CIRP expression on growth and productivity of CHO cells

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Abstract — **Mammalian cell culture is typically operated at the physiological temperature of 37°C. Low temperature cell culture at 30-33°C, in particular for CHO cells, increased the specific productivity of many recombinant proteins amongst many other benefits. However, the cell density is lower, thus reducing the total protein yield. Of the 17 mammalian cold-stress genes reported to be up- or down-regulated at low temperature, CIRP shows potential as a gene target for improving recombinant protein production, as its expression levels were reported to affect both growth and specific productivity. In this study, it was shown that over-expression of the cold-stress gene CIRP did not cause growth arrest in CHO cells, in contrast to previous reports. However, over-expression of CIRP successfully improved the specific productivity and total yield of a recombinant interferon-γ CHO cell-line at 37^{0}C by 25%.**

***Index Terms* — CIRP, cold-stress, interferon-γ, productivity.**

I. Introduction

Developments in mammalian cell culture and recombinant technology has allowed for the production of recombinant proteins for use as human therapeutics. One of the issues that is still being addressed is, improvement of the yield of recombinant proteins whilst maintaining product quality and efficacy[1, 2]. Methods of choice include process control of bioreactor conditions, and the use of genetic manipulation[1]. The change of temperature is a simple means by which an operating parameter for cell-culture can be altered. Conventional mammalian culture is carried out at the physiological temperature of 37^{0}C, but operating mammalian cell-culture at low temperatures of 30-33^{0}C brings about many changes in the characteristics of the cells which are of interest to the field of biotechnology. Recent reports discuss about the various beneficial traits observed during low temperature cell culture of Chinese hamster ovary (CHO) cells, such as enhanced cell longevity in batch culture[3, 4] and retention of proper glycosylation[5, 6]. CHO cells are one of the principal mammalian cell-lines used in industrial production of recombinant proteins[1], and low temperature culture is potentially useful in improving production in terms of yield and quality.

More importantly, the specific productivity of many CHO recombinant lines is increased 2-5 times[4-8] as compared to the same cells grown at 37^{0}C. However, CHO cells generally grow at less than half the rate at low temperatures as compared to 37°C, and up to 90% of the cells arrest in the G1 phase of the cell cycle[3, 5, 7, 9]. The total yield of a recombinant protein depends on both cell density and specific productivity. Despite the multi-fold increase in specific productivity, the total yield is actually decreased 30-50%, and twice the amount of time is actually needed to produce the same amount of recombinant protein at 32^{0}C compared to 37^{0}C[7, 10]. Most authors adopt a biphasic strategy[3, 5, 8, 9] by first building up a high cell density at 37^{0}C, then shifting the temperature down to 30-32^{0}C to harvest the recombinant protein product at high specific productivity.

Maintaining mammalian cells at low temperature elicits a cellular response akin to other external stresses like pressure, osmotic stress and hypoxia[5]. This "cold-stress response" has been observed in all cells, and the gene expression pattern in the cells changes as they adapt to the drop in temperature[11-14]. The changes in cell characteristics such as growth or productivity at low temperature are likely to be modulated genetically. Out of the 17 reported genes that show differential expression with cold stress[14], CIRP[15-17] (cold-inducible RNA-binding protein) is a well-characterised cold-stress gene that shows potential as a gene target to be modified to change growth or productivity. It is a RNA-binding protein that was first found to be highly expressed in mouse BALB/3T3 cells at 32^{0}C[15]. Over-expression of CIRP caused the growth arrest of mouse BALB/3T3 cells, and increased the percentage of cells in cell-cycle arrest. Moreover, transient knockdown of CIRP doubled cell density in mouse BALB/3T3 cells. Knock-down of CIRP at low temperature can potentially increase CHO recombinant protein production by allowing higher cell densities with high specific productivity at low temperature.

However, it was reported that there was no change in growth when CIRP was over-expressed in human cells[18]. Furthermore, transient co-transfection of CIRP and CAT reporter vectors increased CAT production through stabilisation of CAT mRNA transcripts, up to a 1.4-fold increase[18]. For a recombinant CHO cell line producing

human interferon-γ, CHO-IFNγ, the increase in specific productivity at low temperature was attributable to increased interferon-γ mRNA levels[10]. It would thus be fruitful to investigate whether increased CIRP expression is one of the factors that promote mRNA stability at low temperature, and hence increase the specific productivity.

This study was carried out to investigate where CIRP expression has a specific effect on growth or productivity of CHO cells. The end goal was to genetically modify CIRP to improve total production yield of recombinant CHO cells.

II. Materials and Methods

Extraction of RNA and cDNA synthesis RNA was extracted from cell pellets collected from adherent culture was washed with PBS and extracted using the TriZOL reagent (Invitrogen. Conversion of mRNAs into cDNAs was carried out using the ImprompII reverse transcription system (Promega) according to manufacturer's instructions. A 28 base pair docked poly-T oligonucleotide (T_{26}VN, Proligo) added to anneal and target poly-A mRNAs specifically.

Vector construction The open-reading frame of Chinese hamster CIRP was amplified using primers (Proligo) which introduce flanking XhoI and XbaI restriction enzyme sites, and spliced into expression vector pcDNA3.1(+) or pcDNA3.1(-) (Invitrogen) for an antisense vector.

Transfection Vector DNA was introduced into CHO cells by electroporation, using the Nucleofector T reagent pack (VCA-1002) on a Amaxa Nucleofector. The electroporated cells were given a recovery period of 24 hours before G418 (Sigma) was added at 800ug/ml to culture media for every passage for 2 weeks. Limiting dilution was then carried out on the selected cells to arrive at single clones after another 4 weeks.

Cell culture Adherent CHO-K1 (CRL61) was from ATCC. Adherent CHO-IFNγ was obtained from Dr Fiers[19]. It is an amplified CHO cell line secreting human interferon-γ secreting cell-line. Both types of adherent cells were grown in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), at 37^0C or 32^0C in humidified incubators with 5% CO_2.

For time-course studies, duplicates of each cell sample were sacrificed at each time point. Cell density was determined on a haemocytometer using the Trypan-blue exclusion method. Results were presented as mean ± S.D of all replicate readings.

Real-time PCR Reactions were performed in 96-well plates using SYBR-Green chemistry (ABI). Primers specific to 100-200 base pair regions of human interferon-γ, and hamster beta-actin was used. Samples were run in duplicates on an ABI 7000 Genetic Analyzer. The baseline was set to 0.02 and threshold cycles were normalised to the number of cycles of the flat part of the fluorescence against C_T graph. Interferon-γ mRNA levels were normalised to beta-actin levels using the $\Delta\Delta C_T$ method[20], and expressed as a ratio of fold increase of interferon-γ mRNA levels of CIRP over-expressing cells over control cells.

IFNγ ELISA

For quantification of human interferon-γ in culture media, a human IFNγ ELISA test kit (HyCult, Cat #HK030M) was used according to manufacturer's instructions. Results were presented as mean ± S.D of 2 readings.

Western blotting

Cells pellets harvested from culture were washed twice with PBS and added with lysis buffer (4% w/v CHAPS, 40 mM Tris, 0.1M PMSF), and then incubated for 5 minutes at room temperature. Centrifugation was then carried out to remove any cell debris. Extracted cell-lysates were quantified with the Coomassie Plus reagent (Pierce), using BSA as a standard. 25μg of each cell-lysate sample was loaded and separated on 12% SDS-PAGE gels using the Laemmli method. The SDS-PAGE gels were then electroblotted onto Hybond nitrocellulose membranes (Amersham). The blots were incubated in primary antibody at 1:1000 dilution and in secondary antibody at 1:10000 dilution. Detection was carried then out using ECL reagent (Amersham).

Anti-CIRP antibody was custom-made (Open Biosystems) by immunising rabbits with the polypeptide GSYRDSYDSYATHNE (corresponding to the C-terminus end of CHO CIRP) conjugated to through a terminal cysteine. Rabbit anti-β-actin antibody (Santa Cruz) was used to detect actin as a loading control. HRP (horseradish peroxidase) conjugated anti-rabbit Fab fragments (Jackson Immunosystems) were used as secondary antibodies.

III. Results

Cloning of the CHO homologue of CIRP. The mRNA sequence for the Chinese hamster CIRP homologue was first obtained to facilitate the construction of vectors to later modify CIRP expression levels in CHO cells. The sequence for the open reading frame (ORF) fragment was derived by PCR from cDNA of CHO-K1 grown at 32^oC, based on primers designed from conserved sequences of the human (accession number NM001280), rat (accession number NM031147) and mouse (accession number NM007705) CIRP homologues. The PCR product was sequenced and translated to arrive at the corresponding protein sequence needed to make anti-CIRP antibodies. The predicted molecular weight of the CIRP protein is 18.6kD. Both DNA and protein sequences were checked against the NCBI database, and the closest homologues were those of the rat and mouse. This ORF was inserted into expression vector pcDNA3.1(+) for an expression vector, and also in pcDNA3.1(-) for a knockdown vector.

Effects of CIRP over-expression and knockdown on the growth of CHO-K1 cells. CIRP is not expressed in CHO cells growing at 37^0C. CHO-K1 cells were transfected with the CIRP expression vector to over-express CIRP at 37^0C. Stable single clones were selected and screened by Western blotting. Positive CIRP over-expressing clones showed a 20kD band (Figure 2, clones 1, 6, 27 and 35) when probed on a Western blot. Negative clones were also isolated (Figure 2, clones 2, 3, 8-16, 18, 19) that did not show a 20kD band. Actin was

detected as a loading control, and showed an even intensity for all samples.

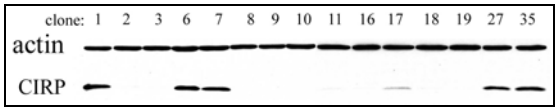

*Figure 2: (**A**) Western blot image for selection of positive single clones. CIRP was detected as a 20kD band. Clones 1, 6, 27 and 35 showed CIRP over-expression.*

The growth kinetics for four positive CIRP over-expressing clones (1, 6, 27 and 35) against four control clones (2, 3, 8, and 19, which are transfected, but not expressing CIRP) at 37^0C were determined. Over-expression of CIRP at 37^0C had no effect on the growth rates (Table 1) of the positive CIRP over-expressing clones compared to control clones.

Table 1: Summary of doubling times of control and positive clones

Control Clones	*Doubling time (hr)*	*Positive Clones*	*Doubling time (hr)*
Clone 2	**24.1**	Clone 1	**24.7**
Clone 3	**22.2**	Clone 7	**26.2**
Clone 8	**27.4**	Clone 27	**24.5**
Clone 19	**22.9**	Clone 35	**22.7**
Average	**24.1±2.3**	*Average*	**24.5±1.4**

CIRP is highly expressed in CHO cells at 32^0C. CHO-K1 cells were transfected with the CIRP knockdown vector to reduce the levels of CIRP at 32^oC. Stable single clones were isolated and screened by Western blotting. A representative null vector clone was used as a comparison as all selected clones showed positive knockdown of CIRP. All positive CIRP-knockdown clones did not show a 20kD band [Figure 4] when probed on a Western blot, but the null vector control clone showed a 20kD band. Actin was detected as a loading control, and showed an even intensity for all samples.

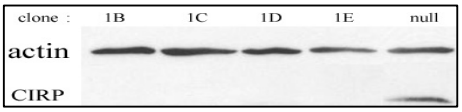

Figure 4: Western blot image for CIRP knockdown clones. CIRP was detected as a 20kD band. Positive knockdown clones 1B-1E did not show CIRP expression, while the null vector control clone showed CIRP expression.

The growth kinetics for four positive CIRP knockdown clones against the null vector control were determined. Knockdown of CIRP at 32^0C had no effect on the growth rates [Table 2] of the positive CIRP knock-down clones compared to the control clone.

Table 2: Summary of doubling times of positive knockdown clones and null vector control.

Knockdown Clones		*Doubling time (hr)*
Clone 1B		**34.7**
Clone 1C		**34.5**
Clone 1D		**33.2**
Clone 1F		**34.0**
	Average	**34.2±0.7**
Null control		**32.24**

Effect of CIRP over-expression on the productivity of CHO-IFNγ cells. The effect of CIRP over-expression on the productivity of CHO cells was investigated, using CHO-IFNγ cells as a model cell-line. CIRP expression vector or null vector was transfected into adherent CHO-IFNγ cells growing at 37^OC. G418 was then used to select the pools of CIRP-expressing or control CHO-IFNγ cells. A batch culture of the selected pools in 6-well plates was performed over a period of typical culture time of 5 days at 37^0C. The growth curves [Figure 6A] showed that CIRP over-expression does not affect growth rate of CHO-IFNγ, in agreement to previous data for CHO-K1 cells. ELISA results [Figure 6B] and real-time PCR results [Figure 6C] showed corresponding increases of in the protein and mRNA levels of interferon-γ, which resulted in a 25% increase in volumetric IFNγ production when CIRP was over-expressed. Since the total production was increased without negligible change to growth, the increase in specific productivity parallels that of the increase of total production. This was calculated to be $1.33x10^7$ μg/cell.hr for the CIRP over-expressing CHO-IFNγ cells, a 30% improvement over control CHO-IFNγ cells, at $1.02x10^7$ μg/cell.hr

IV. Discussions

The CHO growth data obtained in this study was compared to the BALB/3T3 growth data[15] [Table 3]. It could be clearly seen that over-expression or knockdown of CIRP did not change the doubling time of CHO cells. In contrast, over-expression or knockdown of CIRP expression changed the doubling time of BALB/3T3 cells by 8-9hrs. The results also support the observation[18] that over-expression of CIRP did not affect the growth of human cells. This result was not unusual as the functional domains of CIRP do not match any known domains that are related to growth, and RNA-binding proteins in general are not reported to have an effect on growth[21]. Furthermore, RBM3 (RNA-binding motif 3), which shares 80% homology with CIRP and contains the same domains, was reported to have no effect on growth[22, 23].

Table 5: Summary of growth data for CIRP over-expressing and knockdown CHO-K1 clones, with reference to (15)

CHO-K1 cells	Doubling time (hr)	
	BALB/3T3 cells	CHO-K1 cells
At 37^0C		
CIRP-overexpressing clones	**23.5±2.9**	**24.5±1.4**
Control clones	**17.6±1.1**	**24.1±2.3**
At 32^0C		
Knockdown clones	**19.1***	**34.2±0.7**
Null control	**28.6**	**32.24**

**calculated from (15)*

It was previously reported that CIRP over-expression stabilised and increased the amount of CAT reporter mRNA[18]. It was shown that similarly, CIRP over-expression helped to increase the amounts of mRNA of interferon-γ in CHO-IFNγ, resulting in corresponding

increases in interferon-γ protein production. This proof of concept gives motivation into further work with CIRP on other recombinant CHO-cell lines, especially in suspension culture so as to be industrially relevant. Furthermore, identifying cold-stress genes that affect other areas like glycosylation quality and cell longevity, using similar methodology to the work done for CHO CIRP, are potentially beneficial in improving the quality and productivity of recombinant CHO proteins. However, the current limited knowledge on mammalian cold stress genes, in particular that for CHO, first needs to be expanded. The use of high-throughput screening methods such as microarrays and two-dimensional gel electrophoresis would help greatly to elucidate the genomic and proteomic profiles of CHO and other mammalian cells at low temperature to uncover useful genes for subsequent genetic modifications.

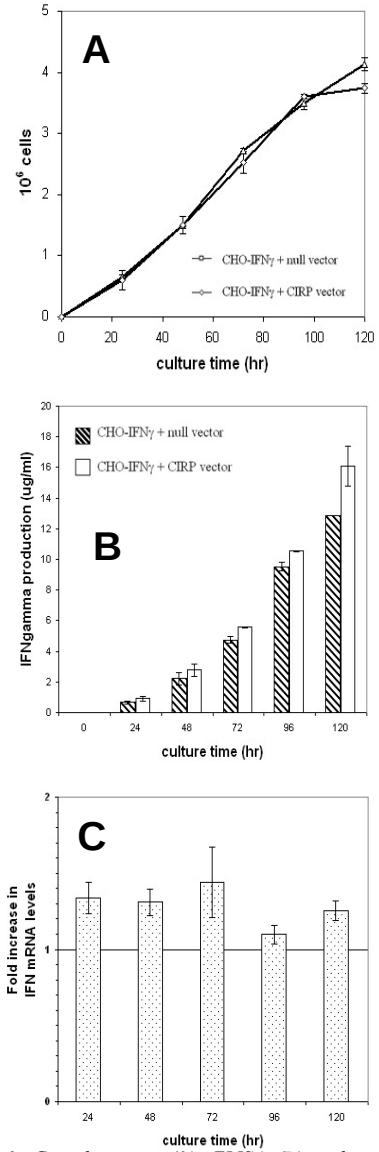

*Figure 6: Growth curves (**A**), ELISA (**B**) and real-time PCR (**C**) comparisons for adherent CHO- IFNγ cells* with CIRP vector / null vector grown in 6-well plates.

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