrease the overall cellular translation and induce ER-stress (Kilberg *et al.*, 2005). Nonetheless, excessive amino acid addition increases media osmolarity and accumulation of by-products such as ammonia, therefore resulting in reduced cell growth (González-Leal *et al.*, 2011). For this reason, to achieve healthy CHO cell cultures, most amino acid levels have to be maintained within specific ranges. Moreover, optimal amino acid supplementation levels vary between cell lines, culture systems and compositions of the recombinant protein being produced (Fomina-Yadlin & McGrew, 2014). Several studies have demonstrated the effectiveness of adjusting amino acid supplementation to achieve high titer and viable cell densities. For example, threonine has been reported to alleviate stressing conditions such as high ammonium, dissolved CO_2 and osmolality effects, resulting in increased VCD and titer profiles (Chen and Harcum, 2005).

Some amino acids have relatively low solubility and stability, such as tryptophan, glutamine, cysteine and tyrosine. To improve their properties and to avoid accumulation of toxic substances derived from their breakage, storage of media protected from light participated in maintaining tryptophan levels (Mcelearney *et al.*, 2016). The replacement of such amino acids for derivatives has been also performed, such as tyrosine-containing dipeptides to improve tyrosine solubility (Kang *et al.*, 2012) or s-sulfocysteine, reported to replace cysteine and act as an anti-oxidant in CHO culture media (Hecklau *et al.*, 2016).

1.1.2.6. Lipids

Lipids are an essential constituent of membrane structure acting as cellular stabilizers. Moreover, lipids can serve as an energy supply and roles in cellular transport and signalling have also been previously described (Ritacco, Wu and Khetan, 2018b). Exogenous addition of lipid is not essential for CHO culture. However, increased viable cell density and viability profiles are achieved with the addition of the mitogenic lipid lysophosphatidic acid, in the absence of growth factors (Miki and Takagi, 2015). Supplementation with ethanolamine phospholipids, major constituents of the cellular membrane, has been described to enhance cell proliferation of CHO cells in serum-free media formulations (Zhang *et al.*, 2013). Increased CHO growth has been also reported with the

supplementation of phosphatidylcholine in serum-free formulations (Sung *et al.*, 2004; Kim and Lee, 2009), which may have an important role in maintaining the integrity of the cellular membrane (Castro *et al.*, 1996). Addition of linoleic acid has been previously reported to stimulate growth of CHO cells and supporting cultures for a minimum of 4 subcultures (Eun Jung Kim, Kim and Lee, 1998). However, linoleic acid is typically supplemented bound to BSA in most of the publications (Liu, Chu and Hwang, 2001b; Liu and Chang, 2006). Addition of fatty acids and cholesterol can be also performed by using soluble derivates or including small amounts of ethanol. However, high concentrations may induce lipotoxicity in CHO cells (Ritacco, Wu and Khetan, 2018b).

1.1.2.7. Vitamins

Vitamins are essential media additives required in small amounts for cell culture. Vitamins have been described to act as enzyme cofactors, biological antioxidants, prosthetic groups or even as hormones (Schnellbaecher *et al.*, 2019). Due to their vital roles, vitamins are normally supplemented in basal media formulations, such as DMEM-F12, DMEM or RPMI 1640 (Van Der Valk *et al.*, 2010). More interestingly, modification in vitamin content has been also reported to enhance growth and titer of CHO cell lines (Kim *et al.*, 2005; Zhang *et al.*, 2013).

Importantly, several factors such as light, heat, oxygen, or reactive oxygen species (ROS) can compromise the stability of some vitamins. For this reason, protection of media from the light and heat are essential considerations for the proper maintenance of media (Schnellbaecher *et al.*, 2019).

1.1.2.8. Surfactants

In suspension, CHO-derived cell lines can spontaneously aggregate forming large clumps of cells. This effect causes detrimental effects due to the limited availability of oxygen and nutrients for the inner cells of the clump (Jing *et al.*, 2016). As a result, several anti-clumping agents have been described in the literature which seek to inhibit cell clumping in serum-deprived conditions. Several mechanisms of cellular aggregation in suspension culture have been described: DNA released from apoptotic cells (Renner *et al.*, 1993), extracellular cell adhesion molecules such as NCAM (neural cell adhesion molecule) (Zanghi *et al.*, 1998; Yamamoto *et al.*, 2000) and high amounts of calcium (Peshwa *et al.*, 1993). Some examples of the most commonly used additives include Pluronic F68, poly vinyl alcohol (PVA), dextran sulphate and suramin (Michaels and Papoutsakis, 1991; Zanghi *et al.*, 2000; Jing *et al.*, 2016). Pluronic F68 has been also reported to improve CHO cell growth, viability and productivity (Clincke *et al.*, 2011).

1.1.2.9. Polyamines

The polyamines spermine, spermidine and putrescine are vital for cell growth and cell cycle progression and also participate in pathway signalling and apoptosis protection (Igarashi and

Kashiwagi, 2019). Intracellularly, polyamines are found interacting mainly with RNA but also with DNA, participating in the regulation of gene expression and protein transcription (Igarashi and Kashiwagi, 2019). In mammalian cells, polyamine production starts with arginase, an enzyme catalysing the conversion of L-arginine to L-ornithine, which is the substrate for ornithine decarboxylase to generate putrescine (Fig. 4). The latter is then used for the synthesis of spermine and spermidine. In CHO, supplementation of putrescine to serum-free formulations is essential to sustain healthy cell growth due to the lack of expression of arginase (Hölttä and Pohjanpelto, 1982). It is also worth noting that further investigation by Hölttä and Pohjanpelto (1982) revealed arginase activity in several sources of serum, masking the vital requirement of polyamines for culture progression.

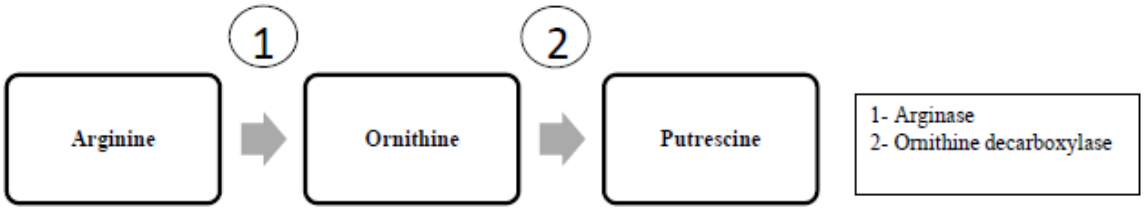

Fig. 4. Schematic of the initial stages of polyamine biosynthesis: arginase and ornithine decarboxylase.

Some basal media such as DMEM-F12 already contain traces of putrescine. However, the level is not sufficient to support CHO growth, requiring further supplementation (Hölttä and Pohjanpelto, 1982). Spermine and spermidine have been also shown to enhance growth rate and viability of CHO cells (Spearman *et al.*, 2016). However, high intracellular polyamine content increases activation of catabolism pathways, accumulating toxic by-products that can trigger endoplasmic reticulum stress and eventually apoptosis (Zahedi *et al.*, 2017; Dever and Ivanov, 2018). Consequently, polyamine content in media has to be controlled below toxic levels (at least, lower than 1mM) (Pastorian and Byus, 1997).

1.2. Media additives strategies for CHO optimisation

Optimisation of media formulation has been a key strategy over many years to enhance CHO performance. Some of the strategies to improve CHO viable cell densities, titer and specific productivities include the use of peptones and hydrolysates, alternative carbon sources, supplementations at increased concentrations or the addition of small molecules and epigenetic modifiers (Table 1). In the following section, some of these approaches are further detailed.

Table 1. Some media strategies reported to enhance CHO performance in terms of growth, viability and/or productivity.

Supplementation strategy	Cell Line	Phenotype	Reference
Peptones and hydrolysates			
Yeast hydrolysates	DUKX-B11 expressing human thrombopoietin	Increased specific productivity rate, extended culture longevity, higher viability	Y. H. Sung, Chung, Lee, & Lim, 2004
Soy protein hydrolysates	DG44	Enhanced cell growth and viability	hun, Kim, Lee, & Chung, 2006
Wheat peptones	rCHO-K1 expressing human interferon-γ	Improved cell growth and productivity	Burteau et al., 2003
Combinations of wheat, soy and casein peptones	DG44 expressing an IgG mAb	Increased volumetric productivity and cell number	Davami, Baldi, Rajendra, & M Wurm, 2014
Yeast extract	rCHO-K1 expressing Fc-fusion protein	Increased specific productivity	Hu et al., 2015
Combinations of yeastolate, soy and wheat gluten hydrolysates	DG44 and DUKX-B11 expressing mAbs	Increased cell growth (soy and wheat) or specific productivity (yestolate)	S. H. Kim & Lee, 2009
Yeast peptones	CHO-AMW expressing mAb	Increased cell growth rate, high cell densities and improved production	Mosser et al., 2013
Small molecules			
Sodium butyrate (NaBu)	rCHO expressing recombinant human thryrotropin	Increased volumetric productivity	Damiani, Almeida, Oliveira, Bartolini, & Ribela, 2013
Lithium chloride (LiCl)	DUKX-B11 expressing Fc-fusion protein	Increased specific productivity	Ha, Kim, & Lee, 2014
Aurintricarboxylic acid (ATA)	rCHO expressing Fc-fusion protein	Induced proliferation under insulin-free conditions	Liu, Chu, & Hwang, 2001
Valeric acid	rCHO-K1 expressing mAb	Increased culture longevity and specific productivity	Park, Noh, Woo, Kim, & Lee, 2016
Valproic acid	DHFR-derived CHO cell line expressing a mAb	Increased titer	Yang et al., 2014
Trace metals			
Iron Citrate	rCHO expressing a mAb	Enhanced titer	Bai et al., 2011
Copper	DXB11 expressing a mAb	Increased product titers, shift to lactate consumption	Yuk et al., 2015
Copper	rCHO expressing a mAb	Increased VCD and titer, shift to lactate consumption	Qian *et al.*, 2011

Copper and iron	DG44 expressing a mAb	Increased VCD, viability and titer	Qian *et al.*, 2014
Manganese	CHOK1SV and DUXK-B11 expressing mAb	Galactosylation patterns	Grainger & James, 2013 Pacis, Yu, Autsen, Bayer, & Li, 2011
Copper and manganese	rCHO expressing a mAb	Increased product quality (reduced tryptophan oxidation)	Hazeltine et al., 2016
Zinc and manganese	DG44 expressing a mAb	Galactosylation patterns	Prabhu, Gadre and Gadgil, 2018
Zinc	DG44 expressing a mAb	Increased titer	B. G. Kim & Park, 2016
Alternative carbon sources			
Combinations of glucose, galactose and/or glutamate	rCHO Tissue plasminogen activator	Increased viability and productivity	Altamirano *et al.*, 2000, 2004
Glucose and galactose	DHFR-derived expressing a mAb	Increased viable cell density and specific productivity	Sun *et al.*, 2013
Others			
Rapamycin	rCHO-K1 and DG44 expressing a mAb	Extended viability profiles and increase mAb titer	Lee and Lee, 2012; Dadehbeigi and Dickson, 2015
Dichloroacetate (DCA)	rCHO-K1 expressing a mAb	Extended lifespan, increased titer and viable cell density	Buchsteiner *et al.*, 2018

1.2.1. Peptones

Enhancement of growth and productivity profiles has been pursued with a range of feed/media additives. Plant peptones and hydrolysates derived from sources such as rice, wheat, soy, pea and yeast hydrolysates have been added to serum-free media (SFM) in CHO cell lines, demonstrating subsequent enhancement of viable cell density (Chun *et al.*, 2006), as well as specific-productivity and/or final titer of recombinant proteins such as IFN-γ (Burteau *et al.*, 2003), human thrombopoietin (Sung *et al.*, 2004) and a range of mAbs (Kim and Lee, 2009; Mosser *et al.*, 2013; Davami *et al.*, 2014; Hu *et al.*, 2015). However, supplementation with these products introduces undefined elements to the media, leading to possible batch-to-batch variability that may affect cellular performance in the bioreactor (McGillicuddy *et al.*, 2018).

1.2.2. Small molecules

A more "chemically-defined" approach has seen the use of chemical reagents, including the extensively reviewed sodium butyrate (NaBu) as well as several other reagents (valeric acid, valproic acid (VPA), lithium chloride, dimethyl sulphoxide (DMSO)) (Liu, Chu and Hwang, 2001a; Damiani

et al., 2013; Ha, Kim and Lee, 2014; Yang *et al.*, 2014; Park *et al.*, 2016), with the aim of improving titer of several therapeutically-relevant proteins in CHO cultures. However, the specific productivity effects observed with most of these chemicals (NaBu, DMSO, VPA) is frequently associated with cytotoxic effects resulting in reduced cellular growth (Sung, Hwang and Lee, 2005; Park *et al.*, 2016).

1.2.3. Trace metals

Supplementation of media with certain metals has revealed great potential to induce beneficial CHO performance features such as improved growth, specific productivity and viability profiles in culture. Increased mAb titers have been reported in CHO supplemented with iron-citrate (Bai *et al.*, 2011), while supplementation with copper has been extensively reported to increase product titer and to extend the lifespan of CHO cultures due to a shift to lactate consumption (Luo *et al.*, 2012; Zagari *et al.*, 2013b; Kang *et al.*, 2014b; Nargund, Qiu and Goudar, 2015b; Yuk, Russell, Tang, W.-T. Hsu, *et al.*, 2015). Co-supplementation of copper and iron during the scale-up stage has been observed to improve cell growth, viability and IgG productivity of CHO cultures. This effect was a consequence of the upregulated expression of hypoxia-inducible factor 1 alpha induced by copper (Qian *et al.*, 2014). Manganese supplementation has been used to modulate the glycosylation patterns of mAb (Gawlitzek *et al.*, 2009b; Pacis *et al.*, 2011; Grainger and James, 2013). Reduced tryptophan oxidation by manganese and copper supplementation in an IgG4-biopharmaceutical producing CHO culture (with no effects on titer or VCD) has also been reported (Hazeltine *et al.*, 2016). Moreover, high zinc supplementation to a DG44 culture growing in an in-house and commercial PFM and CDM media has been reported to induce up to 6.5-fold increased mAb titer (Kim and Park, 2016). However, a suitable ratio between zinc and manganese concentrations has to be achieved in order to obtain proper galactosylation patterns (Prabhu, Gadre and Gadgil, 2018).

1.2.4. Reducing by-product accumulation

As described in the previous sections, accumulation of toxic by-products – primarily lactate and ammonium from glucose and glutamine metabolism - is detrimental for CHO cultures (Lao and Toth, 1997) and approaches to reduce their production have been thus pursued. Combination of the slowly-metabolised carbon source galactose with glutamate have been reported to dramatically reduce lactate and ammonia accumulation, but at a cost of decreased CHO viable cell density profiles (Altamirano *et al.*, 2000; Altamirano, Cairó and Gòdia, 2001). In a follow-up study, alternation of the carbon source between glucose and galactose alongside the replacement of glutamine by glutamate was observed to increase viability, longevity and t-PA production (Altamirano *et al.*, 2004). Similarly, Sun et al. reported increased cell density, specific productivity and a lactate switch following glucose-galactose feeding (Sun *et al.*, 2013). Indirect increased expression of pyruvate dehydrogenase using the inhibitor dichloroacetate (DCA) was reported to result in increased viable cell density, culture

length and final mAb yield, which was observed to be as a consequence of decreased lactate production and glucose consumption profiles (Buchsteiner *et al.*, 2018).

2. Culture strategies

As biopharmaceutical manufacturing culturing strategies developed, batch was the first and the easiest mode of operation; where cells are cultured until the available nutrients are exhausted, closely followed by cell viability and growth drops, when product is harvested and purified through downstream processes (Bielser *et al.*, 2018). A second system is fed-batch. This mode of operation is currently the main workhorse in the biopharmaceutical industry, with feeding strategies targeting the control of by-product accumulation (such as lactate or ammonia) and supplementation of nutrient levels to suffice cell metabolic requirements (Kelley, Kiss and Laird, 2018; Mellahi et al. 2019). Consequently, typical fed-batch culture duration is longer than in batch mode and consequently reaches higher product yields at similar volumes. A third system (continuous/perfusion) is described by constant feed of media and removal of bioreactor content, with a retention of cells into the bioreactor (Bielser *et al.*, 2018). A recent combinatorial approach of perfusion and fed-batch systems has also been described, with sustained increased viable cell densities leading to almost 2-fold higher titers compared to fed-batch production, which was tested in five mAb-producer CHO cell lines (Hiller *et al.*, 2017). Recently, perfusion culture system has shown potential achieving increased cell densities and high volumetric productivities compared to fed-batch production (Bielser *et al.*, 2018). This intensified culture performance is so dramatic that it can result in the design of smaller production plants for similar output levels (Bielser *et al.*, 2018). Moreover, continuous removal of recombinant protein from the culture vessel can also help increase the stability of the products (Kelley, Kiss and Laird, 2018). However, possible technical issues (such as microbial contamination and technical failure), difficulties in mixing high cell density vessels and downstream train bottlenecks are still issues that need to be fully addressed to facilitate the successful application of continuous production systems (reviewed in Bielser *et al.*, 2018; Kelley, Kiss and Laird, 2018).

2.1. Culture parameters

CHO cells are very robust, adapting easily to changes in pH, temperature, osmolality, oxygen and pressure. This has allowed development of strategies to increase titre and cell growth by altering some of the parameters described above (Gagnon *et al.*, 2011; Nasseri *et al.*, 2014). One of the most extensively-studied strategies consists of the use of mild hypothermia (30 - 35^0C) or "cold-shock" at advanced stages of the culture in order to induce increased specific productivities (Becerra *et al.*, 2012; Bedoya-López *et al.*, 2016; Zhou *et al.*, 2018). However, variability in product quality attributes such as glycosylation and charge heterogeneity have been reported in low temperature cultures, which must be considered when applying this approach (Zheng *et al.*, 2018). Additionally, induction of

metabolic switch to lactate consumption has been related to increased productivity profiles (Le *et al.*, 2012). A pH – glucose feeding strategy has also been developed, generally resulting in higher peak viable cell densities and process productivity (Gagnon *et al.*, 2011). The method, named as HiPDOG (Hi-end pH delivery of glucose), involves the continuous monitoring and control of glucose concentrations at very low levels based on changes in pH (Gagnon *et al.*, 2011). Enhancing CHO performance through various culture strategies has been facilitated by the development of process analytical technologies providing *in situ* adjustment of the essential parameters (pH, CO_2, oxygen) (Teixeira *et al.*, 2009), enabling more controlled production processes.

3. Cell line development

In the biopharmaceutical industry, production of therapeutics relies on the generation of stable cell lines following two main technologies: dihydrofolate dehydrogenase (DHFR) and glutamine synthetase (GS). The DHFR-deficient DG44 and DUXB11 cell lines are commonly used for DHFR-selection system, while the GS system can be equally used in GS-deficient or parental CHO cells (Dumont *et al.*, 2016), in which case methionine sulfoximine (MSX) selection pressure is applied. To achieve high producing populations, the use of methotrexate (MTX), a DHFR inhibitor, is included to increase the stringency of the selection. Similarly, for the GS system, MSX inhibitor is supplemented. However, several rounds of incrementing MTX concentrations are usually required, resulting in a 5-6 months selecting process (Goh and Ng, 2018). Compared to DHFR, the GS system, selection in glutamine-free medium reduces the accumulation of ammonia in the medium and thus is advantageous in maintaining healthy cultures (Edmonds *et al.*, 2006).

The process begins with the design of an expression vector encoding for the recombinant protein and the complimentary marker (DHFR or GS) (Fig. 5). Selection of transfected populations is then performed in media depleted of either thymidine, hypoxanthine (DHFR system) or glutamine (for GS system), resulting in only the survival of the transfected population. An amplification step is then performed in order to obtain high producer populations, using the enzyme inhibitors MTX or MSX. In order to survive, cells suffer multiple genomic rearrangements, resulting in increased copy number of DHFR or GS (depending on the system used) and also of the heterologous protein. Consequently, an heterogeneous pool of cells expressing at different levels and rates is obtained and clonal isolation is then performed. Hundreds of clones are screened and only the ones displaying the highest specific productivity and growth rates are selected to be expanded. Clonal-derived populations are then cultured for several passages and re-assessed for growth and productivity to finally select the best performing cell line for large scale production (Lai, Yang and Ng, 2013; Le *et al.*, 2015). Development of stable cell lines is a laborious, time-consuming and costly process, generally lasting between 6 to 12 months (Gutiérrez-Granados *et al.*, 2018).

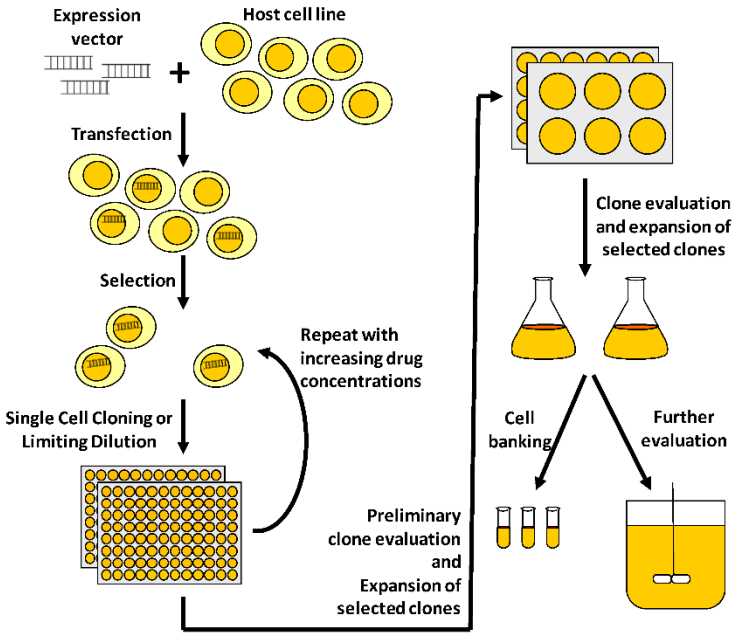

Fig. 5 Stable cell line development. Image obtained from (Lai, Yang and Ng, 2013).

Advances in cell line development technologies are mainly focused on reducing the timeline of the process. Random transgene integration into transcriptionally active loci is one of the major challenges. To enhance the expression of heterologous protein, several approaches have been developed. For example, site-directed integration into productive chromosomal spots ("hot spots") (using e. g. CRISPR-cas or Cre/Lox systems) (Le *et al.*, 2015; Kelley, Kiss and Laird, 2018) or inclusion of chromosomal elements results in an enhancement of transgene transcription (e. g. matrix attachment regions (MARS) and ubiquitous chromatin opening elements (UCOEs)) (Lai, Yang and Ng, 2013). Other strategies focus on increasing the stringency of the selection process in order to increase gene copy number, using attenuated selection markers or controlling the expression of the selection marker by an internal ribosome entry site (IRES) (Le *et al.*, 2015; Zhu and Hatton 2018).

Assurance of clonal-derived cell lines is demanded by regulatory agencies to ensure reproducibility over the production process of product quality attributes. For this reason, several rounds of limiting-dilution cloning or validating the use of a single cell by image analysis are common strategies during the single-cell cloning step (Kelley, Kiss and Laird, 2018; Welch and Arden, 2019). Automated high-throughput platforms have been also developed, enabling the screening of large numbers of plates (Priola *et al.*, 2016).

Large amounts of recombinant protein (at gram level) can be also produced by transient gene expression (TGE). This approach consists on the transfection of an exogenous recombinant protein with no subsequent selection, resulting in very short production processes with recombinant protein expressed after 7-14 days. However, lack of selection pressure leads to an eventual loss of vector expression. Currently, TGE technology plays an important role during the early stages of

biopharmaceutical development, but still presents some limitations for large scale production (such as regulatory acceptance, media suitable for transfection and production, large quantities of DNA required) (Zhu, 2012).

3.1. Cell line engineering – tools and strategies

Some of the genetic engineering strategies for the enhancement of CHO culture performance have focused on the modification of pathways related to cell growth and protein production. For instance, overexpression of genes involved in cell proliferation, cell cycle, anti-apoptotic responses or the mTORC1 pathway have resulted in increased lifespan of cultures, increased growth and final product yields (D. C. F. Wong *et al.*, 2006; Kuystermans and Al-Rubeai, 2009; Dreesen and Fussenegger, 2011). A major bottleneck for CHO recombinant protein production is protein processing and secretion, suggested from the lack of correlation between gene copy number and final protein titer reported. Thus, modifications in the unfolded protein response, endoplasmic reticulum chaperones and vesicle transporters have also been extensively studied in CHO (reviewed in (Zhou *et al.*, 2018)). Metabolomic engineering has been also employed in an attempt to decrease toxic by-product accumulation. For example, strategies to decrease lactate production have been achieved by the overexpression of fructose transporter (GLUT5) a component of the malate-aspartate shuttle (Aralar1) or pyruvate carboxylate (PYC2) (reviewed in Pereira, Kildegaard and Andersen, 2018).

Over the past few years, enormous advances in genome-editing technologies have been achieved, facilitating the generation of modified producer CHO cell lines. These include miRNAs and sponge tools, CRISPR/cas9 and RNAi knock-out technologies, zinc fingers nucleases (ZFN) and transcription activator-like effector nucleases (TALENs) (reviewed in Fischer, Handrick and Otte, 2015). Together with the availability of the CHO-K1 and Chinese hamster genome sequences (Brinkrolf *et al.*, 2013; Lewis *et al.*, 2013; Xu *et al.*, 2011), these advances in the field have provided new opportunities for further genetic improvement of these cell lines.

References

Altamirano C, Paredes C, Cairó JJ and Gòdia F (2000) Improvement of CHO cell culture medium formulation: simultaneous substitution of glucose and glutamine. Biotechnol Prog. 16(1):69-75. DOI: 10.1021/bp990124j

Altamirano C, Paredes C, Illanes A, Cairó JJ and Gòdia F (2004) Strategies for fed-batch cultivation of t-PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium. J Biotechnol. 110(2):171-9. DOI: 10.1016/j.jbiotec.2004.02.004

Altamirano C, Cairó JJ and Gòdia F (2001) Decoupling cell growth and product formation in Chinese hamster ovary cells through metabolic control. Biotechnol Bioeng. 76(4):351-60. DOI: 10.1002/bit.10096

Bai Y, Wu C, Zhao J, Liu YH, Ding W and Ling WL (2011) Role of iron and sodium citrate in animal protein-free CHO cell culture medium on cell growth and monoclonal antibody production. Biotechnol Prog. 27(1):209-19. DOI: 10.1002/btpr.513

Becerra S, Berriosa J, Osses N and Altamirano C (2012) Exploring the effect of mild hypothermia on CHO cell productivity. Biochemical Engineering Journal. 60:1-8. DOI: 10.1016/j.bej.2011.10.003

Bedoya-López A, Estrada K, Sanchez-Flores A, Ramírez OT, Altamirano C, Segovia L, Miranda-Ríos J, Trujillo-Roldán MA and Valdez-Cruz NA (2016) Effect of Temperature Downshift on the Transcriptomic Responses of Chinese Hamster Ovary Cells Using Recombinant Human Tissue Plasminogen Activator Production Culture. PLoS One. 11(3):e0151529. DOI: 10.1371/journal.pone.0151529

Beery R, Haimsohn M, Wertheim N, Hemi R, Nir U, Karasik A, Kanety H and Geier A (2001) Activation of the insulin-like growth factor 1 signaling pathway by the antiapoptotic agents aurintricarboxylic acid and evans blue. Endocrinology. 142(7):3098-107. DOI: 10.1210/endo.142.7.8265

Bielser JM, Wolf M, Souquet J, Broly H and Morbidelli M (2018) Perfusion mammalian cell culture for recombinant protein manufacturing - A critical review. Biotechnol Adv. 36(4):1328-1340. DOI: 10.1016/j.biotechadv.2018.04.011

Brinkrolf K, Rupp O, Laux H, Kollin F, Ernst W, Linke B, Kofler R, Romand S, Hesse F, Budach WE, Galosy S, Müller D, Noll T, Wienberg J, Jostock T, Leonard M, Grillari J, Tauch A, Goesmann A, Helk B, Mott JE, Pühler A and Borth N (2013) Chinese hamster genome sequenced from sorted chromosomes. Nat Biotechnol. 31(8):694-5. DOI: 10.1038/nbt.2645

Buchner DA, Charrier A, Srinivasan E, Wang L, Paulsen MT, Ljungman M, Bridges D and Saltiel AR (2015) Zinc finger protein 407 (ZFP407) regulates insulin-stimulated glucose uptake and glucose transporter 4 (Glut4) mRNA. J Biol Chem. 290(10):6376-86. DOI: 10.1074/jbc.M114.623736

Buchsteiner M, Quek LE, Gray P and Nielsen LK (2018) Improving culture performance and antibody production in CHO cell culture processes by reducing the Warburg effect. Biotechnol Bioeng.115(9):2315-2327. DOI: 10.1002/bit.26724

Burteau CC, Verhoeye FR, Mols JF, Ballez JS, Agathos SN and Schneider YJ (2003) Fortification of a protein-free cell culture medium with plant peptones improves cultivation and productivity of an interferon-gamma-producing CHO cell line. In Vitro Cell Dev Biol Anim. 39(7):291-6. DOI: 10.1290/1543-706X(2003)039<0291:FOAPCC>2.0.CO;2

Butler M and Meneses-Acosta A (2012) Recent advances in technology supporting biopharmaceutical production from mammalian cells. Appl Microbiol Biotechnol. 96(4):885-94. DOI: 10.1007/s00253-012-4451-z

Butler M and Spearman M (2014) The choice of mammalian cell host and possibilities for glycosylation engineering. Curr Opin Biotechnol. 30:107-12. DOI: 10.1016/j.copbio.2014.06.010

Camakaris J, Petris MJ, Bailey L, Shen P, Lockhart P, Glover TW, Barcroft C, Patton J and Mercer JF (1995) Gene amplification of the Menkes (MNK; ATP7A) P-type ATPase gene of CHO cells is associated with copper resistance and enhanced copper efflux. Hum Mol Genet. 4(11):2117-23. DOI: 10.1093/hmg/4.11.2117

Castro PM, Ison AP, Hayter PM and Bull AT (1995) CHO cell growth and recombinant interferon-γ production: Effects of BSA, Pluronic and lipids. Cytotechnology. 19(1):27-36. DOI: 10.1007/BF00749752

Chaderjian WB, Chin ET, Harris RJ and Etcheverry TM (2005) Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed. Biotechnol Prog. 21(2):550-3. DOI: 10.1021/bp0497029

Chen P and Harcum SW (2005) Effects of amino acid additions on ammonium stressed CHO cells. J Biotechnol. 117(3):277-86. DOI: 10.1016/j.jbiotec.2005.02.003

Chun BH, Kim JH, Lee HJ and Chung N (2007) Usability of size-excluded fractions of soy protein hydrolysates for growth and viability of Chinese hamster ovary cells in protein-free suspension culture. Bioresour Technol. 98(5):1000-5. DOI: 10.1016/j.biortech.2006.04.012

Chun C, Heineken K, Szeto D, Ryll T, Chamow S and Chung JD (2003) Application of factorial design to accelerate identification of CHO growth factor requirements. Biotechnol Prog. 19(1):52-7. DOI: 10.1021/bp025575+

Clincke MF, Guedon E, Yen FT, Ogier V, Roitel O and Goergen JL (2011) Effect of surfactant pluronic F-68 on CHO cell growth, metabolism, production, and glycosylation of human recombinant IFN-γ in mild operating conditions. Biotechnol Prog. 27(1):181-90. DOI: 10.1002/btpr.503

Cotter TG, al-Rubeai M (1995) Cell death (apoptosis) in cell culture systems. Trends Biotechnol. 13(4):150-5. DOI: 10.1016/S0167-7799(00)88926-X

Crowell CK, Grampp GE, Rogers GN, Miller J and Scheinman RI (2007) Amino acid and manganese supplementation modulates the glycosylation state of erythropoietin in a CHO culture system. Biotechnol Bioeng. 96(3):538-49. DOI: 10.1002/bit.21141

Dadehbeigi N and Dickson AJ (2015) Chemical manipulation of the mTORC1 pathway in industrially relevant CHOK1 cells enhances production of therapeutic proteins. Biotechnol J. 10(7):1041-50. DOI: 10.1002/biot.201500075

Damiani R, Almeida BE, Oliveira JE, Bartolini P and Ribela MT (2013) Enhancement of human thyrotropin synthesis by sodium butyrate addition to serum-free CHO cell culture. Appl Biochem Biotechnol. 171(7):1658-72. DOI: 10.1007/s12010-013-0467-9

Davami F, Baldi L, Rajendra Y and M Wurm F (2014) Peptone Supplementation of Culture Medium Has Variable Effects on the Productivity of CHO Cells. Int J Mol Cell Med. 3(3):146-56.

Dever TE and Ivanov IP (2018) Roles of polyamines in translation. J Biol Chem. 293(48):18719-18729. DOI: 10.1074/jbc.TM118.003338

Dickson AJ (2014) Enhancement of production of protein biopharmaceuticals by mammalian cell cultures: the metabolomics perspective. Curr Opin Biotechnol. 30:73-9. DOI: 10.1016/j.copbio.2014.06.004

Dreesen IA and Fussenegger M (2011) Ectopic expression of human mTOR increases viability, robustness, cell size, proliferation, and antibody production of chinese hamster ovary cells. Biotechnol Bioeng. 108(4):853-66. DOI: 10.1002/bit.22990

Dumont J, Euwart D, Mei B, Estes S and Kshirsagar R (2016) Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit Rev Biotechnol. 36(6):1110-1122. DOI: 10.3109/07388551.2015.1084266

Eagle H (1955) Nutrition needs of mammalian cells in tissue culture. Science. 16;122(3168):501-14. DOI: 10.1126/science.122.3168.501

de la Cruz Edmonds MC, Tellers M, Chan C, Salmon P, Robinson DK, Markusen J (2006) Development of transfection and high-producer screening protocols for the CHOK1SV cell system. Mol Biotechnol. 34(2):179-90. DOI: 10.1385/mb:34:2:179

Evans VJ, Bryant JC, McQuilkin WT, Fioramonti MC, Sanford KK, Westfall BB and Earle WR (1956) Studies of nutrient media for tissue cells in vitro. II. An improved protein-free chemically defined medium for long-term cultivation of strain L-929 cells. Cancer Res. 1956 Jan;16(1):87-94.

Ezaki O (1989) IIb group metal ions (Zn2+, Cd2+, Hg2+) stimulate glucose transport activity by post-insulin receptor kinase mechanism in rat adipocytes. J Biol Chem. 264(27):16118-22.

Fischer S, Handrick R and Otte K (2015) The art of CHO cell engineering: A comprehensive retrospect and future perspectives. Biotechnol Adv. 33(8):1878-96. DOI: 10.1016/j.biotechadv.2015.10.015

Fomina-Yadlin D and McGrew JT (2014) Are nonessential amino acids not so redundant for Chinese hamster ovary cell lines? Pharm. Bioprocess, 2(3):211–213. DOI: 10.4155/pbp.14.14

Gagnon M, Hiller G, Luan YT, Kittredge A, DeFelice J and Drapeau D (2011) High-end pH-controlled delivery of glucose effectively suppresses lactate accumulation in CHO fed-batch cultures. Biotechnol Bioeng. 108(6):1328-37. DOI: 10.1002/bit.23072

Gawlitzek M, Estacio M, Fürch T and Kiss R (2009) Identification of cell culture conditions to control N-glycosylation site-occupancy of recombinant glycoproteins expressed in CHO cells. Biotechnol Bioeng. 103(6):1164-1175. DOI: 10.1002/bit.22348.

Genzel Y, Ritter JB, König S, Alt R and Reichl U (2005) Substitution of glutamine by pyruvate to reduce ammonia formation and growth inhibition of mammalian cells. Biotechnol Prog. 21(1):58-69. DOI: 10.1021/bp049827d

Goh JB and Ng SK (2018) Impact of host cell line choice on glycan profile. Crit Rev Biotechnol. 38(6):851-867. DOI: 10.1080/07388551.2017.1416577

Gong P, Hu B, Stewart D, Ellerbe M, Figueroa YG, Blank V, Beckman BS and Alam J (2001) Cobalt induces heme oxygenase-1 expression by a hypoxia-inducible factor-independent mechanism in Chinese hamster ovary cells: regulation by Nrf2 and MafG transcription factors. J Biol Chem. 276(29):27018-25. DOI: 10.1074/jbc.M103658200

González-Leal IJ, Carrillo-Cocom LM, Ramírez-Medrano A, López-Pacheco F, Bulnes-Abundis D, Webb-Vargas Y and Alvarez MM (2011) Use of a Plackett-Burman statistical design to determine the effect of selected amino acids on monoclonal antibody production in CHO cells. Biotechnol Prog. 27(6):1709-17. DOI: 10.1002/btpr.674

Gospodarowicz D and Moran JS (1976) Growth factors in mammalian cell culture. Annu Rev Biochem. 1976;45:531-58. DOI: 10.1146/annurev.bi.45.070176.002531

Gowtham YK, Saski CA and Harcum SW (2017) Low glucose concentrations within typical industrial operating conditions have minimal effect on the transcriptome of recombinant CHO cells. Biotechnol Prog. 33(3):771-785. DOI: 10.1002/btpr.2462

Graham RJ, Bhatia H and Yoon S (2019) Consequences of trace metal variability and supplementation on Chinese hamster ovary (CHO) cell culture performance: A review of key mechanisms and considerations. Biotechnol Bioeng. 116(12):3446-3456. DOI: 10.1002/bit.27140

Grainger RK and James DC (2013) CHO cell line specific prediction and control of recombinant monoclonal antibody N-glycosylation. Biotechnol. Bioeng. 110 (11): 2970-2983. DOI: 10.1002/bit.24959.

Gstraunthaler G (2003) Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX. 20(4):275-81.

Gutiérrez-Granados S, Cervera L, Kamen AA and Gòdia F (2018) Advancements in mammalian cell transient gene expression (TGE) technology for accelerated production of biologics. Crit Rev Biotechnol. 38(6):918-940. DOI: 10.1080/07388551.2017.1419459

Ha TK, Kim YG and Lee GM (2014) Effect of lithium chloride on the production and sialylation of Fc-fusion protein in Chinese hamster ovary cell culture. Appl Microbiol Biotechnol. 98(22):9239-48. DOI: 10.1007/s00253-014-6012-0

Ha TK and Lee GM (2015) Glutamine substitution: the role it can play to enhance therapeutic protein production. Pharmaceutical Bioprocessing. 3(3):249–261. DOI: 10.4155/pbp.15.6

Ham RG (1963a) Albumin replacement by fatty acids in clonal growth of mammalian cells. Science. 140(3568):802-3. DOI: 10.1126/science.140.3568.802

Ham RG (1963b) Putrescine and related amines as growth factors for a mammalian cell line. Biochemical and Biophysical Research Communications. 14(1):34-38. DOI: 10.1016/0006-291X(63)90206-7.

Ham RG (1965) Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc Natl Acad Sci U S A. 53(2):288-293. DOI: 10.1073/pnas.53.2.288

Hayashi I and Sato GH (1976) Replacement of serum by hormones permits growth of cells in a defined medium. Nature. 259(5539):132-4. DOI: 10.1038/259132a0

Hazeltine LB, Knueven KM, Zhang Y, Lian Z, Olson DJ and Ouyang A (2016) Chemically defined media modifications to lower tryptophan oxidation of biopharmaceuticals. Biotechnol Prog. 32(1):178-88. DOI: 10.1002/btpr.2195

Hecklau C, Pering S, Seibel R, Schnellbaecher A, Wehsling M, Eichhorn T, Hagen Jv and Zimmer A (2016) S-Sulfocysteine simplifies fed-batch processes and increases the CHO specific productivity via anti-oxidant activity. J Biotechnol. 218:53-63. DOI: 10.1016/j.jbiotec.2015.11.022

Hiller GW, Ovalle AM, Gagnon MP, Curran ML and Wang W (2017) Cell-controlled hybrid perfusion fed-batch CHO cell process provides significant productivity improvement over conventional fed-batch cultures. Biotechnol Bioeng. 114(7):1438-1447. DOI: 10.1002/bit.26259

Hölttä E and Pohjanpelto P (1982) Polyamine dependence of Chinese hamster ovary cells in serum-free culture is due to deficient arginase activity. Biochim Biophys Acta. 721(4):321-7. DOI: 10.1016/0167-4889(82)90085-4

Hong JK, Cho SM and Yoon SK (2010) Substitution of glutamine by glutamate enhances production and galactosylation of recombinant IgG in Chinese hamster ovary cells. Appl Microbiol Biotechnol. 2010 Oct;88(4):869-76. DOI: 10.1007/s00253-010-2790-1

Hossler P, Khattak SF and Li ZJ (2009) Optimal and consistent protein glycosylation in mammalian cell culture. Glycobiology. 19(9):936-49. DOI: 10.1093/glycob/cwp079

Hossler P, Racicot C and McDermot S (2015) Targeted Shifting of Protein Glycosylation Profiles in Mammalian Cell Culture through Media Supplementation of Cobalt. J Glycobiol, 03(01). DOI: 10.4172/2168-958X.1000108.

Hu D, Sun Y, Liu X, Liu J, Zhang X, Zhao L, Wang H, Tan WS and Fan L (2015) Understanding the intracellular effects of yeast extract on the enhancement of Fc-fusion protein production in Chinese hamster ovary cell culture. Appl Microbiol Biotechnol. 99(20):8429-40. DOI: 10.1007/s00253-015-6789-5

Huang EP, Marquis CP and Gray PP (2007) Development of Super-CHO protein-free medium based on a statistical design. Journal of Chemical Technology and Biotechnology. 82(5) DOI: 10.1002/jctb.1670.

Igarashi K and Kashiwagi K (2019) The functional role of polyamines in eukaryotic cells. International Journal of Biochemistry and Cell Biology. 107:104–115. DOI: 10.1016/j.biocel.2018.12.012.

Imamoto Y, Tanaka H, Takahashi K, Konno Y and Suzawa T (2013) Advantages of AlaGln as an additive to cell culture medium: use with anti-CD20 chimeric antibody-producing POTELLIGENT™ CHO cell lines. Cytotechnology. 65(1):135-43. DOI: 10.1007/s10616-012-9468-8

Jagušić M, Forčić D, Brgles M, Kutle L, Šantak M, Jergović M, Kotarski L, Bendelja K and Halassy B (2016) Stability of Minimum Essential Medium functionality despite L-glutamine decomposition. Cytotechnology. 68(4):1171-83. DOI: 10.1007/s10616-015-9875-8

Jayme DW and Smith SR (2000) Media formulation options and manufacturing process controls to safeguard against introduction of animal origin contaminants in animal cell culture. Cytotechnology. 33(1-3):27-36. DOI: 10.1023/A:1008133717035

De Jesus M and Wurm FM (2011) Manufacturing recombinant proteins in kg-ton quantities using animal cells in bioreactors. Eur J Pharm Biopharm.78(2):184-8. DOI: 10.1016/j.ejpb.2011.01.005.

Jing Y, Zhang C, Fu T, Jiang C, Ma K, Zhang D, Hou S, Dai J, Wang H, Zhang X, Kou G and Guo Y (2016) Combination of dextran sulfate and recombinant trypsin on aggregation of Chinese hamster ovary cells. Cytotechnology. 68(2):241-8. doi: 10.1007/s10616-014-9774-4

Kaewpila S, Venkataraman S, Buettner GR and Oberley LW (2008) Manganese superoxide dismutase modulates hypoxia-inducible factor-1 alpha induction via superoxide. Cancer Res. 68(8):2781-8. DOI: 10.1158/0008-5472.CAN-07-2635

Kang S, Mullen J, Miranda LP and Deshpande R (2012) Utilization of tyrosine- and histidine-containing dipeptides to enhance productivity and culture viability. Biotechnol Bioeng. 109(9):2286-94. DOI: 10.1002/bit.24507

Kang S, Xiao G, Ren D, Zhang Z, Le N, Trentalange M, Gupta S, Lin H and Bondarenko PV (2014) Proteomics analysis of altered cellular metabolism induced by insufficient copper level. J Biotechnol. 189:15-26. DOI: 10.1016/j.jbiotec.2014.08.001

Kaufman RJ, Swaroop M and Murtha-Riel P (1994) Depletion of manganese within the secretory pathway inhibits O-linked glycosylation in mammalian cells. Biochemistry. 33(33):9813-9. DOI: 10.1021/bi00199a001

Keenan J, Horgan K, Clynes M, Sinkunaite I, Ward P, Murphy R and O'Sullivan F (2018) Unexpected fluctuations of trace element levels in cell culture medium in vitro: caveat emptor. In Vitro Cell Dev Biol Anim. 54(8):555-558. DOI: 10.1007/s11626-018-0285-z

Keenan J, Pearson D and Clynes M (2006) The role of recombinant proteins in the development of serum-free media. Cytotechnology. 50(1-3):49-56. DOI: 10.1007/s10616-006-9002-y

Kelley B, Kiss R and Laird M (2018) A Different Perspective: How Much Innovation Is Really Needed for Monoclonal Antibody Production Using Mammalian Cell Technology? Adv Biochem Eng Biotechnol. 165:443-462. DOI: 10.1007/10_2018_59

Kilberg MS, Pan YX, Chen H and Leung-Pineda V (2005) Nutritional control of gene expression: how mammalian cells respond to amino acid limitation. Annu Rev Nutr. 2005;25:59-85. DOI: 10.1146/annurev.nutr.24.012003.132145

Kim BG and Park HW (2016) High zinc ion supplementation of more than 30 μM can increase monoclonal antibody production in recombinant Chinese hamster ovary DG44 cell culture. Appl Microbiol Biotechnol. 100(5):2163-70. DOI: 10.1007/s00253-015-7096-x

Kim DY, Lee JC, Chang HN and Oh DJ (2005) Effects of supplementation of various medium components on chinese hamster ovary cell cultures producing recombinant antibody. Cytotechnology. 47(1-3):37-49. DOI: 10.1007/s10616-005-3775-2

Kim DY, Lee JC, Chang HN and Oh DJ (2006) Development of serum-free media for a recombinant CHO cell line producing recombinant antibody. Enzyme and Microbial Technology. 39(3):426-433. DOI: 10.1016/j.enzmictec.2005.11.047

Kim EJ, Kim NS and Lee GM (1998) Development of a serum-free medium for the production of humanized antibody from Chinese hamster ovary cells using a statistical design. In Vitro Cell Dev Biol Anim. 34(10):757-61. DOI: 10.1007/s11626-998-0029-6

Kim EJ, Kim NS and Lee GM (1999) Development of a serum-free medium for dihydrofolate reductase-deficient Chinese hamster ovary cells (DG44) using a statistical design: beneficial effect of weaning of cells. In Vitro Cell Dev Biol Anim. 35(4):178-82. DOI: 10.1007/s11626-999-0024-6

Kim JY, Kim YG and Lee GM (2012) CHO cells in biotechnology for production of recombinant proteins: current state and further potential. Appl Microbiol Biotechnol. 93(3):917-30. DOI: 10.1007/s00253-011-3758-5

Kim SH and Lee GM (2009) Development of serum-free medium supplemented with hydrolysates for the production of therapeutic antibodies in CHO cell cultures using design of experiments. Appl Microbiol Biotechnol. 83(4):639-48. DOI: 10.1007/s00253-009-1903-1

Köhrl J, Brigelius-Flohé R, Böck A, Gärtner R, Meyer O and Flohé L (2000) Selenium in biology: facts and medical perspectives. Biol Chem. 381(9-10):849-64. DOI: 10.1515/BC.2000.107

Kuystermans D and Al-Rubeai M (2009) cMyc increases cell number through uncoupling of cell division from cell size in CHO cells. BMC Biotechnology, 9(1):76. DOI: 10.1186/1472-6750-9-76.

Lai T, Yang Y and Ng SK (2013) Advances in Mammalian cell line development technologies for recombinant protein production. Pharmaceuticals (Basel).6(5):579-603. DOI: 10.3390/ph6050579.

Lalonde ME and Durocher Y (2017) Therapeutic glycoprotein production in mammalian cells. J Biotechnol 251:128-140. DOI: 10.1016/j.jbiotec.2017.04.028

Lao MS and Toth D (1997) Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture. Biotechnol Prog 13(5):688-91. DOI: 10.1021/bp9602360

Le H, Kabbur S, Pollastrini L, Sun Z, Mills K, Johnson K, Karypis G and Hu WS (2012) Multivariate analysis of cell culture bioprocess data--lactate consumption as process indicator. J Biotechnol. 162(2-3):210-23. DOI: 10.1016/j.jbiotec.2012.08.021

Le H, Vishwanathan N, Jacob NM, Gadgil M and Hu WS (2015) Cell line development for biomanufacturing processes: recent advances and an outlook. Biotechnol Lett. 37(8):1553-64. DOI: 10.1007/s10529-015-1843-z

Lebkowski JS, Schain LR and Okarma TB (1995) Serum-free culture of hematopoietic stem cells: a review. Stem Cells. 13(6):607-12. DOI: 10.1002/stem.5530130605

Lee GM, Kim EJ, Kim NS, Yoon SK, Ahn YH and Song JY (1999) Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design. J Biotechnol.69(2-3):85-93. DOI: 10.1016/s0168-1656(99)00004-8

Lee JS and Lee GM (2012) Rapamycin treatment inhibits CHO cell death in a serum-free suspension culture by autophagy induction. Biotechnol Bioeng.109(12):3093-102. DOI: 10.1002/bit.24567.

Lee TY, Lin HH, Chen CL, Hwang SM and Tseng CP (2015) Inhibitory Effect of Excessive Glucose on Its Biochemical Pathway and the Growth of Chinese Hamster Ovary (CHO) Cells. Journal of Carbohydrate Chemistry. 34(1):1-11. DOI: 10.1080/07328303.2014.977908

Lewis NE, Liu X, Li Y, Nagarajan H, Yerganian G, O'Brien E, Bordbar A, Roth AM, Rosenbloom J, Bian C, Xie M, Chen W, Li N, Baycin-Hizal D, Latif H, Forster J, Betenbaugh MJ, Famili I, Xu X, Wang J and Palsson BO (2013) Genomic landscapes of Chinese hamster ovary cell lines as revealed by the Cricetulus griseus draft genome. Nat Biotechnol. 31(8):759-65. DOI: 10.1038/nbt.2624.

Lim UM, Yap MG, Lim YP, Goh LT and Ng SK (2013) Identification of autocrine growth factors secreted by CHO cells for applications in single-cell cloning media. J Proteome Res. 12(7):3496-510. DOI: 10.1021/pr400352n

Liu CH and Chang TY (2006) Rational development of serum-free medium for Chinese hamster ovary cells. Process Biochemistry. 41(11):2314–2319. DOI: 10.1016/j.procbio.2006.06.008

Liu C, Chu I and Hwang S (2001) Aurintricarboxylic acid exerts insulin-like growth stimulating effects on Chinese hamster ovary cells under serum-free conditions. J Biosci Bioeng. 91(6):576-80. DOI: 10.1263/jbb.91.576

Liu CH, Chu IM and Hwang SM (2001b) Enhanced expression of various exogenous genes in recombinant Chinese hamster ovary cells in presence of dimethyl sulfoxide. Biotechnol. Letters. 23(20):1641–1645. DOI: 10.1023/A:1012466112116

Luo J, Vijayasankaran N, Autsen J, Santuray R, Hudson T, Amanullah A and Li F (2012) Comparative metabolite analysis to understand lactate metabolism shift in Chinese hamster ovary cell culture process. Biotechnol Bioeng. 109(1):146-56. DOI: 10.1002/bit.23291

Luo Y and Pierce KM (2012) Development toward rapid and efficient screening for high performance hydrolysate lots in a recombinant monoclonal antibody manufacturing process. Biotechnol Prog. 28(4):1061-8. DOI: 10.1002/btpr.1568

Marreiro DD, Cruz KJ2, Morais JB, Beserra JB, Severo JS and de Oliveira AR (2017) Zinc and Oxidative Stress: Current Mechanisms. Antioxidants (Basel). 29;6(2). DOI: 10.3390/antiox6020024

McElearney K, Ali A1, Gilbert A1, Kshirsagar R and Zang L (2016) Tryptophan oxidation catabolite, N-formylkynurenine, in photo degraded cell culture medium results in reduced cell culture performance. Biotechnol Prog. 32(1):74-82. DOI: 10.1002/btpr.2198

McGillicuddy N, Floris P, Albrecht S and Bones J (2018) Examining the sources of variability in cell culture media used for biopharmaceutical production. Biotechnol Lett. 40(1):5-21. DOI: 10.1007/s10529-017-2437-8

McKeehan WL, Hamilton WG and Ham RG (1976) Selenium is an essential trace nutrient for growth of WI-38 diploid human fibroblasts. Proc Natl Acad Sci U S A. 73(6):2023-7. DOI: 10.1073/pnas.73.6.2023

Mellahi K, Brochu D, Gilbert M, Perrier M, Ansorge S, Durocher Y and Henry O (2019) Assessment of fed-batch cultivation strategies for an inducible CHO cell line. J Biotechnol. 298(10):45-56. DOI: 10.1016/j.jbiotec.2019.04.005

Michaels JD and Papoutsakis ET (1991) Polyvinyl alcohol and polyethylene glycol as protectants against fluid-mechanical injury of freely-suspended animal cells (CRL 8018). J Biotechnol. 19(2-3):241-57. DOI: 10.1016/0168-1656(91)90062-z

Miki H and Takagi M (2015) Design of serum-free medium for suspension culture of CHO cells on the basis of general commercial media. Cytotechnology. 67(4):689-97. DOI: 10.1007/s10616-014-9778-0

Morris AE and Schmid J (2000) Effects of insulin and LongR(3) on serum-free Chinese hamster ovary cell cultures expressing two recombinant proteins. Biotechnol Prog. 16(5):693-7. DOI: 10.1021/bp0000914

Mosser M, Chevalot I, Olmos E, Blanchard F, Kapel R, Oriol E, Marc I and Marc A (2013) Combination of yeast hydrolysates to improve CHO cell growth and IgG production. Cytotechnology. 65(4):629-41. DOI: 10.1007/s10616-012-9519-1

Murakami H, Masui H, Sato GH, Sueoka N, Chow TP and Kano-Sueoka T (1982) Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component. Proc Natl Acad Sci U S A. 79(4):1158-62. DOI: 10.1073/pnas.79.4.1158

Nargund S, Qiu J and Goudar CT (2015) Elucidating the role of copper in CHO cell energy metabolism using (13)C metabolic flux analysis. Biotechnol Prog. 31(5):1179-86. DOI: 10.1002/btpr.2131

Nasseri SS, Ghaffari N, Braasch K, Jardon MA, Butler M, Kennard M, Gopaluni B and Piret JM (2014) Increased CHO cell fed-batch monoclonal antibody production using the autophagy inhibitor 3-MA or gradually increasing osmolality. Biochemical Engineering Journal, 91(15):37–45. DOI: 10.1016/j.bej.2014.06.027

Pacis E, Yu M, Autsen J, Bayer R and Li F (2011) Effects of cell culture conditions on antibody N-linked glycosylation--what affects high mannose 5 glycoform. Biotechnol Bioeng. 108(10):2348-58. DOI: 10.1002/bit.23200

Parampalli A, Eskridge K, Smith L, Meagher MM, Mowry MC and Subramanian A (2007) Development of serum-free media in CHO-DG44 cells using a central composite statistical design. Cytotechnology. 54(1):57-68. DOI: 10.1007/s10616-007-9074-3

Park JH, Noh SM, Woo JR, Kim JW and Lee GM (2016) Valeric acid induces cell cycle arrest at G1 phase in CHO cell cultures and improves recombinant antibody productivity. Biotechnol J. 11(4):487-96. DOI: 10.1002/biot.201500327

Pastorian KE and Byus CV (1997) Tolerance to putrescine toxicity in Chinese hamster ovary cells is associated with altered uptake and export. Exp Cell Res. 231(2):284-95. DOI: 10.1006/excr.1996.3467

Pereira S, Kildegaard HF and Andersen MR (2018) Impact of CHO Metabolism on Cell Growth and Protein Production: An Overview of Toxic and Inhibiting Metabolites and Nutrients. Biotechnol J. 13(3):e1700499. DOI: 10.1002/biot.201700499

Peshwa MV, Kyung YS, McClure DB and Hu WS (1993) Cultivation of mammalian cells as aggregates in bioreactors: effect of calcium concentration of spatial distribution of viability. Biotechnol Bioeng. 41(2):179-87. DOI: 10.1002/bit.260410203

Piret JP, Lecocq C, Toffoli S, Ninane N, Raes M and Michiels C (2004) Hypoxia and CoCl2 protect HepG2 cells against serum deprivation- and t-BHP-induced apoptosis: a possible anti-apoptotic role for HIF-1. Exp Cell Res. 295(2):340-9. DOI: 10.1016/j.yexcr.2004.01.024

Piret JP, Mottet D, Raes M and Michiels C (2002) CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. Ann N Y Acad Sci. 973:443-7. DOI: 10.1111/j.1749-6632.2002.tb04680.x

Prabhu A, Gadre R and Gadgil M (2018) Zinc supplementation decreases galactosylation of recombinant IgG in CHO cells. Appl Microbiol Biotechnol. 102(14):5989-5999. DOI: 10.1007/s00253-018-9064-8

Priola JJ, Calzadilla N, Baumann M, Borth N, Tate CG and Betenbaugh MJ (2016) High-throughput screening and selection of mammalian cells for enhanced protein production. Biotechnol J. 11(7):853-65. DOI: 10.1002/biot.201500579

Qian Y, Khattak SF, Xing Z, He A, Kayne PS, Qian NX, Pan SH and Li ZJ (2011) Cell culture and gene transcription effects of copper sulfate on Chinese hamster ovary cells. Biotechnol Prog. 27(4):1190-4. DOI: 10.1002/btpr.630

Qian Y, Xing Z, Lee S, Mackin NA, He A, Kayne PS, He Q, Qian NX and Li ZJ (2014) Hypoxia influences protein transport and epigenetic repression of CHO cell cultures in shake flasks. Biotechnol J. 9(11):1413-24. DOI: 10.1002/biot.201400315

Renner WA, Jordan M, Eppenberger HM and Leist C (1993) Cell-cell adhesion and aggregation: Influence on the growth behavior of CHO cells. Biotechnol Bioeng. 41(2):188-93. DOI: 10.1002/bit.260410204

Ritacco FV, Wu Y and Khetan A (2018) Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: History, key components, and optimization strategies. Biotechnol Prog. 34(6):1407-1426. DOI: 10.1002/btpr.2706

Schnellbaecher A, Binder D, Bellmaine S and Zimmer A (2019) Vitamins in cell culture media: Stability and stabilization strategies. Biotechnol and Bioeng. 116(6). DOI: 10.1002/bit.26942

Schomburg L, Schweizer U and Köhrle J (2004) Selenium and selenoproteins in mammals: extraordinary, essential, enigmatic. Cell Mol Life Sci. 61(16):1988-95. DOI: 10.1007/s00018-004-4114-z

Schröder M, Matischak K and Friedl P (2004) Serum- and protein-free media formulations for the Chinese hamster ovary cell line DUKXB11. J Biotechnol. 108(3):279-92. DOI: 10.1016/j.jbiotec.2003.12.005

Spearman M, Chan S, Jung V, Kowbel V, Mendoza M, Miranda V and Butler M (2016) Components of yeast (Sacchromyces cervisiae) extract as defined media additives that support the growth and productivity of CHO cells. J Biotechnol. 233:129-42. DOI: 10.1016/j.jbiotec.2016.04.031

Sun Y, Zhao L, Ye Z, Fan L, Liu X and Tan WS (2013) Development of a fed-batch cultivation for antibody-producing cells based on combined feeding strategy of glucose and galactose. Biochem Eng Journal. 81:126-35. DOI: 10.1016/j.bej.2013.10.012

Sung YH, Lim SW, Chung JY and Lee GM (2004) Yeast hydrolysate as a low-cost additive to serum-free medium for the production of human thrombopoietin in suspension cultures of Chinese hamster ovary cells. Appl Microbiol Biotechnol. 63(5):527-36. DOI: 10.1007/s00253-003-1389-1

Sung YH, Hwang SJ and Lee GM (2005) Influence of down-regulation of caspase-3 by siRNAs on sodium-butyrate-induced apoptotic cell death of Chinese hamster ovary cells producing thrombopoietin. Metab Eng. 7(5-6):457-66. DOI: 10.1016/j.ymben.2005.08.001

Sunstrom NA, Gay RD, Wong DC, Kitchen NA, DeBoer L and Gray PP (2000) Insulin-like growth factor-I and transferrin mediate growth and survival of Chinese hamster ovary cells. Biotechnol Prog. 16(5):698-702. DOI: 10.1021/bp000102t

Tan EL, Williams MW, Schenley RL, Perdue SW, Hayden TL, Turner JE and Hsie AW (1984) The toxicity of sixteen metallic compounds in Chinese hamster ovary cells. Toxicol Appl Pharmacol. 74(3):330-6. DOI: 10.1016/0041-008x(84)90286-2

Tang X and Shay NF (2001) Zinc has an insulin-like effect on glucose transport mediated by phosphoinositol-3-kinase and Akt in 3T3-L1 fibroblasts and adipocytes. J Nutr. 131(5):1414-20. DOI: 10.1093/jn/131.5.1414

Taylor GA and Blackshear PJ (1995) Zinc inhibits turnover of labile mRNAs in intact cells. J. Cell. Physiol.162(3) DOI: 10.1002/jcp.1041620310

Teixeira AP, Oliveira R, Alves PM and Carrondo MJT (2009) Advances in on-line monitoring and control of mammalian cell cultures: Supporting the PAT initiative. Biotechnol Adv. 27(6):726-732. DOI: 10.1016/j.biotechadv.2009.05.003

Torii S, Kurihara A, Li XY, Yasumoto K and Sogawa K (2011) Inhibitory effect of extracellular histidine on cobalt-induced HIF-1alpha expression. J Biochem. 149(2):171-6. DOI: 10.1093/jb/mvq129

van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker FH, Prieto P, Thalen M and Baumans V (2004) The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol In Vitro. 18(1):1-12. DOI: 10.1016/j.tiv.2003.08.009

van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, Hickman JJ, Hohensee C, Kolar R, Liebsch M, Pistollato F, Schulz M, Thieme D, Weber T, Wiest J, Winkler S and Gstraunthaler G (2018) Fetal Bovine Serum (FBS): Past - Present - Future. ALTEX. 35(1):99-118. DOI: 10.14573/altex.1705101

van der Valk J, Brunner D, De Smet K, Fex Svenningsen A, Honegger P, Knudsen LE, Lindl T, Noraberg J, Price A, Scarino ML and Gstraunthaler G (2010) Optimization of chemically defined cell

culture media--replacing fetal bovine serum in mammalian in vitro methods. Toxicol In Vitro. 24(4):1053-63. DOI: 10.1016/j.tiv.2010.03.016

Vergara M, Torres M, Müller A, Avello V, Acevedo C, Berrios J, Reyes JG, Valdez-Cruz NA and Altamirano C (2018) High glucose and low specific cell growth but not mild hypothermia improve specific r-protein productivity in chemostat culture of CHO cells. PLoS One. 16;13(8):e0202098. DOI: 10.1371/journal.pone.0202098

Walsh G (2014) Biopharmaceutical benchmarks 2014. Nat Biotechnol. 32(10):992-1000. DOI: 10.1038/nbt.3040

Walsh G (2018) Biopharmaceutical benchmarks 2018. Nat Biotechnol. 36(12):1136-1145. DOI: 10.1038/nbt.4305

Welch JT and Arden NS (2019) Considering "clonality": A regulatory perspective on the importance of the clonal derivation of mammalian cell banks in biopharmaceutical development. Biologicals. 62:16-21. DOI: 10.1016/j.biologicals.2019.09.006

Chee Furng Wong D, Tin Kam Wong K, Tang Goh L, Kiat Heng C and Gek Sim Yap M (2005) Impact of dynamic online fed-batch strategies on metabolism, productivity and N-glycosylation quality in CHO cell cultures. Biotechnol Bioeng. 89(2):164-77. DOI: 10.1002/bit.20317

Wong DC, Wong KT, Nissom PM, Heng CK and Yap MG (2006) Targeting early apoptotic genes in batch and fed-batch CHO cell cultures. Biotechnol Bioeng. 95(3):350-61. DOI: 10.1002/bit.20871

Wong VV, Nissom PM, Sim SL, Yeo JH, Chuah SH and Yap MG (2006) Zinc as an insulin replacement in hybridoma cultures. Biotechnol Bioeng. 93(3):553-63. DOI: 10.1002/bit.20746

Wong VV, Ho KW and Yap MG (2004) Evaluation of insulin-mimetic trace metals as insulin replacements in mammalian cell cultures. Cytotechnology.45(3):107-15. DOI: 10.1007/s10616-004-6173-2

Xu J, Yan FR, Li ZH, Wang D, Sheng HL and Liu Y (2014) Serum-free medium optimization based on trial design and support vector regression. Biomed Res Int. 2014:269305. DOI: 10.1155/2014/269305

Xu S, Hoshan L and Chen H (2016) Improving lactate metabolism in an intensified CHO culture process: productivity and product quality considerations. Bioprocess Biosyst Eng. 39(11):1689-702. DOI: 10.1007/s00449-016-1644-3

Xu X, Nagarajan H, Lewis NE, Pan S, Cai Z, Liu X, Chen W, Xie M, Wang W, Hammond S, Andersen MR, Neff N, Passarelli B, Koh W, Fan HC, Wang J, Gui Y, Lee KH, Betenbaugh MJ, Quake SR, Famili I, Palsson BO and Wang J (2011) The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line. Nat Biotechnol. 29(8):735-41. DOI: 10.1038/nbt.1932

Yamamoto S, Matsuda H, Takahashi T, Xing XH, Tanji Y and Unno H (2000) Aggregate formation of rCHO cells and its maintenance in repeated batch culture in the absence of cell adhesion materials. J Biosci Bioeng. 89(6):534-8. DOI: 10.1016/s1389-1723(00)80052-3

Yang WC, Lu J, Nguyen NB, Zhang A, Healy NV, Kshirsagar R, Ryll T and Huang YM (2014) Addition of valproic acid to CHO cell fed-batch cultures improves monoclonal antibody titers. Mol Biotechnol. 56(5):421-8. DOI: 10.1007/s12033-013-9725-x

Yao T and Asayama Y (2017) Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol. 16(2):99-117. DOI: 10.1002/rmb2.12024

Yoon SO, Kim MM, Park SJ, Kim D, Chung J and Chung AS (2002) Selenite suppresses hydrogen peroxide-induced cell apoptosis through inhibition of ASK1/JNK and activation of PI3-K/Akt pathways. FASEB J. 16(1):111-3. DOI: 10.1096/fj.01-0398fje

Yu Y, Kovacevic Z and Richardson DR (2007) Tuning cell cycle regulation with an iron key. Cell Cycle. 6(16):1982-94. DOI: 10.4161/cc.6.16.4603

Yuk IH, Zhang JD, Ebeling M, Berrera M, Gomez N, Werz S, Meiringer C, Shao Z, Swanberg JC, Lee KH, Luo J and Szperalski B (2014) Effects of copper on CHO cells: insights from gene expression analyses. Biotechnol Prog. 30(2):429-42. DOI: 10.1002/btpr.1868

Yuk IH, Russell S, Tang Y, Hsu WT, Mauger JB, Aulakh RP, Luo J, Gawlitzek M and Joly JC (2015) Effects of copper on CHO cells: cellular requirements and product quality considerations. Biotechnol Prog. 31(1):226-38. DOI: 10.1002/btpr.2004

Zagari F, Jordan M, Stettler M, Broly H and Wurm FM (2013) Lactate metabolism shift in CHO cell culture: the role of mitochondrial oxidative activity. N Biotechnol. 30(2):238-45. DOI: 10.1016/j.nbt.2012.05.021

Zahedi K, Barone S, Destefano-Shields C, Brooks M, Murray-Stewart T, Dunworth M, Li W, Doherty JR, Hall MA, Smith RD, Cleveland JL, Casero RA Jr and Soleimani M (2017) Activation of endoplasmic reticulum stress response by enhanced polyamine catabolism is important in the mediation of cisplatin-induced acute kidney injury. PLoS One. 12(9):e0184570. DOI: 10.1371/journal.pone.0184570

Zanghi JA, Mendoza TP, Knop RH and Miller WM (1998) Ammonia inhibits neural cell adhesion molecule polysialylation in Chinese hamster ovary and small cell lung cancer cells. J Cell Physiol.177(2):248-63. DOI: 10.1002/(SICI)1097-4652(199811)177:2<248::AID-JCP7>3.0.CO;2-N

Zanghi JA, Renner WA, Bailey JE and Fussenegger M (2000) The growth factor inhibitor suramin reduces apoptosis and cell aggregation in protein-free CHO cell batch cultures. Biotechnol Prog. 16(3):319-25. DOI: 10.1021/bp0000353

Zeng H and Combs GF Jr (2008) Selenium as an anticancer nutrient: roles in cell proliferation and tumor cell invasion. J Nutr Biochem. 19(1):1-7. DOI: 10.1016/j.jnutbio.2007.02.005

Zhang H, Wang H, Liu M, Zhang T, Zhang J, Wang X and Xiang W (2013) Rational development of a serum-free medium and fed-batch process for a GS-CHO cell line expressing recombinant antibody. Cytotechnology. 65(3):363-78. DOI: 10.1007/s10616-012-9488-4

Zhang J, Robinson D and Salmon P (2006) A novel function for selenium in biological system: selenite as a highly effective iron carrier for Chinese hamster ovary cell growth and monoclonal antibody production. Biotechnol Bioeng. 95(6):1188-97. DOI: 10.1002/bit.21081

Zheng C, Zhuang C, Qin J, Chen Y, Fu Q, Qian H, Wu T, Wang Y, Wu X and Qi N (2018) Combination of temperature shift and hydrolysate addition regulates anti-IgE monoclonal antibody charge heterogeneity in Chinese hamster ovary cell fed-batch culture. Cytotechnology. 70(4):1121-1129. DOI: 10.1007/s10616-018-0192-x

Zhou Y, Raju R, Alves C and Gilbert A (2018) Debottlenecking protein secretion and reducing protein aggregation in the cellular host. Curr Opin Biotechnol. 53:151-157. DOI: 10.1016/j.copbio.2018.01.007

Zhu, J. (2012) Mammalian cell protein expression for biopharmaceutical production. Biotechnol Adv. 30(5):1158–1170. DOI: 10.1016/j.biotechadv.2011.08.022

Zhu J, Wooh JW, Hou JJ, Hughes BS, Gray PP and Munro TP (2012) Recombinant human albumin supports single cell cloning of CHO cells in chemically defined media. Biotechnol Prog. 28(3):887-91. DOI: 10.1002/btpr.1549

Zhu J and Hatton D (2018) New Mammalian Expression Systems. Adv Biochem Eng Biotechnol. 165:9-50. DOI: 10.1007/10_2016_55

Preface to the Chapters

In-house serum-free media development

Over several years, CHO product yields and culture performance have remarkably improved following directed development efforts by the biopharmaceutical industry. A key strategy driving these improvements has been the development and optimisation of media formulations. However, due to commercial sensitivities around disclosing formulations, industry knowledge regarding the efficacy of particular additives or combinations have remained confidential and progress in this area is typically not disclosed. Consequently, knowledge of media additives routinely used in commercial media is limited in the research sector, reducing efficiency during investigation of media additives for CHO culture enhancement or product quality and resulting in potentially repetitive, time-consuming processes. Moreover, the addition of certain media supplements can negatively impact basic culture techniques, such as transfection efficiencies or affect proteomic sample analysis in mass spectrometry and these negative impacts tend to be unknown to wider research community. For these reasons, a substantial portion of my research focused on the study of media development for CHO and the growth- and production-enhancing effects of selected additives.

During the initial steps of my research project, development of an in-house serum-free medium for CHO cell growth was performed following two approaches described in Fig. 6 and 7. The CHO-K1 parental cell line was chosen as a model cell line and growth was analysed in 5 ml working volume cultures in batch conditions. Cultures were adapted to each testing media prior to cell growth and viability assessment (2 to 3 passages), to ensure reproducibility of the results.

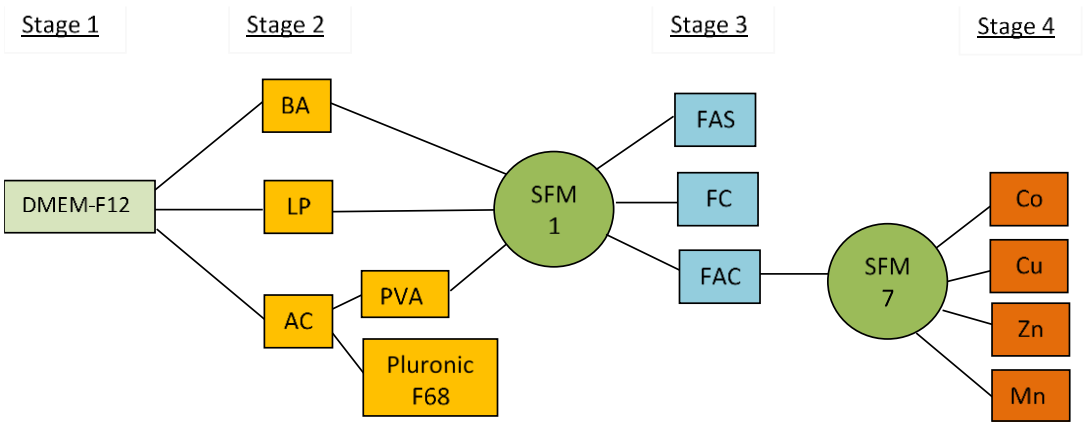

Fig. 6. Workflow for the development of an in-house serum-free medium (SFM) for growth. The process was divided into 4 stages: 1. Basal media; 2. Initial media; 3. Iron Sources; 4. Trace Elements (Preliminary tests). The following abbreviations are indicated: basic additives (BA), lipids/polyamines (LP), anticlumping agents (AC), ferric ammonium sulphate (FAS), ferric ammonium citrate (FAC), ferric citrate (FC), alternative anti-clumping (alternative AC).

1. Approach 1 –Addition - SFM development

Following a thorough literature survey focusing on serum-free, protein-free and chemically-defined media development for CHO, an in-house SFM was designed. DMEM-F12 medium was used as basal medium (J Van Der Valk et al. 2010; C.-H. Liu and Wu 2009; Egorova-Zachernyuk, Bosman, and DeGrip 2011) and eight commonly used supplements (D. Y. Kim et al. 2006; G. M. Lee et al. 1999; H. Zhang et al. 2013) were added in three different groups (Fig. 6 - Stage 2): (i) basic additives (BA) – considered essential to support cell growth – comprising NEAA, glutamine, rhInsulin and NaSe, and (ii) lipids/polyamines (LP) – common additives reported in the literature – comprising linoleic acid, ethanolamine, putrescine and phosphatidylcholine. Supplementation with anti-clumping agents (AC) was also assessed comparing the widely used pluronic F68 with polyvinyl alcohol (PVA) (Costello et al. 2017; Michaels and Papoutsakis 1991; Clincke et al. 2011). Formulation SFM-1, containing both BA, LP and the anticlumping agent PVA was identified as the "best performing" medium (Fig. 8).

At stage 3, supplementation of iron sources was performed (Fig. 6 – stage 3). Three iron sources were compared: Ferric Ammonium Citrate (FAC), Ferric Citrate (FC) and Ferric Ammonium Sulphate (S. H. Kim and Lee 2009b; Schröder, Matischak, and Friedl 2004b; H. Zhang et al. 2013; Rodrigues et al. 2013; Bai et al. 2011; Y. H. Sung, Lim, et al. 2004), resulting in a 2.5-fold increased VCD with the addition of FAC. This formulation was named SFM-7 (Fig. 8).

Finally - at stage 4 – individual supplementation with a set of four trace metals (cobalt, copper, zinc, manganese) was performed (Yuk et al. 2014; Chaderjian et al. 2008b; Gong, Hu, Stewart, Ellerbe, Figueroa, Blank, et al. 2001; J.-P. Piret et al. 2004; B. G. Kim and Park 2016; Tan et al. 1984), but no significant changes were observed in CHO-K1 growth. For this reason, addition of these trace metals was not performed.

This process led us with a final in-house serum-free formulation (SFM-7) that sustained the growth of CHO-K1 cultures for 6 days with viabilities over 90% during that period of time (Fig. 8).

2. Approach 2 - Removal – media simplification and cost reduction

At this stage, we aimed to simplify the formulation in order to obtain a cost-effective product for possible commercialisation while identifying essential additives for CHO-K1 growth (Fig. 7). The cost of production of 1L of SFM-7 (€32.15) was mainly derived from three components: DMEM-F12, rhInsulin and phosphatidylcholine. DMEM-F12 medium was the basal medium used for the SFM-7 formulation and we did not consider that a cheaper substitute was feasible. However, the more expensive phosphatidylcholine and rhInsulin additives had been supplemented in the medium in groups of supplements (BA, LP – Fig. 6 -stage 2), indicating that the essentiality of each of these individual supplements in our medium had not yet been proven. The removal of phosphatidylcholine induced a rapid but shorter growth profile that nevertheless resulted in similar peak VCD to that

obtained for SFM-7 (Fig. 8). As the phosphatidylcholine supplement comprised 25 % of the total cost of formulating SFM-7 (and considering the similar peak obtained between SFM-7 and phosphatidylcholine-removed medium), it was decided (for cost reasons solely) to eliminate this supplement from the medium (SFM-8, referred as BCR-F12 in Chapter 4). This formulation was further simplified with the removal of linoleic acid (no changes in VCD), resulting in SFM-9.

Development of a chemically-defined and protein-free formulation was then approached with the removal of rhInsulin and replacement with two cost-effective mitogenic agents, zinc and aurintricarboxylic acid (ATA) (V. V. T. Wong, Ho, and Yap 2004a; C.-H. Liu, Chu, and Hwang 2001b; Miki and Takagi 2015). Supplementation with either ATA (15 - 30mg/L) or zinc (0.5 – 2.5mg/L) resulted in an enhanced VCD and viability profiles compared to SFM-9 (referred as SFM-F12 in Chapter 2 and 3), resulting in two new CDM products: CDM-ATA (referred as CDM-A in Chapter 1) and CDM-Zn, respectively. A peak VCD of $4x10^6$ cells/ml was observed with ATA, while a maximal VCD of $3.5x10^6$ cells/ml was displayed in zinc-supplemented medium (Fig. 8).

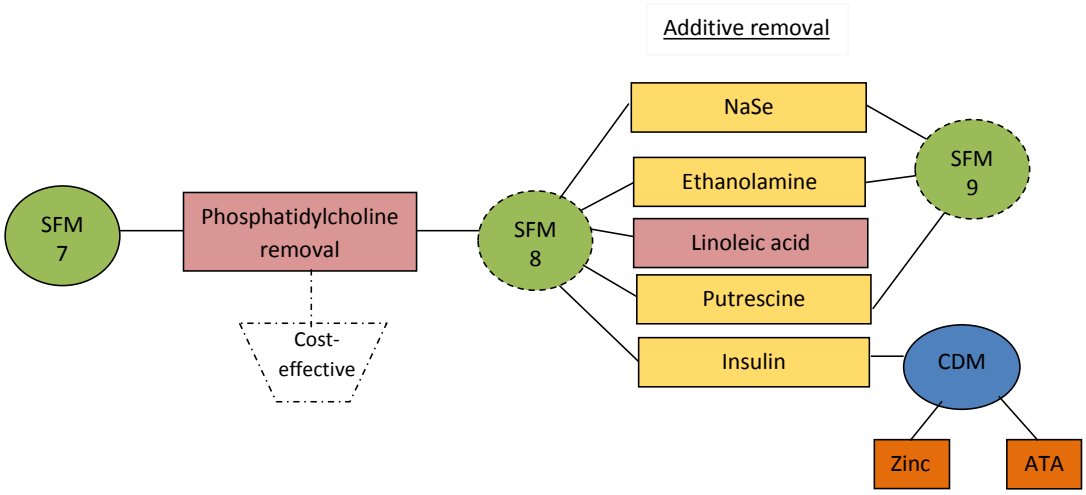

Fig. 7 Workflow for the simplification of SFM-7 formulation and development of CDM products.

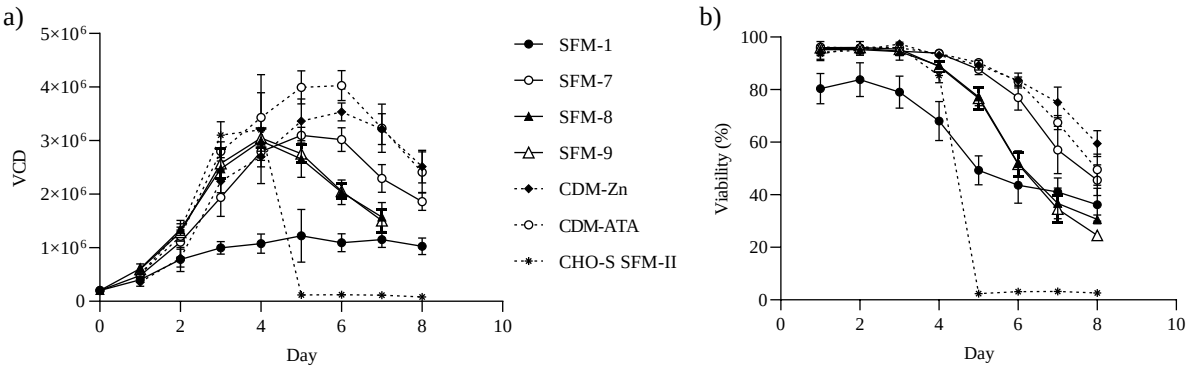

Fig. 8. Viable cell density (VCD) (a) and viability (%) (b) of CHO-K1 cells in the SFM and CDM developed: SFM-1 (stage 1 product), SFM-7 (stage 2 product), SFM-8 (phosphatidylcholine removal, referred as BCR-F12 in Chapter 4), SFM-9 (linoleic acid removal, referred as SFM-F12 in Chapter 2 and 3), CDM-ATA (ATA-supplemented, referred as CDM-A in Chapter 1), CDM-Zn (zinc-supplemented). The commercial CHO-S SFM-II was also added as comparison.

Table 2. Media formulations developed

Additive	Concentration	SFM-8	SFM-9	CDM-ATA	CDM-Zn
NEAA	1.75X	x	x	x	x
L-glutamine	4mM	x	x	x	x
rhInsulin	10mg/l	x	x		
NaSe	6.7µg/l	x	x	x	x
Linoleic acid	0.08mg/l	x			
Ethanolamine	2.5mg/l	x	x	x	x
Putrescine	1mg/l	x	x	x	x
PVA	2%	x	x	x	x
ATA	30mg/l			x	
Zinc Sulphate	2.5mg/l				x

CHAPTER 1

Zinc supplementation increases protein titer of recombinant CHO cells

Berta Capella Roca, Antonio Alarcon Miguez, Joanne Keenan, Srinivas Suda, Niall Barron, Donal O'Gorman, Padraig Doolan*, Martin Clynes*
*Both authors contributed equally

Article published in Cytotechnology (doi: 10.1007/s10616-019-00334-1)

Preface to the paper – zinc as a titer-enhancer for CHO cells

During the initial stages of this research project, a chemically-defined medium was developed on the basis of CHO-K1 cell growth (described in section 2). In CDM containing aurintricarboxylic acid (ATA), cultures displayed the highest VCD and viability profiles among the SFM and CDM formulations developed. However, assessment of recombinant protein production of two CHO cell lines, an IgG-producing DP12 cell line and an in-house EPO-secreting rCHO-K1 (named SK15), revealed low final titers (15 mg/L IgG and 3 mg/L EPO, respectively). For this reason, we aimed to increase the productivity of the cells by supplementing with media additives to increase the expression of recombinant protein. Several additives have been described in the literature to increase specific productivity of CHO cultures, such as the use of peptones and hydrolysates (S. H. Kim and Lee 2009a; Mosser et al. 2013; Hu et al. 2015; Davami et al. 2014) and small molecules such as sodium butyrate (NaBu) or valproic acid (Damiani, Almeida, Oliveira, Bartolini, & Ribela, 2013; Ha, Kim, & Lee, 2014; Liu, Chu, & Hwang, 2001). However, the variable and non-specific composition when hydrolysates are used and the cytotoxic effects reported with NaBu led us to investigate a different approach. In 2016, titer-enhancing effects following zinc supplementation were reported in DG44 cells (B. G. Kim and Park 2016). At the same time, insulin-mimicking effects by zinc-supplementation have been described in several mammalian and some CHO cell lines (at 1.5mg/L Zn) (Wong, Ho, & Yap, 2004;Tang & Shay, 2001; Wong et al., 2006). Considering these two features, supplementation of high zinc concentrations was pursued, resulting in subsequent increased IgG and EPO titers. As we had full knowledge of the formulation of our in-house medium, the specific productivity of both cell lines could be further enhanced by removing a selected additive - ATA - from the zinc-supplemented cultures. Moreover, we could further identify that the productivity-enhancing effects of zinc was also related to an increase in the mRNA levels of both recombinant proteins, indicating a possible effect at transcriptional level. Finally, indirect assessment of the metabolic potential of the cells revealed a connection between cultures displaying enhanced titers, due to the presence of high zinc concentrations, and increased oxidative respiration metabolism. Whether this effect is a consequence of the high productivity profiles or because of the presence of zinc needs further investigation. At high levels of intracellular zinc, localisation of this metal into the mitochondria has been reported (Lu et al. 2016). However, divergence observations into the stimulation of oxidative respiration have been reported in different cell types (Masayoshi, Masatsugu, and Shoji 1982; Dineley, Votyakova, and Reynolds 2003; Dakubo et al. 2006).

Zinc is an essential micronutrient that interacts with hundreds of proteins, being involved in several functions. This trace metal is essential to form the secondary structures of zinc finger motifs, participating in their stabilisation. In this conformation, these proteins are reported to have high affinity for DNA, playing an important role in transcription regulation (Cummings and Kovacic 2009). Moreover, zinc finger proteins have been also described to participate in mRNAs decay, with

reported stabilization of some mRNAs, such as c-fos and Glut4 (Buchner et al. 2015; Taylor and Blackshear 1995). At the same time, zinc has been described as a powerful antioxidant agent, protecting against ROS species produced during cellular respiration due to its structural role in Cu/Zn-superoxide dismutase (Marreiro et al. 2017). Altogether this array of effects on diverse cellular processes indicates that zinc is a vital media additive for supporting CHO cell culture.

In parallel, the extensively studied metal, copper, was also assessed (Luo et al., 2012; Nargund, Qiu, & Goudar, 2015; Yuk et al., 2015), reporting cell line-specific effects in our conditions (Table 3).

Table 3. Summary of copper supplementation effects in SK15 and DP12 compared to CDM+ATA without the metal

Cell line	**Titer effects**	**VCD and Viability effects**
SK15	60-90% increase	No changes
DP12	20% decrease	2-25% lower peak and decrease in viability by day 4 (above 13.7 mg/L Cu)

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3. Section 1: Candidate's details	
Candidate's Name	Berta Capella Roca
DCU Student Number	15212896
School	National Institute for Cellular Biotechnology (NICB), School of Biotechnology
Principal Supervisor	Padraig Doolan
Title of PhD by Publication Thesis	Investigating Media Supplements to Enhance Chinese Hamster Ovary Cells Culture
4. Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Zinc supplementation increases protein titer of recombinant CHO cells
Publication Status	Published
ISSN and link to URL (where available)	Online ISSN: 1573-0778 https://link.springer.com/article/10.1007%2Fs10616-019-00334-1
This paper is one of [4] co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
5. Section 3: Candidate's contribution to the paper	
Provide details below of the **nature** and **extent of your contribution** to the paper (include both your intellectual and practical contributions) and your overall contribution in **percentage terms**: I was the lead author and key contributor of the paper, involved in data curation; investigation; methodology; writing-original draft. Overall 60%	
Where a paper has joint or multiple authors, list the names of all other authors who contributed to the work (this can be appended in a separate document, where necessary)**:** Antonio Alarcon Miguez, Joanne Keenan, Srinivas Suda, Niall Barron, Donal O'Gorman, Padraig Doolan, Martin Clynes	

[1] 'Guidelines for candidates, supervisors and examiners on the 'PhD by Publication' format': https://www.dcu.ie/graduatestudies/A_Z-of-GSO-Policies.shtml

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I confirm that the following statements are true:

(a) the information I have provided in this form is correct

(b) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: _____ Date: 25-11-19

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Padraig Doolan Date: 25-11-19

In some cases, it may be appropriate for verification to be given by both the principal supervisor **and** the lead/corresponding author of the work (where the lead/corresponding author of the work is not the candidate or the principal supervisor):

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ABSTRACT

In order to study the impact of zinc and copper on the titer levels of mAb and recombinant protein in CHO cells, the IgG-expressing (DP12) and EPO-expressing (SK15) cell lines were cultured in chemically-defined media with increasing concentrations of either metal. Supplementation with 25 mg/L in CDM media resulted in a significant increase in EPO (1.7-fold) and IgG (2.6-fold) titers compared to control (no added zinc). Titers at this Zn concentration in CDM containing the insulin replacing agent aurintricarboxylic acid (ATA) (CDM+A), showed a 1.8-fold (EPO) and 1.2-fold (IgG) titers increase compared to control. ATA appeared to also reduce the specific productivity (Qp) enhancement induced by Zn-25, with up to 4.9-fold (DP12) and 1.9-fold (SK15) Qp increase in CDM compared to the 1.6-fold (DP12) and 1.5-fold (SK15) Qp increase observed in CDM+A. A 31 % reduced Viable Cell Density (VCD) in DP12 was observed in both Zn-supplemented media ($3x10^6$ cells/ml vs $4.2x10^6$ cells/ml, day 5), whereas SK15 Zn-25 cultures displayed a 24 % lower peak only in CDM+A ($2.2x10^6$ cells/ml vs $3.2x10^6$ cells/ml, day 5). Supplementation with copper at 13.7-20 mg/L resulted in less significant cell line/product-type dependent effects on titer, VCD and Viability. Analysis of the energetic phenotype of both cell lines in 25 mg/L Zn-supplemented CDM medium revealed a 2-fold increase in the oxygen consumption rate (OCR) compared to non-supplemented cells. Together, these data suggest that high zinc supplementation may induce an increase in oxidative respiration metabolism that results in increased Qp and titers in suspension CHO cultures.

1. Introduction

Enhancement of Chinese Hamster Ovary (CHO) culture performance profiles (titer, specific productivity (Qp), peak cell density, Viability) is a key goal of the biopharmaceutical sector. Industrial production of human therapeutics requires the use of serum-free formulations, mainly protein-free and chemically-defined media, due to biosafety concerns (Gstraunthaler, 2003) and to facilitate downstream processing. Consequently, in order to improve CHO performance in serum-free culture, a range of additives have been evaluated in basal serum-free formulations to optimise growth and productivity profiles.

Supplementations with chemical reagents such as sodium butyrate or valeric acid (Damiani et al., 2013; Park et al., 2016) have been extensively tested, offering a chemically-defined additive alternative for improving specific productivity of several therapeutically-relevant proteins in CHO cultures. However, the specific productivity effects observed with some of these chemicals (sodium butyrate, lithium chloride, valproic acid) have also been frequently associated with apoptosis (NaBu, reviewed by Kim et al., 2013) and/or low growth profiles (Park et al., 2016).

More recently, metal supplementation has shown substantial potential in improving CHO performance features in serum-free culture. For instance, supplementation with iron-citrate has been observed to increase mAb titer by 30-40 % (Bai et al. 2011), while extended lifespan of CHO cultures and increased product titer have been reported following copper supplementation (Yuk et al., 2015; Luo et al., 2012). Additionally, manganese supplementation has been related to the modulation of glycoforms patterns of several recombinant products, including mAb (Grainger & James, 2013). More recently, Kim and Park (2016) reported the titer-associated benefits of high zinc supplementation of a DG44 culture growing in an in-house and commercial media, with up to 6.5-fold increase in mAb titer observed (Kim & Park, 2016).

In this study, we aimed to examine the effects of supplementation of a chemically-defined medium with copper or zinc on the titer, VCD and Viability profiles of two CHO cell lines producing different products in serum-free suspension culture: an IgG-expressing (DP12) and an EPO-expressing CHO-K1 (SK15).

2. Materials and Methods

2.1. In-house chemically-defined media development

As commercially prepared media products frequently do not disclose their exact components, two chemically-defined media (CDM+A and CDM) were developed based on an in-house serum-free medium formulation: DMEM-F12 (D3487) supplemented with sodium selenite (S5261), recombinant insulin (I9279), ethanolamine (E0135), ammonium iron (III) citrate (F5879), poly vinyl alcohol, L-glutamine (Gibco (Dublin, Ireland), 25030024), NEAA (Gibco (Dublin, Ireland), 11140035) and

putrescine dihydrochloride (P7505). For the development of chemically-defined and protein-free formulation, insulin was replaced with 30 mg/L aurintricarboxylic acid (ATA) (CDM+A medium). Due to the chelating nature of ATA, which might mask the effects of the supplemented metals, ATA-removed medium (CDM) was evaluated. All supplements were purchased from Sigma Aldrich (Wicklow, Ireland) unless otherwise stated.

2.2. Cell culture

Over the course of this study, two suspension producer CHO cell lines were used: CHO-DP12 (a recombinant human anti-IL-8 producer, ATCC CRL-12445 clone#1934) and SK15 (an in-house CHO-K1 (ATCC CCL-61) derived cell line, expressing recombinant human erythropoietin (EPO) in a pcDNA3.1 vector (Invitrogen) modified with puromycin resistance as selection system) (Costello et al., 2019). Both cell lines were maintained in in-house protein-free chemically-defined medium supplemented with increasing concentrations of zinc sulphate heptahydrate (1, 10, 15, 25 and 30 mg/L) or copper sulphate pentahydrate (1, 7.5, 13.7 and 20 mg/L) (added to the basal levels found in DMEM-F12: 0.432 mg/L Zn and 1.3 µg/L Cu). Cells were routinely split and re-seeded at $2x10^5$ cells/ml in 5 ml working volume. At least 2 passages were allowed for adaptation before each test. DP12 and SK15 cells were pulsed every second passage with 200 nM MTX (Sigma, M8407) (DP12) or 10 µg/ml puromycin (Gibco, A11138-03) (SK15). Viable Cell Density (VCD) and Viability were analysed in triplicate using the ViaCount on a Guava easyCyte HT benchtop cytometer (Merck Millipore, UK).

2.3. Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme Linked Immunosorbent assay (ELISA) was performed in order to determine the levels of mAb and EPO. For mAb detection, the protocol described in the Human IgG ELISA Quantitation Set from Bethyl Laboratories Inc. (E80-104) was followed. For EPO detection, the protocol previously described by Costello et al. (2019) was followed, including modifications on incubation times for both samples (1.5 h) and capture antibody (overnight). Statistical analysis of the average of each biological triplicate of ELISA data obtained was performed in Microsoft excel software using Fischer's Exact Test to determine variance and the two-tailed T-test tools to generate p-values. Cell specific productivity (Qp; pg protein/cell/day) was determined as per Clarke et al. (2011).

2.4. RNA isolation

RNA samples from SK15 and DP12 cultures in Zn-25 and CDM control media were collected at day 5 from 30ml cultures in shake flasks. Between $1\text{-}5x10^6$ cells were collected, centrifuged at 1000rpm for 5min and resuspended in 1ml Trizol reagent (Thermo Scientific). RNA was extracted following the Trizol protocol as per manufacturer's instructions. Quantity and quality of the extracted samples

were analysed by Nanodrop (Thermo Scientific). To remove potential genomic DNA contamination, DNaseI treatment (Sigma Aldrich) was applied as per manufacturer's protocol.

2.5. RT-qPCR

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was followed as per manufacturer's protocol to generate cDNA from total RNA samples. cDNA was used for mRNA level quantification by qPCR using Fast SYBR Green Master Mix (Applied Biosystems) in a 7500 (Applied Biosystems). 2XSYBR master mix was prepared with 400nM primers, 200ng cDNA and nuclease-free water made up to 20µL per reaction well. Relative quantification was measured by the delta delta Ct method with Gapdh as an endogenous control. Each biological replicate was measured in technical triplicate wells. The sequences of the primers used were as follows (5' -> 3'): IgG-LC Fwd – CATGTCCCGCTCACGTTT, IgG-LC Rev – CAGGCACACAACAGAAGCA (Beckmann et al. 2012); IgG-HC Fwd – ACGGTGTCGTGGAACTCAG, IgG-HC Rev – ACGCTGCTGAGGGAGTAGAG (Haredy et al. 2013); hEPO Fwd – GCATGTGGATAAAGCCGTCA, hEPO Rev – GCAGTGATTGTTCGGAGTGG; Gapdh Fwd – TGGCTACAGCAACAGAGTGG, Gapdh Rev – GTGAGGGAGATGATCGGTGT.

2.6. Energetic phenotype: Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR)

An Agilent Technologies XF96 Analyzer was used to analyse the metabolic potential of both cell lines. Suspension cells were immobilised prior to analysis with the XF96 Seahorse using the "Immobilization of non-adherent cells with Cell-Tak for Assay on the Seahorse XF/XF96" protocol (Agilent Technologies, Technical overview, Publication Part Number: 5991-7153EN, 2016). Some modifications were applied (as per Kelly et al. 2019): 20 µL Cell-Tak was used per well and plates were placed in a non-CO_2 incubator at 37 ^{o}C for 1 h. Plates were then washed twice and air-dried for an hour at room temperature before cell plating. The test used was the Agilent Seahorse XFp Cell Energy Phenotype Test Kit (Agilent Technologies, 103275-100) with a final seeding density of 20,000 cells/well and a final concentration of Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and oligomycin of 1 µM, as per manufacturer's instructions. Basal ("Baseline levels") levels of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were first measured under standard conditions (no inhibitors supplemented). The cells were then "Stressed" following injection of oligomycin (ATP synthase inhibitor) and FCCP (potent uncoupler of mitochondrial oxidative phosphorylation). This supplementation results in the maximum cellular respiration state (maximum Electron Transport System) ("Stressed levels") which corresponds to the OCR and ECAR levels of the cells attempting to restore the proton gradient loss from the mitochondrial inhibitors supplemented. Consequently, by the continuous monitoring of

oxygen concentration changes in the medium (OCR) and pH (ECAR), the XF96 Seahorse instrument allows direct quantification of the mitochondrial respiration and glycolysis of the cells (Plitzko & Loesgen 2018).

In order to avoid interference by dead cells, SK15 and DP12 cells were analysed at day 4. A total of 2 biological replicates for each cell line, with analysis of 11-21 wells/condition per replicate, were performed.

3. Results and Discussion

3.1. Zinc supplementation of CDM+A results in enhanced IgG titer and Qp but lower peak VCD in CHO

Significantly enhanced EPO and IgG titer profiles were observed at Zn-25 medium (Fig. 1), displaying a 40-50 % increase in IgG titer (by day 2-4; Fig. 1b) and a 1.8-fold increase in EPO titer (by day 8; Fig. 1a) compared to the non-supplemented CDM+A control. No significant effects on titer were observed with any of the lower zinc supplementations at later stages of culture.

Results for Qp are presented in Table 1. A 1.5-fold (SK15) and 1.6-fold (DP12) significant increase in Qp was observed at Zn-25 compared to control medium (Table 1). No positive effects were displayed at lower zinc concentrations.

Decreases in peak VCD in both cell lines were observed at 25 mg/L zinc supplementation; a 31 % drop in SK15 and a 24 % drop in DP12 at day 5 (Fig. 1c,d). DP12 VCD was also negatively affected in Zn-15 medium, with a 12 % lower peak VCD observed (Fig. 1d). Supplementation at lower zinc concentrations did not display any effect on VCD in either cell line. Viability profiles were observed to be similar between supplemented and control CDM+A medium at all concentrations (Fig. 1e,f).

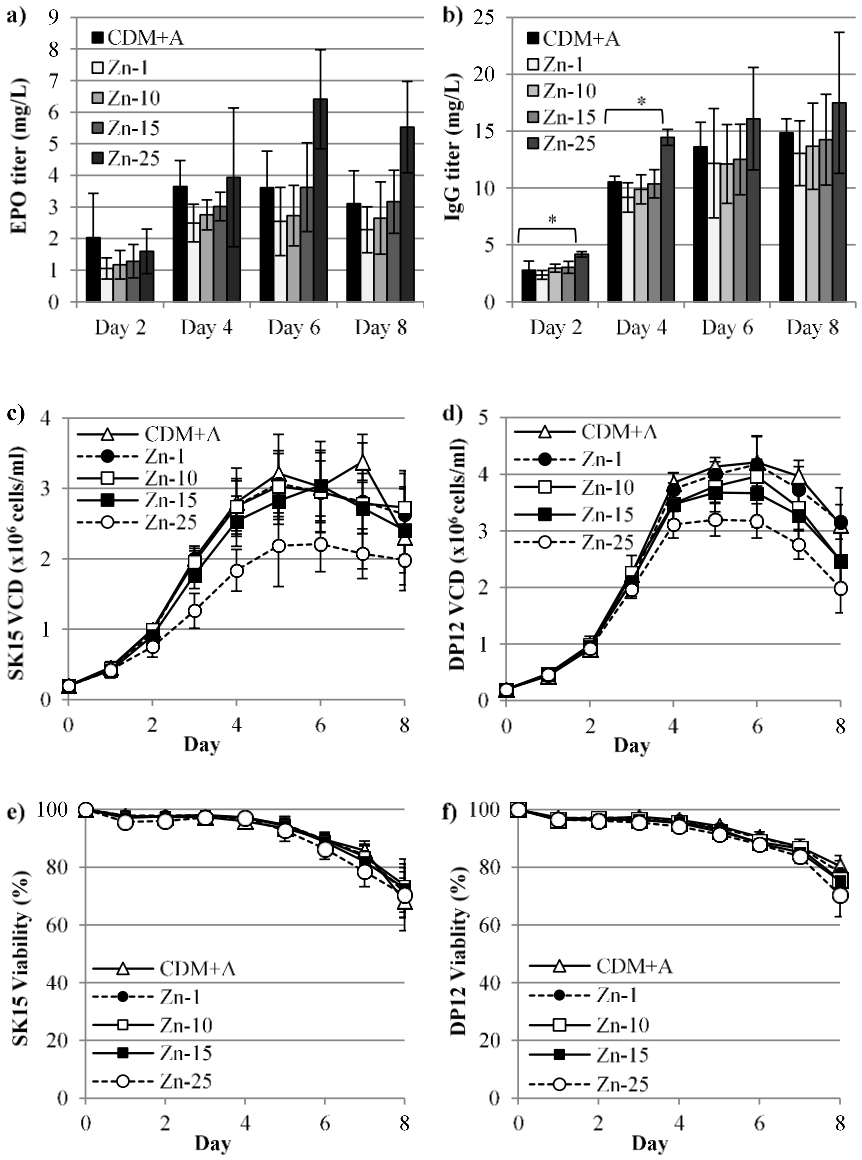

Fig. 1 Titer (a, b), Viable Cell Density (VCD) (c, d) and Viability (e, f) of SK15 (a, c, e) and DP12 (b, d, f) cells grown in suspension in in-house chemically-defined medium CDM+A supplemented with zinc at: 1 mg/L (Zn-1), 10 mg/L (Zn-10), 15 mg/L (Zn-15) and 25 mg/L (Zn-25). Statistical differences of titer data compared to the control (CDM+A) are represented as: p-value <0.05 (*)

3.2. Copper increases EPO titer in SK15 cells

Compared to the CDM+A control, supplementation with copper resulted in different effects in the two CHO cell lines; with EPO titers increased by 80-90 % (at 13.7 mg/L and 20 mg/L Cu, respectively) and 65 % (at 1 mg/L and 7.5 mg/L Cu) media, while IgG final titers decreased by 20 % following copper supplementation at all concentrations (Appendix A, Supplementary Figure 1a,b). Copper-supplemented SK15 cultures displayed similar VCD and Viability profiles to non-supplemented control CDM+A media for all concentrations; with peak VCD of 3-3.2x10^6 cells/ml

and Viabilities above 91 % until day 5 observed (Appendix A, Supplementary Figure 1c,e). Similar to the results obtained for titer profiles (Appendix A, Supplementary Figure 1a,b), DP12 cells were observed to be negatively affected, with peak VCD drops of 2-24 % displayed as the concentrations of copper increased (Appendix A, Supplementary Figure1d). While similar Viability profiles (to the CDM+A control) were observed with supplementations up to 7.5 mg/L Cu, concentrations above 13.7 mg/L resulted in detrimental effects, with a 25 % drop in DP12 Viability from day 4 to day 6 (Appendix A, Supplementary Figure 1f).

Previous studies have reported enhanced titer and VCD profiles following copper supplementation in serum-free CHO culture (Yuk et al., 2015, Xu et al., 2016). However, similar to our observations, cell line-dependant outcomes have also been reported by Luo et al (2012), with increased VCD and mAb titers in two (of three) DUXKB11 CHO suspension cell lines following high copper supplementation in a proprietary CDM, but zero effect on either phenotype in the 3rd subline studied. Additionally, previous studies supplementing with copper concentrations equivalent to the higher levels tested here reported induced DNA damage and reduced Viabilities of CHO-K1 parental cells in a cytotoxic study performed in serum-supplemented media (Grillo et al., 2010), which may explain the decreased Viabilities observed in the DP12 results presented here (Appendix A, Supplementary Figure 1f).

Table 1. Specific productivity (Qp)(pg/cell/day) of SK15 and DP12 cell lines (day 0 - day 4) in CDM+A and CDM supplemented with zinc at: 0 mg/L (Ctl), 1 mg/L (Zn-1), 10 mg/L (Zn-10), 15 mg/L (Zn-15), 25 mg/L (Zn-25) and 30 mg/L (Zn-30). Statistical differences of titer data compared to the respective control are represented as: p-value <0.05 (*), <0.01 (**), <0.001 (***).

		SK15	**DP12**
CDM+A	**Ctl**	1.7 ± 0.5	3.5 ± 0.4
	Zn-1	1.1 ± 0.4	3.1 ± 0.6
	Zn-10	1.3 ± 0.3	3.6 ± 0.6
	Zn-15	1.5 ± 0.3	3.6 ± 0.4
	Zn-25	2.4 ± 1.6	5.8 ± 0.4**
CDM	**Ctl**	3.5 ± 0.4	2.2 ± 0.6
	Zn-1	6.1 ± 0.2	1.6 ± 0.7
	Zn-10	8.1 ± 1.9	6 ± 0.5***
	Zn-25	11.4 ± 1*	10.4 ± 2.7**
	Zn-30	11.7 ± 2*	16 ± 7.4**

3.3. Removal of ATA from CDM+A further increases zinc-induced enhancement of titer and Qp

Similar to zinc, ATA has previously been used as an insulin-replacement additive in the development of PFM for CHO cells (Miki & Takagi, 2015) and its molecular structure provides strong metal chelating ability (Kumar Sharma et al. 2000), acting by forming a coat on the cell surface interacting with IGF-1R (Beery et al. 2001). To avoid possible interference by chelating activity, a medium without ATA (CDM) was formulated and used in order to identify (i) whether addition of zinc by itself could replace the growth-stimulatory effects of ATA while simultaneously improving titer and (ii) if ATA was masking the maximum positive effects of zinc on product titer due to chelating interaction. Moreover, an additional 30 mg/L Zn concentration was also included to evaluate the potential for further titer enhancement at higher concentrations.

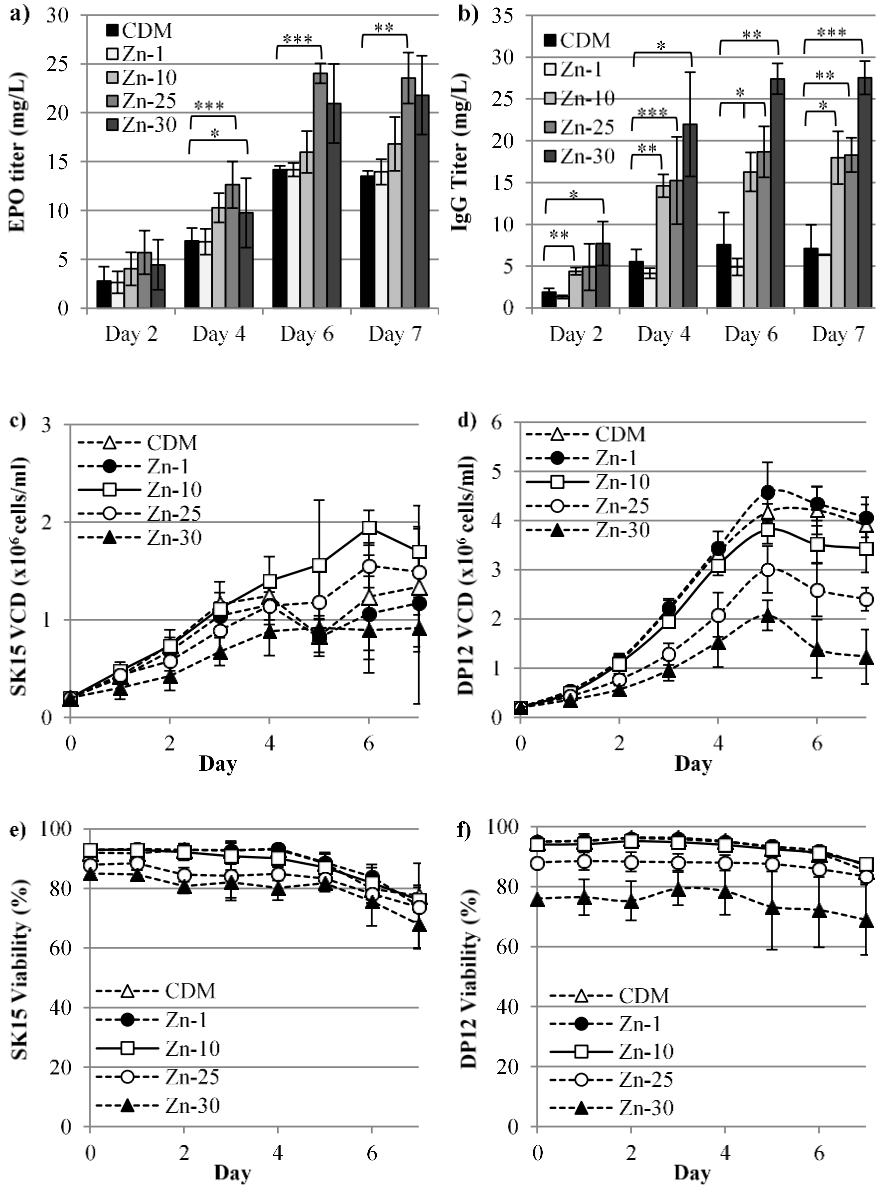

Fig. 2 Titer (a, b) Viable Cell Density (VCD) (c, d) and Viability (e, f) of SK15 (a, c, e) and DP12 (b, d, f) cells grown in suspension in in-house chemically-defined medium CDM supplemented with zinc at: 1 mg/L (Zn-1), 10 mg/L (Zn-10), 25 mg/L (Zn-25) and 30 mg/L (Zn-30). Statistical differences of titer data compared to the control (CDM) are represented as: p-value <0.05 (*), <0.01 (**), <0.001 (***)

Removal of ATA resulted in enhanced EPO and IgG titer profiles following Zn supplementation in the concentration range of 10-30 mg/L; displaying a maximal increase of 1.7-fold EPO titer (in Zn-25) and 3.9-fold IgG titer (in Zn-30) compared to the non-supplemented control (Fig. 2a,b). Final IgG yield was also increased by 2.6-fold with 25 mg/L zinc supplementation (vs. 7.1 mg/L in CDM control) (Fig. 2b).

Specific productivity of both CHO cell lines increased as the concentration of zinc increased, reaching up to 11.4 pg EPO/cell/day (SK15) and 16 pg IgG/cell/day (DP12) at Zn-30 (Table 1). Supplementation with 25 mg/L zinc resulted in a 1.9-fold (SK15) and 4.8-fold (DP12) increase in Qp compared to non-supplemented medium.

VCD and Viability results for each Zn concentration tested are displayed in Fig. 2 (c-f). No effects on SK15 VCD (relative to CDM control) were observed at 25 mg/L zinc supplementation (Fig. 2c). For DP12, negative effects on maximal VCD were observed in Zn-25, causing a drop of 29 % in peak VCD (Fig. 2d). At this zinc concentration, both cell lines displayed lower Viability profiles compared to the non-supplemented CDM control medium (90-83 % vs. 94-88 %; Fig. 2e,f).

Interestingly, the beneficial effects of zinc supplementation on titer were also observed at the transcriptional level, with a 25.6-fold (heavy chain) and 4.3-fold (light chain) increase in IgG mRNA levels in DP12 cells and a 1.8-fold increase in hEPO mRNA levels in SK15 cells, following supplementation at 25mg/L (Fig. 3). This result is in accordance with the IgG expression cassette used for the development of DP12 cell line, which enhances IgG heavy chain expression relative to light chain as a result of MTX selection and amplification, since the DHFR and heavy chain sequences share the same promoter (Gonzalez et al. 2000).

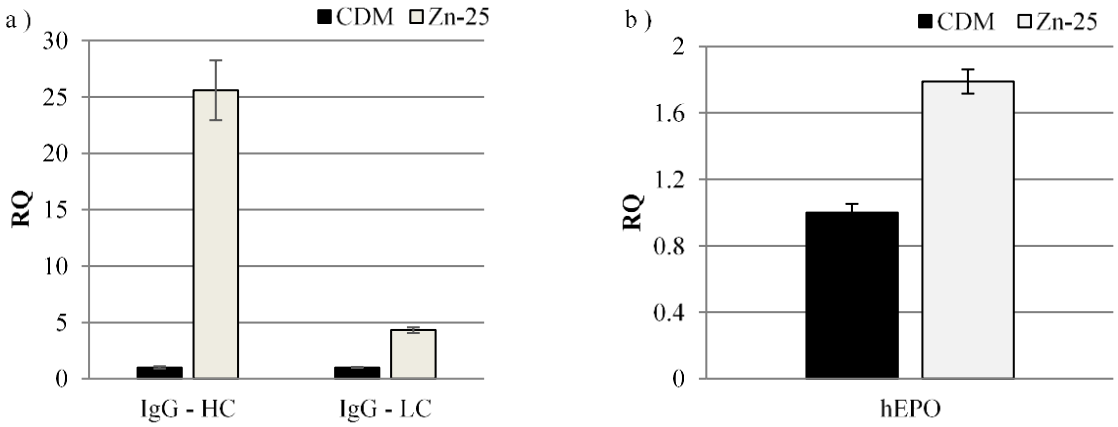

Fig. 3 Relative quantification (RQ) of (a) heavy and light chain of IgG (anti-IL-8) antibody in DP12 cells and (b) hEPO mRNA levels in SK15 cells in CDM Zn25 medium normalized to the mRNA levels of the cells in CDM control medium. The Gapdh endogenous gene was used to standardize the results. RNA samples analysed were collected at day 5 from 30ml cultures.

Only a few studies have reported beneficial effects of high zinc supplementation on enhancing recombinant protein production in CHO. Zuquelis et al. (2006) observed an 8-fold increase of IFN-β1a titer following supplementation with 150 µM zinc and a lower (2-fold) increase at 25-50 µM Zn (both in adherent CHO-K1 cultures grown in 0.5 % FBS) (Zuquelis et al., 2006). More recently, zinc supplementation at 30-60 µM in PFM and CDM in-house formulations increased mAb titer by a maximum of 6.5-fold and peak VCD by 1.2-fold in DG44 suspension cultures (Kim & Park, 2016). Moreover, Kim et. al (2016) also reported enhanced mAb titer at 90 µM Zn supplementation in CDM, which was associated with a lower VCD peak, a finding which is similar to the results presented here at Zn-25 (equivalent to 86.93 µM Zn). However, supplementation with zinc concentrations above 100 µM in CHO suspension cultures has been shown to impact final mAb quality (reduction in galactosylation patterns), although the effects can be reversed by addition of manganese (Prabhu et al., 2018).

From the results displayed here, strategies involving supplementation of 25mg/L zinc on commercial media may be considered as credible approaches focused on increasing titer in suspension CHO cultures utilising commercial media. However, it is important to note that the different additives present in each formulation may influence the positive effects observed with zinc (as was observed here when zinc was co-supplemented in the presence of ATA (Fig. 1a,b)). Interestingly, Kim et. al (2016) have shown increases of 1.2 – 1.5-fold in mAb titer with zinc supplementation to a range of three commercial media (Power CHO-2CD (Lonza), CDM4CHO (Hyclone) and EXCELL CD CHO (SAFC Bioscience)), although the concentrations used (60µM, 17.25mg/L) were lower than the optimal concentration described here. Consequently, due to the lack of disclosure on composition of

commercial media formulations, it may be necessary to deploy several zinc concentration and supplementation strategies to achieve titer enhancement in other culture systems.

3.4. Zn-25 increases the oxidative respiration of DP12 and SK15 cells

Zinc is involved in the folding, stability and/or activity of hundreds of proteins, being essential for several cellular functions such as DNA and RNA synthesis, mRNA stability and protection against apoptosis. Moreover, it also participates in the activation of glutathione and antioxidant enzymes such as, superoxide dismutase and catalase, hence protecting against ROS species produced during cellular respiration (Kloubert & Rink, 2015). However, little is known about its function as a possible additive for improving production of therapeutic proteins.

Batch cultures typically display a stationary phase where growth slows and a production profile is observed, correlated with a switch to oxidative respiration (Dickson 2014a). A maximum induction of Oxygen Consumption Rate (OCR: indicator of mitochondrial respiration) was observed following supplementation at Zn-25, displaying a substantial 1.8-fold (SK15) and 2.1-fold (DP12) increased oxygen consumption compared to the CDM non-supplemented medium (Fig. 4a,b). OCR levels were also affected at lower zinc concentrations, although to a lesser degree; with an increase of 1.2-fold in Zn-10-supplemented SK15 and a 1.3-fold (Zn-1) - 1.4-fold (Zn-10) increase in DP12. Cellular Extracellular Acidification Rate (ECAR: indicator of glycolysis) levels were not substantially affected following Zn supplementation under stressed conditions (Fig. 4c,d).

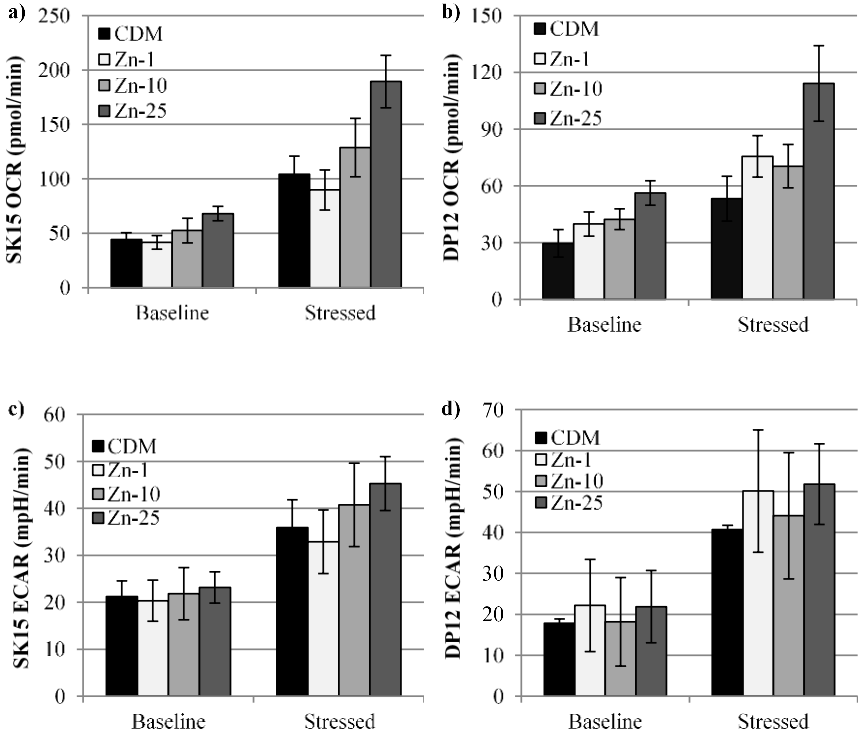

Fig. 4 Oxygen Consumption Rate (OCR) (a, b) and Extracellular Acidification Rate (ECAR) (c, d) of SK15 (a, c) and DP12 (b, d) cells in CDM supplemented with zinc at: 0mg/L (CDM), 1 mg/L (Zn-1), 10 mg/L (Zn-10) and 25 mg/L (Zn-25) at day 4."Baseline" indicates OCR and ECAR levels measured under normal growing conditions; "Stressed" indicates OCR and ECAR levels measured following supplementation of FCCP and oligomycin, which induces maximum cellular respiration state (maximum Electron Transport System) of the cells by blocking mitochondrial function. Consecutively the mitochondrial respiration and glycolysis of the cells are quantified by the changes on the oxygen consumption and pH in both Baseline and Stressed levels. OCR are indicated as pmol Oxygen consumed per min whereas ECAR is indicates as mpH/min.

Disrupted homeostasis by high zinc concentrations results in the sequestration by metallothionein or internalization into organelles, including mitochondria (Qiping et al., 2016). Zinc has been observed to stimulate oxidative phosphorylation and the electron transport chain (ETC) in rat hepatic mitochondria (Masayoshi et al., 1982). Increased ATP production and mitochondrial biogenesis has been also displayed in melanocytes after zinc supplementation (Rudolf& Rudolf, 2017). However, divergent observations have been also reported in rat neurons and prostate epithelial cells, with reduced mitochondrial energy production observed following Zn-supplemented conditions (Dineley et al. 2005; Dakubo et al., 2006), which might indicate possible tissue-specific effects. While the role of zinc in the regulation of energy metabolism in suspension CHO cells is still unclear, the results presented here suggest that zinc supplementation strategies at stationary phases on the cultures might be suitable for enhancing CHO final titers due to the increased OCR levels and titers displayed.

4. Conclusions

We have found that supplementation of protein-free media with zinc at 25 mg/L (86.93 µM) resulted in a significant increase of both recombinant EPO and IgG titers in two CHO cell lines; SK15 and DP12. Although lower peak VCD was also displayed following supplementation, viabilities were maintained above 80 % throughout. Increased oxidative respiration was also observed to correlate with the increased titer profiles in both cell lines. Together, these data indicate that zinc supplementation strategies may be a viable mechanism for increasing specific productivity in CHO cell lines.

5. References

Beery, R., et al. (2001). Activation of the Insulin-Like Growth Factor 1 Signaling Pathway by the Antiapoptotic Agents Aurintricarboxylic Acid and Evans Blue. Endocrinology, https://doi.org/10.1210/endo.142.7.8265

Beckmann, T.F., Krämer, O., Klausing, S., Heinrich, C., Thüte, T., Büntemeyer, H., Hoffrogge, R. & Noll, T. (2012). Effects of high passage cultivation on CHO cells: a global analysis. Appl Microbiol Biotechnol https://doi.org/10.1007/s00253-011-3806-1

Clarke, C.,et al. (2011). Predicting cell-specific productivity from CHO gene expression. J. Biotechnol, https://doi.org/10.1016/j.jbiotec.2010.11.016

Costello, A., Lao, N.T., Barron, N.& Clynes, M.. (2019). Improved yield of rhEPO in CHO cells with synthetic 50 UTR. Biotechnol Lett., https://doi.org/10.1007/s10529-018-2632-2

Costello, A., Lao, N.T., Gallagher, C., Capella Roca, B., Julius, L.A.N., Suda, S., Ducrée, J., King, D., Wagner, R., Barron, N. & Clynes, M. (2019). Leaky Expression of the TET-On System Hinders Control of Endogenous miRNA Abundance. Biotechnol J https://doi-org.dcu.idm.oclc.org/10.1002/biot.201800219

Dakubo, G. D., Parr, R. L., Costello, L. C., Franklin, R. B., & Thayer, R. E. (2006). Altered metabolism and mitochondrial genome in prostate cancer. J Clin Pathol, https://doi.org/10.1136/jcp.2005.027664

Damiani, R., et al. (2013). Enhancement of Human Thyrotropin Synthesis by Sodium Butyrate Addition to Serum-Free CHO Cell Culture. Appl Biochem Biotechnol, https://doi.org/10.1007/s12010-013-0467-9

Dickson, A. J. (2014). Enhancement of production of protein biopharmaceuticals by mammalian cell cultures: the metabolomics perspective. Curr Opin in Biotechnol, https://doi.org/10.1016/j.copbio.2014.06.004

Dineley, K. E., et al. (2005). Zinc causes loss of membrane potential and elevates reactive oxygen species in rat brain mitochondria. Mitochondrion, https://doi.org/10.1016/J.MITO.2004.11.001

Gonzalez, T.N., Leong, S.R. & Presta, L.G. (2000). Humanized anti-IL-8 monoclonal antibodies. US6133426 Retrived from:
http://patft.uspto.gov/netacgi/nph-
Parser?Sect2=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/search-
bool.html&r=1&f=G&l=50&d=PALL&RefSrch=yes&Query=US-6133426-A

Grainger, R. K., & James, D. C. (2013). CHO cell line specific prediction and control of recombinant monoclonal antibody N -glycosylation. Biotechnol Bioeng, https://doi.org/10.1002/bit.24959

Grillo, C. A., Reigosa, M. A., & Fernández Lorenzo de Mele, M. A. (2010). Does over-exposure to copper ions released from metallic copper induce cytotoxic and genotoxic effects on mammalian cells? Contraception, https://doi.org/10.1016/J.CONTRACEPTION.2009.12.003

Gstraunthaler, G. (2003). Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX, Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14671707

Haredy, A.M., Nishizawa, A., Honda, K., Ohya, T., Ohtake, H. & Omasa, T. (2013). Improved antibody production in Chinese hamster ovary cells by ATF4 overexpression. Cytotechnology https://doi.org/10.1007/s10616-013-9631-x

Kelly, P.S., Dorival-García, N., Paré, S., Carillo, S., Ta, C., Alarcon Miguez, A., Coleman, O., Harper, E., Shannon. M., Henry, M., Connolly, L., Clynes, M., Meleady, P., Bones, J. & Barron, N. (2019). Improvements in single-use bioreactor film material composition leads to robust and reliable Chinese hamster ovary cell performance. Biotechnol Prog https://doi.org/10.1002/btpr.2824

Kim, B. G., & Park, H. W. (2016). High zinc ion supplementation of more than 30 μM can increase monoclonal antibody production in recombinant Chinese hamster ovary DG44 cell culture. Appl Microbiol Biotechnol, https://doi.org/10.1007/s00253-015-7096-x

Kim, Y.J., Baek, E., Lee, J.S. & Lee, G.M. (2013). Autophagy and its implication in Chinese hamster ovary cell culture.Biotechnol Lett, https://doi.org/10.1007/s10529-013-1276-5

Kloubert, V. & Rink, L. (2015) Zinc as a micronutrient and its preventive role of oxidative damage in cells. Food Funct, https://doi.org/10.1039/c5fo00630a

Kumar Sharma, R., et al. (2000). Aurine tricarboxylic acid, a potent metal-chelating inhibitor of NFκB–DNA binding. Bioorg Med Chem,https://doi.org/10.1016/S0968-0896(00)00109-7

Luo, J., et al. (2012). Comparative metabolite analysis to understand lactate metabolism shift in Chinese hamster ovary cell culture process. Biotechnol Bioeng, https://doi.org/10.1002/bit.23291

Masayoshi, Y., Masatsugu, K., & Shoji, O. (1982). Role of zinc as an activator of mitochondrial function in rat liver. Biochem Pharmacol, https://doi.org/10.1016/0006-2952(82)90018-1

Miki, H., & Takagi, M. (2015). Design of serum-free medium for suspension culture of CHO cells on the basis of general commercial media. Cytotechnology, https://doi.org/10.1007/s10616-014-9778-0

Plitzko, B. & Loesgen, S. (2018). Measurement of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) in Culture Cells for Assessment of the Energy Metabolism. Bio-protocol https://doi-org.dcu.idm.oclc.org/10.21769/BioProtoc.2850

Park, J.H., et al. (2016). Valeric acid induces cell cycle arrest at G1 phase in CHO cell cultures and improves recombinant antibody productivity. BiotechnolJ, https://doi.org/10.1002/biot.201500327

Prabhu, A., Gadre, R., & Gadgil, M. (2018). Zinc supplementation decreases galactosylation of recombinant IgG in CHO cells. Appl Microbiol Biotechnol, https://doi.org/10.1007/s00253-018-9064-8

Qiping, L., Hariprakash, H., Kira, G.S., Christian, S. & Yang, V.L. (2016). Intracellular zinc distribution in mitochondria, ER and the Golgi apparatus. Int J Physiol Pathophysiol Pharmacol. 8(1):35–43.

Rudolf, E. & Rudolf, K.(2017). Increases in Intracellular Zinc Enhance Proliferative Signaling as well as Mitochondrial and Endolysosomal Activity in Human Melanocytes. Cell Physiol Biochem.,https://doi.org/10.1159/000480306

Xu, S., Hoshan, L., & Chen, H. (2016). Improving lactate metabolism in an intensified CHO culture process: productivity and product quality considerations.Bioprocess Biosyst Eng, https://doi.org/10.1007/s00449-016-1644-3

Yuk, I. H., et al. (2015). Effects of copper on CHO cells: Cellular requirements and product quality considerations. Biotechnol Prog https://doi.org/10.1002/btpr.2004

Zuqueli, R., Prieto, C., Etcheverrigaray, M., & Kratje, R. (2006). Effect of sodium butyrate and zinc sulphate supplementation on recombinant human IFN-β production by mammalian cell culture. Lat Am Appl Res (Vol. 36). Retrieved from http://www.scielo.org.ar/pdf/laar/v36n4/v36n4a18.pdf

CHAPTER 2

An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals

Berta Capella Roca, Nga Lao, Niall Barron, Padraig Doolan, Martin Clynes

Article published in Journal of Biological Chemistry (doi: 10.1074/jbc.RA119.011162)

Preface to the paper – Recombinant protein producer selection

During the development of the in-house SFM (described in: *Research performed – serum-free media development and study of media additives* section), we aimed to simplify the formulation in order to reduce cost of production and identify essential supplements. Individual removal of each additive was performed, identifying the polyamine putrescine as a vital supplement. In putrescine-deprived media, a dramatic negative effect on cell growth and viability was observed. Even though the commercial basal medium DMEM-F12 contains traces of putrescine (81µg/L), this level was revealed to be insufficient for survival of CHO cells. Polyamines are essential for cell proliferation, playing key roles in several cellular processes, including DNA replication, protein expression, cell cycle progression and protection against oxidative stress (Heby, O., 1981, Rhee et al 2007, Pegg, 2016). In 1981, Pohjanpelto *et al* reported an inhibition of CHO-K1 growth when cells were cultured in serum-free media (Pohjanpelto *et al.* 1981). Further investigation of this phenotype revealed an endogenous lack of arginase activity in CHO (Hölttä *et al.* 1982), first enzyme of the polyamine biosynthesis pathway. Consequently, CHO cells display a polyamine-dependent phenotype in serum-free formulations deprived of putrescine.

Currently, generation of biopharmaceutical producing cell lines is mainly focused on two selection systems: the dihydrofolate reductase (DHFR) and the glutamine synthetase (GS) (Costa *et al.* 2010). Both systems are based on the essential function of each enzyme for healthy cell proliferation. Consequently, selection is performed by combining CHO cell lines deficient in expression of either of these enzymes and transfection with a vector co-expressing the gene of interest and the complementary essential enzyme. Selection occurs by culturing the cells in the absence of the vital supplement (thymidine and hypoxanthine for DHFR-deficient cells and glutamine for the GS system), impeding the growth of non-transfected populations (Costa *et al.* 2010). Gene-amplification can be performed in both systems with the use of either methotrexate (MTX), a DHFR inhibitor, or MSX, a GS inhibitor, resulting in highly producing cell lines. However, alterations in the desired product may be introduced as a consequence of long amplification processes, principally with MTX (Guo *et al.* 2010). Moreover, as the number of generations increases, unpredictable drops in specific productivity profiles are reported in both GS and DHFR generated cell lines (Kim *et al.* 2011, Chusainow *et al.* 2009, Kim *et al.* 1998). While not onerous, the limitations of these existing systems highlight the commercial relevance of developing alternative selection systems that can circumvent these challenges and improve this time-consuming and costly step in upstream CHO cell line development. For this reason, we aimed to develop an alternative cytotoxic-free selection system based on the arginase-deficiency phenotype of CHO. To achieve this aim, we designed a bicistronic vector co-expressing GFP or hEPO and arginase and transfected into CHO-K1 cells. Cultures were then placed in selecting conditions - low putrescine and putrescine-deprived media. Analysing the expression of recombinant protein (GFP or hEPO), producer populations were successfully obtained in both

conditions. Single-cell derived populations in in-house selecting medium stably expressed GFP or hEPO for a total of 42 generations, with no changes in growth and viability profiles. To further characterise the system, hEPO-expressing clones were grown in putrescine-containing medium as well as commercial media (BalanCD and CHO-S SFM-II), reporting recombinant protein expression for at least nine passages (27 generations), with a minimal decrease in hEPO titer by the end of the culture.

The arginase-based selection system designed offers an alternative, drug-free, cost-effective and easy-to-apply method for CHO but also other arginase-deficient mammalian cell lines, which may be used in conjunction with DHFR or GS methods. Due to the novelty, efficacy and applicability of the system designed, intellectual property protection has been filed for the work described in this chapter under patent application number 1911023.8 (titled: Transfection selection and polypeptide or RNA expression).

Declaration of Authorship

Candidates are required to submit a separate **Declaration of Authorship** form for each co-authored paper submitted for examination as part of a PhD by Publication thesis. Further information is available from the accompanying guideline document.[2]

6. Section 1: Candidate's details	
Candidate's Name	Berta Capella Roca
DCU Student Number	15212896
School	National Institute for Cellular Biotechnology (NICB), School of Biotechnology
Principal Supervisor	Padraig Doolan
Title of PhD by Publication Thesis	Investigating Media Supplements to Enhance Chinese Hamster Ovary Cells Culture
7. Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals
Publication Status	Accepted
ISSN and link to URL (where available)	http://www.jbc.org/content/early/2019/10/30/jbc.RA119.011162.long
This paper is one of [4] co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
8. Section 3: Candidate's contribution to the paper	
Provide details below of the **nature** and **extent of your contribution** to the paper (include both your intellectual and practical contributions) and your overall contribution in **percentage terms**: I was the lead author and key contributor of the paper, involved in data curation; investigation; methodology; writing-original draft. Overall 70%	
Where a paper has joint or multiple authors, list the names of all other authors who contributed to the work (this can be appended in a separate document, where necessary)**:** Berta Capella Roca, Nga Lao, Niall Barron, Padraig Doolan, Martin Clynes	

[2] 'Guidelines for candidates, supervisors and examiners on the 'PhD by Publication' format': https://www.dcu.ie/graduatestudies/A_Z-of-GSO-Policies.shtml

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Signature of PhD Candidate: _____ Date: 25-11-19

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Padraig Doolan Date: 25-11-19

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ABSTRACT

Polyamines have essential roles in cell proliferation, DNA replication, transcription, and translation processes, with intracellular depletion of putrescine, spermidine, and spermine resulting in cellular growth arrest and eventual death. Serum-free media for CHO-K1 cells require putrescine supplementation, as these cells lack the first enzyme of the polyamine production pathway, arginase. On the basis of this phenotype, we developed an arginase-based selection system. We transfected CHO-K1 cells with a bicistronic vector co-expressing GFP and arginase and selected cells in media devoid of L-ornithine and putrescine, resulting in mixed populations stably expressing GFP. Moreover, single clones in these selective media stably expressed GFP for a total of 42 generations. Using this polyamine starvation method, we next generated recombinant CHO-K1 cells co-expressing arginase and human erythropoietin (EPO), which also displayed stable expression and healthy growth. The EPO-expressing clones grew in commercial media, such as BalanCD and CHO-S SFM-II, as well as in a defined serum-free, putrescine-containing medium for at least nine passages (27 generations), with a minimal decrease in EPO titer by the end of the culture. We observed lack of arginase activity also in several CHO cell strains (CHO-DP12, CHO-S, and DUXB11) and other mammalian cell lines, including BHK21, suggesting broader utility of this selection system. In conclusion, we have established an easy-to-apply alternative selection system that effectively generates mammalian cell clones expressing biopharmaceutically relevant or other recombinant proteins without the need for any toxic selective agents. We propose that this system is applicable to mammalian cell lines that lack arginase activity.

1. Introduction

The Chinese Hamster Ovary cell line (CHO) is the workhorse for the production of therapeutic proteins in the biopharmaceutical industry due to their safety record, ability to grow in large-scale suspension cultures and extensive knowledge about their genome, thus facilitating genetic manipulation. Moreover, introduction of essential post-translational human-type modifications (especially glycosylations) are also achievable with this platform. Media formulations, feed strategies, bioprocess development and gene expression modifications have driven significant increases in final yields over the past years, reaching titer levels of 5-10g/L (1, 2). However, stable cell line development and clonal selection still remain as a costly, labour- and time-consuming step (3).

Development of CHO producer cell lines is mainly based on two selection markers: dihydrofolate reductase (DHFR), an enzyme required for nucleotide metabolism, and glutamine synthetase (GS), essential for intracellular glutamine production (4). In both systems, selection occurs in the absence of a vital supplement (thymidine and hypoxanthine for DHFR-deficient cells and glutamine for the GS system), preventing the growth of non-transfected populations. Compared to DHFR selection, the GS-system can be equally used to derive parental cells lacking GS activity (such as NS0 myeloma or SP2 hybridoma cell lines) or expressing glutamine synthetase (such as CHO cells), in which case methionine sulphoximine (MSX) selection pressure is applied. However, more stringent selection achieved with GS knock out CHO cells have been reported to double mAb bulk culture productivity compared to parental CHO-K1 (5). Similarly, the -/-DHFR DG44 and DUXB11 cell lines are commonly used for DHFR-selection system. In both cases, highly producing populations are generated by increasing the stringency of the selection process with either methotrexate (MTX), a DHFR inhibitor, or MSX, a GS inhibitor, thus inducing transgene co-amplification. However, several rounds of incrementing MTX concentrations are usually required, resulting in a 5-6 months selecting process (6). To note, increased mutation rates have been reported from the long amplification process involving mutagenic chemicals, resulting in occasions in variations in the amino acid sequence of the desired product (7). Hence, cell line development and pharmaceutical manufacturing in drug-free systems is desirable to satisfy safety concerns and achieve more efficient and cost-effective processes. Moreover, removal of selection pressure has to guarantee stable specific productivity of the clones, a vital attribute for the pharmaceutical industry. However, several reports have suggested unstable specific productivity profiles in both GS and DHFR generated cell lines, exposing the limitations of these systems (8, 9, 10).

Putrescine, spermidine and spermine are essential polyamines for cellular development, playing key roles in DNA replication, RNA expression, protein synthesis, protection against oxidative stress, regulation of apoptosis and cell differentiation (11, 12, 13). Historically, eukaryotic polyamine production has been related to arginase, an enzyme catalysing the conversion of L-arginine to L-

ornithine, and ornithine decarboxylase, decarboxylating the L-ornithine to finally generate putrescine (11). However, in some mammalian tissues (such as ovine conceptuses (14) and rat neurons (15) and liver (16)) an alternative pathway, already described in plants and prokaryotes, has been recently reported. Polyamine homeostasis is tightly regulated by internal systems (such as antizyme-antizyme inhibitor) and membrane transport, enabling the uptake or release of these essential polycations for healthy growth development (17). In the early 1980s, putrescine-dependence was first identified in CHO-K1 when culturing in polyamine-free and serum-free media, resulted in a detrimental cellular growth profile due to disorganization of actin bundles and microfilaments (18). Later characterization of the phenotype revealed a lack of arginase activity in CHO cells (19), which was also observed to induce an arrest in the S phase of the cell cycle (20). Further investigation of polyamine metabolism has resulted from the generation of knock out cell lines and use of enzyme inhibitors (such as α-difluoromethylornithine (DFMO) and 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (AbeAdo)), exposing the vital role of these polycations for healthy cell proliferation (21, 22, 23, 24). Based on the polyamine-dependence phenotype of CHO cells and due to the limitations still remaining for efficient generation of stable cell lines, we aimed to develop an alternative cytotoxic-free selection system for producer cell lines.

2. Experimental procedures

2.1. Media development

Two serum-free media (SFM) were developed: a low putrescine (SFM-F12 medium) and a putrescine-free formulation (SFM-F10 medium). The commercial DMEM-F12 medium (Sigma-Aldrich, D8437) was used as a basal medium for SFM-F12 medium. Due to the presence of putrescine in DMEM-F12 medium formulation (81µg/L), a mixture of DMEM high glucose (D5671) and Nutrient Mixture Ham's F10 (Sigma-Aldrich, N2147) (1:1) was used for SFM-F10 medium. The latter was further supplemented with HEPES, linoleic acid (L1376) and glucose in order to mimic DMEM-F12 formulation. Both basal media were further supplemented with sodium selenite (S5261), recombinant insulin (I9279), ethanolamine (E0135), ammonium iron (III) citrate (F5879), poly vinyl alcohol, L-glutamine (Gibco, 25030024), NEAA (Gibco, 11140035) and putrescine dihydrochloride (P7505) (25). When indicated, 100µM L-ornithine or 1mg/L putrescine was added to each media. All media and additives were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Cell Culture

The parental CHO-K1 cell line (ATTC CCL-61) was cultured in SFM-F12 medium supplemented with putrescine. Cells were maintained in suspension culture in an ISF1-X (Climo Shaker) Kuhner incubator at 37ºC, 170rpm, 5% CO_2 and 80% humidity. Cells were routinely split every 3-4 days and

re-seeded at $0.2x10^6$ cells/ml in 50ml spin tubes (Sartorius, DF-050MB-SSH) in a 5 ml working volume. Biological triplicates were analysed for viable cell density (VCD) and viability using the ViaCount on a Guava easyCyte HT benchtop cytometer (Merck Millipore, UK). Measurements were performed in technical duplicates (unless stated).

For arginase activity tests, DUXB11 (kindly donated by Chasin, L., Columbia University) were cultured in MEM alpha medium (Thermo Fisher, 12561056) supplemented with 0.5-2% foetal bovine serum (FBS) in T-75 flasks; CHO-DP12 (ATCC, clone #1934) and CHO-S cells were cultured in BalanCD Growth A medium (Irvine Scientific) supplemented with 8mM L-glutamine (Thermo Fisher, 2503008) in 50ml spin tubes (Sartorius, DF-050MB-SSH) in a 5ml working volume; HEK293 were cultured in MEM (Sigma Aldrich, M5650) supplemented with 2mM L-glutamine (Thermo Fisher, 2503008), 0.1mM MEM non-essential amino acid (Biosciences, 11140050), 1mM sodium pyruvate (Thermo Fisher, 11360070) and 10% heat-inactivated horse serum in T-75 flasks; SP2 (Immune Systems) were cultured in DMEM Glutamax (Gibco, 10566-016) supplemented with 10% heat inactivated FBS in T-25 flasks; BHK-21 (Flow Laboratories Irvine) were cultured in MEM (Sigma Aldrich, M5650) supplemented with 2mM L-glutamine (Thermo Fisher, 2503008), 1% MEM non-essential amino acid (Thermo Fisher, 11140050), 1% sodium pyruvate (Thermo Fisher, 11360070) and 5% fetal calf serum (FCS) in T-25 flasks; the pancreatic cell lines BxPC-3 (ATCC, CRL-1687), MiaPaca-2 (ATCC, CRL-1420), PANC-1 (ATCC, CRL-1469), Capan-2 (DSMZ, ACC 244) were cultured in DMEM high glucose (Sigma-Aldrich, D5671) supplemented with 5% FCS and 2% L-glutamine (Thermo Fisher, 2503008) in T-75 flasks.

Isolation of clonal cells was performed by dilution. A volume of 100µl per well was added in a 96-well plate at 5 cells/ml. Plates were then incubated uninterruptedly at 37ºC and 5% CO2 up to day 7-10, when plates were inspected to identify and mark wells presenting single colonies. Plates were then re-incubated at 37ºC. At day 14, 60-70% confluence was observed and colonies were picked and expanded to larger volumes. First, 1ml cultures were performed in non-adherent suspension 24-well plates (Greiner Bio-one, 662102). After 2-3 days, successful clones were then placed in 50ml spin tubes (Sartorius, DF-050MB-SSH) in a 5ml working volume for stability tests.

For stability tests, the doubling time and generation number of each clone was calculated as per (37). Clones were assessed for a total of 41-53 generations (for GFP-expressing clones) and 40-51 generations (hEPO-expressing clones), depending on the clone. For simplicity, a doubling time of 24h was assumed for data discussion, resulting in a total of 42 generations experiment.

2.3. Vectors

Phusion High-Fidelity PCR master mix (Thermo Scientific) was used to obtain the mouse arginase coding sequence (CDS) from pcDNA3.1-mArg1, an internal ribosome entry site (IRES) sequence from pINDUCER10 vector (kindly gifted by Dr. Stephen Elledge, Harvard Medical School Centre of

Genetics and Genomics, Boston, U.S.A) and the human erythropoietin (hEPO) CDS from a pLenti6.36.3hEPO. The protocol was followed as per manufacturer's recommendations, with 10ng vector used in a total of 50µL reaction volume. Both arginase and IRES fragments were first cloned downstream of a green fluorescence protein (GFP) from a modified GFP-expressing vector N44-CSanDI-Hyg (derived from pcDNA5 CMV-d2eGFP (Addgene, 26164), resulting in a GFP-IRES-Arg bicistronic vector controlled by a CMV promoter. For the hEPO expressing vector, the hEPO CDS replaced the GFP in the GFP-IRES-Arg vector, resulting in a hEPO-IRES-Arg bicistronic vector.

The pcDNA3.1-mArg1 was a gift from Peter Murray (Addgene, plasmid #34573), pInducer10-mir-RUP-PheS was a gift from Stephen Elledge (Addgene plasmid #44011) (38) and pLenti6.3-hEPO was a gift from Juan Melero-Martin (Addgene plasmid #50436) (39).

Proof of concept tests were performed using the pcDNA3.1-mArg1 vector.

2.4. Transfection and selection

Vector transfections were performed in 1ml suspension cultures in 24-well tissue-untreated plates (Greiner Bio-one, 662102) SFM-F12 medium with putrescine. The TransIT PRO transfection agent (Mirus Bio, Mir 5740) was used as per manufacturer's recommendations with minimal changes. Briefly, cells were counted and re-seeded at 0.5-1x10^6cells/ml in fresh media 24h prior transfection. A total of 500ng vector were mixed with 1µL TransIT PRO and 100µl media and incubated for 10min at room temperature. Cells were re-suspended in fresh media at 2x10^6cells/ml and 900µl were seeded per well. A total of 100µl of vector-TransIT PRO complex suspension was then added to each well. Plates were parafilmed and incubated at 37oC, 170rpm with 5% CO2 and 80% humidity in an ISF1-X (Climo Shaker) Kuhner incubator. A negative control for transfection was also included (no vector was added during transfection). Biological triplicate were performed per each transfections. Selection was performed in putrescine-free SFM-F12 medium. Cells were also placed in putrescine-containing SFM-F12 medium as negative controls.

For the GFP-expressing vector, the efficiency of the process was assessed 24h post-transfection by vector Green Fluorescent Protein (GFP) expression using the Express Plus software of GUAVA easyCyte HT benchtop cytometer (Merck Millipore, UK). To determine the amount of fluorescent cells, negative control cells (zero fluorescence) were gated and these settings were then used to identify the GFP-positive populations. Fluorescence of dead cells and debris were excluded to avoid false positive results. Viable cell density and viability were also assessed as previously described.

2.5. Arginase activity

One million cells were collected per sample and centrifuged at 1000 rpm. Pellets were then washed with PBS and finally stored at -80°C until assayed. The arginase activity kit (Sigma-Aldrich,

MAK112) was used. Pellets were lysed for 10min in 100µl of lysis buffer as per manufacturer's recommendations, with pepstatin A (Sigma Aldrich, P5318) and leupeptin (Sigma Aldrich, L9783). To determine arginase activity, manufacturer's protocol was followed. Arginase activity (U) was determined with the following equation:

$$Activity = \frac{Abs\ Sple - Abs\ B}{Abs\ Std - Abs\ W}\ x\ \frac{1mM\ x\ 50\ x\ 1000}{Sple\ vol\ x\ react\ time}$$

Being:

Abs Sple: absorbance sample at 430nm

Abs B: absorbance blank at 430nm

Abs Std: absorbance standard at 430nm

Abs W: absorbance water at 430nm

Sple vol: sample volume (µl)

React time: reaction time (min)

Where one unit of arginase corresponds to the amount of enzyme that will convert 1µmole of L-arginine to ornithine and urea per minute at pH 9.5 and 37°C.

Biological triplicates were analysed per each condition and cell line except for pancreatic cell lines, which were assessed in biological duplicates.

2.6. RNA isolation and RT-qPCR performance

RNA was collected by centrifuging 1-5x10^6cells at 1000rpm, 4°C for 5min. Pellets were re-suspended in 1ml Trizol reagent (Thermo Fisher, 15596018) and stored at -80°C until assayed. Total RNA isolation was performed as per manufacturer's recommendations (Thermo Fisher). RNA quantification and quality were evaluated by NanoDrop (Thermo Scientific). To remove contaminating DNA, RNA samples were treated with DNase I (Sigma Aldrich) as per manufacturer's protocol and stored at -80°C. cDNA was obtained using the High Capacity cDNA Reverse Transcription Kits (Applied Biosciences) which was followed as per manufacturer's recommendations. A total of 200ng cDNA per reaction well was used for qRT-PCR using Fast SYBR Green Master Mix (Applied Biosystems) and run in a 7500 (Applied Biosystems). The 2XSYBR master mix was prepared with 400nM primers and nuclease-free water made up to 16µL per reaction. Relative quantification was measured by the ddCt method with Gapdh as an endogenous control. Technical triplicate wells were run for each clone sample. The sequences of the primers used are as follows (5'->3'): Gapdh-Fw TGGCTACAGCAACAGAGTGG, Gapdh-Rv GTGAGGGAGATGATCGGTGT, Arg-Fw ACAAGACAGGGCTCCTTTCA, Arg-Rev TGCCGTGTTCACAGTACTCT, hEPO-Fw GCATGTGGATAAAGCCGTCA, hEPO-Rv GCAGTGATTGTTCGGAGTGG, d2GFP-Fw GACGACGGCAACTACAAGAC, d2GFP-Rv TCCTTGAAGTCGATGCCCTT.

2.7. ELISA

For hEPO detection, the protocol previously described by Costello et al. (2019) (40) was followed with overnight incubation for capturing antibody and samples (1.5 h).

3. Results

3.1. CHO-K1 cells require supplementation of putrescine or L-ornithine for healthy growth

During the development of a serum-free medium for CHO cells (SFM-F12) (25), reduction of putrescine supplementation to less than 200µg/L was observed to have a dramatic negative effect on cell growth, resulting in a consistent drop on viability to less than 55% at concentrations lower than 100µg/L. In SFM-F12 with increased putrescine supplementation up to 200µg/L, a substantial negative effect in VCD and viabilities was still observed (Appendix B, Fig. S1).

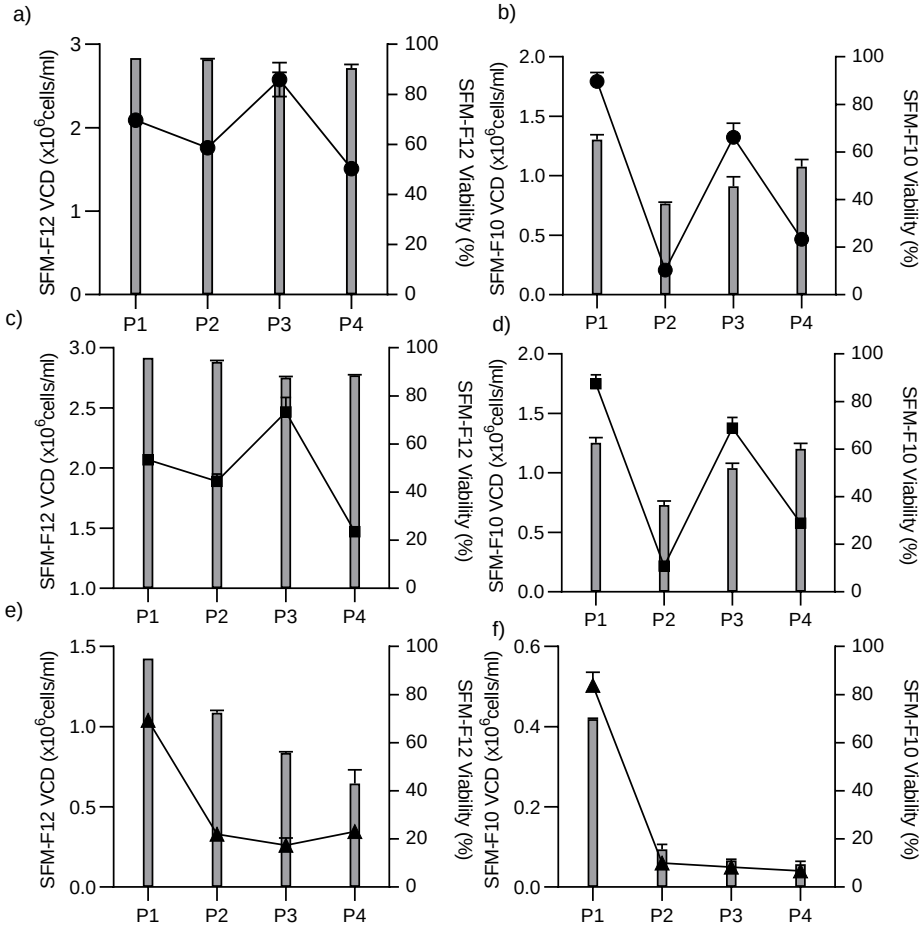


Figure 1. CHO-K1 cells require putrescine or L-ornithine supplementation for healthy growth. CHO-K1 viable cell density (VCD) (lines) and viability (bars) in SFM-F12 medium (81µg/L putrescine) (a, c, e) and SFM-F10 medium (no putrescine) (b, d, f) supplemented with: 1mg/L putrescine (a, b, circles), 100µM ornithine (c, d, squares) or without any supplementation (e, f, triangles). VCD and viability displayed are per each passage (P). Technical triplicates were performed for each condition

except for passage 1 in SFM-F12 which was performed in a single tube. Triplicates were seeded for subsequent passages in SFM-F12 and SFM-F10.

Intracellular putrescine production requires the conversion of arginine to L-ornithine, which is then decarboxylated to generate putrescine. To assess which of the two reactions was causing the putrescine-dependent phenotype observed, CHO-K1 cells were cultured for 4 passages in three conditions: (i) SFM-F12 medium lacking putrescine, (ii) SFM-F12 medium supplemented with L-ornithine (100µM) and (iii) SFM-F12 control medium (containing 1.08mg/L putrescine). Due to the presence of traces of putrescine (81µg/L) in the DMEM-F12 formulation (basal medium for SFM-F12 medium), an SFM-F10 medium (based in DMEM:F10 medium (1:1 v/v) - 0 µg/L putrescine) was developed and tested under the same conditions as SFM-F12 to compare effects of media fully depleted of putrescine. In SFM-F12 (Fig. 1a, c, e), CHO-K1 cells were observed to display a similar growth profile in both L-ornithine- and putrescine- supplemented media with consistent viabilities (87-95%) maintained among the 4 passages. In contrast, cultures in non-supplemented SFM-F12 (Fig. 1e) displayed negligible growth. Similar to the SFM-F12 results, SFM-F10 L-ornithine- and putrescine- supplemented cultures displayed similar profiles, with growth observed at each passage and viabilities increased from P2 to P4 (Fig 1b, d). Zero growth was observed in CHO-K1 cells in SFM-F10 non-supplemented medium, with viability decreasing to 15% by P2 (Fig. 1f). It is noteworthy that CHO-K1 cells were grown in SFM-F12 medium supplemented with putrescine prior to the start of the SFM-F10 test, with no previous adaptation to SFM-F10 medium. This may explain the similar growth profiles observed in all three SFM-F10 testing media at P1 and P2 (Fig. 1b, d, f).

3.2. Replenishment of arginase activity results in healthy growth and can be used to select CHO-K1 cells

In 1982, Hölta et al. (19) reported a lack of arginase activity in a CHO-K1 cell line in serum-free conditions, resulting in a polyamine-dependent phenotype. In accordance with their findings, it was hypothesized that a lack of arginase expression could be also occurring in our parental cell line. Based on that and, in order to exploit this phenotype, we designed a selection system for CHO-K1 producer cell lines. As a proof of concept, a commercial arginase-expressing vector, pcDNA3.1-mArg (Addgene), was first transfected and selected in media lacking putrescine (NoP) compared to media with putrescine (P). A negative control, consisting of wild type untransfected cells, was also included. CHO-K1 cells transfected and selected in SFM-F12 medium lacking putrescine resumed cellular growth by passage 6 (day 20), finally reaching VCD and viability profiles similar to the parental control cells in putrescine-containing medium by passage 8 (day 26) (Fig 2a, b). Correspondingly, an increase in arginase activity was also detected in the transfected and selected cultures (NoP-Arg) from P5 (Fig 2c). In contrast, expected minimal arginase activity levels were detected in parental cells,

with a cessation of growth in medium without putrescine (NoP-Neg) from passage 2 (day 9) and viabilities of 60-69% observed until the end of the culture. Phenotypic characterization of the selected mixed populations (NoP-Arg) over a 7-day culture period revealed almost identical VCD and viability profiles as parental cells in SFM-F12 medium with putrescine (Appendix B, Fig. S2).

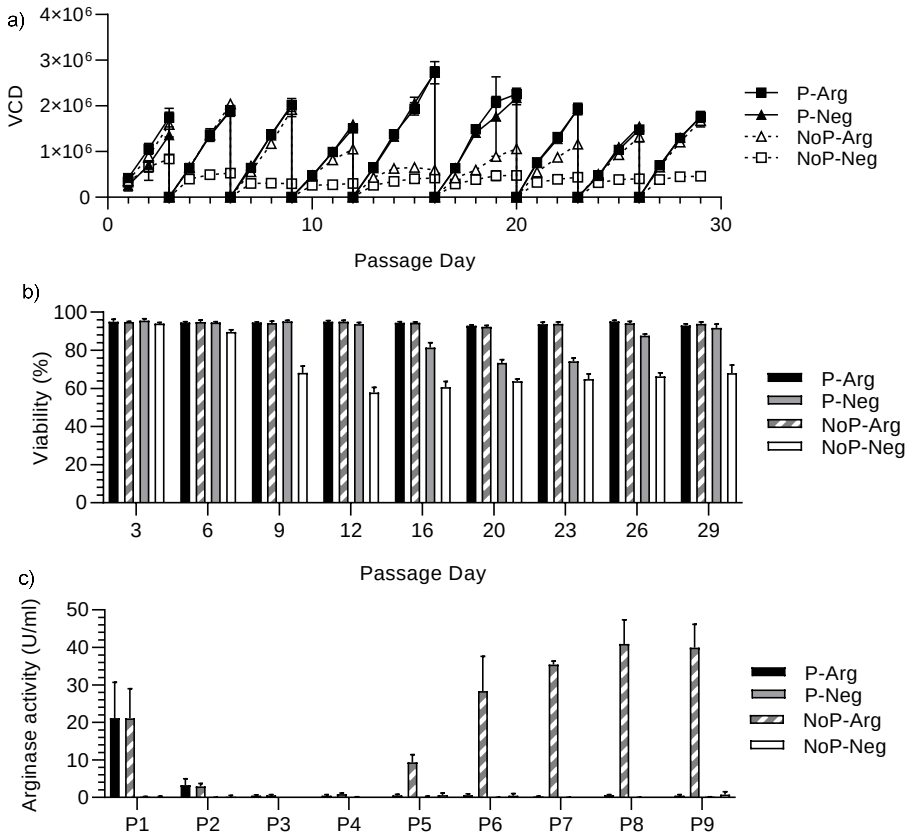


Figure 2. Replenishment of arginase activity in CHO-K1 parental cells enables growth in polyamine-free media. Viable cell density (VCD) (a), viability (b) and arginase activity (c) of CHO-K1 transfected with 500ng pcDNA3.1-mArg1 vector (P-Arg, NoP-Arg). A negative control of cells with no DNA transfected (P-Neg, NoP-Neg) was included. Transfected and non- transfected cells were placed in either SFM-F12 medium supplemented with putrescine (P) or selective SFM-F12 medium (without putrescine, NoP). For arginase activity analysis, samples for NoP-Neg could not be collected at P3, P4 and P7. Triplicate wells were transfected per each condition.

3.3. GFP-expressing clones successfully selected in low putrescine containing media

After having demonstrated the ability to select CHO-K1 cells transfected with an arginase expressing vector in media lacking putrescine, the next step was to determine whether the system could be applied to the selection of cells expressing a gene of interest. To achieve this aim, green fluorescence protein (GFP) was selected for initial tests due to the ease traceability during the transfection process.

A bicistronic vector expressing GFP and arginase linked by an IRES and under the control of a CMV promoter was designed for the purposes of this experiment (Appendix B, Fig. S3).

In SFM-F12 medium, healthy mixed populations were successfully obtained by passage 8, recovering VCD and viability profiles similar to the parental control cells (Fig 3a, b). GFP expression was first detected at passage 7, with 14.5% GFP expressing cells observed in mixed population 1, 2.9% in mixed population 2 and 8.1% in mixed population 3. Interestingly, the GFP-expressing population was observed to increase over each passage, reaching an average of 27.2% cells by the end of the experiment (P12) (Fig. 3c). In contrast and as expected from previous results, the VCD and viability of the untransfected cultures in SFM-F12 medium without putrescine (NoP-Neg) dropped by passage 2, displaying an average VCD of 0.3-0.6x10^6cells/ml and viabilities between 57-64% until the end of the culture (NoP-Neg). Variability in the viability of the three replicates for the negative control was observed by the end of the experiment (P11 and P12), where replicate 2 and 3 displayed viabilities of 44-49% but 79% viable cells were detected in replicate 1.

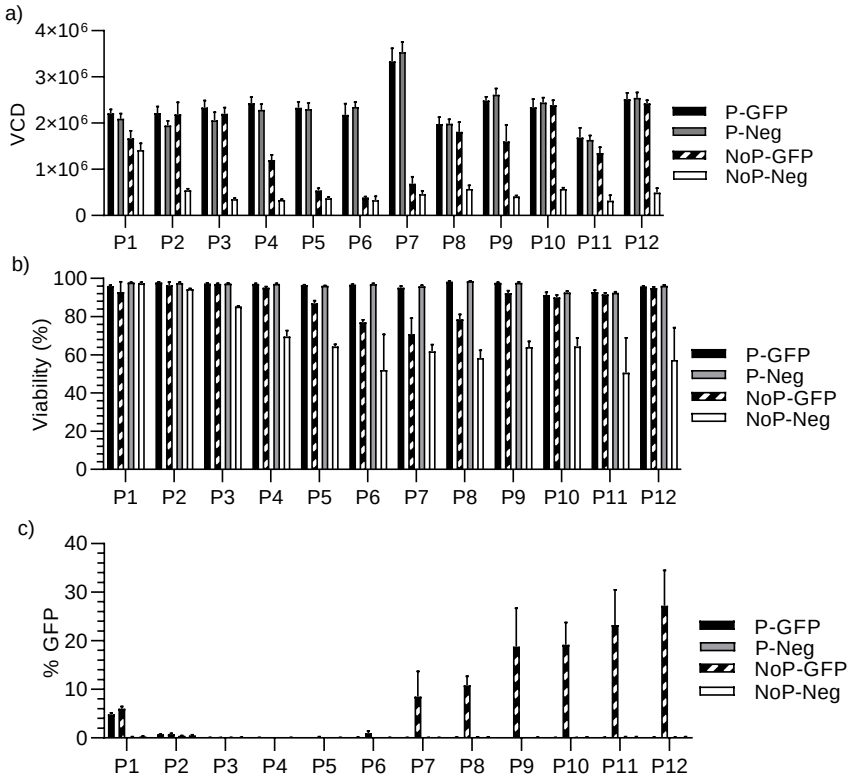


Figure 3. GFP-expressing populations can be isolated in polyamine-free media. Viable cell density (VCD) (a), viability (b) and percentage of GFP expressing (c) CHO-K1 cells transfected with 500ng GFP-IRES-Arg vector (P-GFP, NoP-GFP). A negative control of cells with no DNA transfected (P-Neg, NoP-Neg) was included. Transfected and non- transfected cells were placed in either SFM-F12 medium supplemented with putrescine (P) or selective SMF-F12 medium (without putrescine, NoP). Triplicate wells were transfected per each condition.

Interestingly, selection performed in parallel in putrescine-depleted SFM-F10 medium displayed similar profiles to those performed in SFM-F12, with recovery of mixed populations observed at P8 (Appendix B, Fig. S 4a, b). In this case, a higher percentage of GFP-expressing populations were recovered (reaching on average up to 70% by P9), which may be related to the increased stringency of this medium (Appendix B, Fig. S4c). It is noteworthy that mixed population 2 was observed to unstably express GFP from P6 to P10, displaying a dramatic drop in the percentage of GFP-expressing cells by P11, finally resulting in cellular death (P12). Hence, the large error bars displayed at P10 and P11.

Although SFM-F10 medium was observed to outperform SFM-F12 selective medium in terms of percentage of GFP-expressing populations obtained, differences in the composition of both media resulted in maximal viabilities of 80-85% in control parental cells cultured in SFM-F10 medium (P-T, P-Neg) (Appendix B, Fig. S4b), while viabilities over 90% were consistently observed in SFM-F12 media. For this reason, further testing was performed only on SFM-F12 medium.

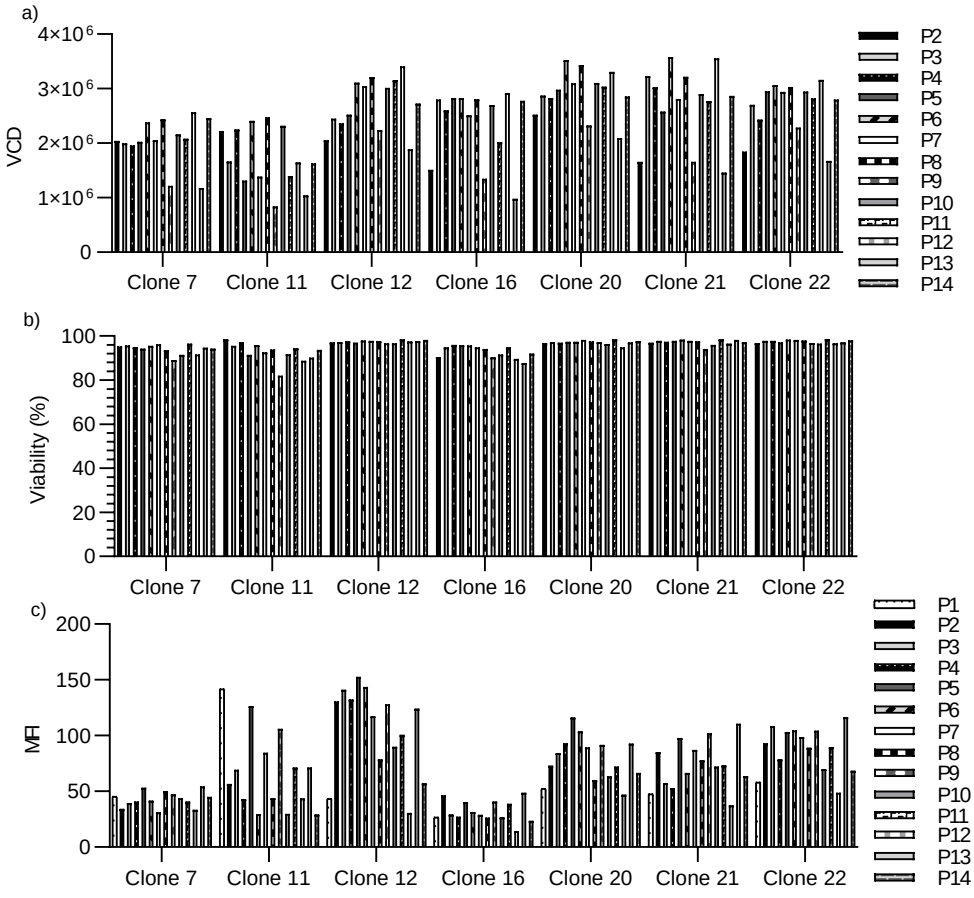


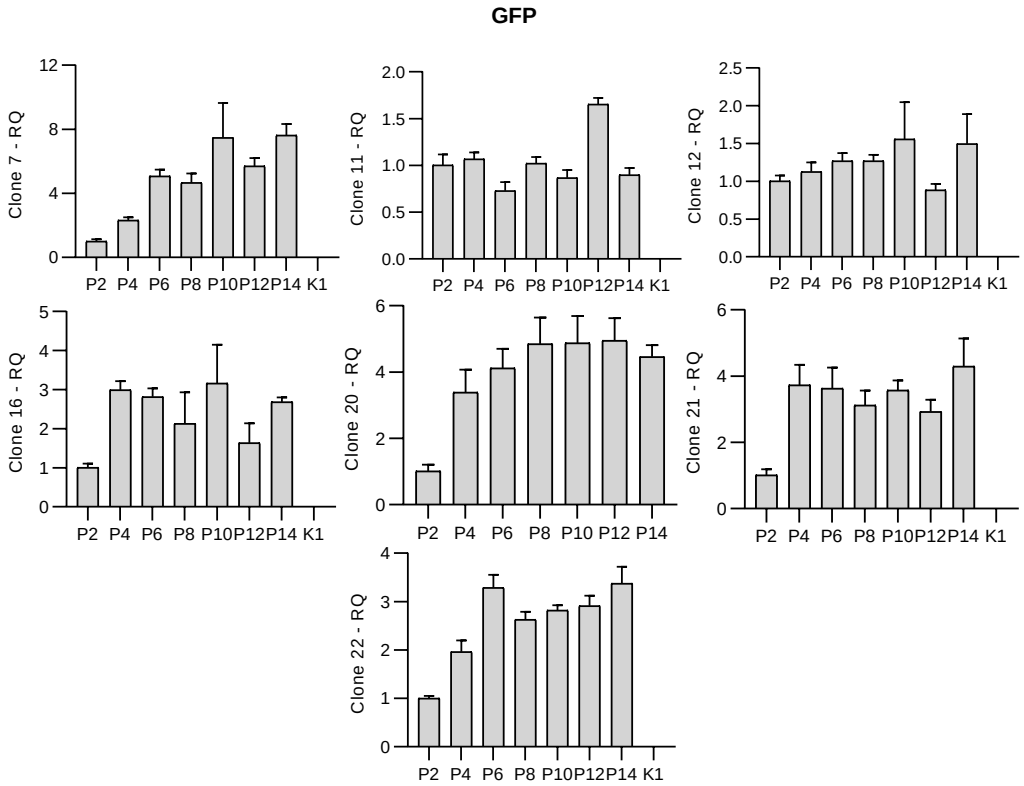
Figure 4. Long-term growth and stable expression of isolated GFP-expressing clones in selective media. Viable cell density (VCD) (a), viability (b) and mean fluorescence intensity (MFI) (c) of seven GFP-expressing clones isolated by limited dilution growing in selective medium (SFM-F12 without

putrescine) for 14 passages (42 generations). Each clone was maintained in a single tube. Duplicate technical readings were performed for each phenotype.

3.4. Long term stably expressing clones can be generated by polyamine and L-ornithine starvation

To assess the stability of the GFP-expressing populations generated, seven clones were isolated and placed in SFM-F12 medium lacking putrescine in order to monitor their growth (VCD), viability and GFP expression in terms of mean fluorescence intensity (MFI) and at a mRNA level (relative quantification, RQ) for a total of 42 generations (14 passages).

Consistent growth and healthy viabilities were displayed over the 42 generations (Fig. 4a, b). Clones 7 and 16 were observed as low producers while clone 12 displayed remarkably increased MFI levels. Nevertheless, GFP expression was detected throughout the 42 generations, with an increase in MFI levels relative to passage 1 observed in all clones except clone 11 (Fig. 4c). Stable expression was also confirmed at a transcriptional level (Fig. 5). Due to the design of the expression vector, almost identical GFP and arginase RQ profiles were observed. Interestingly, from passage 4 (12 generations), an increase in the RQ of both GFP and arginase were observed with all clones except clone 11. This effect might be related to the transference of clones from putrescine-containing media (during single-cell cloning) to SFM-F12 medium depleted of putrescine, i.e. increased stringency of selection.



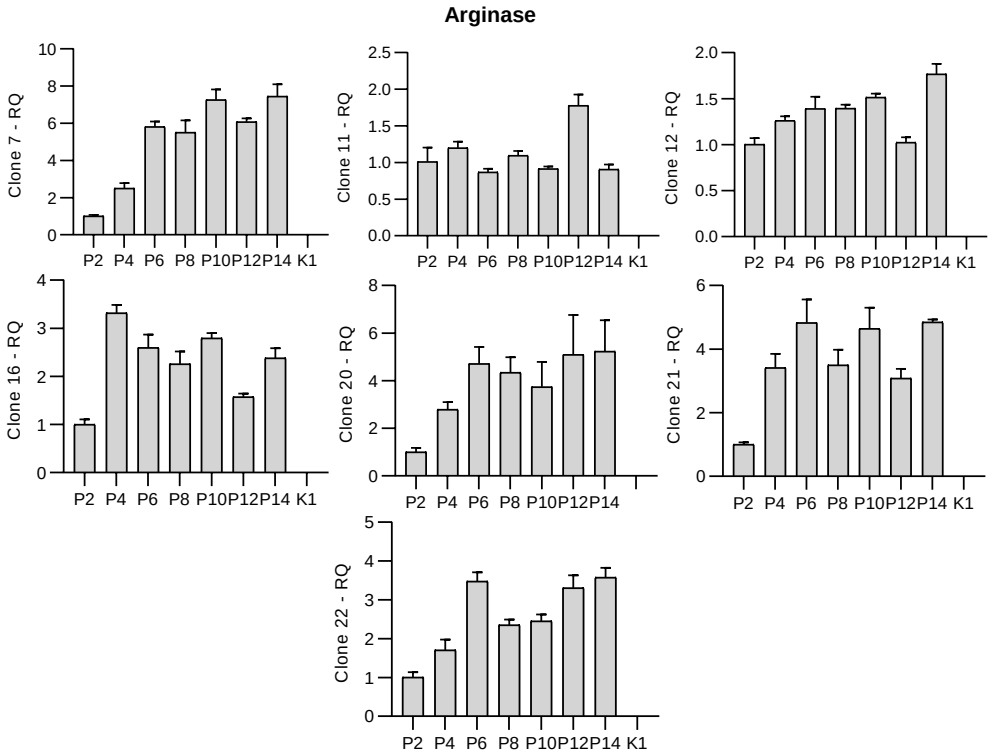


Figure 5. Long-term stable GFP and arginase mRNA expression of isolated GFP-expressing clones in selective media. Relative quantification (RQ) of GFP and arginase mRNA levels in seven GFP-expressing clones growing in selective media for 14 passages (42 generations) normalized to the mRNA levels in passage 2. The Gapdh endogenous gene was used to standardize the results.

3.5. Single clones stably expressing a therapeutically-relevant recombinant protein can be generated in polyamine and L-ornithine-free media

To confirm whether the selection system designed would support the generation and preferential survival of clones expressing pharmaceutically relevant therapeutics, a bicistronic vector expressing human erythropoietin (hEPO) was designed and transfected into CHO-K1 cells; cultures were then plated in selective medium (putrescine-free).

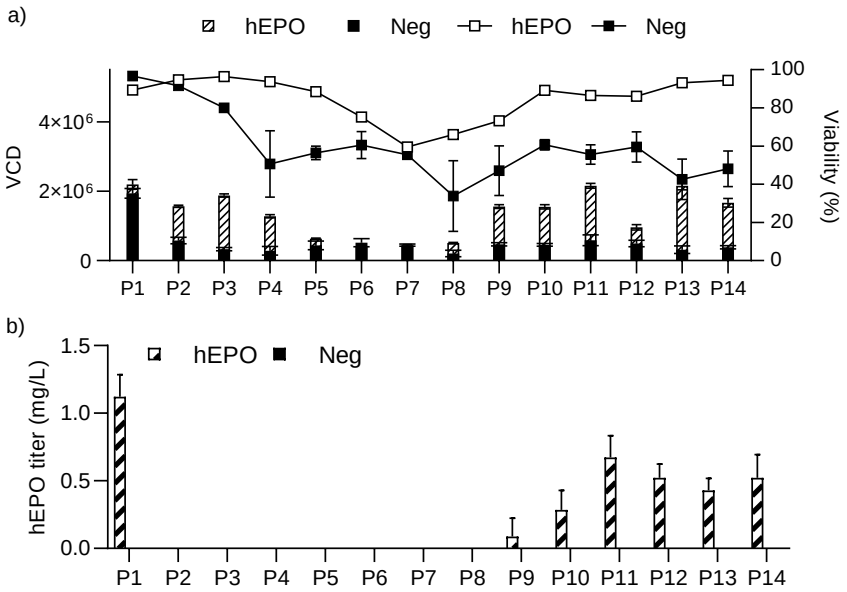


Figure 6. hEPO-expressing populations can be isolated in polyamine-free media. Viable cell density (bars, VCD) and viability (lines) (a) and hEPO titer (mg/L) (b) of CHO-K1 cells transfected with 500ng hEPO-IRES-Arg vector (hEPO, stripes) in selective medium (SFM-F12 without putrescine). A negative control of cells with no DNA transfected (Neg, black) was included. Triplicate wells were transfected per each condition.

Successful mixed populations were selected by passage 9, as observed for the detection of hEPO (0.26mg/L) and the recovery of VCD and healthy viabilities (Fig. 6). Clones were isolated and seven of them were randomly selected to assess stability in selective media for a total of 42 generations (14 passages). Stable titer expression was confirmed over the 42 generations (Fig. 7c). Assessment of hEPO and arginase mRNA expression relative to passage 2 was performed on three phenotypically divergent clones: clone 4 (low producer but fast growing), clone 10 (high producer but moderate growth) and clone 18 (medium producer with moderate growth). Both clones 10 and 18 were found to be stable, displaying levels of expression similar to those in passage 2 for at least 36 generations (P12) (Fig. 8). In contrast, the low producer clone 4 displayed a 20% decrease on hEPO mRNA expression at 18 generations (P6), dropping to less than 50% relative to the expression at passage 2 by passage 8. Nonetheless, both hEPO and arginase expression were detected over the 42 generations (P14) (Fig. 7).

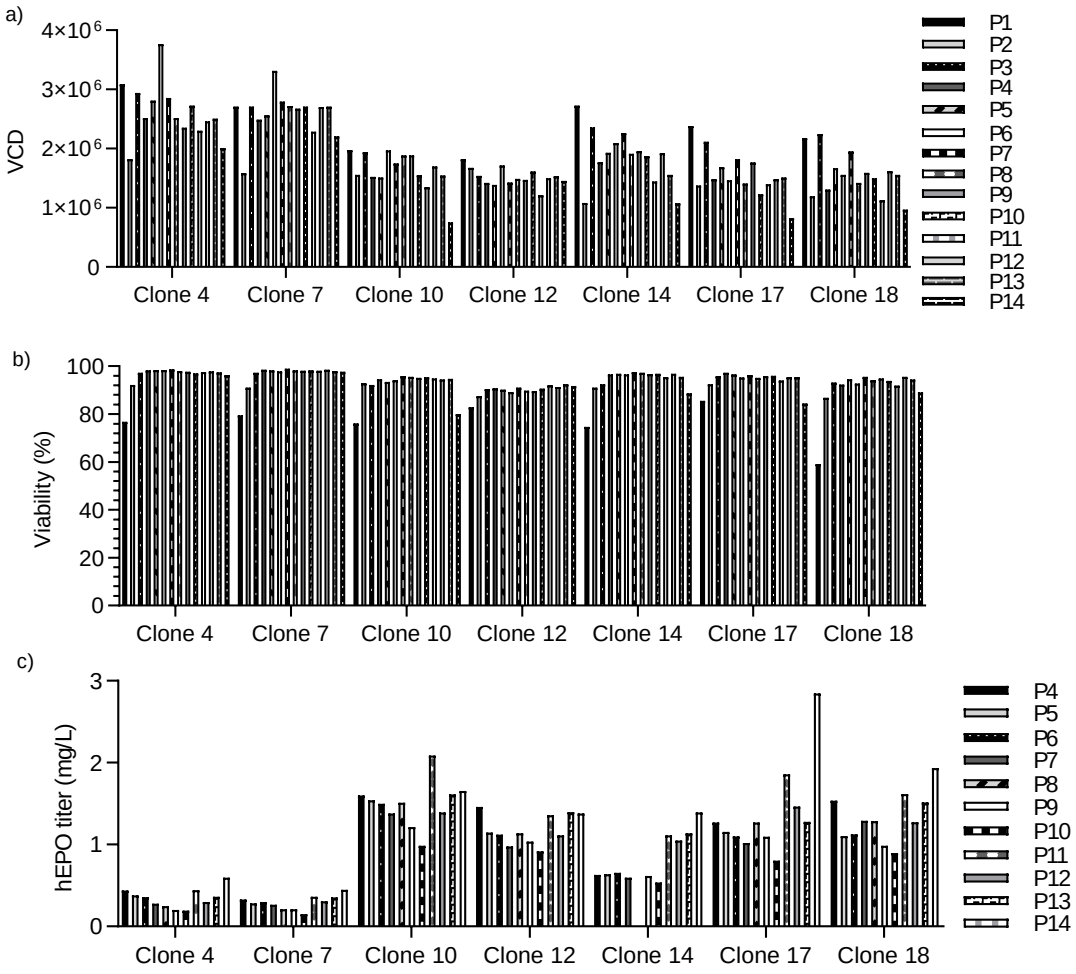


Figure 7. Long-term growth and stable expression of isolated hEPO-expressing clones in selective media. Viable cell density (VCD) (a), viability (b) and hEPO titer (mg/L) (c) of seven hEPO-expressing clones isolated by limited dilution growing in selective media (SFM-F12 without putrescine) for 14 passages (42 generations). Each clone was maintained in a single tube. Duplicate (VCD, viability) or triplicate (titer) technical readings were performed for each phenotype.

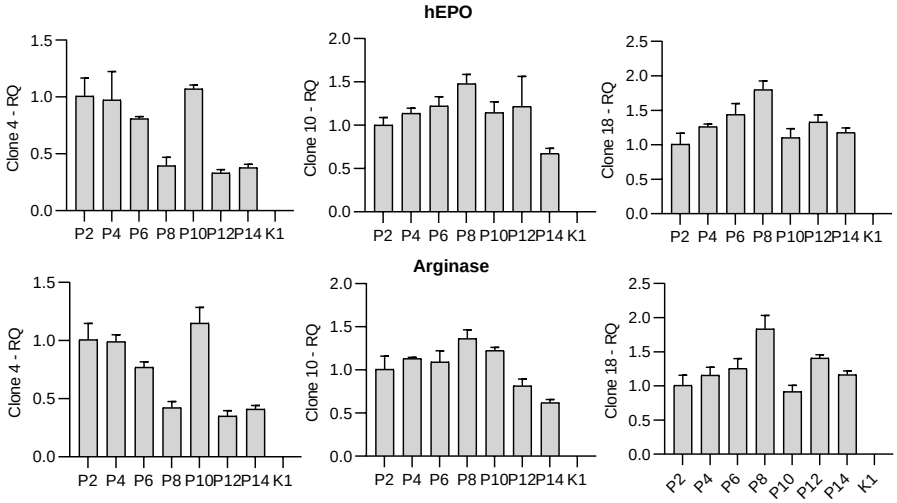


Figure 8. Long-term stable hEPO and arginase mRNA expression of isolated hEPO-expressing clones in selective media. Relative quantification (RQ) of hEPO and arginase mRNA levels in three hEPO-expressing clones growing in selective media for 14 passages (42 generations) normalized to the mRNA levels in passage 2. CHO-K1 (K1) parental cells were also assessed as control. The Gapdh endogenous gene was used to standardize the results.

In order to determine whether the application of this selection system may be limited to CHO-K1 cells, several mammalian cell lines (HEK293, SP2, BHK21, Vero, BxPC-3, Capan-2, MiaPaca-2 and PANC-1), including three CHO cell lines CHO-DP12, CHO-S and DUXB11, were assessed for arginase activity (Fig. 9). Neither BHK21 nor any of the CHO cell lines tested displayed arginase activity, indicating that the system may be applicable to several parental CHO cell lines. Conversely, the HEK293, Vero lines and the pancreatic BxPC-3 and MiaPaca-2 cell lines displayed arginase activity levels similar to the pcDNA3.1-Arg transfected CHO-K1 cells (Fig. 2c), making these parental cell lines unsuitable for the system. Finally, low levels of activity were observed in SP2, Capan-2 and PANC-1. Whether this activity is low enough to allow application of the arginase-based selection system requires further investigation.

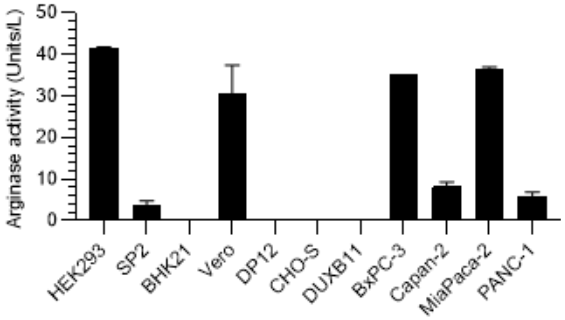


Figure 9. Arginase activity of mammalian cell lines. HEK 293, SP2, BHK21, Vero, DP12, CHO-S and DUXB11 cells were analysed in triplicate. The pancreatic cell lines BxPC-3, Capan-2, MiaPaca-2 and PANC-1 were analysed in duplicates.

3.6. hEPO clones display stable expression in putrescine-containing media

Due to the vital role played by polyamines in the culture of CHO cell lines in serum-free media (19), commercial and chemically-defined formulations contain putrescine levels sufficient to supply the metabolic requirements of CHO cells. To address whether clones generated following our polyamine-starvation method would maintain expression in non-selective conditions, clone 4, 10 and 18 were adapted to SFM-F12 medium containing putrescine as well as two commercially available formulations of undisclosed composition: a serum-free medium, CHO-S SFM-II (Gibco), and a chemically-defined medium, BalanCD Growth A (Irvine). Clones cultured in SFM-F12 and BalanCD media displayed constant VCD and viability profiles (Fig. 10a, b). In contrast, SFM-II medium was less supportive of healthy cultures, resulting in a decrease in growth from passage 2 (6 generations) in two of the clones, reaching consistent VCDs by passage 6 ($2.3x10^6$cells/ml, clone 4) and passage 5 ($1.5x10^6$cells/ml clone 10). Moreover, in this medium, the viability of clone 10 dropped to 70% by

passage 6 and to 60% in clone 18 by passage 7. Stable hEPO expression was observed in BalanCD and SFM-F12 medium for clone 10 (up to P9, 27 generations), while decreased (23-30% lower) hEPO titers were displayed by passage 8 in clone 18 and clone 4 (40-30% lower) by passage 9 (Fig. 10c). In SFM-II medium, both clone 10 and 18 performed similarly to the other two media. However, clone 4 was less stable, with hEPO titer dropping by 60% at passage 6 but maintaining this level until the end of the culture.

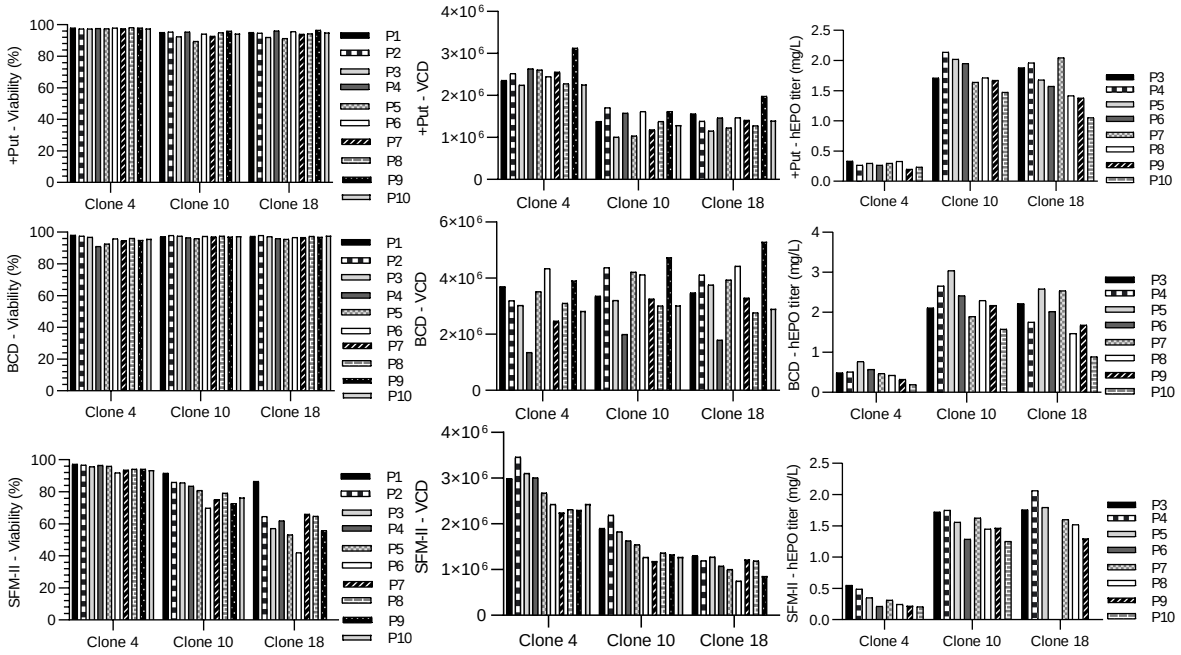


Figure 10. Long-term growth and stable expression of isolated hEPO-expressing clones in commercial and in-house media containing putrescine. Viability, viable cell density (VCD) and hEPO titer (mg/L) of three hEPO-expressing clones isolated by limited dilution growing in SFM-F12 medium with putrescine (+Put), BalanCD Growth A supplemented with L-glutamine (BCD) and CHO-S SFM-II supplemented with PVA (SFM-II) for 10 passages (30 generations). Each clone in SFM-II was maintained in a single tube. Duplicate tubes were performed for SFM-F12 and BalanCD. Duplicate technical readings were performed for each condition.

To further characterize the capabilities of the system, all three clones were each cultured in both commercial media and SFM-F12 with putrescine for a total of 7 days to allow for high hEPO and, consequently, arginase expression. Healthy growth and hEPO expression was supported at different levels in all media tested, indicating that an increase in the arginase expression had no detrimental effect on CHO cell development (Appendix B, Fig. S5).

4. Discussion

With the results reported here, we have demonstrated the efficacy of a newly developed auxotrophic selection system for CHO cells which exploits an arginase-deficiency phenotype observed in several

mammalian cells. The method was shown to sustain the survival and growth of arginase-expressing transfected cell pools in a polyamine and L-ornithine-free environment, supporting the isolation of GFP and recombinant therapeutic protein (erythropoietin) expressing single-cell derived populations. Moreover, initial evidence of clonal stability was observed, with the maintenance of VCD, viability and production profiles for at least 42 generations in selective media (14 passages). The clones were also observed to express and grow in non-selective commercial media for at least 27 generations.

The routinely used DHFR and GS expression systems, although being described as auxotrophic selection methods, require MTX and MSX drug-selection pressure for the generation and isolation of high producer cell lines (4), resulting in time-consuming amplification processes (reaching up to 5-6 months) mainly for the DHFR system (6), as several rounds are required. Moreover, the DHFR-expressing system is predominately used with CHO cells lacking DHFR expression, thus enabling selection in nucleoside-depleted media (26). On the other hand, despite endogenous expression of glutamine synthetase in CHO cells, selection of parental producer colonies have been isolated in glutamine-free media by MSX pressure. However, the endogenous GS gene has been suggested to display activity at a sufficient levels to allow non-producer CHO cells survival, reducing the stringency of the GS system (27). Consequently, use of GS-KO CHO-K1 has been found to be optimal (5). In contrast, the arginase-expression system proposed here is a drug-free method applicable, but not limited to, CHO-K1 parental cells due to their inherent lack of arginase activity, which has been further observed as an intrinsic phenotype of several CHO cell lines and some mammalian cells. Interestingly, arginase activity was reported by Hölttä et al (1982) in several sources of serum (19). As FBS is still a common supplement used in mammalian cultures, arginase-deficiency may be masked in some cell lines, which indicates the possibility of a broader applicability of the system described here.

Development of cell lines with stable production phenotypes is an essential attribute in the pharmaceutical industry. Hence, several studies have been focused on the characterization of the two predominant selection systems. In 2006, Jun et al. reported decreases of 33-62% in specific productivity of GS-derived high producer clones over the first batch, reaching further lower expression up to passage 6 (28). Similarly, monoclonal antibody was observed to decrease during the 30 passages assessment of CHO-GS clones in selective and non-selective media (29), while lower titer levels were observed after 20 days in a DHFR-derived cell line (10). Further investigation studies have revealed loss of gene copies, epigenetic modifications and inefficient or decreased mRNA transcription (8, 9, 30, 21, 32) as the main causes of clonal instability, being observed as a widespread issue. Compared to GS and DHFR studies, clones isolated with the arginase system proposed here have displayed stable GFP and EPO expression at protein and mRNA levels for over 40-53 generations (depending on the clone - 14 passages) in drug-free selection media. Other alternative drug-free selection systems such as OSCAR^{tm} (based on knock out cell lines for hypoxanthine

phosphoribosyl transferase (HPRT)), have reported lower stability with a rapid mAb expression decay displayed after 3 weeks in culture (33). Although clonal stability monitoring in industry is performed for periods over 60 generations, initial evidence for stable production have been described for the arginase-expressing method, with further characterization to be performed.

Due to the essential roles of polyamines in the maintenance of cellular wellbeing, it is not surprising to observe regulatory pathways to compensate imbalanced intracellular polyamine content. In response to critical levels of spermidine, spermine and putrescine, active transport mechanisms participate in the maintenance of polyamine homeostasis. In mammals, polyamine transport systems are still poorly understood but three models have been suggested: (1) polyamine uptake by unidentified membrane permeases, (2) interaction with heparin sulphate and glypican 1 and (3) putrescine uptake by a caveolin-1-dependent endocytosis mechanism, which has been associated to SLC3A2 (17). Other solute carrier transporters are also under investigation (reviewed in 41) and a diamine exporter (DAX) has been identified to have the ability to export putrescine in CHO (42). In plants and microorganisms, putrescine can be alternatively produced by arginine decarboxylase and agmatinase. In this pathway, arginine is first decarboxylated to agmatine, which is then used as a substrate to generate putrescine (11). Recent evidence of agmatinase activity has been reported in rat liver (16) and kidney (34) as well as brain and other tissues of several mammalian species (35) where agmatine has been described as a potent biological active substance (e.g. acting as a neurotransmitter (15), modulator of nitric oxide synthesis or interacting with several receptors, ion channels or membrane transporters (35)). Consequently, agmatine degradation to putrescine has been suggested as regulatory system rather than an "alternative" pathway for the production of polyamine (36). In 2014, ornithine decarboxylase knock-out ovarian conceptuses (ODC-KO) were generated and observed to compensate polyamine-deficiency conditions by agmatine production. However, this phenotype was only observed in half of the ODC-KO ovarian conceptuses while the other half displayed lack of agmatine production resulting in cessation of cellular development (14). Similarly, we have here observed that removal of putrescine and L-ornithine from media results in a drop of viability and VCD of CHO-K1 parental cells and this altered phenotype is maintained for at least 40-53 generations (depending on the clone - 14 passages). As a result, investigation of agmatine expression was not performed. Further research is necessary to determine whether the agmatine-derived pathway is active and contributes to polyamine production in CHO cells.

Similar to the findings reported here, lack of arginase activity and subsequent decreases in cellular growth has been previously reported with CHO cells cultured in absence of putrescine, with cellular death observed after 8-14 days (18, 19) due to the intracellular depletion of the secondary polyamines spermine and spermidine (3). As expected, more stringent conditions obtained in putrescine-depleted media were here observed to outperform the selection efficiency of the low putrescine-containing media. However, traces of this polyamine in media did not impede isolation of arginase-expressing

clones, suggesting potentially easy applicability of this system when paired with common basal media, such as DMEM-F12. It is important to note that the use of this medium would require clonal isolation in order to remove background populations not expressing the gene of interest (such as cells only expressing arginase or non-transfected populations); in any case, this is a step that is commonly performed to achieve stable high producer clones.

In conclusion, we have presented here evidences for an alternative method for the generation of stable producer CHO-K1 cell lines using an arginase-expressing system in polyamine- and ornithine-free media. We have conclusively demonstrated that arginase-expressing selection is efficient, offering a drug-free, cost-effective and easy-to-apply method for a range of parental mammalian cells displaying lack of arginase activity. The system may be also used in conjunction with GS or DHFR methods, offering an alternative to antibiotic-based selection for the generation of double transfectants avoiding possible secondary effects from drug selection. To note, the aim of the investigation presented here was to prove the feasibility of the system. Further analysis focused on the isolation of high producing clones and improvement of the system with modifications such as using an attenuated arginase gene, the use of a weak promoter or knock out of polyamine transporters may lead to a more efficient system and will be further investigated.

5. References

1- Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R. and Amanullah1, A. (2010) Cell culture processes for monoclonal antibody production. mAbs. 2(5), 466-477.

2- Fan, L., Frye, C. C. and Racher, A. J. (2013) The use of glutamine synthetase as a selection marker: recent advances in Chinese hamster ovary cell line generation processes. Pharm. Bioprocess. 1(5), 487–502.

3- Wurm, F. M. (2004) Production of recombinant protein therapeutics in cultivated mammalian cells Nat. Biotechnol. 22(11), 1393-8.

4- Costa, A. R., Rodrigues, M. E., Henriques, M., Azeredo, J. and Oliveira, R. (2010) Guidelines to cell engineering for monoclonal antibody production. Eur J Pharm Biopharm. 74(2), 127-38.

5- Fan, L., Kadura, I., Krebs, L. E., Hatfield, C.C., Shaw, M. M. and Frye, C. C. (2012) Improving the efficiency of CHO cell line generation using glutamine synthetase gene knockout cells. Biotechnol Bioeng. 109(4), 1007-15

6- Jayapal, K. P., Wlaschin, K. F., Hu, W. S. and Yap, M. C. S. (2007) Recombinant protein therapeutics from CHO cells – 20 years and counting. Chem. Engin. Progr. 103 (10), 40–47.

7- Guo, D., Gao, A., Michels, D. A., Feeney, L., Eng, M., Chan, B., Laird, M. W., Zhang, B., Yu, X. C., Joly, J., Snedecor, B. and Shen, A. (2010) Mechanisms of unintended amino acid sequence changes in recombinant monoclonal antibodies expressed in Chinese Hamster Ovary (CHO) cells. Biotechnol Bioeng. 107(1), 163-71

8- Kim, M., O'Callaghan, P. M., Droms, K.A. and James, D. C. (2011) A mechanistic understanding of production instability in CHO cell lines expressing recombinant monoclonal antibodies. Biotechnol Bioeng. 108(10), 2434-46

9- Chusainow, J., Yang, Y. S., Yeo, J. H., Toh, P. C., Asvadi, P., Wong, N. S. and Yap, M. G. (2009) A study of monoclonal antibody-producing CHO cell lines: what makes a stable high producer? Biotechnol Bioeng. 102(4), 1182-96

10- Kim, N. S., Kim, S. J. and Lee, G. M. (1998) Clonal variability within dihydrofolate reductase-mediated gene amplified Chinese hamster ovary cells: stability in the absence of selective pressure Biotechnol Bioeng. 60(6), 679-88.

11- Heby, O. (1981) Role of polyamines in the control of cell proliferation and differentiation. Differentiation. 19(1), 1-20.

12- Rhee, H. J., Kim, E. J. and Lee, J. K. (2007) Physiological polyamines: simple primordial stress molecules. J Cell Mol Med. 11(4), 685-703.

13- Pegg, A. E. (2016) Functions of polyamines in mammals. J Biol Chem. 291(29):14904-12

14- Wang, X., Ying, W., Dunlap, K. A., Lin, G., Satterfield, M. C., Burghardt, R.C., Wu, G. and Bazer, F.W. (2014) Arginine decarboxylase and agmatinase: an alternative pathway for de novo biosynthesis of polyamines for development of mammalian conceptuses. Biol Reprod. 90(4), 84

15- Iyo, A. H., Zhu, M. Y., Ordway, G. A and Regunathan, S. (2006) Expression of arginine decarboxylase in brain regions and neuronal cells. J Neurochem. 96(4), 1042–1050.

16- Horyn, O., Luhovyy, B., Lazarow, A., Daikhin, Y., Nissim, I., Yudkoff, M. and Nissim, I. (2005) Biosynthesis of agmatine in isolated mitochondria and perfused rat liver: studies with 15N-labelled arginine. Biochemical Journal 388 (2), 419-425

17- Baea, D-H., Laneb, D. J. R., Janssona, P. J. and Richardson, D. R. (2018) The old and new biochemistry of polyamines. Biochim Biophys Acta Gen Subj. 1862(9), 2053-2068.

18- Pohjanpelto, P., Virtanen, I. and Hölttä, E. (1981) Polyamine starvation causes disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO cells. Nature. 293(5832), 475-7.

19- Hölttä, E. and Pohjanpelto, P. (1982) Polyamine dependence of Chinese hamster ovary cells in serum-free culture is due to deficient arginase activity. Biochim Biophys Acta. 721(4), 321-7.

20- Anehus, S., Pohjanpelto, P., Baldetorp, B., Långström, E. and Heby, O. (1984) Polyamine starvation prolongs the S and G2 phases of polyamine-dependent (arginase-deficient) CHO cells. Mol Cell Biol. 4(5), 915-22.

21- Pohjanpelto, P., Hölttä, E. and Jänne, O. A. (1985) Mutant strain of Chinese hamster ovary cells with no detectable ornithine decarboxylase activity. Mol Cell Biol. 5(6), 1385-90.

22- Steglich, C. and Scheffler, I. E. (1982) An ornithine decarboxylase-deficient mutant of Chinese hamster ovary cells. J Biol Chem. 257(8), 4603-9.

23- Byers, T. L., Wechter, R. S., Hu, R. H. and Pegg, A. E. (1994) Effects of the S-adenosylmethionine decarboxylase inhibitor, 5'-{[(Z)-4-amino-2butenyl]methylamino}-5'-deoxyadenosine, on cell growth and polyamine metabolism and transport in Chinese hamster ovary cell cultures. Biochem J. 303 (Pt 1), 89-96.

24- Mamont, P. S., Böhlen, P., McCann, P. P., Bey, P., Schuber, F. and Tardif, C. (1976) α-Methyl ornithine, a potent competitive inhibitor of ornithine decarboxylase, blocks proliferation of rat hepatoma cells in culture. Proc Natl Acad Sci U S A. 73(5), 1626-30.

25- Capella Roca, B., Alarcón Miguez, A., Keenan, J., Suda, S., Barron, N., O'Gorman, D. and Doolan, P., Clynes, M. (2019) Zinc supplementation increases protein titer of recombinant CHO cells. Cytotechnology. doi: 10.1007/s10616-019-00334-1

26- Cacciatore, J. J., Chasin, L. A. and Leonard, E. F. (2010) Gene amplification and vector engineering to achieve rapid and high-level therapeutic protein production using the Dhfr-based CHO cell selection system. Biotechnol Adv. 28(6), 673-81.

27- Sanders, P. G. and Wilson, R. H. (1984) Amplification and cloning of the Chinese hamster glutamine synthetase gene. EMBO J. 3(1), 65-71.

28- Jun, S. C., Kim, M. S., Hong, H. J. and Lee, G. M. (2006) Limitations to the development of humanized antibody producing Chinese hamster ovary cells using glutamine synthetase mediated gene amplification. Biotechnol Prog. 22(3), 770-80.

29- Noh, S. M., Shin, S. and Lee, G. M. (2018) Comprehensive characterization of glutamine synthetase-mediated selection for the establishment of recombinant CHO cells producing monoclonal antibodies. Sci Rep. 8(1), 5361.

30- Barnes, L. M., Bentley, C. M. and Dickson, A. J. (2004) Molecular Definition of Predictive Indicators of Stable Protein Expression in Recombinant NS0 Myeloma Cells. Biotechnol Bioeng. 85(2), 115-21.

31- Yang, Y., Mariati, Chusainow, J. and Yap, M. G. (2010) DNA methylation contributes to loss in productivity of monoclonal antibody-producing CHO cell lines. J Biotechnol. 147(3-4):180-5.

32- Brown, M. E., Renner, G., Field, R. P. and Hassell, T. (1992) Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system. Cytotechnology. 9(1-3), 231-6.

33- Costa, A. R., Rodrigues, M. E., Henriques, M., Melton, D., Cunnah, P., Oliveira, R. and Azeredo, J. (2012) Evaluation of the OSCAR system for the production of monoclonal antibodies by CHO-K1 cells. Int J Pharm. 430(1-2), 42-6.

34- Lortie, M. J., Novotny, W. F., Peterson, O. W., Vallon, V., Malvey, K., Mendonca, M., Satriano, J., Insel, P., Thomson, S. C. and Blantz, R. C. (1996) Agmatine, a bioactive metabolite of arginine. Production, degradation, and functional effects in the kidney of the rat. J Clin Invest. 97(2), 413-20.

35- Piletz, J. E., Aricioglu, F., Cheng, J. T., Fairbanks, C. A., Gilad, V. H., Haenisch, B., Halaris, A., Hong, S., Lee, J. E., Li, J., Liu, P., Molderings, G. J., Rodrigues, A. L., Satriano, J., Seong, G. J., Wilcox, G., Wu, N. and Gilad, G. M. (2013) Agmatine: clinical applications after 100 years in translation. Drug Discov Today. 18(17-18), 880-93.

36- Peters, D., Berger, J., Langnaese, K., Derst, C., Madai, V. I., Krauss, M., Fischer, K. D., Veh, R. W. and Laube, G. (2013) Arginase and arginine decarboxylase – where do the putative gate keepers of polyamine synthesis reside in rat brain? PLoS One. 8(6):e66735.

37- Chida, Y., Takagi, K. and Terada, S. (2013) Establishment of a mammalian cell line suitable for industrial production of recombinant protein using mutations induced by high-energy beam radiation. Cytotechnology. 65(6), 955–965.

38– Meerbrey, K. L., Hu, G., Kessler, J. D., Roarty, K., Li, M. Z., Fang, J. E., Herschkowitz, J. I., Burrows, A. E., Ciccia, A., Sun, T., Schmitt, E. M., Bernardi, R. J., Fu, X., Bland, C. S., Cooper, T. A., Schiff, R., Rosen, J. M., Westbrook, T. F. and Elledge, S. J. (2011) The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo.. Proc Natl Acad Sci U S A. 108(9), 3665-70.

39- Lin, R. Z., Dreyzin, A., Aamodt, K., Li, D., Jaminet, S.C., Dudley, A. C., Melero-Martin, J. M. (2011) Induction of erythropoiesis using human vascular networks genetically engineered for controlled erythropoietin release. Blood. 118(20), 5420-8.

40- Costello, A., Lao, N.T., Barron, N. and Clynes, M. (2019) Improved yield of rhEPO in CHO cells with synthetic 5′ UTR. Biotechnol Lett 41(2), 231–239.

41- Abdulhussein, A. A. and Wallace, H. M. (2014) Polyamines and membrane transporters. Amino Acids 46, 655–660.

42- Uemura, T., Yerushalmi, H. F., Tsaprailis, G., Stringer, D. E., Pastorian, K. E., Hawel, L., Byus, C. V. and Gerner, E. W. (2008) Identification and Characterization of a Diamine Exporter in Colon Epithelial Cells. J Biol Chem. 283(39), 26428–26435.

Chung et al. (2003) Reduction of ammonia accumulation and improvement of cell viability by expression of urea cycle enzymes in Chinese Hamster ovary cells. Journal of Microbiology and Biotechnology 13(2):217-224

Claudia Altamirano, C, Berrios, J, Vergara, M and Becerra, S (2013) Advances in improving mammalian cells metabolism for recombinant protein production. Electronic Journal of Biotechnology doi: 10.2225/vol16-issue3-fulltext-2

CHAPTER 3

Altered gene expression in CHO cells following polyamine starvation

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Manuscript accepted (subject to reviewer's comments) in Biotechnology Letters (manuscript ID:BILE-D-19-00932)

Preface to the paper – Polyamine functions

The design of a selection system for producer CHO cells based on polyamine-dependence, highlighted the vital role of these amines in viable cell proliferation in CHO. Intracellularly, polyamines are polycations that interact with nucleic acids, ATP and phospholipids, affecting multiple cellular processes (Igarashi K et al. 2019). Hence, the function of these amines is not only limited to cell proliferation but it is also related to DNA synthesis, RNA expression, protein synthesis, cell cycle progression and apoptosis among many other relevant functions. By binding with DNA, polyamines can cause chromatin condensation or/and induction of Z-DNA conformation, being reported to increase the expression of some genes (Thomas et al. 1995; Dever and Ivanov 2018, Liu *et al.* 2001). Increases on the intracellular polyamine levels have been observed to increase the expression of transcription factors, while deprivation of these amines can have an effect on mRNA stability (Zou et al. 2006; L. Liu et al. 2009). As a consequence, polyamines regulate the expression of multiple genes, being essential for cell survival.

In CHO, polyamines are an absolute vital supplement due to the lack of endogenous arginase activity of these cells, phenotype firstly reported by Höltä et al in 1982 (Hölttä E et al. 1982). Since then, studies on the polyamine metabolism in CHO were performed by generating knock out cell lines and/or using polyamine biosynthesis enzymes inhibitors (such as α-difluoromethylornithine (DFMO) and 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (AbeAdo)) (Pohjanpelto, Hölttä and Jänne 1985; Steglich and Scheffler 1982; Byers *et al.* 1994). However, even though polyamines display major roles on key parameters for the biopharmaceutical area (cell proliferation and protein expression), little recent investigation has been carried out (Hyvönen 1989; Jan O. Fredlund and Oredsson 1997a; Berntsson, Alm and Oredsson 1999; Pastorian, Hawel, and Byus 2000), with last reports in polyamines effects in CHO metabolism from 2008 (Uemura *et al.* 2008).

Leading on from the described arginase-based selection system for CHO (Chapter 2) and given the critical role these compounds play in various cellular pathways, the impact of polyamine-deprivation in CHO was investigated. Due to the broad effects of polyamines, the development of -omic technologies have provided new tools for the study of these multifunction amines. For this reason, changes on gene expression were assessed by using Affymetrix CHO whole transcriptome microarray chips. Moreover, based on the polyamine-dependence of CHO cultures, the use of chemical inhibitors such as DFMO or AbeAdo was avoided, eliminating possible off-target effects.

CHO-K1 cells were starved for putrescine for three days, when samples were collected. Phenotypic assessment displayed a drop on cell proliferation while analysis of the cell cycle by flow cytometry revealed a substantial increase on the S-phase population over the three days culture. At transcriptional level, five major pathways revealed changes in the mRNA levels, being cell cycle, p53, spliceosome, protein processing in endoplasmic reticulum and Fanconi anaemia pathway.

Moreover, changes in proteins localised in the cellular membrane were observed to display major fold-changes between putrescine-containing and starved cultures.

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7. Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Altered gene expression in CHO cells following polyamine starvation
Publication Status	Accepted (subject to reviewer's comments) (ID: BILE-D-19-00932)
ISSN and link to URL (where available)	
This paper is one of [4] co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
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I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Padraig Doolan Date: 25-11-19

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ABSTRACT

Aim: To investigate the impact of polyamine deprivation on the transcriptome of CHO cells

Results: Polyamines play a central but poorly-understood role in cell proliferation. Most studies to date have utilised chemical inhibitors to probe polyamine function. Here we exploit the fact that CHO cells grown in serum-free medium have an absolute requirement for putrescine supplementation due to their deficiency in activity of the enzyme arginase. A gene expression microarray (Affymetrix) analysis of CHO-K1 cells starved of polyamines for 3 days showed that cessation of growth, associated with increased G1/S transition and decreased M/G1 transition, was accompanied by increased mRNA levels of mitotic complex checkpoint genes (*Mad2l1*, *Tkk*, *Bub1b*) and of the transition of G1- to S-phase (such as *Skp2* and *Tfdp1*). mRNAs associated with DNA homologous recombination and repair (including Fanconi's anaemia-related genes) and with RNA splicing were consistently increased. Alterations in mRNA levels for genes related to protein processing in the ER, to ER stress, and to p53-related and apoptosis pathways were also observed. mRNAs showing highest levels of fold-change included several which code for membrane-localised proteins and receptors (*Thbs1*, *Tfrc1*, *Ackr3*, *Extl1*).

Conclusions: Growth-arrest induced by polyamine deprivation was associated with significant alterations in levels of mRNAs associated with cell cycle progression, DNA repair, RNA splicing, ER trafficking and membrane signalling as well as p53 and apoptosis-related pathways.

1. Introduction

The polyamines spermine, spermidine and putrescine play multiple roles in cell growth, survival and differentiation, being essential for healthy cell proliferation. Due to their cationic state at physiological pH, polyamines mainly exist as polyamine-RNA complexes, being described to induce ribosome shunting, displacing translation inhibitors and stabilising RNA structures, suggesting an important role in the regulation of protein synthesis (Igarashi and Kashiwagi 2019). DNA structural changes are also induced by polyamine interaction, increasing chromosomal condensation or inducing B- to Z- DNA conformational changes, thus regulating gene expression (Casero, Murray Stewart, and Pegg 2018; Igarashi and Kashiwagi 2019). Activation of transcription factors has been also observed to be affected by polyamine levels. Consequently, polyamines have an impact on gene expression at transcriptional and translational level, affecting the proliferation and viability of mammalian cells.

In 1982, Hölta et al. reported growth arrest of a CHO-K1 cell line in serum-free and polyamine-free media, which was associated with a lack of expression of the first enzyme of polyamine production pathway (arginase) (E Hölttä and Pohjanpelto 1982). Since then, several studies focused on understanding polyamines essential role were performed by generating mutants or supplementing polyamine biosynthesis inhibitors (Anehus et al. 1984; Jan O. Fredlund and Oredsson 1997b). However, little investigation has been carried out in CHO since the early 2000s. Recently, we have described a novel selection system for the generation of recombinant protein producing CHO cells by combining the use of an arginase-expressing vector and selection in putrescine-free media (Capella Roca et al. 2019). Lack of arginase activity was further confirmed in a panel of three CHO cell lines (CHO-S, DP12 and DUXB11), confirming the importance of putrescine for CHO cells. This unusual property of CHO cells provides an interesting opportunity to study the effects at a cellular and molecular level of polyamine deprivation, without the need to use chemical inhibitors of polyamine biosynthesis which although quite specific may also display off-target effects. Given the critical role these compounds play in various cellular pathways we therefore decided to investigate the impact of polyamine removal on gene expression in CHO cells using Affymetrix CHO whole transcriptome microarray chips

2. Materials and methods

2.1. Cell culture

A parental CHO-K1 cell line (ATTC CCL-61) was cultured in in-house serum-free SFM-F12 medium containing 1mg putrescine/L (Capella Roca et al. 2019). To study the effects of polyamine deprivation, cells were cultured both with and without putrescine in another in-house SFM-F10 medium, which has a similar formulation to SFM-F12 but lacks putrescine (Capella Roca et al. 2019).

Biological triplicates were analysed for viable cell density (VCD) and viability using the ViaCount on a Guava easyCyte HT benchtop cytometer.

2.2. Cell Cycle analysis

For cell cycle analysis, samples were prepared using the Guava Cell Cycle reagent containing propidium iodide, following manufacturer's instructions, and read on the Guava easyCyte HT benchtop cytometer. Data was analysed using the ModFit LT 3.2 software.

2.3. RNA extraction

Cell pellets from biological triplicates were collected at day 3 from putrescine-deprived and control (putrescine-containing) cultures. They were re-suspended in 1ml Trizol reagent and total RNA was then isolated following manufacturer's recommendations. NanoDrop was used to evaluate RNA quantification and quality. DNase I treatment was performed as per manufacturer's protocol and samples were stored at – 80 °C until assessed.

2.4. Microarrays

RNA quality was assessed using an Agilent 2100 Bioanalyzer. A total of 300 ng was added to each Affymetrix CHOGene 2.0 ST array, as per manufacturer's instructions. Data pre-processing and analysis of microarray data was carried out using the Transcriptome Analysis Console (TAC) 4.0.1 (Applied Biosystems). Only differentially-expressed (DE) genes between putrescine-deprived and control cells with a fold-change cut off of 1.5 and a Benjamini-Hochberg FDR p-value ≤ 0.05 were considered. Enrichment analysis was carried out for the differentially-expressed mRNAs via the DAVID interface (http://david.abcc.ncifcrf.gov) for two databases (Kyoto Encyclopedia of Genes and Genomes (KEGG)). The microarray data files have been deposited at Gene Expression Omnibus (GEO).

2.5. RT-qPCR

Samples were prepared using the High Capacity cDNA Reverse Transcription Kits and Fast SYBR Green Master Mix, as per manufacturer's recommendations. RT-qPCR were performed in an 7500 (Applied Biosystems) as per Capella et al. 2019. Relative quantification was measured by the comparative ddCt method (Livak and Schmittgen 2001). *Gapdh* was used as endogenous control, as it was by Veress et al. (2000) but note that they reported stabilisation of *Gapdh* mRNA following polyamine-inhibition treatment. The Ct values for *Gapdh* from both cultures (with and without putrescine) were observed in between the range of 15-16 Ct. It was then considered that the RQ data was obtained was accurate, although our qPCR may underestimate fold change of mRNAs showing

differential expression (this does not apply to the microarray results). Technical triplicate wells were run per each biological triplicate. The sequences of the primers used are available in Supplementary Table 1 (Appendix C).

3. Results and Discussion

Exploiting the lack of arginase activity of CHO-K1 cells (E Hölttä and Pohjanpelto 1982), the effects of polyamine withdrawal in these cells was assessed in serum-free medium. Hence, polyamine biosynthesis inhibitors (e. g. α-difluoromethylornithine (DFMO)), commonly used for the study of polyamine-deprivation, were not needed, eliminating the danger of possible off-target effects (Mamont et al. 1978). Initial assessment of cell proliferation following polyamine starvation revealed a decrease in CHO K1 growth, with viability only slightly reduced (Fig. 1a), in agreement with previous research (Anehus et al. 1984; E Hölttä and Pohjanpelto 1982; Capella Roca et al. 2019).

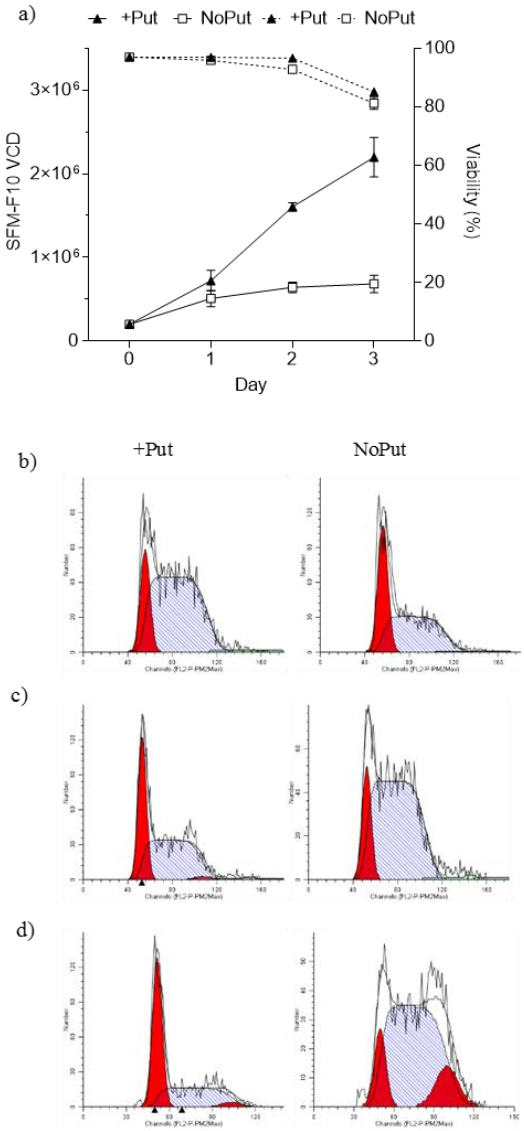

Fig. 1 (a) Viable cell density (VCD, black lines) and viability (dotted lines) of CHO-K1 cells in SFM-F10 without putrescine (NoPut) and supplemented with putrescine (+Put). (b, c, d) Distribution of cell cycle population of cultures with putrescine (+Put) and without (NoPut) at day 1 (b), day 2 (c) and day 3 (d), being populations at G1 (first peak, left) and G2 (second peak, right) represented in red and S-phase cells in stripped lines. Biological triplicates and technical duplicates were performed for each condition (cell cycle represented corresponds to the biological triplicate 3).

3.1. Gene expression altered in CHO K1 cells following polyamine-deprivation

To investigate impact of putrescine deprivation on mRNA levels in CHO cells, RNA samples taken at day 3 (when growth arrest had been maintained for 2 days) were hybridised to the Affymetrix CHOGene 2.0 ST array. A total of 2,171 mRNAs were found to be differentially-expressed (DE) with 1,194 mRNAs up- and 977 down-regulated. It should be noted that our methods do not distinguish between transcriptional activation of genes and stabilisation of existing mRNAs; in fact as discussed by Veress et al (2000) it is likely that polyamine deprivation results in selective stabilisation of certain mRNAs since eIF5A, which requires polyamines for an essential posttranslational modification, is believed to play a central and selective role in RNA degradation. Our finding that many mRNAs involved in RNA splicing are present at higher levels suggests that increased saturation of pre-mRNAs may also play a role.

Initial analysis of the data focused on changes in the expression of polyamine metabolism genes in order to validate the experiment. An induction of polyamine biosynthesis pathway-related genes (ornithine decarboxylase (*Odc1*) and antizyme inhibitor 1) and down-regulation of genes involved in polyamine catabolism (ornithine decarboxylase antizyme 2 and N1-acetyltransferase 1 (*Sat1*)) was observed as expected from the literature (Igarashi and Kashiwagi 2019). These results suggest that even though CHO-K1 cells lack endogenous ornithine production pathways (E Hölttä and Pohjanpelto 1982; Baumgartner 2000), a response to restore polyamine levels by *Odc1*overexpression is still present in polyamine-deprived CHO cells.

From a list of 20 mRNAs displaying the largest fold-changes in both directions (10 up and 10 down, Table 1 - for top 50 up- and down- see Appendix C, Supplementary Table 2) we chose 6 based on their relevance to cell proliferation and involvement with polyamines (*Thbs1*, *Ackr3*, *Tfrc*, *Pacrg*, *Tbc1d2* and *Extl1)* for RT-qPCR validation using primers listed in Supplementary Table 1 (Appendix C). All six genes were observed to display RQ changes on the same direction as the microarray data (Appendix C, Supplementary Figure 1) providing strong validation of the microarray data. The polyamine modulated factor 1 (*PMF-1*) gene, not detected in the microarray, was also included in the RT-qPCR analysis due its reported expression-response to high polyamine levels (Igarashi and Kashiwagi 2019). However, a small up-regulation of *PMF-1* was observed, although a target gene (*Sat1*) and the co-transcription factor required for its function (*Nrf-2*) were here observed to be down-regulated.

Table 1. List of the 10 most compelling up-regulated and down-regulated differentially-expressed genes considering fold-changes.

Gene symbol	Description	Fold-change	FDR p-val
Thbs1	thrombospondin 1	7.79	4.25E-05
Ackr3	atypical chemokine receptor 3	6.97	2.06E-05
Tfrc	transferrin receptor	6.62	1.07E-05
Chst2	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 of the Golgi network	4.58	0.0002
Loc103162148	uncharacterized LOC103162148	4.45	0.0002
Znf483	zinc finger protein 483	4.24	2.13E-05
Rnft2	ring finger protein, transmembrane 2 – E3 ubiquitin ligase	3.95	1.62E-05
Arhgef16	Rho guanine nucleotide exchange factor (GEF) 16 – involved in phagocytosis of apoptotic cells	3.93	1.80E-05
Pacrg	PARK2 co-regulated	3.89	8.71E-05
Slc17a6	solute carrier family 17 (vesicular glutamate transporter), member 6	3.72	0.0021
Cornifin-a	cornifin alpha	-13.85	2.37E-05
Tbc1d2	TBC1 domain family, member 2	-7.85	2.13E-05
Cd68	CD68 molecule	-7.81	2.13E-05
Trib3	tribbles pseudokinase 3 – mTORC2/AKT pathway inhibitor	-7.54	1.07E-05
Ypel3	yippee-like 3 (Drosophila) – apoptosis and cell cycle arrest inducer	-6.84	1.62E-05
Slc6a9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	-6.84	1.62E-05
Gtpbp2	GTP binding protein 2	-6.39	6.82E-06
Atf3	activating transcription factor 3 – ER stress responsive	-6.19	2.06E-05
Chac1	ChaC, cation transport regulator homolog 1 (E. coli) - ATF3/4 activated under ER stress	-5.7	1.07E-05
Extl1	exostosin-like glycosyltransferase 1	-5.63	2.06E-05

Thrombospondin 1 (*Thbs1*) was the mRNA with greatest increase in level. Increased expression of this gene has been already reported in DFMO-treated breast cancer cell lines (Manni et al. 2003; Verderame et al. 2007). Thbs1 has been described to induce cell cycle arrest and decreased growth in human umbilical vein endothelial cells (Yamauchi, Imajoh-Ohmi, and Shibuya 2007). Substantial up-regulation (6.69 fold-change) of CXCR7 (*Ackr3*) was also observed. Several polyamine-derived inhibitors (such as Quinazoline-triazole based antagonists) targeting the chemokine receptor CXCR4 have been reported (Tsou et al. 2018) while affinity for this receptor by natural polyamines has been also described (Smith et al. 2017). Singh et al. (2013) reported that crosstalk can occur between the chemokine receptors CXCR 3,4 and 7 via CXCR 11 and 12.

Due to the essential role of iron during DNA replication, increased expression of Tfrc during the S-phase of the cell cycle has been reported (Neckers and Cossman 1983). Correlation between iron

levels and expression of polyamine metabolism enzymes has been also observed (Lane et al. 2018), while uptake of iron by the polyamine transport system has been also observed (Gaboriau et al. 2004). In our microarray data, an increase in *Tfrc* expression was observed, further suggesting a possible relationship between iron and polyamines in cell cycle progression. It is also worth noting that a substantial down-regulation of *Tbc1d2* expression was also detected (-7.8 fold-change). Low expression of this gene has been related to a decrease in endocytic receptor recycling, observed with lack of Tfrc recycling, which might indicate possible increased *Tfrc* mRNA detected due to a dysregulation on the recycling pathway (Serva et al. 2012).

The Parkin co-regulated gene (Pacrg), regulated by the same promoter as Parkin, was also considered of possible significance due to the involvement of Parkin as a tumor suppressor inducing cell cycle arrest (M. H. Lee et al. 2015).

Regulation of the expression of the heparan sulphate glycosyltransferases EXT1 and EXT2 has been previously described to be mediated by polyamines (Imamura et al. 2016) and in our study, down-regulation of *Extl1* was similarly associated with polyamine starvation in CHO.

All together these data indicate that polyamines cause an impact on the expression of cell membrane proteins and receptors involved in cell proliferation, which might contribute to activation of proliferating pathways or polyamine transport.

A decrease in levels of several apoptosis-inducing mRNAs was observed with *Ypel3, Trib3, ATF3, Chac1, Casp 8 and Bax,* which correlates with the high percentage of viability maintained even after growth arrest (Fig. 1a).

From the 2,171 DE transcripts list, enriched pathway analysis using the DAVID interface was performed, revealing five statistically significant pathways (Benjamini-Hochberg FDR-corrected p-value ≤ 0.05) to be differentially-regulated following putrescine deprivation (Fig. 2). The most significant areas which emerged were cell proliferation, Spliceosome, p53-related pathways, Fanconi's Anaemia/DNA repair and Protein Processing in Endoplasmic Reticulum (ER).

In relation to the cell cycle, the expression of genes related to S-phase and mitosis included increased mRNA levels *Skp2* and *Tfdp1* (participating in the transition to S-phase) and *Mad2l1* (part of the mitotic complex checkpoint). Supporting these findings, analysis of the cell cycle of the cultures in medium without putrescine displayed a continuous decrease on G1 population with a subsequent increase in S-phase populations over the three days culture. At the same time an accumulation of cells in the G2-phase was also observed by day 3 (Fig. 1 b,c,d). These results suggest arrest caused by polyamine-deprivation at possibly 3 points; the G1/S transition, mitosis and possibly also at the G2/M transition point. Similar to our findings, CHO cultures deprived of polyamines have been reported to display prolonged S-phase (Jan O. Fredlund and Oredsson 1997b; J. O. Fredlund and Oredsson 1996; Anehus et al. 1984). However, cell cycle effects on other mammalian cell lines have been reported to arrest at different stages depending on the cell line, such as G0/G1 (NIH3T3 mouse fibroblasts)

(Landau et al. 2012), G1/S (Hela and MALME-3M) (Yamashita et al. 2013; Kramer et al. 1999) or G2/M (MALME-3M) (Kramer et al. 1999). Polyamine deprivation has been reported to induce structural changes on microtubules, resulting in delayed cytokinesis in Hela (Yamashita et al. 2013) and CHO (Pohjanpelto, Virtanen, and Hölttä 1981). A peak on ODC activity is also detected at late stage of the cell cycle (Oredsson 2003), suggesting an involvement of polyamines for proper mitosis progression.

In response to polyamine-deprivation, we observed lower levels of some mRNAs involved in ER stress, with the significant lower expression of *Ddit3* apoptosis-inducer gene being validated by RT-qPCR (Fig. 2, Appendix C, Supplementary Figure 1). Increased polyamine catabolism produces toxic by-products that can trigger ER stress and eventual apoptosis, as reported by the overexpression of Sat1 and SMOX in acute kidney injuries (Zahedi et al. 2017). Similarly, CCl_4 treatment of liver increased Sat1 expression, inducing tissue damage (Zahedi et al. 2012). Consequently, decreased expression of genes related to ER-stress observed in our study might be associated with down-regulation of *Sat1*. DFMO treated epidermal IEC-6 cells and NIH3T3 displayed lack of apoptosis pathway activity (Li et al. 1999) (effect also observed in our system), although increased ER-stress was observed in the NIH3T3 cultures (Landau et al. 2012).

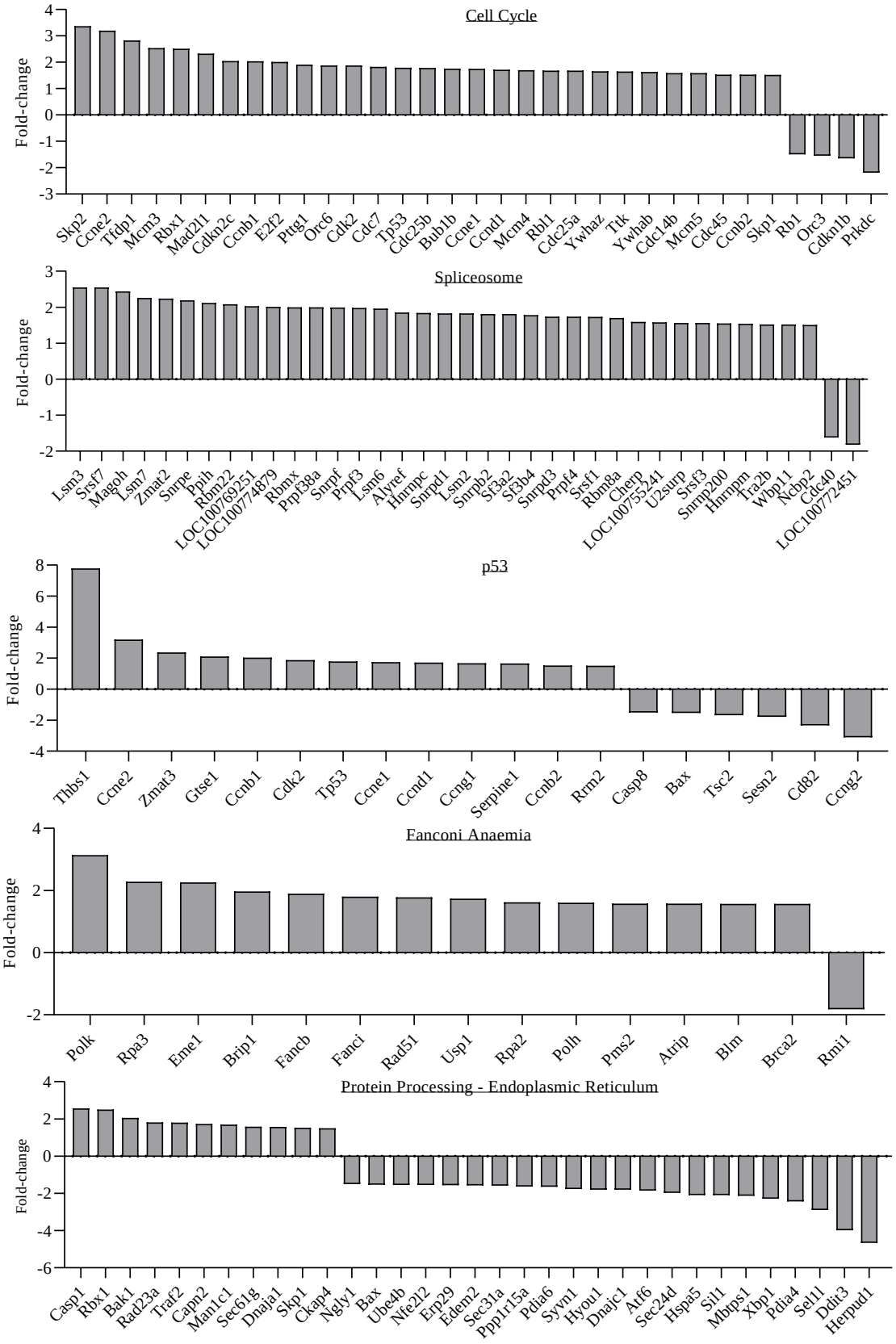

Fig. 2 Fold-changes of DE genes from enriched pathways DAVID interface analysis.

4. Conclusions

The research described here is the first step in exploiting the CHO cell requirement for exogenous polyamine to investigate cellular roles of polyamines by nutritional depletion rather than by using chemical inhibitors.

Insights have been gained into alterations in mRNA levels for specific genes and pathways, notably relating to cell cycle, RNA splicing, DNA homologous recombination and repair, p53-related pathways, membrane receptors and protein processing in ER. We realise that much remains to be discovered using this inhibitor-free system unique to CHO cells by examining, for example, changes at the proteomic and functional levels. In this paper we have demonstrated the power of the serum-free CHO system to generate new knowledge on polyamine biology, still poorly understood, and we hope that the data presented supposes a first step for further investigation in the area.

5. References

Anehus, S. *et al.* (1984) Polyamine starvation prolongs the S and G2 phases of polyamine-dependent (arginase-deficient) CHO cells. Mol Cell Biol. 4(5), 915–22. doi: 10.1128/mcb.4.5.915

Baumgartner, M. R. et al. (2000) Hyperammonemia with reduced ornithine, citrulline, arginine and proline: a new inborn error caused by a mutation in the gene encoding Delta1-pyrroline-5-carboxylate synthase. Hum Mol Genet. 9(19), 2853–2858. doi: 10.1093/hmg/9.19.2853

Berntsson PS, Alm K and Oredsson SM.(1999) Half-lives of ornithine decarboxylase and S-adenosylmethionine decarboxylase activities during the cell cycle of Chinese hamster ovary cells. Biochem Biophys Res Commun. 263(1):13-6. doi:10.1006/bbrc.1999.1293

Byers, T. L., Wechter, R. S., Hu, R. H. and Pegg, A. E. (1994) Effects of the S-adenosylmethionine decarboxylase inhibitor, 5'-{[(Z)-4-amino-2butenyl]methylamino}-5'-deoxyadenosine, on cell growth and polyamine metabolism and transport in Chinese hamster ovary cell cultures. Biochem J. 303 (Pt 1), 89-96.

Capella Roca, B. *et al.* (2019) An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals. J Biol Chem. doi: 10.1074/jbc.RA119.011162

Casero, R. A., Murray Stewart, T. and Pegg, A. E. (2018) Polyamine metabolism and cancer: treatments, challenges and opportunities. Nat Rev Cancer. 18 (11) 681–695. doi: 10.1038/s41568-018-0050-3

Dever, T. and Ivanov, I. P. (2018) Roles of polyamines in translation. J Biol Chem 293, 18719-18729 doi: 10.1074/jbc.TM118.003338

Fredlund, J. O. and Oredsson, S. M. (1996) Normal G_1/S transition and prolonged S phase within one cell cycle after seeding cells in the presence of an ornithine decarboxylase inhibitor. Cell Prolif. 29(8), 457–465. doi: 10.1111/j.1365-2184.1996.tb00988.x

Fredlund, J. O. and Oredsson, S. M. (1997) Ordered Cell Cycle Phase Perturbations in Chinese Hamster Ovary Cells Treated with an S-Adenosylmethionine Decarboxylase Inhibitor. Eur J Biochem. 249(1), 232–238. doi: 10.1111/j.1432-1033.1997.00232.x

Gaboriau, F. *et al.* (2004) Polyamine modulation of iron uptake in CHO cells. Biochem Pharmacol. 67(9), 1629–1637. doi: 10.1016/j.bcp.2003.12.033

Hölttä, E. and Pohjanpelto, P. (1982) Polyamine dependence of Chinese hamster ovary cells in serum-free culture is due to deficient arginase activity. Biochim Biophys Acta. 721(4), 321–7. doi: 10.1016/0167-4889(82)90085-4

Hyvönen T (1989) Excretion of acetylated and free polyamines by polyamine depleted Chinese hamster ovary cells. Int J Biochem. 21(3):313-6. doi: 10.1016/0020-711x(89)90189-4

Igarashi, K. and Kashiwagi, K. (2019) The functional role of polyamines in eukaryotic cells. Int J Biochem Cell Biol. Elsevier Ltd, 107, 104–115. doi: 10.1016/j.biocel.2018.12.012

Imamura, M. *et al.* (2016) Polyamines release the let-7b-mediated suppression of initiation codon recognition during the protein synthesis of EXT2. Sci Rep. 6:33549. doi: 10.1038/srep33549

Kaushik, P, Henry, M, Clynes, M and Meleady, P (2018) The Expression Pattern of the Phosphoproteome Is Significantly Changed During the Growth Phases of Recombinant CHO Cell Culture. Biotechnol J.13(10):e1700221. doi: 10.1002/biot.201700221

Kramer, D. L. et al. (1999) Polyamine Analogue Induction of the p53-p21 WAF1/CIP1-Rb Pathway and G 1 Arrest in Human Melanoma Cells. Cancer Res. 59(6):1278-86.

Landau, G. *et al.* (2012) Expression profiling and biochemical analysis suggest stress response as a potential mechanism inhibiting proliferation of polyamine-depleted cells. J Biol Chem. 287(43), 35825–35837. doi: 10.1074/jbc.M112.381335

Lane, D. J. R. et al. (2018) Coupling of the polyamine and iron metabolism pathways in the regulation of proliferation: Mechanistic links to alterations in key polyamine biosynthetic and catabolic enzymes. Biochim Biophys Acta Mol Basis Dis.1864(9), 2793–2813. doi: 10.1016/j.bbadis.2018.05.007

Lee, M. H. et al. (2015) Parkin induces G2/M cell cycle arrest in TNF-α-treated HeLa cells. Biochem Biophys Res Commun. 464(1), 63–69. doi: 10.1016/j.bbrc.2015.05.101

Li, L. *et al.* (1999) Inhibition of polyamine synthesis induces p53 gene expression but not apoptosis. Am J Physiol. 276(4 45-4). doi: 10.1152/ajpcell.1999.276.4.c946

Liu R *et al.* (2001) Regulation of CSF1 promoter by the SWI/SNF-like BAF complex. Cell. 06(3):309-18. doi: 10.1016/s0092-8674(01)00446-9

Liu L et al. (2009) Polyamines regulate c-Myc translation through Chk2-dependent HuR phosphorylation. Mol Biol Cell. 20(23):4885-98. doi: 10.1091/mbc.E09-07-0550

Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25(4), 402–408. doi: 10.1006/meth.2001.1262

Mamont, P. S. et al. (1978) Anti-proliferative properties of DL-α-difluoromethyl ornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase', Biochem Biophys Res Commun. 81(1), 58–66. doi: 10.1016/0006-291X(78)91630-3

Manni, A. *et al.* (2003) Effects of α-difluoromethylornithine on local recurrence and pulmonary metastasis from MDA-MB-435 breast cancer xenografts in nude mice. Clin Exp Metastasis. 20(4):321-5. doi: 10.1023/a:1024055522067

Neckers, L. M. and Cossman, J. (1983) Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc Natl Acad Sci U S A. 80(11), 3494–3498. doi: 10.1073/pnas.80.11.3494

Oredsson, S. M. (2003) 'Polyamine dependence of normal cell-cycle progression. Biochem Soc Trans. 31(2):366-70. doi: 10.1042/BST0310366

Pastorian KE, Hawel L and Byus CV (2000) Identification of putrescine-responsive mRNAs in Chinese hamster ovary cells using representational difference analysis. Biochim Biophys Acta. 2000 Dec 15;1524(2-3):131-42

Pohjanpelto, P., Virtanen, I. and Hölttä, E. (1981) Polyamine starvation causes disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO cells. Nature. 293(5832), 475–477. doi: 10.1038/293475a0

Pohjanpelto, P., Hölttä, E. and Jänne, O. A. (1985) Mutant strain of Chinese hamster ovary cells with no detectable ornithine decarboxylase activity. Mol Cell Biol. 5(6), 1385-90.

Serva, A. *et al.* (2012) miR-17-5p Regulates Endocytic Trafficking through Targeting TBC1D2/Armus. *PLoS ONE*. Edited by K. Ghoshal, 7(12), e52555. doi: 10.1371/journal.pone.0052555

Smith, N. et al. (2017) Natural amines inhibit activation of human plasmacytoid dendritic cells through CXCR4 engagement. Nat Commun. 8:14253 doi: 10.1038/ncomms14253

Steglich, C. and Scheffler, I. E. (1982) An ornithine decarboxylase-deficient mutant of Chinese hamster ovary cells. J Biol Chem. 257(8), 4603-9.

Thomas T, Gallo MA, Klinge CM, Thomas TJ. (1995) Polyamine-mediated conformational perturbations in DNA alter the binding of estrogen receptor to poly(dG-m5dC).poly(dG-m5dC) and a plasmid containing the estrogen response element. J Steroid Biochem Mol Biol.54(3-4):89-99. doi: 10.1016/0960-0760(95)00126-k

Tsou, L. K. et al. (2018) Harnessing CXCR4 antagonists in stem cell mobilization, HIV infection, ischemic diseases, and oncology. Med Res Rev. 38(4), 1188–1234. doi: 10.1002/med.21464

Uemura T et al. (2008) Identification and characterization of a diamine exporter in colon epithelial cells. J Biol Chem 283(39):26428-35. doi: 10.1074/jbc.M804714200.

Verderame, M. et al. (2007) Effects of α-difluoromethylornithine on thrombospondin-1 production by human breast cancer cells. Int J Oncol. 31(5):1187-91. doi: 10.3892/ijo.31.5.1187

Yamashita, T. et al. (2013) Role of polyamines at the G1/S boundary and G2/M phase of the cell cycle. Int J Biochem Cell Biol. 45(6), 1042–1050. doi: 10.1016/j.biocel.2013.02.021

Yamauchi, M., Imajoh-Ohmi, S. and Shibuya, M. (2007) Novel antiangiogenic pathway of thrombospondin-1 mediated by suppression of the cell cycle. Cancer Sci. 98(9), 1491–1497. doi: 10.1111/j.1349-7006.2007.00534.x.

Zahedi, K. *et al.* (2012) Hepatocyte-specific ablation of spermine/spermidine-N1-acetyltransferase gene reduces the severity of CCl_4-induced acute liver injury. Am J Physiol Gastrointest Liver Physiol. 2012 Sep 1;303(5):G546-60 doi: 10.1152/ajpgi.00431.2011

Zahedi, K. *et al.* (2017) Activation of endoplasmic reticulum stress response by enhanced polyamine catabolism is important in the mediation of cisplatin-induced acute kidney injury. PLoS One. 12(9):e0184570 doi: 10.1371/journal.pone.0184570

Zou T *et al.* (2006) Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. J Biol Chem. 281(28):19387-94. doi: 10.1074/jbc.M602344200

CHAPTER 4

Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO cells

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Article accepted in Biotechnology Progress (doi: 10.1002/btpr.2954)

Preface to the paper – A case study on the relevance of media formulation for transfections

From the previous chapters, media composition has been determined to be a relevant factor to achieve enhanced culture performance of CHO cells (growth or productivity). However, alterations in media formulation have also a major impact on other bioprocess-relevant parameters, such as transfection efficiency. In this last chapter, to further showcase the importance of media additives, the impact of SFM-8 (described in *Preface to the Chapters – in-house serum-free media development)* in the transfection of CHO cells was assessed. In this case study, three different chemically-based transfection reagents (cationic lipid, cationic polymers and lipopolyplexes) were included to consider possible differences between each mechanism and how they may be differently affected by the same media additive. Moreover, the effects were confirmed in three CHO cell lines, accounting for possible cell line-specific results. Transfection efficiency was monitored using a GFP-expressing vector. The levels of percentage of GFP-expressing population as well as the intensity of fluorescence were assessed to determine the efficacy of transfection. Initial tests revealed a lack of GFP detection in all cell lines tested with two of the transfection reagents and very low efficiencies with lipopolyplexes. In order to identify the impeding additive, a step-wise removal protocol was followed revealing ferric ammonium citrate (FAC) as an inhibitory supplement for chemical transfection. With the removal of FAC, successful transfections were achieved, although viabilities were negatively impacted due to the vital role this additive plays in CHO cell culture. To circumvent this effect, an alternative transfection protocol was developed, combining transfection in FAC-depleted medium with replenishment prior 24h post-transfection.

Inhibitory FAC effects were observed during the initial steps of transfection, as supplementation of this iron source 30 min post-transfection did not impede GFP expression. For this reason, we decided to characterise the DNA-delivery particles in order to gain insight into the mechanistic inhibitory effect of FAC by assessing their zeta potential and particle size. These parameters have become standard in the study of transfection efficiencies, being sensitive to changes in the pH, viscosity, temperature or ionic strength of the media (Mandal *et al.* 2018; Yu *et al.* 2019; Smith *et al.* 2017). Consequently, changes in the particle size of each of the three DNA-reagent complexes were monitored over 1 h incubation in media with and without FAC. Effects in three commercial media were also assessed. The results displayed increases in the particles size over the 1h incubation, indicating a potential FAC-mediated aggregation or destabilization. However, this effect was not observed with polymers transfection complexes.

Zeta potential gives an indication of the surface charge of the particle under specific conditions (pH, temperature, viscosity) (Fig. 10) (Smith *et al.* 20017; Bhattacharjee 2016). Chemical-based transfection rely, in part, to the electrostatic interaction between cellular membrane and transfection-complex (Kim and Eberwine 2010). For this reason, "negatively charged" complexes may result in lack of transfection. Positive zeta potentials were here detected in the absence of FAC in two of the

complexes, while liposomes displayed negative zeta potential values. The differences observed in zeta potential and particle size between the three transfection reagents indicated that investigation of the different mechanistic DNA uptake of each the three transfection reagents requires investigation to further understand the negative effects of FAC in transfection.

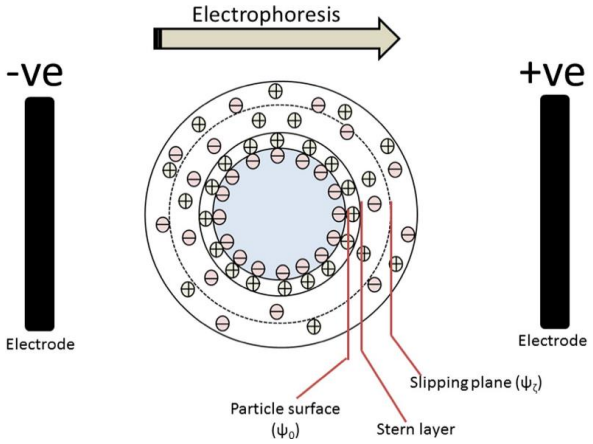


Fig. 10 Determination of the zeta potential on a negatively charged particle. Immediately on top of the particle surface there is a strongly adhered layer (Stern layer) comprising of ions of opposite charge i.e. positive ions in this case. Beyond the Stern layer, a diffuse layer develops consisting of both negative and positive charges. These two layers of tightly and loosely associated ions are collectively referred to as the electrical double layer (EDL). During electrophoresis the particle with adsorbed EDL moves towards the electrodes (positive electrode in this case) with the slipping plane becoming the interface between the mobile particles and dispersant. The zeta potential is the electrokinetic potential at this slipping plane. Image and legend (adapted) from (Bhattacharjee 2016).

Declaration of Authorship

Candidates are required to submit a separate **Declaration of Authorship** form for each co-authored paper submitted for examination as part of a PhD by Publication thesis. Further information is available from the accompanying guideline document.[4]

6. Section 1: Candidate's details	
Candidate's Name	Berta Capella Roca
DCU Student Number	15212896
School	National Institute for Cellular Biotechnology (NICB), School of Biotechnology
Principal Supervisor	Padraig Doolan
Title of PhD by Publication Thesis	Investigating Media Supplements to Enhance Chinese Hamster Ovary Cells Culture
7. Section 2: Paper details	
Title of co-authored paper included in the thesis under examination	Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO cells
Publication Status	Accepted
ISSN and link to URL (where available)	
This paper is one of [4] co-authored papers to be submitted as part of the PhD by publication thesis submitted for examination	
8. Section 3: Candidate's contribution to the paper	
Provide details below of the **nature** and **extent of your contribution** to the paper (include both your intellectual and practical contributions) and your overall contribution in **percentage terms**: I was the lead author and key contributor of the paper, involved in data curation; investigation; methodology; writing-original draft. Overall 60%	
Where a paper has joint or multiple authors, list the names of all other authors who contributed to the work (this can be appended in a separate document, where necessary)**:** Lao, N., Clynes, M., Doolan, P.	

[4] 'Guidelines for candidates, supervisors and examiners on the 'PhD by Publication' format': https://www.dcu.ie/graduatestudies/A_Z-of-GSO-Policies.shtml

Section 4: Signature and Validation

I confirm that the following statements are true:

(a) the information I have provided in this form is correct

(b) this paper is based on research undertaken during my candidature at DCU

Signature of PhD Candidate: _____ Date: 25-11-19

I confirm that the information provided by the candidate is correct:

Signature of Principal Supervisor: Padraig Doolan Date: 25-11-19

In some cases, it may be appropriate for verification to be given by both the principal supervisor **and** the lead/corresponding author of the work (where the lead/corresponding author of the work is not the candidate or the principal supervisor):

Signature of Lead/Corresponding Author _____ **Date** _____

Nature of Current Post/Responsibilities _____

Home institution _____

ABSTRACT

While reliable transfection methods are essential for Chinese Hamster Ovary (CHO) cell line engineering, reduced transfection efficiencies have been observed in several commercially-prepared media. In this study, we aimed to assess common media additives that impede efficiency mediated by three chemical transfection agents: liposomal-based (Lipofectamine 2000), polymer-based (TransIT-X2), and lipopolyplex-based (TransIT-PRO). An in-house GFP-expressing vector and serum-free medium (BCR-F12: developed for the purposes of this study), were used to analyse transient transfection efficiencies of three CHO cell lines (CHO-K1, DG44, DP12). Compared to a selection of commercially-available media, BCR-F12 displayed challenges associated with transfection in vendor-prepared formulations, with no detection when liposomal-based methods were used, reduced (<3 %) efficiency observed when polymer-based methods were used and only limited efficiency (25 %) with lipopolyplexes. Following a stepwise removal protocol, ferric ammonium citrate (FAC) was identified as the critical factor impeding transfection, with transfection enabled with the liposomal- and polymer-based methods and a 1.3- to 7-fold increased lipopolyplex efficiency observed in all cell lines in FAC-depleted medium (-FAC), although lower viabilities were observed. Subsequent early addition of FAC (0.5-5 h post-transfection) revealed 0.5 h post-transfection as the optimal time to supplement in order to achieve transfection efficiencies similar to -FAC medium while retaining optimal cellular viabilities. In conclusion, FAC was observed to interfere with DNA transfection acting at early stages in all transfection agents and all cell lines studied and a practical strategy to circumvent this problem is suggested.

1. Introduction

Transfection is a core technique in molecular biology studies which is used to investigate the role of proteins and genes in cellular systems. In the field of biopharmaceutical manufacturing, transfection efficiency is a critical early step in upstream cell line development to produce complex proteins, typically in Chinese Hamster Ovary cells (CHO), as efficient transient gene expression offers the possibility of producing large amounts of recombinant protein rapidly. For this reason, optimization of transfection efficiency, accompanied with minimal cellular perturbation is a desirable goal of industry, examining the critical parameters affecting this process: cell type, cell line, expressing vector, transfection method and cultivation media, among others (*1*).

DNA-delivery systems can be broadly classified as biological (virus-mediated), chemical (vector-assisted) and physical (*2, 3*). Chemical transfection methods work on the principle of electrostatic interaction; forming positively charged complexes with nucleic acid, enabling interaction with the cellular membrane and finally releasing the genetic material into the cells (*2*). Chemical methods can be sub-classified into 3 types: (i) lipid-based (lipoplexes; cationic lipid-based), (ii) polymer-based (polyplexes) and (iii) lipid mixed with polymer or peptides (lipopolyplexes). Currently, a wide range of transfection reagents are commercially available (e. g. Lipofectamine 2000, FuGENE 6, PEI, TransIT-X2, TransIT-PRO, TransIT-LT1). Compared to more cost-effective reagents such as calcium phosphate or DEAE-Dextran, commercial agents have been advantageously applied to highly differentiated cell lines (such as CHO) and serum-free conditions achieving high transfection efficiencies (*4, 5*). Among polymer cationic methods, polyethylenimine (PEI) is the most common transfection system used for CHO and HEK293 transient transfection, offering a cost-efficient, easy-to use and efficient methodology (*6*). As a result, multiple studies have focused on understanding and improving this transfection system (*7, 8*), resulting in the development of several PEI-derivate agents (*9, 10*). However, other polymer-based methods reported for successful CHO cells transfection (e.g. TransIT-X2 (*11*)) have received less investigation. Cationic lipids are comprised of hydrocarbon chains, neutral helpers and a cationic head group (*12*). Structural changes such as the type of lipid and composition of the complex plays a crucial role on final DNA delivery. Thus, several lipids have been tested, mainly to improve gene delivery, such as palmstearin, which have been reported to be successful in CHO cells, increasing efficiency of a commercial control lipofectamine RNAiMAX (*13*). Improvements in cell transfection have also been observed with the novel use of tomatidine, recently described by Rangasami et al (2019), which was reported to induce cell permeability when used as a helper lipid (*14*). Liposomes have frequently been shown to efficiently generate transiently-expressing (*15*) and stably-expressing (*16, 17)* CHO cell lines. However, negative effects on cellular viabilities (*18*) have been described using this method, while poor endosomal escape and subsequent increased lysosomal degradation have also been reported (*19*). To overcome this hurdle, a second-generation of DNA-delivery systems consisting of ternary structures of cationic lipids, polycations

(polymers or peptides) and nucleic acids have been developed, characterized by high transfection efficiencies due to the highly condensed DNA complexes together with the generated smaller particle sizes (*20*). Described by Caracciolo, G. et al (2011), lipopolyplexes composed by DNA/protamine coated by DOTAP have been observed to outperform the lipid-based DOTAP transfection efficiencies in mammalian cells, including CHO-K1 (*21*). Similarly, TransIT-PRO has been reported to display increased transfection efficiency, DNA expression and lower toxicity than PEI and Liposome 2000 in CHO-S cells (*22).*

One of the key challenges that impact the efficiency of DNA delivery into mammalian cells is the composition of the culture media. Previous studies examining the efficiency of several transfection methods in combination with commercially-prepared media formulations have reported substantially decreased or even fully impeded DNA transfection in HEK293 and CHO cell lines, the two most prominent cell lines used in industry for mammalian cell production of biopharmaceuticals *(23, 24).* As the media components in these formulations are frequently not disclosed by the manufacturer due to commercial sensitivities, identification of medium components that may interfere with DNA-delivery remains a challenge to optimization of transfection efficiency in mammalian culture. While some media additives have been identified as having a detrimental impact on transfection efficiency (*1, 25, 26, 27*), further investigation is still required to enhance the efficacy of the process. In order to assess the ability of media additives to support or impede transfection efficiency, we developed a serum-free (SFM) medium (BCR-F12) containing common media supplements and used it to test three different chemical-based transfection methods: (i) Lipofectamine 2000 (liposomes/lipoplexes/cationic lipid-based), (ii) TransIT-X2 (polyplexes/polymer-based) and (iii) TransIT-PRO (lipopolyplex system) in three CHO cell lines (DG44, DP12, CHO-K1), measuring the percentage of GFP expressing cells from an in-house vector. Following the identification of a single media component as a transfection inhibitor, cell culture performance (viability and growth) was then assessed in the absence and presence of the interfering additive, in order to develop an efficient transfection protocol that simultaneously maintained minimal cellular perturbance. Finally, in order to gain insight in the mechanistic inhibitory effects observed, complexes generated with all three transfection reagents in each media tested were assessed for size and zeta potential changes.

2. Materials and methods

2.1. Cell culture

Three CHO cell lines, CHO-DP12 (a recombinant human IgG-producer, ATCC CRL-12445 clone#1934), an in-house DG44 derived cell line and a CHO-K1 parental (ATTC CCL-61) fully adapted to grow in suspension in serum-free media were cultured and maintained in three commercial media: a chemically-defined and animal-free medium (BalanCD CHO Growth A medium (Irvine

Scientific), a serum-free medium (CHO-S-SFM-II (Thermo Fisher Scientific), and a protein-free medium (ProCHO-5 (Lonza)). Cells were also cultured in an in-house serum-free medium (BCR-F12). The BalanCD Growth A medium lacks hypoxanthine, thymidine, antibiotics and antimycotic and it was further supplemented with 8 mM L-glutamine (Gibco). The CHO-S SFM-II is a low-protein formulation containing hypoxanthine, thymidine, L-glutamine, sodium pyruvate and sodium bicarbonate. The ProCHO5 medium contains 0.1 % Pluronic F-68 but lacks thymidine, hypoxanthine and phenol red. This medium was further supplemented with 5 mM L-glutamine (Gibco). The in-house BCR-F12 medium was developed based on DMEM-F12 (1:1, v/v) (Sigma Aldrich, D8437) supplemented with sodium selenite, recombinant human insulin, ethanolamine, ferric ammonium citrate, poly vinyl alcohol (PVA), L-glutamine (Gibco), NEAA (Gibco), linoleic acid and putrescine dihydrochloride (*28*). The only growth factor supplement in the BCR-F12 serum-free medium is insulin, which has been extensively shown to be sufficient to support CHO cell growth in culture (*29*). All supplements were purchased from Sigma Aldrich unless otherwise stated. Cells were grown in each media for at least 3 passages, ensuring that similar VCDs were reached at each passage before performing transfection tests. Cells were routinely split every 3-4 days and re-seeded at $2x10^5$ cells/ml in 250 ml shake flasks in 20-30 ml working volume. Cultures were maintained at 37 ^oC in suspension culture in an ISF1-X (Climo Shaker) Kuhner incubator with a speed of 170 rpm, 80 % Humidity and 5 % CO_2. Viable cell density (VCD) and viability were analysed using the ViaCount reagent (Guava Technologies, 4000-0041) on a Guava easyCyte 75 HT benchtop cytometer (Merck Millipore, UK). This reagent is a mixture of two DNA-binding dyes (propidium iodide and LDS-751) which distinguishes between non-viable and live cells. LDS-751 is membrane permeable dye (30) that stains all nucleated cells, while propidium iodide stains non-viable cells due to the lack of integrity of their membranes (31). The detection of viable cells is recorded when the fluorescence signal is accompanied by the signal of forward light scatter (FSC) in the appropriate intensity (in the photomultiplier tube 2, PM-2), which is related to cell size. If a lower FSC intensity is detected, the signal is recorded as cell debris. For this study, in order to exclude GFP detection from the VCD and viability fluorescence signal, the ExpressPro software was used and cell gating was performed using healthy cultures following the easyCyte™ System User Guide (0110-8493 Rev B). Statistical analysis of the VCD and viability data obtained was performed in Microsoft excel software using two-tailed homoscedastic student t-test to generate p-values. This is the form of Student's t-test which is appropriate to use when comparing sets of data which have similar variances - for the type of data presented here, it is reasonable to assume a normal distribution.

$$t = \frac{x1 - x2}{\frac{s1}{\sqrt{n1}} + \frac{s2}{\sqrt{n2}}}$$

Described in (***32***).

2.2. Transfections

An in-house GFP expressing vector (named as N44), based on CMV-d2eGFP pcDNA5 vector (Addgene) (modified with a functional hygromycin selection and modifications in the cloning site) was used for the purposes of this study due to the expression of destabilized GFP (d2GFP). Despite its lowered stability profile, the d2GFP vector was utilised in order to monitor minor effects on transfection efficiency between cell lines, transfection reagents and media – these minor effects would have been missed if the more stable eGFP vector and protein was used. Vector DNA was extracted from 5 ml Component Escherichia coli DH5α (Invitrogen) transformed cultures using the GeneJET Plasmid MiniprepKit (Thermo Fisher Scientific) as per the manufacturer's instructions. Vector extractions were assessed for quality and quantity using the NanoDrop ND-100 (NanoDrop Technologies). Three transfection systems were used in this study: (i) a lipoplexes/cationic lipid-based transfection method (Lipofectamine 2000 (Thermo Fisher Scientific)), (ii) a polymer-based/polyplexes system (TransIT-X2 (Mirus Bio), not Polyethyleneimine (PEI)-based) and iii) a lipopolyplex-mediated system (TransIT-PRO (Mirus Bio)). Transfections were performed following manufacturer's recommendations for CHO cells with no adaptation of the protocol per each cell line. Briefly, cells were counted and re-seeded at $1x10^6$ cells/ml in fresh media 24 h prior transfection. For TransIT-PRO, a total of 500 ng vector was mixed with 1 µL reagent and 100 µl media and incubated for 10 min at room temperature. For Lipofectamine 2000, 800 ng vector was diluted in 50 µl media. In parallel, 1 µL Lipofectamine 2000 was mixed with 50 µl media. Both mixtures were incubated for 5 min and then gently mixed and incubated for an additional 20 min at room temperature. For TransIT-X2, 500 ng vector was mixed with 50 µl media and 1 µl TransIT-X2 was mixed in 50 µl media. Both were then combined, gently mixed and incubated for 30 min at room temperature. All complexes were generated in basal DMEM-F12 medium (Sigma Aldrich, D8437). Cells were re-suspended in fresh media without PVA at $2x10^6$ cells/ml (TransIT-PRO) and $1x10^6$ cells/ml (TransIT-X2) and 900 µl were seeded per well. For Lipofectamine 2000, cells were also re-suspended in media without PVA at $1.6x10^6$ cells/ml and 500 µl were seeded per well. A total of 100 µl of vector-complex suspension was then added to each well per each transfection reagent. Plates were parafilmed and incubated at 37 ^oC in an ISF1-X (Climo Shaker) Kuhner incubator. Media change was not performed. For TransIT-PRO, the PRO boost reagent was not used. A negative control for transfections was also prepared following the same protocols as per TransIT-X2, TransIT-PRO and Lipofectamine 2000. However, DNA was not added to prepare the complexes. Each transfection condition tested was performed in triplicate wells. For the negative control, one to two wells were used for each testing condition. Viable cell density (VCD) and viability were assessed 24 h post-transfection using the ViaCount on a Guava easyCyte HT benchtop cytometer (Merck Millipore, UK). Technical replicates were performed for each transfected and non-transfected well.

2.3. Detection of GFP expression

The detection of GFP-expressing cells was performed 24 h post-transfection by flow cytometry using the Express Plus software for the Guava easyCyte benchtop cytometer (Merck Millipore, UK). To determine the amount of fluorescent cells, negative control cells (cells that did not express GFP fluorescence above autofluorescence levels) were gated by modifying the voltage in the applicable channel to remove autofluorescence signal - these settings were then used to identify the GFP-positive populations. Fluorescence of dead cells and debris were excluded to avoid false positive results. Technical replicates were performed for each transfected and non-transfected well. Statistical analysis of the transfection data obtained was performed in Microsoft excel software using two-tailed homoscedastic student *t*-test to generate p-values (as per VCD and viability data).

2.4. Measurement of size and zeta potential

A Malvern Zetasizer Ultra, using ZS XPLORER version 1.2, (Malvern Panalytical Ltd, UK) was used for measuring the size of the complexes using Dynamic light scattering (DLS) at 37 °C. Triplicate measurements were performed.

Samples for zeta potential measurements were placed in a ZEN1002 dip cell cuvette and measurements were obtained using Phase Analysis Light Scattering at 25 °C, using the Smoluchowski model. Zeta potential values were obtained using monomodal mode due to the dispersant conductivity. Between two to five replicates were performed per sample.

3. Results

3.1. BCR-F12 transfection efficiencies perform similarly to commercial media preparations

A serum-free formulation (BCR-F12) was developed and compared to a range of three commercially available media (BalanCD Growth A, CHO-S-SFM-II and ProCHO5) to assess whether its transfection efficiency profile was representative of the issues observed in commercial formulations. Figure 1 displays the efficiencies observed in three CHO cell lines (DG44, DP12 and CHO-K1), measured as percentage of GFP-expressing populations (excluding autofluorescence signal based on non-transfected control cells), at 24 hours post-transfection using a range of three transfection agents; Liposome 2000 (Liposomes), TransIT-X2 (Polymers) and TransIT-PRO (Lipopolyplexes). The N44 vector expressing the destabilized GFP (d2GFP) was used to assist in monitoring minor changes between media and transfection agents.

In BalanCD, transfection was not supported with any of the transfection agents or cell lines used, while in contrast, 44-60% GFP-expressing populations were detected in SFM-II with all three DNA-delivery systems and in all cell lines (Fig 1). In ProCHO5, all 3 cell lines displayed transfection

efficiencies between 31 % - 65 % using polymers or lipopolyplexes agents; however, GFP expression was not observed in any of the 3 cell lines when liposomes were used. The GFP expression levels observed following transfection in the candidate BCR-F12 medium indicated that it is a suitable model for studying medium-related transfection disruption; only the lipopolyplex method returned GFP-expressing populations (25 %) in all 3 cell lines, while polymer-based transfections resulted in < 3 % efficiency and GFP expression was not detected with liposomes.

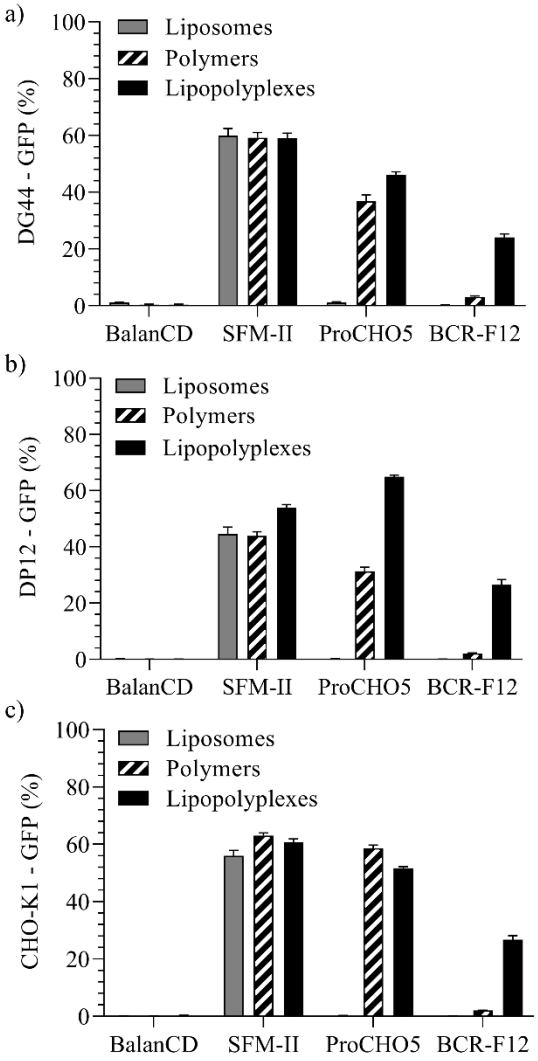


Fig. 1 Transfection efficiency in commercial and in-house media. Percentage of GFP-expressing DG44 (a), DP12 (b) and CHO-K1 (c) cells 24 h post-transfection with d2GFP in three commercial media: BalanCD growth A (CDM), CHO-S SFM-II (SFM) and ProCHO5 (PFM) and an in-house SFM (BCR-F12). Three transfection systems were tested: Liposomes (Lipofectamine 2000), Polymer-based (TransIT-X2) and Lipopolyplexes (TransIT-PRO). Triplicate transfections were carried out for each media, cell line and transfection method combination. Two negative control wells were included for each media, cell line and transfection method, displaying between 0.0 - 1.7 % GFP (data not shown).

3.2. SIFA additives interfere with BCR-F12 transfection with all transfection systems studied

As our in-house BCR-F12 medium formulation was observed to display transfection issues representative of those seen in commercial media formulations (Fig. 1), identification of the BCR-F12 medium additives that may contribute to the decreased transfection efficiencies was attempted. To achieve this aim, all the additives supplemented to DMEM-F12 basal medium to develop BCR-F12 were removed in two groups. The first group comprised lipids and polyamines - linoleic acid, ethanolamine and putrescine which were suspected to inhibit transfection or interfere with DNA-complex formation (*25, 26, 33, 34*) – this is the "LP" formulation. The second, "SIFA" formulation comprised the rest of the additives - sodium selenite, insulin, ferric ammonium citrate and non-essential amino acids supplements. Basal medium (DMEM-F12) used for the development of BCR-F12 formulation was also included as a control in order to assess maximal transfection efficiency achievable without supplemental additives, while DG44 cells were used as an initial model cell line, as transfection efficiencies for the different methods and in the different media were observed to be representative for all three cell lines (except for ProCHO5 medium) (Fig 1). Compared to BCR-F12 medium, transfection efficiencies in Basal media increased from 1 % to 16 % in liposomes and increased 6.3-fold (5 % to 31 %) when using polymers (Fig 2). No significant differences were observed between transfections performed in LP and BCR-F12 media with any of the 3 methods, indicating that these formulations are practically identical with regard to transfection performance. However, in SIFA medium, efficiencies were observed to be restored to similar levels observed in Basal medium (31 - 33 % GFP-expressing cells in polymer-based transfection and 15 - 16 % GFP positive cells in liposomes), indicating that the major impediment additive to transfection in liposomes and polymer-based methods was removed (Fig 2). Conversely, a small but significant effect was observed in lipopolyplexes-mediated transfection efficiencies, displaying slightly lower efficiencies in SIFA and Basal media than in BCR-F12 (19 % compared to 21 %).

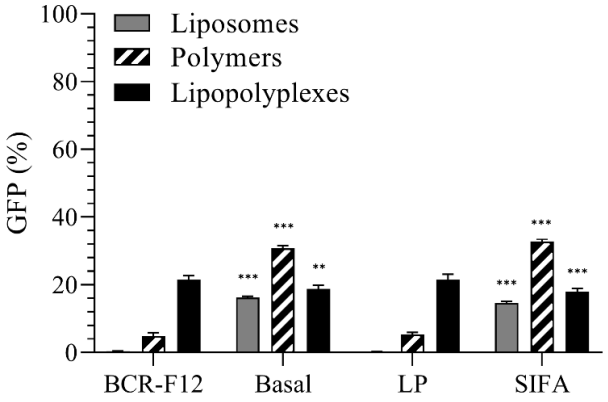


Fig. 2 Transfection efficiency in BCR-F12, LP, SIFA and Basal media. Percentage of GFP expressing DG44 cells 24 h post-transfection with d2GFP in DMEM-F12 (Basal), in-house formulation BCR-F12, BCR-F12 lacking linoleic acid, ethanolamine and putrescine (LP) and BCR-

F12 lacking sodium selenite, insulin, FAC, NEAA and L-glutamine (SIFA). Three transfection system were used: Liposomes (Lipofectamine 2000), Polymer-based (TransIT-X2) and Lipopolyplexes (TransIT-PRO). Triplicate transfections were set for each media and transfection method combination. A negative control well was included for each media and transfection method, displaying between 0.1 - 1.5 % GFP (data not shown). Statistical differences between transfection efficiencies observed in LP and SIFA conditions compared to the ones in BCR-F12 are represented as: $p< 0.001$ (***), $p<0.01$ (**).

3.3. FAC reduces transfection efficiency of liposomes and polymer-based agents in DG44 cells

Individual removal of each component of the SIFA group was then performed in DG44 cells in order to identify whether the enhancement of transfection efficiencies observed was a result of single or combinatorial components. As larger effects were observed in polymer- and liposomal-mediated transfection (Fig. 2), we first focused on these two agents. Following this step-wise removal approach, ferric ammonium citrate (FAC) was identified as a key additive interfering with both systems, as its removal resulted in a dramatic increase in the percentage of GFP-expressing cells observed (15 % compared to no detection when liposomes were used and 25 % compared to 3 % when polymers were used) relative to transfections in BCR-F12 (Fig. 3a). By contrast, individual removal of the other four additives (L-glutamine, insulin, sodium selenite, NEAA) did not show any positive effect on transfection efficiencies when liposomes were used (Fig. 3a). Polymer-mediated transfection of the d2GFP-expressing vector resulted in a small but significant ($p< 0.001$) improvement in transfection efficiencies (10 %, 9 %, 4 % and 5 %) observed following the removal of insulin, sodium selenite, L-glutamine and NEAA (respectively) relative to the transfections in BCR-F12 (Fig. 3a). In the control Basal medium, lower GFP-expressing populations were achieved compared to the previous comparison Figure 2, which was likely associated with random biological variability between experiments.

As individual removal of components was anticipated to affect cell line performance, viable cell density (VCD) and viability assessments 24 h post-transfection were also carried out, revealing that supplement removal resulted in significantly decreased VCD levels in all cases except for DG44 transfected with polymers in medium lacking NaSe, in which VCD was observed to be unaffected. FAC removal had a considerable impact, with VCD decreased by 1.8-fold (liposomes) and 1.5- fold (polymers) compared to DG44 cells in BCR-F12 medium and viabilities decreased to 84 % (liposomes) and 74 % (polymers) compared to 95 - 96 % in BCR-F12 (Fig. 3b). VCD and viability results 24 h post-transfection in Basal medium were poorer than those observed in -FAC medium when using the polymer-based agent (VCD decreased by 56 % and viabilities reduced to 61 %). In this medium, transfections mediated by liposomes displayed a significant ($p< 0.05$) VCD decrease of 11 %, while viability reported similar values to that observed in -FAC medium. Viability tests showed

that individual removal of L-glutamine, insulin, non-essential amino acids and sodium selenite did not significantly impact this parameter (Fig. 3b), except for liposomes-mediated transfection, displaying a decrease to 91 % DG44 viability when transfected in medium without insulin.

In a separate experiment, FAC removal was shown to have a similar effect when the lipopolyplex transfection method was used in the DG44 cell line, with the percentage of GFP-expressing cells 24 h post-transfection almost doubling (from 20 % to 38 %) when transfected in medium without FAC (Fig. 3c); this result was also associated with a drop in viability (89 % to 74 %) and (interestingly) a small (9 %) increase in VCD (Fig. 3d).

Data on the mean fluorescent intensity (MFI) (Appendix D, Supplementary Fig. 1a) revealed no relationship between GFP expression and transfection efficiency in BCR-F12 medium, as removal of FAC resulted in both increased MFI and % GFP-expressing populations in all three transfection reagents.

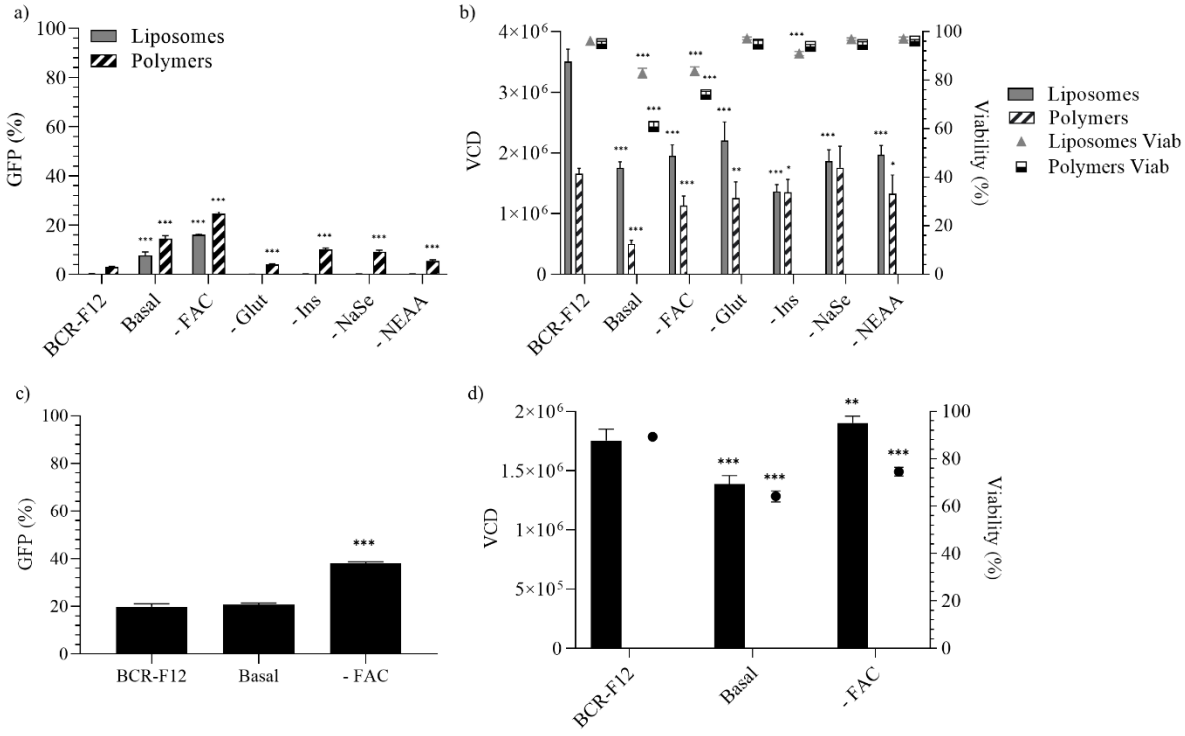


Fig. 3 Transfection efficiency, viable cell density and viability obtained with individual supplement removal. Percentage of GFP expressing DG44 cells (a) and viable cell density (VCD, bars) and viability (lines) (b) 24 h post-transfection with d2GFP vector in BCR-F12 medium (Basal with added FAC, L-glutamine, insulin, sodium selenite and non-essential amino acids), in Basal medium (DMEM-F12) and in BCR-F12 medium with individual removal of: FAC (-FAC), L-glutamine (- Glut), insulin (- Ins), sodium selenite (- NaSe) and non-essential amino acids (- NEAA). DMEM-F12 (Basal) and BCR-F12 were included as a control media. Two transfection systems were tested: liposomes (triangles) and polymers (squares). Percentage of GFP expressing DG44 cells (c) and VCD (bars) and viability (symbols) (d) are represented for lipopolyplexes transfection in BCR-

F12 medium and BCR-F12 without FAC (-FAC). Triplicate transfections were set for each media and transfection method combination. A negative control well was included for each media and transfection method, displaying between 0.1 - 1.3 % GFP (data not shown) and viabilities between 92 – 97 % for all three transfection methods (data not shown). Statistical differences between transfection efficiencies observed in each testing media compared to the ones in BCR-F12 are represented as: $p< 0.001$ (***), $p< 0.01$(**) and $p< 0.05$(*).

3.4. FAC removal facilitates transfection in DP12 and CHO-K1 cell lines

To confirm this FAC-depleted result on transfection efficiency was not cell line-dependent, CHO-K1 and DP12 cells were transfected in FAC-depleted medium (-FAC) using all 3 methods. Similar to the results shown for DG44 (Fig. 3), transfection efficiencies were enhanced in both cell lines for all three transfection agents used, making the -FAC medium very similar to Basal medium in the DP12 cell line (Fig. 4a) and superior to Basal for the CHO-K1 cell line (Fig. 4b). In DP12, GFP-expressing populations were successfully generated in -FAC medium 24 h post-transfection when using liposomes (12 % efficiency), while an 8.5-fold increase in transfection efficiency was observed with polymer-mediated transfection compared to BCR-F12 medium and a small but significant ($p< 0.001$) increase (26 % to 34 %) in transfection efficiency for the lipopolyplexes system (Fig. 4a). In CHO-K1, transfections performed in -FAC medium also resulted in higher efficiencies for all 3 methods (Fig. 4b). Similar to DP12, GFP-expressing populations were detected in -FAC medium when using liposomes (15 %), while a 5.2-fold increase in transfection efficiency was observed with polymers compared to BCR-F12. Similar to the results observed for DG44 but in a marked contrast to that seen in DP12, the largest transfection efficiency increase was observed with the lipopolyplexes system in CHO-K1, displaying an almost 5-fold increase in GFP-expressing populations (8 % compared to 38 %; Fig. 4b). However, it is worth noting that the transfection efficiencies for BCR-12 medium presented in Figure 4b are much lower than those presented for BCR-12 medium in Figure 1. As a result, the possibility that these lower efficiencies adversely complicate interpretation of the transfection efficiency increase observed with the CHO-K1 lipopolyplexes system cannot be fully excluded. CHO-K1 transfections in -FAC medium displayed a higher number of GFP-expressing populations (1.7-fold in polymer-based transfection and 2.4-fold with liposomes) compared to Basal medium for all three methods (Fig. 4b), while in -FAC medium DP12 performed very similarly to the cells in Basal medium (Fig. 4a).

In the absence of FAC, increased MFI levels were also observed in both cell lines with the three transfection agents, except for lipopolyplexes-mediated CHO-K1 transfection, which reported lower expression than in FAC-containing medium (Appendix D, Supplementary Fig. 1c). As expected from the transfection efficiencies data (Fig. 1), comparison with ProCHO5, SFM-II and BalanCD revealed substantially lower expression in the in-house serum-free medium. Interestingly, among the three

transfection reagents and media conditions tested, polymer-mediated transfection in SFM-II medium was observed as the only transfection condition to display high MFI levels across the three CHO cell lines (Appendix D, Supplementary Fig. 1a,b,c). While lipopolyplexes were observed to be optimal for high GFP expression in DP12 (independent of media), CHO-K1 and DG44 cell lines reported increased MFI levels when transfected with polymers in both ProCHO5 and SFM-II.

As in the DG44 cell line, removal of FAC resulted in decreased viability profiles compared to BCR-F12 in both DP12 and CHO-K1 with all 3 transfection agents tested (Fig. 4c,d), underlining the importance of this element to culture performance. Compared to the cells in BCR-F12, VCDs for DP12 in -FAC medium were lower for all three methods. CHO-K1 cells demonstrated similar VCDs (to BCR-F12) in -FAC medium when liposomes were used, but lower VCD for polymers and lipopolyplexes (Fig. 4c,d). It is important to also note that cells transfected in -FAC medium generally displayed unchanged or slightly improved VCD and viability profiles compared to those observed in Basal medium (Fig. 4d).

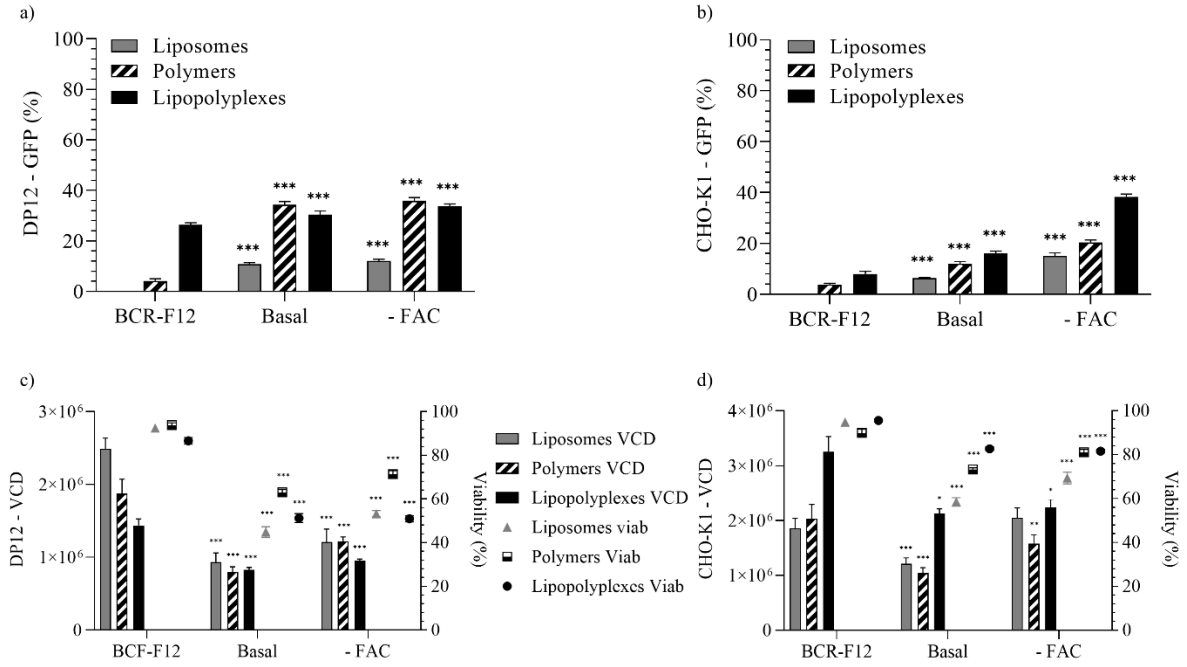


Fig. 4 Transfection efficiency with FAC removal. Percentage of GFP expressing DP12 (a) and CHO-K1 (b) cells 24 h post-transfection with d2GFP vector in BCR-F12, DMEM-F12 (Basal) and BCR-F12 without FAC (- FAC) media. Viable cell density (VCD, bars) and viabilities (symbols) of DP12 (c) and CHO-K1 (d) cells 24 h post-transfection are also represented. Three transfection system were used: Liposomes (Lipofectamine 2000) (triangles), Polymer-based (TransIT-X2) (squares) and Lipopolyplexes (TransIT-PRO) (circles). Triplicate transfections were set for each cell line in each condition (media and transfection method combination). A negative control well was included for each media and transfection method, displaying between 0.0 - 0.5 % GFP (data not shown) and viabilities between 86 – 93 % (DP12) and 90 – 96 % (CHO-K1) for all three transfection methods

(data not shown). Statistical differences between transfection efficiencies observed in -FAC medium compared to the ones in BCR-F12 are represented as: $p< 0.001$ (***), $p< 0.01$(**) and $p< 0.05$(*).

3.5. Ferric ammonium citrate affects zeta potential and size of some transfection reagents

Zeta potential and particle size have become standard analytical parameters to characterise DNA-delivery complexes and study transfection efficiency (*35*, *36*, *37*). Both of these physicochemical characteristics are dependent on the environment, thus being sensitive to several parameters such as pH, viscosity or ionic strength (*38*). In order to gain insight into the mechanistic inhibitory effect of FAC, the zeta potential and size of the three DNA-complex particles used here (liposomes, polymers and lipopolyplexes) were characterised in each testing medium.

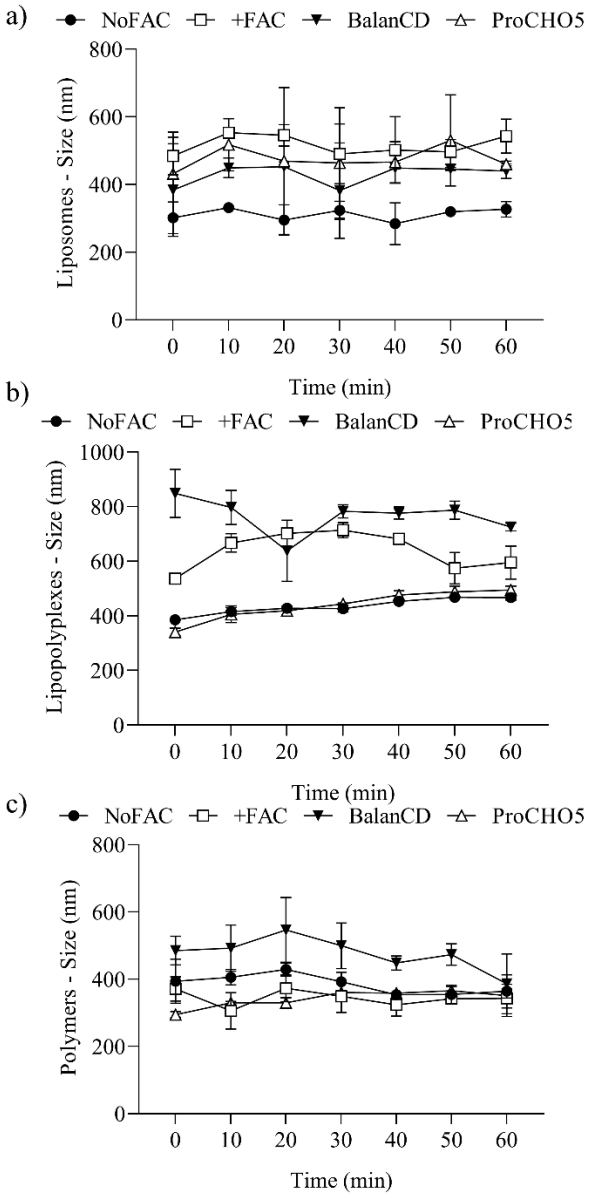


Fig. 5. Size of liposomes, lipopolyplexes and polymers complexes in different media. Liposomes (a), lipopolyplexes (b) and polymers (c) were placed in BCR-F12 without FAC (NoFAC), BCR-F12 with FAC (+FAC), ProCHO5 and BalanCD and the size (nm) was analysed every 10 min during an hour incubation in the Malvern Zetasizer X. Each measurement was performed in triplicate.

Particle size was analysed in intervals of 10 min over an hour incubation in order to identify possible particle destabilization (such as aggregation) over time. For comparison, size was also analysed in complex formation medium (DMEM-F12). Interestingly, in DMEM-F12 all three complexes reported similar sizes, with 367 nm +/- 18 observed in liposomes, 377 nm +/- 11.8 in lipopolyplexes and 406 nm +/- 51 in polymers. As the size of these complexes in DMEM-F12 was only assessed at time 0h, to compare with the media-effects during transfection, this data is not represented in figure 5. However, when diluted in some of the transfection media, increased sizes of the complexes were observed. Liposomes became bigger over time when diluted in BCR-F12+FAC (484 - 542 nm), ProCHO5 (431 - 460 nm) and BalanCD (389 - 439 nm), while in the absence of FAC complexes of 301 to 326 nm were observed (Fig. 5a). Lipopolyplexes displayed two distinguishable behaviours depending on the media (Fig. 5b), as follows. In both ProCHO5 and BCR-F12 without FAC, similar particle sizes (to DMEM-F12) were observed at the beginning, slightly increasing over the incubation time. Conversely, in media containing FAC and in BalanCD, big particle sizes of 536 nm and 848 nm were already observed at time zero. In these media, indication of particle aggregation were also detected due to prompt drops and increases in size observed over the incubation time, possibly as a consequence of the sedimentation of these big particles. Similar to that observed for liposomes and lipopolyplexes, polymer complexes in BalanCD were observed to increase in size to 485 nm compared to the particles in DMEM-F12, BCR-F12 with and without FAC and ProCHO5, which were observed to be 398 nm, 371 nm and 294 nm, respectively (Fig. 5c). In all four media, polyplexes sizes were increased over the 1 h incubation, with signs of aggregation or increased variability in sizes observed in both FAC and NoFAC media as well as BalanCD.

Zeta potential does not correspond to the particle charge but gives an indication of the surface charge determined by the electrophoretic mobility of the complexes under specific conditions (pH, temperature, viscosity), thus being influenced by media composition (*37*, *38*). Liposomes in DMEM-F12, BCR-F12 NoFAC and BCR-F12+FAC were observed to behave similarly, with zeta potential values ranging from -35 to -30 mV (Fig. 6a). In contrast, the presence of FAC had a substantial effect on the zeta potential of polymers and lipopolyplexes, inducing negative zeta potentials of -17 mV and -6.9 mV, respectively, while measurements in DMEM-F12 and BCR-F12 without FAC displayed positive zeta potentials (11 – 17 mV, polymers and 11 – 10 mV lipopolyplexes) (Fig. 6b,c).

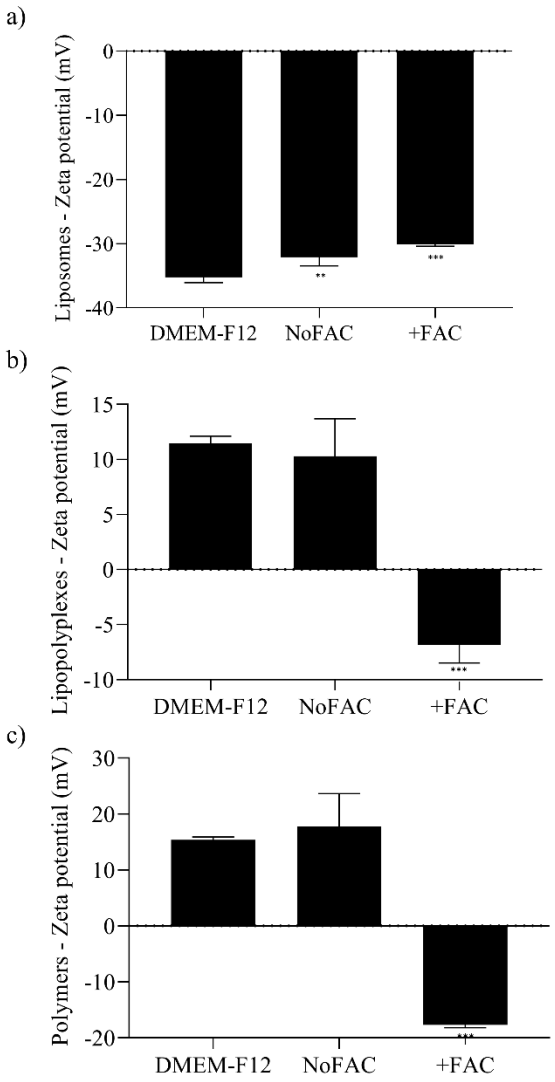


Fig. 6. Zeta potential of liposomes, lipopolyplexes and polymers complexes in different media. Liposomes (a), lipopolyplexes (b) and polymers (c) were placed in DMEM-F12, BCR-F12 without FAC (NoFAC) and BCR-F12 with FAC (+FAC) and the zeta potential was determined by Phase Analysis Light Scattering in the Malvern Zetasizer X. Each measurement was performed between two to five times (depending on the conductivity of the media). Statistical differences observed in zeta potential in NoFAC and +FAC media compared to the ones in DMEM-F12 are represented as: $p< 0.001$ (***) and $p< 0.01$(**).

3.6. Ferric ammonium citrate inhibits transfection during the initial stages of the process

Due to the importance of iron for the maintenance of cell culture performance (*39*), supplementation of FAC at early stages post-transfection was assessed in order to determine the optimal time to schedule FAC addition for maximal culture performance while not adversely affecting transfection efficiency. The manufacturer's instructions suggested that supplementation with additional additives should not be performed earlier than 4 h (polymers and liposomes) and 24 h post-transfection

(lipopolyplexes). A 4 h gap in iron supplementation was observed to have an adverse effect mainly on viability for all 3 cell lines (Appendix D, Supplementary Fig. 2) and for this reason, a period of 0.5 - 5 h post-transfection was examined to determine the transfection-inhibitory effects of FAC supplementation. Interestingly, supplementation with 100 µM FAC at 0.5 h post-transfection was sufficient to restore transfection efficiencies for all three methods; dramatically increasing efficiencies for liposomes (from no detection to 22 %) and polymers (3 % to 28 %), while also increasing transfection efficacy (27 % to 38 %) for the lipopolyplexes method (Fig. 7). Supplementation of FAC at timepoints later than 0.5 h resulted in similar (or slightly worse) transfection efficiencies in almost all cases examined, reaching similar percentages of DG44 GFP-expressing populations (Fig. 7). Following on from this comprehensive test in DG44 cells, a simplified protocol, examining just the 0 h, 0.5 h and 4 h timepoints, identified that 0.5 h was the optimal supplementation timepoint for DP12 and CHO-K1 as well; the single exception being later (4 h) supplementation optimal for lipopolyplexes-based efficiency in CHO-K1, increased to 38 % at 4 h, compared to 25 % for the same method at 0.5 h (Appendix D, Supplementary Fig. 3 (b.)) Supplementation with FAC at 0.5 h also resulted in higher viabilities for all three cell lines and all methods tested (Appendix D, Supplementary Table 1), with the single exception of the lipopolyplexes method in DG44 cells, where viabilities increased slightly (from 83 % to 84 %) when FAC was supplemented at the later 4 h timepoint.

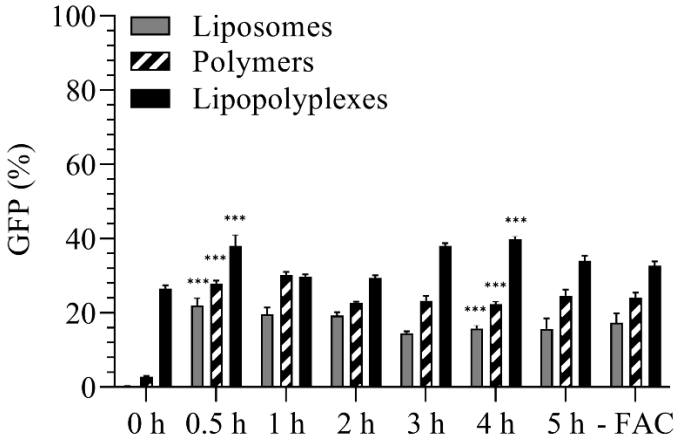


Fig. 7. Effect of FAC supplementation at 0- 5 h post-transfection on the percentage of GFP expressing DG44 cells 24 h post-transfection. Three transfection agents were tested: liposomes, polymers and lipopolyplexes. Transfections were performed in BCR-F12 medium without FAC. Supplementation of 100 µM FAC was performed at different time points (0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h and 5 h) post-transfection. Non-supplemented media (- FAC) was added as a control. Triplicate transfections were carried out for each condition (timepoint and transfection method combination). A negative control well was included for each condition and transfection method, displaying between 0.1 – 0.4 % (lipofectamine), 0.1 – 2.4 % (polymers) and 0.2 – 0.7 % GFP (lipopolyplexes) (data not shown). The 2.4 % GFP-expression detected only in polymer-transfection in -FAC medium was

suspected to be a consequence of cross-contamination between the transfected and non-transfected wells, as all other negative controls in each transfection reagents and media combinations displayed % GFP below 1.3 %. Transfection efficiencies observed in FAC-supplemented medium at each time point (0.5 – 5 h) compared to the ones at 0 h control were statistically significantly different with a significance of $p< 0.001$, represented as (***) per 0.5 h and 4 h timepoints.

4. Discussion

Due to the importance of achieving optimal transfection efficiencies for the generation of stably-expressing clones and/or rapid recombinant protein production by transient gene expression in CHO cells, continued efforts to enhance the efficiency of this process are essential. Following our development of an in-house serum-free medium where the formulation was known, ferric ammonium citrate (FAC) was identified as an inhibitory supplement for all cell lines and chemical transfection methods used (Liposome 2000, TransIT-X2 and TransIT-PRO in CHO-K1, DG44 and DP12 cells). With the aim of obtaining GFP-expressing populations while maintaining healthy cultures, we showed that transfection in -FAC medium followed by replenishment of FAC 30 min post-transfection (rather than 4 h or 24 h) facilitates high transfection efficiencies and delivers satisfactory CHO culture viabilities.

Driven by regulatory and process concerns, the removal of serum from routine use in mammalian culture media has resulted in the development of a wide variety of commercially-available, chemically-defined serum-free formulations, optimized for high cell culture performance in terms of growth and volumetric productivity. However, the use of these SFM formulations has also frequently been associated with poor transfection efficiencies. For instance, Ye, J., et al (2009) observed media-dependent effects in PEI-mediated transfections of CHOK1SV cells in a range of eight commercial protein-free and chemically-defined media, with less than 1mg/L mAb titer detected 4 days post-transfection in ProCHO5 (also analysed in this study) but high yields obtained in ProCHO4 and UltraCHO media (*24*). Similarly, we have shown differences between the serum-free, protein-free and chemically-defined commercial media examined. In certain media, these differences may be absolute, displaying zero transfection in BalanCD but high transfection efficiencies in CHO-S-SFMII, with all three cell lines and transfection agents. In other media the differences are mixed - as observed in ProCHO5 - with zero transfection detected with liposomes, but high transfection efficiencies in both polymers and lipopolyplexes. In either case, the presence or absence of transfection-inhibitory molecules in the media formulations are likely to be the key factors contributing to this outcome. Cell proliferation has been suggested to play a role in transgene expression and thus, cultures displaying increased growth rates have been observed to achieve increased transfection efficiencies (*40, 41*). In this study, although specific growth rate (μmax) was not calculated, all three cell lines were observed to perform differently in each media as per VCD levels achieved on passage day (day 3) during cell

maintenance (data not shown), which is an indicator of different growth profiles in each media. By comparing VCD levels, in this study we have not observed a direct linear relationship between increased VCD and transfection efficiency. A similar effect has been previously reported in transfections mediated by cationic-lipids and PEI complexes, which were observed to achieve efficient transgene expression independently of cell proliferation (*42, 43*), indicating possible alternative pathways influencing efficient transgene expression. Further analysis on specific growth rate would provide more information to characterize this possible effect.

Since the composition of many available media is typically not disclosed by manufacturers, we developed an in-house SFM formulation to study the impact on transfection efficiency following removal of various standard supplements. The presence of media additives such as polymers and anti-clumping agents (dextran and heparin sulphate) have been suggested to hinder PEI mediated transfection, affecting the stability and uptake of the DNA complexes formed (*1*). In 2001, a study focused on the effects of serum and transfection efficiency in five mammalian cell lines (including CHO) revealed that the lipid part of serum (mainly cholesterol, LDL, HDL and phospholipids) caused inhibition of liposomal-mediated transfection. Interestingly, Son et. al (*25*) further reported successful transfection and liposomal-complex formation in delipidated-serum medium, thus demonstrating the critical role of lipid content in transfection media. In HEK293, a source-dependent effect of peptone supplementation was described to alter PEI transfection efficiencies, describing beneficial transfection effects with 0.5% gelatine peptones but inhibition of DNA delivery when casein-derived peptones were added (*26*). In this study, FAC has been observed as an inhibitory element for transfection and/or GFP expression of three CHO cell lines (DG44, DP12 and CHO-K1) and three chemical transfection methods (liposomal-based (Lipofectamine 2000), polymer-based (TransIT-X2), and lipopolyplex-based (TransIT-PRO)). Ferric citrate (Iron (III) citrate) has been previously identified to impede PEI-mediated transfection of CHO-S and DXB11 cell lines when supplemented over 50 µM (*27*). A subsequent study (*44*) identified that the decreased transfection efficiencies may be due to a net negative zeta potential detected in the complexes formed in the presence of iron, leading to lower interaction of PEI:DNA complexes with CHO-S cellular membranes in ferric citrate-supplemented CD-CHO medium. In this work, we have built on these two previously published studies to demonstrate that the inhibiting effect of FAC is not limited to PEI, but also affects another polymer-based method (TransIT-X2), as well as cationic lipid-based methods (lipofectamine 2000) and the mixed lipid: polymer lipopolyplexes method (TransIT-PRO). In accordance with previous reported data for PEI (*27*, *44*), the zeta potential of lipopolyplexes and polymer complexes studied here were adversely affected in the presence of FAC, with negative zeta potentials detected. Interestingly, lipid-mediated complexes displayed equal negative zeta potential in all media tested, independent of the presence of FAC, which indicates a possible alternative inhibitory mechanism. Chemical agents deliver DNA to cells by hydrophobic and electrostatic interactions with the

negatively charged cellular membrane and thus positive zeta potential has been reported for successful DNA delivery (*45, 46*). However, the negative lipid-mediated complexes formed here were capable of generating GFP-expressing populations, an effect also reported by Son et al. (*47, 25*). The published literature on the influence of zeta potential on successful lipid-mediated transfection has shown mixed results, with some studies reporting positive zeta potentials (*45, 46, 48)*, mainly measured in water, PBS or diluted media, but negative zeta potentials in lipid-mediated transfection have also been demonstrated, measured in culture media (*47*).

We have here observed that in the absence of iron (BCR-F12 NoFAC), smaller lipopolyplexes and lipid-mediated complexes were obtained, relating to increased transfection efficiencies compared to FAC-containing medium. These results indicate a potential FAC-mediated influence on the encapsulation of DNA into lipopolyplexes and liposome complexes and/or an increase in the aggregation of the complexes, leading to a decreased transfection efficiency caused by decreased cellular uptake. However, this effect was not observed in polymer-DNA complexes, which displayed increased sizes in -FAC medium compared to medium with FAC. The influence of complex size on cellular uptake has been reported in the literature, but without unanimity as to its importance, with some studies defining this parameter as essential (*49, 50*) while others reporting no effect on achieving successful transfection (*51*). Several steps are involved in the generation of producer cells, which include successful transport through the cellular membrane, but also efficient DNA-carrier complex formation, cytoplasm transport, and nuclear localization (*52*), which could be disrupted by FAC. Internalisation of DNA-delivery particles has been described to be mainly mediated by endocytosis, with endosomal escape one of the critical steps to achieve DNA expression. Among the three transfection agents used here, different strategies have been proposed for successful endosomal release, such as the proton sponge strategy (mostly described in polyplexes), the membrane fusion strategy (described in liposomes) and membrane disruption and pore formation (mediated by peptide or/and polymer complexes) (*53*). Recently, an alternative random Brownian motion was described for intracellular trafficking of lipofectamine-containing vesicles (*54*), indicating that further investigations are still required to fully understand the mechanisms involved with each type of transfection agent. In our study, GFP was used as an indirect measure of traceability of efficient transfection (*55, 56*). Future work to confirm that the different conditions/additives impact the actual transfection efficiency and not GFP expression could be carried out by labelling the plasmid DNA with (e.g.) rhodamine dye (*57*) to follow membrane interaction and intracellular vesicles trafficking and could further investigate the mechanistic effect of FAC in lipoplex, polymer and liposome DNA-delivery.

Iron is vital for healthy growth of mammalian cells, with limited availability of this metal resulting in poor cell densities and eventual cellular death (*39*). Moreover, supplementation with iron citrate forms (such as FAC) has been also shown to be essential for increased mAb titers in CHO culture

(*58*). For these reasons, we aimed to identify the optimal timepoint for FAC supplementation post-transfection in -FAC medium which would deliver satisfactory outcomes in both cellular viability and transfection efficiencies. Eberhardy, et al. (2009) had previously reported that FAC interference occurs at early stages of transfection and interestingly, FAC supplementation during the initial 4 h was reported to decrease PEI-mediated transfection in adherent DUXB11 cultures (*27*). In this study, supplementation as early as 30 min post-transfection has been shown to positively impact transfection efficiency mainly in polymers and liposomes mediated transfection, with slight enhancement in lipopolyplexes. Moreover, FAC supplementation at this timepoint has delivered beneficial cellular viability in all three cell lines and with all three transfection methods studied.

During the course of this study, differing transfection efficiencies between cell lines (2 to 3-fold lower for BCR-F12 than for SFM-II and ProCHO5), transfection agents and media were observed. The in-house serum-free medium underperformed in terms of transfection efficiency and GFP expression (MFI levels) compared to two of the commercial media (ProCHO5 and SFM-II), which was likely due to differences in media formulation additives interfering with, or impeding, efficient transfection (also reported previously *1, 24, 25, 26*). Moreover, from in-house experimentation (data not shown), the BCR-F12 medium is defined as a low-producing medium, in which DP12 have been observed to reach up to 51 % less mAb titer than in the SFM-II commercial medium. This aspect of our in-house medium may have contributed to the low MFI levels detected even in -FAC medium. Interestingly, from the combined data of MFI and percentage of GFP expressing cells, it was revealed that high transfection efficiencies and expression can be achieved by using the polymer-based TransIT-X2 transfection agent in combination with the commercial medium CHO-S SFM-II (in our conditions).

In a recent study, transfections performed under the same conditions on a range of ten cell lines were observed to differently perform in terms of transfection efficacy (*18*), an effect that has been here similarly observed in the in-house -FAC medium. It is worth noting that all three CHO cell lines were transfected using the manufacturer's recommendations for CHO cells but adaptation of the protocol was not performed per each cell line. The use of this standardised approach may explain some of the variability in transfection efficiencies observed between cell lines in the different media. Even though it is possible the transfections were performed in non-optimal conditions for each cell line, the inhibitory effects of FAC are not considered to be dependent on a suboptimal transfection condition, as removal of FAC enabled CHO transfection with all reagents tested.

In conclusion, FAC was observed to interfere with DNA transfection mediated by the three chemical agents studied, acting at early stages of the process. Although previously reported to interfere with the interaction between DNA-complexes with the cell membrane, the effects of FAC on the zeta potential and complex sizes were here observed to be dependent on the transfection reagent, indicating that further investigation examining other essential steps of the DNA-delivery process (such as endosomal escape) is needed to fully understand FAC inhibition effect. To circumvent this

issue, a practical strategy to recover successful transfection while increasing CHO culture viabilities post-transfection was devised, involving transfection in -FAC medium followed by replenishment of FAC just 30 min post-transfection.

5. References

1. Dyson, M. R. (2016) Fundamentals of Expression in Mammalian Cells. Adv Exp Med Biol. 217–224.

2. Kim, T. K. and Eberwine, J. H. (2010) Mammalian cell transfection: the present and the future. Anal Bioanal Chem. 397, 3173–3178

3. Slivac, I., Guay, D., Mangion, M., Champeil, J., & Gaillet, B. (2016) Non-viral nucleic acid delivery methods. Expert Opinion on Biological Therapy, 17(1):105–118

4. Hunter, M., Yuan, P., Vavilala, D., & Fox, M. (2019) Optimization of protein expression in mammalian cells. Current Protocols in Protein Science, 95,e77.

5. Guo, L., Wang, L., Yang, R., Feng, R., Li, Z., Zhou, X., Dong, Z., Ghartey-Kwansah, G., Xu, M., Nishi, M., Zhang, Q., Isaacs, W., Ma. J. and Xu, X. (2017) Optimizing conditions for calcium phosphate mediated transient transfection. Saudi J Biol Sci. 24(3):622-629

6. Gutiérrez-Granados, S. , Cervera, L., Kamen, A. A. and Gòdia, F. (2018) Advancements in mammalian cell transient gene expression (TGE) technology for accelerated production of biologics Crit Rev Biotechnol 38(6), 918-940

7. Rajendra Y1, Kiseljak D, Baldi L, Wurm FM, Hacker DL. (2015) Transcriptional and post-transcriptional limitations of high-yielding, PEI-mediated transient transfection with CHO and HEK-293E cells Biotechnol Prog. 31(2):541-9.

8. Rajendra, Y., Balasubramanian, S., Hacker, D. L. (2017) Large-Scale Transient Transfection of Chinese Hamster Ovary Cells in Suspension. Methods Mol Biol. 1603:45-55

9. Bansal, R., Gupta, K. C. and Kumar, P. (2015) Biodegradable and versatile polyethylenimine derivatives efficiently transfer DNA and siRNA into mammalian cells. Colloids Surf B Biointerfaces. 135:661-8

10. Giron-Gonzalez, M. D., Salto-Gonzalez, R., Lopez-Jaramillo, F. J., Salinas-Castillo, A., Jodar-Reyes, A. B., Ortega-Muñoz, M., Hernandez-Mateo, F. and Santoyo-Gonzalez, F. (2016) Polyelectrolyte Complexes of Low Molecular Weight PEI and Citric Acid as Efficient and Nontoxic Vectors for in Vitro and in Vivo Gene Delivery. Bioconjug Chem. 27(3):549-61.

11. Costello A., Lao N., Clynes M., Barron N. (2017) Conditional Knockdown of Endogenous MicroRNAs in CHO Cells Using TET-ON-SanDI Sponge Vectors, in Molecular Biology, vol 1603: Heterologous Protein Production in CHO Cells (Meleady P. eds) Humana Press, New York, NY: pp 87-100

12. Kaestner, L., Scholz, A. and Lipp, P. (2015) Conceptual and technical aspects of transfection and gene delivery. Bioorganic & Medicinal Chemistry Letters 25 6):1171-1176

13. Dharmalingam, P., Rachamalla, H. K. R., Lohchania, B., Bandlamudi, B., Thangavel, S., Murugesan, M. K., Banerjee, R., Chaudhuri,A., Voshavar, C. and Marepally, S. (2017) Green Transfection: Cationic Lipid Nanocarrier System Derivatized from Vegetable Fat, Palmstearin Enhances Nucleic Acid Transfections. ACS Omega 2 (11):7892-7903

14. Rangasami, V. K., Lohchania, B., Voshavar, C., Rachamalla, H. R., Banerjee, R., Dhayani, A., Thangavel, S., Vemula, P. K. and Marepally, S. (2019) Exploring membrane permeability of Tomatidine to enhance lipid mediated nucleic acid transfections. Biochim Biophys Acta Biomembr. 1861(1):327-334.

15. Rahimpour, A., Ahani, R., Najaei, A., Adeli, A., Barkhordari, F. and Mahboudi, F. (2016) Development of Genetically Modified Chinese Hamster Ovary Host Cells for the Enhancement of Recombinant Tissue Plasminogen Activator Expression. Malays J Med Sci. 23(2): 6–13.

16. Chen, K., Li, D., Li, H., Li, B., Li, J., Huang, L., Li, R., Xu, X., Jiang, L., Jiang, C., Gu, H. and Fang, J. (2017) Genetic analysis of heterogeneous sub-clones in recombinant Chinese hamster ovary cells. Appl Microbiol Biotechnol. 101(14):5785-5797

17. Zhao, C. P., Guo, X., Chen, S. J., Li, C. Z., Yang, Y., Zhang, J. H., Chen, S. N., Jia, Y. L. AND Wang, T Y. (2017) Matrix attachment region combinations increase transgene expression in transfected Chinese hamster ovary cells. Scientific Reports 7:42805

18. Wang, T., Larcher, L. M., Ma, L. and Veedu, R. N. (2018) Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides. Molecules 23(10), 2564

19. Ewe, A., Schaper, A., Barnert, S., Schubert, R., Temme, A., Bakowsky, U. and Aigner A. (2014) Storage stability of optimal liposome-polyethylenimine complexes (lipopolyplexes) for DNA or siRNA delivery. Acta Biomater 10(6), 2663-73

20. Rezaee, M., Oskuee, R. K., Nassirli, H. and Malaekeh-Nikouei, B. (2016) Progress in the development of lipopolyplexes as efficient non-viral gene delivery systems J Control Release 28, 236:1-14

21. Caracciolo, G., Pozzi, D., Capriotti, A. L., Marianecci, C., Carafa, M., Marchini, C., Montani, M., Amici, A., Amenitsch, H., Digman, M. A., Gratton, E., Sanchez, S. S. and Laganà, A. (2011) Factors determining the superior performance of lipid/DNA/protammine nanoparticles over lipople xes. J Med Chem. 54(12), 4160-71

22. Sou, S. N., Polizzi, K. M. and Kontoravdi, C. (2013) Evaluation of transfection methods for transient gene expression in Chinese hamster ovary cells. Adv in Biosci and Biotechnol. 4, 1013-1019

23. Cervera, L., Gutiérrez-Granados, S., Martínez, M., Blanco, J., Gòdia, F. and Segura, M. M. (2013) Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J Biotechnol. 166(4), 152-65.
24. Ye, J., Kober, V., Tellers, M., Naji, Z., Salmon, P. and Markusen, J. F. (2009) High-level protein expression in scalable CHO transient transfection. Biotechnol Bioeng.103(3),542-51
25. Son, K. K., Tkach, D. and Rosenblatt, J. (2001) Delipidated serum abolishes the inhibitory effect of serum on in vitro liposome-mediated transfection. Biochim Biophys Acta. 1511(2), 201-5
26. Pham, P. L., Perret, S., Doan, H. C., Cass, B., St-Laurent, G., Kamen, A. and Durocher, Y. (2003) .Large-scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency. Biotechnol Bioeng. 84(3),332-42.
27. Eberhardy, S. R., Radzniak, L. and Liu, Z. (2009) Iron (III) citrate inhibits polyethylenimine-mediated transient transfection of Chinese hamster ovary cells in serum-free medium. Cytotechnology 60(1-3), 1
28. Capella Roca, B., Alarcón Miguez, A., Keenan, J., Suda, S., Barron, N., O'Gorman, D., Doolan, P. and Clynes, M. (2019) Zinc supplementation increases protein titer of recombinant CHO cells. Cytotechnology. 71(5):915-924.
29. Ritacco, F. V., Wu, Y. and Khetan, A. (2018) Cell culture media for recombinant protein expression in Chinese Hamster Ovary (CHO) cells: history, key components, and optimization strategies. Biotechnol Prog., Vol. 34, No. 6
30. Snyder, D. S. and Small, P. L. (2001) Staining of cellular mitochondria with LDS-751. J Immunol Methods. 257(1-2):35-40.
31. Yang, Y., Xiang, Y. and Xu, M. (2015) From red to green: the propidium iodide-permeable membrane of Shewanella decolorationis S12 is repairable. Sci Rep. 5:18583.
32. Gauthier, T. D., & Hawley, M. E. (2007). Statistical methods. Introduction to Environmental Forensics, 129–183
33. Qi, M., Duan, S., Yu, B., Yao, H., Tian, W. and Xu, F. J. (2016) PGMA-based supramolecular hyperbranched polycations for gene delivery. Polym. Chem. 7, 4334-4341
34. Thomas, T. J., Tajmir-Riahi, H. A. and Thomas, T. (2016) Polyamine–DNA interactions and development of gene delivery vehicles. Amino Acids. 48(10):2423-31.
35. Mandal, H., Katiyar, S. S., Swami, R., Kushwah, V., Katare, P. B., Kumar Meka, A., Banerjee, S. K., Popat, A. and Jain, S. (2018) ε-Poly-l-Lysine/plasmid DNA nanoplexes for efficient gene delivery in vivo. Int J Pharm. 542(1-2):142-152
36. Yu, Q., Zhang, B., Zhou, Y., Ge, Q., Chang, J., Chen, Y., Zhang, K., Peng, D. and Chen, W. (2019) Co-delivery of gambogenic acid and VEGF-siRNA with anionic liposome and polyethylenimine complexes to HepG2 cells. J Liposome Res. 29(4):322-331.

37. Smith, M. C., Crist, R. M., Clogston, J. D. and McNeil, S. E. (2017) Zeta potential: a case study of cationic, anionic, and neutral liposomes. Anal Bioanal Chem. 409(24):5779-5787
38. Bhattacharjee, S. (2016) DLS and zeta potential – What they are and what they are not? Journal of Controlled Release 235:337–351
39. Yu, Y., Kovacevic, Z. and Richardson, D.R. (2007) Tuning cell cycle regulation with an iron key. Cell Cycle. 6(16):1982-94.
40. Tseng, W. C., Haselton, F. R. and Giorgio, T.D. (1999) Mitosis enhances transgene expression of plasmid delivered by cationic liposomes. Biochim Biophys Acta. 1445(1):53-64.
41. Mortimer, I., Tam, P., MacLachlan, I., Graham, R. W., Saravolac, E. G and Joshi, P. B. (1999) Cationic lipid-mediated transfection of cells in culture requires mitotic activity. Gene Ther. 6(3):403-11.
42. Matz, RL. ., Erickson, B., Vaidyanathan, S., Kukowska-Latallo, J. F., Baker, J. R. Jr., Orr, B. G. and Banaszak Holl, M. M. (2013) Polyplex exposure inhibits cell cycle, increases inflammatory response, and can cause protein expression without cell division. Mol Pharm. 10(4):1306-17
43. Kirchenbuechler, I., Kirchenbuechler, D. and Elbaum, M. (2016) Correlation between cationic lipid-based transfection and cell division. Exp Cell Res. 345(1):1-5
44. Mozley, O. L., Thompson, B. C., Fernandez-Martell, A. and James, D. C. (2014) A mechanistic dissection of polyethylenimine mediated transfection of CHO cells: to enhance the efficiency of recombinant DNA utilization Biotechnol Prog. 30(5), 1161-70
45. Romøren, K., Thu, B. J., Bols, N. C. and Evensen, Ø. (2004) Transfection efficiency and cytotoxicity of cationic liposomes in salmonid cell lines of hepatocyte and macrophage origin. Biochim Biophys Acta 1663(1-2):127-34.
46. Asasutjarit, R., Lorenzen, S., Sirivichayakul, S., Ruxrungtham, K., Ruktanonchai, U. and Ritthidej, G. C. (2007) Effect of Solid Lipid Nanoparticles Formulation Compositions on Their Size, Zeta Potential and Potential for In Vitro pHIS-HIV-Hugag Transfection. Pharm Res. 24(6):1098-107
47. Son, K. K., Patel, D. H., Tkach, D. and Park, A. (2000) Cationic liposome and plasmid DNA complexes formed in serum-free medium under optimum transfection condition are negatively charged. Biochim Biophys Acta. 1466(1-2):11-5.
48. Rajendra, Y., Kiseljak, D., Manoli, S., Baldi, L., Hacker, D. L. and Wurm F. M. (2012) Role of Non-Specific DNA in Reducing Coding DNA Requirement for Transient Gene Expression With CHO and HEK-293E Cells. Biotechnol Bioeng. 109(9):2271-8
49. Rejman, J., Oberle, V., Zuhorn, I. S. and Hoekstra, D. (2004) Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis. Biochem J 377(Pt 1):159-69.

50. Sang, Y., Xie, K., Mu, Y., Lei, Y., Zhang, B., Xiong, S., Chen, Y. and Qi, N. (2015) Salt ions and related parameters affect PEI–DNA particle size and transfection efficiency in Chinese hamster ovary cells. Cytotechnology. 67(1):67-74.

51. Kim, B.K., Hwang, G. B., Seu, Y. B., Choi, J. S., Jin, K. S. and Doh, K. O. (2015) DOTAP/DOPE ratio and cell type determine transfection efficiency with DOTAP-liposomes. Biochim Biophys Acta.1848(10 Pt A):1996-2001

52. Bai, H., Lester, G. M. S., Petishnok, L. C. and Dean, D. A. (2017) Cytoplasmic transport and nuclear import of plasmid DNA. Biosci Rep. 37 (6)

53. Selby, L. I., Cortez-Jugo, C. M., Such, G. K. and Johnston, A. P. R. (2017) Nanoescapology: progress toward understanding the endosomal escape of polymeric nanoparticles. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 9(5).

54. Cardarelli,F., Digiacomo, L., Marchini, C., Amici, A., Salomone, F., Fiume, G., Rossetta, A., Gratton, E. Pozzi, D., and Caraccioloa, G. (2016) The intracellular trafficking mechanism of Lipofectamine-based transfection reagents and its implication for gene delivery. Sci Rep. 6: 25879.

55. Metta, M. K., Kunaparaju, R. K. and Tantravahi1, S. (2016) Rapid amplification system for recombinant protein production in Chinese Hamster Ovary (CHO) Cells. Cell. Mol. Biol. 62 (2), 101-106

56. Peng, L., Xiong, W., Cai, Y., Chen, Y., He, Y., Yang, J., Jin, J. and Li, H. (2017) A simple, rapid method for evaluation of transfection efficiency based on fluorescent dye. Bioengineered. 8(3):225-231

57. Shi, J., Xu, Y., Wang, X., Zhang, L., Zhu, J., Pang, T. and Bao, X. (2015) Synthesis and evaluation of a novel Rhodamine B pyrene [2]rotaxane as an intracellular delivery agent for doxorubicin. Org. Biomol. Chem., 13:7517-7529

58. Bai, Y., Wu, C., Zhao, J., Liu, Y. H., Ding, W. and Ling, W. L. (2010) Role of iron and sodium citrate in animal protein-free CHO cell culture medium on cell growth and monoclonal antibody production Biotechnol Prog. 27(1), 209-19

5. Summary and Conclusions

The research work undertaken in this manuscript in preparation for a PhD. thesis by Publication aimed to focus on two clear elements of the 3 main strategies for improvement in CHO-based bioprocesses: namely (i) medium optimisation and (ii) CHO cell line engineering. With regard to (i), the impact of media additives on CHO performance has been comprehensively highlighted over the course of this research project. From the development of an in-house serum-free medium as a working tool, the titer-enhancing properties of the trace metal zinc were studied to better understand its potential role in improving CHO bioreactor performance. The research work then further built on the knowledge gained to investigate other important additives for CHO survival, eventually resulting in (ii), the design of a novel selection system to potentially rival the already well-established DHFR and GS selection systems. This novel innovative method offers the prospect of successful upstream cell line development without the use of cytotoxic drugs. As part of this invention discovery, the relevance of polyamines as essential components for CHO survival was established. Even though the absolute requirement of polyamines for CHO survival in serum-free conditions was first described in the early eighties, the vital role these amines play in CHO cell proliferation is still not fully understood. Exploiting this dependent phenotype, the effects of this multifunctional additive at transcriptomic level were investigated. Finally, the role media formulation additives play in a key step – transfection – in the generation of producer cell lines was analysed and an alternative method to optimise this essential process was recommended.

Conclusions specific to each chapter are discussed below

<u>Chapter 1: Zinc supplementation increases protein titer of recombinant CHO cells</u>

- High zinc supplementation at 25mg/L improves the specific productivity of two producer CHO cell lines, increasing product titer but negatively affecting VCD profiles.
- Building on previous reported work (Kim and Park 2016), our work has expanded the available knowledge in the field by demonstrating that the titer-enhancing effects of high zinc supplementation may be independent of cell line specificity (result demonstrated in two producer CHO cell lines: DP12 and SK15 (rCHO-K1)) and product type-specificity (supplementation increased titer of both IgG mAb and hEPO).
- The titer-enhancing effects of zinc were attenuated by the presence of ATA in the medium (CDM+A). This results indicates that the positive effects of zinc may be offset by the presence of other additives.
- At 25mg/L zinc, increased mRNA levels of both hEPO and IgG proteins were observed, which may be linked to higher transcriptional rates or increased mRNA stability, or a combination of both.

- By contrast, copper supplementation displayed cell line-specific effects at the range of concentrations tested. While SK15 displayed increased hEPO titers with no changes in VCD and viability profiles, DP12 cells growing in medium supplemented with copper were observed to be negatively impacted in both of these parameters.
- Increased oxygen consumption rate (OCR) – indicative of oxidative respiration metabolism – in zinc-supplemented producing cultures was observed. Increased oxidative respiration was also observed to correlate with the increased titer profiles in both cell lines.
- Zinc increases specific productivity but decreases cell growth. This result suggest that zinc supplementation strategies at stationary/production phases on the cultures might be suitable for enhancing CHO final titers.
- The work described above was published in Cytotechnology (doi: 10.1007/s10616-019-00334-1)

Chapter 2: An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals

- Based on the polyamine-dependence phenotype of CHO cells and due to the limitations (lack of stability and toxicity of amplifying/selecting agents) in the current selection systems used by the biopharmaceutical industry (GS and DHFR) for the generation of stable cell lines, an alternative cytotoxic-free selection system was developed.
- The efficacy of the newly developed auxotrophic selection system was reported. The method was shown to sustain the survival and growth of arginase-expressing transfected cell pools in a polyamine- and L-ornithine-free environment, supporting the isolation of GFP and recombinant therapeutic protein (hEPO) expressing single-cell derived populations.
- This arginase-based system avoids the use of toxic selective agents and selection can be performed in low putrescine medium, such as the commercial DMEM-F12. Consequently, this method offers an easy-to-apply, cost-effective alternative to the GS and DHFR selection, with no need for customised media.
- Clones generated from this system can be maintained for at least 42 generations in selecting media, demonstrating considerable stability in their production and growth profiles. Although clonal stability monitoring in industry is performed for periods over 60 generations, these results indicate initial evidence for stable production, which is of direct value to potential biopharma scientists in industry and academia.
- Clones can be also maintained in putrescine-containing media and in commercial formulations. In both cases, productivity can be maintained for at least 27 generations. This

indicates that cell lines can be generated in putrescine-free media but modifications in media formulations for bioreactor-scale production cultures are not required.

- The system may also present broader applicability than CHO, as other biopharmaceutical relevant cell lines displaying low arginase were identified (e.g. SP2).
- Due to the lack of drug treatment and easy-to-use method designed, the system may be also applied to non-biopharmaceutical relevant cell lines that display low or lack of arginase (Capan-2, PANC-1) for the generation of recombinant cell lines.
- The system may be also used in conjunction with GS or DHFR methods, offering an alternative to antibiotic-based selection for the generation of double transfectants.
- In response to critical levels of intracellular polyamine, active transport mechanisms participate in the maintenance of polyamine homeostasis decreasing or increasing the intracellular levels by exporting/importing polyamines from the environment. This effect may result in a mixed population of non-transfected and transfected cells due to the overproduction of polyamines in high expressing clones and the uptake from non-transfected cells. For this reason, single-cell cloning is essential. Moreover, it has to be considered that the possible lack of stability of clonal-derived populations may lead to inefficient mixed populations.
- Compared to the GS/DHFR system, the arginase-based system attempts to avoid the use of cytotoxic drugs. However, this may lead to the obtaining of lower producers compared to the amplified GS/DHFR system.
- From the activity of arginase, L-ornithine and the by-products urea are generated, intracellular accumulation of which could lead to cytotoxic effects.
- Following further investigations on the selection system, a single resistant parental CHO culture was obtained, displaying increased arginase activity. This effect indicated that arginase in CHO may be epigenetically repressed, but under high external pressure (with lack of polyamines) the repression may be reversed.
- The work described above was published in Journal of Biological Chemistry (doi: 10.1074/jbc.RA119.011162).
- Due to the novelty, efficacy and applicability of the system designed, intellectual property protection has been filed for the work described under patent application number 1911023.8 (Titled: Transfection selection and polypeptide or RNA expression; Authors: Berta Capella Roca, Martin Clynes, Niall Barron and Padraig Doolan). The research outlined has been the result of the work performed with my supervisors (Prof. Martin Clynes and Dr. Padraig Doolan) and with the collaborative support of Prof. Niall Barron (NIBRT). This process has been possible to the collaboration with DCU's KTI (Invent) and Jonathan Myers (Barker

Brettell LLP) that have contributed to the preparation of the invention disclosure documents and novelty research.

Chapter 3: Altered Gene expression in CHO cells following polyamine starvation

- Polyamines play a central but still not fully understood role in cell proliferation and even less knowledge is available on the role they play in CHO. Due to the lack of arginase activity of these cells, CHO provide an interesting platform to study the effects at a cellular and molecular level of polyamine deprivation, without the need for chemical inhibitors of polyamine biosynthesis. An investigation of the impact of polyamine removal on gene expression was therefore performed.
- Due to the multiple effects of polyamines at both transcription and translational levels, the use of an -omics platform (Affymetrix CHO whole transcriptome microarray chips) was used to gain insights into the vital cellular functions polyamines play in CHO.
- Starving CHO-K1 cells of the essential culture additive putrescine resulted in an accumulation of cell populations at two stages of the cell cycle: S-phase and G2-phase. This effect was correspondingly observed at transcriptomic level, with the overexpression of genes related to S-phase transition and arrest in M/G1.
- Increased expression of genes involved in DNA repair (such as RAD51, BRCA2) and RNA splicing (genes from the spliceosome complex) was also observed to be correlated with the large amount of cells observed to be found in the S-phase following putrescine starvation.
- Removal of polyamines was also significantly associated with a decreased expression of genes related to ER stress and apoptosis pathways.
- Polyamine-deprivation displayed a major impact on the increased expression of genes previously reported to be affected by polyamine levels or suggested to interact with these amines (transferrin receptor, thrombospondin 1, CXCR7, EXTL1). These genes were also described as cell membrane proteins and receptors involved in cell proliferation. These data suggest that these genes may contribute to activation of proliferating pathways or polyamine transport.

Chapter 4: Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO cells

- An in-house SFM supplemented with common media additives was used to assess the inhibitory effects of these supplements in transfections mediated by three chemical

transfection agents: liposomal-based (Lipofectamine 2000), polymer-based (TransIT-X2), and lipopolyplex-based (TransIT-PRO) were assessed.

- Removal of FAC from the medium resulted in increased/successful transfections in all three transfection methods but lower viabilities were observed. To circumvent this negative effect early addition of FAC (0.5-5 h post-transfection) was tested, revealing 0.5 h post-transfection as the optimal time to supplement in order to achieve transfection efficiencies similar to -FAC medium, while retaining acceptable cellular viability profiles.
- From three commercial media formulations examined, differences in the efficiency and support of transfection were observed between formulations and transfection reagents. BalanCD medium did not support transfection with any of the reagents while CHO-S-SFMII supported transfection with all three transfection reagents. In ProCHO5, zero transfection was detected with liposomes, but high transfection efficiencies in both polymers and lipopolyplexes. From the results observed, the presence or absence of transfection-inhibitory molecules in the media formulations are likely to be the key factors contributing to this outcome.
- In BalanCD, analysis of the size of the DNA-complexes generated with the three transfection reagents were observed to be the biggest compared to the other media tested. However, transfections were not achieved with any of the agents tested in this medium. These results suggests a destabilization or aggregation of transfection complexes, possibly related to the media formulation.
- FAC affects the size of the DNA-complexes generated with liposomes and lipopolyplexes, increasing their sizes. This result suggest that for liposomes- and lipopolyplexes-mediated transfections, size may matter. This effect was not observed with DNA-polymers complexes.
- In medium containing FAC, the zeta potential of complexes formed with lipopolyplexes and polymers was affected, displaying negative potentials that could induce a lack of complex-cell membrane interaction. In liposomes-complexes, a negative zeta potential was detected independent of the presence of FAC. This result indicates that liposomes might interact with the cell membrane with a separate system to electrostatic interactions.
- Differing effects of FAC on the zeta potential and size particles observed between the three transfection reagents indicates that the mechanistic inhibitory effect of FAC may be different for each transfection reagent, most likely based on the underlying chemistry of each.
- From combined data of MFI and percentage of GFP expressing cells, the polymer-based TransIT-X2 transfection agent in combination with the commercial medium CHO-S SFM-II (in our conditions) was identified to display the highest transfection efficiencies and expression levels.

6. Future work

Chapter 1:. Zinc supplementation increases protein titer of recombinant CHO cells

- As one of the implications of this research is that zinc should be considered as a beneficial additive in the formulation of a suitable media for CHO culture, product quality from cells cultured in zinc-supplemented medium should be assessed in terms of glycosylation patterns due to the involvement of this trace metal as a co-factor of glycosyltransferases. As per previously published research (Prabhu, Gadre and Gadgil, 2018), it is possible that lower galactosylation patterns might be a consequence of zinc supplementation - in this case, a double supplementation with increased manganese (strong co-factor of galactosylatses) could be attempted to recover galactosylation patterns
- At concentrations of zinc of 25mg/L, titer was enhanced but VCD and viability were negatively affected. Similar to Zn-25, supplementation of 10mg/L (second highest concentration tested) resulted in increased titer compared to non-supplemented cultures. However, at this concentration, little or zero (depending on cell line) negative effects were observed in viability and VCD profiles. These results suggest that study of the effects of zinc supplementation in the range of 10-25mg/L may identify an optimised concentration to achieve high biomass, high specific productivity and extended viability profiles.
- Zinc enhances the final titer of the cultures but decreases cell growth. The effect suggest that supplementation of zinc at later stages of the culture may increase final titers. To test this possible titer-enhancing strategy in a fed-batch operation mode, CHO cells could be cultured in growth media (devoid of zinc) until reaching high cell densities. At this stage, feeding with media containing zinc could result in increased titers.
- Increased mRNA levels of both products (IgG and hEPO) were observed following high zinc supplementation. To investigate whether this effect was a result of increased mRNA stability, cultures growing in zinc-supplemented medium could be treated with actinomycin D (a transcription inhibitor). Samples should be then collected at several time-points post-treatment (e.g. every 30min or 1h) to analyse the differences in mRNA levels (e.g. by RT-qPCR) of both recombinant proteins over time. The results should be compared to a non-zinc supplemented control culture.
- Intracellular zinc homeostasis is regulated by several systems, such as the expression of transporters that distribute free zinc ions among cells. Increased OCR levels (indirectly associated to an increased oxidative respiration metabolism) were observed following zinc-treatment. Identifying if zinc accumulates in the mitochondria could assist in the understanding if a possible link between zinc and the oxidative respiration metabolism exists.

In order to gain insight in the mechanistic function of zinc, this study could be performed in two ways. One, samples of zinc-supplemented and non-supplemented cultures could be collected for the analysis of zinc transporters at proteomic level by using western blots. Alternatively, the localisation of intracellular supplemented zinc could be followed by using fluorescently labelled zinc (such as Zinpyr-1). After treatment with this labelled zinc, the mitochondria could be dyed (such as MitoFluor Red 589) and by using fluorescence microscopy, co-localisation of both fluorophores could be assessed.

- The increased titer profiles were associated with higher OCR levels, an indication of increased oxidative respiration metabolism (OXPHOS) and were also correlated with high zinc supplementation. For this reason, there may be a potential connection between the three parameters (titer, OXPHOS and zinc). For example, it could be that zinc induces a switch to OXPHOS metabolism and this results in increased titers. A second hypothesis is that zinc increases recombinant protein production (e. g. increased mRNA stability) which leads to a switch in the metabolism to reach the energetic levels required for high production. To gain insight into the mechanistic effect of zinc, a metabolomic approach could be taken by analysing changes in the intracellular and extracellular levels of metabolites by Gas-chromatography-mass spectrometry (GC-MS). This analysis should be performed in non-producer and very high producer cells, to identify titer-enhanced effects or metabolism-switch effects (or both). If changes in the oxidative respiration of non-producers are observed in zinc-supplemented medium, an indication that zinc can induce a switch of metabolism would be obtained. Similarly, if very high producers are used (in which the supplementation of zinc has no effect on productivity) and changes in the metabolism are observed, zinc could be indicative of this effect. With this approach, further insights on the mechanism and possible implications of zinc as titer-enhancer that could be used for optimisation of the culture parameters would be obtained. Moreover, information on the changes of metabolites would also give an indication of possible limiting metabolites to improve the process (in terms of VCD and/or viability), which could lead to increased titers.

<u>Chapter 2: An arginase-based system for selection of transfected CHO cells without the use of toxic chemicals</u>

- In order to isolate highly producing clones while avoiding the use of cytotoxic agents, an altered arginase marker displaying low activity should be used. The increased stringency of this selection strategy would force the cells to increase the gene copy number or integrate the maker (and the recombinant protein) into a chromosomal “hot spot” – highly expressed site.

Consequently, this strategy would ultimately result in high producer clones. The arginase could be modified by including random mutations or using a less efficient codon code. The suitable modified arginase to be used should be based on the activity levels displayed, prioritising low activity levels but sufficient to support growth.

- The use of a different expression vector strategy could also increase the expression of recombinant protein and enable the isolation of high producers, following the same principle as per the attenuated arginase strategy described above. This strategy would consist of having the expression of arginase and the recombinant protein controlled by two different promoters: a strong promoter for the expression of recombinant protein (such as cytomegalovirus (CMV) or human elongation factor 1α promoter (EF1A)) and a weak promoter controlling the expression of arginase (such as thymidine kinase promoter (TK) or human ubiquitin C promoter (UbC)). Comparison of this approach with the attenuated arginase should be performed to determine the best strategy to achieve high production.
- Arginase generates L-ornithine to be converted into putrescine by ornithine decarboxylase (ODC). ODC is a rate-limiting enzyme that participates in the regulation of intracellular polyamine content. At high arginase expression, increased production of polyamines may be induced, leading to the inhibition of ODC activity. This effect may then lead to the accumulation of L-ornithine, which can cause cytotoxic effects. At the same time, the conversion of arginine to L-ornithine generates the by-product urea, accumulation of which is also toxic for the cells. For this reason, the intracellular levels of L-ornithine should be assessed to determine whether accumulation is occurring (e.g. using the Ornithine Assay Kit (Fluorometric), Biovision). If confirmed that L-ornithine accumulation is in fact occurring as a consequence of using the technology, this result would highlight a possible limitation of the described novel system. Intracellularly, detoxification of ornithine and urea is achieved though the urea cycle. However, a previous study reports that there is a lack of expression of this pathway in CHO (Altamirano *et al.* 2013). Hence, to overcome the possible accumulation of L-ornithine and urea, the use of CHO cell lines overexpressing the urea cycle (such as the reported CHO-OTC1-A19 (Chung *et al.* 2003), expressing carbamoyl-phosphate synthase 1 (CSP1) and ornithine transcarbamylase (OTC)) may be necessary, potentially resulting in the detoxification of these toxic by-products.
- The system developed is not limited to just biopharmaceutical production; offering an easy-to-apply, cost-effective and drug-free alternative to the still widely used antibiotic-based selection system in the academic sector. For this reason, assessment of the arginase activity of other mammalian cell lines and generation of arginase knock-out cell lines should be performed to extend the applicability of the system. Moreover, the threshold arginase activity tolerated for the system to successfully achieve the isolation of expressing-populations should

be performed in different cell lines to determine whether knock-out are necessary. To achieve this, mammalian cell lines (such as the pancreatic Panc-1 and Capan-2 described in this study) should be placed in serum-free media lacking polyamines and L-ornithine and growth and survival should be assessed. Parental cells being unable to support growth would be then suitable for selection. Combination of the arginase activity and survival data in polyamine-free media would then help indicate the threshold arginase activity levels needed to determine whether a cell line requires or not an arginase knock-out to be used with our system.

- Due to the possibility to obtain resistant colonies, an arginase knock-out CHO cell line (using e. g. CRISPR/cas9 technology) should be developed.
- Improvement of the SFM-F10 medium (completely devoid of putrescine content) should be performed in order to increase the stringency of the selection process, while achieving high VCD and viabilities of the expressing populations. This process should be performed by adjusting the composition of SFM-F10 (based on DMEM:F10) to a similar formulation to the SFM-F12, used for the development of producer cell lines during this study (based on DMEM-F12).
- Possible mixed populations of non-transfected and transfected cells may be obtained due to the release of polyamines by high expressing clones and their uptake from non-transfected cells. For this reason, attenuation of the polyamine transport system should be performed by knocking out (CRISPR/cas9) some of the transporters. Knowledge on the polyamine transport systems is still limited, but at least three transporters have been identified (SLC22A16, SLC3A2, DAX). Due to the importance of polyamine homeostasis to maintain cell survival, individual knock-outs (instead of all three combined in a single KO) should be performed, which would result in an attenuation of the transport system. This process could ultimately result in a decrease of the non-transfected background populations.

Chapter 3: Altered gene expression in CHO cells following polyamine starvation

- From the data obtained by microarray analysis, the expression of genes involved in pathways related to cell proliferation (cell cycle, DNA repair, RNA synthesis, apoptosis and ER) were observed to be significantly affected. Due to the relevance of these pathways to explain the phenotype observed when removing polyamines (lack of growth), further validation of these results should be performed by RT-qPCR in order to confirm the results observed.
- Intracellularly, polyamines are mainly found interacting with RNA, being described to regulate the translation of several mRNAs. For this reason, proteomic profiling by mass-spectrometry would be interesting to further understand the involvement of polyamines in

CHO proliferation and, at the same time, validate the results on cell cycle observed at mRNA level.

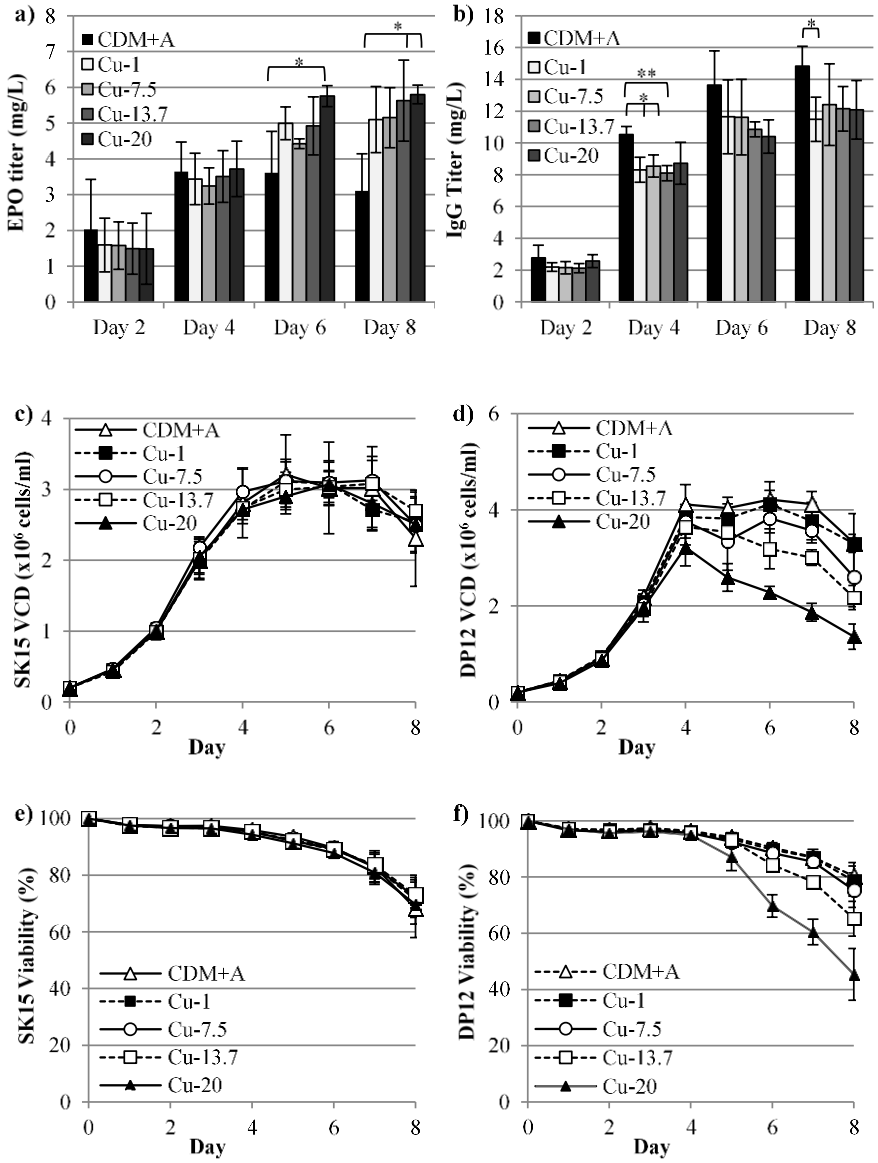
- The pathways identified to be affected during the microarray analysis are regulated by phosphorylation. This phenotype indicates that study not only of the overall proteome expression but focus on the changes in the phosphorylation state of the proteins should be assessed to correlate the changes with possible activation/deactivation of the pathways. This approach could be achieved by analysing the samples in mass-spectrometry using protocols for phosphopeptide enrichment (as described in Kaushik *et al* 2018).
- Analysis of the changes at miRNA should be also performed (with microarrays), due to the regulatory role of this small RNAs on the expression of mRNAs.

Chapter 4: Investigation and circumvention of transfection inhibition by ferric ammonium citrate in serum-free media for CHO cells

- Although a protocol was designed to circumvent the negative effects of FAC during transfection, a possible further optimisation of the process could be achieved by testing whether alternative iron sources (such as iron sulphate) are also inhibitory of cell transfection. This assessment should be tested with the three transfection agents. Moreover, iron sources to be used have to display similar cell performance (in terms of growth and viability) to FAC in order to avoid media changes between cell culture and transfection processes.
- To gain more insight into the inhibitory-effects of FAC, use of labelled DNA (with e.g. rhodamine or fluorescein) in order to follow the transfection process and determine whether the complexes in FAC-containing medium interact with the membrane, get into the cells, achieve endosomal escape and/or get into the nucleus. Combining this approach with the GFP results could give indications on whether FAC has an impact on the expression of the DNA.

Appendices

Appendix A (Chapter 1 supplementary material)



Supplementary Figure 1. Titer (a, b), VCD (c, d) and Viability (e, f) of SK15 (a, c, e) and DP12 (b, d, f) cells grown in suspension in in-house chemically-defined medium CDM+A supplemented with copper at: 1 mg/L (Cu-1), 7.5 mg/L (Cu-7.5), 13.7 mg/L (Cu-13.7) and 20 mg/L (Cu-20). Statistical differences in titer data compared to the control (CDM+A) are represented as: p-value <0.05 (*) and <0.01 (**).

Appendix B (Chapter 2 supplementary material)

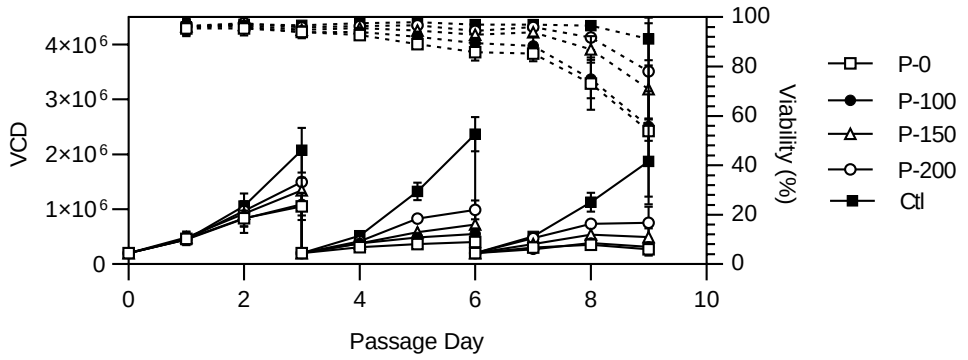


Fig. S1. CHO-K1 cells require concentrations over 200µg/L putrescine for healthy growth. Viable cell density (continuous lines) and viability (discontinuous lines) of parental CHO-K1 cells in SFM-F12 medium supplemented with different concentrations of putrescine: 0µg/L (Put-0), 100µg/L (Put-100), 150µg/L (Put-150) and 200µg/L (Put-200) compared to optimal putrescine concentration (control, 1mg/L). Triplicates were performed per each condition.

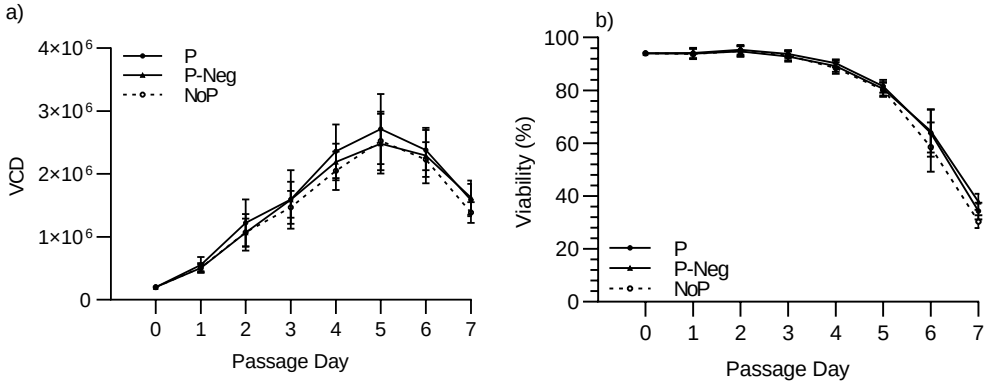


Fig. S2. CHO-K1 cells expressing arginase recover growth profiles similar to parental cells in putrescine media. Viable cell density (VCD) (a) and viability (b) of CHO-K1 arginase expressing populations in SFM-F12 medium without putrescine (NoP) and medium with putrescine (P) compared to parental CHO-K1 cells in SFM-F12 medium containing putrescine (P-Neg). Triplicates were performed per each condition.

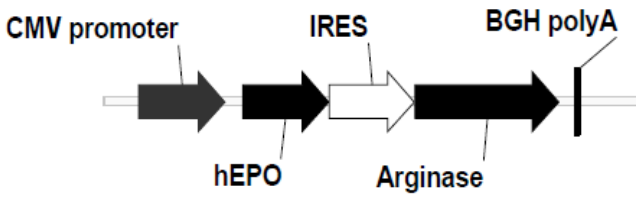


Fig. S3. hEPO-IRES-Arg Bicistronic map (generated in Vector NTI Advance10 (Invitrogen)).

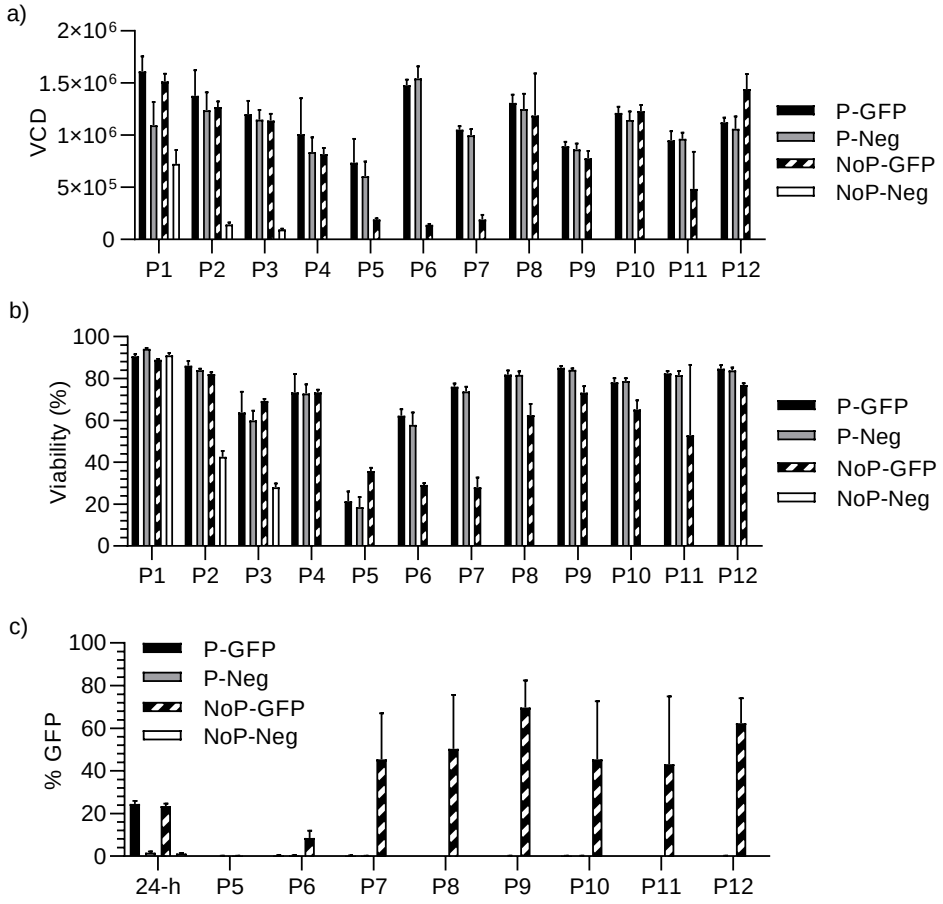


Fig. S4. GFP-expressing populations can be isolated in polyamine-free SFM-F10 medium. Viable cell density (VCD) (a), viability (b) and percentage of GFP expressing (c) CHO-K1 cells transfected with 500ng GFP-IRES-Arg vector (P-GFP, NoP-GFP). A negative control of cells with no DNA transfected (P-Neg, NoP-Neg) was included. The NoP-GFP culture was discontinued at P4 as viabilities dropped to less than 30%. Transfected and non- transfected cells were placed in either SFM-F10 medium supplemented with putrescine (P) or selecting SFM-F10 medium (without putrescine, NoP). Triplicate wells were transfected per each condition.

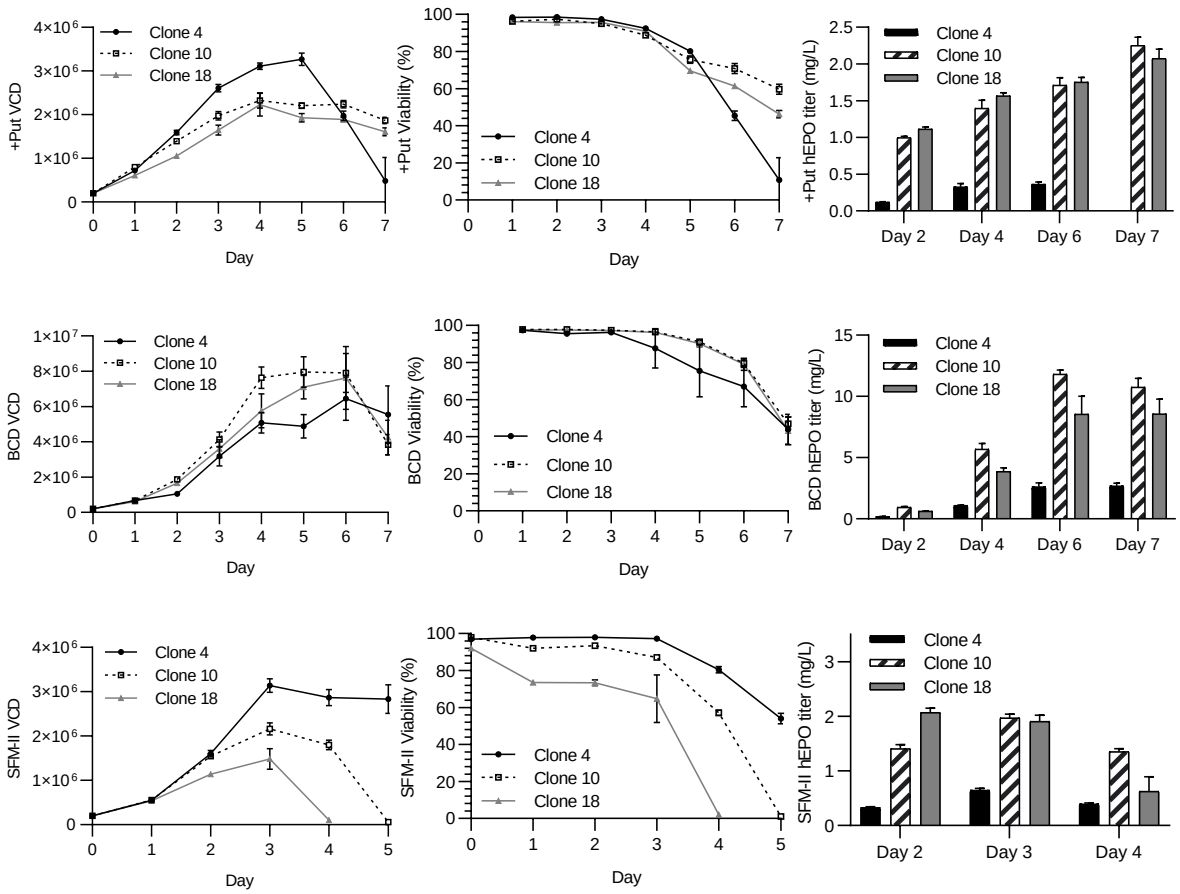


Fig. S5. Characterisation of hEPO-expressing clones in commercial and SFM-F12 media containing putrescine in batch cultures. Viable cell density (VCD), viability and hEPO titer (mg/L) of three hEPO-expressing clones in SFM-F12 medium with putrescine (+Put), BalanCD Growth A supplemented with L-glutamine and CHO-S SFM-II supplemented with PVA in 5ml batch cultures for 7 days. Biological triplicates and duplicate (VCD, viability) or triplicate (titer) technical readings were performed for each phenotype.

Appendix C (Chapter 3 supplementary material)

Supplementary Table 1. Primers used for qRT-PCR validation

Gene	Fwd (5'-> 3')	Rev (5'-> 3')
Skp2	GGTCCTTTATGGAGCAACCA	CCACTGCAGATTCGGAAAAT
Tfdp1	CCTCCCAACTCTGTCATCCA	CTTCAGCAGAACAGTTCCCC
Mad2l1	GGAAGAATCAGGACCCCAGT	AGGGATTTTGTAGGCCACCA
Ddit3	CTAGACTCCGCATCCCTAGC	ACTGACCACTCTGTTTCCGT
Thbs1	TTCCTGTTGCATGTGTGTGG	GGGAATCAGGTTGGCGTTTT
Ackr3	ATCTTGAACCTGGCCATTGC	CTGGTTATGCTGCACGAGAC
Tfrc	CACTTCCTGTCACCCTACGT	AAAGCCGTGAGAGTGTGAGA
Pacrg	GCATCTAGTTGTGTCAGCGG	TCGCCAATGTTCTCCCTCTT
PMF-1	TCCTGAGGTGACAAAGTGGG	CCAGGGAGTTCAAGACAGCT
Tbc1d2	CATACTGCCGGCTGAGTACT	TCACTGATGAGAGAGTCGGC
Extl1	GATGGTGGGCTTTCTGACAC	AGTGGGTAAAGAGGGTGTGG
Gapdh	TGGCTACAGCAACAGAGTGG	GTGAGGGAGATGATCGGTGT

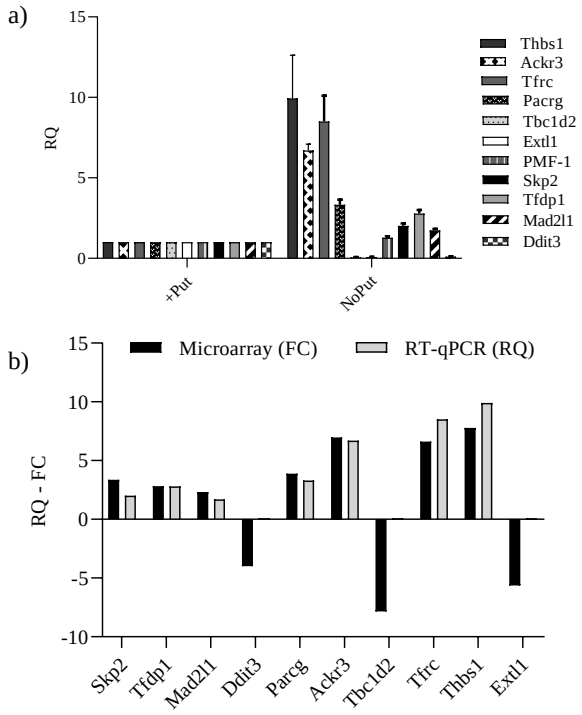
Supplementary Table 2. List of the 50 most compelling up-regulated and down-regulated differentially-expressed genes considering fold-changes.

Gene Symbol	Description	Fold Change	FDR P-val
Thbs1	thrombospondin 1	7.79	4.25E-05
Ackr3	atypical chemokine receptor 3	6.97	2.06E-05
Tfrc	transferrin receptor	6.62	1.07E-05
Chst2	carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2	4.58	0.0002
LOC103162148	uncharacterized LOC103162148	4.45	0.0002
Znf483	zinc finger protein 483	4.24	2.13E-05
Rnft2	ring finger protein, transmembrane 2	3.95	1.62E-05
Arhgef16	Rho guanine nucleotide exchange factor (GEF) 16	3.93	1.80E-05
Pacrg	PARK2 co-regulated	3.89	8.71E-05
Slc17a6	solute carrier family 17 (vesicular glutamate transporter), member 6	3.72	0.0021
E2f8	E2F transcription factor 8	3.59	2.06E-05
LOC100757526	putative P2Y purinoceptor 10	3.55	6.54E-05
Rab7b	RAB7B, member RAS oncogene family	3.52	4.17E-05
LOC100774651	cysteine-rich protein 1	3.47	2.06E-05
Rab7b	RAB7B, member RAS oncogene family	3.44	1.98E-05
Immp1l	IMP1 inner mitochondrial membrane peptidase-like (S. cerevisiae)	3.42	2.89E-05
Arhgef37	Rho guanine nucleotide exchange factor (GEF) 37	3.4	4.40E-05

Skp2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	3.37	0.0002
LOC100774336	von Willebrand factor A domain-containing protein 5A-like	3.33	0.0001
Serpinb1	serpin peptidase inhibitor, clade B (ovalbumin), member 1	3.33	1.80E-05
LOC100753285	uncharacterized LOC100753285	3.29	0.0001
Col6a1	collagen, type VI, alpha 1	3.23	0.0002
Ccne2	cyclin E2	3.2	6.25E-05
LOC100761037	serpin B6-like	3.19	0.0013
Pdss1	prenyl (decaprenyl) diphosphate synthase, subunit 1	3.16	1.87E-05
Mid1	midline 1 (Opitz/BBB syndrome)	3.16	5.03E-05
Polk	polymerase (DNA directed) kappa	3.14	0.0002
Eda2r	ectodysplasin A2 receptor	3.12	0.0107
Col3a1	collagen, type III, alpha 1	3.12	4.60E-05
Gtf2f1	general transcription factor IIF, polypeptide 1, 74kDa	3.1	2.06E-05
Rgs16	regulator of G-protein signalling 16	3.08	3.79E-05
Il6	interleukin 6	3.08	0.0001
Col12a1	collagen, type XII, alpha 1	3.05	6.76E-05
Atp2b4	ATPase, Ca++ transporting, plasma membrane 4	3.05	1.98E-05
Apool	apolipoprotein O-like	3.04	2.89E-05
Traip	TRAF interacting protein	3.04	2.13E-05
Cdh9	cadherin 9, type 2 (T1-cadherin)	3.04	4.51E-05
Zwint	ZW10 interacting kinetochore protein	2.99	2.06E-05
Taf9b	TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa	2.97	0.0001
Hspg2	heparan sulphate proteoglycan 2	2.96	5.60E-05
Pcdh7	protocadherin 7	2.95	6.18E-05
Crip1	cysteine-rich protein 1 (intestinal)	2.94	8.45E-05
Fus	fused in sarcoma	2.93	2.89E-05
Rad51ap1	RAD51 associated protein 1	2.91	2.37E-05
LOC100773941	low-density lipoprotein receptor-related protein 1B	2.9	2.13E-05
Dna2	DNA replication helicase/nuclease 2	2.89	5.30E-05
Clspn	claspin	2.87	5.21E-05
Nqo2	NAD(P)H dehydrogenase, quinone 2	2.86	3.97E-05
Gpc6	glypican 6	2.86	2.89E-05
Adm	adrenomedullin	2.85	7.91E-05
Cornifin-a	cornifin alpha	-13.85	2.37E-05
Tbc1d2	TBC1 domain family, member 2	-7.85	2.13E-05
Cd68	CD68 molecule	-7.81	2.13E-05
Trib3	tribbles pseudokinase 3	-7.54	1.07E-05
Ypel3	yippee-like 3 (Drosophila)	-6.84	1.62E-05

Slc6a9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9	-6.84	1.62E-05
Gtpbp2	GTP binding protein 2	-6.39	6.82E-06
Atf3	activating transcription factor 3	-6.19	2.06E-05
Chac1	ChaC, cation transport regulator homolog 1 (E. coli)	-5.7	1.07E-05
		-5.65	1.62E-05
Extl1	exostosin-like glycosyltransferase 1	-5.63	2.06E-05
		-5.31	4.52E-05
		-5.27	1.80E-05
		-5.16	9.51E-05
Exd1	exonuclease 3-5 domain containing 1	-5.03	1.80E-05
LOC100750381; LOC103162814	interferon-inducible protein AIM2; uncharacterized LOC103162814	-4.82	2.06E-05
LOC100762031	interferon-induced GTP-binding protein Mx2	-4.71	2.13E-05
Abhd6	abhydrolase domain containing 6	-4.7	4.40E-05
		-4.68	1.62E-05
Herpud1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	-4.66	1.17E-05
Rnf157	ring finger protein 157	-4.57	4.51E-05
LOC100773612	ribosomal protein S6 kinase alpha-2	-4.48	1.80E-05
Atf3	activating transcription factor 3	-4.45	8.05E-05
Ccbe1	collagen and calcium binding EGF domains 1	-4.31	3.43E-05
		-4.29	0.0003
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-4.25	3.43E-05
Klf8	Kruppel-like factor 8	-4.23	1.62E-05
LOC103161775	uncharacterized LOC103161775	-4.22	3.95E-05
Fat4	FAT atypical cadherin 4	-4.09	6.79E-05
Dgat2	diacylglycerol O-acyltransferase 2	-3.99	2.89E-05
Klhl24	kelch-like family member 24	-3.98	1.98E-05
Ddit3	DNA-damage-inducible transcript 3	-3.98	1.80E-05
Bfar	bifunctional apoptosis regulator	-3.86	1.80E-05
Uso1	USO1 vesicle transport factor	-3.85	1.98E-05
		-3.79	1.62E-05
LOC100754734	25-hydroxycholesterol 7-alpha-hydroxylase	-3.78	7.89E-05
Tmem140	transmembrane protein 140	-3.76	1.87E-05
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1	-3.75	3.95E-05
Mroh1	maestro heat-like repeat family member 1	-3.73	2.89E-05
LOC103161640	NKG2D ligand 1-like	-3.67	0.0002
Rwdd3	RWD domain containing 3	-3.66	0.0001
Elk3	ELK3, ETS-domain protein (SRF accessory protein 2)	-3.58	2.76E-05
Amdhd2	amidohydrolase domain containing 2	-3.54	0.0001
Pdk1	pyruvate dehydrogenase kinase, isozyme 1	-3.52	2.13E-05

Clybl	citrate lyase beta like	-3.41	2.06E-05
Sat1	spermidine/spermine N1-acetyltransferase 1	-3.41	2.76E-05
Gabarapl1	GABA(A) receptor-associated protein like 1	-3.39	4.51E-05
C3ar1	complement component 3a receptor 1	-3.39	2.89E-05
Cln3	ceroid-lipofuscinosis, neuronal 3	-3.39	1.98E-05

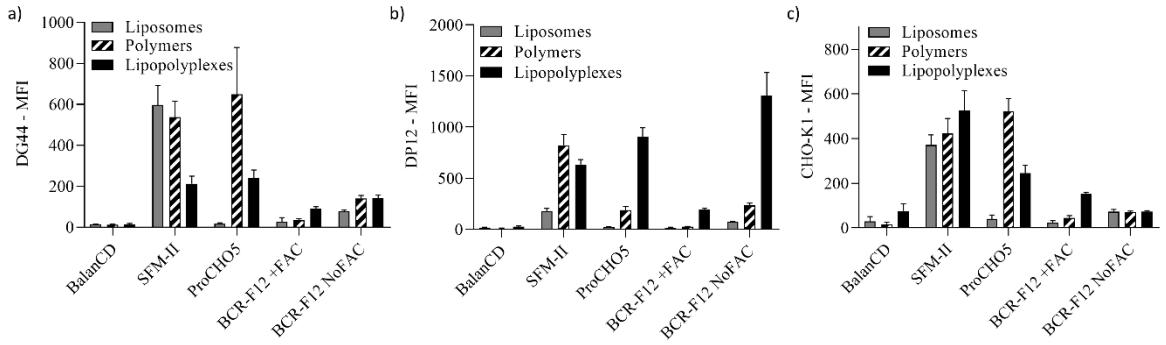


Supplementary Figure 1. (a) RT-qPCR of DE genes chosen for validation: *Skp2, Tfdp1, Mad2l1, Ddit3, Parcg, Ackr3, Tbc1d2, Tfrc, Thbs1, Extl1*. Biological and technical triplicates were performed. The endogenous gene *Gapdh* was used. (b) Comparison between fold-changes (FC) from microarray data and relative quantification (RQ) data from RT-qPCR for DE genes validated. The y-axis represents the values for FC and RQ of DE genes. Fold-changes and relative quantification are from DE genes expressed in polyamine-deprived media relative to the genes expressed in polyamine-containing conditions.

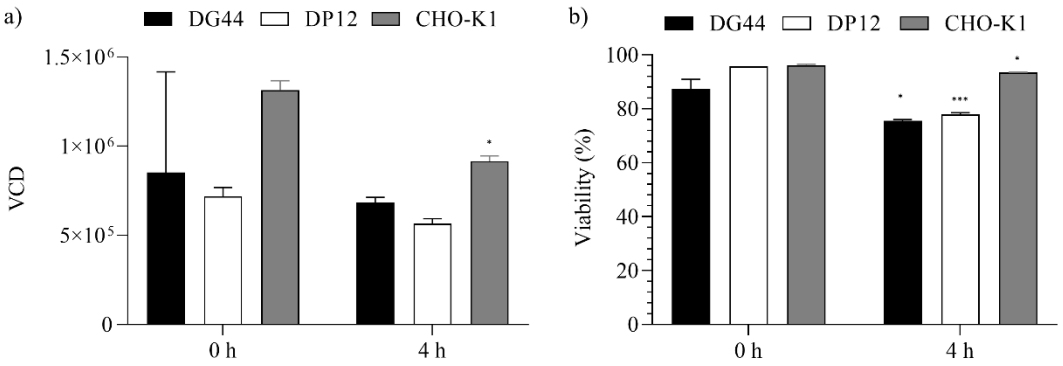
Appendix D (Chapter 4 supplementary material)

Supplementary Table 1. Effect of FAC supplementation at 0, 0.5 and 4 h post-transfection on the viability of DG44, CHO-K1 and DP12 cells 24-hours after transfection. Three transfection agents were tested: liposomes, polymers and lipopolyplexes. Transfections were performed in BCR-F12 medium without FAC. Supplementation of 100 µM FAC was added at 0 h, 0.5 h and 4 h post-transfection. Triplicate transfections were carried out for cell line in each condition (timepoint and transfection method combination). The BCR-F12 medium without FAC (- FAC) was used as control.

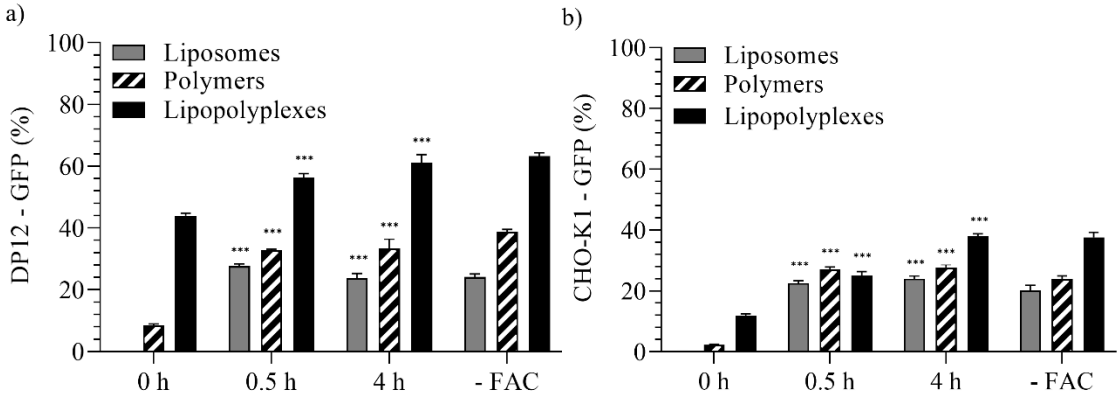
		FAC added at 0h post-transfection	**FAC added at 0.5h post-transfection**	**FAC added at 4h post-transfection**	**- FAC**
Liposomes	DG44	80 ± 1.5	67 ± 2.7	49 ± 2.8	48 ± 5.7
	CHO-K1	97 ± 0.6	84 ± 1.8	77 ± 2	66 ± 3.2
	DP12	91 ± 1	54 ± 1.1	46 ± 6.4	46 ± 3.8
Polymers	DG44	86 ± 2.1	83 ± 3.8	80 ± 1.2	71 ± 1.7
	CHO-K1	93 ± 0.4	85 ± 0.9	82 ± 1	77 ± 1.5
	DP12	90 ± 3.4	85 ± 0.9	75 ± 3.8	76 ± 1.5
Lipopolyplex	DG44	88 ± 1.1	83 ± 2.5	84.1± 1.6	84 ± 0.8
	CHO-K1	95 ± 0.8	91 ± 1.3	82 ± 3.1	77 ± 1.3
	DP12	88 ± 1	79 ± 1.3	71 ± 1.4	65 ± 3.5



Supplementary Fig 1. Mean fluorescent intensity (MFI) levels in different media. DG44 (a), DP12 (b) and CHO-K1 (c) cells 24-hours after transfection. Three transfection agents were tested: liposomes, polymers and lipopolyplexes. Transfections were performed in BalanCD, SFM-II, ProCHO5 and in-house BCR-F12+FAC (with FAC) and BCR-F12 NoFAC (without FAC). Triplicate transfections were carried out for each cell line in each condition (media and transfection method).



Supplementary Fig 2. VCD and viability of CHO cells with FAC supplementation at 4 h. VCD (a) and viability (b) of DG44, DP12 and CHO-K1 cells in BCR-F12 supplemented with FAC at 4 h post-seeding compared to cells in medium containing FAC (0 h). Statistical differences between VCD and viabilities observed at the 4 h FAC-supplemented medium compared to those in BCR-F12 with FAC (0 h) are represented as: $p < 0.001$ (***) and $p < 0.05$ (*).



Supplementary Fig 3. Effect of FAC supplementation at 0, 0.5 and 4 h post-transfection on the percentage of GFP expressing cells. DP12 (a) and CHO-K1 (b) cells 24-hours after transfection. Three transfection agents were tested: liposomes, polymers and lipopolyplexes. Transfections were performed in BCR-F12 medium without FAC. An amount of 100 µM FAC was supplemented at 0 h, 0.5 h and 4 h post-transfection. The BCR-F12 medium without FAC (- FAC) was used as control. Triplicate transfections were set for cell line for each condition (timepoint and transfection method combination). A negative control well was included for each condition, displaying between 0.0 - 0.2 % GFP (data not shown). Statistical differences between transfection efficiencies observed in medium supplemented with FAC at 0.5 and 4 h compared to the ones supplemented at timepoint 0 h are represented as: $p < 0.001$ (***).