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Characterization of cellular states of CHO-K1 suspension cell culture through cell cycle and RNA-sequencing profiling

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ABSTRACT

Chinese hamster ovary (CHO) derived cell lines are the preferred host system for the production of therapeutic proteins. The aim of this work was to explore the regulation of suspension-adapted CHO-K1 host cell line bioprocesses, especially under a temperature gradient from 37 °C to 31 °C. We analyzed cell cycle behavior through flow cytometry of propidium iodide stained cells and high throughput transcriptome dynamics by RNA sequencing.

We found a cell culture state characterized by G0/G1 synchronization, mainly during the late exponential growth phase and towards the last days of the stationary phase. We successfully identified key genes and pathways connected with the particular culture states, such as response to low temperature, modulation of the cell cycle, regulation of DNA replication and repair, apoptosis, among others. The most important gene expression changes occurred throughout the stationary phase when gene up-regulation markedly prevailed.

Our RNA-seq data analysis enabled the identification of target genes for mechanism-based cell line engineering and bioprocess modification, an essential step to translate gene expression data from CHO-K1 host cells into bioprocess-related knowledge. Further efforts aim at increasing desirable phenotypes of CHO cells, and promoting efficient production of high quality therapeutic proteins can highly benefit from this type of studies.

1. Introduction

The production of therapeutic recombinant proteins is one of the fastest growing areas within the pharmaceutical industry (Durocher and Butler, 2009; Rodney and Chien, 2014). At present, mammalian cells are the leading production system for these proteins since they can produce complex post-translational modifications (e.g. glycosylation) that are necessary for proper secretion, drug efficacy and stability.

Chinese hamster ovary (CHO) cells are the main platform commonly used for the industrial production of therapeutic proteins. Nearly 70% of biologics are produced in these cells (Jayapal et al., 2007; Butler and Spearman, 2014), because it has demonstrated several major benefits. CHO cells can be easily manipulated so as to achieve greater recombinant protein yields. Besides, these cells are capable of growing in suspension culture for large-scale production and in serum-free conditions, which generates a safety profile (Kim et al., 2012; Lai et al., 2013). Furthermore, this expression system is highly tolerant to variations in temperature, pressure, pH or oxygen levels throughout manufacturing (Ghaderi et al., 2012; Kim et al., 2012; Lai et al., 2013; Yang et al., 2014).

Nonetheless, the increasing need for biopharmaceuticals demands enhanced productivity. Current approaches involve optimization of culture conditions, medium formulation, reactor design and cell engineering (Kumar et al., 2008). However, despite its wide use, many aspects of CHO cell biology that could lead to improved protein production remain poorly understood. In this context, genome-scale technologies lead to a system-level analysis in order to understand the complex molecular basis of protein production in mammalian cells,

Abbreviations: CHO, Chinese hamster ovary; RNA-seq, RNA sequencing; TALENs, transcription activator-like effector nucleases; ZFNs, zinc-finger nucleases; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated protein-9 nuclease; FPKM, reads per kilobase per million mapped reads; PCA, principal component analysis; GO, gene ontology

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offering new perspectives for further process optimization.

In the past few years, based on the rapid advances in DNA sequencing technologies, many studies have expanded the -omics data for CHO cells. Those comprise the recent availability of CHO genome references (Xu et al., 2011; Brinkrolf et al., 2013; Lewis et al., 2013), transcriptome and proteome data (Baik et al., 2006; Baycin-Hizal et al., 2012; Becker et al., 2011; Ernst et al., 2006; Farrell et al., 2014; Nissom et al., 2006; Yee et al., 2008, 2009). They have led to new areas of study for better understanding of CHO cells metabolic behavior, with the long-term goal of developing new biologics.

CHO engineering work has made remarkable progress in optimizing cells with desired features. This is particularly interesting for host cell lines which can be used for product transgene introduction. Thus, the engineering of metabolic, secretory and growth control pathways of host cell lines plays an important role for increasing their growth and product secretion. Rapid and accurate modification of mammalian cell machinery has lately been facilitated by emerging genome editing technologies, for instance transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system (Kim and Kim, 2014). While empirical approaches for cell lines and processes development will remain essential for the industry, the need to deeply understand the relationships between cells and bioprocesses will increase.

Biphasic cultures are used in several bioprocesses (especially at industrial scale), in which the initial phase of rapid cell growth at 37 °C is followed by a growth arrest phase, induced by the reduction of the culture temperature (30–32 °C). This inhibition is associated with G1-phase cell cycle arrest and it is connected with many positive properties, such as improved productivity (by prolonging the stationary/production phase) and desirable cell viability. For this reason, the G1-phase is considered the appropriate stage for increasing the production of recombinant proteins. This approach has been used in CHO cell lines and hybridomas (al-Rubeai et al., 1992; al-Rubeai and Emery, 1990; Du et al., 2015; Dutton et al., 2006; Fussenegger et al., 1998; Ibarra et al., 2003; Kaufmann et al., 1999, 2001; Moore et al., 1997; Trummer et al., 2006; Vergara et al., 2014). Nevertheless, the mechanisms that regulate these phenotypes during mild hypothermia are still not completely understood.

While some studies have been focused on producer cell lines, limited transcriptomic information is available for CHO cells with regard to the effects of the variables applied during bioprocessing —especially low temperature— at industrial scale.

Thus, we studied the distribution of cell cycle phases by flow cytometry and we performed transcriptome analysis using next-generation RNA sequencing (RNA-seq), during typical industrial bioprocesses of suspension-adapted CHO-K1 cells. We explored the global changes in gene expression of biphasic cultures, i.e. following a reduction of the culture temperature from 37 °C to 31 °C. Our results indicate that the exposure of CHO-K1 cells to sub-optimal temperatures triggers a coordinated response, comprising the modulation of the cell cycle, energy metabolism and cell proliferation, and biological processes, such as DNA replication and repair, protein metabolism, cell communication, cell signaling and apoptosis. All the genes that exhibited significant expression changes can serve as potential targets for cellular and metabolic engineering with the purpose of generating a host cell line with desired features for product transgene introduction, improving CHO cell bioprocessing both at laboratory and at industrial scale.

2. Materials and methods

2.1. Cell culture

Firstly, two CHO-K1 independent cell cultures were carried out for cell cycle study. Suspension-adapted CHO-K1 cells were seeded at 6.0×10^5 cells/ml in 1 L bioreactors (Biostat Q-plus, Sartorius). The first culture lasted 22 days and the second culture, 18 days. The temperature was gradually decreased from 37 °C to 31 °C during the bioprocesses. Particularly, during the second cell culture the temperature was reduced earlier (on day 3). The first culture reached a maximum cell density of 3.0×10^7 cells/ml and the second culture 1.1×10^7 cells/ml, both maintaining around 90% viability. Since high cell concentrations were achieved during the first culture, three cell bleeds were necessary to maintain proper dissolved oxygen, glucose and lactic acid concentrations. A perfusion regime (spin-filter) with Ex-Cell 302 serum-free medium (Sigma) was applied.

Secondly, three replicates of CHO-K1 suspension-adapted cells were cultured in 1 L bioreactors (Biostat Q-plus, Sartorius) for RNA-seq analysis. Each bioreactor was inoculated with 6.0×10^5 cells/ml. A perfusion regime (spin-filter) of 1 L/day with Ex-Cell 302 serum-free medium (Sigma) was applied. One of the cultures lasted 12 days, and the other two cultures lasted 17 days. Identical operating conditions were applied (aeration, agitation, pH and foam control) and equilibrium in lactate and glucose concentrations was observed by the end of the processes. To avoid excessive cell proliferation, the temperature was gradually decreased from 37 °C to 31 °C, starting on day 3.

2.2. Cell cycle analysis

Daily samples were taken and DNA content was determined by propidium iodide (PI) staining (Darzynkiewicz et al., 2001) followed by flow cytometry analysis, using the BD FACSAria III system (BD Biosciences). DNA QC Particles kit (BD Biosciences) was used to perform quality control of the flow cytometer.

Each day 30,000 events were recorded inside the 'singlet' population gate defined by a PI-W vs. PI-A plot, thus excluding debris, doublets and aggregates. Cell cycle phase distribution was estimated using FlowJo v10 (Tree Star Inc.) and ModFit LT v5.0 (Verity). G0/G1 peak was detected automatically, while G2/M peak was adjusted by visual inspection, and the S phase was modeled as a rectangle of three equally spaced compartments.

2.3. RNA purification and cDNA sequencing library preparation

Bioreactor samples were collected on day 1, and during the exponential (days 7 and 8) and the stationary (days 14 and 15) growth phases from each culture replicate. RNA was extracted using the RNEasy Mini kit (QIAGEN), following the manufacturer's protocol. RNA concentration and quality were measured by NanoDrop2000 (Thermo Fisher) and Agilent2200 TapeStation (Agilent), respectively. All extracted RNA samples had RNA Integrity Number (RIN) > 8.5, and 13 samples were used for subsequent RNA-seq study. A DNase treatment step was carried out using a DNA-free kit (Ambion).

Messenger RNA was subsequently isolated from total RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs) and the indexed cDNA sequencing libraries were constructed using the NEBNext Ultra Directional RNA Library Prep kit (New England Biolabs) and the NEBNext Multiplex Oligos dual Index Primers Set 1 (New England Biolabs), according to the manufacturer's instructions. The concentration and quality of the libraries were measured by Qubit2.0 (Thermo Fisher Scientific) and Agilent2200 TapeStation (Agilent), respectively.

2.4. RNA sequencing and data analysis

Libraries were sequenced on the HiSeq 2500 instrument (Illumina), using a 1×50 bp single-end configuration. Quality control was performed using FASTQC v0.11.3 (Andrews, 2010) and quality trimming and adapter filtering with Trimmomatic v0.33 (Bolger et al., 2014).

An index of the currently available CHO-K1 reference genome (CriGri_1.0 RefSeq assembly accession **GCF_000223135.1**) was built using Bowtie v1.1.2, and the filtered reads were mapped to the genome

using STAR v2.5 (Dobin et al., 2013). Gene expression estimation (FPKM normalized values) and differential expression (DE) analysis between exponential and stationary phases were performed using Cufflinks package v2.2.1 (Trapnell et al., 2012) and HOMER v4.8 (Heinz et al., 2010), applying DESeq2.

Scatter plots of gene expression values between biological replicates were built in R software environment v3.2.5. Also, Gene Cluster v3.0 (de Hoon et al., 2004) and Java TreeView v1.1.6 (Saldanha, 2004) were used for hierarchical clustering analysis and cummeRbund R package (Goff et al., 2014) was used for Principal Component Analysis (PCA) of normalized expression data.

Gene Ontology (GO) enrichment and pathways analysis were carried out using PANTHER (Thomas et al., 2003) and Pathview (Luo et al., 2013) to visualize data on KEGG pathways (Kanehisa and Goto, 2000) for *Mus Musculus* organism, respectively. Significant differentially expressed genes (log2-fold-change ≥ 1 and FDR-adjusted p-value (Benjamini Hochberg) ≤ 0.05) from exponential and stationary phase, both compared to inoculation day, were taken into consideration.

2.5. Quantitative real-time PCR

To confirm CHO-K1 RNA-seq data, reverse transcription of RNA samples was performed using the SuperScript III Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions. Specific primers for 11 transcripts were designed to target exon-intron boundaries by Primer-BLAST (Supplementary Table S1). A standard curve was performed for each primer set to assess its efficiency, using a 5-fold serial dilutions of pooled cDNA and water as non-template control.

Quantitative real-time PCR was conducted in triplicate using SYBR green based qPCR amplification (SYBR green Master Mix, Thermo Fisher Scientific) on the Stratagene MX3005 P (Agilent) equipment with the following parameters: 50 °C for 2 min, 95 °C initial denaturation for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence was detected at the end of each cycle at 60 °C. GAPDH, a commonly used housekeeping gene, was found to be significantly regulated on the RNA-seq data. Therefore, Eif3i was chosen as an internal reference. Data analysis was performed using the delt A-D elta Ct method (Livak and Schmittgen, 2001).

3. Results and discussion

3.1. Cell cycle analysis shows a marked tendency towards G0/G1 synchronization of CHO-K1 cells cultured in 1 L bioreactors when a temperature gradient is applied

To analyze cell cycle behavior during a typical industrial bioprocess of suspension-adapted CHO-K1 cells we used two different bioreactors whose cell growth characteristics and culture parameters are described in Fig. 1.

On the one hand, the first culture exhibited a prolonged lag phase followed by a pronounced exponential growth phase, rapidly reaching cell concentrations around 3.0×10^7 cells/ml. Since the accelerated cell growth exceeded the ability to properly control the bioprocess, cell bleeds were performed on days 12 (1.5×10^7 cells/ml), 13 and 17 (1.0×10^7 cells/ml). The reduction of the culture temperature contributed to the system stabilization by progressively diminishing the metabolic activity. Cellular viability showed a stable behavior around 90% throughout the culture.

On the other hand, after a short lag phase, the second culture showed a fast growing exponential phase. Unlike the previous one, this bioprocess was rapidly stabilized (around 1.1×10^7 cells/ml) and cell bleed was avoided, probably because the temperature was controlled earlier. Through this process, cell viability remained around 90% and a gradual consumption of glucose was observed, without falling below detectable limits. In addition, lactate levels remained low.

Both bioprocesses characteristics are reflected in the cell cycle analysis. Cell growth profiles and daily percentages of cell cycle phases modeled using ModFit LT are illustrated in Fig. 2. Moreover, Supplementary Fig. S1 shows the representative DNA histograms on cell cycle profile of the second culture generated by ModFit LT (number of events vs PI-A).

During the first culture the maximum percentage of cells in G0/G1 phase (81%) was reached on the last day of the bioprocess, when the culture temperature had been at 32 °C for the previous 9 days. Meanwhile, in the second culture the maximum proportion of cells in G0/G1 (89.5%) was achieved early, on day 11 (at the beginning of the stationary phase), after only two days of having used a culture temperature of 31 °C.

For this last bioprocess, cells in S phase were gradually reduced as culture progressed, reaching a minimum percentage of 3.4% on day 10. Thus, a strong tendency towards G0/G1 synchronization was identified, particularly in the late exponential growth phase and during the last days of the stationary phase. More than 80% of the cells were in G0/G1 during the whole stationary phase. This is probably due to the early marked temperature gradient applied during the bioprocess to properly control metabolites (glucose and lactate), applying a lower perfusion volume and avoiding cell bleed.

Furthermore, after cell bleeds during the first culture (days 12, 13 and 17), we observed that the percentage of S phase cells slightly increased each time before declining again. This could be due to the fact that cell proliferation is boosted by the available nutrients being incorporated into the non-conditioned medium. In addition, the percentage of cells in the G2/M phase was relatively constant in the first culture while in the second one a slight reduction of cells in G2/M during the late exponential phase was also observed.

An evident trend in all these results is that the earlier the temperature gradient is applied, the sooner cells arrest in G0/G1 phase, resulting in a major bioprocess performance control by shortening

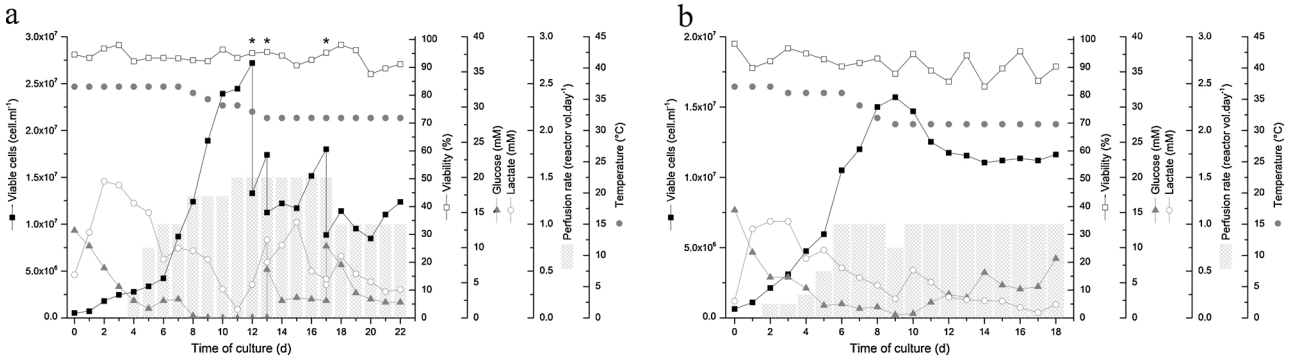

Fig. 1. CHO-K1 cell cultures in 1 L bioreactors in perfusion mode. Profiles of cell density, cell viability, glucose, lactate, perfusion rate and temperature are shown. Asterisk characters indicate cell bleeds.

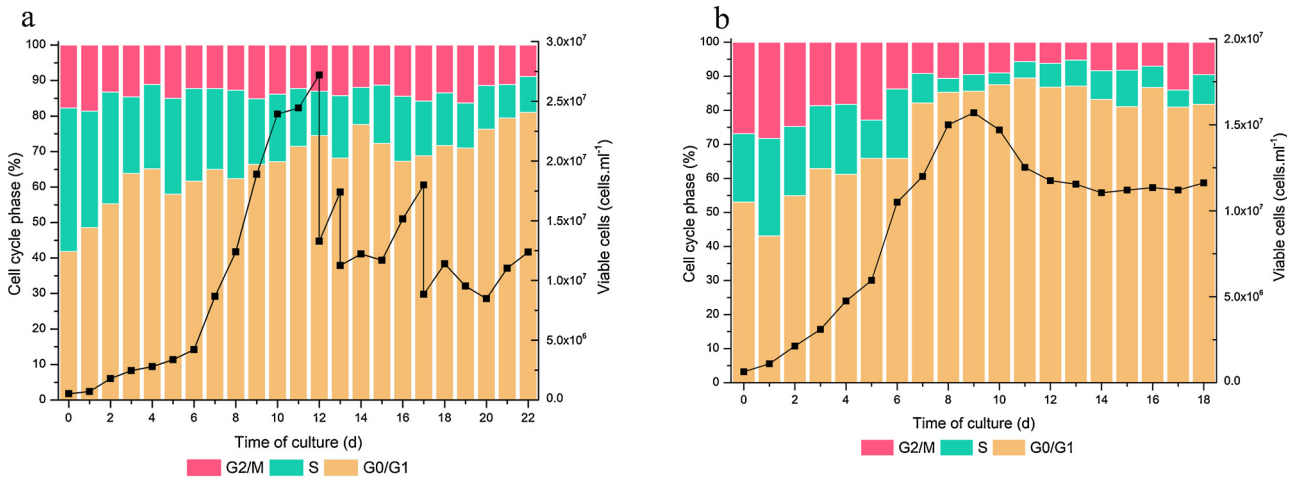

Fig. 2. Cell growth profiles and daily percentages of cell cycle phases obtained with ModFit LT of: A. First suspension-adapted CHO-K1 cells culture, and B. Second suspension-adapted CHO-K1 cells culture.

culture time, diminishing cell proliferation and improving culture longevity. Altogether, these factors may render higher specific rates of recombinant protein production.

3.2. Genome wide expression profiling through RNA sequencing reveals distinctive transcriptomic CHO-K1 cellular states throughout bioprocessing

3.2.1. RNA-seq sample preparation

To analyze transcriptome dynamics in suspension-adapted CHO-K1 cells, three replicates were cultured in 1 L bioreactors, in which culture conditions were similar to those utilized in the second cell cycle culture. The growth and metabolites curves along with process parameters are shown in Fig. 3. The three bioreactors were inoculated at a relatively high cell density (6.0×10^5 cells/ml) in order to minimize the lag phase. Also, to avoid excessive cell proliferation, we applied a decreasing temperature gradient since the first days of the exponential phase. Detectable concentrations of glucose and acceptable levels of lactate were achieved.

RNA samples were collected on day 1 and during the exponential (days 7 and 8) and the stationary (days 14 and 15) growth phases, from each culture replicate (Fig. 3). Purity and integrity analysis of these samples led to a successful outcome, with RNA Integrity Number (RIN)

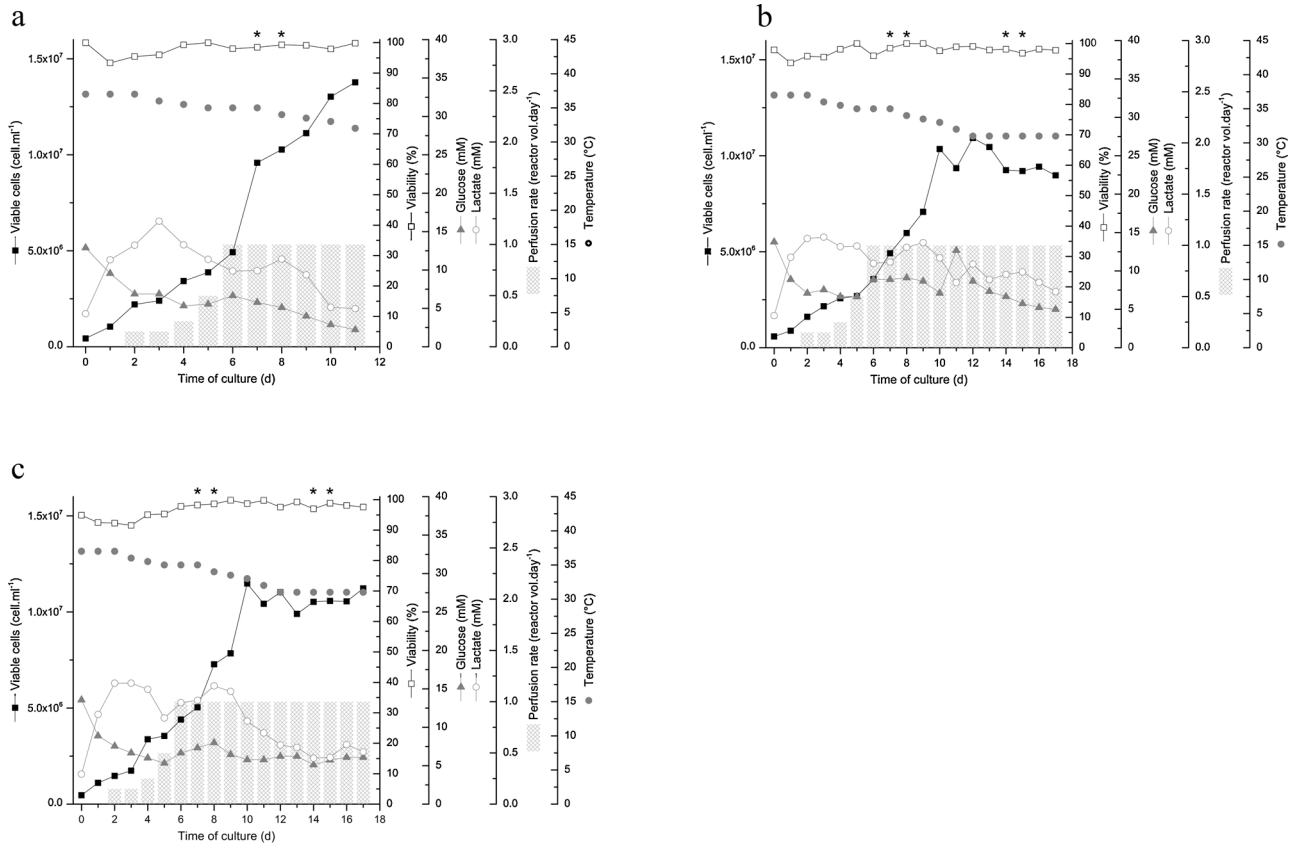

Fig. 3. Replicates of CHO-K1 cell cultures in 1 L bioreactors operated in perfusion mode. Profiles of cell density, cell viability, glucose, lactate, perfusion rate and temperature are shown. RNA extraction points are indicated by asterisk characters.

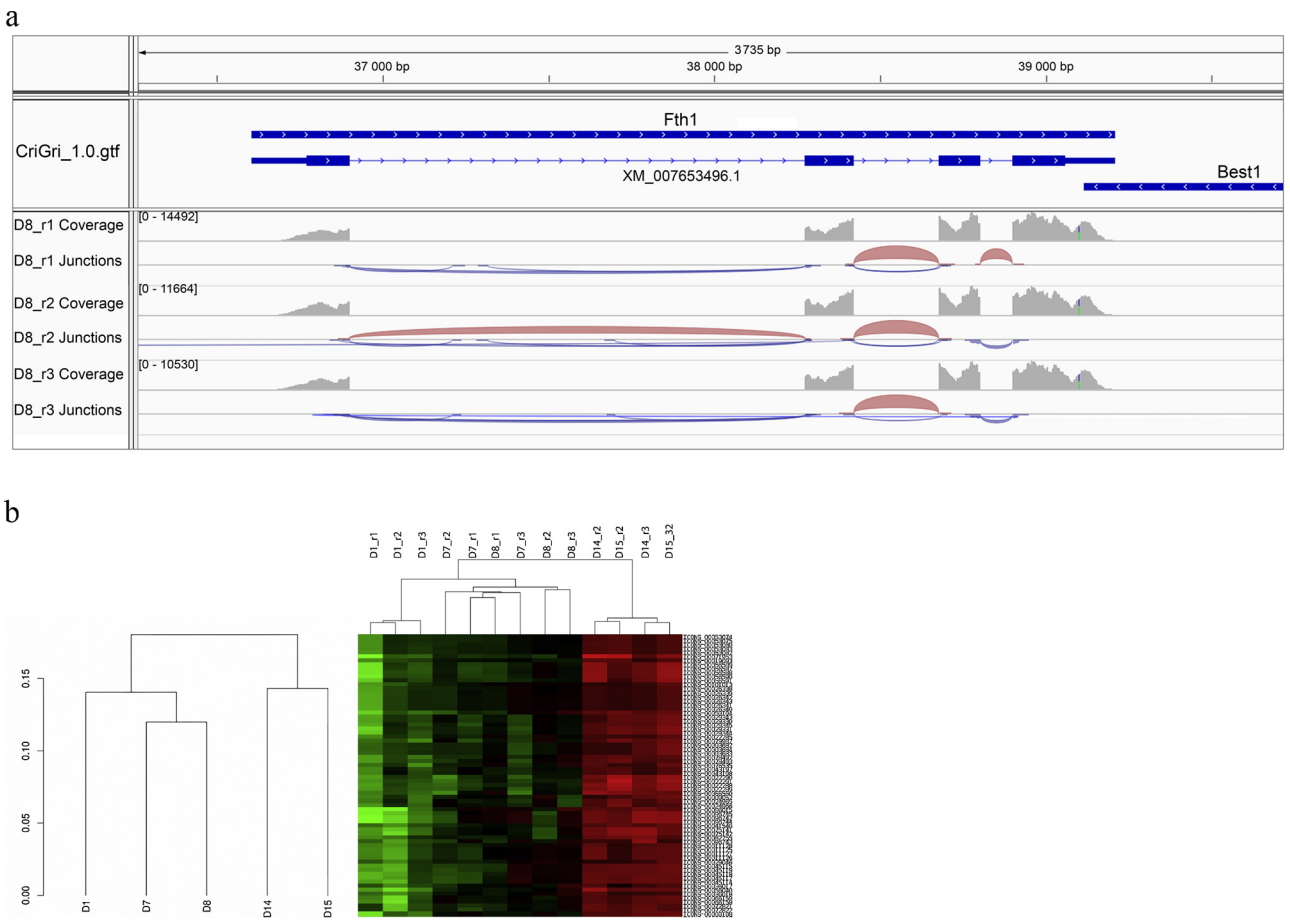

Fig. 4. CHO-K1 RNA-seq data analysis. A. Example of biological replicates mapping to the reference for Fth1 gene on day 8 (coverage and splice junctions tracks displayed by IGV). B. Unsupervised Hierarchical Clustering analysis: biological replicates are grouped together (left) and a subset of genes is shown and biological replicates are considered separately (right). C. Example of scatter plots of normalized gene expression values between samples (day 1 vs. day 8; day 1 vs. day 15 and day 8 vs. day 15).

values above 8.5.

3.2.2. Transcriptome sequencing and data validation

Strand-specific RNA sequencing was performed and a total of 525 million sequencing reads were obtained, with 40 million reads on average generated from each individual library. After trimming adapter sequences and removing low quality bases, around 90% of the sequences were uniquely mapped to the CHO-K1 draft genome sequence. An example of reads mapping is presented in Fig. 4A, which shows day 8 replicates mapped to Fth1 gene.

Unsupervised Hierarchical Clustering analysis (Fig. 4B) revealed that the data from day 1 grouped with the exponential phase data, and the days corresponding to the stationary phase produced a different cluster. Thus, it is evident that the major expression changes occurred during the stationary phase in relation to the beginning of the culture.

In order to assess the reproducibility between biological replicates, scatter plots were made (Supplementary Fig. S2) and pairwise correlation coefficients were calculated, demonstrating a high degree of reproducibility between biological replicates ($R^2 > 0.98$). Fig. 4C illustrates the correlation between day 1 replicates. Besides, scatter plots comparing the normalized gene expression values between samples are shown (day 1 vs. day 8; day 1 vs. day 15 and day 8 vs. day 15). In addition, Principal Component Analysis (PCA) showed three distinct groups when comparing PC1 and PC2, which also indicates a high correlation between biological replicates (Supplementary Fig. S3).

In order to validate the accuracy of our RNA sequencing based gene expression quantification, we randomly selected 10 genes with variable expression identified from the RNA-seq data: Fth1, Ctsz, Hmox1, Lgmn, Lamp1, Lgals1, Vim, Anxa2, Actb and Ybx1, to confirm their expression using qRT-PCR. Supplementary Fig. S4 A-D shows that the expression patterns obtained through qRT-PCR were largely consistent with the RNA-Seq data. Moreover, Supplementary Fig. S4E-H presents linear regression analyses of $\log_2$ fold change data per day, compared to day 1. A high correlation was found between RNA-seq and RT-qPCR results for these 10 genes ($R^2 > 0.90$). Overall, these analyses indicate that gene expression observed in CHO-K1 suspension cell bioprocess transcriptome was highly reliable.

3.2.3. Differential expression analysis between exponential and stationary phases in CHO-K1 suspension cell cultures

To better understand the cellular mechanisms underlying the observed phenotypes, an analysis of the changes in global gene expression from inoculation (day 1) throughout exponential and stationary phases was performed. Only genes with a calculated log2-fold-change ≥ 1 and a FDR-adjusted p-value ≤ 0.05 (Benjamini Hochberg) were taken into consideration. As shown in Fig. 5A, the number of differentially expressed (DE) genes between exponential phase vs. inoculation compared to the number of DE genes between stationary phase vs. inoculation day reveals that the most important regulatory changes happened throughout the stationary phase when gene up-regulation markedly prevailed. The low number of DE genes between exponential phase vs. inoculation may reflect the exponential growth that already existed at the time of inoculation (a short lag phase can be appreciated within the growth curves in Fig. 3). Moreover, CHO-K1 cells in a

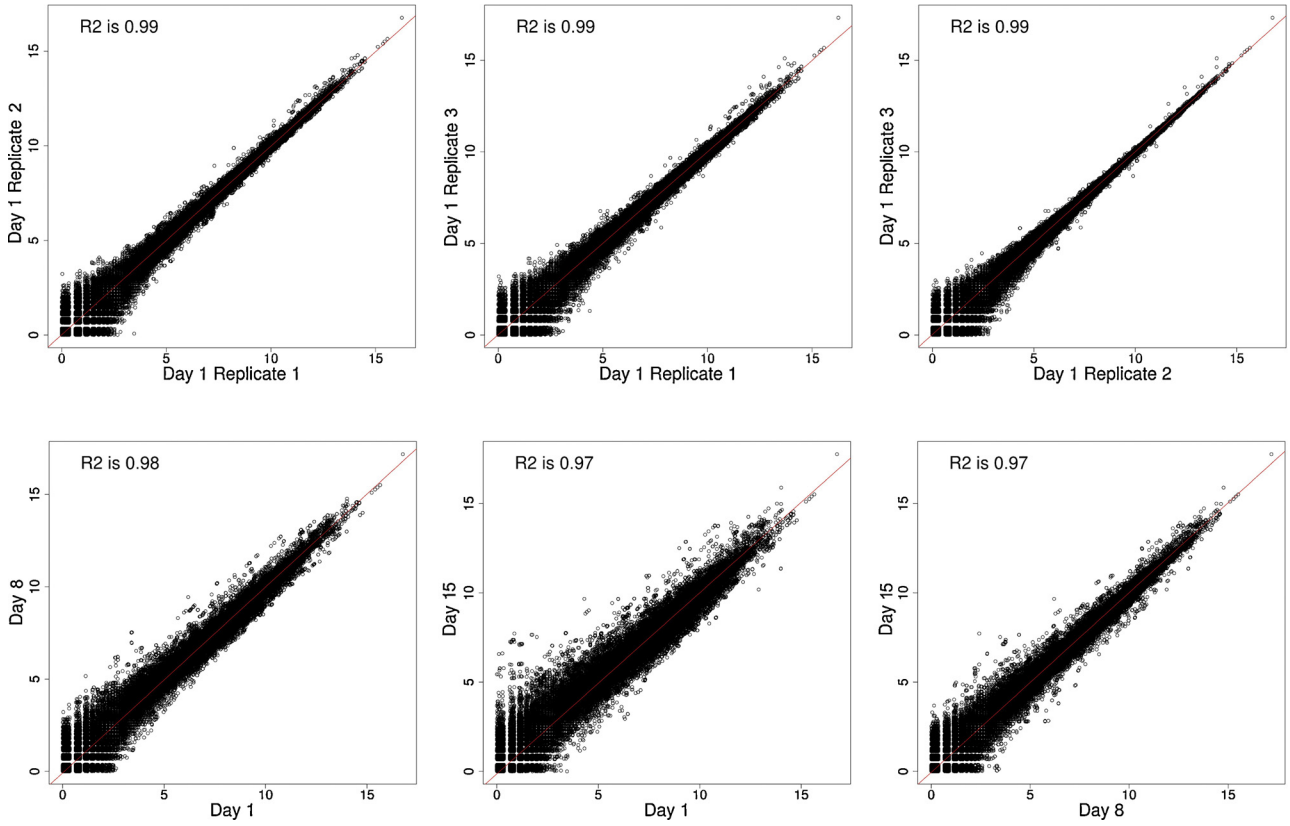

Fig. 4. (*continued*)

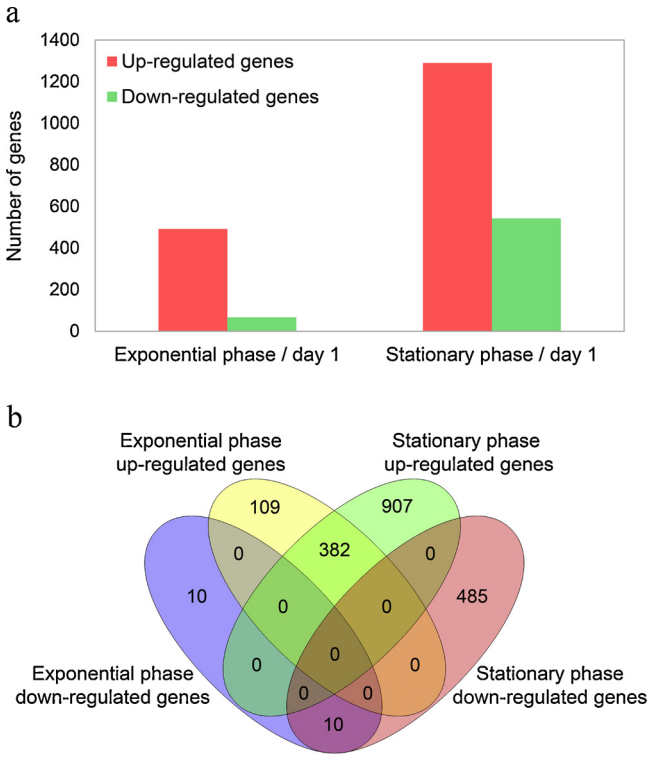

Fig. 5. Differential expression analysis of exponential phase and stationary phase vs. inoculation day. A calculated log2-fold-change ≥1 and a FDR-adjusted p-value ≤0.05 were taken into consideration. A. Red bars represent the amount of up-regulated genes and green bars indicate the down-regulated genes in each culture phase. B. Venn diagram showing overlap of the up-regulated and down-regulated genes during cell culture stages.

suspension bioprocess need to rearrange their expression pattern towards the last days of the culture, in a more drastic manner than cells during the exponential phase. Therefore, cells activate a greater amount of genes in order to continue proliferating and responding to culture conditions.

To identify the genes that are uniquely up/down regulated in each phase of the culture we sorted the DE genes using Venn diagrams. As shown in Fig. 5B, within the 1289 up-regulated genes in the stationary phase, 907 DE genes are stationary phase-specific while 382 genes were already activated during the exponential phase. Likewise, within the 495 down-regulated genes in the stationary phase, only 10 were already down-regulated in the exponential phase.

The amount of DE genes in the exponential and stationary phases represents 2.0% and 6.4%, respectively, from the total number of genes expressed. These subsets of DE genes constitute a very specific group of genes with significant regulatory changes, making them promising targets for genetic engineering in order to improve CHO-K1 bioprocesses.

The incorporation of a temperature reduction step is commonly used during production cell cultures in the biopharmaceutical industry for simultaneously inducing growth arrest and extending long term culture viability (thus increasing recombinant protein productivity and yield). Consequently, we decided to examine the expression of genes associated with low temperature stress responses. Up-regulated genes, such as CIRP (Cold-inducible RNA-binding protein) and RBM3 (RNA-binding motif protein 3), have been widely studied in mild hypothermia conditions (Chappell et al., 2001; Danno et al., 2000; Dresios et al., 2005; Nishiyama et al., 1997; Tan et al., 2008). Both are implicated in the modulation of transcription and translation by acting as RNA chaperones. RBM3 facilitates cap-independent protein synthesis at low temperatures and we found it to be more than 3.0 fold up-regulated in the exponential phase (at 35 °C) and more than 5.5 fold during the stationary phase (at 31 °C). CIRP is highly expressed at low temperature but not at 37 °C; and in this study it was 2.7 fold up-regulated during

the stationary phase (without expression changes in the exponential phase compared to inoculation day). This protein improves recombinant protein productivity and can arrest cell growth in a cell line-specific manner. Tan et al. (2008) have revealed that overexpression of CIRP at physiological temperature can increase the recombinant-protein titer in CHO cells by 25% in adherent culture and up to 40% in suspension culture. Hence, these cold shock genes are prominent targets for engineering CHO cells.

Vim and GAPDH have been observed differentially expressed following temperature shift. We found Vim was 2.1 fold up-regulated in the stationary phase, in accordance with a previous proteomic analysis in CHO cells (Kumar et al., 2008) upon temperature reduction. Conversely, while in that study GAPDH, which has a key role within glycolysis and gluconeogenesis pathways, was found up-regulated, we saw a 1.9 fold GAPDH down-regulation in the stationary phase. This could be linked to the reduced energy metabolism observed during that stage in our CHO-K1 cultures.

3.2.4. GO enrichment and KEGG pathway analysis of differentially expressed genes

Subsequently, we performed Gene Ontology searches for significant GO biological process terms enriched in the stationary phase-specific DE genes (1289 up-regulated and 495 down-regulated genes). From this analysis, we only examined the terms with a p-value ≤ 0.05.

Whereas among the enriched terms for down-regulated genes a clear tendency for terms associated with DNA replication and repair and cell cycle processes was observed, the enriched biological process terms for up-regulated genes exhibited a greater variation. Tables 1 and 2 show the enriched terms by PANTHER Overrepresentation Test, their corresponding GO accession number, fold enrichment (compared to *Mus musculus* reference database) and p-value.

We also performed pathway enrichment analysis using the KEGG pathways database on genes up/down regulated ≥ 1 log2-fold-change and a FDR-adjusted p-value ≤ 0.05 so as to ensure identification of all relevant pathways, as significance can be obtained also for slight variations for multiple genes within a pathway, which together may significantly affect a biological process.

We first examined the cell cycle pathway. As shown in Fig. 6A, we identified down-regulated genes implicated in DNA replication during the exponential phase. However, in the stationary phase (Fig. 6B) we observed many more down-regulated genes and only three up-regulated genes. Specifically, 26% of cell cycle pathway genes were found to be down-regulated in the stationary phase, in contrast with 4% during the exponential phase (on average these genes were 1.4 fold more down-regulated in the stationary phase).

In accordance with our cell cycle analysis, cyclins E and A were down-regulated in the stationary phase, resulting in G1-phase cell cycle arrest. Cyclin E is essential for progression through G1 and for the initiation of DNA replication, and this promotes cyclin A expression and allows the cell to enter and progress through the S phase. We also found genes that were more than 2.0 fold down-regulated genes, which are essential for the initiation of DNA replication, such as Cdc6 and Cdc45, along with genes that comprise the minichromosome maintenance protein complex (MCM) and the origin recognition complex (ORC). All these genes are main regulators at the early steps of DNA replication, prior and during the S phase of the cell cycle.

Moreover, genes Cip1 (p21) and GADD45 were up-regulated during the stationary phase. Microarray-based studies report that p21 expression positively correlates with the suppression of genes that are important for cell cycle progression and the induction of genes associated with senescence (Chang et al., 2000). In addition, Cip1 inhibits apoptosis and GADD45 is implicated in stress signaling responses to various physiological or environmental stressors. It is possible that the interaction of GADD45 with Cip1 plays a key role in G1 cell cycle arrest, in agreement with our finding of more cells in the G1 phase in the stationary phase (on average 80% in G1 and 10% in S). Interestingly, the effect of Cip1 has been associated with an increase in recombinant protein productivity (Bi et al., 2004). In addition, cyclin B and its counterpart CDK1 were significantly down-regulated. Cyclin B is necessary for the progression of cells into and out of M phase of the cell cycle. Other genes with critical functions in the M phase were also down-regulated, for example MPS1, which is a critical mitotic checkpoint gene for precise chromosomes segregation during mitosis.

A critical aspect in mammalian cell cultures is the problem of cell death, which can result from nutrient depletion, product accumulation and other stresses, signaling the cell to die through apoptosis or programmed cell death. The use of low temperatures reduces cellular metabolism and in consequence the cells do not undergo apoptosis (Moore et al., 1997). In our CHO-K1 cell cultures, relatively constant and very high percentages of cell viability were observed, mainly in the stationary phase (Figs. 1 and 3).

When analyzing the apoptosis pathway in the stationary phase we found differentially up-regulated genes in both signaling ways that initiate apoptosis. For example, the gene TRAIL, which is a death receptor that promotes the extrinsic apoptotic pathway, was 4.6 fold up-regulated, and BIM, that is involved in the intrinsic pathway initiated by mitochondrial events, was 3.0 fold up-regulated. JNK has been observed to play a central role in both of these ways, and it was found to

Table 1
Gene Ontology biological process terms enriched in the stationary phase data for down-regulated genes with a log2-fold-change ≥ 1 and FDR-adjusted p-value ≤ 0.05. Only terms with a p-value ≤ 0.05 obtained by PANTHER Overrepresentation Test are shown.

	Reference (*Mus musculus*)	Down-regulated genes (Stationary phase dataset)			
GO Biological process	Number of Genes	Number of genes	Expected value	Fold Enrichment	P-value
DNA replication initiation (GO:0006270)	25	10	0.3	31.8	1.44E-08
mitotic chromosome condensation (GO:0007076)	13	7	0.2	42.8	4.28E-06
positive regulation of cytokinesis (GO:0032467)	31	8	0.4	20.5	7.32E-05
centromere complex assembly (GO:0034508)	20	7	0.3	27.8	8.09E-05
chromosome separation (GO:0051304)	27	7	0.3	20.6	6.14E-04
nuclear DNA replication (GO:0033260)	17	6	0.2	28.1	8.99E-04
regulation of double-strand break repair via homologous recombination (GO:0010569)	29	7	0.4	19.2	9.91E-04
regulation of sister chromatid cohesion (GO:0007063)	21	6	0.3	22.7	3.06E-03
DNA integrity checkpoint (GO:0031570)	96	10	1.2	8.3	4.59E-03
positive regulation of chromosome segregation (GO:0051984)	24	6	0.3	19.9	6.61E-03
mitotic G2/M transition checkpoint (GO:0044818)	27	6	0.3	17.7	1.30E-02
microtubule depolymerization (GO:0007019)	15	5	0.2	26.5	1.41E-02
centromeric sister chromatid cohesion (GO:0070601)	7	4	0.1	45.4	1.96E-02
chromatin remodeling at centromere (GO:0031055)	8	4	0.1	39.8	3.30E-02
positive regulation of cell cycle phase transition (GO:1901989)	73	8	0.9	8.7	4.40E-02

Table 2
Gene Ontology biological process terms enriched in the stationary phase data for up-regulated genes with a log2-fold-change ≥1 and FDR-adjusted p-value ≤0.05. Only terms with a p-value ≤0.05 obtained by PANTHER Overrepresentation Test are shown.

	Reference (*Mus musculus*)	Down-regulated genes (Stationary phase dataset)			
GO Biological process	Number of Genes	Number of genes	Expected value	Fold Enrichment	P-value
cell adhesion (GO:0007155)	756	47	17.0	2.8	5.40E-06
negative regulation of cellular protein metabolic process (GO:0032269)	950	51	21.3	2.4	1.21E-04
cellular response to oxygen-containing compound (GO:1901701)	713	41	16.0	2.6	5.79E-04
cell surface receptor signaling pathway (GO:0007166)	1698	73	38.1	1.9	7.60E-04
negative regulation of cell proliferation (GO:0008285)	645	38	14.5	2.6	9.79E-04
regulation of cytokine secretion (GO:0050707)	176	18	4.0	4.6	1.48E-03
regulation of peptidase activity (GO:0052547)	369	26	8.3	3.1	4.24E-03
regulation of protein modification process (GO:0031399)	1645	68	37.0	1.8	8.91E-03
negative regulation of intracellular signal transduction (GO:1902532)	503	30	11.3	2.7	1.70E-02
negative regulation of catalytic activity (GO:0043086)	678	36	15.2	2.4	2.27E-02
negative regulation of extrinsic apoptotic signaling pathway (GO:2001237)	97	12	2.2	5.5	2.53E-02
positive regulation of secretion by cell (GO:1903532)	422	26	9.5	2.7	4.68E-02

be 2.0 fold up-regulated during the stationary phase. Nevertheless, genes implicated in evading apoptosis were over-expressed, favoring cell survival. For example, NGF was almost 9.0 fold up-regulated in the stationary phase (and 3.0 fold up-regulated during the exponential phase). In its absence, cells undergo apoptosis, and consequently activate the phosphatidylinositol-3-kinase (PI3K) gene that plays a central role in the PI13K-Akt pathway, which was up-regulated in the stationary phase (about 15% of the genes in the pathway were over-expressed and only 3% were down-regulated). PI3K signaling pathway is crucial in many aspects of cell growth and survival. In fact, no gene that leads to apoptosis was activated in this pathway, and those related to cell cycle and cell survival were up-regulated.

Moore et al. (1997) observed that the global rate of metabolism was reduced in CHO cell culture at low temperature. When analyzing glycolysis and gluconeogenesis pathway, we found 5 genes significantly down-regulated more than 2.0 fold in the stationary phase: TPI1, GAPDH, PGK1, PCK1 and ACSS2. This metabolism reduction could be enough to extend culture viability by a decrease in toxic metabolites and/or a limitation of nutrient deprivation.

The inefficient use of glucose for ATP production is a well-known characteristic of CHO cells (Mulukutla et al., 2010), which converts most of the available glucose into lactate and produces ATP from an aerobic glycolysis process. The dependence of these cells on anaerobic glycolysis was also observed in some cancer cells (Diaz-Ruiz et al., 2011). The accumulation of lactate in culture has long been recognized as an inhibitory factor for cell growth and recombinant protein production (Zhou et al., 1995, 1997). Lactate dehydrogenase A (LDHA) catalyzes the reversible conversion of pyruvate to lactate. This isoenzyme of lactate dehydrogenase is highly expressed in CHO cell lines. In the present transcriptome analysis, this gene did not exhibit any expression change throughout cell culture; in fact, it was expressed at constant and relatively low levels during the whole bioprocess. Also, there is evidence that in the late phases of fed-batch or continuous cultures, mammalian cells shift their metabolism from lactate production to lactate consumption (Europa et al., 2000; Mulukutla et al., 2012). In contrast, quiescent cells metabolize glucose at a slower rate and most of the glucose consumed is converted to biomass. Thus, cells in culture may or may not shift their metabolism to consume lactate according to glucose and growth rate of the cells. Since we observed a reduction of lactic acid concentration in the culture medium when cells entered into the stationary phase, from the pathways analysis it is not clear if lactate is being consumed or if it is not being produced by CHO-K1 cells.

We further analyzed DNA processing pathways and we found a general down-regulation in both the homologous recombination (HR) and DNA replication pathways, especially during the stationary phase. It has been reported (Kostyrko et al., 2015) that the lack of several genes, such as Rad51B, Rad51C, BRCA1 and Rad50 involved in the HR pathway, lead to the arrest of cells in the G0/G1 cell cycle phase, even in the absence of DNA damage. We observed that gene Rad51C, which is essential for this pathway, was 2.2 fold down-regulated during the stationary phase. Also, BARD1 was 3.9 fold down-regulated and this gene interacts with the N-terminal region of BRCA1 which was 2.3 fold down-regulated. The BRCA1-BARD1 heterodimer coordinates a wide range of cellular pathways, such as DNA damage repair, ubiquitination and transcriptional regulation to maintain genomic stability. Other essential genes for the HR pathway were down-regulated in the stationary phase: BRIP1 (2.3 fold), PALB2 (2.5 fold), BRCA2 (2.4 fold), Rad54 L (3.5 fold), POLD1 (2.6 fold) and BLM (2.0 fold).

Likewise, genes that play an important role in DNA replication were down-regulated during the stationary phase of the CHO-K1 bioprocesses. DNA polymerase delta subunit 1 (POLD1) and DNA polymerase epsilon subunit 1 (POLE) were down-regulated by 2.6 and 2.0 fold, respectively. MCM complex genes were found to be down-regulated too: MCM2 (2.0 fold), MCM3 (2.8 fold), MCM (3.1 fold) and MCM7 (2.4 fold). This complex serves as DNA helicase and it has a role in both the initiation and the elongation phases of eukaryotic DNA replication, precisely in the formation and elongation of the replication fork. Furthermore, gene FEN1, an endonuclease essential for repairing double-strand breaks in the microhomology-mediated end joining (MMEJ) pathway, was 2.3 fold down-regulated.

The marked variations in these pathways were observed as the culture progressed to the stationary phase in agreement with our cell cycle studies. The number of cells in G0/G1 increased, the DNA machinery was down-regulated and the cells were not able to progress through the cell cycle. This dynamic may be at least in part due to the activation of signaling ways for stress response, such as p53 signaling and AMPK signaling pathways, both up-regulated during the stationary phase. On the one hand, it is well-known that p53 induces cell cycle arrest, cellular senescence or apoptosis. On the other hand, once AMPK is activated, it leads to a connected inhibition of energy-consuming biosynthetic pathways.

As regards protein processing in the endoplasmic reticulum (ER) pathway, we found that normal folding, quality control and secretion steps, as well as stress and unfolded protein responses ways were up-regulated in the stationary phase. Specifically, genes UGGT2, ERLEC1, SEC31B and JNK were found to be 2.7, 2.0, 3.4 and 2.0 fold up-regulated, respectively. Harreither et al. (2015) found that, in highly productive cell lines, some parts of the secretory machinery were up-regulated in response to high protein content. We examined CHO-K1 host cell line and similar results were obtained, suggesting that the regulation of protein processing is related not only to the recombinant protein production but also to the bioprocess conditions, such as low temperature. They also observed that apoptosis/anti-apoptosis, as well

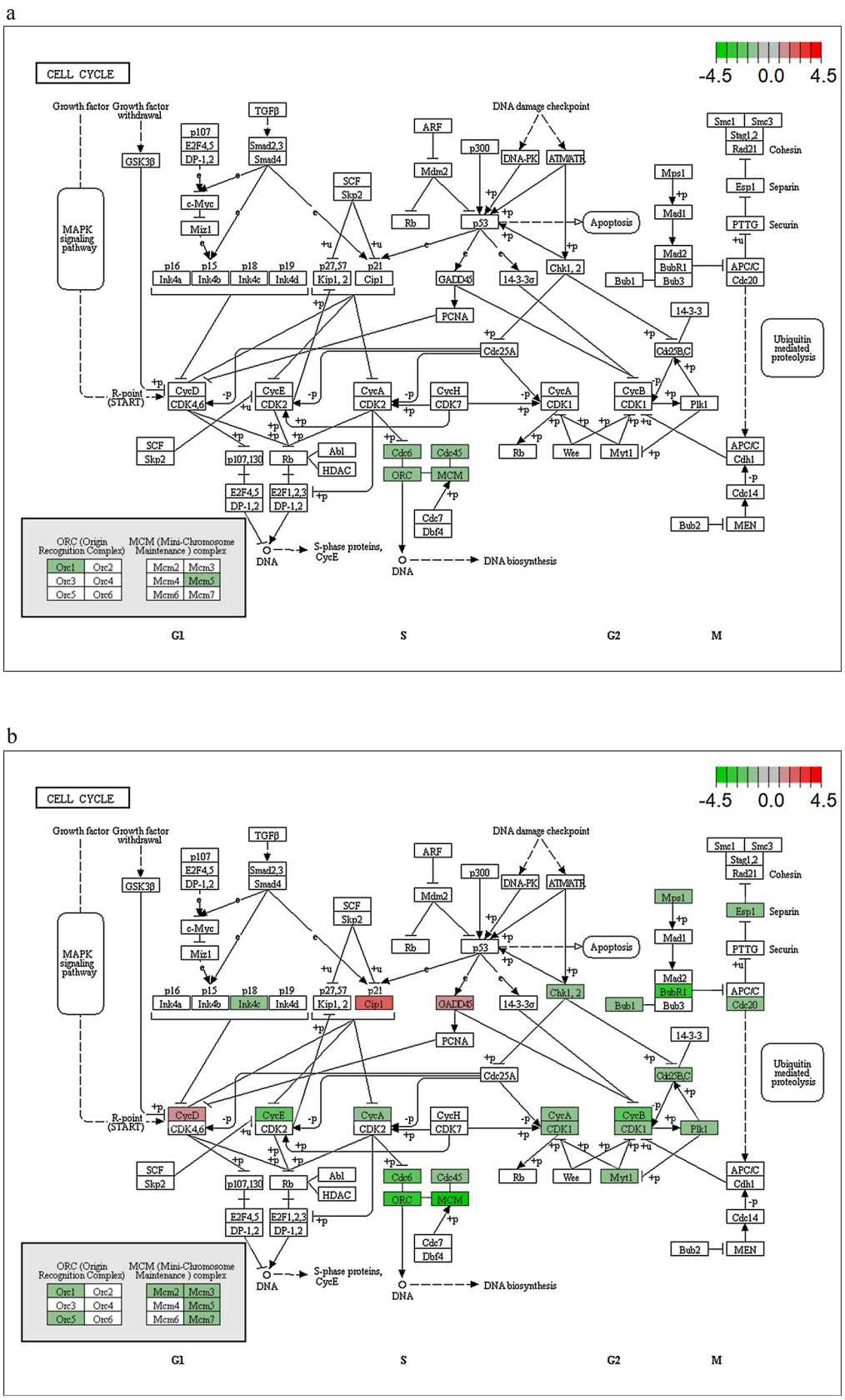

Fig. 6. Differentially expressed genes mapped to KEGG pathway for cell cycle. Red boxes represent up-regulated genes and green boxes indicate down-regulated genes. The color scale representing the log2-fold-change expression values is shown in the top-right corner. A. DE genes during the exponential phase. B. DE genes in the stationary phase.

as UPR/degradation ways were up-regulated, especially the regulatory control proteins CHOP and ATF4. In our work, ATF4 was only differentially expressed during the exponential phase compared to inoculation day (2.0 fold up-regulated), and CHOP was expressed at very low levels through the culture, revealing that the ER stress levels were lower and the cells were not entering apoptosis.

A critical aspect for a successful mammalian cell culture process is the correct glycosylation of the recombinant product. Low productivity and inconsistent protein glycosylation are common problems in biopharmaceutical production. Previous studies described that post-translational modifications, particularly glycosylation, were sustained or even improved at low temperature (Bollati-Fogolín et al., 2008; Yoon et al., 2003). When analyzing the expression of genes involved in glycosylation pathways, we could not see significant global changes, neither in the exponential phase nor in the stationary phase. The only gene related to glycosylation that significantly changed its expression was GALNT6, which is part of the N-acetylgalactosaminyltransferase (GalNAc-T) family of enzymes. It initiates mucin-type O-linked glycosylation in the Golgi apparatus by catalyzing the transfer of GalNAc to serine and threonine residues on target proteins. We found it 3.0 fold up-regulated during the exponential phase and 10.5 fold up-regulated in the stationary phase. These results indicate that the O-glycosylation is enhanced in these CHO cells under the culture conditions previously described.

Some genes inside the regulation of actin cytoskeleton pathway were up-regulated in the stationary phase; e.g. growth factor FGF13 and growth factor receptor FGFR2 were 3.0 fold and 4.3 fold up-regulated, respectively, and the latter was similarly up-regulated in the exponential phase. Some integrin genes were also up-regulated (ITGA8 gene was 2.0 fold up-regulated in the exponential phase and 2.9 fold up-regulated during the stationary phase, and ITGA5 was only up-regulated in the stationary phase by 2.0 fold). Interestingly, gene IQGAP3 which controls cell adhesion was 2.8 fold down-regulated during the stationary phase. Walther et al. (2016) found that the expression of integrins on the cell surface of two differently suspension-adapted CHO cells played a key role in cell signaling processes and cell proliferation, demonstrating the importance of integrins for cell survival even in a ligand-free environment.

Furthermore, we found differentially expressed genes in cancer related pathways, with a prevailing up-regulation during the stationary phase. Lee et al. (2016) studied significantly up-regulated cancer related genes, based on the fact that CHO-K1 cells adapted to suspension culture show similar phenotypes as cells during metastasis of cancer. These genes were used as genetic engineering targets for accelerating adaptation of CHO cells to suspension culture. Besides, Pourhassan-Moghaddam et al., (2012) proposed the use of promoter regions of cancer genes, as they may have a high rate of transcription, with the purpose of designing new expression vectors.

Overall, these findings suggest that the exposure of the CHO-K1 cells to sub-optimal temperatures triggers a coordinated response comprising the modulation of the cell cycle, energy metabolism, cell proliferation, cytoskeleton reorganization and biological processes, such as DNA replication and repair, transcription, protein metabolism, cell communication and apoptosis.

In this work we performed a detailed cell cycle analysis throughout CHO-K1 host cell line bioprocesses at industrial scale. We found a noticeable G0/G1 synchronization in the late exponential phase and mainly during the stationary growth phase. This may be due to the temperature reduction applied during the bioprocesses, as part of a typical biphasic cell culture. These findings correlate with RNA-seq data, which showed a pronounced downregulation of cell cycle and DNA regulation pathways, particularly during the stationary phase (at 31 °C). Several key genes that played an important role in arresting cells in G0/G1 cell cycle phase were identified in each culture stage.

All the genes reported which exhibit significant expression changes can be used as potential targets for cellular and metabolic engineering by means of the currently most applied techniques, such as TALENs or CRISPR/Cas9. In this way, CHO-K1 cell bioprocessing can be further improved, both at laboratory and at industrial scale.

4. Conclusions

Recombinant therapeutic proteins have revolutionized the field of medicine since their introduction more than thirty years ago. Mammalian cells are the preferred system for the production of biotherapeutics, and CHO-derived cells (such as CHO-K1) have been the most commonly used hosts for large-scale commercial production. However, the lack of understanding of the regulatory behavior of these cells results in several issues that modify culture performance, including inefficient glucose metabolism and improper post-translational modifications of the recombinant product. The increasing demand for biotherapeutics is the main driver to improve current production systems.

The regulation of cell cycle and, therefore, proliferation has allowed significant improvements in cell characteristics and protein production. Sub-physiological temperature cultivation of mammalian cells is a broadly accepted method for this purpose and it is linked to G1-phase cell cycle arrest that has been positively correlated with increased productivity. To understand and harness the mechanisms by which reduced temperature can potentially result in enhanced recombinant protein yield, it is firstly necessary to consider cell response to low temperature cultivation.

An open question in the field of CHO cell engineering is whether fluctuations in gene expression that are observed in high producing cell lines originate from the high protein content of these cells or whether some cells intrinsically have an improved capacity to produce large amounts of protein, based on their global gene expression pattern. In consequence, analyses focused on host cell lines are of vital importance.

Here we analyzed the distribution of cell cycle phases and the global gene expression dynamics of a suspension-adapted CHO-K1 cell line to provide a systematic view of CHO cell physiology under variable experimental conditions, especially following the reduction of culture temperature from 37 °C to 31 °C. This is the first report combining cell cycle and transcriptome analysis of CHO-K1 host cells during typical culture bioprocesses at industrial scale.

We found a cell culture state characterized by G0/G1 synchronization (as expected), mainly during the late exponential growth phase and towards the stationary phase (Fig. 2). This may be due to the progressive temperature reduction, considering no chemical or physical agent was used for cell arrest. Consequently, controlling the percentage of S phase cells at a level below 30% in perfusion continuous cell culture led to a better bioprocess performance control, shortening the culture time in order to achieve the maximum cell concentration required, diminishing excessive cell proliferation and improving culture longevity, which may cause higher specific rates of recombinant protein production. Thereby, the method used for CHO-K1 cell cultures could serve as a standard protocol for the production of biotherapeutics.

We successfully identified key genes and pathways connected with the particular culture states. The most important gene expression changes occurred throughout the stationary phase when gene up-regulation markedly prevailed. Our RNA-seq data analysis enabled the identification of target genes for mechanism-based cell line engineering and bioprocess modification, an essential step to translate gene expression data from CHO-K1 host cells into bioprocess-related knowledge. Further efforts that aim at increasing desirable phenotypes of CHO cells and promoting efficient production of high quality therapeutic proteins can highly benefit from this type of studies.

Conflict of interest

The authors declare that they have no conflict of interests.

Author contribution

I.T. designed and performed the experiments, analyzed the data and wrote the manuscript. F.J.L-D. and C.C.P. contributed to experiments planning and supervised the research and the manuscript. R.K. supervised the manuscript. All authors have approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2018.09.007.

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