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*.

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

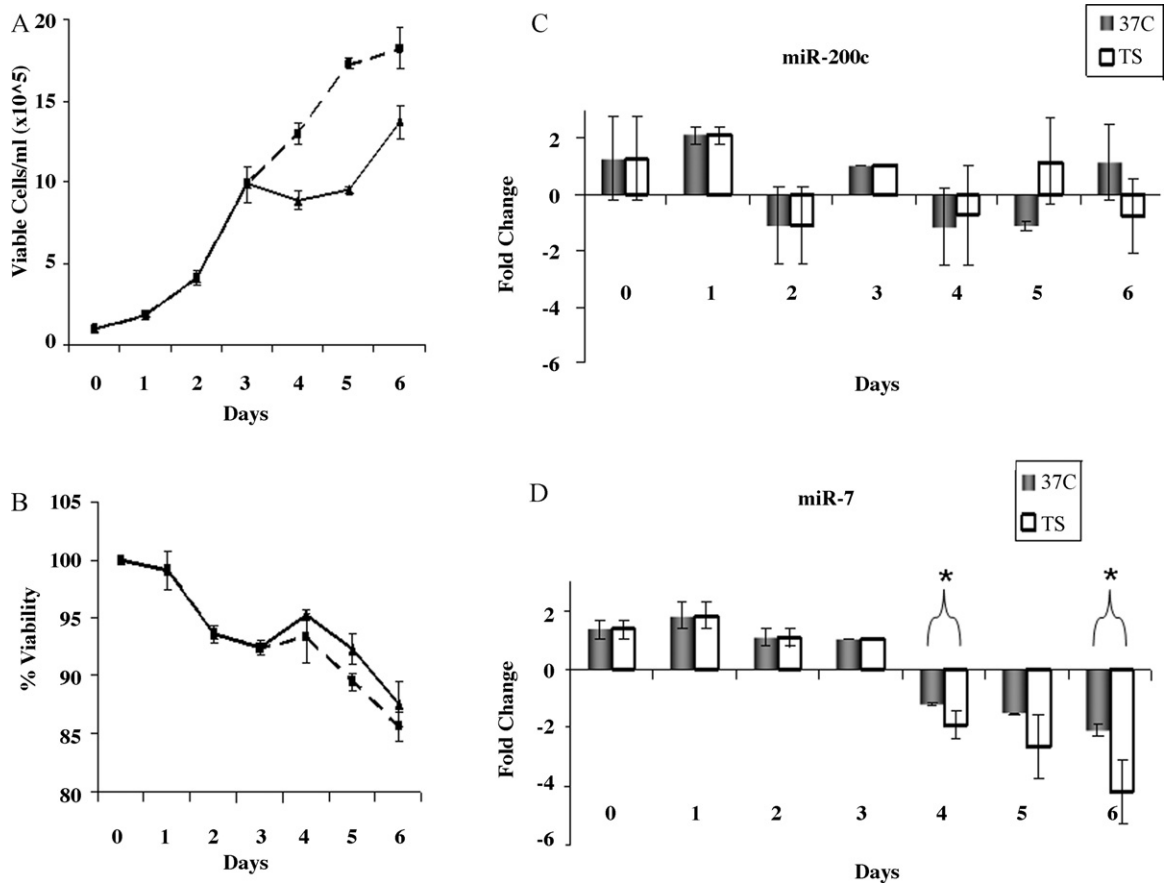


Fig. 1. Growth (A) and viability (B) of CHO-K1 cells in batch, suspension culture at 37 °C (–) or following Day 3 temperature shift (– –). Samples were taken from triplicate cultures daily. qRT-PCR analysis of miR-200c (C) and miR-7 (D) expression in temperature shifted (open bars) and isothermal culture (shaded bars) over 6 days. Fold change at each timepoint is expressed relative to the level on Day 3 in the 37 °C sample. All samples were normalised to let-7a (error bars = SD, $^*p < 0.02$, $n = 3$).

there were 4 miRNAs whose expression was either switched on (miR-30e, miR-489 and miR-345) or switched off (miR-119b) after the temperature shift. In 3 out of 4 cases the $\Delta\Delta$Ct value was <1.5 indicating a switch from off to weakly expressed or vice versa. Mir-30e had a $\Delta\Delta$Ct value = −3 indicating a significant expression level upon temperature shift to 31 °C, having been from undetectable at 37 °C.

To verify the results of the TLDA analysis, a more detailed experiment was designed to analyse the expression of two miRNAs in cells grown in parallel batch cultures over 6 days with or without a temperature shift from 37 °C to 31 °C on Day 3. miR-7 was chosen as the most significantly down-regulated target on the list. miR-200c was chosen as a control target whose expression was unchanged in the TILDA results, as further verification of the accuracy and reliability of the profiling data—i.e. as proof that reducing temperature did not cause a generic reduction in all miRNA expression. Once again, both cultures displayed the expected growth patterns and viability profiles as before (Fig. 1A and B). The expression analysis showed good agreement with the TLDA profiling; miR-200c levels fluctuated only slightly (±1 fold change) throughout the culture period except for a slight increase at Day 2 (Fig. 1C) while miR-7 expression gradually decreased over time (Fig. 1D). The magnitude of the drop in miR-7 levels in the temperature shifted culture (2-fold at Day 4, 4-fold by Day 6) was not as great as reported in the TLDA experiment (>8-fold). It was interesting to note the difference in expression between isothermal (37 °C) and biphasic cultures (37–31 °C); while miR-7 expression gradually declined with the progression of both cultures, it did so to a significantly greater extent in the temperature-shifted cells.

3.2. Bioinformatics prediction of potential targets of temperature-sensitive miRNAs

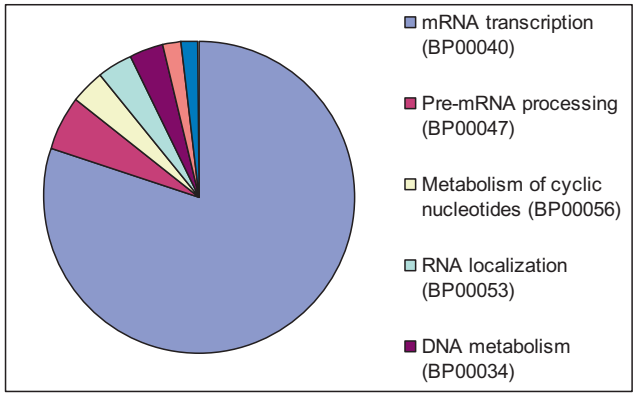
In silico prediction of miRNA targets is possible using several freely available software programs. Typically, the output from these programs can run to several hundred potential targets per miRNA. Several recent publications have demonstrated that combining the output of these programs may be more effective in predicting true biological targets than any of them individually (Megraw et al., 2007).

As an alternative, we employed another software program, ElMMo, (http://www.mirz.unibas.ch/ElMMo3/) that uses more than one miRNA as an input in order to predict likely human mRNA targets. The algorithm is designed to use the output of miRNA profiling experiments and is based on the hypothesis that any group of miRNAs whose expression changes in response to a particular signal (e.g. temperature) may be involved in co-ordinated targeting of a similar set of genes or pathways. As such, target genes rank higher if their 3′UTR contains binding sites for more than one of the miRs on the input list. The output of this analysis again provides a list of genes that might be impacted by co-ordinate action of several miRNAs. Implementing this approach the software generated a highly redundant list of >17000 potential target transcripts which were ranked according to their enrichment for one or more binding sites for the list of miRNAs in Table 1. From the top 300 hits we generated a non-redundant, annotated list of 173 genes which were subsequently subjected to gene ontology (GO) analysis in the PANTHER on-line functional annotation software program (http://www.pantherdb.org/) (Table 2a). The

Table 2
(a) Biological process functional annotation of 173 genes predicted to be targeted by more than one of the 10 differentially expressed miRNAs from the profiling experiment. 124 of the 173 genes were annotated to at least one known biological process (BP), while 49 genes were listed as "unclassified" with regard to functional annotation using PANTHER analysis (http://www.pantherdb.org/). (b) Nucleic acid metabolism (BP00031), accounted for 30% of the genes on the input list and within this category, 80% of the gene could be further sub-catagorised into mRNA transcription (BP00040).

Biological process (Accession)	No. of genes	% of total # genes
(a)		
Nucleoside, nucleotide and nucleic acid metabolism (BP00031)	52	30.1
Biological process unclassified (BP00216)	49	28.3
Signal transduction (BP00102)	38	22.0
Developmental processes (BP00193)	37	21.4
Protein metabolism and modification (BP00060)	20	11.6
Cell proliferation and differentiation (BP00224)	11	6.4
Transport (BP00141)	11	6.4
Cell cycle (BP00203)	9	5.2
Neuronal activities (BP00166)	8	4.6
Cell structure and motility (BP00285)	7	4.0
Immunity and defense (BP00148)	6	3.5
Oncogenesis (BP00281)	6	3.5
Cell adhesion (BP00124)	6	3.5
Apoptosis (BP00179)	4	2.3
Intracellular protein traffic (BP00125)	4	2.3
Lipid, fatty acid and steroid metabolism (BP00019)	4	2.3
Homeostasis (BP00267)	3	1.7
Sensory perception (BP00182)	3	1.7
Other metabolism (BP00289)	2	1.2
Amino acid metabolism (BP00013)	2	1.2

(b)



most significantly represented biological process was Nucleic Acid Metabolism (BP00031), accounting for 30% of the genes on the input list and within this category, 80% of the gene could be further sub-categorised into mRNA Transcription (BP00040) (Table 2b).

3.3. Disregulation of miR-7 expression impacts CHO cell growth and productivity

The observation that miR-7 was down-regulated in growth-arrested cells led us to investigate if this miRNA had a role in switching the growth behavior of the cells in response to temperature shift or if modifying its expression in CHO cells could mimic or augment the impact of temperature reduction on proliferation. We transiently transfected SEAP-expressing CHO-K1 cells with either an exogenous miR-7 mimic (pre-mir) or antagonist (anti-mir) to investigate how perturbing its expression would impact on cell behavior. Cells were grown as described at 37 °C for 24 h after transfection and either maintained at 37 °C or shifted to 31 °C which, as expected, reduced cell proliferation (Fig. 2A). Negative anti-mirs (AM-Cont) and pre-mirs (PM-Cont), which should not target any known endogenous sequence, were used to control for non-specific effects of the procedure and had negligible impact on growth compared to the untreated (cells only) or transfection reagent-treated cells (Tfx only). The specific targeting (knockdown) of endogenous miR-7 with anti-mir (AM-7) had little impact compared to AM-Cont in both cultures even though the profiling data suggested that this intervention would be expected to reduce growth. On the other hand, upregulation of miR-7 with pre-mir-7 (PM-7) had a considerable negative (8-fold) impact on viable cell density at 37 °C, effectively arresting cell growth. It was notable that PM-7 treatment reduced the cell density to levels similar to those observed in the temperature shifted cultures. MiR-7 up-regulation by PM-7 transfection was confirmed by qRT-PCR (Fig. 2B). Knockdown of endogenous miR-7 with AM-7 was less dramatic (~40%). However, accurately estimating target knockdown by anti-mirs can be difficult using qRT-PCR due to the mode of action of anti-mirs, i.e. competitive binding of the endogenous miRNA, which may be reversed upon RNA extraction from cells. Effective transfection was confirmed under these conditions using a siRNA against valosin-containing protein (VCP) which reduced cell proliferation by 60–80% (Doolan et al., 2010).

In view of the importance of CHO cells as recombinant protein factories we also investigated the impact of these treatments on SEAP production in the cultures (Fig. 3). As expected the total yield of secreted recombinant protein in batch cultures maintained at 37 °C was higher simply due to the higher cell densities in these tubes, except in the case of PM-7 transfection where the temperature-shifted cells yielded more secreted SEAP than PM-7-treated cultures maintained at 37 °C. However, the normalised productivity was significantly higher in all temperature-shifted cul-

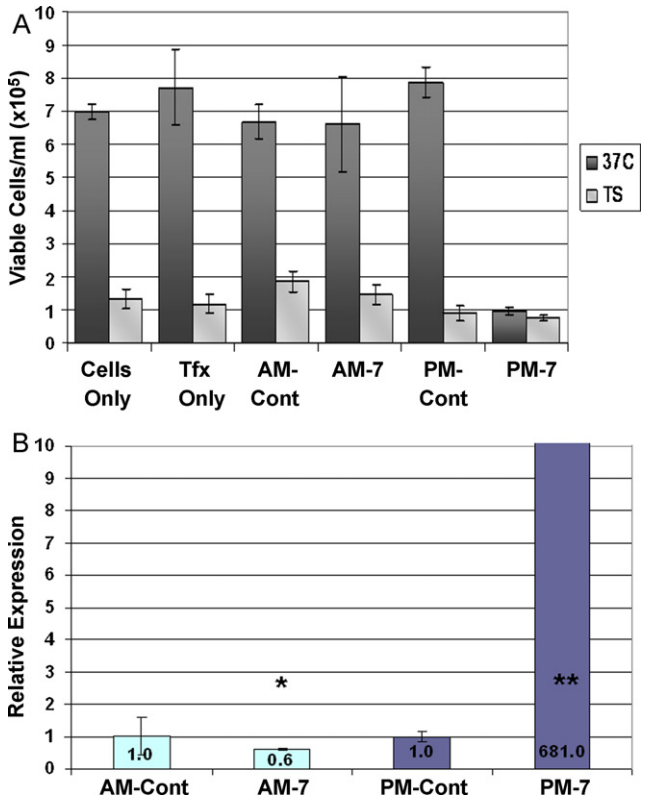


Fig. 2. (A) Effect of exogenous up-regulation with pre-mir-7 (PM-7) and down-regulation with anti-mir-7 (AM-7) on growth of SEAP-secreting CHO-K1 cells at constant 37 °C or with a temperature shift. Triplicate cell cultures were transfected on Day 0 and grown for 24 h at 37 °C. Half the cultures were then shifted to 31 °C and the remainder left at 37 °C. Viable cell number was measured after a further 72 h. (*Tfx Only*) Transfection reagent only as a technical control; (*AM-Cont*) anti-mir negative, non-mir-targeting sequence control; (*PM-Cont*) Pre-mir negative, scrambled control; (*TS*) temperature shifted culture. (B) qRT-PCR verification of miR-7 overexpression and repression in transiently transfected CHO-SEAP cells. Fold-change is indicated by figure inside column ($n = 3$, error = ±SD, $^*p = 0.31$, $^{**}p < 0.001$).

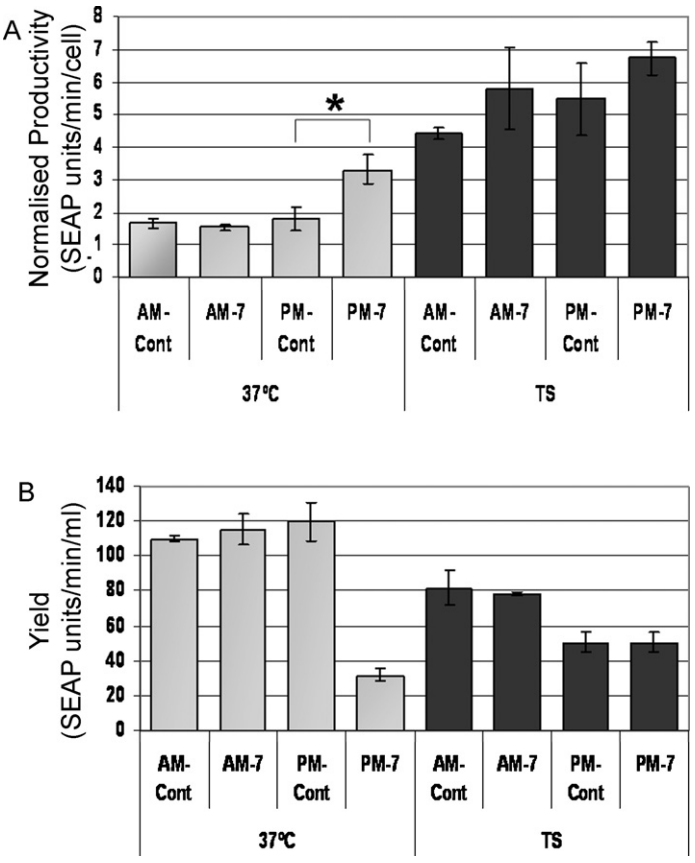


Fig. 3. Effect of exogenous up- and down-regulation of miR-7 expression on (A) normalised and (B) total productivity of SEAP-secreting CHO-K1 cells at 37 °C and with temperature shift (TS). SEAP activity in the culture medium was measured as increase in absorbance (colorimetric assay) per minute. Normalised productivity is calculated as change in absorbance per minute *per cell* in the original culture. (*Tfx Only*) Transfection reagent only; (*AM-Cont*) Anti-mir negative control; (*PM-Cont*) Pre-mir negative control; (*TS*) temperature shifted culture; (*NP*) non-SEAP secreting CHO cell line. $^*p < 0.005$, from biological triplicates.

tures, again as expected from cells that can commit resources to protein synthesis rather than proliferation. More notably, the cultures that were transiently transfected with PM-7 demonstrated an increase in normalised productivity – significantly in the case of 37 °C cultures ($p < 0.005$) – but not in total yield due to reduced cell numbers.

3.4. Hamster miR-7 is identical to orthologues

Most miRNA sequences are highly conserved across species though there may be occasional single base-pair differences. This indicates how fundamental these molecules are in biology—being found in viruses, plants and the animal kingdom. The first *Criteculus griseus* miRNA sequence (*cgr-mir-21*) was deposited in the Sanger miRBase database 2 years ago by this group (MI0005725). We were interested to know if miR-7 was similarly conserved. Cloning primers were designed to flanking regions of the genomic locus after alignment of orthologous sequences (mouse, rat and human) and amplified by PCR. The product of the PCR reaction was approximately of the expected size, again based on orthologues and sequencing revealed that the mature miRNA region was exactly the same as other species (Fig. 4A). The flanking pre-mir sequences were less conserved, again agreeing with what has been reported in species comparisons. This sequence been registered with the Sanger database (MI0016998) as *cgr-mir-7* based on its homology to the mouse and rat orthologues (Fig. 4B).

4. Discussion

We have previously demonstrated that the miRNA expression profile in CHO cells is altered subsequent to temperature-induced growth arrest (Gammell et al., 2007). At that time we compared the profile of cells grown at 37 °C for 3 days to cells that were shifted to growth at 31 °C and subsequently grown for another 3 days. In the work presented here we were interested in identifying earlier, acute miRNA expression responses to reduced temperature and so shortened the sampling time after the temperature shift to 24 h. We also employed qPCR-based Taqman Low Density Arrays (TLDA) for this analysis instead of the hybridization arrays used previously. The TLDAs are designed for profiling human samples but the high degree of evolutionary conservation demonstrated amongst miRNAs made it likely that they would be suitable for CHO profiling also. Indeed, recent information coming from next-generation sequencing of CHO miRNAs indicates that the conservation observed across other species is similarly maintained in hamster (Johnson et al., 2010). In our experiments we detected a similar percentage of the total (~350) miRNAs present in CHO-derived RNA as we did in human samples (35–42%). In addition, we cloned and sequenced a *miR-7* from Chinese Hamster, which we have designated *cgr-miR-7*, further demonstrating the mature sequence conservation amongst species at the genomic level at least. This reflected the findings with cross-species hybridization arrays (Gammell et al., 2007). One notable difference was that the commonly used human

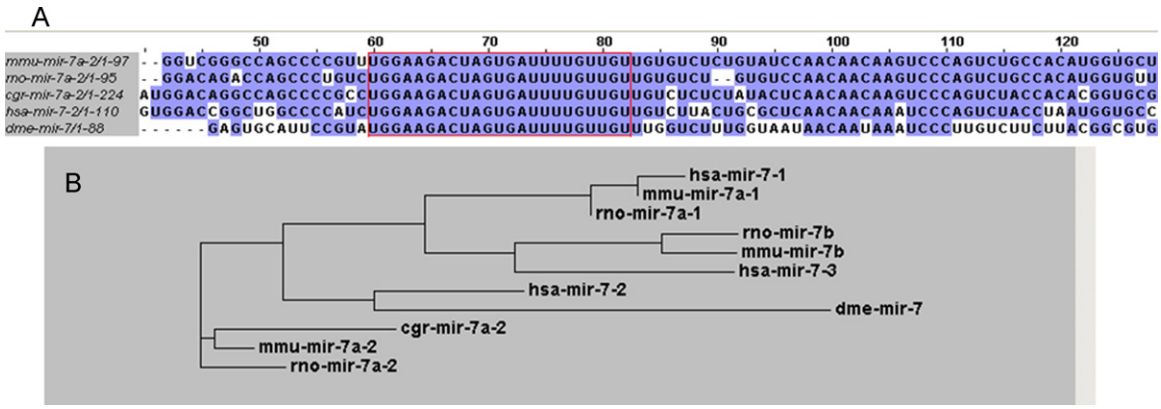


Fig. 4. Sequence alignment of *cgr-miR-7a-2* with orthologues (A). Darker blue denotes regions of higher similarity. The mature miRNA sequence in each case is denoted by a red box. (B) Dendrogram illustrating sequence similarity between species and miR-7 family members. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

endogenous control, RNU6, was not detected in any of the CHO samples using the TLDA cards. Let-7a was chosen as an alternative because it showed very consistent expression across the samples. Of the 10 differentially regulated miRNAs identified, 7 have previously been associated with cancer phenotypes (Reddy et al., 2008; Schaar et al., 2009; Friedman et al., 2009; Garzia et al., 2009; Wong et al., 2008; Liu et al., 2009; Cervigne et al., 2009). Somewhat surprisingly, the 4 miRNAs that were down-regulated after the temperature shift, including miR-7, have all been reported to mediate onco-suppressive effects in other systems. Likewise, miR-345 was activated subsequent to the change in culture temperature but has been shown to be causative in progressing leukoplakia to oral cancer (Cervigne et al., 2009). Given that the most obvious effect of reducing the temperature of a culture is to slow or arrest growth one would assume that miRNAs displaying increased expression under these conditions might be involved in mediating this phenotypic response, i.e. anti-proliferative. One possibility as to why we see the opposite trend, based on known functions of these miRNAs in other cell types, might be that the anti-proliferative role of these molecules is not required under conditions of low temperature culture, i.e. cold stress. Alternatively, it may also be due to the cell/tissue-specific nature of miRNA function (Cheng et al., 2005). In addition, it has been shown that, depending on factors such as cell cycle stage, miRNAs can either activate or repress translation of their target proteins (Vasudevan et al., 2007).

Comparing these findings to our previous study (Gammell et al., 2007) revealed that there was only one miRNA common to both lists, miR-320, but the direction of the change was different. The discord between the lists is not surprising given the different sampling times involved – 72 h versus 24 h post-temperature shift – and probably reflects the dynamic nature of miRNA expression in different stages of the fermentation cycle.

In silico prediction of miRNA target genes is possible with several on-line software tools (e.g. http://diana.cslab.ece.ntua.gr/microT/, http://www.targetscan.org/). These are based on either evolutionary conservation across species or straightforward miRNA:target homology searches. These analyses typically yield hundreds of potential targets per miRNA. In the case of profiling experiments where several miRNAs are potentially acting in unison to impact a cellular phenotype it may be more useful to try to identify genes that represent targets of more than one miRNA in the list. By applying this strategy and looking at the biological processes potentially impacted by targets of miRNAs reacting positively or negatively to TS, we found that Nucleic Acid Metabolism and mRNA Transcription were the most significantly over-represented processes. This makes sense given that cold shock has previously been shown to inhibit transcription in cells generally. The mechanism of this reduction in global transcription is poorly understood, although reduced culture temperature has been demonstrated to impact on expression of transcription factors leading to inhibition of transcription (Sonna et al., 2002; Roobol et al., 2009). In addition, low temperature is known to stabilize the secondary structures of RNA and DNA resulting in reduced DNA replication, transcription and translation (Phadtare et al., 1999). Furthermore, certain proteins demonstrate an ability to bind their own mRNA to destabilize RNA polymerase in prokaryotes causing attenuation of transcription and translation (Bae et al., 1997). Our analysis suggests that miRNA action may represent a further means by which transcription is attenuated in response to TS. In contrast, increased mRNA levels of specific genes have also been observed at low temperature suggesting that cells transcribe genes selectively at low temperature (Marchant et al., 2008; Fox et al., 2005; Yoon et al., 2005). Our data shows that disregulating miR-7 expression results in increased specific expression of SEAP, in effect mimicking the impact of TS in control cultures.

The use of TLDA technology, which employs a multiplex reverse transcription step, prompted us to undertake some validation of relative gene expression in single-target quantitative RT-PCR. The results for two miRNAs (miR-7 and miR-200c) demonstrated good correlation with the TLDA results in terms of directionality, though the magnitude, in the case of miR-7, was somewhat different. This discord might be due to differences in the RT step in each analysis whereby a megaplex (primers to all miRNAs) reaction is used for the TLDAs as opposed to a target specific primer being used in the individual PCR assays.

MiR-7 was chosen for functional investigation as the most down-regulated miRNA and due to its recognised role in modifying the expression of important cellular proteins such as α-synuclein, Pak1 and IGF-1R (Li and Carthew, 2005; Reddy et al., 2008; Junn et al., 2009; Jiang et al., 2010). The observation that exogenously increasing miR-7 expression caused a decrease in cell growth was unexpected based on the profiling data, where endogenous miR-7 decreased as the culture entered the stationary phase of growth after Day 3 and more so upon reduction of the culture temperature. This would suggest that artificially increasing miR-7 levels would reverse this decline in growth rate rather than reduce it further. Likewise accelerated depletion of cellular miR-7 by treatment with the inhibitor, AM-7, would be expected to cause a decrease in growth. However, the phenotypic impact subsequent to treatment with PM-7 is in keeping with the reported functions of miR-7 in human cancers and cancer cell lines. Intriguingly, in a recent very elegant study, miR-7 has been proven to be crucial in conferring robustness in biological systems (Li et al., 2009). This study demonstrated how miR-7 was instrumental in maintaining a nor-

mal developmental phenotype in *D. melanogaster* eggs and larvae when exposed to periodic temperature fluctuations. In the absence of wild type miR-7, the flies were significantly developmentally compromised as a result of the treatment leading the authors to conclude that miR-7 attenuated the more dramatic molecular responses to environmental flux, essentially smoothing out the potentially catastrophic effects of aberrant gene expression during development. In our system it may be that, although endogenous miR-7 levels drop in response to a temperature shift, artificially reducing miR-7 does not cause a similar physiological response, i.e. reduced growth. This would suggest that low endogenous miR-7 expression is not instrumental in slowing cell growth but it merely a consequence of it. However, artificially high levels of miR-7 have been shown to slow proliferation dramatically via down-regulation of the EGF receptor—an effect that has been observed already in cancer cells (Kefas et al., 2008). This pathway is unlikely to be the route to growth arrest in CHO cells given their lack of EGFR expression (Shi et al., 2000).

Exogenously increasing cellular miR-7 levels resulted in increased *per cell* productivity in 37 °C cultures compared to controls. This was despite achieving similar cell densities at either 37 °C or 31 °C, suggesting that miR-7 expression may impact on some aspect of protein translation or secretion in a temperature-dependent manner. It may also be the case that the increase in specific productivity was as a result of reduced cell growth in the PM-7 treated cultures. The establishment of a stable miR-7 over-expressing cell line may provide further insight into this effect, though the impact on proliferation might make clone generation somewhat challenging. In vivo, miR-7 is highly and, almost exclusively, expressed in specialised neuro-secretory cells, particularly pancreatic beta cells, suggestive of an involvement in some aspect of the secretory phenotype (Correa-Medina et al., 2009; Joglekar et al., 2009).

In conclusion, we provide the first report of exogenously dis-regulated miRNA expression as a potential tool to modify CHO cell bioprocess phenotypes. Its applicability in larger, fed-batch processes is yet to be investigated. As a gene engineering target for proliferation control miR-7 would have to be carefully utilized. The most obvious application would be to build cell density to optimal levels at 37 °C first, before increasing cellular miR-7 levels via an appropriate inducible system. Alternatively, supplying PM-7 to the cells transiently after the growth phase could be envisaged given appropriate optimization. This switch might be implemented instead of a drop in culture temperature to arrest proliferation and prolong cell viability to potentially improve overall process yield.

Acknowledgement

This work was supported by funding from Science Foundation Ireland.

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