Journal of Biotechnology 151 (2011) 204–211

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Engineering CHO cell growth and recombinant protein productivity by overexpression of miR-7

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ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form
22 November 2010
Accepted 7 December 2010
Available online 15 December 2010

Keywords:
CHO cell engineering
miRNA
Recombinant protein production
Expression profiling

ABSTRACT

The efficient production of recombinant proteins by Chinese Hamster Ovary (CHO) cells in modern bioprocesses is often augmented by the use of proliferation control strategies. The most common method is to shift the culture temperature from 37 °C to 28–33 °C though genetic approaches to achieving the same effect are also of interest. In this work we used qRT-PCR-based expression profiling using TLDA™ cards to identify miRNAs displaying differential expression 24 h after temperature-shift (TS) from 37 °C to 31 °C. Six miRNAs were found to be significantly up-regulated (mir-219, mir-518d, mir-126, mir-30e, mir-489 and mir-345) and four down-regulated (mir-7, mir-320, mir-101 and mir-199). Furthermore, qRT-PCR analysis of miR-7 expression over a 6 day batch culture, with and without TS, demonstrated decreased expression over time in both cultures but to a significantly greater extent in cells shifted to a lower culture temperature. Unexpectedly, when miR-7 levels were increased transiently by transfection with miR-7 mimic in CHO-K1 cells, cell proliferation at 37 °C was effectively blocked over a 96 h culture period. On the other hand, transient inhibition of endogenous miR-7 levels using antagonists had no impact on cell growth. The exogenous overexpression of miR-7 also resulted in increased normalised (per cell) production at 37 °C, though the yield was lower than cells grown at reduced temperature. This is the first report demonstrating a functional impact of specific miRNA disregulation on CHO cell behavior in batch culture and provides some evidence of the potential which these molecules may have in terms of engineering targets in CHO production clones. Finally, we report the cloning and sequencing of the hamster-specific *cgr-miR-7*.

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1. Introduction

One of the challenges associated with modifying the behavior of cells in a bioprocess setting is the complex nature and range of desirable phenotypes. Targeting the expression of one gene or protein may not be sufficient to alter the phenotype of a cell unless it is a rate-limiting factor in a critical pathway or, as demonstrated more recently, a transcription factor whose expression impacts on numerous downstream molecules (Tigges and Fussenegger, 2006; Ohya et al., 2008). The current interest in miRNAs has been highlighted as a potential opportunity to engineer networks of genes and proteins in order to achieve complex phenotypic changes in mammalian cells (Müller et al., 2008). MiRNAs are short, non-coding RNA molecules that are expressed in plant and animal cells in a similar manner to protein-encoding genes. They can be found in intergenic regions of the genome or embedded in intron sequences. In addition, their transcription is mediated by binding of transcription factors to an upstream promoter and recruitment of RNA PolII. They may be co-transcribed with other locally situated miRNAs or individually depending on the sequence structure. The primary transcribed unit is enzymatically processed and transported to the cytoplasm where it binds and directs the RISC complex to a specific subset of target mRNAs. The mechanism by which miRNAs prevent translation is the subject of intensive study but it has been shown to be via transcript destabilization leading to degradation of the message or inhibition of translation without cleavage of the bound mRNA (Carthew and Sontheimer, 2009). In addition, most miRNAs would appear to impact on the expression of tens or hundreds of mRNAs but usually to a modest degree (Selbach et al., 2008).

Research in numerous biological systems, in particular the study of cancer, has revealed the roles of these regulatory molecules in biological processes such as proliferation, apoptosis, stress response, angiogenesis and secretion. Furthermore, the availability of miRNA microarray expression profiling technologies, including 2nd generation sequencing, has been utilized to measure the changes in miR expression under different conditions (Koh et al., 2009; Kantardjieff et al., 2009). Our laboratory was first to report

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doi:10.1016/j.jbiotec.2010.12.005

the use of human/mouse miRNA hybridization arrays to profile miRNA expression in CHO cells, demonstrating that commercially available miRNA tools could be applied to CHO cells, unlike the situation with conventional mRNA profiling. This led to the cloning and sequencing of the first, and to date only, hamster miRNA in the Sanger miRbase—*cgr-mir-21* (Gammell et al., 2007). In this study we utilized qRT-PCR expression profiling in an attempt to identify which, if any, miRNAs are differentially expressed 24 h after a temperature shift. We then investigated the potential of modifying specific, cellular miRNA expression levels as a potential alternative or complement to temperature shift in controlling CHO cell growth in batch culture.

2. Materials and methods

2.1. Cell lines and transfection

CHO-K1 cells (ATCC Cat#:CCL-61) were stably transfected with an expression vector encoding human secreted alkaline phosphatase (pSPORT-SEAP) (kind gift from M. Fussenegger, ETH). After selection in 800 μg/ml G418 for 2 weeks and limited dilution cloning, a panel of clones were assessed for consisted high SEAP secretion over 6–8 passages in culture. The highest SEAP expressing cell line was identified and maintained in low-serum (0.5%, v/v) DMEM/HAM12 medium or CHO-S-SFMII, serum-free medium (Invitrogen Cat#:12052) in suspension culture. Low-serum stock cultures were routinely maintained in 100 ml medium in a 250 ml spinner vessel at 60 rpm and 37 °C with 5% CO_2. Cells were routinely counted using a haemocytometer and viability estimated using the Trypan Blue exclusion method.

Transient transfection assays were performed in 50 ml filter-topped tubes (Cultiflask, Sartorius). Typically, 50 nM miRNA mimic (pre-mir) or antagonist (anti-mir) were complexed with 2 μl NeoFx transfection reagent (Ambion) and added to 2 ml of cells seeded at 1×10^5 cells per ml. Pre-mirs and anti-mirs specific to miR-7, as well as non-specific controls, were sourced from Applied BioSystems (PM10737, AM10737). Cell growth and viability was measured on a Guava Benchtop Cytometer after staining with Viacount™ (Guava Technologies). SEAP activity was measured using the phosphatase assay as described previously (Lipscomb et al., 2005). Samples were diluted to ensure readings fell within the appropriate absorbance limits. SEAP was reported in terms of activity (unit/min/ml) rather than protein quantity. Normalised activity referred to the measured activity divided by the cell number per unit volume.

2.2. Taqman low density arrays

Human TaqMan Array MicroRNA Cards V1.0 (TLDAs) were run as per the manufacturer's guidelines (Applied BioSystems). These consist of 384-wells containing primers designed against individual human miRNAs. 100 ng of total RNA was reverse transcribed in 8 individual multiplex reactions. These cDNA mixes were then used to seed 48 mini-PCR cells (1 μl each) via 8 access ports on the card. PCR was performed on an AB7900 real time instrument with 10 min at 95 °C followed by 40 cycles of 30 s at 97 °C and 1 min at 60 °C. Mir-let7a expression was used to normalise across the samples and differential expression was calculated using the Statminer™ software programme (Integromics). A cycle threshold (Ct) cut-off of 35 was applied and p-value ≤ 0.05 was chosen for significantly changing miRNAs using the LIMMA parametric test, across duplicate TLDA cards per sampling point.

2.3. Quantitative RT-PCR

Cellular concentrations of miRNAs were measured using pre-designed assays from Applied Biosystems (000268). Briefly, total RNA was extracted from cell pellets using a miRVana kit (Ambion) and 10 ng was reverse transcribed using a primer specific for the mature target miRNA. The resulting cDNA was then amplified in triplicate per sample ($n = 3$) in an AB7500 Real Time PCR instrument and quantified using the $2^{-\Delta\Delta Ct}$ method. The assays were normalised to the levels of endogenous U6 RNA.

2.4. Cloning miR-7

Primers were designed based on consensus sequence alignments using orthologous sequences from human, mouse, rat, etc. The mature sequence of miR-7 plus flanking sequence was amplified from genomic CHO DNA with the following primers: Sense: 5′-AATAGAATTCAGAGGCAGGAAACTCAGGTGTCA-3′; Antisense: 5′-TATTGCGGCCGCATGTCCTTGTTTCTGGAGAAG TCC-3′. The PCR product was gel purified and cloned into the vector pMF111 for sequencing (Eurofins-MWG).

2.5. Bioinformatics analysis and literature mining

Potential target genes of the differentially expressed miRNAs were predicted using ElMMo (http://www.mirz.unibas.ch/ElMMo3/). Ontological analysis of the genes predicted to be targeted by more than one of the differentially expressed miRNAs from the profiling experiment was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) (http://www.pantherdb.org/).

3. Results

3.1. Differential expression of miRNAs in CHO cells subjected to temperature shift

CHO-K1 cells were seeded in duplicate spinner flasks and grown for 3 days at 37 °C. A sample was taken from each flask and the cells grown for a further 24 h at 31 °C at which time the second sample was taken. The cultures demonstrated a typical growth profile during this time with the temperature-shift resulting in reduced growth and slight improvement in viability compared to a control culture maintained at 37 °C (data not shown). MiRNA expression was analysed using one TLDA™ card per sample, i.e. biological duplicates after 3 days at 37 °C and after a further 24 h at 31 °C. This identified 10 significantly differentially expressed miRNAs (Table 1) ranging from 8-fold down-regulated (miR-7) to 5-fold up-regulated (miR-219) subsequent to the temperature shift. Included in this list, and based on a cycle threshold cut-off of 35 (above which the signal is considered too low to detect reliably),

Table 1
List of differentially regulated miRNAs identified by TLDA profiling. A Ct cutoff of 35 was applied and p-value ≤ 0.05 was chosen for significantly changing miRs using the LIMMA parametric test. Data was normalised to let-7a expression levels. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. Two independent cultures were profiled for each condition.

Name	p-Value	Fold Change
miR-219	0.002	+5.4
miR-518d	0.002	+3.5
miR-126	0.008	+3.1
miR-7	0.020	−8.4
miR-320	0.028	−2.8
miR-101	0.045	−1.7
miR-199b	0.010	On–off
miR-30e	0.015	Off–on
miR-489	0.020	Off–on
miR-345	0.047	Off–on

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