Investigation of the molecular mechanisms regulating growth and recombinant protein productivity in suspension-adapted CHO-K1 cells

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By

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ (Candidate) **ID No.:** 54166454

Date:

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Abstract

Chinese Hamster Ovary (CHO) cells are the primary system for the production of recombinant proteins for therapeutic use. Protein productivity is directly proportional to viable biomass, viability and culture longevity. Identification of proteins and microRNAs (miRNAs) associated with regulation of growth and viability of CHO cells could improve recombinant protein productivity.

Low temperature cultivation of CHO cells can reduce growth and improve viability and productivity of culture by extending the viable culture period. CHO-K1 cells grown in temperature-shifted culture (initially maintained at 37^0C to maximize biomass and then shifted to 31^0C to arrest cell growth) and standard culture at 37^0C were analysed using 2D-DIGE. From the total of 53 identified differentially expressed proteins, 23 proteins were specifically differentially expressed in temperature-shifted culture and were involved in regulation of variety of cellular functions such as growth (HNRPC), cap-independent translation (EIF4A) and cytoskeleton (PFN2). The inhibition of HNRPC, EIF4A and PFN2 using siRNAs was found to reduce growth and increase specific productivity (Qp) of SEAP in CHO-K1 cells suggesting them as potential target to improve productivity of cells.

miRNA profiles of CHO cells grown in biphasic culture were established using Ambion miRNA-bioarray and miRNA TaqMan® Low Density Arrays (miRNA-TLDA) to identify miRNAs associated with regulation of cell growth and viability at low temperature. Two known growth regulatory miRNAs, miR-7 (down regulated) and miR-24 (up regulated), were observed to be differentially expressed following temperature-shift. Inhibition and over-expression studies showed that over-expression of miR-7 and miR-24 arrested growth (88% and 56% respectively) and increased Qp (116% and 68% respectively) of SEAP in

CHO-K1 cells in a temperature-independent fashion. These miRNAs represent attractive targets for regulating cell growth and improving productivity in CHO cells.

Secreted proteins and peptides by CHO cells into culture medium can modulate growth and viability of culture as well as quality of product. The secretome of CHO-K1 cells was profiled using IMAC30 and Q10 chip surfaces with SELDI-ToF MS to investigate differential expression of proteins/peptides over time. While not identified yet, some of these peaks appear at different phases of cell cycle (i.e. lag, log and stationary) and may provide markers for monitoring proteolytic activity and health of the culture to maximize recombinant protein therapeutic production.

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Abbreviations

%	-	Percentage
AM	-	Anti-mir
ATCC	-	American Tissue Culture Collection
BSA	-	Bovine Serum Albumin
cDNA	-	Complementary DNA
Da	-	Daltons
DMEM	-	Dulbecco's Minimum Essential Medium
DMSO	-	Dimethyl sulfoxide
DNase	-	Deoxyribonuclease
DNA	-	Deoxyribonucleic Acid
dNTP	-	Deoxynucleotide triphosphate (N= A, C, T, G or U)
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetracetic acid
FCS	-	Fetal Calf Serum
GAPDH	-	Glyceraldehyde-6-phosphate dehydrogenase
Hrs	-	Hours
IMS	-	Industrial Methylated Spirits
kDa	-	kilo Daltons
Min.	-	Minute
miRNA	-	microRNA
mRNA	-	Messenger RNA
M.W.	-	Molecular Weight Marker
NCTCC	-	National Cell & Tissue Culture Centre
NFX	-	neoFX
OD	-	Optical Density
Oligos	-	Oligonucleotides
P	-	Passage
PBS	-	Phosphate Buffered Saline

PCR	-	Polymerase Chain Reaction
PM	-	Pre-mir
RNA	-	Ribonucleic Acid
RNase	-	Ribonuclease
RNasin	-	Ribonuclease Inhibitor
rpm	-	Revolution(s) Per Minute
RT-PCR	-	Reverse Transcriptase-PCR
SCR	-	Scrambled
SDS	-	Sodium Dodecyl Sulphate
sec(s)	-	Second(s)
SF	-	Serum-Free
SFM	-	Serum-Free Medium
siRNA	-	Small interfering RNA
TBS	-	Tris Buffered Saline
TE	-	Tris-EDTA
TEMED	-	N, N, N', N'-Tetramethyl-Ethylenediamine
Tris	-	Tris(hydroxymethyl)aminomethane
UHP	-	Ultra high pure water
v/v	-	volume/volume
w/v	-	weight per volume

Section 1.0

Introduction

1.1 Chinese Hamster Ovary Cells

Although various expression systems (prokaryotes, yeast and insects, etc) are capable of producing large amounts of protein, the decision to employ mammalian cells has been driven by the need for biologically active proteins. The biological activity of proteins can depend on a number of complex protein modifications including proper folding and post-translational modifications such as glycosylation. Lower organisms such as prokaryotes and yeast cells, cannot perform these complex post-translational modifications and hence restricts their use in the production of pharmaceutical bio-products.

Mammalian cells, in particular the Chinese Hamster Ovary (CHO) cell line, have emerged as the preferred host for the large scale production of recombinant proteins for therapeutic use. A large number of recombinant protein products, such as interferons (IF-γ and IF-β), tissue plasminogen activator, antibodies, growth factors (VEGF) and viruses (sindbis), are being industrially produced using CHO cells. CHO cells are epithelial-like (sometimes also described as 'fibroblast-like') cells that were established in 1957 by TT Puck and colleagues at the Department of Biophysics, University of Colorado Medical School in Denver, USA from an ovarian biopsy of an adult Chinese hamster (Puck *et al.* 1958). The CHO cell lines were found to have the following attributes that make it suitable for recombinant protein production:

- Easy to handle and culture (CHO are a very fast growing cell line)
- Highly adaptable to a variety of growth conditions including serum-free media
- Exogenous DNA can be readily transfected into CHO cells
- Genetic manipulation of CHO cells is simplified by virtue of their functionally haploid state (Siminovitch 1985).

Many commercially available subclones of the parental CHO and/or derivatives of CHO cells have been constructed to exhibit certain features that may help to improve the efficiency of production processes (Table 1).

Table: 1 Commercially available subclones of the CHO cell line

Cell line	**Description**
A2	Overproduces ornithine decarboxylase in response to polyamine deprivation
A2H	A recline of "Clone A" established by Puck (the "H" denotes MRC Harwell)
CHO-DHFR or CHO-DUKX-B11	Dehydrofolate reductase negative subclone of the original CHO cell line
CHO-K1	A subclone from the parental CHO cell line
CHO MT^+	A subclone of the parental CHO cell line
Pro-5	The cells are proline auxotrophs and must be grown in a medium that contains proline (40 mg/L)
CHO-SSF	A subclone of CHO-DUKX-B11
CHO-protein free	The cells have been adapted to grow in protein-free medium
SuperCHO	A subclone of CHO-K1 expressing insulin, transferrin and IGF-1
ViggieCHO	A subclone of DUKX-B11 adapted to grow in the absence of exogenous growth factors

Over the last two decades, significant improvements in recombinant protein productivity in CHO cells have taken place. This improvement is mainly due to improved media formulation, cell engineering approaches and reactor design (Wurm 2004). Until 1986, CHO cells typically reached a maximal density of ~$2x10^6$ cells/mL, specific productivity ~10pg/cell/day and product titre ~50mg/l in a batch process production phase of about 7 days. By 2004, cells were observed to rapidly grow >$10x10^6$ cells/mL with specific

productivity ~90pg/cells/day and product titre ~4.7 g/L and can maintain high viability for up to 3 weeks.

1.2 CHO Cell Culture

Although each clone has its own specific media requirement, CHO cells can be generally grown as an adherent culture with commercially available media such as DMEM, α-MEM or Ham's F-12, usually in the presence of 10% fetal calf serum in a humidified incubator at 37^0C and 5% CO_2. However CHO cells can readily be adapted to grow in serum-free or protein-free medium. CHO cells usually grow quickly, so sub-culturing at least twice-a-week is recommended. CHO cells can be frozen for storage in culture medium supplemented with 5% DMSO in liquid nitrogen.

1.2.1 Types of CHO Cell Cultivation

CHO cells can be cultured as adherent or suspension culture.

1.2.1.1 Suspension Culture (non-anchorage dependent cells)

Cells that do not require a surface to attach for growth are generally grown in suspension culture. However due to the absence of the cell wall, mammalian systems are prone to damage by shear forces and therefore the development of novel techniques to cultivate mammalian suspension cultures has focussed on the need to avoid creating high shear stresses. Suspension cultures are adaptable, homogenous and scalable systems. Their engineering is thoroughly understood and standardized at large scale, and automation and cleaning procedures are well established. Suspension systems offer the possibility of quick implementation of production protocols due to their ability to be scaled easily once the basic culture parameters are understood. The scale of operation of freely suspended

mammalian cell culture has been increasing and in order to meet the demand for recombinant therapeutic products, this increase is likely to continue (Wurm 2004). One of the great advantages of suspension culture is the opportunity it provides to study interactions of metabolic and production phenomena in chemostat or turbidostat steady-state systems. Furthermore, in suspension cultures from which cell number and cell mass measurements are very easy to obtain, rigorous and quantitative estimations of the effects of growth conditions or perturbations of metabolic homeostasis can be made. Such studies speed up the development of optimal processes (Wurm 2004). CHO cells are very amenable to suspension culture and are generally grown at 60-100 rpm agitation speed, 20-80% DO, 5% CO_2, 7.2-7.4 pH and 37^0C (either in the presence or absence of serum).

1.2.1.2 Anchorage dependent cells

Anchorage-dependent cells require a surface substratum for growth. The routinely used surfaces, i.e. petri dishes, carrel flasks, T-flasks and roux bottles that are used for small scale cultures, are not amenable to scale-up since they provide only a limited growth surface to volume ratio. The first challenge for the production of recombinant protein products from anchorage dependent CHO cells was to increase the surface to volume ratio for attainment of maximal cell densities in the bioreactor. In the 1950s, many industrial vaccine production plants started using roller bottles which provide an inner surface as the substratum for growth. The roller bottles were rotated mechanically at optimized speed and each rotation ensures that the cells bound to the inner surface are exposed to gases and nutrients from the culture medium for growth. The disadvantage of such a process is that it

is labour intensive, expensive and the risks of contamination are enhanced because of the degree of handling necessary.

1.2.1.3 Suspension culture of anchorage dependent cells

The traditional approaches for the production of recombinant protein products from adherent cell culture were based on roller bottle cultures. The discovery of microcarriers combined the potentials of attached with suspension culture and opened the possibility of growing anchorage-dependent cells in suspension. The first microcarrier culture was diethylaminoethyl (DEAE) Sephadex A50 which was originally designed for ion exchange chromatography (van Wezel 1967). Most conventional microcarriers have a diameter of approximately 200- 250μm when suspended in medium and a specific density of 1.02-1.03g/cm^3, slightly higher than that of the medium. This allows minimum agitation to be used to keep them in suspension. It also allows a quick settlement after agitation is turned off, which is ideal for culture harvest or exchange of medium for supporting higher cell growth. The potential for increasing cell growth surface area was further advanced by the development of macroporous microcarriers. These are highly porous convoluted spheres with spacious internal cavities for cell attachment and growth. The cells in the interior of the macroporous microcarriers are also shielded from detrimental shear effect. The ease of cell retention in microcarriers in culture also enables the use of perfusion system.

1.2.2 Modes of cell culture in Bioreactors

The following modes of cultivation are available for cells grown in bioreactors:

1.2.2.1 Batch Culture

Batch culture is a closed system in which cells and medium are added once to the reactor at the beginning of culture and harvested after an appropriate time interval. The physical as

well as chemical environment in batch culture varies with time. Proper agitation and aeration are required for good cell growth and production. Nutrient depletion and waste accumulation are the major critical factors determining cell growth in batch culture. Batch culture was the method of choice for pharmaceutical production until recently. It is economical, flexible for different products or cell types with lower risk of contamination or cell mutation because of the relatively short cultivation time. On the other hand, it is time and labour consuming, requires a higher cost of inoculum preparation, needs instrumentation more frequently (i.e. autoclave and washer, etc) and is less productive.

1.2.2.2 Fed-batch Culture

Fed-batch culture is a modified version of batch culture to improve productivity. In fed-batch culture, small volumes of medium or selected nutrients are fed to the culture during the growth cycle to enhance growth and productivity of the culture. The culture is subsequently harvested and the product is recovered. In this type of culture method, accumulation of waste products is the major limiting factor for cell growth and recombinant protein productivity. Fed-batch cultures are more productive and can maintain higher viable cell densities for longer time periods compared to the batch mode.

1.2.2.3 Perfusion Culture

In perfusion cultures, cells are physically retained in the vessel while the spent medium is withdrawn continuously from the culture system and an equal volume of fresh medium is added. The perfusion culture production requires reliable cell retention and proper feed rate specifications. The rate of medium replacement (perfusion) is controlled by the nutritional requirements of the cells. They are highly automated, consistent and productive with reduced labour and instrumentation requirements. However, they are less flexibile and

difficult to monitor cell, require uniformity of raw material and high cost due to continuous replenishment of medium, and have a higher risk of contamination and cell mutation.

1.3 Generation of suitable production clones

For the production of a recombinant protein, the gene coding for the protein of interest needs to be stably transfected into CHO cells. Lipofection, using cationic lipid-based transfection reagents e.g. Lipofectamine-2000 (Invitrogen), is the most commonly used method for high transfection efficiency (Gamper and Shapiro 2003). However, calcium phosphate-mediated method is a cheap and reliable method.

The gene of interest is expressed using selectable and amplifiable expression systems to increase recombinant protein production. There are a number of expression systems available to express the protein of interest in CHO cells. These include dihydrofolate reductase (DHFR), neomycine phosphotranspherase (NPT)-G418, glutamine synthetase (GS) and metallothionein (MT) expression systems. The use of selectable marker (e.g. DHFR, NPT, GS or MT) enables survival of only those cells which produce increased amounts of the marker gene product (due to exogenous plasmid) in presence of selection pressure (methotrexate, G418, reduced glutamine or increased metal) in culture medium. This facilitaes easy isolation of recombinant variants.

All expression systems also have issues with their stability in expression which is an important aspect to consider when selecting expression systems. The presence of selective pressure at an optimized concentration is a constant requirement for stable production of recombinant proteins (Bailey *et al.* 2002). A drop in recombinant protein production has also been observed in the presence of selection pressure over time (Strutzenberger *et al.*

1999). An increase in selection pressure above optimum concentration can increase the gene copy number in the genome of rCHO but it may not significantly enhance the productivity due to limited transcription in cells (Fann *et al.* 2000). The removal of selection pressure in culture results in reduced gene copy number with time and this results in reduced production (Barnes *et al.* 2003). Also, the co-amplification of marker and recombinant genes can result in reduced cell growth due to increased metabolic load on the cell (Yallop and Svendsen 2001, Gu *et al.* 1995). Apart from this, the site of incorporation of the recombinant gene within the host cell genome and clonal variation can also affect stability and transcription of recombinant genes (Barnes *et al.* 2003, Kim *et al.* 2001). Therefore, a better understanding of these processes will be helpful to engineer cells for improved cell growth and productivity.

1.4 Culture Medium

Culture medium is an important compartment in designing any culture process since productivity is directly proportional to biomass and viability of producer cells. Each culture medium contains a large number of components i.e. amino-acids (essential and non-essential), vitamins, nucleic acids, lipids, inorganic salts, growth promoters (IGF, EGF and FGF, etc) and an energy source, and vary from each other in their composition. Based on composition, culture medium should be able to satisfy all the nutritional needs of cells throughout the culture. At the same time, culture medium should not increase osmolality or encourage generation of excess metabolic waste products as these issues affect both quantity and quality of product (Castro *et al.* 1992, Lao and Schalla 1996). Recent developments in media formulation can be broadly divided into following categories.

1.4.1 Serum supplemented medium

Serum, a non-cellular portion of blood that remains after removal of blood cells and clotting proteins, is one of the most important basic requirements for most mammalian cell lines during *in vitro* culture. It contains an ill-defined mixture of plasma proteins and polypeptides, lipids and hormones, nutrients, minerals and a variety of other unknown substances that may or may not be advantageous for cell culture (Sato 1983). Serum exerts shear-protective and anti-apoptotic effects on cells (Zanghi *et al.* 1999). However, serum can show relatively high batch-to-batch variation and has also been considered as a great source of microbial contaminations such as viruses, mycoplasmas or prions.

1.4.2 Serum-free medium formulation

Elimination of serum from culture medium is an important factor when a product is for therapeutic purposes since serum may be a source of contaminants (e.g. viruses, mycoplasma or prions), is hard to standardize (batch to batch variation), and increases the cost of down stream processing. A number of serum-free media formulations for CHO cells have been developed by various researchers using different approaches. These includes GC3 (Gasser *et al.* 1985), WCM5 (Keen and Rapson 1995), CHO-T1-SF (Schroder *et al.* 2004), ExCell (Wyatt DE 1994) or UC203 (Ii *et al.* 1995). A number of animal component-free and chemically-defined serum-free media are also commercially available for CHO cell growth i.e., ExCell CD (Sigma).

1.4.3 Protein-free medium formulation

The elimination of proteins from medium would be a major achievement in pharmaceutical protein production as it allows a further reduction in downstream processing costs. A number of protein-free media formulations are available to date, including MCDB301

(Hamilton and Ham 1977) and CHO-T1-PF (Schroder *et al.* 2004). A few animal component-free and chemically-defined protein-free media are also available for CHO cell growth, such as CHO-PF-AF (Sigma).

1.5 Translation

Decoding of messenger RNA (mRNA) to produce a specific protein (or polypeptide) according to the rules specified by the genetic code is called translation. Translation is the first stage of protein biosynthesis and occurs in the cytoplasm at ribosomes. Ribosomes are made of a small and large subunit. During translation, mRNA sequence provides a template to guide the synthesis of a chain of amino acids that form a protein. Eukaryotic translation proceeds in three phases: activation, initiation, elongation and termination.

1.5.1 Eukaryotic translation initiation

The amino acid is joined by its carboxyl group to the 3' OH of the transfer RNA (tRNA) by an ester bond and therefore decoding of mRNA during translation proceeds in the 5'→ 3' direction. The small ribosomal subunit has to be recruited to the mRNA at the 5'end for translation initiation. The eukaryotic translation initiation is one of the major control points in translation. Two principle pathways, cap-dependent and cap-independent, are available for attachment of the ribosome in eukaryotes for translation initiation. Cap-dependent translation pathway is preferable translational mechanism, however cells may also use the cap-indepndent translation mechanism under various stress conditions such as hypoxia or hypothermia (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006).

1.5.1.1 Cap-dependent translation initiation

The cap-structure is added to the 5' end of the pre-mRNA during transcription. Cap-structure consists 7-methylguanosine linked to the first nucleoside via a 5'-5' triphosphate bridge added during the synthesis of the primary transcript. It facilitates slicing, polyadenylation, nuclear export, stability and recognition of mRNA for translation.

Cap-dependent translation initiation involves large numbers of eukaryotic translation initiation factors (eIFs) and other proteins that participate in the stepwise assembly of the 80S ribosome initiation complex (translation initiation complex) (Holcik *et al.* 2000) and can be explained in following 5 specific steps (Figure 1.5.1):

1. The ribosome must dissociate into 40S and 60S subunits;
2. The pre-initiation complex is formed when eIF2 binds Met-tRNA with GTP (eIF2.Met-tRNA.GTP), which then binds 40S subunit to form 43S ribosomal subunit. tRNAs carry the amino acids to the actively translating ribosome during protein synthesis;
3. The 7'-Methyl-Gppp cap-structure at 5'-UTR of mRNA is recognised by eIF4E which is part of the cap-binding protein complex eIF4F (composed of the RNA helicase eIF-4A, the scaffold protein eIF4G, and the cap-binding protein eIF4E). eIF-4B and eIF-4H also joins this complex. This complex melts the secondary structure of near the 5' end of mRNA;
4. eIF-3 of 43S pre-initiation complex binds to eIF-4G at cap-structure and searches for the translation initiation signal. The eukaryotic signal to begin translation is an AUG codon in a particular context and as such, all proteins begin with a Methionine (encoded by AUG and recognised by t-Met) that is later cleaved. Binding of pre-

initiation complex to AUG releases the associated translation initiation factors which are used in further translation initiation cycles;

5. The 60S subunit then associates with the pre-initiation subunit to form the 80S initiation complex.

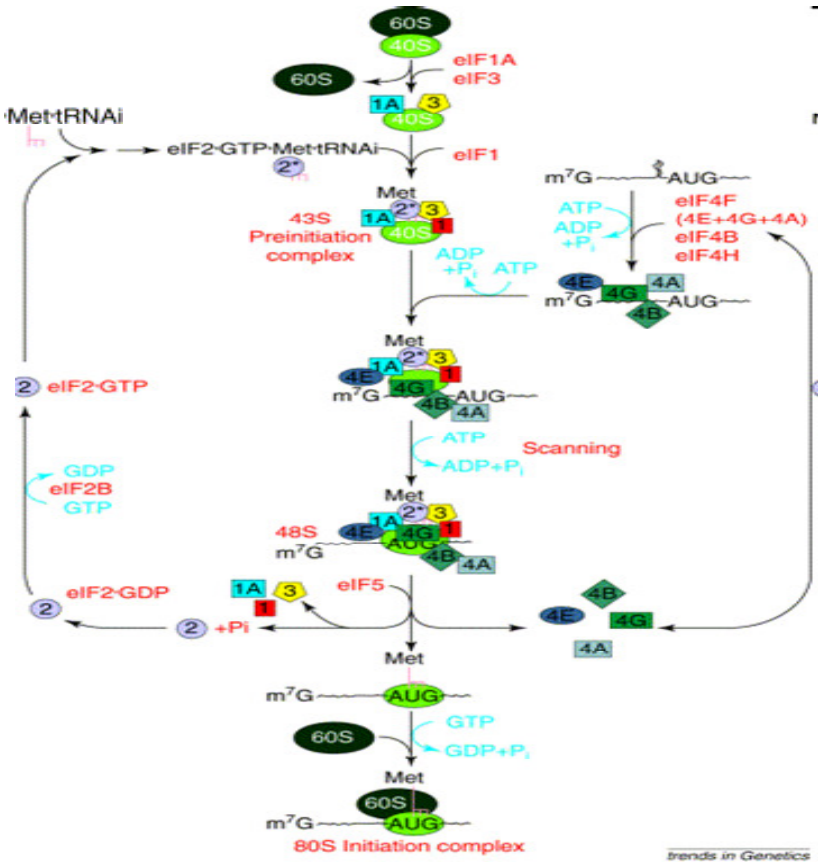

Figure 1.5.1: Schematic diagram of cap-depndent translation initiation.

1.5.1.2 Cap-independent translation initiation

The cap-independent translation initiation involves recruiting and binding of the small ribosomal subunit (40S) to a complex secondary structure element within the mRNA, known as the internal ribosomal entry site (IRES) (Pelletier and Sonenberg 1988). IRES elements are often found in characteristically long structured regions on the 5'-UTR of an mRNA that may or may not have regulatory upstream open reading frames (uORFs). Both

of these features on the 5'-end of the mRNA hinder ribosomal scanning, and thus promote a cap-independent translation initiation mechanism. The molecular mechanism of cap-indepdent translation is not known. A number of proteins (represented by Factor X), such as FUBP2 (Lin *et al.* 2009), HNRPL (Hwang *et al.* 2009), and HNRPD (Paek *et al.* 2008), have been observed to bind with IRES elements which could be involved in cap-independent translation initiation (Figure 1.5.2). The variant of eIF-4G (or other members of eIF-4G familiy such as p97) could also be expected to facilitate the IRES-mediated interaction between mRNA and 40S ribosomal unit as the truncated form of eIF-4G has been observed to stimulate translation of some of the viral mRNAs containing an IRES (Borman *et al.* 1997). In cap-dependent translation initiation, eIF-4G is responsible to bring together the capped end of the mRNA (via cap-binding protein eIF-4E) with the 40S ribosomal subunit (via eIF-3). eIF-4G is a subunit of the trimolecular eIF-4F complex, together with eIF-4E and eIF-4A. Once 40S subunit finds initiation codons, the 60S subunit joins this IRES-mediated pre-initiation complex and facilitates translation.

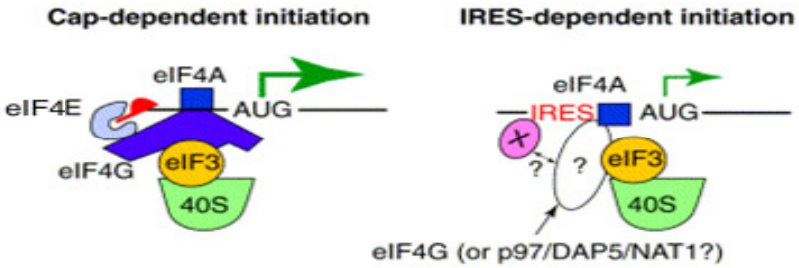

Figure 1.5.2 IRES-mediated cap-independent translation initiation.

1.5.2 Translation elongation

Each translation elongation cycle adds one amino acid at a time to a growing polypeptide according to the sequence of codons found in the mRNA. The translation initiation codon

(AUG) facilitates binding of t-RNA containing methionine amino acid at the P-site. The extension of the polypeptide chain occurs when a specific aminoacyl-tRNA, as determined by the template mRNA, binds to ribosome at the A-site. The peptidyl transferase catalyses the formation of a peptide bond between the incoming amino acid at the A-site and peptide at P-site resulting the transfer of naïve peptide to t-RNA at the A-site. Thereafter peptidyl-tRNA at their A-site physically translocates to the P-site and another cycle of elongation starts to add further amino acids into the growing peptide.

1.5.3 Translation termination

Termination of the polypeptide happens when the ribosome encounters a stop codon (UAA, UAG, or UGA). No tRNA can recognize stop codons. Releasing factors recognize these nonsense codons (stop codons) and cause release of the polypeptide chain.

1.6 Regulation of cell proliferation and productivity

An increase in the number of cells as a result of cell growth and cell division is called cell proliferation. Recombinant protein yields from CHO cell fermentations are directly correlated with cell number and culture longevity. Cell growth in standard production culture systems can be divided into four phases, i.e. lag, log, stationary and decline phase. Long lag phases (slow growth rate) are undesirable. High growth rates are necessary essential at the beginning of a process to achieve sufficient cells for maximal production. Uncontrolled proliferation beyond a certain desired cell density is also undesirable due to nutrient and oxygen depletion, toxic metabolite accumulation, cell death and degradation of the products (al-Rubeai and Singh 1998, Zeng *et al.* 1998, Zeng and Deckwer 1999). Regulation of cell proliferation in production cultures has been a topic of interest for researchers. Proliferation control strategies are typically implemented in the mid- or late-

phases of exponential growth and have been shown to improve both the productivity and duration of production processes. Regulation of cell proliferation can generally be achieved through cell cycle arrest or through regulation of nutrients availability.

1.6.1 Cell cycle arrest based approaches

Protein production is dependent on the phase of the cell-cycle and several genes such as those involved in ribosome biogenesis and protein translation are expressed highly in the G1 phase (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002). Cells arrested at the end of G1-phase of cell cycle are metabolically more active and bigger in size than non-arrested cells (Bi *et al.* 2004, Carvalhal *et al.* 2003). For these reasons, the G1-phase of the cell cycle is considered the ideal time for increased production of recombinant proteins and G1 arrest has been used to increase the productivity in a number of commercially relevant cell lines such as hybridomas and CHO cells (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002). Some studies have reported the S phase as the optimal production phase (Lloyd *et al.* 2000, Fox *et al.* 2005), an example being the increased production of human interferon-gamma (IFN-γ) upon increasing the percentage of CHO in S phase (Fox *et al.* 2005). There are a number of G1 phase arrest based approaches to regulate the cell growth and productivity and are discussed in the following sections.

1.6.1.1 Cell engineering based approaches

Currently most cell engineering based approaches to control proliferation and enhance productivity are at the proof of concept stage and do not form a major part of most industrial production runs. These studies have allowed researchers to specifically identify molecules which are important for regulation of proliferation (Table 2).

Table 2: Effect of cell engineering on cell cycle and productivity

Contributor	**Cell line and recombinant product**	**Effectors**	**Productivity (fold change)**	
			Qp	**Y**
(Fussenegger *et al.* 1997)	CHO Secreted alkaline phosphatase (SEAP)	$p21^{CIP1}$, $p27^{KIP1}$ or p53175P	10	ND
(Fussenegger *et al.* 1998)	CHO SEAP	$p21^{CIP1}$	10 to 15	ND
(Mazur *et al.* 1998)	CHO SEAP	$p21^{CIP1}$, $p27^{KIP1}$ or p53175P	10 to 15	
(Fussenegger *et al.* 1998)	CHO SEAP	$p21^{CIP1}$ + Bcl-2	30	ND
(Kaufmann *et al.* 2001)	CHO SEAP	$p27^{KIP1}$ and reduced temperature culture (30^0C)	2 to 3	ND
(Watanabe *et al.* 2002)	NS0 human-mouse chimeric IgG4 antibody	$p21^{CIP1}$	>4	ND
(Meents *et al.* 2002)	CHO soluble intercellular adhesion molecule (sICAM)	$p27^{KIP1}$	5	NC
(Ibarra *et al.* 2003)	NS0 Chimeric antibody	$p21^{CIP1}$	4	ND
(Carvalhal *et al.* 2003)	CHO SEAP	$p27^{KIP1}$	4	NC
(Bi *et al.* 2004)	CHO human-mouse chimeric IgG4 antibody	$p21^{CIP1}$	~4	NC

1.6.1.1.1 p21

Cyclin dependent kinases (CDKs) are key components of the pathways that control cell cycle transition (Hengst *et al.* 1994, Sugimoto *et al.* 2002) and therefore are important targets for achieving control of cell proliferation. Cyclins, phosphorylation and the formation of ternary complexes with cyclin dependent kinase inhibitors (CKI), such as $p21^{CIP1}$ and $p27^{KIP1}$, direct CDK activity (Grana and Reddy 1995). $p21^{CIP1}$ can be induced by either tumour suppressor gene p53 or tumor growth factor-β (TGF-β) (Reynisdottir *et al.* 1995). At elevated intracellular concentrations, $p21^{CIP1}$ binds to cyclin-CDK complexes and inhibits the progression of cell cycle at the G1/S transition phase (Bi *et al.* 2004). $p21^{CIP1}$-arrested CHO cells were ~4-fold bigger than proliferating CHO cells, metabolically more active and demonstrated a corresponding increase in protein synthesis (Bi *et al.* 2004). Similarly, specific productivity of chimeric IgG4 antibody was found to be increased by 4-fold in NS0 cells following $p21^{CIP1}$ arrest (Watanabe *et al.* 2002, Ibarra *et al.* 2003).

1.6.1.1.2 p27

$p27^{KIP1}$ evokes G1-cell cycle arrest by binding to cyclin E/CDK2, Cyclin A/CDK2 and cyclinD/CDK4, all of which are required for transition of cells from G1 to S phase (Toyoshima and Hunter 1994). TGF-β-treated Mv1Lu mink lung epithelial cells and human keratinocytes were reported to have increased $p27^{KIP1}$ levels resulting in cell cycle arrest (Reynisdottir *et al.* 1995, Sgambato *et al.* 2000). The use of $p27^{KIP1}$ under the control of the Tet-switch (tetracycline-responsive promoter phCMV–1) facilitated a growth-arrested extended production phase of at least 7 days and enhanced volumetric SEAP production in CHO cells (Mazur *et al.* 1999). $p27^{KIP1}$ arrest-induced increase in specific productivity was linked to higher expenditure of cellular energy as demonstrated by increased nutrient

consumption (oxygen, glutamine and glucose) and waste production (lactate and ammonia) (Carvalhal *et al.* 2003).

1.6.1.1.3 p53

p53, besides promoting $p21^{CIP1}$ expression, also induces other effectors of cell-cycle arrest such as GADD45 and the insulin-like growth factor binding protein 3 (IGF-BP3), all of which exert a cell-cycle block at G1-phase (Mazur *et al.* 1998, el-Deiry *et al.* 1994, Buckbinder *et al.* 1995, Rowan *et al.* 1996) making p53 one of the most important growth suppressors in eukaryotes. However it also induces apoptosis in some cell lines (el-Deiry *et al.* 1993, Ko and Prives 1996). A p53 mutant, p53175P, has been generated which causes cell-cycle arrest but not apoptosis in primary rat embryo fibroblasts (Rowan *et al.* 1996). p53175P has been shown to cause cell cycle arrest and improve cell specific productivity comparable to $p21^{CIP1}$ or $p27^{KIP1}$ (Fussenegger *et al.* 1997, Mazur *et al.* 1998).

1.6.1.2.4 Bcl2

Apart from CDKs and CKIs, expression of Bcl-2 can also cause cell cycle arrest in G1-phase of murine hybridoma cells (Kim *et al.* 2001, Simpson *et al.* 1999). About 80% of hybridoma cells expressing Bcl-2 accumulated in G1 phase of the cell cycle (Simpson *et al.* 1999). The duration of G1-phase was increased by 75% due to Bcl-2 expression in NS0 cells, although cell cycle arrest occurred in the G2 phase (Tey and Al-Rubeai 2005). Bcl-2 is known for its anti-apoptotic activity, however its cell cycle modulation activity has been reported to be distinct from its anti-apoptotic domain (Huang *et al.* 1997).

1.6.1.2 Low temperature cultivation of mammalian cells

Low culture temperature has been shown to reduce the growth rate and increase culture longevity and specific productivity in CHO cells expressing a wide range of recombinant proteins (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002). A shift in the proportion of cells from the S to the G1 phase of the cell cycle has been observed at reduced temperatures which results in a state close to growth arrest and has been shown to improve productivity (Table 3). For example, 80% of secreted alkaline phosphatase (SEAP) and 76% of erythropoietin (EPO) producing CHO lines accumulate in G0/G1 phase of the cell cycle following reduction of the culture temperature to 30^0C with associated increases in productivity (Kaufmann *et al.* 1999, Yoon *et al.* 2003). The quality of recombinant products, with regard to isoform pattern, sialic acid content, and in vivo biological activity, is maintained at reduced temperatures (Hendrick *et al.* 2001, Clark *et al.* 2004, Bollati-Fogolin *et al.* 2005), while proteases activity appears unaltered (Clark *et al.* 2004).

Low culture temperature results in reduced metabolism (glucose/medium consumption, oxygen uptake, lactate production and ammonia production) and shear sensitivity (Moore *et al.* 1997, Chuppa *et al.* 1997, Jorjani and Ozturk 1999, Marchant *et al.* 2008). The specific glucose and oxygen uptake rates were observed to be reduced extensively at low temperatures compared to 37°C (Marchant *et al.* 2008, Sureshkumar and Mutharasan 1991, Fox *et al.* 2004). The reduced metabolic rates at low temperature contribute to reduced lactic acid (and ammonia) formation in the culture over time. This enabled significant reductions in the amount of $NaHCO_3$ required for pH control and consequently, in pCO_2 and osmolality levels in culture which often reach inhibitory levels in high density cultures. Thus, reduced metabolic activity contributes to delayed initiation of apoptosis and helps in

extending the duration of stationary (or production) phase at reduced temperature (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002). Apart from this, the effect of reduced temperature on cells is likely to be multifactorial as it also combines the effect of changes in oxygen concentration due to the higher dissolved oxygen concentration at reduced temperatures (Ohsaka *et al.* 2002).

For the above reasons, many processes employ a biphasic culture process for recombinant protein production (Kaufmann *et al.* 1999, Baik *et al.* 2006, Trummer *et al.* 2006). This culture process consists of two phases: an initial proliferation phase designed to maximize biomass at 37^0C, followed by an extended production phase, at reduced temperature (28–33^0C), during which the cells remain growth arrested while the production of heterologous proteins is maintained at a high and stable level (Table 3).

Table 3: Effect of reduced temperature on cell cycle and productivity in CHO

Contributor	Product expressed in CHO	Media Type	Culture Vessel	Reduced Temperature (^{0}C)	Cell cycle distribution in G0/G1 (%)	Productivity (fold change)	
						Qp	Y
(Moore *et al.* 1997)	Chimeric Fab	DMEM/HAM's F-12 (1:1 mixture) + 1g/L Pluronic F68, 0.2ml/L antifoam emulsion	2L Bioreactor	30[a]	87	ND	ND
(Kaufmann *et al.* 1999)	SEAP	FMX-8 medium + 10% FCS	T-25 flask	30[a]	80	1.7	3.4
(Yoon *et al.* 2003)	Anti-4-1BB Antibody	IMDM+10%FBS replaced with SF2 SFM in exponential phase followed by temperature-shifts	T-25 flask	30[b]	77	NC	NC
			T-25 flask	33[b]	67	NC	3.9D
(Yoon *et al.* 2003)	EPO	IMDM+10%FBS replaced with SF2 SFM in exponential phase followed by temperature-shifts	T-75 flask	30[a]	76	5.6	2.5
			T-75 flask	33[a]	67	4	0.86D
(Fogolin *et al.*	hGM-CSF	ZKT-1 medium + 5% FCS	Spinner	33[a]	65	2.1	2.3

2004)			flasks				
(Trummer *et al.* 2006)	Epo-Fc	DMEM/HAM's F-12 (1:1 mixture) + 0.58 g/L L-glutamine, an in-house developed protein-free supplement, 0.25% soy peptone, 0.1% Pluronic F68, and 0.096 mM MTX	0.5L Bioreactor	30[a]	65	1.85	1.3
(Yee *et al.* 2008)	IgG	DMEM/HAM's F-12 (1:1 mixture) + 0.3 g/L ethanolamine, 19 mg/L L-ascorbic acid, 4.84 mg/L sodium selenite, 6.2 mM putrescine, 1.85 mg/L mercaptoethanol, and 5 mg/L apotransferin.	Spinner flasks	33[a]	ND	1.4	1.2

[a]: Temperature-shift from 37^0C; [b]: Continuous Reduced Temperature Culture; **Qp**: Cell Specific Productivity; **Y**: Overall Volumetric Yield; **ND**: Not Determined; **NC**: No Change; **D**: Down Regulated

The mechanisms whereby cells at lower temperatures improve productivity are still poorly understood. The recent studies have demonstrated that reduced culture temperature invokes a highly coordinated response in transcription and translational machinery to synthesize selective proteins which may be involve in regulation of cell growth, viability and protein productivity of cells (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002).

1.6.1.2.1 Effects of low temperature on transcription

Reduced culture temperature alters expression of transcription factors leading to the global inhibition of transcription (Sonna *et al.* 2002, Roobol *et al.* 2009). The mechanism of this reduction in global transcription is poorly known. 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). Moreover, a few proteins could bind to its own mRNA to destabilize the elongation complex of RNA polymerase which causes attenuation of transcription and translation (Bae *et al.* 1997).

Increased mRNA levels of specific genes have been observed at low temperature suggesting that cells transcribe genes selectively at low temperature (Sonna *et al.* 2002, Yoon *et al.* 2003, Marchant *et al.* 2008). However, the mechanism of this selective transcription is unknown. The expression of interferon-γ mRNA was found to be increased in CHO cells due to its enhanced transcription at low temperature (Fox *et al.* 2005). Similarly, the level of EPO mRNA was also observed to be increased by ≥2 fold at low temperatures (31 and 33^0C) due to increased transcription compared to standard culture (37^0C) in the lag and log phases of culture and this contributed into increased productivity (Yoon *et al.* 2003). The increased mRNA stability has also observed at low temperature in

CHO cells (Sonna *et al.* 2002). The mRNA for cold shock proteins have been observed to contain point mutations in their 5'UTR regions which provide resistance to the RNAase and stabilize mRNAs at low temperature (Fang *et al.* 1997). The cold shock proteins, such as CIRP, has also been observed to bind to the succeptible mRNAs at 3' UTR and expected to facilitate increased stability at low temperature (Yang and Carrier 2001). This suggests that the increased level of gene of interest either due to increased transcription or mRNA stability is one of the crucial factors for the enhanced cell specific productivity at low culture temperature.

A shift in the transcription of splice variants of amyloid precursor protein has been observed in a normal human fetal astrocytic cell line (CC2565) following heat shock (Shepherd *et al.* 2000). A number of proteins has been identified at multiple location on the 2D-gel in response to hypothermic conditions which could be a alternative splice variant of proteins rather than being a post-translationally modified variants (Sonna *et al.* 2002, Baik *et al.* 2006). Therefore, CHO cells could also be suspected to perform the alternative splicing of proteins at low temperature (Sonna *et al.* 2002).

1.6.1.2.2 Effects of low temperature on translation

Low temperature has been observed to inhibit global cap-dependent protein translation (Roobol *et al.* 2009, Phadtare *et al.* 1999, Wassmann *et al.* 1998). The expression of number of proteins, including translation initiation factors (e.g. EIF3i) and molecular chaperons (e.g. CCT), was observed to be reduced in CHO cells at low temperature (Roobol *et al.* 2009). Cells remain metabolically active at low temperature and induce the synthesis of small number of proteins using alternative translation pathway(s), possibly by cap-independent translation, enabling cells to more effectively tolerate hypothermic

conditions (Al-Fageeh *et al.* 2006, Kaufmann *et al.* 1999, Baik *et al.* 2006, Yee *et al.* 2008). Cap-independent translation has been observed to be activated under various stress conditions, such as hypoxia or hypothermia, however its molecular mechanism is still unknown. These include cold-inducible RNA binding protein (CIRP, also known as hnRNP A18) (Nishiyama *et al.* 1997b) and RNA binding motif protein 3 (RBM3) (Yee *et al.* 2008, Danno *et al.* 2000).

CIRP and RBM3 have been studied extensively. Both are involved in the modulation of transcription and translation by acting as RNA chaperones (Sonna *et al.* 2002, Nishiyama *et al.* 1997b, Danno *et al.* 2000, Dresios *et al.* 2005). Both proteins are highly similar and belong to the glycine rich RNA-binding protein family that are characterized by a consensus sequence RNA-binding domain (CS-RBD) at the N-terminus and a glycine-rich domain at the C-terminus (Derry *et al.* 1995). CIRP was the first protein identified that was induced following reduction of temperature in mammalian cells (Nishiyama *et al.* 1997b). Apart from CHO, CIRP has been detected in mouse, rat and human cells (Nishiyama *et al.* 1997a, Xue and Rao 1981). The expression of CIRP has also been observed to increase when cells are exposed to other stress conditions, such as ultraviolet radiation (Yang and Carrier 2001) or hypoxia (Fujita 1999), although heat stress does not affect CIRP levels (Nishiyama *et al.* 1997b). Inhibition of CIRP expression using antisense oligonucleotides allowed the reversal of cold induced growth arrest in mouse fibroblasts cells (BALB/3T3) indicating that CIRP is at least a component of the low temperature induced growth suppression (Nishiyama *et al.* 1997b). CIRP also specifically binds to the 3' untranslated region (UTR) of susceptible RNAs protecting and restoring their native confirmation during stressful conditions and this binding could improve both the efficiency of IRES-

mediated cap-independent translation and RNA stability (Yang and Carrier 2001, Chappell *et al.* 2001). In recent studies, 4-fold increases were observed in CIRP expression in both EPO and FSH expressing CHO cells at 32^0C in comparison to standard 37^0C culture (Yoon *et al.* 2006). No significant permanent change (decrease or increase) in CIRP expression was observed through further adaptation of both cell lines to reduced temperature, which may be because the effect of cold shock was eliminated during the adaptation process. The growth rates of low temperature-adapted CHO cells were improved by 73% and 20%, respectively, despite static CIRP levels. It is clear therefore that other factors are involved in the control of cell growth at reduced temperature (Yoon *et al.* 2006).

RBM3 is another well-studied protein that is induced in mammalian cells at reduced temperature. The RBM3 gene resides on the Xp11.2 region in humans and codes for a protein very close in structure to other human RNA-binding proteins and shares 94% identity in amino acid sequence with mouse RBM3 (Danno *et al.* 2000, Derry *et al.* 1995). RBM3 is increased at reduced temperature in various cells, i.e. HepG2, NC65, HeLa, T24, K562, TAMA26 and CHO cells (Yee *et al.* 2008, Danno *et al.* 2000). RBM3 has been found to be involved in cytokine-dependent proliferation (Baghdoyan *et al.* 2000), poxvirus replication (Wright *et al.* 2001), development of cancer (Baldi *et al.* 2003) and apoptosis (Kita *et al.* 2002). Expression of RBM3 at reduced temperature was shown to result in higher relative levels of 80S monosomes and polysomes consistent with an increased association of ribosomal subunits during the initiation step of protein synthesis (Dresios *et al.* 2005). A tight physical association of RBM3 with 60S ribosomal subunits that did not require RNA or nascent polypeptide chains has also been reported. Several deletion and mutational analysis experiments on a putative internal ribosome entry site (IRES) within

the 720 bp 5' leader sequence of the RBM3 mRNA revealed at least nine discrete cis-acting sequences, including a 22-nt IRES module (a 10-nt enhancer and two inhibitory sequences), and four of them probably bind specifically to distinct cytoplasmic proteins (Chappell and Mauro 2003). As a result of these findings, it is thought that the 5'-UTR of RBM3 mRNA contains a number of specialized sequences that facilitate cap-independent translation to ensure mRNA translation upon cold shock despite general (cap-dependent) mRNA translation being compromised (Chappell *et al.* 2001, Chappell and Mauro 2003). Decreased levels of microRNAs were also reported in mouse N2a neuroblastoma cells expressing an RBM3-c-Myc fusion protein, indicating that RBM3 can interact with the miRNA fraction and hence could regulate global levels of protein synthesis (Dresios *et al.* 2005). RBM3, like CIRP, can be regulated by changes in oxygen levels which further indicates a link between the low-temperature and oxygen-response pathways (Wellmann *et al.* 2004).

Therefore, the proteins synthesized by cells at low temperature using IRES-mediated cap-independent translation may facilitate transcription and translation of selective proteins by acting as chaperones or translational factors (Sonna *et al.* 2002, Roobol *et al.* 2009). Increased stability of proteins has been observed at subphysiological temperatures and this further help to regulate low temperature-induced phenotypes (Roobol *et al.* 2009). The half-life of p53 was observed to be ≥2 fold improved at low temperature in CHO cells which could contribute to p53-mediated cell cycle arrest in hypothermia (Roobol *et al.* 2009). The mutations in the low-temperature-induced proteins could be one of the possible mechanisms used by cells to improve the stability of proteins without compromising activity at hypothermic conditions (Roobol *et al.* 2009, Schindler *et al.* 1998). The

proteases activity in culture medium at reduced temperature has also been observed to be very similar to cultures maintained at standard temperature (37^0C) suggesting the independency of protein stability at low temperature with protease activity (Clark *et al.* 2004). Therefore, selective translation and improved stability of proteins are one of the critical factors that help to regulate various cellular phenotypes, such as growth, viability, secretion and productivity, at low temperature.

The comparison of cell cycle arrest through either reduced culture temperature or heterologous gene expression has revealed that temperature-shift is currently superior and it is also a more practical approach. Direct comparisons include the example where p27-mediated growth arrest greatly enhances specific productivity (~15-fold) while low temperature ensures complete and sustained growth arrest leading to a higher overall volumetric productivity (Mazur *et al.* 1998, Kaufmann *et al.* 2001). In studies where the expression of p27 was carried out at low temperature, it was found that the temperature effect was dominant (Kaufmann *et al.* 2001). Arresting cells through reduction of the temperature also has the advantage that all cells are similarly affected whereas engineering approaches allow for the possibility of clones which escape growth suppression (Mazur *et al.* 1998). Mutation or loss of the genes necessary for proliferation control may render the situation worse and could provide a substantial growth advantage to mutant cells which can lead to overgrowth of the arrested cells and subsequent loss in product yield (Fussenegger *et al.* 1997, Fussenegger *et al.* 1998, Kaufmann *et al.* 2001). Although the effects of low culture temperature on the cellular productivity of recombinant proteins have been observed to be variable and depends on the temperature, cell line, clone, expression system

and/or protein, reduced cell growth was observed in cases suggesting it as a universal phenomenon (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Sonna *et al.* 2002). Therefore use of temperature-shift for recombinant protein production is the method of choice employed in industrial processes.

1.6.1.3 Chemical approaches for cell cycle arrest

1.6.1.3.1 NaBu

Sodium butyrate (NaBu) has been used to improve recombinant protein production in mammalian cells (Hendrick *et al.* 2001, Kim and Lee 2002, Lee and Lee 2003). It exerts various effects on cells including G1-phase cell cycle arrest (Hendrick *et al.* 2001, Kim and Lee 2002), cell differentiation (Garcia-Bermejo *et al.* 1997) and apoptosis (Kim and Lee 2002). Approximately 67% of HeLa cells and 54% of CHO cells were found to accumulate in the G1-phase following NaBu treatment (Xue and Rao 1981, Hendrick *et al.* 2001). The specific and volumetric productivity were also increased (Hendrick *et al.* 2001, Lee and Lee 2003, Palermo *et al.* 1991, Kim and Lee 2001). However increased apoptosis was also observed in these cultures therefore undermining the beneficial effects of improved productivity (Xue and Rao 1981, Kim and Lee 2001 & 2002, Chang *et al.* 1999). The use of anti-apoptotic factors such as Bcl-2 and caspase inhibitors have been shown to improve productivity in the presence of NaBu (Kim and Lee 2001 & 2002, Li and Elsasser 2005).

1.6.1.3.2 Pentanoic Acid

Pentanoic acid has been reported as an alternative to NaBu as it has comparable enhancing effects on protein production with lower associated growth suppression and apoptosis compared with NaBu (Liu *et al.* 2001). The molecular mechanisms underlying the stimulation of protein expression and growth suppression effects by pentanoate are not

clear. NaBu and pentanoate could have similar mechanism as they share similar carboxyl carbon conformation, inhibit histone deacetylase, and suppress cell growth (Liu *et al.* 2001).

1.6.1.3.3 DMSO

Dimethyl sulfoxide (DMSO) has been shown to induce an efficient and reversible G1 phase arrest in CHO cells (Fiore *et al.* 2002), human lymphoid cell lines (Sawai *et al.* 1990) and mouse fibroblasts (Srinivas *et al.* 1991). DMSO-induced growth arrest is associated with restored contact inhibition of cell growth and prevention of density dependent-apoptosis (Fiore and Degrassi 1999). Addition of 1–2% DMSO to cultures efficiently and reversibly arrested up to 90% of CHO cells (Fiore and Degrassi 1999) and 85% of hybridoma cells in G1-phase (Ponzio *et al.* 1998). Addition of DMSO to CHO cells has been shown to increase CDK and/or CKI activity and $p27^{KIP1}$ and Bcl-2 expression and this results in growth arrest, improved cell-adhesion and reduced apoptosis (Fiore and Degrassi 1999). However, higher concentrations of DMSO (30%) caused mutagenic effects (Hakura *et al.* 1993).

1.6.1.3.4 Quinidine and thymidine

Quinidine and thymidine, both cell cycle inhibitors, have also been used for regulating the cell cycle distribution of CHO cells (Alrubeai *et al.* 1992, Andersen *et al.* 2000). Quinidine and thymidine inhibit potassium channels in cells (Alrubeai *et al.* 1992, Andersen *et al.* 2000, Wang *et al.* 1998). The level of potassium channel activity has been shown to be different in the proliferating and quiescent stage of cell cycle, and reduced potassium channel activity is hypothesized to result in cell cycle arrest (Wang *et al.* 1998). Following quinidine treatment, 67% of tPA-producing CHO cells were arrested in G1-phase of cell

cycle, however 57% cells were arrested in S phase after thymidine treatment (Andersen *et al.* 2000).

1.6.2 Media formulation/nutrient control based approaches

Besides fulfilling the nutritional requirements of cells, media formulation and its utilization have been implemented as strategies for the regulation of cell proliferation and recombinant protein production. Inefficient use (higher uptake than required for normal cell growth) of glucose and glutamine leads to rapid biomass generation, nutrient depletion and by-product accumulation (lactate and ammonium ions, etc.) which often have inhibitory effects on culture longevity and productivity (Ozturk *et al.* 1992, Zeng *et al.* 1998, Zeng and Deckwer 1999). Finding alternative compounds that can be used efficiently can lead to reduced/arrested cell growth and to improved culture longevity and overall productivity (Altamirano *et al.* 2001). The substitution of glucose with galactose (a slow metabolising sugar) and glutamine with glutamate (slow transportation rate in to cell and has only one amino group) reduced the growth rate and improved culture longevity in batch cultures of CHO cells (Altamirano *et al.* 2000). The regulation of the glucose feed rate in culture also increased culture longevity and t-PA yield in CHO cells without affecting cellular specific productivity (Altamirano *et al.* 2001). Therefore, nutritional-shift-based biphasic cultures that include replacement of glucose with galactose, glutamine with glutamate or different feed rates once cells achived high biomass has been shown to increase culture longevity and volumetric productivity (Altamirano *et al.* 2001).

1.6.3 Effect of Micro-environmental Factors on cell growth and productivity

The micro-environmental factors have significant impact on cell growth and protein product and therefore have always been given enough consideration during optimization of a bioproduction process.

1.6.3.1 Dissolved Oxygen (DO)

Oxygen is a basic requirement for all aerobic organisms and O_2 uptake rate has always been a key process optimization variable as nutrient or oxygen limitation can induce apoptosis (Simpson *et al.* 1999, Lai *et al.* 1982). DO ranging from 20% and 80% did not affect growth of a murine hybridoma cell line while the death rates were lowest at 20-50% air saturation (Ozturk and Palsson 1991). DO may affect the productivity of cells as the productivity of human follicle stimulating hormone (hFSH) in CHO cells was observed to greatly vary under different DO levels (10-90%) and of NaBu (0-1.5mM) (Chotigeat *et al.* 1994).

1.6.3.2 Culture pH

Culture pH is recognized as one of the most important process parameters in mammalian cell culture. It has effects on cell growth, metabolism, recombinant protein synthesis and protein quality (Borys *et al.* 1993, Yoon *et al.* 2005). Although close to physiological pH environment is generally suitable for mammalian cell growth, the optimal pH differs slightly among cell lines, and some cell lines are less sensitive to deviations from the optimal pH than others (Kurano *et al.* 1990b).

1.6.3.3 Metabolic byproducts

One goal in the optimization of mammalian cell culture processes is to protect cells from inhibitory conditions that may arise during culture, such as nutrient deprivation, ammonia

and lactate accumulation and elevated osmolality and pCO_2, as they can affect cell growth and production.

Ammonia can readily diffuse across the cell membrane and hence, elevated levels of Ammonia can decrease intracellular pH (pHi) levels which negatively affects both cell growth and recombinant protein productivity. t-PA production has been observed to be reduced under elevated ammonium conditions (Hansen and Emborg 1994). Ammonia also negatively affects the recombinant protein glycosylation profile, thus altering product quality (Chen and Harcum 2005).

Lactate build-up leads to the acidification of the culture environment and could inhibit cell growth in culture (Hassell *et al.* 1991, Lao and Toth 1997). The accumulation of lactate is often a critical limiting factor of a cell culture process especially when the cell density is high. Up to 75% reduction in cell growth rates have been reported with addition of 60mM lactate in CHO cells (Hassell *et al.* 1991, Tsao *et al.* 2005). The increased level of lactate and osmolality have additive inhibitory effects on cell growth (Lao and Toth 1997).

The elevated osmolality has been reported to decrease cell growth mainly due to apoptosis (Zhu *et al.* 2005). Cell culture medium is typically designed to have an osmolality in the range of 260-320 mOsm/kg, mimicking the osmolality of serum (290 mOsm/kg). Information on the response of CHO cells to changes in osmolality is scarce. The combination of pCO_2 and osmolality has been observed to effect cells more extensively in comparison to osmolality alone (Zhu *et al.* 2005, Kimura and Miller 1996, Ryu *et al.* 2000). Elevated pCO_2 can reduce intracellular pH (pHi) and can affect cell growth and recombinant protein productivity. The physiological range for pCO_2 is 31-54mm Hg. Up to five times increased pCO_2 levels have been observed for cultures oxygenated with a low

flow rate and small bubbles of O_2 (Kimura and Miller 1996). Elevated pCO_2 was observed to inhibit glycolysis (Kimura and Miller 1996, deZengotita *et al.* 2002) which reduced the growth and productivity of CHO cells (Kimura and Miller 1996). The quality of product may also be compromised due to alterations in the carbohydrate moeities (*N*-glycolylneuraminic and polysialation) at elevated pCO_2 level (Zanghi *et al.* 1999).

Therefore, optimization of culture conditions for reduced waste accumulation and their consequences could help to improve the growth and productivity of bioprocesses.

1.7 miRNAs: novel regulators of cellular behaviours

MicroRNAs (miRNAs) are endogenous, small (~22 nt), usually single-stranded and non-coding RNAs (ncRNAs) that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression (Bartel 2004). A single miRNA can regulate the expression of a number of gene targets through binding of the 3' untranslated regions (UTRs). For example miRNA-21 has been found to regulate more than one phenotype including apoptosis and proliferation (Cheng *et al.* 2005). In addition, the 3'UTRs of many genes contain putative binding sites for a number of miRNAs. The cold inducible protein, RNA binding motif 3 (RBM3) that increases global translation under conditions of low culture temperature, has been shown to interact with small RNA species suggesting the involvement of miRNA regulation in the low temperature response (Dresios *et al.* 2005). As discussed, low culture temperature has been employed in industry to increase the productivity, viability and stationary phase duration of production CHO cell lines (Al-Fageeh *et al.* 2006, Baik *et al.* 2006). miRNAs do not rely on the cellular translational machinery to exert their effects on the cell and therefore do not cause

metabolic burden on cellular machinery. The expression of miRNAs can be easily regulated by transfecting small genetic constructs (mature miRNAs) or antagomirs, small single-stranded RNA molecules with complementarity to the mature target miRNA. It has been observed that the overall similarity at miRNA level is much higher between species in comparison to proteins (Berezikov *et al.* 2005). For these reasons, miRNAs may offer great potential to regulate cell growth and productivity of CHO cells in production cultures (Gammell 2007, Muller *et al.* 2008).

1.7.1 Discovery of miRNA

The first miRNAs, Lin-4, was identified in *Caenorhabditis elegans* in 1993 (Lee *et al.* 1993). Lin-4 was shown to be involved in disrupting the post-embryonic development in *Caenorhabditis elegans* through repressing the lin-14 (a gene that also works in the same developmental pathway) by binding to 3'UTR of lin-14 with partial complementarity. A second miRNA, let-7, was also discovered in *Caenorhabditis elegans* (Reinhart *et al.* 2000). Similarly to lin-4, let-7 miRNA recognized sequences present in the 3' UTR of lin-41 mRNA and repressed LIN-41 protein levels. These findings encouraged others to investigate the presence of miRNAs in different species and thereafter a number of miRNAs were reported in various species. miRNAs have been found to be highly conserved throughout evolution, from worms, through fly and up to humans (Ambros 2003). According to current *in-silico* estimations, miRNAs appear quite abundant and might account for up to 3% of all genes in the genome (Berezikov *et al.* 2005).

1.7.2 miRNA Biogenesis and Processing

It has been observed that all the elements required for the regulation and initiation of transcription of miRNAs are located within a short fragment of ~800bp length (Lee *et al.*

1993). However, little is known regarding these regulatory transcriptional processes for miRNA genes. Some miRNAs that reside in introns are likely to share their regulatory elements and primary transcript with their pre-mRNA host genes. For the remaining miRNA genes, presumably transcribed from their own promoters, no primary transcripts have been fully defined. Nonetheless, these primary miRNA transcripts, called pri-miRNAs (Lee *et al.* 2002), are generally thought to be much longer than the conserved stem loops currently used to define miRNA genes, as suggested by the following: (1) clustered miRNA stem loops are transcribed from a single primary transcript (Lagos-Quintana *et al.* 2001), (2) matches between miRNAs and lengthy ESTs in the databases (Lagos-Quintana *et al.* 2002), and (3) RT-PCR experiments amplifying large fragments of the long primary miRNAs (pri-miRNAs) (Lee *et al.* 2002).

Most miRNAs are transcribed by RNA polymersase II as pri-miRNAs, though some are transcribed by RNA polymersase III (Ohler *et al.* 2004, Cai *et al.* 2004). The miRNA portion of the pri-miRNA transcript likely forms a hairpin with signals for dsRNA-specific nuclease cleavage. The pri-miRNA is then cleaved at its flanks by a dsRNA-specific nuclear ribonuclease III (RNase III) endonuclease, Drosha, yielding stem loop structure of about 70 nt termed the precursor miRNA (pre-miRNA) (Figure 1.7.2) (Lee *et al.* 2002 & 2003). The pre-miRNAs processed by RNase III contains 5' phosphate and 3' hydroxy termini and 1–4 nt 3' single-stranded overhangs. Exportin 5 (Exp 5) facilitates the export of miRNAs from nucleus to cytoplasm (Yi *et al.* 2003). Once the pre-miRNA is in the cytoplasm, another RNase III endonuclease, Dicer, processes this pre-miRNA and generates the miRNA duplex (~21 nt). Only one of the two strands of miRNA duplex is the mature miRNA. The selection of the active strand from the dsRNA appears to be based

primarily on the stability of the termini of the two ends of the dsRNA (Khvorova *et al.* 2003, Schwarz *et al.* 2003). The strand with lower stability base pairing of the 2–4 nt at the 5' end of the duplex preferentially associates with RNA induced silencing complex (RISC) (an another enzyme that is used, like dicer, for the siRNA directed silencing pathway) and thus becomes the active miRNA (Schwarz *et al.* 2003, Hutvagner and Zamore 2002).

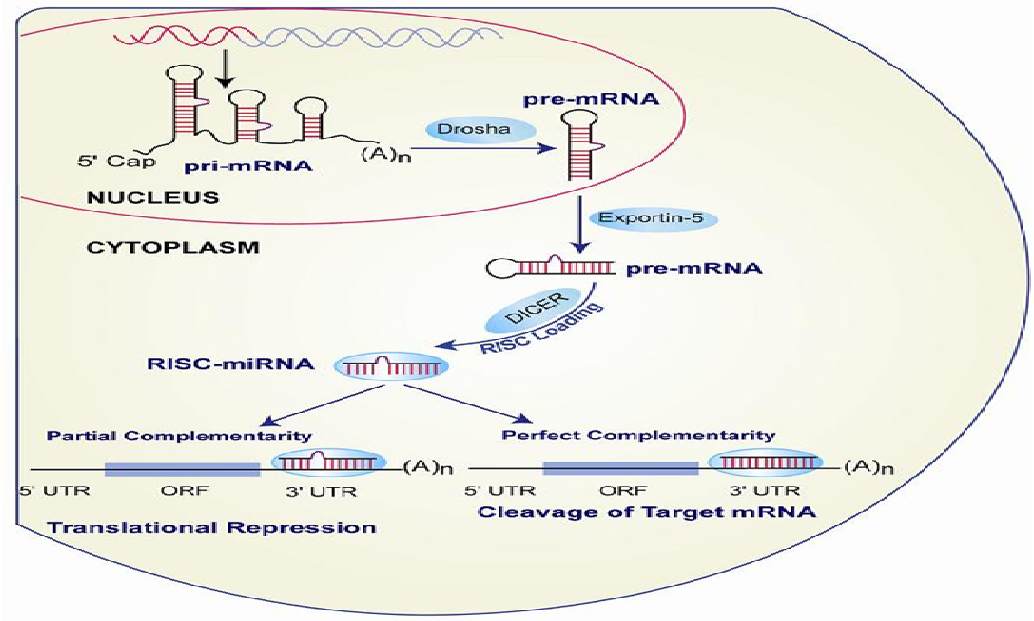

Figure 1.7.2. miRNA biogenesis and bioprocessing.

1.7.3 Mechanism of miRNAs action

miRNAs mediate target silencing using both mRNA cleavage and translational repression (Figure 1.7.2). In plants, it is more common to observe almost perfect homology between miRNA strands and the mRNA target(s), hence the RISC complex behaves as an endonuclease and cleaves the target mRNA between the target nucleotides paired to bases 10 and 11 of the miRNA. The normal situation for animal miRNAs however is that homology between the miRNA and the target mRNA is typically restricted to the 5’ end of

the miRNA (Lewis *et al.* 2003, Lewis *et al.* 2005, Brennecke *et al.* 2005). In this situation, the bound miRNA-RISC complex inhibits translation of the target mRNA either at the level of translational elongation or initiation of translation (Olsen and Ambros 1999, Pillai *et al.* 2005). The molecular mechanism of miRNA-mediated translational repression is unknown.

1.7.4 Effects of miRNAs on cellular phenotypes

Research into the regulation of gene expression through the action of miRNAs has increased hugely over the last 4 years. The number of identified miRNA in the Sanger repository is increasing exponentially. Recently miRNAs have been found to be critical for development (Ambros 2003), cell proliferation (Gammell 2007, Brennecke *et al.* 2003), apoptosis (Xu *et al.* 2007) and metabolism (Gauthier and Wollheim 2006). However, the miRNAs regulating these characteristics have not been functionally validated in CHO cells. The regulation of cell growth, apoptosis and metabolism are all pre-requisite characteristics for improving the recombinant protein production of production cell lines in bioreactors. Therefore miRNAs could serve as a potential targets for this purpose.

1.7.4.1 Regulation of cell growth

Growth control in bioreactors is crucial for efficient protein production. The use of biphasic culture strategies to control growth are commonly employed whereby cells are initially grown in conditions to promote high proliferation rates to maximize viable biomass and subsequently cell division is arrested (typically through reduction of the culture temperature), to encourage productivity and reduce apoptosis (Al-Fageeh *et al.* 2006, Baik *et al.* 2006). A number of miRNAs have been identified that regulate the expression of well known growth regulatory proteins and many of these proteins have already been employed in cell-engineering based approaches to improve growth and productivity of CHO cells.

Let-4 and Let-7 control the timing of proliferation and differentiation in *C. elegans* and abnormalities in miRNAs coding for Let-4 and Let-7 can lead to uncontrolled cell growth and other developmental abnormalities (Lee *et al.* 1993, Reinhart *et al.* 2000, Ambros 2003). The mammalian homologues of these genes are also involved in the regulation of cell proliferation. The depletion of the miR-125b (Lin-4 homologue) resulted in reduced proliferation of human cancer cells (Lee *et al.* 2005), whereas the over expression of Let-7 suppressed the growth of DLD-1 colon cancer and A549 lung adenocarcinoma cells (Akao *et al.* 2006, Takamizawa *et al.* 2004). Let-7 has also been shown to represses Ras and c-Myc expression (Akao *et al.* 2006, Johnson *et al.* 2005). Ras and c-Myc are well known regulators of cell growth. The expression of c-Myc has already been implemented in the regulation of CHO cell growth in production cultures (Ifandi and Al-Rubeai 2005, Katakura *et al.* 1999, Miura *et al.* 2001). c-Myc was also shown recently to activate the expression of miR-17 cluster, including miR-17-5p and miR-20a, and the transcription factor E2F1 (O'Donnell *et al.* 2005). miR-17-5p and miR-20a were observed to negatively regulate the expression of E2F1. E2F1 is involved in the control of cellular proliferation and over expression of E2F1 has been associated with growth arrest (Johnson *et al.* 1993). Therefore the organized function of c-Myc and its associated miRNA and mRNA targets is required to control cell growth in a production culture.

Inhibition of miR-7 has been found to down regulate the cell growth of the lung carcinoma cell line, A549 (Cheng *et al.* 2005). miR-7 exerted its effects on cell growth through EGF receptor (EGFR) signaling that triggers extra-cellular-signal regulated kinases (ERK)-mediated degradation of a transcription factor (Li and Carthew 2005). ERK can be activated by growth factors and has an important role in the regulation of cell proliferation

(Nishimoto and Nishida 2006). miR-143 has also been found to reduce the cell growth of human adipocytes through interacting with ERK5 (Esau *et al.* 2004). ERK5 is a member of the mitogen activated protein kinase (MAPK) family and is similar to ERK1/2. miR-221 and miR-222 are also involved in the inhibition of proliferation of human erythroleukemic cells (Felli *et al.* 2005).

A number of other miRNAs have been identified in a large-scale screening experiment that are involved in the regulation of growth in HeLa and A549 cells (Cheng *et al.* 2005). The effect of miRNAs can be dependent on other factors including cell type. For example inhibition of miR-24 resulted in a significant increase in the specific growth rate of HeLa cells, whereas it down regulated cell growth in the case of A549. The inhibition of miR-21 also resulted in increased growth of HeLa cells (Cheng *et al.* 2005), whereas over expression of miR-21 was found to improve cellular growth of the breast cancer cell line, MCF-7 (Si *et al.* 2007). Similarly, miR-34 induces cell-cycle arrest in G1 phase in both normal and tumor cells (He *et al.* 2007) but supports cell proliferation in renal carcinogenesis induced by oxidative stress in rats (Dutta *et al.* 2007). Although miRNAs are highly conserved among different species and expression of miRNAs can be expected to exert similar type of effect(s) in CHO cells, varying results indicates the need for CHO-specific screening of each individual miRNA target for regulation of growth in specific states of a production process.

1.7.4.2 Regulation of Apoptosis

Apoptosis or programmed cell death is an obstacle to maintain high viable cell densities over time to improve the yield of bioprocess. Apoptosis in bioreactors is a result of a number of stressful conditions that emerge as a result of the production process, including

nutrient limitation, mechanical agitation, oxygen depletion and waste accumulation (al-Rubeai and Singh 1998). Cell death through apoptosis can negatively affect the quality of product and overall productivity (Fussenegger *et al.* 1998). For these reasons, a number of apoptotic regulators have been identified and engineered into CHO cells to improve productivity. However identification of miRNAs regulating these processes may further improve the control of cell death in the production process.

miR-21, apart from its role in cell growth, increases apoptotic cell death in MCF-7 breast cancer cells (Cheng *et al.* 2005, Si *et al.* 2007). Phosphatase and tensin homology deleted from chromosome 10 (PTEN) and Bcl-2 have been found to be the direct targets of miR-21 (Si *et al.* 2007, Ji *et al.* 2007). The inhibition of miR-21 expression was found to up regulate their expression while over expression of miR-21 was found to down regulate the expression of PTEN (Si *et al.* 2007). Bcl-2, a well known anti-apoptotic protein, inhibits apoptosis through the maintenance of mitochondrial membrane integrity and by binding to pro-apoptotic Bcl family members. The expression of Bcl-2 was down regulated upon inhibition of miR-21 and was up regulated after over expression of miR-21 in vascular smooth muscle cells (Ji *et al.* 2007). The inhibition observed in cell growth after inhibition of miR-21 expression has a direct correlation with increased apoptosis associated with down regulation of bcl-2 expression. The tumor suppressor protein, programmed cell death 4 (PDCD4), is another target for miR-21 and down regulation of PDCD4 by miR-21 caused an increase in apoptosis in MCF-7 breast cancer cells (Frankel *et al.* 2008).

Furthermore, miR-15 and miR-16 have been identified as negative regulators of the apoptotic suppressor Bcl-2 at a posttranscriptional level and thus induces apoptosis in malignant B cells (Cimmino *et al.* 2005). The inhibition of miR-7 was shown to increase

apoptosis whereas inhibition of miR-214 decreased the level of apoptosis in HeLa cells (Cheng *et al.* 2005). The expression of miR-1 also affects the level of apoptosis in cardiomyocytes (Xu *et al.* 2007). HSP60 and HSP70 work as direct targets for miR-1 and can be inhibited by miR-1 at a post-translational level without changing their transcript levels. The inhibition of HSP60 and HSP70 could reduce the expression of Bcl-2 proteins and enhance caspase activity leading to apoptosis in culture (Xu *et al.* 2007). miR-133 has anti-apoptotic effects and represses caspase-9 expression at both the protein and mRNA levels. miR-34a can regulate the activity of p53 (Raver-Shapira *et al.* 2007). p53 is a potent tumor suppressor, whose biological effects are largely due to its function as a transcriptional regulator. The depletion of miR-34a expression strongly attenuates p53-mediated apoptosis in cells exposed to genotoxic stress, whereas over expression of miR-34a mildly increases apoptosis. Hence, miR-34a has a direct interaction with p53 and can influence some of the pro-apoptotic biological effects of p53 in cells. The over expression of miR-224 was shown to inhibit the expression of apoptosis inhibitor 5 (API-5) which ultimately increased apoptosis in the human colon cancer cell line, HCT116 (Wang *et al.* 2008).

1.7.4.3 Stress resistance

Stress, depending on its type i.e. micro-environmental (thermal, shear or oxidative), nutritional (amino-acid(s) or nutrient(s) starvation) or physiological (transcriptional, translational or post-translational), as discussed before does not only lead to apoptosis and cell death but, at sub-lethal levels, can also modulate both the output of recombinant protein and its quality. Cells have been observed to induce expression of certain proteins such as CIRP, RBM3, PDI and ER chaperones etc, in response to stress conditions (Wellmann *et*

al. 2004, Sugimoto and Jiang 2008, Zhang *et al.* 2008). A number of miRNAs (miR-15b, miR-16, miR-20a and miR-20b) have been observed to be down-regulated significantly in hypoxic conditions in the human nasopharyngeal carcinoma cell line, CNE (Hua *et al.* 2006). Another investigation revealed the induction of miR-23, miR-24, miR-26, miR-27, miR-103, miR-107, miR-181, miR-210 and miR-213 in response to low oxygen in a panel of four human cancer cell lines (MDA-MB231, MCF7, HT29, and HCT116) (Kulshreshtha *et al.* 2007). The expression of miR-26, miR-107 and miR-210 was found to decrease pro-apoptotic signalling in a hypoxic environment. Interestingly, the vast majority of hypoxia-induced microRNAs are also over expressed in a variety of human tumors.

1.7.4.4 Regulation of cellular metabolism

In a typical production system, glucose is used as an energy source by the producer cells and is converted into lactate. Glutamine is consequently shuffled into the tricarboxylic acid (TCA) cycle, releasing excessive amounts of ammonia. The accumulation of these byproducts may trigger cell death and additionally affects the quality of the secreted product in terms of reduced and incorrect glycosylation (Yang and Butler 2000). Therefore efforts are being consistently made to balance the nutrient supply as well as waste accumulation in culture. A few different miRNAs have been reported to be involved in the regulation of cellular metabolism. miR-375 has been described as a key regulator of glucose homeostasis and insulin secretion in pancreatic cell lines (Gauthier and Wollheim 2006, Poy *et al.* 2007). Over expression of miR-375 in these cells resulted in decreased glucose-induced insulin secretion with no alterations in glucose-mediated production of ATP or a rise in intracellular Ca^{2+} (Poy *et al.* 2007). In addition, repression of miR-375 increased glucose-stimulated secretion of insulin. These results suggested that miR-375

regulates a late step of insulin exocytosis. A correlation between inhibition of miR-122 and the down regulation of genes involved in cholesterol synthesis has also been observed indicating that miR-122 may negatively regulate a transcriptional repressor (Krutzfeldt *et al.* 2005). Furthermore, miR-29b controls the metabolism of amino acids in mammalian cells by regulating the catabolism of branched-chain amino acids (BCCAs) in HEK293 cells (Mersey *et al.* 2005). miR-277 was observed to work as a metabolic switch controlling amino acid catabolism (valine, leucine and isoleucine) in flies (Stark *et al.* 2003). The degradation of these essential amino acids is presumably regulated under conditions of starvation or excess dietary intake. Clearly, a better understanding of miRNA-mediated regulation of metabolism could help to improve the productivity of mammalian cell factories.

1.7.4.5 Regulation of secretory pathways

Optimizing secretion, after optimizing transcription and translation, of the recombinant protein product from the producer cells is another important aspect of improving the product yield. A number of target proteins have been engineered to improve the secretion process in cells. For example, the over expression of XBP-1 regulates several factors involved in secretion and exerts positive effects on productivity. Other potential targets include, coat protein complex I (COPI) and PDI (Barlowe 2000, Pfeffer 2007, Robinson *et al.* 1994). miR-375, miR-124 and let-7b, are also involved in the regulation of secretory pathways (Poy *et al.* 2007). miR-124 and let-7b suppress the translation of mRNA encoding myotrophin (MTPN) (Poy *et al.* 2007). Normally, MTPN interacts with cytoskeletal elements and is thought to open the F-actin mesh, allowing access of the secretory granules to exocytotic sites. miR-124a increases secretion of insulin by regulating

vesicle fusion proteins, such as the target SNARE protein SNAP25, the small GTPase, Rab3A and the secretory regulator, synapsin-1A (Lovis *et al.* 2008). Therefore, these miRNAs might present targets for altering the secretory potential of CHO cells but again, it remains to be determined to what extent these functions are cell-type specific.

1.8 Secretome of CHO-K1 Cells

In 1986, human tissue plasminogen activator (tPA) was the first recombinant therapeutic protein product approved for production in mammalian cells and since then the number of such proteins have increased greatly (Wurm 2004). These recombinant therapeutic protein products are secreted from producer cells. Depending on the nature of the product and culture process, crude recombinant proteins are typically harvested in late exponential or early stationary phase of growth. These crude proteins are then subjected to a multi-step purification process to purify the recombinant product from the culture medium which also contains contaminants such as host cell proteins (HCP) and nucleic acids (de Oliveira *et al.* 1999). HCP includes all the peptides or proteins secreted by viable cells or leaked into the culture medium by cell damage over the culture time.

A number of secretory peptides/proteins from mammalian cells have been reported to play a critical role in the regulation of various cellular phenotypes, i.e. growth and differentiation in culture. Apolipoprotein E (*ApoE*) is involved in cholesterol transport and metabolism and can be both endogenous or secretory in nature (Chen *et al.* 2005, Greenow *et al.* 2005). Recently, inhibition of the expression of ApoE has been show to regulate cell growth by inducing G2-phase cell-cycle arrest and apoptosis in the ovarian cancer cell line, OVCAR3 (Chen *et al.* 2005, Greenow *et al.* 2005). Perlecan (heparan sulfate proteoglycan 2) is a secretory protein observed to regulate cell proliferation in mouse fibroblast cells,

NIH/3T3 (Aviezer *et al.* 1997) and increased levels have been reported in cancer (Sharma *et al.* 1998, Cohen *et al.* 1994). The expression of perlecan has been observed to be induced by transforming growth factor-β (Iozzo *et al.* 1997) and can regulate cell growth by acting as a co-receptor for basic fibroblast growth factor (FGF2) (Sharma *et al.* 1998). The inhibition of perlecan reduced the growth of colon carcinoma cells, whereas exogenous expression was able to reconstitute the effect (Sharma *et al.* 1998). Lysyl oxidase (LOX) is a secreted amino oxidase that oxidizes primary amines to reactive aldehydes (Kirschmann *et al.* 2002). This family of enzymes is involved in the regulation of biogenesis of connective tissue, development, cell growth and tumor suppression. Recently four new proteins designated lysyl oxidase-like proteins have been described (LOXL, LOXL2, LOXL3 and LOXL4) that are functionally similar but genetically distinct from the LOX family of proteins. Fibronectin is one of the well known secretory proteins that promotes cell growth (Williams *et al.* 2008). MCF-10A cells cultured with an excess of fibronectin were prominently growing whereas cells deprived of it were found to be growth arrested (Williams *et al.* 2008). Fibronection can also induce the secretion of matrix metalloproteinase (MMP)-9 in culture (Thant *et al.* 2000) and increased MMPs activity in culture may have potential to alter the recombinant proteins in culture as MMPs can mediate the cleavage of proteins.

Many reports have shown the secretion of transferrin from actively growing primary or established cell lines (Vandewalle *et al.* 1989, Dowling *et al.* 2007, Gronborg *et al.* 2006). Non-dividing cells can have extremely low levels of transferrin receptor expression, whereas rapidly proliferating cells, for example carcinoma cell lines, can express up to 100,000 transferrin receptors per cell (Inoue *et al.* 1993). Transferrin has been linked with

the regulation of cell growth in culture (Laskey *et al.* 1988, Chun *et al.* 2003, Sunstrom *et al.* 2000). It has been observed that transferrin alone does not promote survival or proliferation of CHO cells, however in the presence of IGF-1, it can improve cells survival and proliferation (Sunstrom *et al.* 2000). Exogenous transferrin was also found to improve the growth and survival of CHO DG44 in another study (Chun *et al.* 2003).

Secreted peptides/proteins in culture media can also contribute to the accumulation of waste products in culture and could alter pH and osmolality, specially during the later stage of culture to ultimately initiate apoptosis (Chuppa *et al.* 1997, Zhu *et al.* 2005, Kurano *et al.* 1990a, Olsnes *et al.* 2003). This could result in reduced cell growth in culture. The culture-environment has also been shown to alter post-translational modifications of recombinant protein products and therefore it can be expected that secreted proteins may also be contributing towards the regulation of PTMs in a production process (Yoon *et al.* 2005, Zanghi *et al.* 1999, Kimura and Miller 1997).

The most common method for HCP removal is affinity chromatography and the purified product is assessed for purity using immunoassay (Rathore *et al.* 2003, Krawitz *et al.* 2006, Denizli and Piskin 1995, Ouyang *et al.* 2007). The antibodies used in immunoassays are raised against a total cell protein mixture of non-product-expressing cells and hence these antibodies measure the population of non-product proteins from the host cell. It is possible that the antibody affinity to HCP may vary due to change in nature and composition of HCP at different phases of growth (e.g. logarithmic or stationary) and this may reduce the specificity of product recovery (Denizli and Piskin 1995, Ouyang *et al.* 2007). Therefore investigation of secreted proteins from CHO cells at different phases of culture represents a

valuable resource to design better culture and media for production processes from CHO, hybridoma and/or NS0 cells.

1.9 Recent developments in 'Omics' technology

Within the past decade, Omics have moved from being technology-driven to application-oriented high-output assay systems. The driving force behind this is the quest for increased understanding of molecular mechanisms of biologically-important phenomenon. This has resulted in the development of a number of exciting tools in proteomics (protein-arrays, 2D-DIGE etc.) as well as in genomics (expression microarrays, miRNA-arrays etc.) that has enabled researchers to meet their defined goals more efficiently.

1.9.1 Proteomics Analysis

Genomic technologies such as microarrays are being extensively used to detect the changes in gene expression associated with a specific test condition, for example, diseased versus normal phenotypes. Microarrays contain specific oligodeoxynucleotide probes that bind to specific targets and enable identification of alterations in the expression of targets in response to any treatment. Genomic techniques typically rely on the information of DNA molecules in the nucleus of cells, however several studies have shown that differential mRNA expression does not always correlate with protein expression (Gygi *et al.* 1999, Anderson and Seilhamer 1997). There are number of processes that can regulate a DNA molecule to exert its biological effect on cells, such as rate of transcription and translation, efficient post-translational modification and transportation of protein to its appropriate location in the cell. Investigations at the protein level are beneficial as proteins are directly involved in exerting their effects on cells, although a combination of both approaches could

provide a clearer view of cellular processes. However, the majority of proteomic techniques are costly and labor intensive and also may have problem with sensitivity and specificity (Haynes and Yates 2000).

There are a number of proteomic techniques available to analyse the proteome of cellular systems. For example, Two-dimensional difference gel electrophoresis (2D-DIGE), Surface Enhanced Laser Desorption/Ionisation Time of Flight (SELDI-ToF), isobaric tag labelling technologies such as ICAT (Isotope Coded Affinity Tags), iTRAQ^{TM} (Isobaric Tagging for Relative and Absolute Protein Quantitation), Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS) and Liquid chromatography-mass spectrometry (LC-MS/MS) etc. In this investigation 2D-DIGE coupled with MALDI-ToF MS and SELDI-ToF MS were used.

1.9.1.1 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two-dimensional difference gel electrophoresis (2D-DIGE) allows the quantitative protein expression profiling across many samples in a reproducible, sensitive and high-throughout manner. This is a modified version of conventional Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) combining two different unrelated protein separation steps, i.e. proteins separates according to their isoelectric points (pI) in the first dimension step (isoelectric focusing, IEF), and then on the basis of molecular weight in the second dimension step (SDS-polyacrylamide gel electrophoresis, SDS-PAGE). While 2D-PAGE is the most widely used proteomics technique, challenges remains due to its low sensitivity and reproducibility (Bunai and Yamane 2005). For example, 2D-PAGE shows poor separation of hydrophobic and very acidic, alkaline and larger proteins. Low abundant and small proteins are also very difficult to investigate using 2D-PAGE-based approuches due

to compromised sensitivity. Apart from this, in 2D-PAGE, only one sample can be separated on single gel and this contributes to gel-to-gel variation.

Modified 2D-gel electrophoresis, 2D-DIGE offers great potential due to increased throughput, ease of use, reproducibility, and accurate quantitation of protein expression differences (Unlu *et al.* 1997). This system enables the separation of two or three fluorescently labelled protein samples (Cy2, Cy3 and Cy5) on the same gel (Figure 1.9.1.1) and therefore minimizes gel-to-gel variation. However, 2D-DIGE is also unable to analyse very low or high abundand proteins and very alkaline, acidic and/or hydrophobic proteins. The CyDye DIGE Fluor dyes (Cy2, Cy3 and Cy5) are matched for mass and charge but possess distinct excitation and emission spectra. The Cy dyes minimally label the lysine residues of proteins; the dyes undergo nucleophilic substitution reaction with the epsilon-amino group of lysine residues on the protein resulting in the formation of an amide bond. The dye : protein ratio is optimised so that only 3-5% of the protein is labelled. This method ensures that proteins with a single dye molecule are visualised, resulting in co-migration of proteins originating from separate samples. Consequently, the same protein labelled with any of the dyes will migrate to the same position on the 2D gel. By using different dyes to separately label proteins isolated from normal or treated cells, proteins can be co-separated and quantitated by scanning gels at three different set of wavelengths, i.e. Cy2 at 488nm, Cy3 at 532nm and Cy5 at 633. The inclusion of a pooled internal standard (Cy2), containing every protein from all samples is used to match protein patterns across all of the gels. This feature, reduces inter-gel variation, allows normalization of individual experiments and accurate quantification of differences between samples with statistical significance (Unlu *et al.* 1997, Alban *et al.* 2003, Gharbi *et al.* 2002).

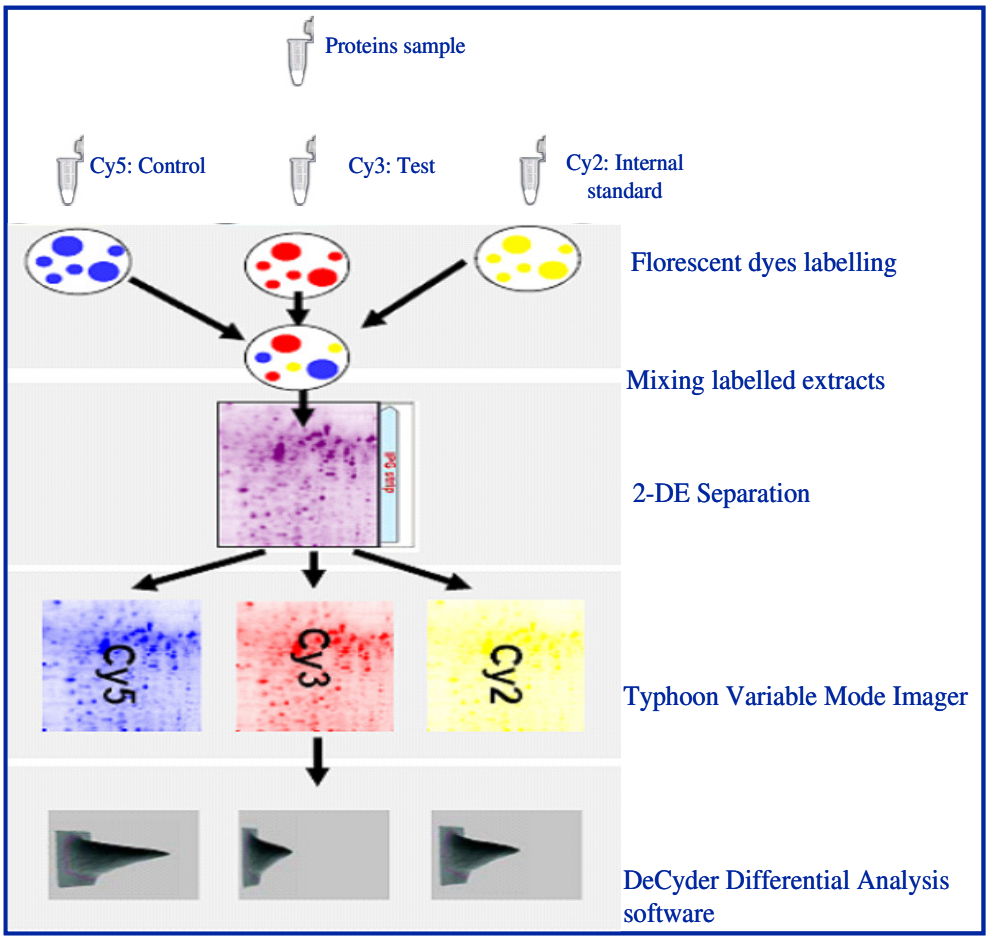

Figure 1.9.1.1. Schematic representation of 2D-DIGE approach.

Advanced image analysis software, e.g. DeCyder, further aid the efficiency of identifying real changes in protein expression as it allows to define landmarks during spot matching and also uses advanced spot normalization algorithms. The biological variation analysis module of DeCyder (BVA) can processes multiple gel images and quantatively compares protein abundance across gels to identify statistically significant difference in control and test conditions. Differentially expressed protein spots observed using DeCyder can be identified by Mass Spectrometry techniques such as MALDI-ToF MS by generating peptide mass fingerprints (PMF) and subsequent database searching.

1.9.1.2 Matrix assisted Laser Desorption/Ionisation Time-of-flight Mass spectrometry (MALDI-ToF MS)

Matrix assisted Laser Desorption/Ionisation Time-of-flight Mass spectrometry (MALDI-ToF MS) involves the precipitation of sample molecules with an excess of matrix material (α-cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid). The precipitant is then bombarded with a laser pulse and imparts energy. The matrix materials have absorbances at the wavelength of the laser and are subject to desorption and ionisation accompanied by fragmentation. The MS measures the mass-to-charge ratio (m/z) of the protein, peptide or peptide fragments. The time-of-flight (ToF) analyzer separates ions according to their m/z ratios by measuring the time it takes for ions to travel through a field-free region known as the flight or drift tube. The laser is set in such a way that it always generates single charged ions and therefore m/z represents the approximate molecular weight of protein target. The smaller ions possess a higher velocity relative to the larger/heavier ions. Separated ion fractions arriving at the end of the drift tube are detected by an appropriate recorder that produces a signal upon the impact of each ion group. The ToF mass spectrum is a recording of the detector signal as a function of time. This peptide mass fingerprint can then be used to search databases such as Profound, Mascot etc, to identify the protein (Blackstock and Weir 1999, Yates 2000).

1.9.1.3 Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-ToF-MS)

SELDI-ToF MS represents the successful combination of retentate chromatography and mass spectrometry. SELDI-TOF-MS can be considered as an extension of the MALDI-ToF-MS as they shares similar mechanisms of action. Proteins to be analyzed are

cocrystallized with UV-absorbing compounds (sinnapinic acid) and vaporized by a pulsed-UV laser beam. Ionized proteins are then accelerated in an electric field, and the mass to charge ratios of the different protein ion species can be deduced from their velocity. The biggest difference is that SELDI uses chromatographic surfaces as sample targets. These surfaces include a variety of chemically (anionic, cationic, hydrophobic, hydrophilic, metal affinity capture, etc.) or bio-chemically (immobilized antibody, receptor, DNA, enzyme, etc.) defined chromatographic surfaces. SELDI allows proteins/peptides to be profiled from different biological origins i.e. cell lysates, conditioned media and serum (Dowling *et al.* 2007, Shiwa *et al.* 2003, Langbein 2008).

SELDI is a highly sensitive technique and therefore a small amount of sample of interest is loaded onto chromatographic arrays. The proteins from crude samples selectively bind to the surfaces by adsorption, partition, electrostatic or affinity based interactions according to their nature. After a short incubation period, unbound proteins and unspecific substances are washed away with an appropriate buffer and water. The ToF reader records the time of flight and calculates the accurate molecular weight of proteins/peptides in the form of a spectral map containing mass to charge ratios (m/z) and intensities corresponding to each bound proteins/peptides. Biomarker wizard software analyses the spectral map and detects differentially expressed protein/peptides with statistical significance. An increase in published research using SELDI-ToF in the past few years itself has demonstrated its potential for the early detection of cancer especially for low molecular weight cancer-associated proteins (Wright 2002, Espejel and Roa 2008). Recently, SELDI-ToF-MS has been used to investigate the efficiency of purification process as well as to ensure the

quality (glycosylation and other post translational modification) of products (Wierling *et al.* 2007).

1.9.1.4 Proteomics and protein production in mammalian cells

A number of proteomics studies have been published recently that mainly focus on how to improve the efficiency of biopharmaceutical production using mammalian cell lines such as CHO or NS0 (Kaufmann *et al.* 1999, Baik *et al.* 2006, Nissom *et al.* 2006, Yee *et al.* 2008, Underhill and Smales 2007, Smales *et al.* 2004). Protein profiling using iTRAQ, nano LC and mass spectrometry have shown a significant increase in expression of proteins related to transcription and translation (Hmgb1), chaperones (Hspd1), and cellular growth (S100a11) in a high producer CHO cell line (Nissom *et al.* 2006). In a another similar investigation, ER luminal chaperones (ENPL, BiP and PDI) and proteins involved in mRNA translation (TCTP) and oxidative stress (PDX1) were observed to be up regulated in NS0 using 2D-PAGE (Smales *et al.* 2004). ENPL, BiP and PDI are known to be involved in recombinant protein synthesis and assembly (Meunier *et al.* 2002). PDI and BiP were also found to be increased with increased levels of Mab productivity in the microsomal fraction from the NS0 cells (Alete *et al.* 2005). Increased levels of the molecular chaperone (BiP) has also been observed in NaBu-treated CHO cells indicating their potential role in improving recombinant protein production in mammalian cells (Van Dyk *et al.* 2003). An abundance of ER chaperone (BiP, PDI, Calr) and non-ER chaperone (CCT2, GRP78, TCP1) proteins have also been observed in the NS0 cells with increased productivity of Mab (Dinnis *et al.* 2006).

A number of proteins involved in the regulation of cell structure (especially vimentin) have recently been observed to be differentially regulated in CHO cells following temperature-

shift from 37^0C to low temperature (Baik *et al.* 2006, Underhill and Smales 2007). Vimentin was down regulated in high producer CHO cells (Meleady *et al.* 2008). ACTB was also observed to be differentially regulated in response to the metabolic shifts in mouse hybridoma cell line MAK (Seow *et al.* 2001), to temperature-shift in CHO cells (Baik *et al.* 2006, Underhill and Smales 2007) and to NaBu treatments in CHO cells (Yee *et al.* 2008). Low temperature and metabolic shift have been used to regulate growth and productivity in culture. The cytoskeletal proteins (ACTB, TUBB5, Cofilin) were observed to be abundant in NS0 cells with increased productivity of Mab (Dinnis *et al.* 2006). Therefore reorganisation of cytoskeleton is critical to improve growth and/or productivity of culture.

Proteomic profiling of CHO cells has also identified a number of metabolic proteins to be associated with the regulation of growth and productivity in culture. GAPDH, a glycolytic enzyme, has been observerd to be up regulated in CHO cells cultured at low temperature (Baik *et al.* 2006, Underhill and Smales 2007) and in MAK cells following metabolic shift (Seow *et al.* 2001). Apart from GAPDH, abundance of various other metabolic proteins (i.e. ENO1 and TPI) have been reported in high producer cells (Dinnis *et al.* 2006). ENO1 was also observed to be up regulated in high producer CHO cells (Meleady *et al.* 2008). Nucleoside diphosphate kinase A (NDKA), a ubiquitous enzyme catalyzing the non-specific interconversion of nucleoside triphosphates was observed to be abundant in high producer NS0 cells (Smales *et al.* 2004). NDKA was also observed to increased in high producer NS0 cells. NDKA is involved in numerous core cellular processes such as DNA/RNA synthesis, protein synthesis, cellular signaling and glycosylation.

The number of proteins associated with transcription and translation have also been observed with respect to increased productivity. The expression of TCTP (translationally

controlled tumor protein) was observed to be increased in high producer NS0 cells (Smales *et al.* 2004). TCTP is involved in facilitating the elongation phase of mRNA translation in association with eEF1A. eEF1 has also been observed to be increased in high producer CHO cells (Meleady *et al.* 2008). Another translation associated protein, alanyl tRNA synthetase, was also observed to be up regulated. This protein is a specific component of the gene-expression machinery by which aminoacyl tRNAs translate mRNA into an amino acid sequence on the ribosome, attaching alanine to its corresponding transfer tRNA during protein synthesis. Therefore increased expression of translation associated proteins have been reported to be associated with increased protein production in culture.

Taken together, the changes observed in the expression of chaperons, structural proteins and metabolic proteins suggests that the cellular cytoskeletal apparatus interacts functionally with the translational apparatus and this highly cordinated interaction plays an important part in improving recombinant proteins production in mammalian cells.

1.9.2 miRNA Arrays

miRNA-arrays are recently developed high quality microarrays that can contain probes for miRNAs from human, rat and mouse. miRNAs are highly conserved during evolution and therefore these probes are believed to specifically bind to and amplify their complementary parts in the samples. Sequences used in the design of the probes and array were mainly taken from miRBase Sequence Database, which is curated by the Sanger Institute (http://microrna.sanger.ac.uk/sequences). Detection of these amplifications enable us to quantify the expression level of miRNAs. Two types of miRNA-arrays (mirVana miRNA bioarray and miRNA TaqMan Low Density Arrays) were used in this investigation. The mirVana^{TM} miRNA bioarrays were the first commercially available miRNA arrays

launched in late 2005 by Ambion. The mirVana miRNA bioarrays include a comprehensive panel of known human, mouse and rat miRNAs as well as Ambion's proprietary, non-published microRNAs, Ambi-miRs. The miRNA-bioarray uses similar technology to gene microarray technology. Both processes include RNA isolation, labeling, hybridization to the array and washing to provide a robust, reproducible platform for expression profile analysis. The results for individual targets can be validated using real-time RT-PCR with TaqMan® MicroRNA Assays. miRNA TaqMan Low Density Arrays (miRNA-TLDA) (Applied Biosystems) are qRT-PCR based highly sensitive, specific and reproducible genomic tools to detect the expression of miRNA genes in a wide variety of samples such as serum or tumors etc. All of the miRNA species in the samples are first transcribed using a pool of primers and then applied to the miRNA-TLDA card to amplify miRNA genes. Eight samples can be analyzed on one card at a time and this minimizes the variation within the samples due to similar processing conditions. miRNA-TLDA is itself an RT-PCR based reaction and therefore validation of expression is not necessary.

miRNA-arrays has been extensively used to investigate the differential expression of miRNA in various diseases i.e. cancer and diabetes etc, however its potential in bioprocess-related research has not been explored yet. The genome of CHO cells has still not been completely sequenced and therefore not available publicly. Due to this, no validated miRNA-array is available to date for CHO cells. Since the miRNA are well conserved during evolution and CHO cells have sequence similarities with human, mouse and rat, miRNAs arrays containing probes from these orthologous species could potentially identify significant changes in miRNA expressions in CHO cells. This information will open the

possibility to recruit miRNAs to regulate growth and recombinant protein productivity of mammalian cells.

Aims of thesis

The specific aims of this investigation were as follows:

- **To establish non-producer and SEAP-producer CHO-K1 cells in low serum supplemented or serum-free medium (SFM) in suspension culture.**

Most of the recombinant protein production in industry is performed in suspension cultures. As such, we wished to adapt adherent CHO-K1 cells to grow in suspension culture as a suitable model system. The presence of serum in culture medium increases the risk of contamination (i.e. viral and mycoplasma, etc) and also the difficulty and cost of recovery of recombinant protein from the culture media. For this reason, we aimed to establish suspension-adapted CHO-K1 cells capable of growing in low serum-supplemented or SFM culture conditions. One of the major goals of this thesis was to identify and investigate the role of selected protein/miRNA targets in the regulation of cell growth and recombinant protein productivity in mammalian cells. Thus, we wished to development of a human recombinant protein-secreting model CHO-K1 cell line, capable of growing in low serum-supplemented or serum-free medium in this work.

- **To identify temperature-dependent and independent changes in expressed proteins/miRNAs in CHO-K1 cells.**

Low temperature cultivation of CHO-K1 cells can reduce cell growth, metabolism and waste accumulation in culture and can maintain viability for extended periods of time. This results in increased recombinant protein production. However, the mechanisms regulating this phenotype are poorly understood. We wished to identify temperature-dependent and

independent differential expression of proteins using 2D-DIGE and miRNAs using hybridization-arrays to gain understanding of the relationship between the low temperature response of mammalian cells and improved recombinant protein productivity in culture.

- **To establish a functional role of selected protein targets in cell growth and recombinant protein productivity of CHO-K1 cells.**

Having identified differentially expressed proteins using 2D-DIGE, we aimed to investigate whether artificial disregulation of these targets impacted on desired cellular phenotypes. The expression of selected protein targets can be inhibited using Small interfering RNA (siRNA) transfection. The knockdown of expression of selected protein targets in low-serum and suspension-adapted SEAP secreting CHO-K1 cells would establish a role for these proteins in the regulation of growth and recombinant protein productivity in culture. The potential identified targets could be engineered into the cells to improve the efficiency of the bioprocess.

- **To establish a functional role of selected miRNA targets in cell growth and recombinant protein productivity in CHO-K1 cells.**

Since one miRNA can regulate the expression of a number of proteins, we wished to investigate effects of selected miRNA targets on growth and productivity of CHO cells. Chemically modified, small RNA molecules (Anti-mir and pre-mir) can be transfected into cells to specifically inhibit or over express these miRNA targets. The knockdown studies in conjunction with over expression studies for selected miRNA targets in low-serum and suspension-adapted SEAP secreting CHO-K1 cells would enable us to identify miRNA

target(s) that regulate growth and productivity in culture. These potential miRNA targets could be useful to design cell engineering approaches to improve growth and productivity.

- **To investigate the CHO-K1 cell secretome at distinct phases of the growth cycle using SELDI-ToF MS.**

The CHO-K1 cell secretome provides a valuable source of information as it contains proteins and peptides that can modulate cell growth in culture. There is a possibility that these secreted proteins could also contribute to alterations of the recombinant protein product as the extra-cellular environment can have an impact on post-translational modifications (PTMs). Moreover, knowledge of secreted host cell proteins could help to improve the efficiency of the product recovery process from culture media. Therefore, we aimed to identify secreted proteins/peptides in different phase of growth cycle i.e. lag, log and stationary phase as it will improve our understanding of the behaviour of CHO cells in production culture and may enable us to identify protein/peptide markers associated with growth, apoptosis and various other stress conditions in culture. This information could then be applied in cell engineering based approaches to improve recombinant protein productivity.

Section 2

Materials & Methods

2.1 Ultrapure water

Ultrapure water was used in the preparation of all media and solutions. For ultra-purification, the water was initially pre-treated which involved activated carbon, pre-filtration and anti-scaling. This water was then purified by a reverse osmosis system (Elga USF Maxima Water Purification System) to a standard of 12 - 18 MΩ/cm resistance.

2.2 Glassware

All glassware and lids were soaked in a 2% (v/v) solution of RBS-25 (VWR International) for at least 1 hour (hr). This is a deproteinising agent, which removes proteineous material from the bottles. Glassware was scrubbed and rinsed several times in tap water. The bottles were then washed by machine using Neodisher detergent, an organic, phosphate-based acid detergent. The bottles were then rinsed twice with distilled water, once with ultrapure water and sterilised by autoclaving. The spinner vessels were additionally treated with 1M NaOH solution over night to further ensure the elimination of cell debris from the inner surface and were washed twice with ultrapure water. Spinners were sealed in autoclave bags (Stericlin, 0775) and were autoclaved.

2.3 Preparation of cell culture media

Ultrapure water (UHP) and glassware were autoclaved before use in cell culture as described in Section 2.1 & 2.2.

ATCC medium (1:1 (v/v) of Dulbecco's modified Eagle medium (DMEM) and F-12 Ham basal media (Sigma, 56495C)) containing glutamine and sodium pyruvate (Gibco-Invirogen, 11360-035) and ExCell serum-free medium (Sigma, 14361C) were used in this

investigation. ATCC medium was prepared in house. 1X media for DMEM and F-12 Ham were prepared as follows- the basal 10X media was added to sterile UHP water, buffered with HEPES (N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid) and $NaHCO_3$ as required and adjusted to pH 7.5 using sterile 1.5 N NaOH or 1.5 N HCl. The media was then filtered through sterile 0.22µm bell filters (Gelman, 12158) and stored in sterile 500mL bottles at 4°C. Sterility checks on all media bottles for bacterial, yeast and fungal contamination were made using Trypto Soya Broth (TSB, Oxoid, CM0129) and Thioglycolate broths (Oxoid, CM173). All samples for sterility checks were then incubated at both 25°C and 37°C. Basal media were stored at 4°C for up to three months in the dark. Complete media was then prepared by adding serum as required (Table 2.3). Complete media was also stored at 4°C for a maximum of one month in the dark. Fetal calf serum (Sigma, F7524), G418 (Sigma, A1720-5G) and Insulin-transferrin-selenium (ITS, Sigma, I3146) were added fresh to media. One mililiter of ITS (100x) was added to 99mL of media. Sterility testing was also performed for all complete culture media and cell culture related solutions by incubating a small aliquot at 37°C for a period of seven days. This ensured that no bacterial or fungal contamination was present in the media or the solutions at the time of their use.

Table 2.3. Preparation of complete media. FCS denotes Fetal Calf Serum, G418 for Geneticin Sulfate and ITS for Insulin-Transferrin-Selenium.

Cell Line	**Recombinant Protein Product**	**Basal Medium**	**FCS (%)**	**G418/ITS**	**Culture Type**
CHO-K1	-	ATCC	10	-	Monolayer
CHO-K1	-	ATCC	10	-	Suspension
CHO-K1	-	ATCC	5	-	Monolayer
CHO-K1	-	ATCC	5	-	Suspension
CHO-K1	-	ATCC	0.5	-	Monolayer
CHO-K1	-	ATCC	0.5	-	Suspension
CHO-K1	SEAP	ATCC	0.5	G418 (1mg/mL)	Monolayer
CHO-K1	SEAP	ATCC	0.5	G418 (1mg/mL)	Suspension
CHO-K1	-	ATCC	-	ITS (1mL/100mL)	Suspension
CHO-K1	-	ExCell	-	-	Monolayer
CHO-K1	-	ExCell	-	-	Suspension
CHO-K1	SEAP	ExCell	-	G418 (1mg/mL)	Monolayer
CHO-K1	SEAP	ExCell	-	G418 (1mg/mL)	Suspension

2.4 Cells and cell culture

All cell culture work was carried out in a class II laminar air-flow (LF) cabinet (Holten). Before and after use, the LF cabinet was cleaned with 70% industrial methylated spirits (IMS). Any items brought into the cabinet were also swabbed down with IMS. At any time, only one clone / cell line was used in the LF cabinet. Upon completion of work with any given clone / cell line, a 15 minute clearance was given to eliminate any possibilities of cross-contamination between the various clones / cell lines. The cabinet was cleaned weekly with Virkon (Antech International) and IMS. Details relating to the cell lines used for the experiments are provided in Table 2.4.1. All cells were routinely maintained at 37°C in an atmosphere of 5% CO_2. In the case of biphasic culture, cells were shifted to 31^0C after growing cells at 37^0C. Cells were fed with fresh media or subcultured in mid-exponential phase of culture at every 3-4 days or as required in order to maintain active cell growth.

Table 2.4.1. Description of CHO-K1 cells and variants developed and used in this thesis

Cell line	**Cell type**	**Source**
CHO-K1	Adherent parental Chinese hamster cells growing in 5-10% FCS supplemented ATCC medium	ATCC
CHO-K1_10s	CHO-K1 adapted to grow in 10%FCS supplemented ATCC medium in suspension culture	NCTCC*
CHO-K1_5s	CHO-K1 adapted to grow in 5%FCS supplemented ATCC medium in suspension culture	NCTCC*
CHO-K1_0.5	CHO-K1 adapted to grow in 0.5%FCS supplemented ATCC medium in monolayer culture	NCTCC*
CHO-K1_0.5s	CHO-K1 adapted to grow in 0.5%FCS supplemented ATCC medium in suspension culture	NCTCC*
CHO-K1_SEAP_0.5	SEAP secreting CHO-K1 adapted to grow in 0.5%FCS supplemented ATCC medium in monolayer culture	NCTCC*
CHO-K1_SEAP_0.5s	SEAP secreting CHO-K1 adapted to grow in 0.5%FCS supplemented ATCC medium in suspension culture	NCTCC*

CHO-K1_SF	CHO-K1 adapted to grow in serum free medium ExCell in monolayer culture	NCTCC*
CHO-K1_SFs	CHO-K1 adapted to grow in serum free medium ExCell in suspension culture	NCTCC*
CHO-K1_SEAP_SF	SEAP secreting CHO-K1 adapted to grow in serum free medium ExCell in monolayer culture	NCTCC*
CHO-K1_SEAP_SFs	SEAP secreting CHO-K1 adapted to grow in serum free medium ExCell in suspension culture	NCTCC*

ATCC: American Type Culture Collection

NCTCC: National Cell and Tissue Culture Centre

* CHO-K1 variants developed during the course of these studies at NCTCC.

2.4.1 Subculturing of cell lines

Cells were grown in adherent and suspension culture. The following method was used to subculture cells.

2.4.1.1 Anchorage dependent cells

Spent cell culture medium was removed from the tissue culture flask and discarded into a sterile bottle. The flask was then rinsed out with 2-5mL of trypsin/EDTA solution (0.25% trypsin (Gibco, 043-05090) and 0.01% EDTA (Sigma, E9884) solution in PBS (Oxoid, BRI4a)) to ensure the removal of any residual media. Depending on the size of the flask, 2-5mL of trypsin was then added and incubated at 37°C for approximately 2-5 minutes until all of the cells detached from the inside surface of the flask. The detachment of cells was monitored by microscopic observation. A 10mL volume of complete media or trypsin

inhibitor solution was added to the flask to deactivate trypsin. The trypsin inhibitor solution was prepared by dissolving trypsin inhibitor (Sigma, T6522) in sterile PBS to achieve concentration of 1mg/mL and was used after filter (0.2µm) sterilization at 1:1 ratio (v/v) to inhibit the trypsin in culture. The cell suspension was removed from the flask and placed in a 30mL sterile universal container (Sterilin, 128a) and centrifuged at 1000rpm for 5 minutes. The supernatant was then discarded from the universal and the pellet was resuspended gently in fresh complete medium. A cell count was performed and an aliquot of cells was used to seed a flask to achieve the required density. All cell waste and media exposed to cells were autoclaved before disposal.

2.4.1.2 Suspension Cells

Cells were sampled from the suspension culture in 30mL sterile universal containers and were centrifuged at 1000rpm for 5 minutes. The supernatant, conditioned medium, was collected in another sterile container(s) and pellet was resuspended in fresh complete culture medium. Cells were counted and were seeded in fresh spinner vessel(s) (Techne) at a seeding density of $2x10^5$ cells/mL in 100mL working volume supplemented with 10% conditioned medium. All cell waste and media exposed to cells were autoclaved before disposal.

2.4.2 Cell adaptation

Cells were adapted to grow in suspension as well as in low or serum free culture medium as this is more relevant cell model to industrial production process.

2.4.2.1 Suspension Culture

Adherent CHO-K1 cells growing in 10% fetal bovine serum (Sigma) supplemented ATCC medium were cultured in 250mL spinner (Techne) with a working volume of 100mL and

maintained at 60rpm in an incubator at 37^0C to adapt to grow in suspension culture. Cultures were gassed each day with 'Mixture of gasess' (Air products, 21179) containing CO_2, O2 and N_2. Cell counts were performed every alternate day. Cells were subcultured once they reached the density of $\geq 1 \times 10^6$ cells/mL. For this, appropriate sample was collected from the spinner and centrifuged at 1000rpm for 5 minutes. The supernatant was used as conditioned media and pelleted cells were resuspended in fresh media and were used to seed the culture at $2x10^5$ cells/mL. Of this cell-free conditioned media, 10% was also added into the culture medium during subculturing. Cells ($1x10^5$ cells/mL) from exponentially growing cultures were inoculated in a separate spinner to monitor the improvement in growth behaviour of cells. Cell counts were performed every day over 144hrs of culture using the trypan blue dye-exclusion method and a hemocytometer.

2.4.2.2 Adherent Culture

For adherent culture, suspension-adapted CHO-K1 cells were seeded at 3×10^5 cells/mL in three T-75 cm^2 culture flasks in 10mL of culture medium. Cell counts were performed every 2^{nd} and 4^{th} day of culture. If cells achieved $\geq 1 \times 10^6$ cells/mL within 4 days, they were transferred to medium with further reduced serum. If cells failed, the medium was replaced in the 3^{rd} flask and once cells became confluent, the work flow was followed until they met the set criteria of adaptation. The used culture medium was collected and centrifuged at 1000rpm for 5min to get cell-free conditioned media. A volume of 10% conditioned medium was included at each subculture. This procedure was repeated until cells were adapted.

2.4.3 Assessment of cell number and viability

Prior to cell counts, cells were prepared by subculturing adherent cells or direct sampling for suspension cells as described in Section 2.4.1. An aliquot of the cell suspension was then added to trypan blue (Gibco, 525) at a ratio of 1:1. The mixture was incubated for 2-3 minutes at room temperature. An aliquot (10μL) was then applied to the chamber of a glass coverslip-enclosed haemocytometer. For each of the four grids of the haemocytometer, cells in the 16 squares were counted. The average of the four grids was multiplied by a factor of 10^4, volume of the grid, and the relevant dilution factor to determine the average cell number per mL in the original cell suspension. Viable cells excluded the trypan blue dye as their membranes remained intact and therefore remained unstained while non-viable cells stained blue. On this basis, the percentage viability of culture was calculated by counting non-viable cells and viable cells in the sample.

2.4.4 Cryopreservation of cells

Cells were preserved with 5% DMSO (Sigma, D5879). For the cells that were being cultured in 10% or 5% FCS supplemented ATCC medium, 10% DMSO solution was made in serum while for the cells that were adapted to grow in low-serum or serum-free culture condition, 10% DMSO solution was prepared in complete culture medium (serum-free or serum-supplemented). The filter (Gelman, 121-58) sterilized 10% DMSO solution and complete medium was then kept at 4^0C for 1 hour (hr). Cells for cryopreservation were harvested in the log phase of growth and counted as described in section 2.2 and 2.3. Cells were centrifuged and pellets were resuspended in a suitable volume of serum or culture medium. An equal volume of a 10% DMSO solution was added drop-wise with mixing to the cell suspension. The suspension was then aliquoted in 1 mL volumes to pre-labelled

cryovials (Greiner, 201151) and immediately placed at -80^{0}C. After 6-12 hrs, cryovials were transferred to the liquid nitrogen tank for long term storage at -196°C.

2.4.5 Thawing of cryopreserved cells

A volume of 10mL of fresh pre-warmed complete medium was added to a sterile universal. The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C quickly. The ice-cold cells were removed from the vials and transferred to the aliquoted medium in the sterile universal. The resulting cell suspension was centrifuged at 1,000rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in fresh pre-warmed complete medium. An assessment of cell viability on thawing was then carried out (section 2.4.3). Thawed cells were then added to the appropriate culture flask, i.e. spinner or T-flask, with a suitable volume of complete culture medium and allowed to grow at 37^{0}C. In the case of adherent cells, flasks were re-fed with fresh media to remove any non-viable cells on the following day.

2.5 Establishment of SEAP-secreting CHO-K1 cells

The SEAP secreting CHO-K1 cells were established as the model cell line to investigate the effects of selected miRNA or protein targets on cell growth and recombinant protein production. For this, a SEAP coding gene was inserted in to pcDNA3.1 and this plasmid was transfected into CHO-K1 cells.

2.5.1 Plasmids

pSEAP2-Control was a kind gift from Professor Martin Fussenegger (Institute for Chemical and Bioengineering, ETH Zurich, Zurich, Switzerland). pcDNA3.1 was obtained from Invitrogen.

2.5.2 Transformation of competent cells

A bacterial cell suspension (100 µl) of competent JM109 (Promega, L2001) was mixed with ~20 ng DNA and placed on ice for 40 min after which the mixture was heat-shocked at 42^{o}C for 90 seconds and then placed on ice for 3 minutes. A volume of 1mL of LB broth ((10 g Tryptone (Oxoid, L42), 5 g Yeast Extract (Oxoid, L21), 5 g NaCl (Merck, K1880814))/litre, autoclaved before use) was added to the competent cell suspension and incubated at 37^{o}C for 1 hour. Different volumes of this suspension were spread on selecting agar plates (LB agar containing 100 mg/mL Ampicillin) and incubated overnight at 37^{o}C. Single colonies that grew on these selecting plates were transferred into 5 mL LB broth containing 100mg/mL of Ampicillin (Invitrogen, Q100-17) and grown overnight with agitation at 37^{o}C. Cells were then subjected to a DNA mini preparation (see section 2.5.3) or inoculated into 250 ml LB broth containing Ampicillin (100mg/mL) in a 1 L baffled flask with shaking overnight at 37^{o}C for large scale preparation (section 2.5.3.4).

2.5.3 DNA miniprep of plasmid DNA

The Qiagen Plasmid DNA Extraction Kit (Qiagen, 12262) was used according to the manufacturer's instructions to isolate plasmid DNA from a 5 mL culture grown overnight in LB broth containing selection antibiotic with shaking at 37^{o}C. After incubation, the cells were pelleted by centrifugation at 8000rpm for 3 minutes. Pelleted cells were resuspended in 250 µL of buffer P1 and the cell suspension was transferred to a microcentrifuge tube. To this solution, 250 µL of buffer P2 were added and mixed gently. A volume of 350 µL of buffer N3 was then added to the solution and the tube was mixed immediately. This mixture was centrifuged for 10 minutes at 13,000 rpm. The supernatant was applied to a QIAprep spin column and centrifuged for 60 seconds. The flow-through was discarded. The

column was washed by adding 500 µL of buffer PB and centrifuged for 60 seconds. Again, the flow through was discarded. This step was repeated with 750 µL of buffer PB. The column was centrifuged for a further 60 seconds to remove the remaining wash buffer and was then placed in a clean microcentrifuge tube. To elute the DNA, 50 µL of buffer EB were added to the column. The column was allowed to stand for 60 seconds and then centrifuged for 60 seconds. The concentration of eluted DNA was determined using a NanoDrop spectrophotometer by measuring the OD_{260nm}. Plasmid was stored at –20°C until required.

2.5.4 DNA maxiprep of plasmid DNA

Plasmid DNA was purified using an Endofree Plasmid Maxi kit (Qiagen, 12362). Following the growth of plasmid culture in 270 mL Ampicillin-containing LB broth in 1L baffled flask with shaking overnight at 37°C, the culture was split into 2 x 250 mL centrifuge bottles and spun at 3,500 rpm for 15 minutes at 4°C. Pellets were processed immediately or stored at –20°C until required. The bacterial pellet was resuspended in 10 mL of buffer P1. To this suspension, 10 mL of buffer P2 was added, mixed gently and incubated at room temperature for 5 minutes. A volume of 10 mL of chilled buffer P3 was then added to the lysate and mixed gently and immediately. The lysate was poured into the barrel of a QIAfilter cartridge and incubated at room temperature for 10 minutes. The cap from the QIA filter outlet nozzle was then removed and the plunger gently inserted into the cartridge. The cell lysate was filtered into a 50 mL tube. To the lysate, 2.5 mL of buffer ER were added and mixed by inverting repeatedly. This mixture was incubated on ice for 30 minutes. A Qiagen-tip 500 column was equilibrated by applying 10 mL of buffer QBT and the column was allowed to empty by gravity flow. The filtered lysate was then applied to

the Qiagen-tip and also allowed to enter the resin by gravity flow. The Qiagen-tip was washed twice with 30 mL of buffer QC. The DNA was then eluted by the addition of 15 mL of buffer QN in a 30 mL tube. The DNA was precipitated by adding 10.5 mL of isopropanol to the DNA solution, which was then mixed and centrifuged at ~10,000 rpm for 30 minutes at 4^0C. The DNA pellet was washed with 5 ml of 70% ethanol and again centrifuged at ~10,000rpm for 10 minutes. The pellet was then air-dried and dissolved in 500 µL buffer TE. The DNA concentration was determined by measuring the OD_{260nm} using a Nanodrop spectrophotometer.

2.5.5 SEAP plasmid construction

A volume of 1 µg of pcDNA3.1-SEAP was digested with 1 µL of HindIII (Biolabs, R0104S, 10X) and SalI (Biolabs, R0138S, 10X) while pcDNA3.1 was digested with 1 µL of HindIII and XhoI (Biolabs, R0146S, 10X) overnight at 37^0C. The digested mixture was separated using 1% (v/v) agarose gel electrophoresis at 60V. The band at ~2 kb and ~5.4 kb were cut out from the gel and DNA was retrieved by centrifugation at 13000rpm for 1 minute. using a GenElute Agarose Spin Column. DNA was then purified using MinElute (Qiagen, 28004). Five volumes of Buffer PB were added to the DNA sample. The mixture was applied to a MinElute column for 1 minute and the supernatant was removed by centrifugation at 13000rpm for 1 minute. The column was washed with 750µL of Buffer PE. The DNA was eluted into a fresh tube using 10µL of Buffer EB. DNA was then quantified using a NanoDrop Spectrophotometer. Fragments of ~2 kb and ~5.4 kb were ligated over night at 37^0C. The ligated plasmid was transformed into the competent bacterial cells. After miniprep as described in Section 2.5.3.3, 1 µg of plasmid was digested

using 1 µL of EcoRI (Biolabs, R0101S, 10X) and BamHI (Biolabs, R0136S, 10X) and were separated by 1% (v/v) agarose gel electrophoresis at 60V.

2.5.6 pcDNA3.1-SEAP transfection

Low serum adapted CHO-K1 cells ($1x10^5$ cells/mL) were seeded in 4x T-25 cm^2 culture flasks. Once they reached 70-80%, the culture medium was replaced ~6 hrs before transfection. In separate round bottom tubes, 1µg of pcDNA3.1-SEAP and of empty vector pcDNA3.1 were incubated at room temperature with 100µL in serum-free medium (OptiMEM, Gibco, 31985) for 5 minutes. A volume of 4µL of Lipofectamine 2000 (Invitrogen, 11668019), transfection reagent, was also incubated in 200µL of OptiMEM in the same conditions and 100µL each was added to both plasmid complexes. Both solutions were combined, mixed gently and allowed to form complexes over a 20 minute time period at room temperature. A volume of 200 µL of pcDNA3.1-SEAP complex was added to one flask and pcDNA3.1 complex to another and were mixed gently. Two non-transfected flasks were considered as controls. The media was changed after 24 hrs of culture and cells were allowed to grow for another 24 hrs.

2.5.7 Selection and isolation of Clones

G418 (400 µg/mL) was added to culture after 48hrs of transfection except to one of the two non-transfected controls. The amount of G418 in the medium was increased every alternate day (600, 800 and 1000 µg/mL) to achieve complete elimination of cells in the non-transfected culture. SEAP Clones were isolated using the limiting dilution method. For this, a suspension of cells containing 100 cells/mL was prepared. A volume of 100µL/well (10 cells/well) of this suspension was inoculated into 8 wells (1 column) of a 96-well culture plate. The suspension was further diluted by a factor 10x by adding 90 µL/well media and

10 µL/well of cell suspension into the next column to achive 1 cells/well. Cells were further diluted in the another column to achive 0.5 cells/well. The concentration of 0.5 cells/well is a condition where cell is either present or absent in a particular well. The cells were allowed to grow. The well containing single cells were identified under microscope. The culture media was replaced routinely after 3-4 days to allow single cell clones to expand further. The confluent cultures were transferred to a 24-well. Conditioned media was collected after 24hrs from cultures growing in media containing 800µg/mL and 1000µg/mL of G418 and after 72hrs of culture from cultures growing in media containing 1000µg/mL of G418 to perform SEAP-assays (as described in section 2.5.7) to identify SEAP-secreting CHO clones. The identified SEAP-secreting cells were allowed to grow further in T-75 flasks.

2.5.7 SEAP Assays

Cells ($1x10^5$ cells/mL) were seeded in 0.5% FCS supplemented ATCC medium containing G418 (800 or 1000µg/mL) in 24-well plate with a 0.5mL of working volume. The conditioned medium was collected from cells at appropriate time intervals, such as for optimizing SEAP-assays after 24hrs and for stability assays after 72hrs or 96hrs of culture.

The enzymatic assay for the quantification of SEAP was adapted from the method reported by Berger *et al.*, (1988) and Lipscomb *et al.*, (2005). The conditioned media was centrifuged at 13000rpm for 15minutes. A volume of 50µL of cell-free conditioned media were transferred to individual wells of a 96 well flat bottom plate. To each sample, 50µL of 2X SEAP reaction buffer (containing 10.50 g diethanolamine (100%), 50 µL of 1 M $MgCl_2$ and 226 mg of L-homoarginine in a total volume of 50 mL) was added. Plates were incubated for 10 min at 37°C and then 10 µL of substrate solution (158 mg of p-

nitrophenolphosphate (Sigma, P4744-10G) in 5 mL of 1X SEAP reaction buffer, made fresh for each use) was then added to each well. The change in absorbance per minute (OD_{405}/min) was considered as an indicator of the amount of SEAP present in the sample. SEAP is a highly glycosylated protein. Therefore, changes observed in this investigation in SEAP amount could be due to changes in the SEAP quantity, quality or both.

2.5.8 Comparison of SEAP-productivity to identify highest and most stable producer clone

To identify the cells with different level of SEAP-secretion and stability in secretion, 0.5mL of $1x10^5$cells/mL from master culture at different passages (time intervals) were seeded in a 24-well plate in 0.5%FCS supplemented ATCC culture medium containing G418 (1000µg/mL). The non-producer CHO-K1 cells growing in 0.5%FCS supplemented ATCC culture medium were used as control. The conditioned media was taken after 72hrs of culture. The viable cells counts were also performed to calculate the cell specific productivity (Qp) in culture. Qp was calculated by dividing the total productivity by the total viable cells in culture.

2.6 Two-dimensional difference gel electrophoresis (2D-DIGE)

Two dimentional differential gel electrophoresis (2D-DIGE) is an advanced version of classical two-dimensional gel electrophoresis (2D-PAGE). In 2D-DIGE, protein samples are labeled with three different fluorescent dyes (Cy2, Cy3 and Cy5) and then separated by 2D-PAGE. Labeling of different protein samples with different fluorescent dyes and then mixing together allows samples to be separated on the same gel and this minimizes gel-to-

gel variation. The following steps were carried out to perform 2D-DIGE experiment in this investigation.

2.6.1 Cell culture

Suspension-adapted CHO-K1 cells were seeded at $1x10^5$ cells/mL in 100mL in 10% FCS supplemented ATCC medium in 250mL spinner with a working volume of 100mL. Cells were maintained in biphasic culture, where cells grown at 37^0C for 72hrs and then shifted to 31^0C for a further 72hrs, or in standard culture at 37^0C for 144hrs. Cultures were rotated at 60rpm and gassed everyday with Mixture of Gasses (N_2, O_2 and CO_2). Cell counts and viability were measured every day as mentioned in section 2.5.3. The cell samples were collected at 72hrs and 144hrs of culture for 2D-DIGE experiment.

2.6.2 Total protein extraction and quantification

Cell samples were harvested from culture as appropriate. Cells were then washed twice with ice-cold PBS. All procedures from this point forward were performed on ice. A total of $3x10^7$ cells were re-suspended in 500µL lysis buffer as described in Table 2.6.1. Re-suspended sample was then put through a 1 mL syringe with a 21 gauge needle 5 times. The sample was placed on an orbital shaker and shaken gently for 1 hour at room temperature.

Table 2.6.1. Preparation of lysis buffer

Contents	Recipe	Required weight/volume for making 25mL of lysis buffer
Urea	7M	10.5 g
Thiourea	2M	3.8 g
CHAPS	4%	1 g
Tris	30mM	0.091 g
Magnesium acetate	5mM	0.027 g
Water		Up to 25mL
pH		8 – 9
Aliquot in 1 mL/eppendorf and store at -20^{0}C		

After incubation, lysates were centrifuged on a bench-top centrifuge at 14,000rpm for 15 minutes at 4°C. The middle layer of the supernatant containing extracted protein was transferred to a fresh chilled eppendorf tube. Samples were then stored in aliquots at -80°C until used. A small aliquot of sample was used for protein estimation.

Protein levels were determined using the Bio-Rad protein assay kit (Bio-Rad, 500-0006) as follows. A 2 mg/mL bovine serum albumin (BSA) solution (Sigma, A9543) was used as a known standard. Dilutions of BSA stock for 0, 0.125, 0.25, 0.5 and 1.0mg/mL was prepared and used for generating a protein standard curve. To each well of a 96-well plate, 240µL/well of thiourea-compatible Bradford protein assay reagent (Bio-Rad, 500-0205) was added, followed by 10µL of protein standard dilution or sample (diluted 1:10). All samples were assayed in triplicate. After 5 minutes incubation, the absorbance was assessed

at 595nm. The concentration of the protein samples was determined from the plot of the absorbance at 595nm versus the concentration of the protein standard.

2.6.3 Labelling of proteins

2.6.3.1 Preparation of CyDye dyes for minimal-DIGE protein labelling

The three CyDye DIGE Fluor Minimal dyes (Cy3, Cy5 and Cy2 (GE Healthcare, 25-8010-65) were thawed from –20^{o}C to room temperature for 5 minutes. To each microfuge tube dimethylformamide (DMF) (Sigma, 22,705-6) was added to generate a concentration of 1 nmol/μL. Each microfuge tube was vortexed vigorously for 30 seconds to dissolve the dye. The tubes were then centrifuged for 30 seconds at 14,000rpm in a microcentrifuge. The reconstituted dyes were stored at –20^{o}C for up to 3 months.

2.6.3.2 Preparation of 10 μL working dye solution (200 pmol/μL)

On thawing, the dye stock solutions were centrifuged in a microcentrifuge for 30 seconds. To make 10 μL of the three working dye solutions, 8 μL of DMF was added to three fresh eppendorfs labelled Cy2, Cy3 and Cy5. A volume of 2 μL of each of the reconstituted dye stock solutions (200 pmol /μL) was added to their respective tubes. The dyes were stored at -20^{o}C in tinfoil in the dark for 3 months.

2.6.3.3 Protein sample labeling

The 2D-DIGE experiment was carried out for biological triplicate samples. Technical duplicates samples were included in this experiment. A volume of 100 μg of protein from 3 control (biological triplicate samples at 72hrs of culture) and 3 treated (biological triplicate samples at 144hrs of culture) protein samples was placed into 6 separate eppendorf tubes (3 for Cy3 and the other 3 for Cy5). They were labelled with the minimal dyes as follows, 2 μL/eppendorf of Cy3 was added to Control samples and 2 μL/eppendorf of Cy5 was added

to Treated samples. From each Control and Treated samples, 50 μg was aliquoted into an eppendorf and 2 μL of Cy2 was added to it. Each tube was mixed by vortexing, centrifuged and then left on ice for 30 minutes in the dark. To stop the reaction, 1 μL of 10 mM lysine (per 200pm of dye) was added. The tubes were mixed, centrifuged briefly and left on ice for 10 minutes in the dark.

2.6.4 Preparing the labelled samples for the first dimension

The protein samples labelled Cy3 (Control) and Cy5 (Treated) from respective biological replicates were combined to get 3 eppendorfs each containing 200 μg of Cy3- and Cy5-labeled protein samples. To each eppendorf, 100 μg of Cy2 labelled internal standard was added. An equal volume of 2X sample buffer (2.5 mL rehydration buffer stock solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer (GE Healthcare, 17-6000-86), 50 mM DTT (Sigma, D9163)), was added to the labelled protein samples. The mixture was left on ice for at least 10 minutes and then applied to Immobiline DryStrips for isoelectric focussing.

2.6.5 First dimension separation - isoelectric focussing methodology

Isoelectric focussing of all samples was carried out using immobiline pH gradient (IPG) strips (18cm, 4-7pH) (GE Healthcare, 17-1233-01). The protective lid was removed from the Immobiline Dry Strip Reswelling tray. The tray was levelled using the spirit level. Rehydration buffer (350μL) was slowly pipetted into the centre of each slot. All air bubbles generated were removed. The cover film from the IPG strip (GE Healthcare) was removed and positioned with the gel side down and lowered. To ensure the entire strip was evenly coated with rehydration buffer, the strip was gently lifted and lowered onto the entire surface of the solution avoiding trapping air bubbles. Each strip was overlaid with about 3

mL IPG Cover Fluid (GE Healthcare, 17-1335-01) starting on both ends of the strip and moving to the centre. The protective lid was then replaced and the strips were left at room temperature to rehydrate overnight (or at least 12 hours).

Following the rehydration procedure, the Manifold (GE Healthcare) was placed onto the IPGphor unit (GE Healthcare) by inserting the "T" shape into the hollow provided. A 9 mL of Cover Fluid was placed into each of the twelve lanes in the tray in order to cover the surface. Two wicks (GE Healthcare, 80-6499-14) per strip were placed on tinfoil and 150 µL of UHP was pipetted onto each one to rehydrate them. The rehydrated strips were placed in the correct orientation (+ to anode) and aligned just below the indented mark, to allow for the wicks to overlap the strip. The rehydrated wicks were then placed over both the cathodic and anodic ends of all the strips. The wicks were checked to ensure they were positioned over the gel portion of the strip and avoiding the indent in the lane so as to guarantee a good contact with the electrodes. The sample cups (GE Healthcare, 80-6498-95) were then positioned approximately 1 cm from the cathodic end of the strip and an insertion tool was used to securely "click" the cups into place. The electrodes were then fitted with their "Cams" open and in direct contact with the wicks. The amount of protein loaded per strip was 150µg for DIGE or 400µg for spot picking. The protein samples were prepared by centrifuging to remove any insoluble material and the appropriate volume was loaded with a pipette tip placed just beneath the surface of the cover fluid. The cover of the IPGphor unit was closed and the desired programme selected. The temperature was set for 20^{o}C with 50µA/strip. The IEF parameters are as follows: step 1: 300 volts for 3 hours (step-and-hold), step 2: 600 volts for 3 hours (gradient), step 3: 1000 volts for 3 hours (gradient), step 4: 8000 volts for 3 hours (gradient). The IEF was left at 500 volts (step-and

–hold) until ready for SDS-PAGE step. On completion of the IEF run, the strips were drained of the cover fluid and stored in glass tubes at –80^{o}C or used directly in the second dimension.

2.6.6 Second Dimension – SDS polyacrylamide gel electrophoresis

2.6.6.1 Casting gels in the ETTAN Dalt-12 gel caster

The 12.5 % acrylamide gel solution was prepared in a glass beaker (acrylamide/bis 30%, 1.5 M Tris pH 8.8, 10 % SDS). Prior to pouring, 10 % ammonium persulfate and neat TEMED were added.

Two types of plates were used, low fluorescent for DIGE experiments and hinged for preparative and silver stained gels. All plates (both normal hinged and low fluorescent) and casting equipment were inspected to ensure they were clean. The gel caster frame was placed on a level bench leaning on its "legs" so that the back of the caster was open and facing the operator. The plates were assembled so that the front and back plates were evenly aligned and all seals and hinges in place. A thin spacer was placed in the gel caster unit followed by an assembled plate and then a thin spacer and then another plate. The plates were positioned in the caster unit so that the lower, front plate was the furthest away from the operator and the spacers packed with their curved edges to the top. This layering was repeated until all 14 plates and spacers were in place. All plates and spacers were checked to ensure they were packed tightly together so as to minimise any gaps and air pockets. When the desired amount of plates had been added, the thicker spacers were placed next to bring the level marginally over the edge of the back of the caster. The backing plate was then added to the caster frame and screwed into place with the 6 screws provided. The silicone tubing was added to the outlet of the glass beaker and the glass tube

was inserted to the other end of the silicone tubing. The glass tube was inserted into the inlet of the reservoir and the glass beaker containing the gel solution was then clamped to a retort stand. The gel solution was held in place using arterial clamps on the top tube and the tube running down from the reservoir to the caster chamber. The top tube was unclamped and the gel solution was allowed to fill the tubing and the reservoir drain. Air bubbles that had been generated were dislodged by flicking the tube. When all air bubbles had been removed the bottom clamp was released allowing the gel solution into the gel caster. When the gel solution reached the indicator line across the top of the caster, the bottom and top tubes were reclamped. The displacement solution (0.375M Tris-Cl 1.5M pH8.8, 30% glycerol, UHP and bromophenol blue) was added to the reservoir and the glass tubing was slowly removed from the reservoir inlet. The clamp was removed from the bottom tube allowing the displacement solution into the tube and forcing the remaining gel solution into the gel caster. The gels were overlaid with 1 mL of saturated butanol or sprayed with 0.1 % SDS solution. The gels were left to set for at least three hours at room temperature. Following this, the caster was gently unlocked and the gels removed and rinsed with distilled water. If the gels were not used immediately they were stored for up to four days in 1X running buffer at 4^{o}C.

If gels were to be used for “spot picking” one of the plates were silanised prior to casting to stick the acrylamide mixture. A volume of 2mL of working bind-silane solution (8mL ethanol, 200µL glacial acetic acid, 10µL bind-silane (GE Healthcare) and 1.8mL UHP) was pipetted over the glass plate and wiped over with a lint free cloth. This was left to air dry for 15 minutes, after which 2mL ethanol and 2 mL UHP were each pipetted over the plate

and wiped off respectively. The plate was left to air dry for approximately 1 hour 30 minutes.

2.6.6.2 Preparing the ETTAN DALT-12 electrophoresis unit

The electrophoresis chamber was prepared by adding 6.48 litres of UHP and 720 mL of 10X SDS running buffer. The pump was then turned on to cool the system to 10^{o}C.

2.6.6.3 Equilibration of focussed Immobiline DryStrips

The SDS equilibration buffer (30% glycerol, 6M urea, 50nm 1.5M Tris-Cl pH 8.8, 2% SDS, bromophenol blue and UHP) which had been prepared, aliquotted into 30 mL volumes and frozen at –20^{o}C was allowed to thaw to room temperature. Two SDS equilibration buffer solutions with DTT (65 mM) or iodoacetamide (240 mM) (Sigma, I1149) were then prepared. Using a forceps, the IPG strips were removed from the IPGphor unit, the cover fluid was drained off by holding the strips at an angle and they were placed into individual glass tubes with the support film toward the wall. Equilibration buffer (10 mL containing DTT) was added to each tube and incubated for 15 minutes with gentle agitation using an orbital shaker. During this equilibration step, the gel cassettes were rinsed with UHP and then the tops rinsed with 1X running buffer. After the first equilibration, DTT containing equilibration solution was removed and 5 mL of the iodoacetamide containing equilibration buffer was added. The strips were incubated for 15 minutes with gentle agitation. During this equilibration step, the agarose overlay solution (0.5% agarose in running buffer) was prepared and 50 mL of 1X running buffer was placed in a glass tube. If the strips had been frozen at this stage they were left at room temperature to thaw before the DTT-containing equilibration solution was added.

2.6.6.4 Loading the focussed Immobiline DryStrips

Using a forceps and holding the anode end, the IPG strips were rinsed in 1X SDS electrophoresis running buffer and placed between the two glass plates of the gel. The strip was pushed down gently using a thin plastic spacer until it came in contact with the surface of the gel. Any air bubbles trapped between the gel surface and the strip were gently removed. Approximately 1 mL of the 0.5 % agarose overlay solution was applied over the IPG strip to seal it in place.

2.6.6.5 Loading gels into the Ettan DALT-12 electrophoresis unit

When the running buffer reached the desired temperature (10^{o}C) the loaded gel cassettes were wetted with UHP and inserted into the tank through the slots provided in the same orientation. When all 12 slots were filled the upper chamber was filled, 2X running buffer was added to the upper chamber until the mark on the side of the chamber was reached. The cover of the unit was replaced and the required running conditions selected. The unit was run for 18–24 hours at 1.5 Watts per gel at 10°C or until the bromophenol blue dye front reached the bottom of the gel. When the run was completed, the gel cassettes were removed from the tank one at a time using the DALT cassette removal tool and rinsed with UHP to remove the running buffer.

2.6.6.6 Method for scanning DIGE labelled samples

The Typhoon Variable Mode Imager (GE Healthcare) was turned on and left to warm up for 30 minutes prior to scanning. The scanning control software was opened and the fluorescence mode was selected. The appropriate emission filters and lasers were then selected for the separate dyes (Cy2 520 BP40 Blue (488), Cy3 580 BP30 Green (532) and Cy5 670 BP30 Red (633)). The first gel was placed in the scanner and pre-scanned at a

1000 pixel resolution in order to obtain the correct photo multiplier tube (PMT) value (to prevent saturation of the signal from high abundant spots). Once the correct PMT value was found, the gel was scanned at 100 pixel resolution, resulting in the generation of three images, one each for Cy2, Cy3 and Cy5. Once the scanning was completed, the gel images were imported into the ImageQuant software. All gels were cropped identically to facilitate spot matching in the Decyder BVA module.

2.6.7 Analysis of gel images

Scanned fluoroscent gel images were analysed using Biological Variation Analysis (BVA) module of DeCyder 6.5 software (GE Healthcare). Images were loaded into DeCyder software using image loader function. Batch Processor Module of DeCyder was used. The experiment design was set up to have all 72hrs samples in one group and 144hrs sample in other group. The spots on the gels were then matched across all gels in the experiment. The software standardises the relative spot intensity of the Cy5 image and Cy3 image in the same or different gels using the intensity of the Cy2 image. The standardised spot intensity was then averaged across the triplicate gels. The BVA module calculated the degree of difference in the standardized protein abundance between 2 spots from different groups and expressed these differences as average ratio. The values by software are displayed in the range of $-\infty$ to -1 for decrease in expression and +1 to $+\infty$ for increase in expression. For example, a two-fold increase and decrease is represented by +2 and -2, respectively (not by 2 and 0.5). The 'average ratio' has been termed as 'fold change' in this thesis. The software also calculated the consistency of the differences between samples across all the gels and applies statistics to associate a level of confidence (p-value) for each of the differences. The spots with statistically significant changes in protein expression ($\pm$1.5 fold with p-value $\leq$

0.05) were considered as differentially expressed proteins. These differentialy expressed proteins observed using DeCyder were picked with the ETTAN Spot Picker (GE Healthcare) for identification using MALDI-ToF MS (GE Healthcare).

2.6.8 Staining Methods

2.6.8.1 Silver staining 2-D Electrophoresis gels

This method is used to screen protein samples prior to labelling with the Cy Dyes. After 2-DE, the gels were removed from the plates by very carefully placing a ruler between the two plates at the top right hand corner and the top plate removed gradually. Using the ruler, the side borders of the gel were cut away in line with the ends of the IPG strip at the top of the gel. The strip and agarose were then removed and the gel was lifted gently and placed in a gel box containing fixing solution (50mL ethanol, 12.5 mL acetic acid (Lennox) and 62.5 mL UHP). The gel boxes were placed on an orbital shaker and fixed for at least 1 hour (usually overnight). After fixing, the solution was drained from the gels. The gels were then washed three times with 150 mL of UHP for 5 minutes each time and drained. The gels were next sensitised (60 mL ethanol, 13.6 g sodium acetate, 0.4 g sodium thiosulfate and UHP in 200 mL) for 30 minutes on the orbital shaker.

The gels were then washed three times (for 10 minutes). Following the washes, 200 mL of silver staining solution (0.5 g silver nitrate, 80µL formaldehyde and 200mL UHP) was added and the boxes returned to the orbital shaker. After 20 minutes the silver solution was drained and the gels were washed twice for 5 minutes each with UHP. After the last wash, 200 ml of developer (5 g sodium carbonate, 40 µL formaldehyde and 200mL UHP) was added to each of the boxes. The gels were placed on the orbital shaker and allowed to develop. When the desired amount of spots appeared the developer was drained into the

silver containing 5 L drum (this precipitated out the silver) and 200 mL of stopping solution (2.92g EDTA and 200 mL UHP) was added. The gels were left on the belly dancer for at least 10 minutes. The gels were then scanned at 300dpi resolution.

2.6.8.2 Brilliant blue G Colloidal Coomassie staining

After electrophoresis, the smaller lower plates that is attached to preparative-gels were placed in the boxes containing fixing solution (7% glacial acetic acid in 40% (v/v) methanol) for at least one hour. During this step a 1X working solution of Brilliant Blue G colloidal coomassie (Sigma, B2025) was prepared by adding 800 mL UHP to the stock bottle. When the fixing step had nearly elapsed a solution containing 4 parts of 1X working colloidal coomassie solution and 1 part methanol was made, mixed by vortexing for 30 seconds and then placed on top of the gels. The gels were left to stain for 2 hours. To destain, a solution containing 10% acetic acid in 25% methanol was poured over the shaking gels for 60 seconds. The gels were then rinsed with 25% methanol for 30 seconds and then destained with 25% methanol for 24 hours.

2.6.9 Spot picking

Preparative gels (400 µg of protein/gel) for spot picking were focussed and run out on SDS-PAGE gels. The gels were then stained with colloidal coomassie (section 2.6.8.2). The glass surface was dried and two reference markers (GE Healthcare, 18-1143-34) attached to the underside of the glass plate before scanning. The resulting image was imported into the ImageMaster software (GE Healthcare) and the spots were detected, normalised and the reference markers selected. While keeping the shift key depressed, all spots of interest were manually selected. The resulting image was saved and exported into the Ettan Spot Picker

software (GE Healthcare). Spots that showed differential protein expression in DeCyder analysis were picked with the ETTAN Spot Picker (GE Healthcare, 18-1145-28).

The stained gel was placed in the tray of the Ettan Spot Picker with reference markers (GE Healthcare) aligned appropriately and covered with UHP. The imported pick list was opened, the syringe primed and the system was set up for picking the spots from the pick list. The spots were robotically picked and placed in 96-well plates, which were stored at 4^{o}C until spot digestion.

2.6.10 Spot digestion and identification with MALDI-ToF

The 96-well plate was placed in the Ettan Digester (GE Healthcare, 18-1142-68) to digest the protein as follows: Step 1 – the gel plugs were washed three times for 20 minutes each with 50µL 50mM ammonium bicarbonate (Sigma, A6141) in 50% methanol. Step 2 – the gel plugs were washed twice for 15 minutes with 50µL 70% acetonitrile (Sigma, 34967). The gel plugs were left to dry for at least 60 minutes. After drying, the individual gel pieces were rehydrated in 10µL digestion buffer (12.5ng sequence-grade trypsin (Promega, V5111) per µL of 10% acetonitrile, 40mM ammonium bicarbonate). Exhaustive digestion was carried out overnight at 37^{o}C. After digestion, the samples were transferred as follows: Step 1 – A volume of 40µL of 0.1% trifluoroacetic acid (Sigma, 302031) in 50% acetonitrile was added to the wells, mixed and left for 20 minutes. A volume of 60µL of this solution was transferred to a fresh 96-well plate. Step 2 - A volume of 30µL of 0.1% trifluoroacetic acid in 50% acetonitrile was added to the wells, mixed and left for 20 minutes. A volume of 50µL of this solution was transferred to a fresh 96-well plate. The liquid in the plate was dried using a Maxi-Dry-Plus vaccum-dryer (Medical Supply Co.). After drying, the 96-well plate was placed in the Ettan Spotter (GE Healthcare, 18-1142-

67) for spotting onto the target plates. A volume of 3µL of 0.5% trifluoroacetic acid in 50% acetonitrile was added to the desiccated peptides and mixed 5 times. A volume of 0.3µL of this mixture was spotted onto the target plate after which a volume of 0.3µL matrix solution (7.5mg/mL a-cyano-4-hydroxycinnamic acid (LaserBio labs, 28166-41-8) in 0.1% trifluoroacetic acid in 50% acetonitrile) was mixed and allowed to dry.

The target plate was placed in the MALDI-ToF MS (GE Healthcare, 11-0010-87) instrument. The system was set up as follows: the target plate was disengaged from the machine. A new empty run list was opened. In the acquisition mode of the "favorites icon, the "spectrum processes", specifically PepMix 4 (LaserBio labs, C104) was picked and "protein digest optimised" were successively selected and dragged to positions 1 and 2-24 respectively on the target slide. Within the identification section of favorites, "protein digest mammalian IAA" was selected and dragged to positions 2-24 on the target slide. The run list for slide 1 was saved and associated to position 1 on the previously disengaged tray. Selecting process and play then started the MALDI instrument.

Mass spectra were recorded operating in the positive reflector mode at the following parameters: accelerating voltage 20 kV; and pulsed extraction: on (focus mass 2500). Internal and external calibration was performed using trypsin autolysis peaks at 842.509 m/z, 2211.104 m/z and PepMix 4 respectively. Calibration using Pep4 was performed as follows: Once two spectra were generated for the PepMix 4 mix (position 1 on the slide), the acquisition of spectra was stopped. The first spectrum of sample one was selected and the calibrant peaks readjusted for accuracy. The five individual peaks cover the 500-3500 Da mass range and include bradykinin fragment 1-5 (573.315), angiotensin II human (1046.5424), neurotensin (1672.9176) and insulin B chain oxidised (3494.6514). Once

calibration was completed it was saved as the new "system calibration". The MALDI was then restarted. The mass spectra generated for each of the proteins were analyzed against all mammalian species using MALDI evaluation software (GE Healthcare). Protein identification was achieved with the PMF Pro-Found search engine for peptide mass fingerprints.

2.6.11 Western blot Analysis

2.6.11.1 Gel electrophoresis

Proteins for analysis by Western blotting were resolved using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Using the Atto dual mini slab kit (AE 6450) the stacking and resolving gels were prepared as illustrated in Table 2.6.11. The gels were set in clean 10cm x 8cm x 0.75cm gel cassettes, which consisted of 2 glass plates separated by a rubber gasket to a width of 1mm. The resolving gel was added to the gel cassette and allowed to set. Once the resolving gel had set, the stacking gel was poured on top. A comb was placed into the stacking gel after pouring, in order to create wells for sample loading (maximum sample loading volume of 15-25µL).

The acrylamide stock in Table 2.6.11 consists of a 30% (29:1) ratio of acrylamide:bis-acrylamide (Sigma). In advance of samples being loaded in to the relevant sample wells, 20µg of protein was diluted in 2X loading buffer (Sigma, S3401). Molecular weight markers (New England Biolabs, P7708S) were loaded along side the samples. The gels were run at 250V and 45mA until the bromophenol blue dye front was found to have reached the end of the gel, at which time sufficient resolution of the molecular weight markers was achieved.

Table 2.6.11. Preparation protocol for SDS-PAGE gels (2 x 0.75mm gels).

Components	**10% Resolving Gel**	**5% Stacking Gel**
30% Acrylamide stock (Sigma, A3574)	4.6mL	670µL
UHP water	5.6mL	2.7mL
1.875 M Tris-HCl pH 8.8	3.5mL	-
1.25 M Tris-HCl pH 6.8	-	500µL
10% SDS (Sigma, L-4509)	140µL	40µL
10% Amonium persulfate* (Sigma, A-1433)	140µL	40µL
TEMED* (Sigma, T-8133)	**5.6µL**	4µL

* Added immediately before pouring

2.6.11.2 Western blotting

Once electrophoresis had been completed, the SDS-PAGE gel was equilibrated in transfer buffer (25mM Tris (Sigma, T8404), 192mM glycine (Sigma, G-7126), pH 8.3-8.5) for approximately 30 minutes. Five sheets of Whatman 3mm filter paper (Whatman, 1001824) were soaked in freshly prepared transfer buffer. These were then placed on the cathode plate of a semi-dry blotting apparatus (Bio-rad). Air pockets were then removed from between the filter paper. Nitrocellulose membrane (GE Healthcare, RPN3032D), which had been equilibrated in the same transfer buffer, was placed over the filter paper on the

cathode plate. Air pockets were once again removed. The gels were then aligned onto the membrane. Five additional sheets of transfer buffer-soaked filter paper were placed on top of the gel, all air pockets removed and excess transfer buffer removed from the cathode plate. The proteins were transferred from the gel to the membrane at a current of 34mA at 15V for 30-40 minutes, until all colour markers had transferred. Following protein transfer, membranes were stained using Ponceau (Sigma, P7170) to ensure efficient protein transfer. The membranes were then blocked overnight using 5% Marvel (Cadburys; Marvel skimmed milk) in PBS at 4°C. The membranes were washed with PBS prior to the addition of the primary antibody. Membranes were incubated with primary antibody overnight at 4°C. Antibodies were prepared in 1% Marvel in TBS. The primary antibody dilutions are illustrated in Table 2.6.11.2. The membranes were then rinsed 3 times with TBS containing 0.5% Tween 20 (Sigma P1379) for a total of 15-30 minutes. Relevant secondary antibody (1/1000 dilution of anti-mouse (Dako Cytomation, P0260) or anti-rabbit (Dako Cytomation, P0448) or anti-goat (Santa Cruz Biotechnology, Sc2098) IgG peroxidase conjugate in 2-5% Marvel-TBS) was added for 1 hour at room temperature. The membranes were again washed three times thoroughly in TBS containing 0.5% Tween for 15 minutes.

Table 2.6.11.2 List of primary antibodies and dilutions

No.	Primary Antibody (supplier)	Source	Dilution	Marvel (%)
1	anti-HNRPC (Sigma, R5028)	Mouse	1/5000	5
2	anti-Vimentin (Sigma, MMS-459S)	Goat	1/1000	5
3	anti-RBM3 (kindly supplied by Peter W. Vanderklish, Scripps Research Institute)	Rabbit	1/1000	2
4	anti-EIF4A (Abcam, ab31217)	Rabbit	1/1000	5
5	anti-PFN2	Mouse	1/500	2
6	anti-Tubulin (Abcam, ab55611)	Mouse	1/1000	2
7	Anti-GAPDH (Sigma, G8795)	Mouse	1/5000	5

2.6.11.3 Enhanced chemiluminescence detection

Immunoblots were developed using an Enhanced Chemiluminescence kit (GE Healthcare, RPN2106), which facilitated the detection of bound peroxidase-conjugated secondary antibody. Following the final washing, membranes were subjected to ECL. A layer of parafilm was flattened over a glass plate and the membrane placed gently upon the plate. A volume of 3 mL of a freshly prepared 1:1 (v/v) mixture of ECL reagent A and B was used

to cover the membrane. The ECL reagent mixture was completely removed after a period of one minute and the membrane were covered with transparent film. All excess air bubbles were removed. The membrane was then exposed to autoradiographic film (Roche, 11666916001) for various times (from 10 seconds to 30 minutes depending on the intensity of the signal). The exposed autoradiographic film was developed for 3 minutes in developer solution (Kodak, LX24, diluted 1:5 in water). The film was then washed in water for 15 seconds and transferred to a fixative solution (Kodak, FX-40, diluted 1:5 in water) for 5 minutes. The film was washed with water for 5-10 minutes and left to dry at room temperature.

2.6.12 siRNA design for selected protein targets

2.6.12.1 Primer design

mRNA sequences for protein targets from orthologous species (human, rat, mouse, chicken, chimpanzee, cow, horse, rhesus monkey, pig and rabbit) were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/) (Table 2.6.12.1). The sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The conserved regions were searched and reverse and forward primers were designed to amplify fragments ranging from ~300 to 1500 base pairs.

Table 2.6.12.1. Sequences for protein targets from orthologous species used for identifying conserved regions for designing PCR Primers.

No.	Protein Target	Orthologous Sequences (Accession Number)
1	EIF4A	NM144958, NM204549, NM199372, NM001416, NM001098575, XM001109801, NM001034228, XM001504774,
2	HNRPC	NM001077442, XM001151919, XM001096688, NM001082038, NM001034395, XM001505154, NM016884, NM001025633
3	Importin-α	NM010655, NM053483, NM002266, XM001170686, NM001034449, XM001500180, NM001006209, XM001093937
4	LGALS1	NM008495, NM019904, NM002305, XM001162066, XM001083427, NM175782, AY604429, NM205495
5	PFN2	NM002628, XM001252022, XM516998, NM001079760, NM019410, NM030873, XM001491165, XM001102817

2.6.12.2 Reverse Transcription

Reverse transcriptase (RT) reactions were set up on benches using micropipettes, which were specifically allocated to this work.

To form the cDNA, the following reagents were mixed to make RT-master-mix in a 0.5 mL eppendorf.

10µL of 5X buffer (100mM-Tris/HCl, pH 9.0, 50mM-KCl, 1% Triton X-100) (Sigma, P-2317)

4 µL 25X dNTPs (10mM of each dNTP) (Sigma, DNTP-100)

5 µL 10X reverse transcriptase (Sigma, M-1302)

5 µL 10X RNAse inhibitors (40U/µL) (Sigma, R-2520)

26 µL DEPC water

A volume of 9µL of RT-master-mix was mixed with 1µL of reverse primer in pre-labeled 0.5 mL eppendorf and then 10µL of RNA sample was added. This was repeated for all protein targets using their respective primer. The solutions were mixed and the RT reaction was carried out by incubating the eppendorfs at 37°C for 1 hour. The RT enzyme was inactivated by heating to 95°C for 3 minutes. The cDNA was stored at -20°C until required for use in PCR reactions.

2.6.12.3 Polymerase Chain Reaction (PCR) amplification of cDNA

The cDNA formed in the above reaction was used for subsequent analysis by PCR. Polymerase chain reaction (PCR) was performed using Platinum Supermix *HiFi* (Invitrogen). As for the RT reactions, 0.5 mL eppendorf tubes were used.

45µL Platinum Supermix HiFi (Invitrogen, 12532-016)

2µL Forward primer

2µL Reverse primer

1µL cDNA

The samples were mixed by pipetting two or three times and were amplified using:

	95°C for 3 min	-	denaturation
35 cycles:	94°C for 30 sec.	-	denaturation

55°C for 30 sec. - annealing

72°C for 1 min. - extension

And finally,

72°C for 7 min. extension

Following amplification, the PCR products were stored at 4°C for analysis by gel electrophoresis after purification as described in Section 2.5.5.

2.6.12.4 DNA Quantitation

Purified DNA samples were quantitated using Nanodrop spectrophotometer. Before applying the DNA sample the pedestal was wiped down using a lint-free tissue dampened with UHP. A volume of 1µL of UHP was then loaded onto the lower measurement pedestal. The upper sample arm was then brought down so as to be in contact with the solution. "Nucleic acid" and "DNA" was selected on the Nanodrop software to read the samples at 260nm. After the equipment was initialised the "blank" option was chosen, and after a straight line appeared on the screen the "measure" option was selected. The concentration of DNA was calculated by software using the following formula:

$$OD_{260nm} \text{ x Dilution factor x } 40 = \mu g/mL \text{ RNA}$$

A_{260}/A_{280} ratio for each sample was also recorded. An A_{260}/A_{280} ratio of 1.8-2 is indicative of pure DNA, although DNA with ratios from 1.7 – 2.1 were routinely observed and used in subsequent experiments.

All sample readings were automatically saved as tab delimited files which could be viewed using Microsoft excel. The upper and lower pedestals were cleaned with a clean dry wipe between samples. When finished, the pedestal was cleaned with a wipe dampened with UHP followed by drying with a dry wipe.

2.6.12.5 Sequencing of amplified cDNA fragments

Purified DNA samples were quantitated using Nanodrop spectrophotometer. These samples and primers used for RT-PCR were then sent to 'Eurofins MWG Operon' for sequencing.

2.6.12.6 siRNA design for protein targets

The sequence information was sent to Applied Biosystems in order to design 2 siRNA molecules for each target (table 2.6.12) using Silencer Select algorithm.

Table 2.6.12 Sequences of siRNAs designed

Target	siRNA	siRNA Sequence
EIF4A	siRNA 1	sense GCAGUAAUCUUUAUCAAUAtt antisense UAUUGAUAAAGAUUACUGCtt
	siRNA 2	sense GGAUUCUUGUCAAGAAAGAtt antisense UCUUUCUUGACAAGAAUCCga
HNRPC	siRNA 1	sense AGAUAAAACAAAAAGUGGAtt antisense UCCACUUUUUGUUUUAUCUga
	siRNA 2	sense AAAGUGGAUUUAAUUCAAAtt antisense UUUGAAUUAAAUCCACUUUtg
PFN2	siRNA 1	sense CACUUUGUCUUAGCAAUUAtt antisense UAAUUGCUAAGACAAAGUGat
	siRNA 2	sense GGAUACUGAUUACUAAAAUtt antisense AUUUUAGUAAUCAGUAUCCca

2.7 miRNA-arrays

miRNA-arrays are high quality microarrays that contains probes from human, mouse and/or rat for miRNA detection in the samples. Total RNA was isolated from CHO cells and after quality assurance was applied to miRNA-arrays to identify differential expression in response to low temperature culture.

2.7.1 Cell Culture

Suspension-adapted CHO-K1 cells were seeded at $1x10^5$ cells/mL in 100mL in 10% FCS supplemented ATCC medium in 250mL spinner with a working volume of 100mL. Cells were maintained in biphasic culture, where cells grown at 37^0C for 72hrs and then shifted to 31^0C for a further 72hrs or 24hrs, or in standard culture at 37^0C for 144hrs or 96hrs. Cultures were rotated at 60rpm and gassed everyday with Mixture of Gasses (N_2, O_2 and CO_2). Cell counts and viability were measured every day as mentioned in section 2.4.3. The cell samples were collected for biphasic cultures at 72hrs, 96hrs and 144hrs of culture.

2.7.2 Total RNA isolation and quantification

Total RNA was isolated using mirVana™ miRNA isolation Kit (Ambion, AM1561). A volume of $0.5\text{-}1x10^6$ cells were sampled and washed twice using ice-cold PBS. The cell lysis was performed in ice. The cells were gently resuspended in 300-600µL of Lysis/Binding Buffer for lysis. A $1/10^{th}$ volume of miRNA Homogenate Additive was added to the cell lysate and incubated for 10min on ice. To each sample, 300-600 µL of Acid-Phenol:Chloroform was added and shaken vigorously for 30 seconds. The sample was centrifuged at 13000rpm for 15 mins at 4^oC in a microfuge. This step separated the mixture into 3 phases with the RNA contained in the colourless upper aqueous layer. The aqueous layer was transferred to a new eppendorf and 1.25 volume of 100% ethanol was added to

each sample and mixed. The sample was filtered by centrifuging at 13000rpm for 10 min at 4°C. This allowed RNA to bind on the membrane of filter. The bound RNA was washed once with miRNA Wash Solution 1 and twice with Wash Solution 2/3. RNA was then eluted using 100µL of Elution Solution in fresh eppendorf.

RNA was quantified using Nanodrop spectrophotometer at 260nm. The method for using Nanodrop spectrophotometer has been described in section 2.6.12.4. RNA samples were diluted to 500 ng/µL and stored at –80°C.

2.7.3 Determination of RNA purity using Bioanalyser

The Agilent 2100 Bioanalyser is a microfluidics-based platform for the analysis of proteins, DNA and RNA. The miniature chips are made from glass and contain a network of interconnected channels and reservoirs. The RNA 6000 Nano LabChip kit enables analysis of samples containing as little as 5ng of total RNA. The channels are firstly filled with a gel matrix and the sample wells with buffer or sample, there are 12 sample wells per chip (Figure 2.7.3). To each well, 1µL of each sample is loaded along with a fluorescent dye (marker). An RNA ladder is loaded into another sample well for size comparison. When all the samples are loaded, the chip is briefly vortexed and loaded onto the bioanalyser machine. The machine is fully automated and electrophoretically separates the samples by injecting the individual samples contained in the sample wells into a separation chamber.

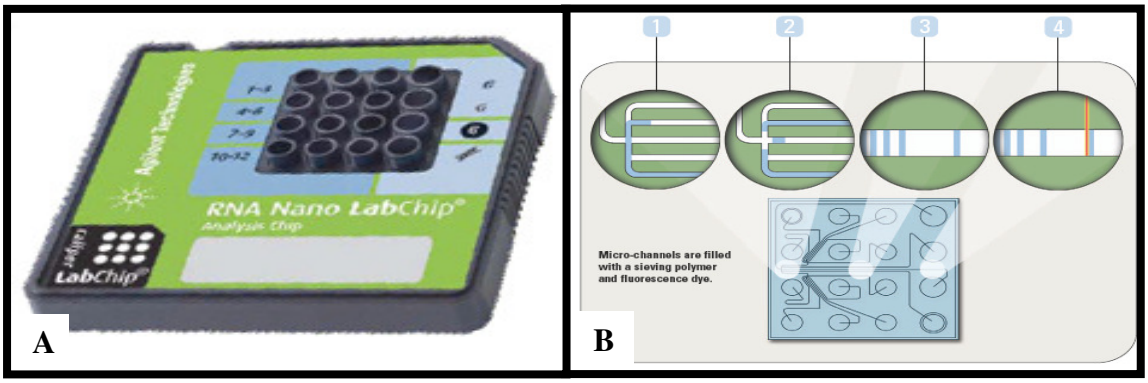

Figure 2.7.3 The RNA 6000 Nano chip, a picture of the front of the RNA Nano chip (A) and a diagram of the microchannels in the chip (B), the sample moves through microchannels (1) and is injected into separation chamber (2) were components are electrophoretically separated (3) and detected by their fluorescence and translated into gel-like images and electropherograms. Both pictures available from http://www.agilent.com/chem/labonachip..

The fluorescensce was measured and presented in the form of peaks for 18S and 28S ribosomal RNA peaks. Higher 28S peaks than the 18S peak is an indication of good quality of RNA. As RNA degrades, the 28S RNA peak decreases and smaller fragments appear.

2.7.4 miRNA bioarray

2.7.4.1 Sample Processing

Samples for miRNA profiling studies were processed by Asuragen according to the company's standard operating procedures. The microRNA enriched fraction was obtained by passing 10µg of total RNA through a flashPAGE™ Fractionator apparatus (Ambion) and cleaned and concentrated using the flashPAGE™ Reaction Clean-Up Kit (Ambion). The 3' ends of the RNA molecules were tailed and labelled using the mirVana™ miRNA

Labeling Kit (Ambion, AM1562) according to the manufacturer's instructions. Amine-modified nucleotides were incorporated during the poly (A) polymerase mediated tailing reaction, and Cy5 succinimide esters (GE Healthcare) were conjugated to the amine moieties on the microRNAs. Hybridization to the mirVana™ miRNA Bioarrays (Ambion, P/N 4392878) was performed using the mirVana™ miRNA Bioarray Essentials Kit (Ambion, AM1567). The Cy5 fluorescence on the arrays was scanned at an excitation wavelength of 635nm using a GenePix 4200AL scanner (Molecular Devices). The fluorescent signal associated with the probes and local background was extracted using GenePix Pro (version 6.0, Molecular Devices).

2.7.4.2 Data analysis

Thresholding and signal scaling were generated using algorithms selected by Asuragen, as implemented as part of the miRNA Standard Service Premium Analysis (miSSP package). The background adjusted fluorescent values generated by GenePix Pro were normalized for each miRNA using a variation stabilization transformation method (Huber *et al.* 2002). Each miRNA-bioarray contains 22 control probes, of which 12 serve as negative controls. These probes aid the normalization of miRNA-bioarray data.

Hierarchical clustering was performed to investigate the relationship between replicate samples and to investigate if all CHO samples are unique in their miRNA profiles in comparison to other human samples tested. The list of miRNA expression data were loaded in to the software and it represented the relationship among samples in the form of dendrogram. This method was useful in its ability to represent varying degrees of similarity and distant relationships among groups of closely related miRNAs.

Pair-wise comparisons were also carried out on differentially expressed genes identified by ANOVA to see how they differ from each other. For each pair of treatments, a two-sample t-test was carried out for every gene and multiplicity correction was followed to control the false discovery rate (FDR) using a step-up approach using an FDR of 5%. This method is referred to as "protected Least Significant Difference (LSD)". The detailed miRNA lists and associated information such as fold-change and *p*-values are reported.

2.7.5 PCR based miRNA Taqman Low Density Arrays

TaqMan® Low Density Arrays (Applied Biosystems, 4334812) are 384-well micro fluidic cards designed for analyzing gene expression patterns of up to 8 samples in parallel across a defined set of gene targets (Figure 2.7.5). TaqMan® Low Density Array is real-time PCR-based highly sensitive and reproducible array that contains probes for human miRNA signatures.

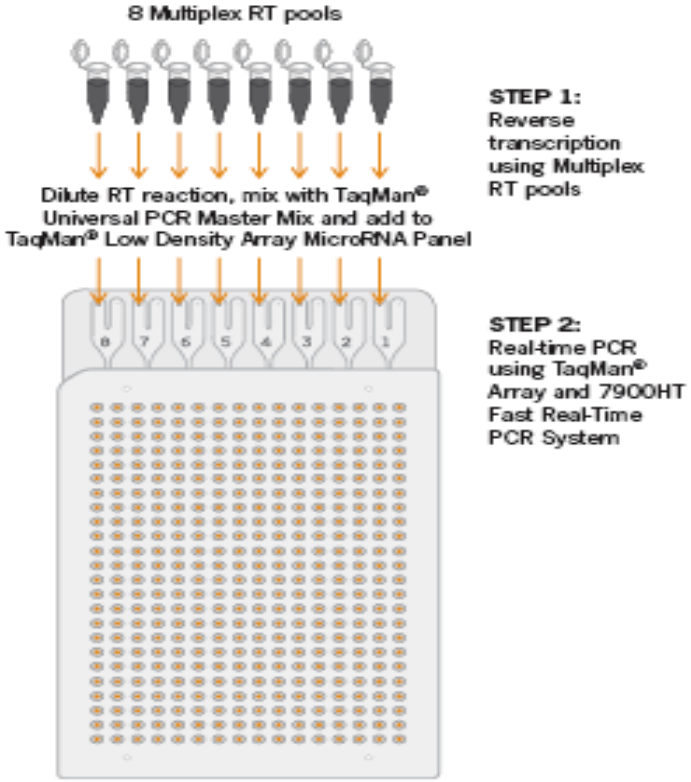

Figure 2.7.5. Schematic representation of using miRNA TaqMan® Low Density Array.

2.7.5.1 Reverse Transcription

The RT-master mix was prepared as described below

Component	**Volume for One RT Reaction (µL)**	**Volume for One Sample (8 RT Reactions) with 12.5% Excess(µL)**
100 mM dNTPs	0.20	2.0
MultiScribe™ Reverse Transcriptase, 50 U/µL	2.00	20
10x Reverse Transcription (RT) Buffer	1.00	10
RNAse Inhibitor, 20 U/µL	0.125	1.25
Nuclease-free water	3.675	36.75
Total	**7**	**70**

A volume of 7 µL of RT-master mix was added to each well (as required) of 396-well of MicroAmp™ optical reaction plate. To each well, 2µL of RNA sample (100ng) and 1µL of Multiplex RT Human Primer Pool (10X) were added. Samples were mixed gently and were incubated on ice for 10 minutes. Multiplex reverse transcription was performed using the thermal cycler to the 9600 Emulation mode and final volume of reaction to 10µL in HT7900 RT-PCR machine, then programmed as follows:

Step Type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

2.7.5.2 PCR

RT reaction was diluted 62.5-fold by adding 615 µL of nuclease-free water to 10 µL RT reaction and thereafter RT reaction-specific PCR mix was prepared for each RT reaction as follows

Component	Volume (µL) per Fill Reservoir
Diluted RT Reaction	50.0
TaqMan 2X Universal PCR Master Mix (No AmpErase® UNG)	50.0
Total Volume	100.0

A volume of 100µl of RT reaction-specific PCR mix was loaded in to the filling ports (Figure 2.7.5) of the TLDA as shown bellow

TaqMan Array	Fill Port 1	Fill Port 2	Fill Port 3	Fill Port 4	Fill Port 5	Fill Port 6	Fill Port 7	Fill Port 8
Multiplex RT pool	1	2	3	4	5	6	7	8

Arrays were incubated for 10min on ice and were loaded on HT7900 real time PCR machine. The following protocol was used.

Step	**AmpliTaq Gold® Enzyme Activation**	**PCR**	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 sec	60 sec
Temp	(°C)	95	95

2.7.5.3 Data Analysis

Data was analysed using Real-Time *StatMiner*™ Software from Integromics. miR-let-7c (human) was used as an endogenous control in this investigation. Cycle time (Ct) ≤30 and p-value ≤0.05 were used as cut-off criteria for identifying differential expression of miRNAs.

2.7.6 qRT-PCR validation

2.7.6.1 qRT-PCR validation using *mir*Vana Assays

For the detection and quantification of specific miRNAs, the *mir*Vana™ qRT-PCR miRNA detection kits (Ambion, AM1558) and primer sets were used according to manufacturer's instructions. In all cases SuperTaq™ (Ambion, AM2052) was used for polymerisation reactions. Per reaction, 2.5ng of RNA was used in qRT-PCR. PCR-primer for 5S endogenous control were diluted by 1/10 factor. Both RT and PCR reactions were carried out using an ABI 7500 Real-Time PCR System (Applied Biosystems) as described in

Section 2.6.4.3 & 2.8.2.1. Detection and normalisation was facilitated using SYBR green and ROX normalisation dyes (Invitrogen). Biological replicate results were checked for statistical significance using a student's t-test with a *p*-value cut-off of ≤0.05.

2.7.6.2 singleplex qRT-PCR validation using Taqman miRNA assays

2.7.6.2.1 Reverse Transcription (RT)

RT-master mix for each reaction was prepared separately using a kit from Applied Biosystems (ABI, 4366596) as follows;

Component	**Master Mix** **15-µL (1 Reaction)**
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10X Reverse Transcription Buffer	1.50
RNase Inhibitor, 20U/µL	0.19
RT primer (RNUB6: ABI-4373381, miR-7: ABI-4373014, miR-200c: ABI-4373096)	3
Nuclease-free water	6.16
Total	12.00

An aliquot of 12 µL of RT-master mix was transferred in to a 0.5mL eppendorf and 3 µL of RNA sample (10ng) was added to each tube. RT was performed using following parameters in hybrid thermocycler

Step Type	**Time(min)**	**Temperature (°C)**
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

2.7.6.2.2 PCR

PCR-master mix was prepared for each reaction as follows

Component Volume (µL)	**20-µL (1 Reaction)**
TaqMan MicroRNA Assay (20X)	1.00
Product from RT reaction (Minimum 1:15 Dilution)	4
TaqMan 2X Universal PCR Master Mix, No AmpErase UNGa (ABI, 4324018)	10.00
Nuclease-free water	5
Total Volume	**16**

To each well of a MicroAmp™ optical reaction plate, 16 µL of PCR-master mix was loaded. From RT reaction, 4 µL of Product was added to PCR-master mix. The plate was

sealed and then incubated for 10min on ice. PCR was performed using 9600 emulsion mode of thermocycler with reaction volume of 20 µL in HT7600 real time PCR machine.

Step	**AmpliTaq Gold® Enzyme Activation**	**PCR**	
	HOLD	CYCLE (40 cycles)	
		Denature	Anneal/Extend
Time	10 min	15 sec	60 sec
Temp	(°C)	95	95

2.7.7 Isolation and cloning of the *Cricetulus griseus* miRNAs

Primers were designed for cloning *C. griseus* miR-21 based on alignment of the corresponding genomic regions flanking the pre-miR-21 sequence from *Mus musculus*, *Rattus norvigicus* and *Homo sapiens*. The primers used were 5′atgtttgctttgctttaaaccctgcctgagca3′ and 5′ctgcaaaccatgatgctgggtaatgtttga3′. Genomic DNA was extracted from approximately 5×10^6 CHO-K1 cells (Whole blood extraction kit, Nucleon, 44100) and eluted in 100 µL water. A volume of 1.5 µL (≈100 ng) DNA was used as a template for PCR. The reaction also contained 400 nM of each primer, 1 µL DMSO and 20.5 µL Platinum Supermix (Invitrogen). Cycling conditions were:

	95°C for 3 min	-	denaturation
30 cycles:	94°C for 30 sec.	-	denaturation
	53°C for 30 sec.	-	annealing
	72°C for 45 sec.	-	extension

And finally,

72°C for 7 min. extension

PCR product was checked on an agarose gel for a specific band of appropriate length (ca. 220 bp) and the remainder of the mix cleaned up (Qiagen PCR cleanup kit, 28704) for sequencing. Sequencing was performed on both strands using the cloning primers (MWG Biotech, Germany).

2.8 Functional Analysis of effects of selected miRNA or protein targets

RNA interference (RNAi) was carried out using small interfering RNAs (siRNAs) to silence the expression of specific genes. Chemically synthesized small RNAs were used to suppress or induce the expression of selected miRNAs. The siRNAs/miRNAs used in this investigation were purchased from Applied Biosystems. These siRNAs/miRNAs were transfected into the cells using the transfection agent siPORT^{TM} *Neo*FX^{TM} (Ambion, AM4511). All transfections were performed using filtered-cap spin tubes (Sartorius) in a 2mL working volume.

2.8.1 siRNA/miRNA transfection

The siRNA used in this investigation were generated as described in Section 2.6.12. miRNA were purchased from Applied biosystems. The details are AM-Control (AM17010), PM-Control (AM17110), AM-miR-7 (AM10047), PM-miR-7 (PM10047), AM-miR-24 (AM10737), PM-miR-24 (PM10737). Solutions of siRNAs and miRNAs at a final concentration of 30nM/mL were prepared in 100µL (per tube) of OptiMEM (Gibco^{TM}, 31985) and incubated at room temperature for 10 minutes. NeoFX solution at 2µL/mL was also prepared in 100 µL(per tube) of OptiMEM in parallel and incubated at room temperature for 10 minutes. A volume of 100µL of the siRNA/miRNA were topped on the

100µL of neoFX solution to form complexes for another 10 min. To each tube, 200µL of complex was added and was mixed with the cell suspension to achieve $1x10^5$cells/mL. The tubes were mixed gently and incubated at 175rpm agitation speed at 37°C for 96hrs or 144hrs, 31^0C for 144hrs or 31^0C for 72hrs after 24hrs of transfection at 37^0C. The efficiency of inhibition or induction of targets were confirmed using Western blot analysis or singleplex qRT-PCR after 96hrs culture.

2.8.2 Guava ViaCount® Assay

The assay provides more accurate than hemocytometer counting or impedence-based counting. The ViaCount Assay distinguishes viable and non-viable cells based on differential permeabilities of two DNA-binding dyes in the Guava ViaCount® Reagent (Guava Technologies, 4000-0041). The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. ViaCount reagent was allowed to come at room temperature. A volume of 100 µL of cell suspension was mixed with 100 µL of ViaCount reagent and incubated for 10 min at room temperature. The samples were then analyzed using Guava flow cytometer.

2.8.3 SEAP Assays

Samples were harvested after 96hrs of transfection and were analyzed for SEAP productivity as described in Section 2.5.7.

2.9 SELDI-ToF MS

SELDI-ToF MS is a highly sensitive proteomics tool being used to identify differential expression of proteins in a given sample. Protein samples from conditioned media of low-

serum adapted CHO-K1 cells representing different phases of growth cycle were analyzed using SELDI-ToF MS to identify secreted growth specific proteins/peptides in culture.

2.9.1 Cell culture

Low serum-adapted cells (0.5% FCS) were seeded at $1x10^5$cells/mL in ATCC medium in T-75 flasks using 10mL of working volume for a period of 144hrs. The conditioned medium (used medium) was collected from four biological replicate samples at every 24hrs and centrifuged at 1000rpm for 15-20 min at 4^0C. The supernatant was carefully collected and stored at -80^0C until used.

2.9.2 Preparation and analysis of chip surfaces

Conditioned media samples were concentrated using 5000 MW cut-off centricons (Millipore, UFC8005). A volume of 10mL of conditioned media was centrifuged at 4000g at 4^0C reduce sample size to ~1mL to concentrate media by a factor of ~10. The protein concentrated in this conditioned media was estimated as described in section 2.6.2. Prior to full profiling analysis, optimization studies were performed with the day 4 sample using 10 μg and 50 μg of extracted protein from conditioned media samples on four different chromatographic surfaces to see which protein concentration and Protein Chip array (Bio-Rad) gave optimal results. The following Protein Chip arrays were selected, an IMAC30 (immobilized metal affinity capture) (Bio-Rad, C730078) activated with copper ions, CM10 (weak cation-exchange) (Bio-Rad, C530075) with 20 mM Tris pH 4.5, Q10 (strong anion-exchange) (Bio-Rad, C5730080) with 50 mM Tris pH 8.0 and H50 (reversed-phase hydrophobic surface) (Bio-Rad, C5730065) with 10% acetonitrile/0.1% trifluoroacetic acid. The IMAC30 and Q10 ProteinChip 8-spot arrays provided better profiles under these conditions in terms of number and resolution of peaks and therefore were selected for the

final profiling studies. IMAC30 arrays were coated with 5 μL of 100 mM $CuSO_4$ for a total of 30 minutes (2x15 minute applications) and then rinsed with high-performance liquid chromatography (HPLC) grade water. The copper ions were charged by applying 50 μL of 100 mM sodium acetate for 5 minutes. Each array was placed in a Bioprocessor (Bio-Rad) and the IMAC-Cu^{2+} arrays were washed twice with 250 μL of 250 mM sodium chloride containing 0.1% Triton X-100, and Q10 arrays were washed twice with 50 mM Tris pH 8.0. From each sample, 10 μg of protein was diluted in binding buffers. To the spots of the arrays, 250 μL of diluted samples were applied. The arrays were placed on a shaker and gently agitated for 90 minutes to allow for interaction of proteins with the array surface. After removing the samples, the IMAC-Cu^{2+} arrays were washed twice with 250 μL of 250 mM sodium chloride containing 0.1% Triton X-100, and Q10 arrays with 50mM Tris pH 8.0 for 5 minutes, followed by a brief HPLC-grade water wash. An aliquot of 0.8 μL of saturated sinapinic acid (SPA (Bio-Rad, C3000002) dissolved in 50% acetonitrile containing 0.5% trifluoroacetic acid) was then added to each of the spots, allowed to dry and repeated. The IMAC-Cu^{2+} and Q10 ProteinChip arrays were then analyzed in a Ciphergen Series PBS-IIC ProteinChip® System, and time-of-flight (ToF) data was generated by averaging a total of 220 laser shots collected at a laser intensity of 200, a detector sensitivity of 8 and molecular mass range from 5–20,000 Da for low molecular weight range and 20,000-100,000 Da for high molecular weight range.

2.9.3 Analysis of differential expression of Proteins/peptides

Molecular weights were calibrated externally using an all-in-1 protein standard (Bio-Rad, N76006). All data was analyzed using Biomarker Wizards Software, version 3.1 (Bio-Rad). After automatic baseline noise correction, all of the spectra were normalized together by the

‘‘total ion content’’ method as described by the manufacturer, i.e. with an *m/z* between 5,000 and 100,000. The peaks with an *m/z* value < 5000 were excluded, as these peaks were mainly ion noise from the matrix (sinapinic acid), and 5kDa cut-off centricons were used to concentrate the samples in this analysis. Peak clusters were generated by automatically detecting qualified mass peaks with a signal to noise ratio (S/N) >5 in the first pass, completed with a second-pass peak selection of S/N >3, with a 0.3% mass error for 5000-20,000 Da, and the same for 20,000-100,000 Da. Statistically significant peaks were considered to be those with $p < 0.05$.

Section 3.0

Results

3.1 Establishment of a recombinant protein-secreting CHO-K1 cells

3.1.1 Cell Adaptation

The industrial production of therapeutically important recombinant protein is mostly performed in suspension culture and preferably using reduced serum or serum-free culture medium. CHO-K1 cells were therefore adapted to grow in suspension and in reduced serum and serum-free culture medium.

Cells typically have a preference to grow in a specific type of culture condition, i.e. monolayer, suspension and/or in the presence of serum or particular nutrients, etc. Cells can respond to the change(s) in their growth environment. When cells are initially exposed to new culture conditions, they react to this new stimulus. This exposure induces a reaction that modifies the cellular machinery to help the survival of the cell in the new environment. However the mechanisms of CHO cell adaptation at the molecular level are not well understood. The intensity of the cellular response to new conditions declines gradually. In this way cells adjust their sensitivity to the new environment in terms of growth and survival, and this process is called adaptation. Cell adaptation is a time-consuming process and its efficiency and success can be cell, clone- or change-specific; for example, cells respond differently to the gradual or immediate removal of serum from culture medium.

3.1.1.1 Suspension Culture

Adherent CHO-K1 cells were obtained from the American type culture collection (ATCC) and were maintained in 10% FCS supplemented ATCC medium (1 : 1 ration (v/v) of DMEM and Ham's F12) in T-75cm^2 flasks at 37^{0}C in humidified incubators containing 5% CO_2. To adapt adherent CHO-K1 cells to grow in suspension, exponentially growing cells

were inoculated at $2x10^5$ cells/mL in spinner vessels at a final volume of 100 mL of culture medium and maintained at 60rpm. The growth behavior of cells was monitored during the process of adaptation by seeding exponentially growing cells at $1x10^5$ cells/mL in a separate spinner vessel. Cell counts were performed using a hemacytometer and viabilities were measured using the trypan blue exclusion method at every 24hrs for 144hrs of this culture. Results indicate that during adaptation, the growth behaviour of the cell improved with time. The cells in passage 1 (P-1) (at early stage of adaptation) were continuously growing through out the culture period and achieved a maximum viable cell density of $1.63x10^6$ cells/mL at 144hrs of culture, whereas the cells at P-42 reached $1.99x10^6$ cells/mL within 96hrs (Figure 3.1.1A). The cultures maintained high viability (≥94%) throughout the adaptation (Figure 3.1.1B). The viability of cells in P-1 was reduced to 98% at 72 hrs of culture and improved >99% at the end of the culture. Comparably, the cells at P-42 were 98% viable at 72 hrs of culture (Figure 3.1.1B).

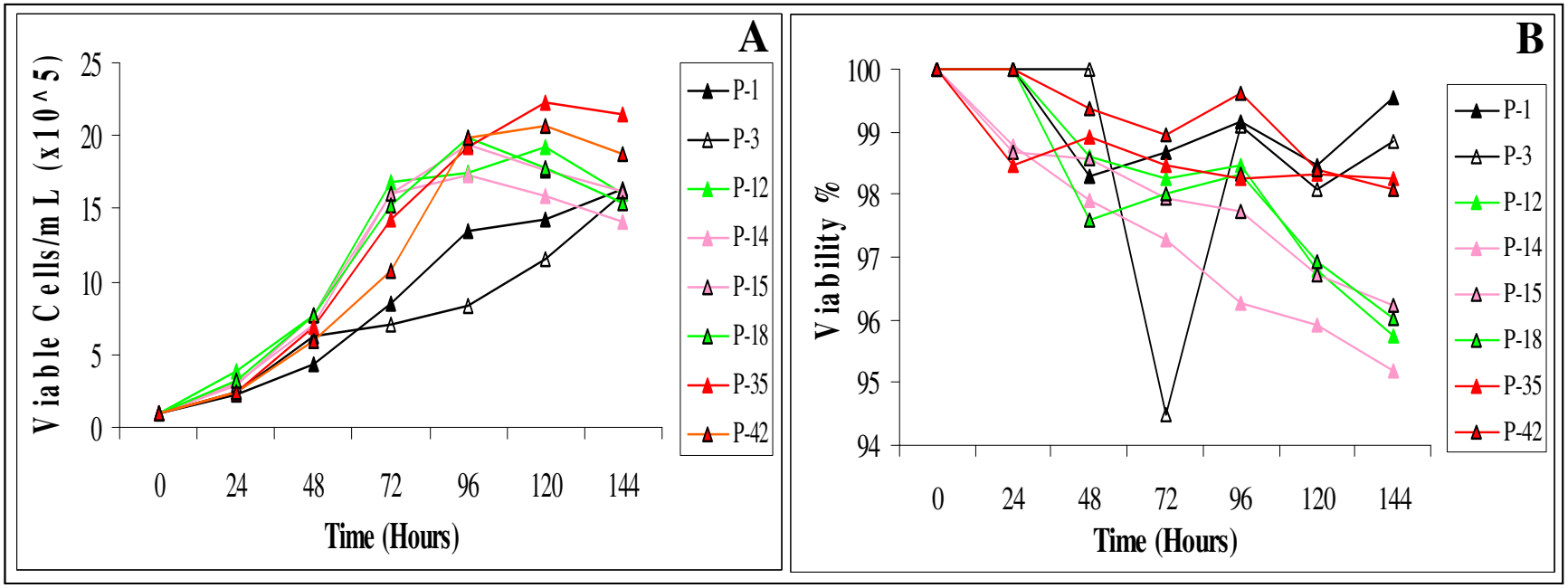

Figure 3.1.1. Comparison of the viable cell number (A) and viability percentage (B) of CHO-K1 cells during the process of cell adaptation to grow in suspension culture (n=1). P represents passage number of cells.

3.1.1.2 Serum-Free Medium (SFM)

To adapt suspension-adapted CHO-K1 cells to grow in serum-free culture conditions, serum was gradually reduced in the culture medium over time. The following work-flow was used to adapt CHO-K1 to serum-free conditions. For adherent culture, cells were seeded at $3x10^5$ cells/mL in three T-75 cm^2 culture flasks in 10mL of culture medium. Cell counts were performed every 2^{nd} and 4^{th} day of culture. For suspension culture, cells were seeded at 2 $x10^5$ cells/mL in spinner flasks and were counted. If cells achieved $\geq 1x10^6$ cells/mL within 4 days, they were transferred to medium with reduced serum. This criterion was selected as these cells had a doubling time of ~24hrs and therefore if cells are able to reach $\geq$ 1x 10^6 cells/mL within 4 days of culture, it is a sign that the cells are healthy and would be able to cope well with the adaptation process. If cells failed to reach $\geq 1x10^6$ cells/mL within 4 days, the medium was replaced in the 3^{rd} flask, and once cells became confluent, the work flow was followed until they met the set criteria of adaptation. The cell-free conditioned medium that contains a number of unknown growth factors secreted by cells was also collected at every sub-culture. A volume of 10% conditioned medium was included at each subculture to support cell growth in stressed conditions which occurred due to the gradual decrease in serum in culture medium during adaptation. The procedure for adaptation was repeated until cells were adapted.

Suspension-adapted CHO-K1 cells, growing in 10% FCS supplemented ATCC culture medium, were used for SFM adaptation. Cells were initially adapted to reduced serum levels in adherent culture and were then re-adapted to suspension culture in reduced serum or serum-free medium. Cells took only one cycle of the above described work flow (4 days) to adjust up to the 1% serum level in culture medium suggesting that even 1% serum in the

medium may provide a sufficient nourishment and protection for the growth of CHO cells. After 1% FCS level in the medium, the time taken by cells to reach the set criteria increased gradually. It took 10 days in 0.5 % FCS and 34 days in a commercially available serum-free medium (ExCell) (Figure 3.1.2). Cells took 22 days to adapt to grow in 0.5% FCS supplemented medium from 10% FCS supplemented medium. Cells adapted to grow in 0.5% FCS supplemented medium were then transferred to a very simplified and low protein containing SFM (Insulin-Transferrin-Selenium (ITS) supplemented ATCC medium) and to ExCell medium. Using this protocol, CHO-K1 cells have been adapted to grow in adherent culture in 0.5% FCS-supplemented ATCC medium and ExCell medium as well as in suspension culture in these both culture media. However, cells did not successfully adapt to grow in ITS supplemented medium in adherent culture and were therefore discarded after ~4 months of culture. Cells could be re-tried to adapt to grow in ITS supplemented medium by using higher initial cell density (>$3x10^6$ cells/mL) or by gradually removing the important nutrient factors one by one from the culture medium in order to slowly adapt the cells to grow in such a simplified medium.

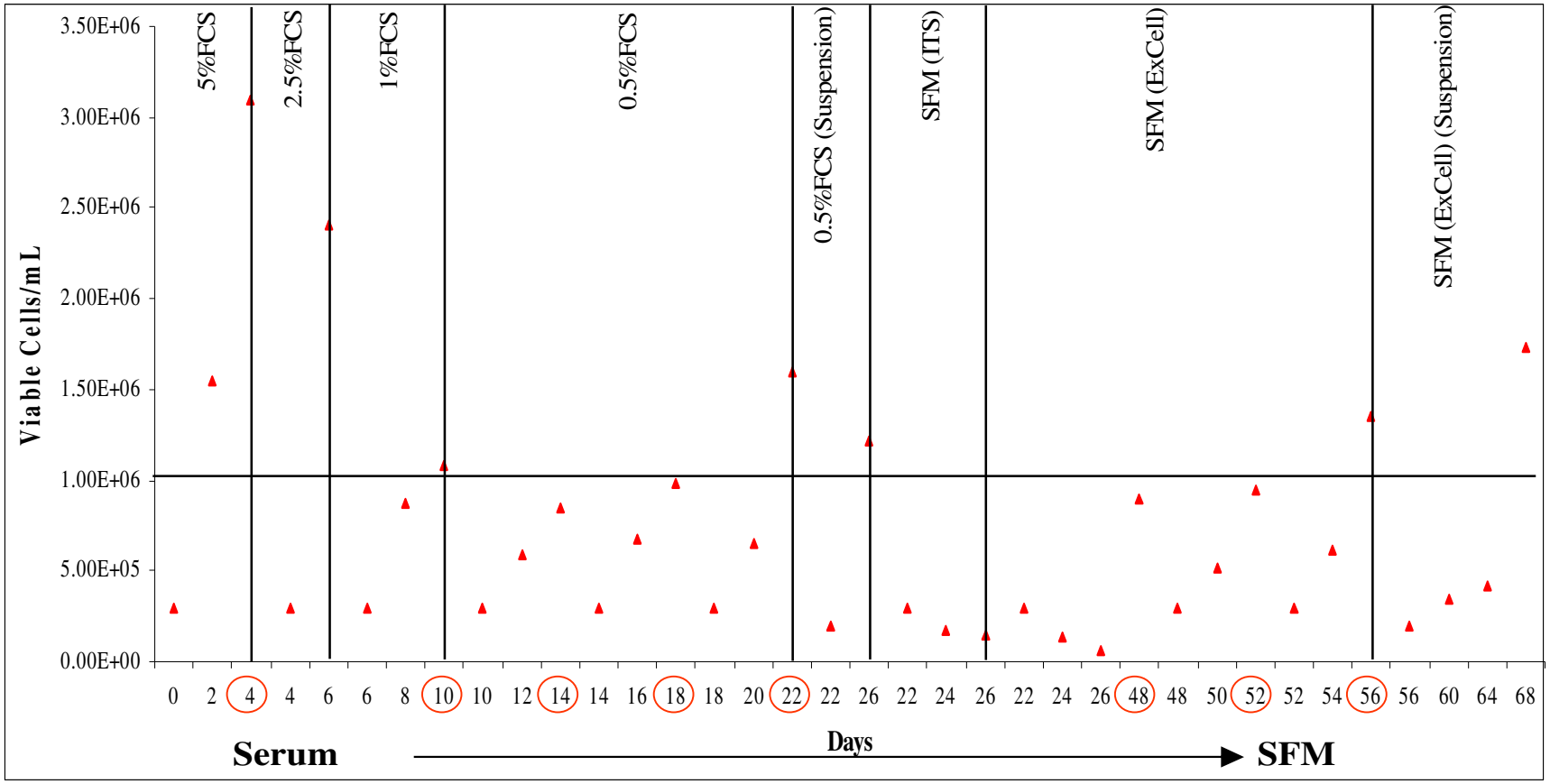

Figure 3.1.2. Adaptation of suspension-adapted CHO-K1 cells to serum-free culture medium (SFM). SFM(ITS) is Insulin-Transferrin-Selenium supplemented serum-free ATCC medium whereas SFM(ExCell) is a commercially available serum-free medium from Sigma. The cell adaptation was performed using adherent and suspension culture. Cells adapted to grow in suspension culture have been marked in the figure. FCS denotes fetal calf serum. Red circle represents subculture points.

3.1.2 Establishment of SEAP-secreting CHO-K1 cells

We were interested to investigate the impact of selected protein and miRNA targets on the growth and recombinant productivity of mammalian cells. CHO-K1 cells were therefore engineered to express a model recombinant protein product, secreted alkaline phosphatase (SEAP). SEAP is a highly glycosylated recombinant protein. The cells secreting SEAP were isolated and adapted to grow in suspension culture and were then used in further investigations.

3.1.2.1 SEAP plasmid construction

The pSEAP2-control vector was a kind gift from Professor Martin Fussenegger (Institute for Chemical and Bioengineering, ETH Zurich, Zurich, Switzerland). pSEAP2-control did not contain an eukaryotic selection marker and therefore was only suitable for transient expression studies (Figure 3.1.2.1A). pcDNA3.1 contains CMV promoter to drive the expression of gene of interest and has an eukaryotic selection marker, neomycin resistance gene (*neo*^{r}) (Figure 3.1.2.1B). The SEAP coding gene was isolated from the pSEAP2-control and was inserted into the pcDNA3.1 to generate pcDNA3.1-SEAP2.

The pSEAP2-control was transformed into competent bacterial cells and was amplified and purified. pSEAP2-control was digested with HindIII and SalI restriction enzymes to isolate the SEAP coding sequence (Figure 3.1.2.2). pcDNA3.1 was also digested with HindIII and XhoI to accept the SEAP insert. Digests of both, pSEAP2-control and pcDNA3.1, were separated on 2% (w/v) agarose gels (Figure 3.1.2.2) and the bands of interest, SEAP-HindIII/SalI (~2kb) and pcDNA3.1-HindIII/XhoI (~5.4kb), were retrieved from the gel and purified. These fragments were quantified using a NanoDrop spectrophotometer and were re-checked on a 2% (w/v) agarose gel for purity. The purified SEAP-HindIII/SalI fragment

and pcDNA3.1-HindIII/XhoI backbone were then ligated to construct pcDNA-SEAP2. Competent cells were transformed and plated out on agar under selection pressure (100µg/mL of ampicillin). Several clones were picked, plasmids were minipreped for digestion using EcoRI/BamHI and then separated on 2% (w/v) agarose gel. As expected, bands at ~0.2, 2 and 3kb were observed which indicated the successful construction of pcDNA-SEAP2. A MaxiPrep was performed to generate large quantities of pcDNA-SEAP2 for stable transfection.

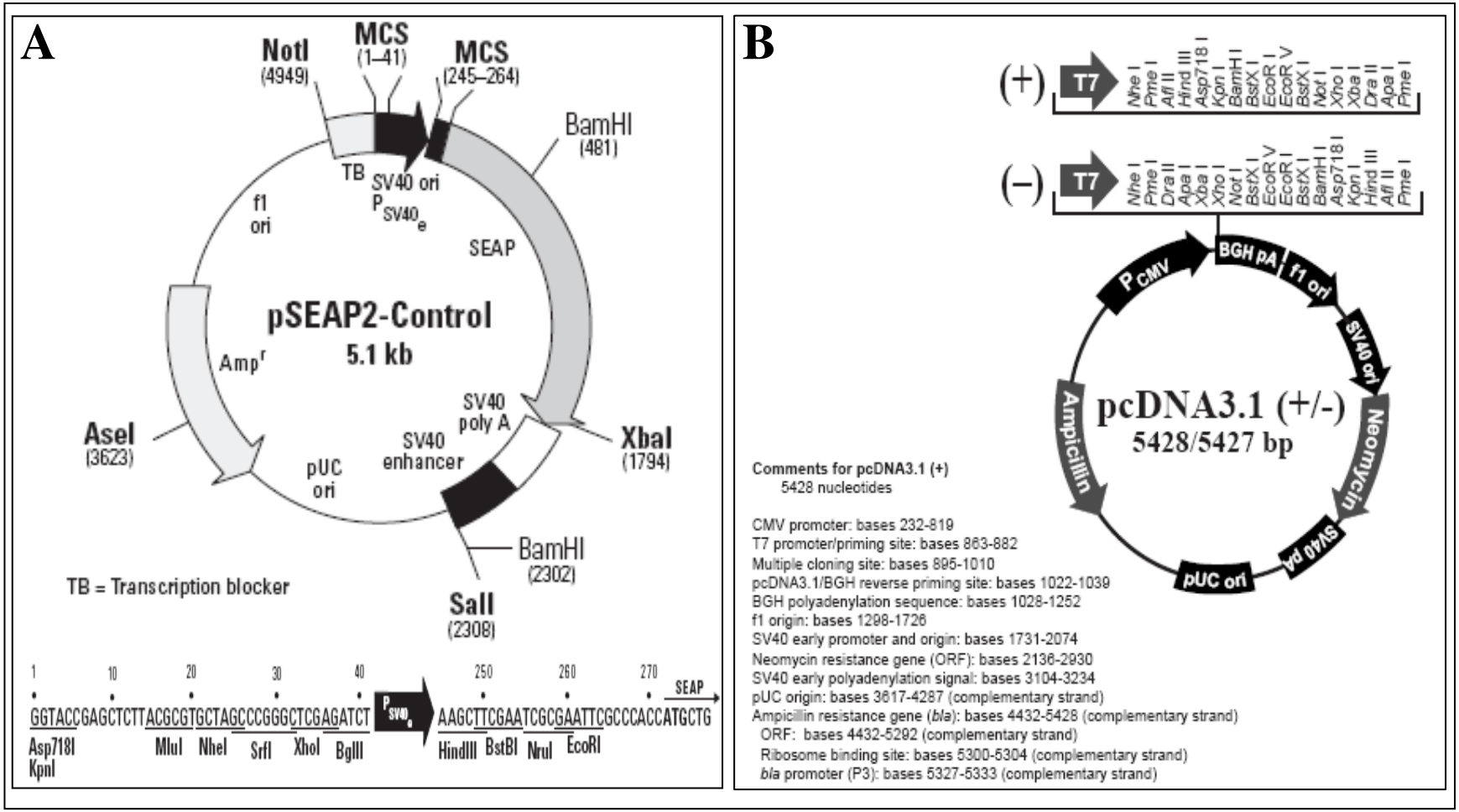

Figure 3.1.2.1. Restriction maps and multiple cloning sites of pSEAP2-Control and pcDNA3.1 (+/-) vectors. **A** represents pSEAP2-Control vector and **B** represents pcDNA3.1 (+/-) vector.

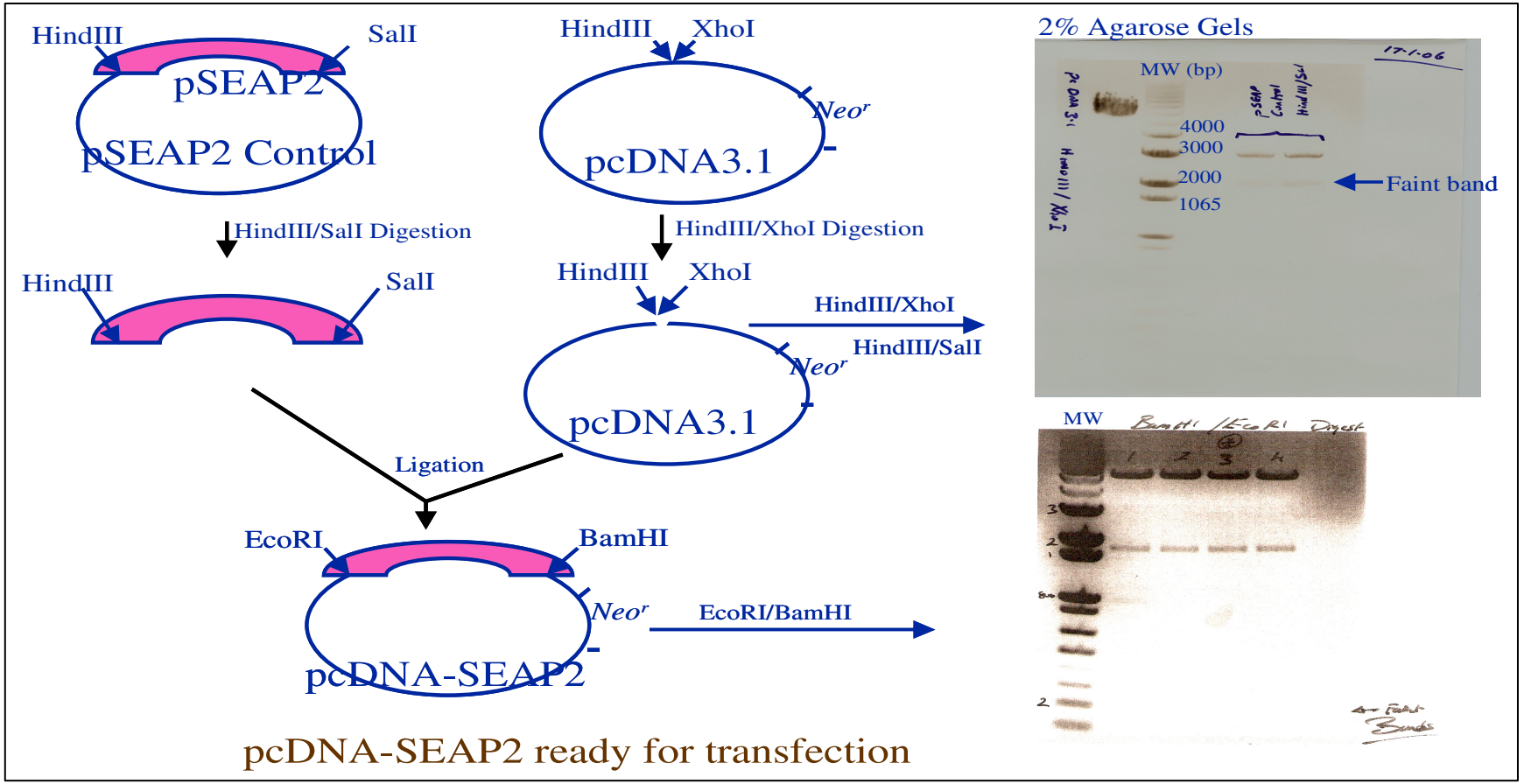

Figure 3.1.2.2. Construction of pcDNA-SEAP2 from pSEAP2-Control and pcDNA3.1. pSEAP2-Control was digested with HindIII and SalI. pcDNA3.1 was digested with HindIII and XhoI. Digests were separated using agarose gel electrophoresis. The purified fragment coding for SEAP was then ligated into the purified pcDNA3.1 backbone to construct pcDNA-SEAP2. Minipreped pcDNA-SEAP2 was also digested with EcoRI and BamHI and was separated on agarose gel again to ensure efficiency of plasmid construction.

3.1.2.2 Transfection of pcDNA3.1-SEAP2 and selection of transfectant CHO-K1 cells

CHO-K1 cells that were adapted to grow in 0.5% (v/v) FCS supplemented medium in adherent culture were transfected with pcDNA-SEAP2. The cells were seeded at $1x10^5$ cells/mL in 4x T-25 culture flasks. Once cells reached 70-80% confluency, the culture media was replaced. One flask was transfected with pcDNA3.1 and one flask with pcDNA-SEAP2 using Lipofectamine 2000 transfection reagent. Two flasks were maintained as non-transfected controls in this investigation. The media was changed after 24 hrs of culture in all flasks and cells were allowed to grow for another 24 hrs. Transfectants were selected using G418 in the culture medium. Clonal populations were clearly visible in transfected culture in the presence of G418 (800µg/mL) in the medium, whereas the cells in non-transfected culture died (Figure 3.1.2.3). This is because pcDNA3.1 and pcDNA-SEAP2 contain *neo*^{r} which enabled transfected cells to survive in the presence of G418. These clonal populations were allowed to grow and were used to isolate single cell clones secreting SEAP in culture.

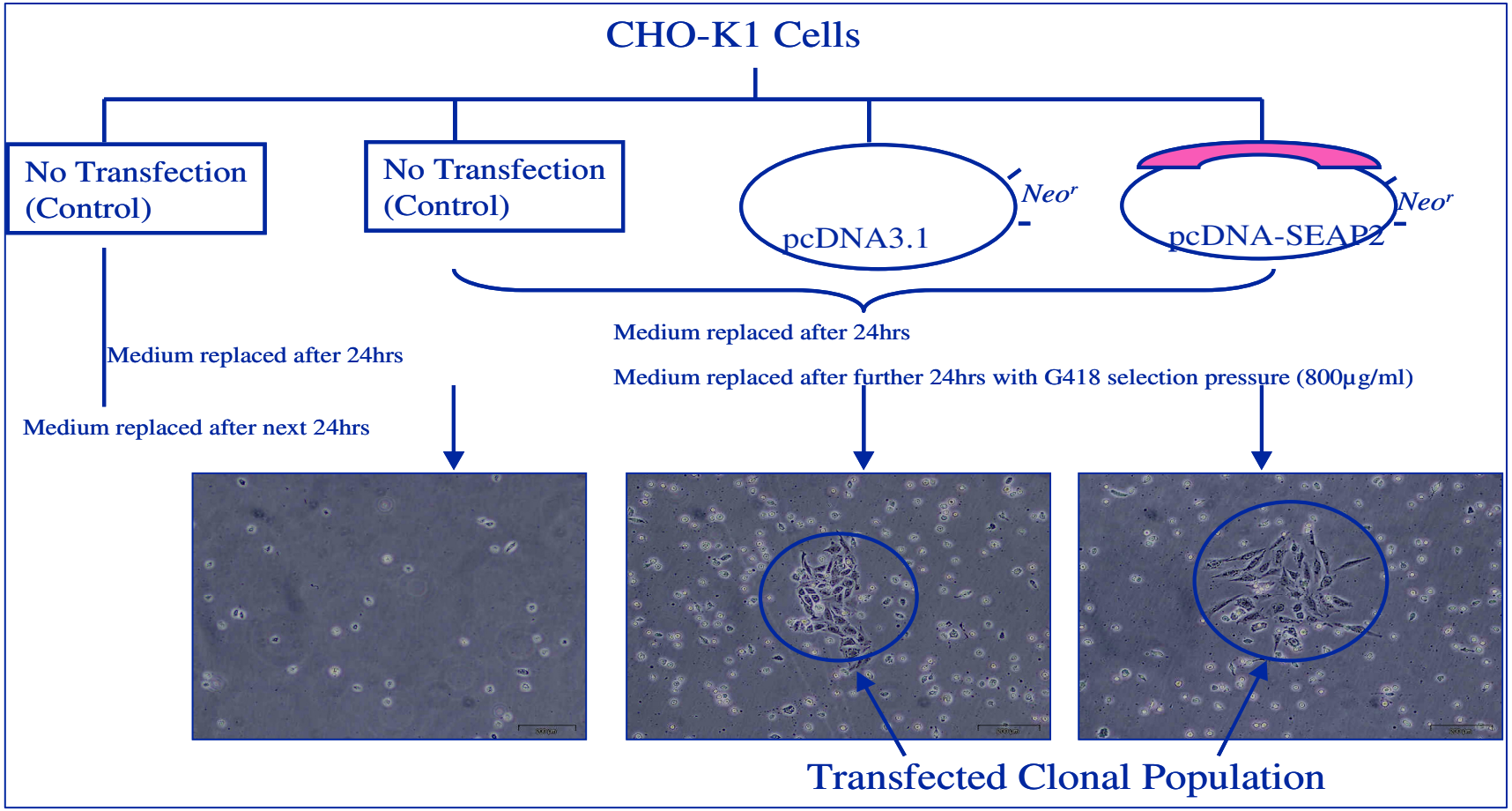

Figure 3.1.2.3. Transfection of pcDNA-SEAP2 in CHO-K1 cells adapted to grow in 0.5% FCS supplemented culture medium in monolayer. Media was replaced after 24hrs of transfection. G418 was added after 24hrs of media replacement. pcDNA3.1 and pcDNA-SEAP2 contain *neo*^{r} gene and therefore clonal populations were only observed in transfected culture.

3.1.2.3 Selection and isolation of suitable clones

Single cell clones were isolated from mixed populations of transfected cells using the limiting dilution method as described in Section 2.5.7. For this, cells were serial-diluted to achieve 1 cell/well in a 96-well culture plate using 0.5% FCS supplemented culture media containing G418 (800µg/mL). Single cell clones were identified using microscope. Twenty-four single cell clones were isolated from the mixed population and were transferred to a 24-well culture plate for further growth and expansion once cultures became confluent in the 96-well plate.

The conditioned media from these clones were collected under different culture conditions (different time points (24 & 72hrs) and G418 concentrations (800 & 1000 µg/mL)) and were analysed for SEAP production (Figure 3.1.2.4). The reason for selecting different time points was directed by the fact that clones may vary in their growth rates and productivity. Slow growing clones may have high productivity but as the productivity is directly dependent on the number of producer cells, it might not be detected at early stage of culture (24hrs). The culture of clones for 72hrs will allow these slow growing cells to grow up to detectable limits and therefore might enable us to identify slow growing high-producer clones. The use of different G418 concentrations (800 & 1000 µg/mL) helped us to improve the productivity as well as ensuring the elimination of non-transfected clones in this investigation. At the same time, the use of different time points and G418 concentration allowed us to optimize the time point for SEAP assays for the cells.

SEAP was quantified by measuring the change in absorbance per minute (OD/min) reflecting alteration in enzymatic activity (Section 2.5.8). SEAP is a glycosylated protein and therefore, alternation in SEAP level observed in this study could be due to change in

SEAP quantity, quality or both. From the 3 tested conditions, 1000 µg/mL of G418 at 72 hrs was found to be the optimum condition for the SEAP assay as 72hrs of culture was sufficient time for CHO cells to grow and secrete SEAP into the culture medium. Six clones (3H3(16), 4A10(19), 4D10(21), 4H9(22), 5A10(23) and 5F12(24)) were identified as SEAP producers (Figure 3.1.2.4). The clone 4A10(19) was observed to be the highest producer in all culture conditions tested in this investigation.

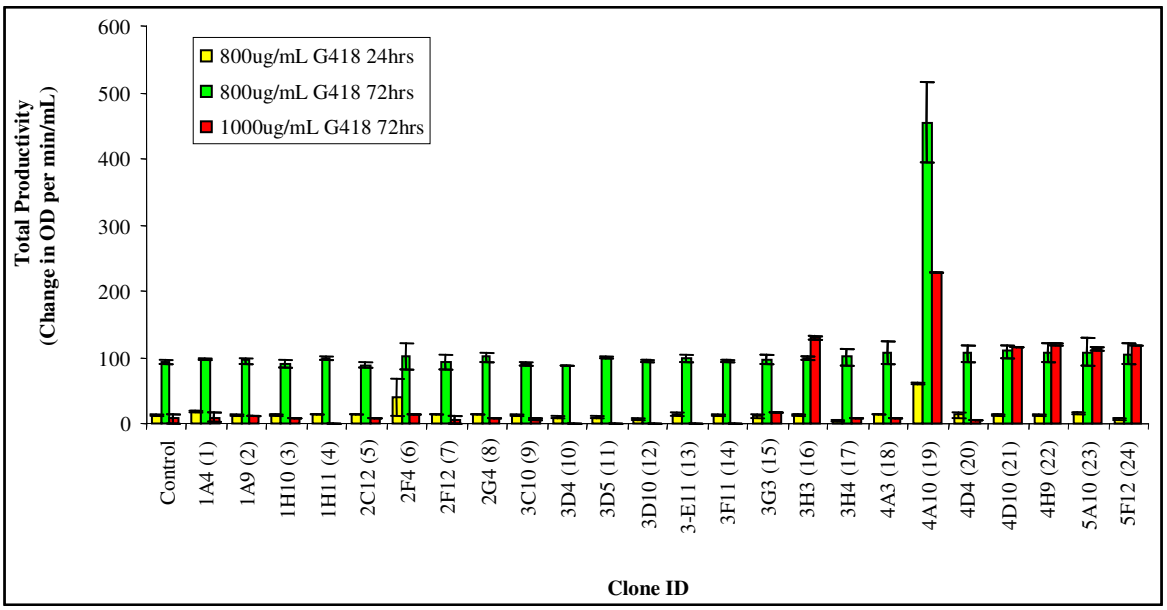

Figure 3.1.2.4. Identification of SEAP producer CHO-K1 clones in the single cell clones isolated from the mixed population. Conditioned medium from non-producer CHO-K1 cells was used as a control. Error bars represent standard deviation calculated from data obtained from three independent biological samples.

These six SEAP-secreting clones were investigated for their SEAP productivity to identify clones with different levels of SEAP productivity for future investigations. For this, all clones were seeded at $1x10^5$ cells/mL in 0.5% FCS supplemented culture medium containing G418 (1000 µg/mL) in a 24-well plate with a working volume of 0.5mL/well. Non-producer CHO-K1 cells were also seeded in 0.5% FCS supplemented culture medium as a control culture. The conditioned media were collected after 72hrs of culture and investigated for SEAP activity. The conditioned medium from a transiently tranfected known SEAP-secreting CHO cells was also used as a positive control in this investigation and this enabled us to identify a suitable producer clone. The SEAP productivity was highest, consistent and comparable to the positive control for only 1 clone, clone-19 (Figure 3.1.2.5). The remaining five clones secreted low levels of SEAP. All clones tested in this investigation were observed, using microscope, to be comparable in biomass (40-60% confluency) at the time of sample collection for SEAP assays.

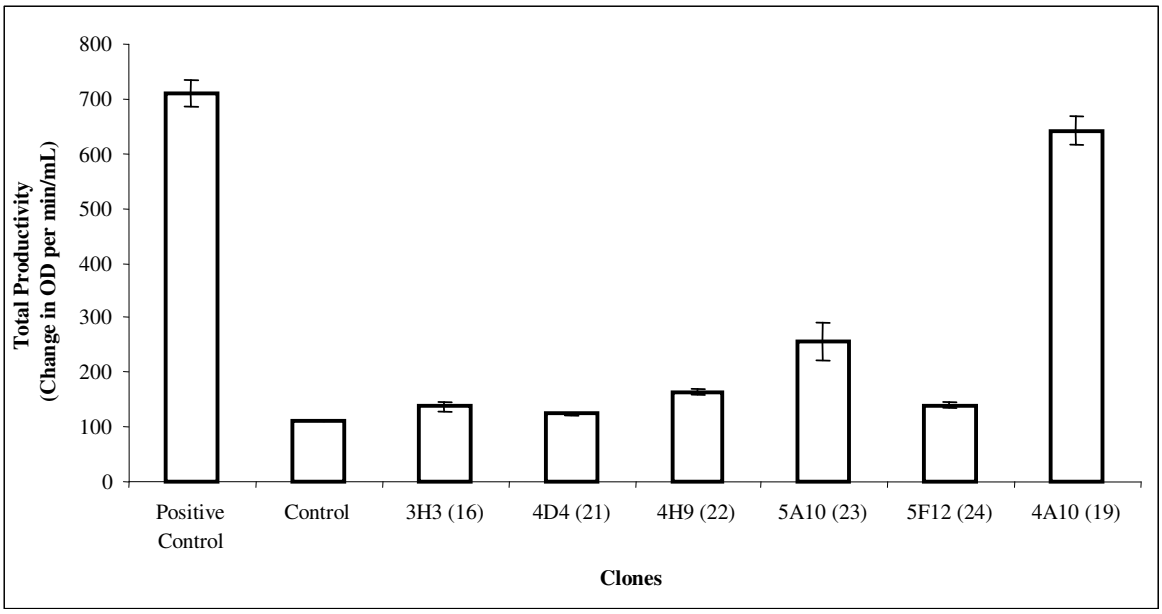

Figure 3.1.2.5. Comparison of SEAP productivity of six SEAP-secreting clones isolated from the mixed population. Conditioned medium from CHO cells transiently transfected with SEAP was used as a positive control. Conditioned medium from non-producer CHO-K1 cells was used as a negative control. Error bars represent standard deviation calculated from data obtained from three independent biological samples.

Since only 1 clone (clone 19) was observed to secrete SEAP in culture, additional single cell subclones were isolated from this clone in order to establish a wide range of SEAP-producing CHO clones, i.e. low and high producer, for future investigations. A total of 16 subclones were isolated from clone 19 using the limiting dilution method (Section 2.5.7) and compared for their SEAP productivity. For this, subclones were seeded at $1x10^5$cells/mL in 24-well plate with a working volume of 0.5mL/well. Non-producer CHO-K1 cells were also seeded in 0.5% FCS supplemented culture medium as a control culture. The conditioned media were collected after 72hrs of culture and were investigated for SEAP activity. From these 16 subclones, 2 subclone 3 (3s) and 12 (12s)) were observed to secrete comparable SEAP into the culture compared with the positive control (conditioned media from transiently transfected SEAP secreting CHO cells) (Figure 3.1.2.5-6). The cell growth was observed visually to be comparable within all subclones (40-60% confluent) at the time of sample collection for SEAP assays. These 2 subclones were also considered for further investigations.

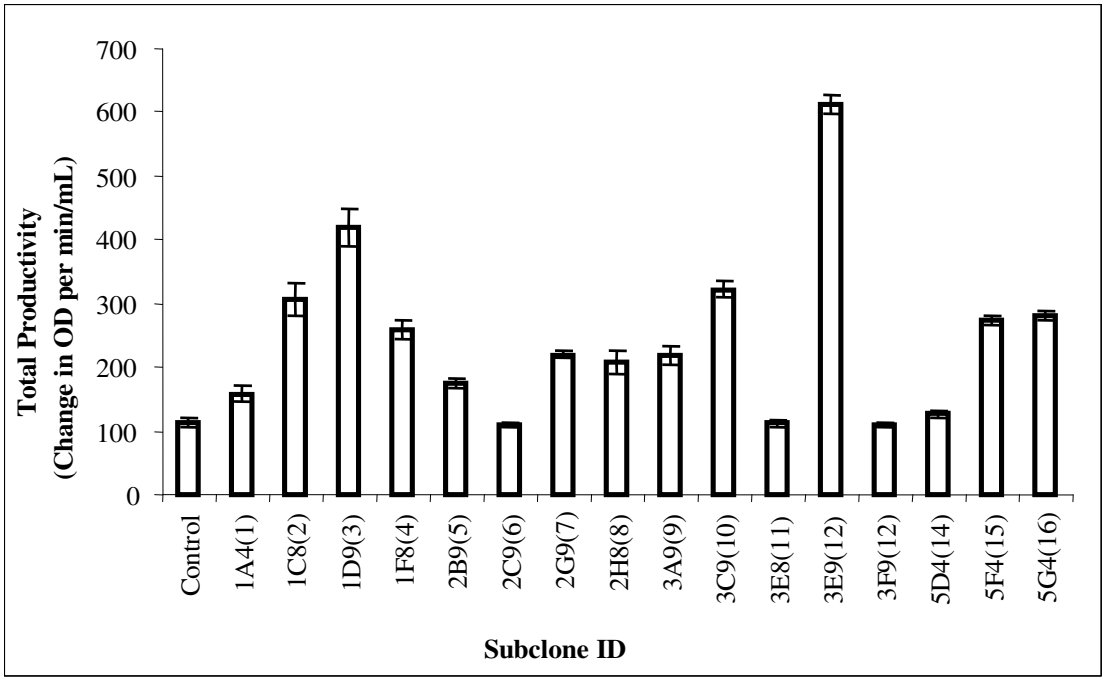

Figure 3.1.2.6. Identification of SEAP producer subclones isolated from clone 19. Conditioned medium from non-producer CHO-K1 cells was used as control. Error bars represent standard deviation calculated from data obtained from three independent biological samples.

3.1.2.4 Identification of clones with different levels of SEAP productivity

All clones (19, 3s and 12s) were then compared for their SEAP productivity in order to identify the highest producer clone for further work (Figure 3.1.2.7). Clones were seeded at $1x10^5$ cells/mL in 0.5% FCS supplemented culture medium containing G418 (1000µg/mL) in a 24-well plate. Non-producer CHO-K1 cells were also seeded in 0.5% FCS supplemented culture medium as a control culture. The working volume was maintained at 0.5mL/well. The conditioned media were collected at 72hrs of culture and were analyzed for SEAP activity. The cell counts were also performed. This experiment was performed using cells from 3 consecutive passages (P-3, P-4 and P-5) to improve the efficiency of the clone identification process. The highest total productivity was observed for subclone 12s followed by subclone 3s and clone 19 respectively at all passages (Figure 3.1.2.7B). A similar pattern was confirmed by analysis of the cell specific productivity (Figure 3.1.2.7C). The cell specific productivity was calculated by dividing the total productivity by cell number. This established subclone 12s as the best producer clone identified in this investigation followed by subclone 3s as a medium producer. Both clones were scaled-up by growing firstly in a 6-well plate and then scaled upto T-75 flasks.

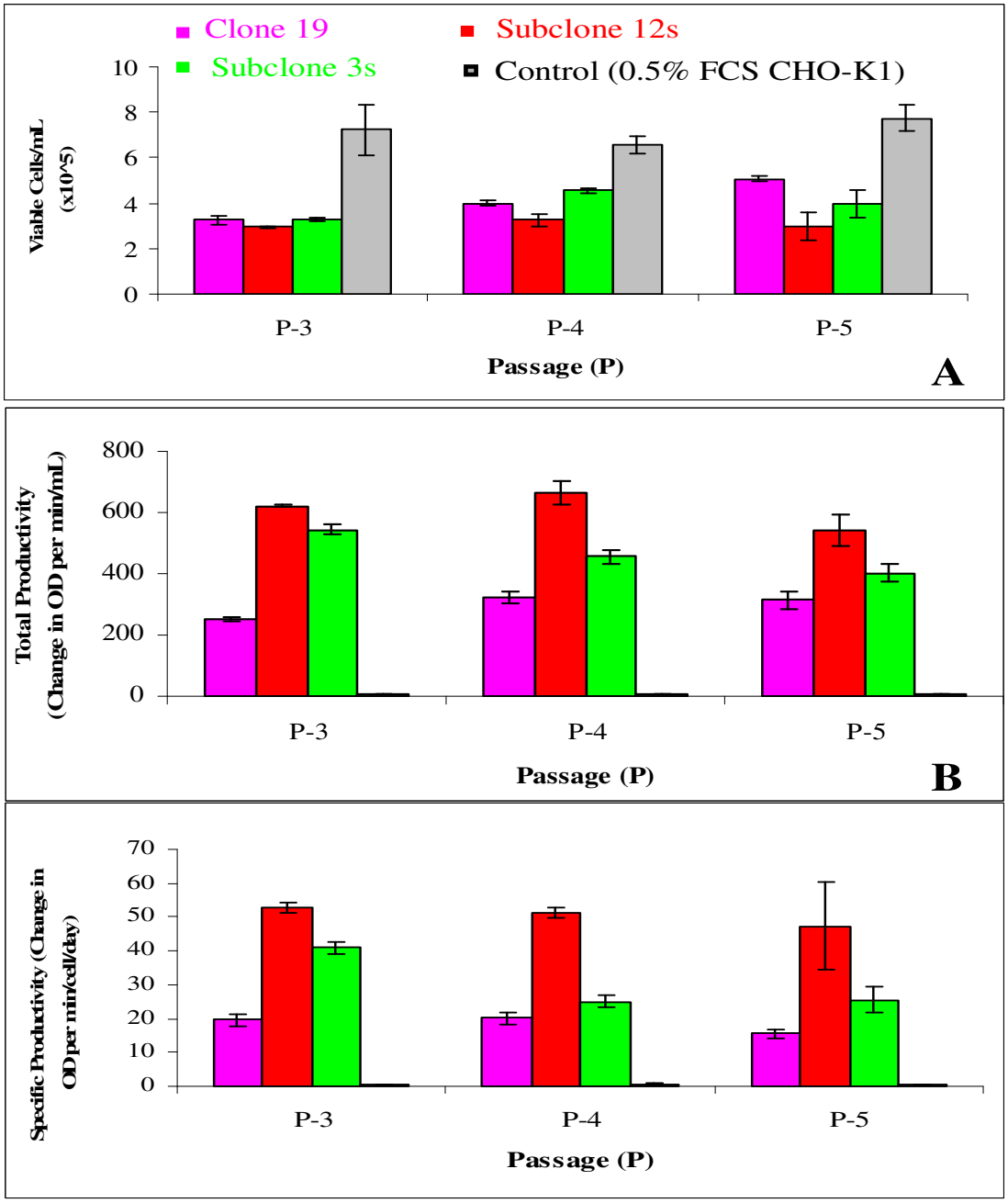

Figure 3.1.2.7. Comparison of SEAP productivity of clone 19 and subclones 3s and 12s to identify different levels of SEAP secreted by the 3 clones over 3 consecutive passages. Non-producer CHO-K1 cells were used as a control. A represents viable cell counts, B total productivity, and C specific productivity of cells. Error bars represent standard deviation calculated from data obtained from three independent biological samples.

3.1.2.5 Investigation of stability in SEAP productivity of clones

SEAP-secreting cells were routinely seeded at $3x10^5$ cells/mL in 0.5% FCS supplemented culture medium in the presence of G418 (1000µg/mL) in T-75 flasks and were subcultured in the exponential phase (3^{rd} or 4^{th} day) of culture. From this master culture, exponentially growing cells were seeded at $1x10^5$ cells/mL in 0.5% FCS supplemented culture medium containing G418 (1000µg/mL) in a 24-well plate with 0.5mL working volume over a period of 27 days to investigate the stability of SEAP productivity of both subclones 12s and 3s. Non-producer CHO-K1 cells were also seeded in 0.5% FCS supplemented culture medium as a control culture. The conditioned media were collected at 72hrs of culture and were analyzed for SEAP activity. Cell counts were also performed at 72hrs of culture. The cell specific productivity was found to be comparable through out the test period (Figure 3.1.2.8) which suggests that both clones are quite stable in their SEAP productivity for the testing period in this study.

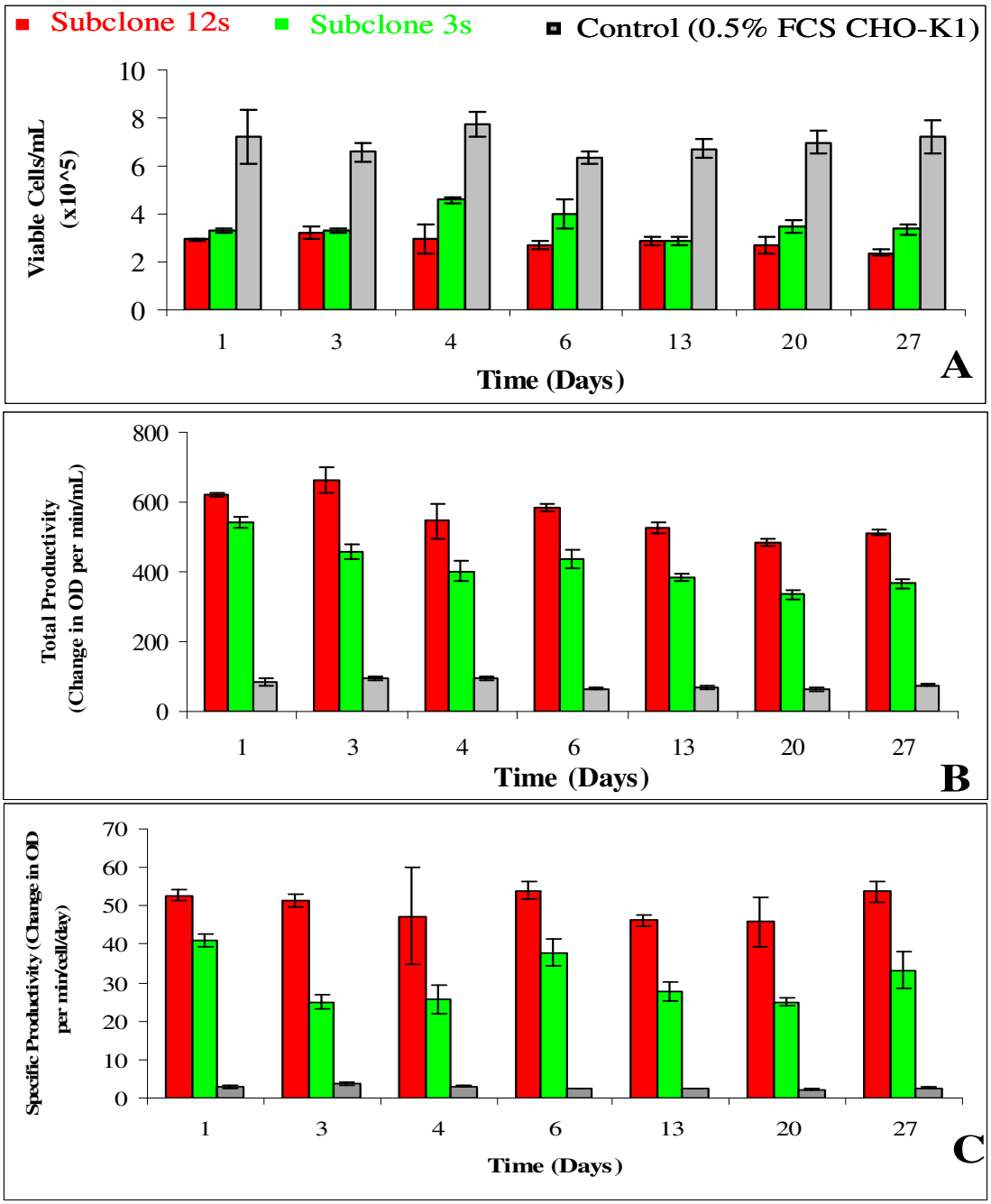

Figure 3.1.2.8. Investigation of the stability of SEAP productivity of subclones 3s and 12s over a period of 27 days. Non-producer CHO-K1 cells were used as a control. A represents viable cell counts, B total productivity and C specific productivity of cells. Error bars represent standard deviation calculated from data obtained from three independent biological samples.

3.1.2.5 Suspension-adaptation of SEAP-secreting clones

Both subclones, 3s and 12s, were subjected to adaption to grow in suspension culture and then to SFM conditions. Cells were seeded to achieve $2x10^5$ cells/mL density in 100mL of 0.5% FCS supplemented culture medium in the presence of G418 (1000µg/mL) in spinner flask. The cells were rotated at 60rpm and were gassed with mixture of gasses (O_2, N_2 and CO_2) everyday. The cell counts were performed on 2nd and 4th day of culture. Both clones achived $\geq 1x10^6$ cells/mL within 4 days and therefore were adapted to suspension culture (Figure 3.1.2.9). Attempts were made to adapt these suspension-adapted clones to grow in serum-free ExCell medium. Both clones were unable to adapt to ExCell medium in the period tested (30 days). These clones could be re-tried for SFM adaption by using a higher initial cell density (seeding density) or by adapting cells first to 0.25% FCS-supplemented medium. Clone 12s that had been adapted to grow in 0.5% serum supplemented medium in suspension culture was selected for further studies.

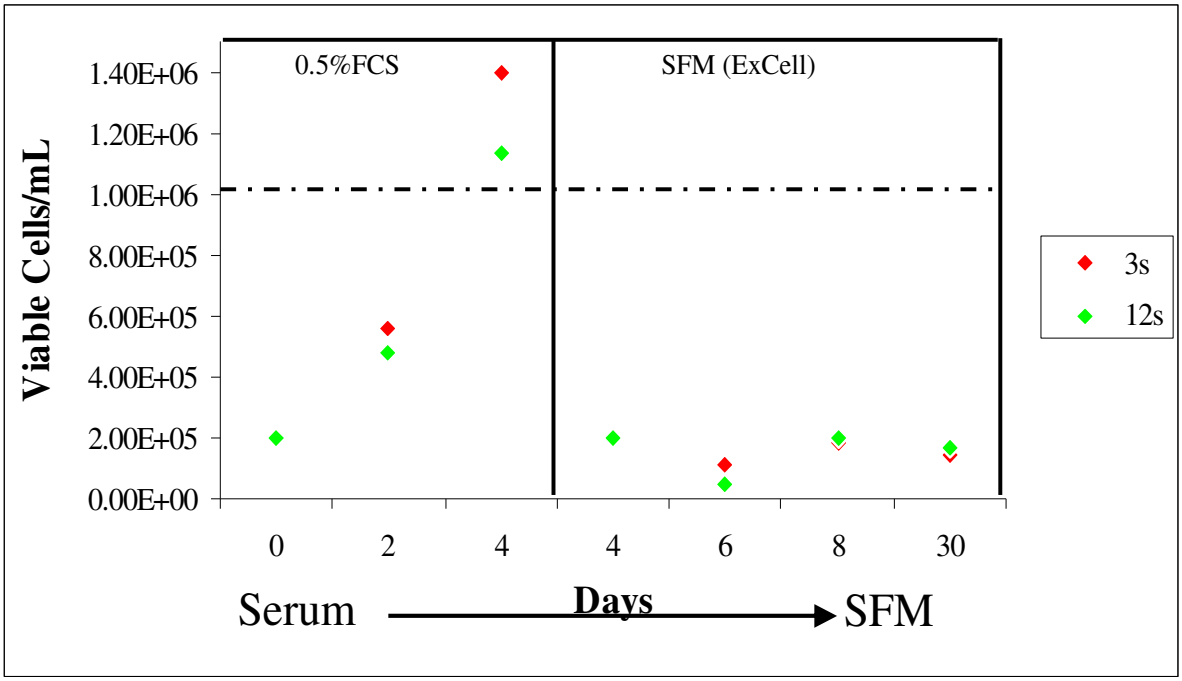

Figure 3.1.2.8. Adaptation of SEAP-secreting CHO-K1 subclones to suspension culture and serum-free culture conditions (SFM). Cells were transferred to SFM (Excell) on 4th day of culture. SFM (ExCell) represents commercially available serum-free medium, ExCell.

3.2 Investigation of low temperature-induced differential expression of proteins in CHO-K1 cells using 2D-DIGE

As described in section 1.5, low temperature cultivation of CHO cells can arrest cell growth and maintain high viability for a longer period of time and this results in an extended production phase and contributes to increased recombinant protein production. However, the mechanisms regulating these cellular phenotypes are still poorly understood. Therefore suspension-adapted CHO-K1 cells were cultured in a low temperature based biphasic culture process and analysed for differential expression of proteins following temperature-shift using 2D-DIGE.

3.2.1 Cell Culture

Suspension-adapted CHO-K1 cells were seeded at $1x10^5$ cells/mL in spinner flasks and maintained at 37^0C for 144hrs or for 72hrs at 37^0C followed by a temperature-shift to 31^0C for a further 72hrs. The temperature-shift was performed after 72hrs of culture because it represents the mid-exponential phase of cell growth. The temperature of 31^0C was selected as it represents the average temperature employed by others performing similar studies, i.e. 28-33^0C. As can be seen in Figure 3.2.1, the cells that were temperature-shifted immediately ceased logarithmic growth and did not exceed a peak viable cell density of $1.45\ x10^6 \pm 0.013$ cells/mL, whereas the cells cultured at 37^0C continued in logarithmic growth for a further 48hrs and achieved a peak viable density of $2.02\ x\ 10^6 \pm 0.11$ cells/mL. The cells that were temperature-shifted displayed a steady, if slightly increasing, viable cell density throughout, whereas the cells maintained at 37^0C had entered the late

stationary/death phase of the growth cycle by 144hrs (Figure 3.2.1A&B). Cultures were sampled at 72hrs and 144hrs for protein extraction from 3 independent experiments.

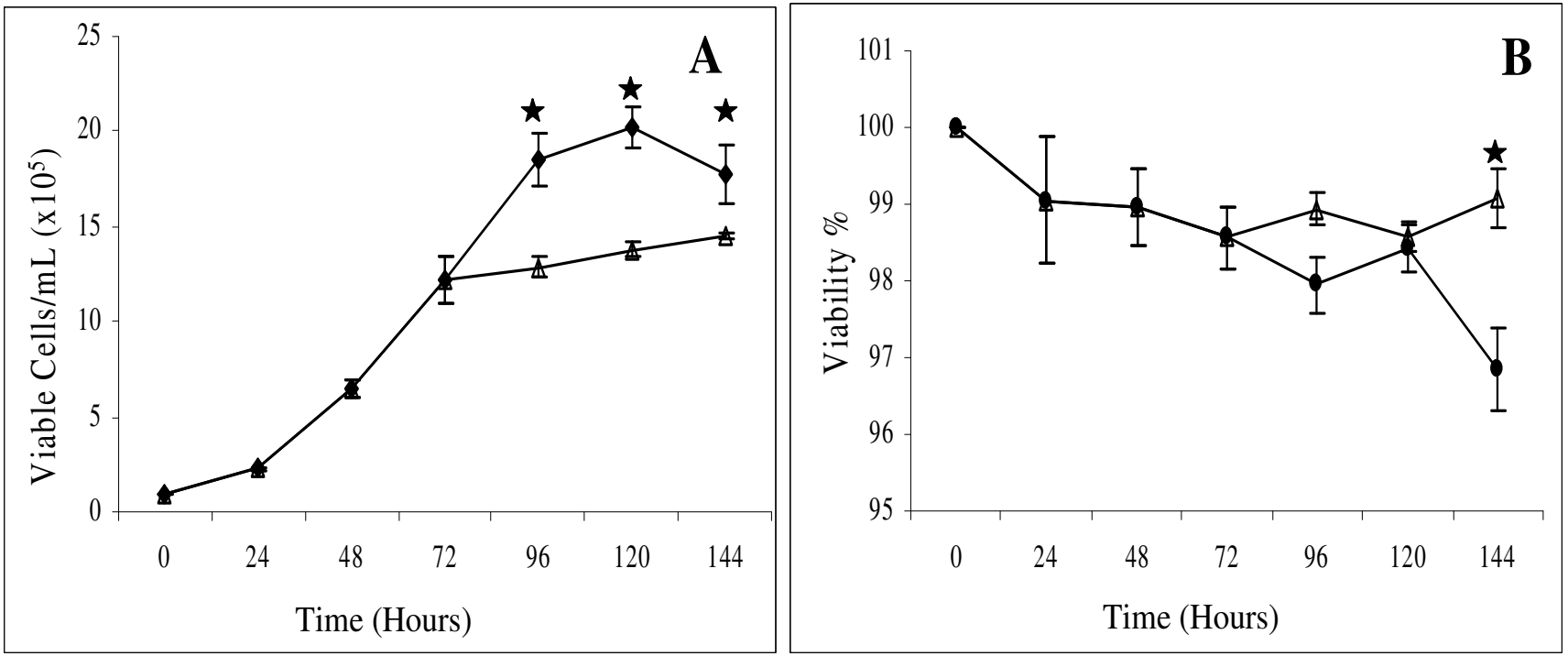

Figure 3.2.1. Comparison of the viable cell number (A) and percentage viability (B) of CHO-K1 cells cultured using either a biphasic temperature-shifted culture (—△—) or using a standard culture at 37^0C (—◆—). Error bars represent the standard deviation calculated from three biological replicate cultures. '*' indicates statistically significant (p-value ≤ 0.02) differences in cell growth and viability.

3.2.2 Quality analysis of Protein Samples

A total of 50 µg of proteins from biological triplicate samples representing 72hrs and 144hrs of culture for temperature-shifted and standard culture conditions were separated using 2D-PAGE. The gels were stained with silver stain initially to check the quality of protein samples before performing 2D-DIGE. Results indicated that the protein samples were of satisfactory quality as the spots were distinctly separated and all biological samples broadly showed a similar initial profile (Figure 3.2.2).

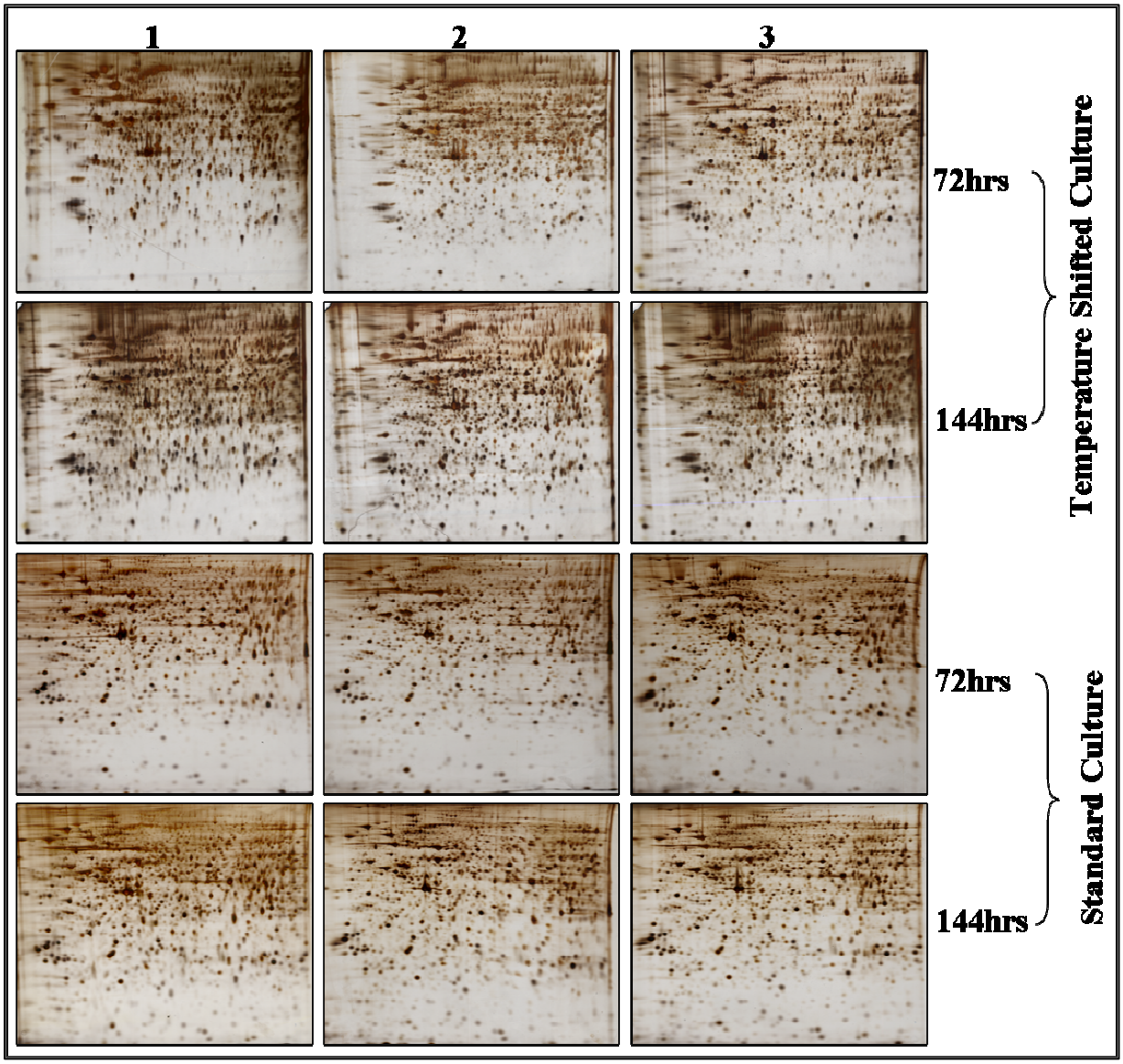

Figure 3.2.2. Quality analysis of protein samples. Figure represents silver stained images of 3 biological proteins samples separated using 2D gel electrophoresis.

3.2.3 Analysis of differential expression of spots

A 2D-DIGE experiment was carried out to identify the differential expression of cellular proteins between temperature-shifted and standard cultures of CHO-K1 cells. For this, 3 independent biological samples representing 72 and 144hrs of culture from CHO-K1 cells grown in temperature-shifted and non-shifted conditions were prepared separately.

A total of 50µg of protein from each of the cell samples were labelled with fluorescent dyes (Table 3.2.3) and were separated using 2D gel electrophoresis (section 2.6.3-6). Protein expression maps (PEMs) for the biological triplicate samples were generated using 2D-DIGE for both temperature-shifted cultures and standard cultures. The 72hrs and 144hrs PEMs were then compared using the Biological Variation Analysis (BVA) module of DeCyder 6.5 software. This was aided by the use of a pooled internal standard labelled with Cy2 which facilitated spot matching and relative spot quantitation while minimizing gel-to-gel variation. Spots originating from dust particles and gel impurities in each gel image were checked and removed manually to improve the quantitation and matching process. Landmarks, which aid the gel-to-gel spot matching process, were also defined in the gel to increase the accuracy of the matching algorithm. Manual checks were carried out to allow for cases where spots were detected more than once (due to spot splits by the spot detection algorithms). These spots were then merged back to improve spot matching. After spot matching and filtering, an average of 2852 and 2885 spots were detected for the temperature-shifted and standard culture experiments respectively. The average ratio, which was considered as fold change in this investigation, was calculated using DeCyder software by comparing the normalized spot abundances between two different groups such as 72hrs of culture vs. 144hrs of culture (Section 2.6.7). Spots that were 1.5 fold up/down

regulated at 144hrs compared to 72hrs with a t-test score of ≤ 0.05 and present on all gels in the experiment were considered differentially expressed (DE). A total of 201 DE spots (118 up regulated and 83 down regulated) were observed in the temperature-shift experiment and 404 DE spots (210 up regulated and 194 down regulated) were observed in the standard culture experiment.

Table 3.2.3. Ettan 2D-DIGE experimental design used for the analysis of differential protein expression for temperature-shifted and standard culture of CHO-K1 cells.

Culture Condition	**Gel number**	**Cy2**	**Cy3**	**Cy5**
Temperature-shift	1	Pooled internal standard	Biological Sample-1 for 72hrs culture	Biological Sample-1 for 144hrs culture
	2	Pooled internal standard	Biological Sample-2 for 72hrs culture	Biological Sample-2 for 144hrs culture
	3	Pooled internal standard	Biological Sample-3 for 72hrs culture	Biological Sample-3 for 144hrs culture
Standard Culture	4	Pooled internal standard	Biological Sample-1 for 72hrs culture	Biological Sample-1 for 144hrs culture
	5	Pooled internal standard	Biological Sample-2 for 72hrs culture	Biological Sample-2 for 144hrs culture
	6	Pooled internal standard	Biological Sample-3 for 72hrs culture	Biological Sample-3 for 144hrs culture

3.2.4 Analysis of differentially expressed proteins

The spots observed differentially expressed in temperature-shifted or standard culture were identified using MALDI-ToF MS. These identified proteins were then analyzed together to identify temperature-dependent and independent changes in expression of proteins. This enabled us to identify proteins that may have a possible role in the regulation of cell growth and apoptosis and hence have the potential to improve recombinant protein production in culture.

3.2.4.1 Spot identification

Following spot picking and tryptic digestion, protein identification was carried out using MALDI-ToF MS (section 2.6.10). All identified proteins had an expectation value of 0.01 or better. The expectation value for proteins was determined by Ettan MALDI-ToF Pro evaluation software (GE Healthcare) using the Profound database search engine for peptide mass fingerprints. An expectation value of 0.01 or better means that there is $\leq 1\%$ chance that the identification is random. Although many spots resulted in high quality spectra, they were not identified which may be due to insufficient amounts of protein, modification and/or variation of the amino acid sequences in CHO proteins or the lack of CHO proteins in the protein databases. Despite this, a total of 53 differentially expressed proteins were identified in this investigation (Figure 3.2.4.1). These proteins were comprised from the list of proteins differentially expressed in three different comparison groups, 72hrs at 37^0C vs. 144hrs at 31^0C (temperature-shifted culture), 72hrs at 37^0C vs. 144hrs at 37^0C (standard culture), and 144hrs at 37^0C vs. 144hrs at 31^0C (stationary culture). The representative spectra of 3 of these identified proteins (vimentin, HNRPC and GAPDH) are presented in Figure 3.2.4.2.

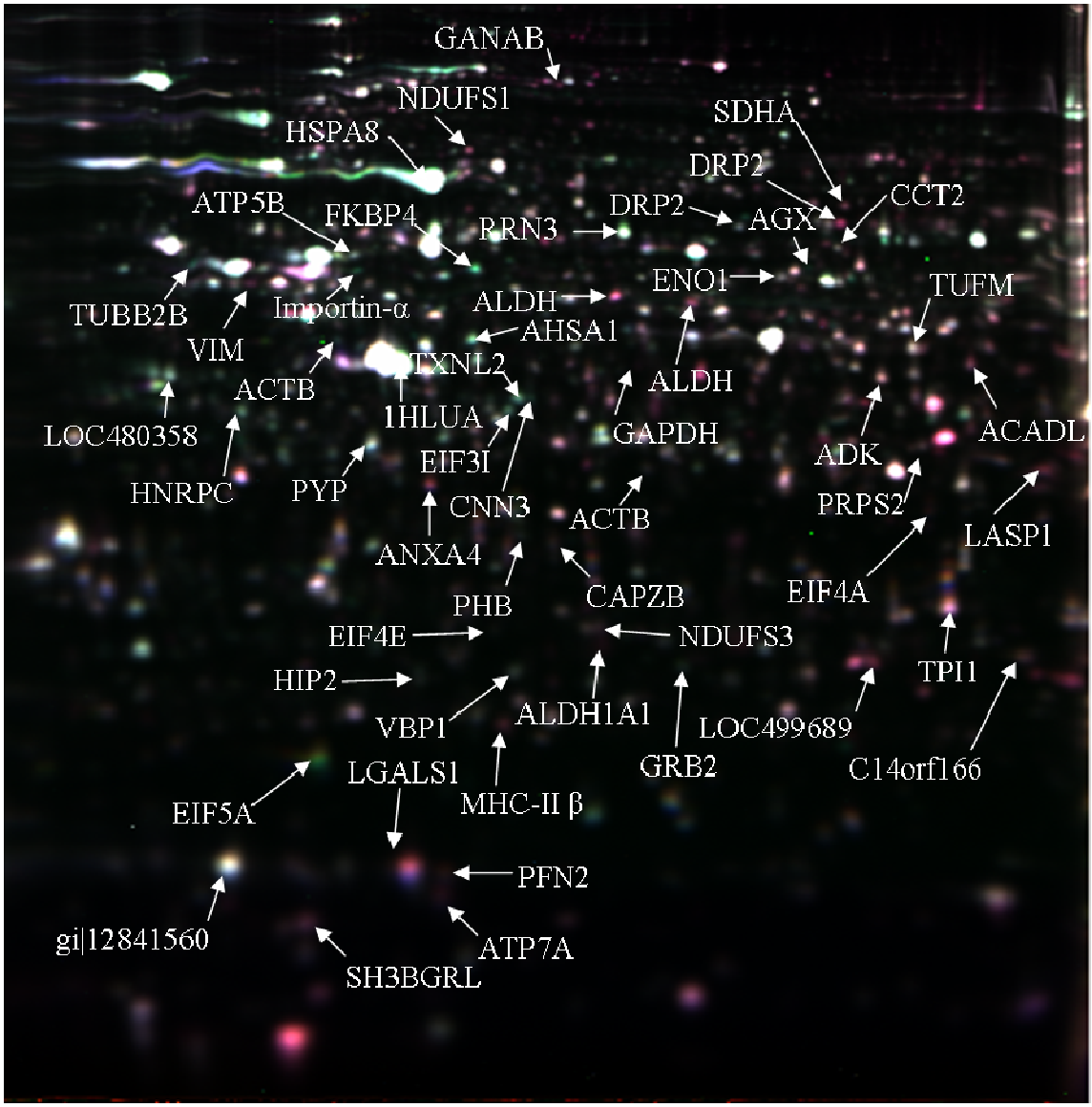

Figure 3.2.4.1. Representative overlapping 2D-DIGE expression map of CHO-K1 proteins labelled with fluorescent dyes (Cy2, Cy3 and Cy5). The location of differentially expressed proteins identified using MALDI-ToF MS are indicated by arrows.

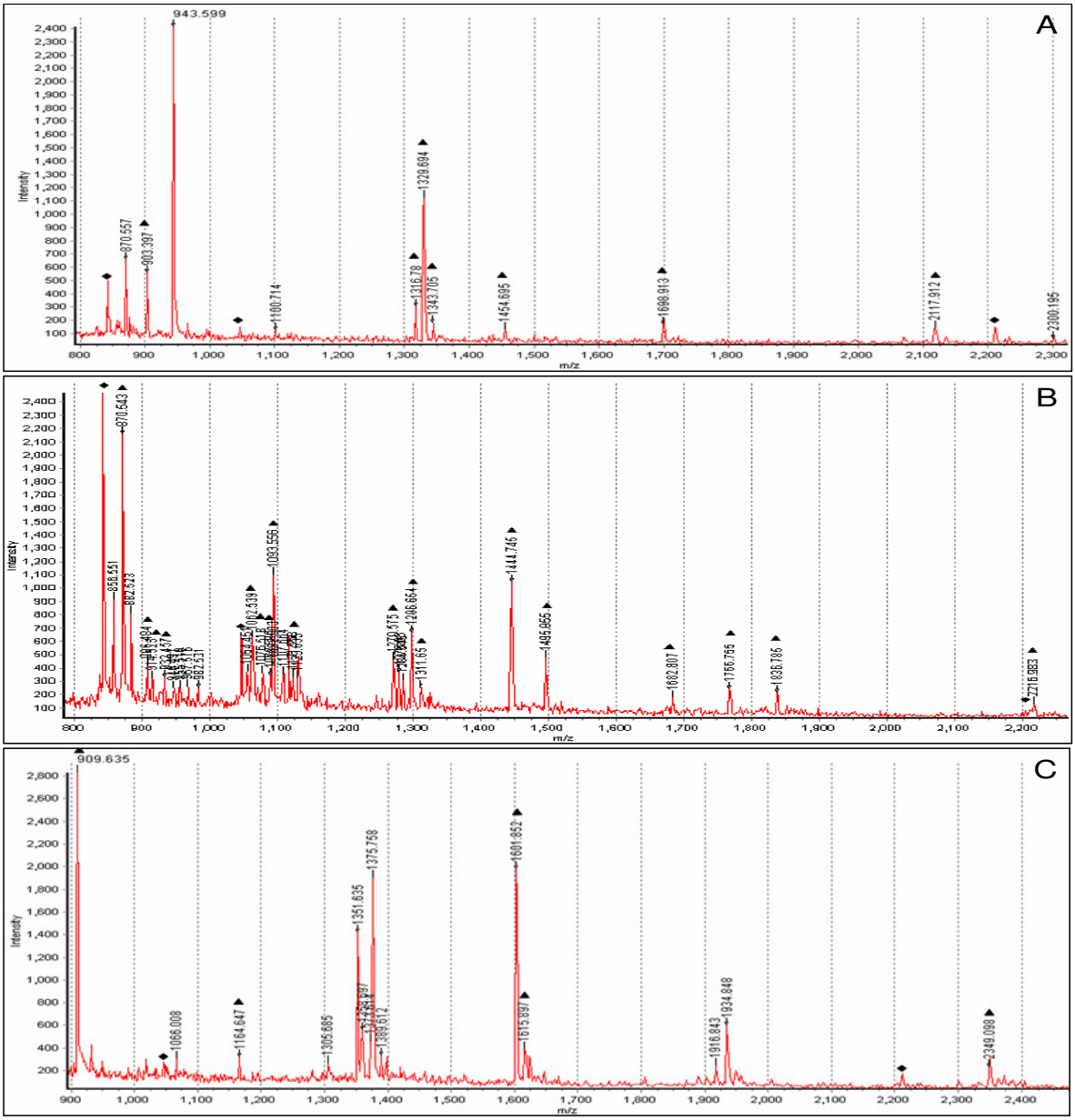

Figure 3.2.4.2. MALDI-ToF generated spectra of heterogeneous nuclear ribonucleoprotein C (HNRPC) (A), Vimentin (VIM) (B) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (C). Trypsin gives peaks at specific molecular weight (842.51, 1045.5 and 2211.1 m/z) that can be used as calibrants in the MS spectra. Peaks other than trypsin are from target proteins. Trypsin calibration peaks are represented by ♦ and peaks belonging to the identified proteins by ▲.

3.2.4.2 Temperature-shifted culture (72hrs at 37^0C vs. 144hrs at 31^0C)

The protein expression maps (PEMs) for cells at 72hrs at 37^0C were compared with the PEMs for cells that were shifted to 31^0C for a further 72hrs after culturing for 72hrs at 37^0C. The differentially expressed proteins identified in this comparison could be associated with the regulation of low-temperature induced phenotypes such as reduced growth and medium consumption and improved viability and recombinant protein productivity in CHO culture.

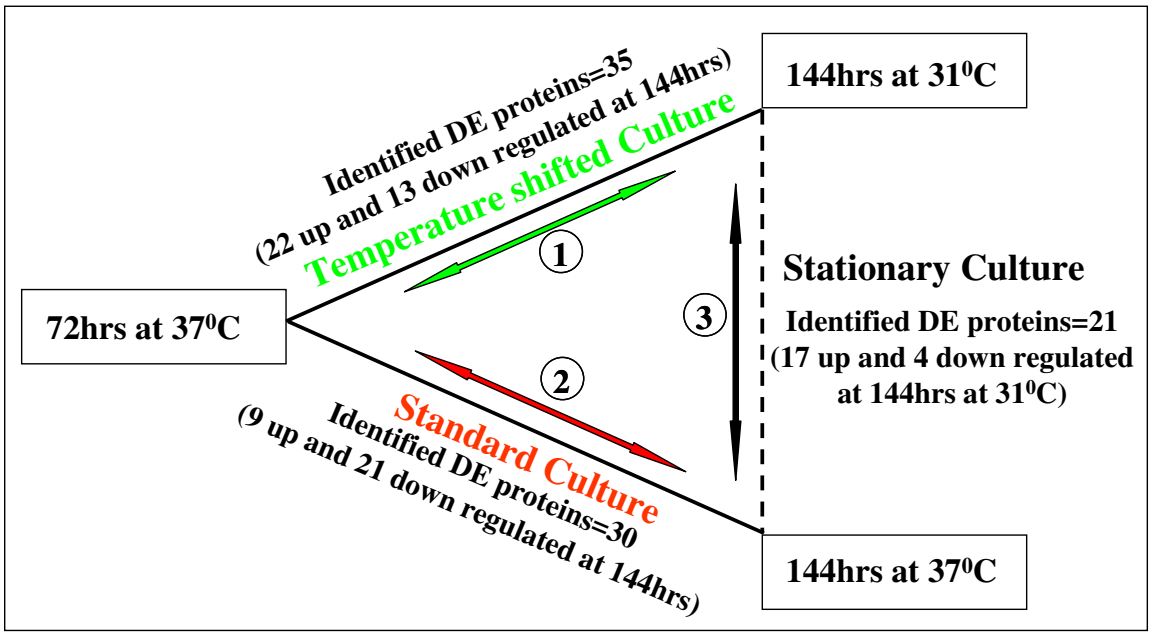

Figure 3.2.4.3. A figure describing the different comparison groups and the number of identified differentially expressed (DE) proteins. The temperature-shifted culture (72hrs at 37^0C vs. 144hrs at 31^0C) are represented by 1, the standard culture (72hrs at 37^0C vs. 144hrs at 37^0C) are represented by 2 and the stationary culture (144hrs at 31^0C vs. 144hrs at 37^0C) by 3.

Of the 53 identified proteins, 35 proteins were differentially regulated in temperature-shifted cells at 144hrs at 31^0C in comparison to cells maintained at 72hrs at 37^0C (Figure 3.2.4.3 and Table 3.2.4), of which 22 proteins were up regulated and 13 were down regulated at 144hrs at 31^0C. The list of differentially expressed proteins observed in temperature-shifted culture (72hrs at 37^0C vs. 144hrs at 31^0C) were compared with the list of differentially expressed proteins observed in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). This enabled us to identify temperature-dependent and -independent protein changes. Of the 35 proteins, 23 proteins were only differentially regulated in temperature-shifted cultures (72hrs at 37^0C vs. 144hrs at 31^0C), of which 18 proteins were up regulated and 5 proteins were down regulated at 144hrs at 31^0C. For example, HNRPC, a protein involved in the regulation of protein translation was reduced by 1.52 fold in cells at 144hrs at 31^0C following reduction of culture temperature in comparison to cells at 72hrs at 37^0C. GANAB, a glycan-processing enzyme that is involved in glycoprotein quality control in the endoplasmic reticulum (Totani *et al.* 2006), was increased by 2.19 fold in cultures at 144hrs at 31^0C following temperature-shift compared to cultures at 72hrs at 37^0C. PFN2 is involved in the regulation of normal actin polymerization in response to thermal stress and this protein was 1.69 fold up regulated in cells at 144hrs at 31^0C in biphasic cultures in comparison to cells at 72hrs at 37^0C. The expression of HNRPC, GANAB and PFN2 was unchanged when the cells were maintained at standard culture (72hrs at 37^0C vs. 144hrs at 37^0C) suggesting these changes to be temperature-induced changes in expression of these proteins.

The identified temperature-dependent proteins were involved in the regulation of a wide range of biological functions such as transcription, translation, cell adhesion, structure,

growth and apoptosis, etc (Table 3.2.4). For example, RRN3 is a transcription factor and its expression was reduced by 1.53 fold in cells at 144hrs at 31^0C following temperature-shift in comparison to cells at 72hrs at 37^0C whereas it was unchanged in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). EIF4A is a translation initiation factor and was increased by 1.81 fold in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C. The expression of EIF4A remained unaltered in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). Importin-α is involved in the regulation of transport of growth- and apoptosis-related factors into the nucleus and was 1.8 fold down regulated in cells at 144hrs at 31^0C in biphasic culture in comparison to cells at 72hrs at 37^0C, whereas it was unchanged in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). LGALS1, a cell adhesion protein, was 1.62 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in biphasic culture and was not changed in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). Vimentin is a structural protein and was increased by 2.05 fold in cells at 144hrs at 31^0C in response to low temperature compared cells at 72hrs at 37^0C while it was not changed in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C).

On the other hand, there were a few proteins that were similarly affected in both temperature-shifted and non-shifted cultures indicating the temperature-independent changes. Activator of 90 kDa heat shock protein ATPase homolog 1 (AHSA1) was differentially expressed in both experiments. It was reduced by 1.69 fold in cells at 144hrs at 31^0C following temperature-shift compared to cells at 72hrs at 37^0C and 1.73 fold down regulated in cells at 144hrs at 37^0C in standard culture in comparison to cells at 72hrs at 37^0C. Similarly, TXNL2 was 1.8 fold down regulated at 144hrs in both temperature-shifted

(72hrs 37^0C vs. 144hrs at 31^0C) and standard culture (72hrs 37^0C vs. 144hrs at 37^0C) and hence these changes could be temperature-independent alterations in protein expression.

Two proteins, ALDH and DPYSL2, were identified at more than one location on the gel. ALDH was 1.98 and 1.63 fold up regulated at both spots respectively in cells at 144hrs at 31^0C in temperature-shifted culture compared to cells at 72hrs at 37^0C and was not differentially regulated in standard culture (72hrs 37^0C vs. 144hrs at 37^0C). DPYSL2 was up regulated at 144hrs at one spot by 2.58 fold in temperature-shifted (72hrs 37^0C vs. 144hrs at 31^0C) and by 1.97 fold in standard culture (72hrs 37^0C vs. 144hrs at 37^0C). At another spot, this protein was 2.51 fold down regulated in cells at 144hrs at 31^0C following temperature-shift in comparison to cells at 72hrs at 37^0C and was unchanged in standard culture (72hrs 37^0C vs. 144hrs at 37^0C). This could be possibly due to some form of post-translational modification (PTM) of these proteins in response to low temperature culture.

Table 3.2.4 MALDI-ToF MS identification of proteins that were differentially expressed at 144hrs compared to 72hrs using either temperature-shifted or standard culture.

Number	Protein ID	Protein Description	Abbreviation	Expectation value[a]	Peptides Matched for Identification	% Coverage	Temperature-shift		Standard Culture	
							Fold Change[b]	t-test score	Fold Change[b]	t-test score
Structural										
1	gil1407651	LIM and SH3 domain protein Lasp-1	LASP1	0.005	6	33.2	2.06	7.20E-09	NDE	NDE
2	gil2078001	Vimentin	VIM	0	19	44.6	2.05	3.70E-07	NDE	NDE
3	gil55391513	Calponin 3, acidic	CNN3	0.008	6	19.7	-1.96	7.20E-09	-3.37	2.80E-10
4	gil55621900	PREDICTED: similar to profilin 2 isoform b	PFN2	0	5	39.3	1.69	1.00E-03	NDE	NDE
5	gil2624850	Chain A, Structure Of Bovine	---	0.002	10	44.8	-1.59	3.60E-08	-1.53	1.20E-11

		Beta-Actin-Profilin Complex (1HLUA)								
6	gi|16304154	Beta actin	ACTB	0.003	5	17.9	NDE	NDE	-1.97	4.50E-09
7[c]	gi|60389477	Beta actin	ACTB	0.001	8	30.7	NDE	NDE	-1.74	4.20E-10
8	gi|54695812	Capping protein (actin filament) muscle Z-line, beta	CAPZB	0.005	8	26.5	NDE	NDE	-1.5	1.90E-05
9	gi|224839	Tubulin T beta15	TUBB2B	0.009	7	18	NDE	NDE	-2.02	2.00E-08
Metabolism										
1	gi|16073616	Aldehyde dehydrogenase	ALDH	0	6	18.7	1.98	5.50E-08	NDE	NDE
2[d]	gi|16073616	Aldehyde dehydrogenase	ALDH	0.004	9	22.1	1.63	5.80E-06	NDE	NDE
3	gi|28386049	Aldehyde dehydrogenase family 1, subfamily A1	ALDH1A1	0.001	8	20	NDE	NDE	-1.64	0.00011
4	gi|2897818	Huntingtin interacting protein-2	HIP2	0.006	5	28.5	1.97	1.40E-06	NDE	NDE
5	gi|56783068	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	0.004	5	22.3	1.8	4.00E-07	NDE	NDE

6	gi|206428	Phosphoribosylphosphate synthetase (PRPS2) precursor	PRPS2	0	5	21.7	1.53	1.6E-06	NDE	NDE
7	gi|55635845	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	NDUFS3	0.007	7	25.5	1.51	2.30E-07	NDE	NDE
8	gi|57110953	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa precursor	NDUFS1	0.008	8	12.2	2	8.40E-08	1.59	3.80E-06
9	gi|76611941	PREDICTED: similar to UDP-N-acetylhexosamine pyrophosphorylase (Antigen X)	AGX	0.002	6	12.8	-1.93	1.70E-06	NDE	NDE
10	gi|31982520	Acetyl-Coenzyme A dehydrogenase, long-chain	ACADL	0.005	10	24.2	1.88	7.8E-008	NDE	NDE
11	gi|6679891	Alpha glucosidase 2, alpha neutral	GANAB	0.009	12	11.9	2.19	5.60E-09	NDE	NDE

		subunit								
12	gil5759173	Succinate dehydrogenase flavoprotein subunit	SDHA	0.003	9	19.3	1.7	5.90E-08	1.87	6.90E-10
13	gil71059735	Pyrophosphatase	PYP	0.001	8	33.9	NDE	NDE	-1.58	3.60E-10
14	gil38512111	Triosephosphate isomerase 1	TPI1	0.001	8	29.8	NDE	NDE	1.51	5.60E-08
15	gil74007151	PREDICTED: similar to alpha enolase	ENO1	0.007	9	30.3	NDE	NDE	1.51	4.80E-07
16	gil73968432	PREDICTED: similar to ATP synthase beta chain, mitochondrial precursor isoform 1	ATP5B	0	10	24.9	NDE	NDE	-2.28	1.60E-06
17	gil25320034	Adenosine kinase	ADK	0.006	6	16.1	NDE	NDE	1.78	3.50E-12
Differentiation										
1	gil57105264	PREDICTED: similar to Dihydropyrimidinase related protein-2	DPYSL2	0.007	15	11.2	2.58	5.90E-08	1.97	3.50E-10

2[e]	gil3122018	Dihydropyrimidinase-related protein 2	DPYSL2	0.009	5	10.5	-2.51	5.50E-07	NDE	NDE
Signal Transduction										
1	gil55742832	Annexin A4	ANXA4	0.001	11	35.4	1.95	7.20E-09	NDE	NDE
2	gil28876	Growth factor receptor-bound protein 2	GRB2	0.002	5	27.5	NDE	NDE	-1.72	2.40E-09
Translation										
1	gil66910561	Heterogeneous nuclear ribonucleoprotein C	HNRPC	0	8	32.9	-1.52	6.9E-07	NDE	NDE
2	gil20987331	Eukaryotic translation initiation factor 3, subunit I	EIF3I	0.008	5	20	-1.72	1.70E-08	-2	7.50E-11
3	gil73978223	PREDICTED: similar to eukaryotic translation initiation factor 4A, isoform 1	EIF4A	0	6	21.7	1.81	7.30E-07	NDE	NDE
4	gil74001995	PREDICTED: similar to	EIF4E	0.005	6	30.4	NDE	NDE	1.67	0.0013

		eukaryotic translation initiation factor 4E isoform 1								
5	gi|109296	Eukaryotic translation initiation factor 5A	EIF5A	0.001	6	34.4	-1.81	1.30E-07	-2.07	7.50E-08
6	gi|56967054	Chain A, Tu translation elongation factor, mitochondrial	TUFM	0.003	7	23.5	NDE	NDE	1.56	4.60E-07
7	gi|73989786	40S ribosomal protein SA (p40)	LOC480358	0	7	41.3	NDE	NDE	-1.9	2.40E-06
Transcription										
1	gi|21706613	Rrn3 protein	RRN3	0.001	3	5.7	-1.53	7.40E-10	NDE	NDE
2	gi|73966295	PREDICTED: similar to prohibitin	PHB	0.002	4	25.2	NDE	NDE	-1.66	4.30E-10
Cell adhesion										
1	gi|12805209	Lectin, galactose binding, soluble 1	LGALS1	0.007	4	32.6	1.62	5.70E-05	NDE	NDE
Stress Response										
1	gi|73963752	PREDICTED: similar to Activator	AHSA1	0.003	8	15.5	-1.69	9.70E-09	-1.73	4.60E-10

		of 90 kDa heat shock protein ATPase homolog 1								
Protein binding										
1	gil78187979	Thioredoxin-like 2	TXNL2	0.009	7	19.3	-1.8	4.60E-08	-1.8	7.90E-09
2	gil5542272	Chain A, Importin Alpha, Mouse	Importin-α	0.007	5	13.5	-1.8	8.80E-05	NDE	NDE
Secretory pathway										
1	gil1698802	Menkes disease gene product	ATP7A	0.005	6	6.2	1.79	9.30E-07	1.6	9.00E-10
Protein Folding										
1	gil13097417	FK506 binding protein 4	FKBP4	0.01	7	13.1	-2.5	7.40E-10	-2.43	1.50E-10
2	gil73954621	PREDICTED: similar to heat shock protein 8 isoform 3	HSPA8	0.003	7	15.7	-1.9	5.60E-09	-2.09	2.20E-09
3	gil74008872	PREDICTED: similar to von Hippel-Lindau binding protein 1 isoform 2	VBP1	0.007	7	26.9	NDE	NDE	-1.8	1.20E-10
4	gil73968675	PREDICTED: similar to	CCT2	0.001	10	29.6	NDE	NDE	-1.64	2.60E-08

		chaperonin containing TCP1, subunit 2 isoform 2								
Miscellaneous										
1	gi|66730313	Hypothetical protein LOC499689	---	0.004	6	23.4	2.2	2.80E-08	NDE	NDE
2	gi|13161222	MHC class II antigen beta chain	MHC II β	0.007	7	62.9	1.87	2.70E-08	NDE	NDE
3	gi|75765278	Chain A, Crystal Structure Of The Human Sh3 Binding Glutamic-Rich Protein Like	SH3BGRL	0	8	74.4	1.7	1.80E-07	NDE	NDE
4	gi|73963786	PREDICTED: similar to Protein C14orf166	---	0.002	8	24.6	1.5	1.80E-07	NDE	NDE
5	gi|12841560	Unnamed protein product	---	0.006	5	43.8	NDE	NDE	-1.54	1.60E-08

Legends: [a] Expectation value is an approximation of "the probability that a certain protein candidate is a random hit".

[b] The fold change was calculated using DeCyder software as an 'Average Ratio' by dividing the spot abundance at 144hrs by that at 72hrs. '-' represents down regulation and '+' up regulation of protein at 144hrs of culture compared to 72hrs of culture.

[c]/[d]/[e] represents the proteins identified at multiple spots.

NDE: proteins that are **N**ot **D**ifferentially **E**xpressed at cut-off criteria of at least 1.5 fold up/down regulated with a t-test score ≤0.05.

'---': Not specified

Of the 35 proteins, 12 were observed to be differentially regulated in both temperature-shifted (72hrs at 37^0C vs 144hrs at 31^0C) and standard culture (72hrs at 37^0C vs 144hrs at 37^0C), of which 4 proteins were up regulated and 8 were down regulated at 144hrs of culture in comparison to cultures at 72hrs (Table 3.2.5). These proteins represent the process-dependent changes in expression of proteins in culture. Calponin 3 (CNN3) was 1.96 fold down regulated in cells at 144hrs at 31^0C in temperature-shifted culture and 3.37 fold down regulated in cells at 144hrs at 37^0C in comparison to cells at 72hrs at 37^0C in standard culture. Comparison of expression of CNN3 at 144hrs at 31^0C vs. 144hrs at 37^0C revealed that this protein was 1.72 fold up regulated at 144hrs in cells maintained at 31^0C. Besides CNN3, the expression of proteins between 72hrs and 144hrs was similar in both temperature-shifted (72hrs at 37^0C vs 144hrs at 31^0C) and standard culture (72hrs at 37^0C vs 144hrs at 37^0C) indicating that these changes are time-dependent changes in culture. For example, TXNL2 was 1.8 fold down regulated at 144hrs in both temperature-shifted (72hrs at 37^0C vs 144hrs at 31^0C) and standard culture (72hrs at 37^0C vs 144hrs at 37^0C) compared to cultures maintained at 72hrs.

Table 3.2.5. Differential expression of process-dependent proteins at 144hrs between temperature-shifted and standard culture conditions.

Number	**Protein Description**	**Abbreviation**	**Fold Change in temperature-shifted culture (at 144hrs at 31^0C compared to 72hrs at 37^0C)**	**Fold Change in standard culture (at 144hrs at 37^0C compared to 72hrs at 37^0C)**	**Fold Change in stationary culture (at 144hrs at 31^0C compared to 144hrs at 37^0C)**
1	Calponin 3, acidic	CNN3	-1.96	-3.37	1.72
2	Chain A, Structure Of Bovine Beta-Actin-Profilin Complex	---	-1.59	-1.53	-1.04
3	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa precursor	NDUFS1	2	1.59	1.26
4	Succinate dehydrogenase flavoprotein subunit	SDHA	1.7	1.87	-1.1
5	PREDICTED: similar to Dihydropyrimidinase related protein-2	DPYSL2	2.58	1.97	1.31

6	Eukaryotic translation initiation factor 3, subunit I	EIF3I	-1.72	-2	1.16
7	Eukaryotic translation initiation factor 5A	EIF5A	-1.81	-2.07	1.14
8	PREDICTED: similar to Activator of 90 kDa heat shock protein ATPase homolog 1	AHSA1	-1.69	-1.73	1.02
9	Thioredoxin-like 2	TXNL2	-1.8	-1.8	1
10	Menkes disease gene product	ATP7A	1.79	1.6	1.12
11	FK506 binding protein 4	FKBP4	-2.5	-2.43	-1.02
12	PREDICTED: similar to heat shock protein 8 isoform 3	HSPA8	-1.9	-2.09	1.1

Legends: '---': Not specified

3.2.4.3 Standard culture (72hrs at 37^0C vs. 144hrs at 37^0C)

The protein expression maps (PEMs) for cells at 72hrs at 37^0C were compared with the PEMs for cells maintained for 144hrs at 37^0C to identify differentially expressed proteins. These differentially expressed proteins could be associated with the regulation of various cellular phenotypes such as structure, metabolism, growth and apoptosis in CHO culture.

Of the 53 identified proteins, 30 proteins were differentially regulated in the comparison of cells at 72hrs at 37^0C vs. 144hrs at 37^0C (Figure 3.2.4.3 and Table 3.2.4), of which 9 proteins were up regulated and 21 proteins were down regulated in cells at 144hrs at 37^0C in comparison to cells at 72hrs at 37^0C. Eighteen proteins were specifically differentially regulated in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C). Four from these 18 proteins were up regulated and 14 proteins were down regulated in cells at 144hrs at 37^0C compared to cells at 72hrs at 37^0C. For example, ENO1 a metabolic protein was 1.51 fold up regulated at 144hrs at 37^0C in comparison to cells maintained at 72hrs at 37^0C in standard culture. This protein remained unchanged in the temperature-shifted cultures (72hrs at 37^0C vs. 144hrs at 31^0C). GRB2, a protein that is involved in signal transduction, was 1.72 fold down regulated at 144hrs in cells maintained at 37^0C compared to cells at 72hrs at 37^0C.

The proteins specifically observed differentially expressed in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C) were mainly associated with the regulation of cell structure and metabolism. For example, TUBB2 and CAPZB were reduced by 2.02 fold and 1.5 fold respectively in cells at 144hrs at 37^0C compared to cells at 72hrs at 37^0C in standard culture. ACTB was identified at two different locations in the gel indicating possible post-translational modification of these proteins. ACTB was down regulated in cells at 144hrs at

37^0C by 1.97 and 1.74 fold at both spots compared to cells at 72hrs at 37^0C. All of these proteins, TUBB2, CAPZB and ACTB, are involved in the regulation of cell shape and structure. A number of proteins involved in cellular metabolism proteins were also altered in stationary phase of culture at 144hrs of culture at 37^0C in comparison to cells exponentially growing at 72hrs of culture at 37^0C. For example, ALDH1A1 and PHB were reduced by 1.64 and 1.66 fold respectively in cells at 144hrs at 37^0C in standard culture compared to cells at 72hrs at 37^0C. Proteins involved in the regulation of protein translation were also observed differentially expressed. EIF4E was 1.67 fold up regulated whereas 40S ribosomal protein SA (p40) was 1.9 fold down regulated in cells at 144hrs at 37^0C in comparison to cells at 72hrs at 37^0C.

3.2.4.4 Stationary culture (144hrs at 31^0C vs. 144hrs at 37^0C)

The expression of identified proteins observed in stationary phase induced by low temperature culture (144hrs at 31^0C) were compared to the stationary phase in standard culture (144hrs at 37^0C). This comparison enabled us to identify changes in protein expression that could be associated with cell death and various stress conditions, i.e. nutrient limitation and/or waste accumulation in culture.

Of the 53 identified proteins, 21 were found to be differentially expressed in the stationary phase of culture (144hrs at 31^0C vs. 144hrs at 37^0C) (Figure 3.2.4.3 and Table 3.2.6). In this comparison, 17 proteins were up regulated and 4 were down regulated at 144hrs of culture at 31^0C compared to 144hrs of culture at 37^0C. For example, importin-α was 2.09 fold up regulated in cells in the stationary phase of culture at 144hrs at 37^0C compared to cultures at 144hrs at 31^0C. Importin-α has been associated with the transport of various apoptotic and growth regulating factors, i.e. p53, into the nucleus. CCT2 was 1.74 fold

down regulated in cells in stationary phase at 144hrs at 37^0C compared to cells at 144hrs at 31^0C. CCT2 is a member of molecular chaperone family and has been associated with protein folding including actin and tubulin proteins. The proteins differentially expressed in this comparison were mainly involved in the regulation of cell structure and metabolism. All structural proteins identified in this investigation were up regulated in cells at stationary phase of culture induced by low temperature in comparison to the cells in stationary phase in standard culture. For example, vimentin, CNN3, ACTB, CAPZB and TUBB2 were 1.9, 1.72, 2.2, 1.88 and 1.76 fold up regulated respectively in cells at stationary phase at 144hrs at 31^0C compared to cells at 144hrs at 37^0C. A number of metabolic proteins were also differentially regulated. ALDH1A1 was 1.76 fold up regulated while ENO1 was 1.51 fold down regulated in cells at stationary phase at 144hrs at 31^0C in comparison to cells at stationary phase at 144hrs at 37^0C.

Table 3.2.6. Differential expression of proteins at 144hrs between temperature-shifted and standard culture conditions.

Number	**Protein Description**	Abbreviation	**Fold Change at 144hrs at 31^0C (144hrs at 31^0C vs. 144hrs at 37^0C)**
1	Vimentin	VIM	1.9
2	Calponin 3, acidic	CNN3	1.72
3	Beta actin	ACTB	2.2
4	Capping protein (actin filament) muscle Z-line, beta	CAPZB	1.88
5	Tubulin T beta15	TUBB2B	1.76
6	Aldehyde dehydrogenase family 1, subfamily A1	ALDH1A1	1.76
7	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.64
8	PREDICTED: similar to UDP-N-acetylhexosamine pyrophosphorylase (Antigen X)	AGX	-2.90
9	Acetyl-Coenzyme A dehydrogenase, long-chain	ACADL	1.71
10	PREDICTED: similar to alpha enolase	ENO1	-1.51
11	PREDICTED: similar to ATP synthase beta chain, mitochondrial precursor isoform 1	ATP5B	1.6
12	Annexin A4	ANXA4	1.9

13	PREDICTED: similar to eukaryotic translation initiation factor 4E isoform 1	EIF4E	-2.42
14	PREDICTED: similar to prohibitin	PHB	2.32
15	Lectin, galactose binding, soluble 1	LGALS1	1.56
16	Chain A, Importin Alpha, Mouse	Importin-α	-2.09
17	PREDICTED: similar to von Hippel-Lindau binding protein 1 isoform 2	VBP1	1.51
18	PREDICTED: similar to chaperonin containing TCP1, subunit 2 isoform 2	CCT2	1.74
19	Hypothetical protein LOC499689	---	1.53
20	MHC class II antigen beta chain	MHC II- β	2.08
21	Chain A, Crystal Structure Of The Human Sh3 Binding Glutamic-Rich Protein Like	SH3BGRL	1.56

Legends: '---': Not specified

3.2.5 Validation of proteomics results by Western blotting

Two proteins, vimentin and HNRPC, were selected from the list of differentially expressed identified proteins for validation by Western blotting. RNA binding motif protein 3 (RBM3) is well known to be induced following shift to low temperature. RBM3 is a small protein and was not identified in our study due to limitation of 2D-gel electrophoresis-based methods in detecting low molecular weight proteins. RBM3 was included in this study as a positive control. α-Tubulin was used as a loading control as it was not identified differentially expressed in our study and has been used previously to show equal protein loading. Western blot analysis (Figure 3.2.5) reflected a similar pattern of expression to those observed using 2D-DIGE (Table 3.2.4).

Vimentin was 2.05 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in temperature-shifted culture and not differentially regulated (1.08 fold up) in standard culture (72hrs at 37^0C vs. 144hrs at 37^0C) from 2D-DIGE analysis. Densitometry analysis of Western blot data revealed that vimentin was 1.9 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in temperature-shifted culture and 1.11 fold down regulated in cells at 144hrs at 37^0C compared to cells at 72hrs at 37^0C in standard culture.

HNRPC was reduced following temperature-shift by 1.52 fold in cultures at 144hrs at 31^0C in comparison to cultures maintained at 72hrs at 37^0C and by 1.26 fold in cultures at 144hrs at 37^0C compared to cultures maintained standard culture at 72hrs at 37^0C from 2D-DIGE analysis. The densitometry analysis of Western blot data showed that HNRPC was 1.33 fold down regulated in cells at 144hrs at 31^0C and 1.07 fold down regulated in cells at 144hrs at 37^0C compared to cells at 72hrs at 37^0C.

The slight difference observed in the expression of vimentin and HNRPC using 2D-DIGE and Western blotting could be due to the difference in the sensitivity of both techniques, i.e. Western blotting is only a semi-quantitative technique in comparison to 2D-DIGE. RBM3 was only expressed in cultures maintained at low temperature (144hrs at 31^0C) confirming the expected induction of expression of this protein following temperature-shift.

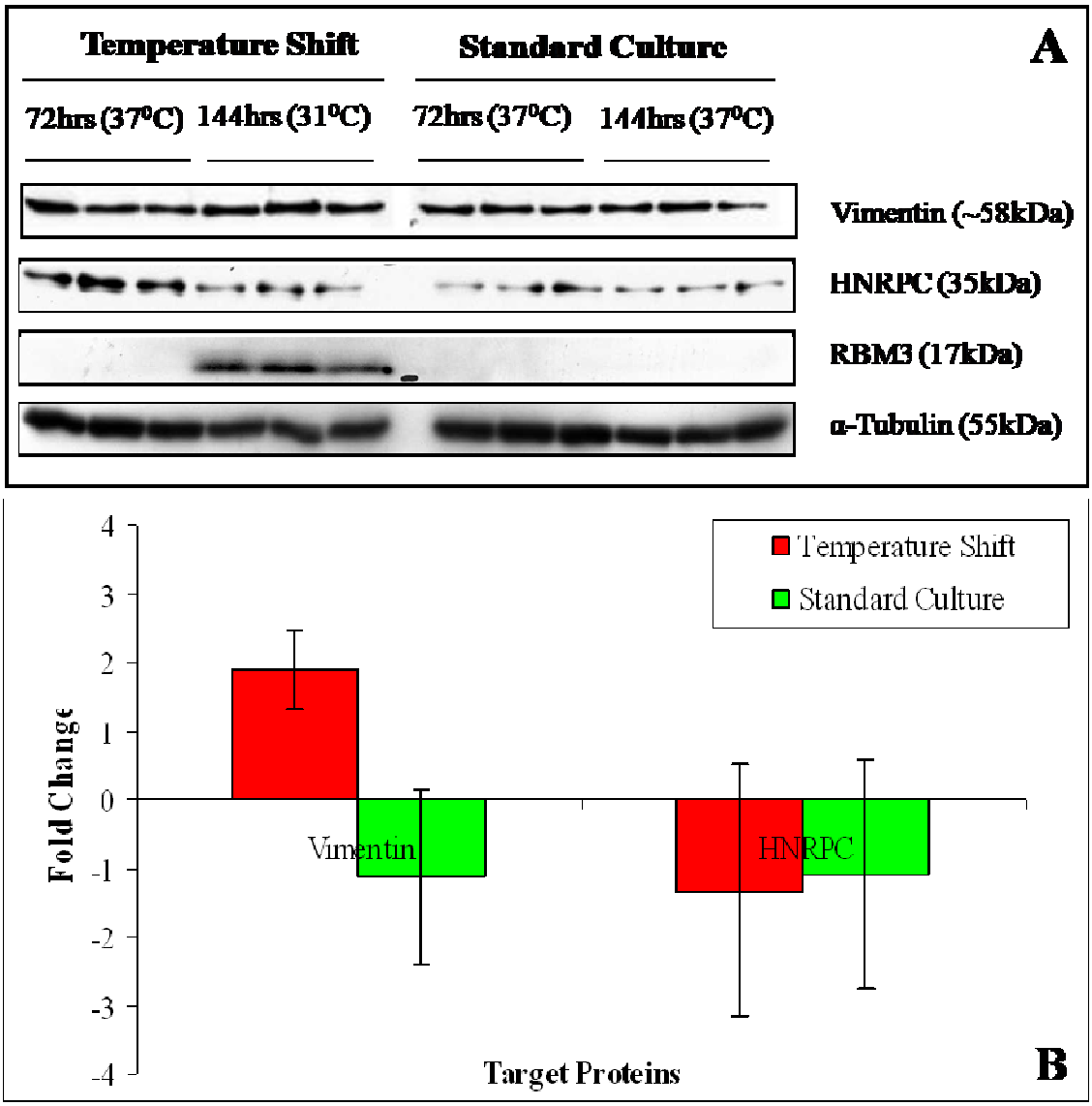

Figure 3.2.5. Comparative Western blot analysis for HNRPC, vimentin and RBM3 proteins between 72hrs and 144hrs of culture in temperature-shifted and standard culture (A). α-tubulin was used as a loading control. The graph indicates the fold change, derived from normalized intensities of bands from densitometry analysis, of vimentin and HNRPC between 72hrs and 144hrs of culture (B). Error bars represent the standard deviation calculated from normalized intensities of bands from three biological replicate samples for vimentin and HNRPC.

3.2.6 Protein target selection for knockdown and siRNA design

Five protein targets (EIF4A, HNRPC, importin-α, LGALS1 and PFN2) were selected for functional validation based on their expression pattern observed in proteomics study and/or potential impact on cellular phenotypes, i.e. cell proliferation, apoptosis and cell structure, etc, that are observed at low temperature.

- EIF4A is a RNA helicase enzyme that catalyzes the unwinding of messenger RNAs (mRNAs) and facilitates cap-dependent and independent translation. EIF4A was 1.81 fold up regulated in cells at 144hrs at 31^0C following temperature-shift in comparison to cells at 72hrs at 37^0C which suggests its potential role in the regulation of protein translation at low temperature.
- HNRPC, a nuclear pre-mRNA binding protein, can regulate pre-mRNA processing and can contribute to the regulation of cell proliferation in culture. HNRPC was 1.52 fold down regulated in cells at 144hrs at 31^0C in comparison to cells at 72hrs at 37^0C and could have a role in the regulation of cell growth and protein translation at low temperature.
- Importin-α is a nuclear import receptor that transports proteins associated with various biological functions, i.e. apoptosis and cell proliferation, in cells. The expression of importin-α was reduced by 1.8 fold in cells at 144hrs at 31^0C following temperature-shift compared to cells at 72hrs at 37^0C. This suggests that importin-α could be associated with reduced cell growth and improved viability in culture at low temperature.
- LGALS1, a cell adhesion protein, acts as a negative regulator of growth in fibroblasts. LGALS1 was 1.62 fold up regulated at 144hrs of culture at 31^0C compared to 72hrs of

culture at 37^0C in temperature-shifted cultures which suggests its potential role in the regulation of cell growth at low temperature.

- PFN2 is involved in the regulation of actin polymerization in response to extracellular signals, including the low temperature inductions. PFN2 was increased by 1.69 fold in cells at 144hrs at 31^0C following temperature-shift compared to cells at 72hrs at 37^0C and could be associated with the maintenance of cell structure and viability in culture at low temperature.

The full genome sequence of hamster is not yet publicly available. As a result, the siRNAs for selected protein targets are not commercially available for CHO-K1 cells. However, partial sequence information can be used to design siRNAs to the selected proteins. Therefore siRNAs were designed by sequencing the fragments of coding regions amplified using PCR primers against orthologus gene sequences.

3.2.6.1 Primer design

Sequences of orthologous genes from other mammalian species were aligned using ClustalW (multiple sequence alignment software). These species included human, rat, mouse, chicken, chimpanzee, cow, horse, rhesus monkey, pig and rabbit. Conserved regions in the sequences were identified and used to design primers for PCR (Table 3.2.6.1).

Table 3.2.6.1. PCR primers for target proteins identified from proteomic study

Target Protein	Forward Primer	Reverse Primer
EIF4A	ggcgtcatcgagagtaactg	accggaactccctcatgatc
HNRPC	acgatctgcagcggagatgt	ctatgtgcttaagagtcatc
Importin-α	attcagtttgaatctgcttg	ctctgcagcctgaaagatat
LGALS1	ggtctggtcgccagcaacct	tctggcagcttgatggtcag
PFN2	gactgcacaatggacatccg	tctgagctactgcaatga

3.2.6.2 RT-PCR amplification of fragments coding DNA sequences for target proteins

Reverse transcription was performed using the reverse primer to generate the cDNAs for target genes from extracted RNA. cDNA was then used as a template for PCR amplification. PCR products were separated on a 2% (w/v) agarose gel to ensure specific amplification of the fragments of interest. As expected bands at approximately 898, 636, 951, 299 and 1209 bp were observed confirming the amplification of fragments of EIF4A, HNRPC, importin-α, LGALS1 and PFN2 respectively (Figure 3.2.6.1).

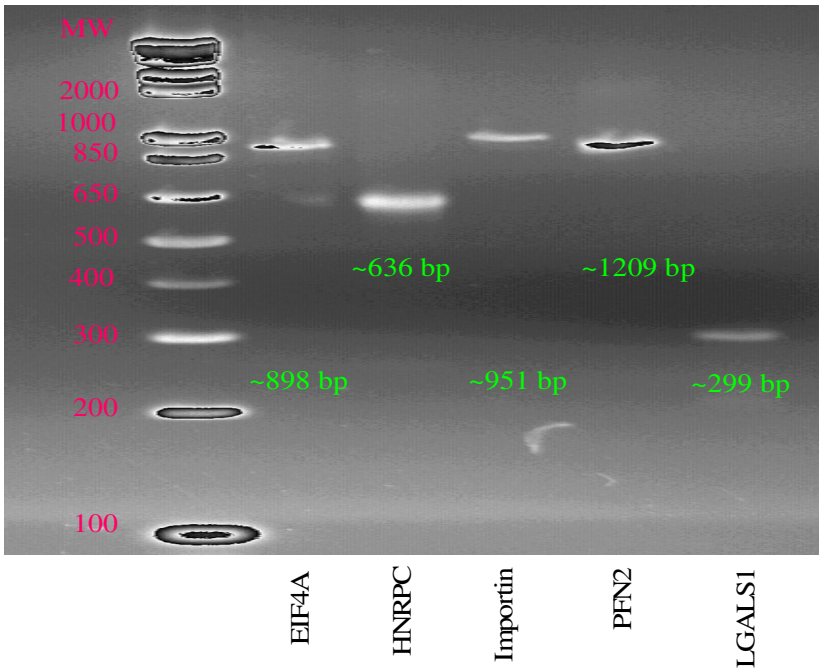

Figure 3.2.6.1. Investigation of amplification of fragments coding DNA for protein targets using RT-PCR product on 2% agarose gel. The expected molecular weight for each target has been included in green.

The purified cDNA fragments were sent out for sequencing (Eurofins MWG Operon). Of the 5 targets, 3 targets, EIF-4A, HNRPC and PFN2, were successfully sequenced (Figure 3.2.6.2).

>EIF4A-CHO
GAGTAACTGGAATGAGATTGTGGATAGCTTTGATGACATGAATCTCTCAGAATCCCTCCT
CCGTGGTATTTATGCCTATGGTTTTGAGAAGCCCTCTGCCATCCAGCAGCGAGCCATTCT
TCCTTGTATCAAGGGTTATGATGTGATTGCTCAAGCCCAGTCTGGGACTGGGAAAACAGC
TACATTTGCCATATCAATTCTGCAGCAGATTGAGTTAGATCTTAAGGCCACTCAGGCTTT
GGTTCTGGCCCCCACTCGTGAGTTGGCTCAGCAGATTCAAAAGGTGGTCATGGCATTAGG
AGACTACATGGGTGCCTCTTGTCATGCCTGTATTGGAGGCACTAATGTGCGTGCTGAGGT
GCAGAAGTTGCAGATGGAAGCTCCCCATATCATCGTGGGCACCCCTGGCCGGGTGTTTGA
TATGCTTAACCGGAGATACCTGTCCCCCAAATACATCAAGATGTTTGTACTGGATGAAGC
AGATGAAATGTTAAGCCGTGGGTTCAAGGACCAGATATATGATATATTCCAAAAGCTCAA
CAGTAACACACAGGTAGTTTTGTTGTCTGCTACAATGCCCTCAGATGTCCTTGAGGTGAC
CAAGAAGTTCATGAGAGACCCCATTCGGATTCTTGTCAAGAAAGAAGAGTTGACCCTGGA
GGGTATCCGTCAATTCTACATCAATGTGGAAAGAGAGGAGTGGAAGCTTGACACATTGTG
TGACTTGTATGAAACCCTGACTATCACCCAAGCAGTAATCTTTATCAATACCAGAAGGAA
GGTGGATTGGCTCACTGAAAAGATGCATGCCCGGGATTTCACTGTTCCGCCATGCATGAG
AT

>HNRPC-CHO
TGACTTTAGCGGGATTACTATGACAGGATGTACAGTTACCCAGCACGTGTTCCTCCTCCT
CCTCCTATTGCTCGAGCTGTGGTGCCTTCTAAACGCCAGCGTGTTTCTGGAAACACCTCT
CGAAGGGGCAAAAGTGGATTTAATTCAAAGAGTGGACAGCGGGGATCATCATCCAAGTCT
GGAAAGTTGAAAGGAGATGACCTTCAAGCCATTAAGAAGGAGCTGACTCAGATAAAACAA
AAAGTGGATTCTCTTCTGGAAAGCCTAGAAAAAATTGAAAAGGAACAGAGCAAACAAGCA
GACTTGTC

>PFN2-CHO
AACCAACCTTTATCTGGCATCATAATTGCAGCACAATAATGATTTGCATGATATCTTGAA
ATTGGGGAAGGGGGCATGCCGAGTTGGGCATCACTTTGTCTTAGCAATTAATGGGATACT
GATTACTAAAATAAGTTAATATTAAGCAAGGTGCCAGTTGTACAATCTCTAATTTGATCA
ATGTCTTTTCAGCACTTTGAGCATTTACTTGGCTCATTTAGTCTTCCTTTTGTAGCGCAT
GGTTGGGAGGAAAAAGTGCATGCATCTTTACTTCACTCTTTTTTCATCCCCTCCCTTTGA
ACATGAGGTATTTGGTTTGCTTCCATTCTCTTTTGTGTAGTGCCTGGTTTATTTAACCTA
ATTAATACCTCCTTTGTTGATGAGCTATTGAAAGCTGCAGTGATTTGCTTTTAATACTAT
GATTGCACTTGAGACAGACTATTTGTAGTGTCTGTAGGATATGAAGAGTGCAACTGTCAA
GAGCAAAGGCATTTCCTCCCATGACCTTACTACAGTAACCATACTATC

Figure 3.2.6.2. The sequence information for the amplified fragments of EIF4A, HNRPC and PFN2.

3.2.6.3 Analysis of cDNA sequences

To confirm the specificity of target amplification, the CHO cDNA sequences were aligned using ClustalW with the respective orthologous sequences from other mammalian species (Figure 3.2.6.3, 3.2.6.4 & 3.2.6.5). Multiple sequence alignment showed that the sequence information generated for CHO cells contained high similarity with respective orthologous

sequences tested and was suitable for siRNA design. Two siRNA molecules for each target were designed using *Silencer Select Algorithm*® by Applied Biosystems.

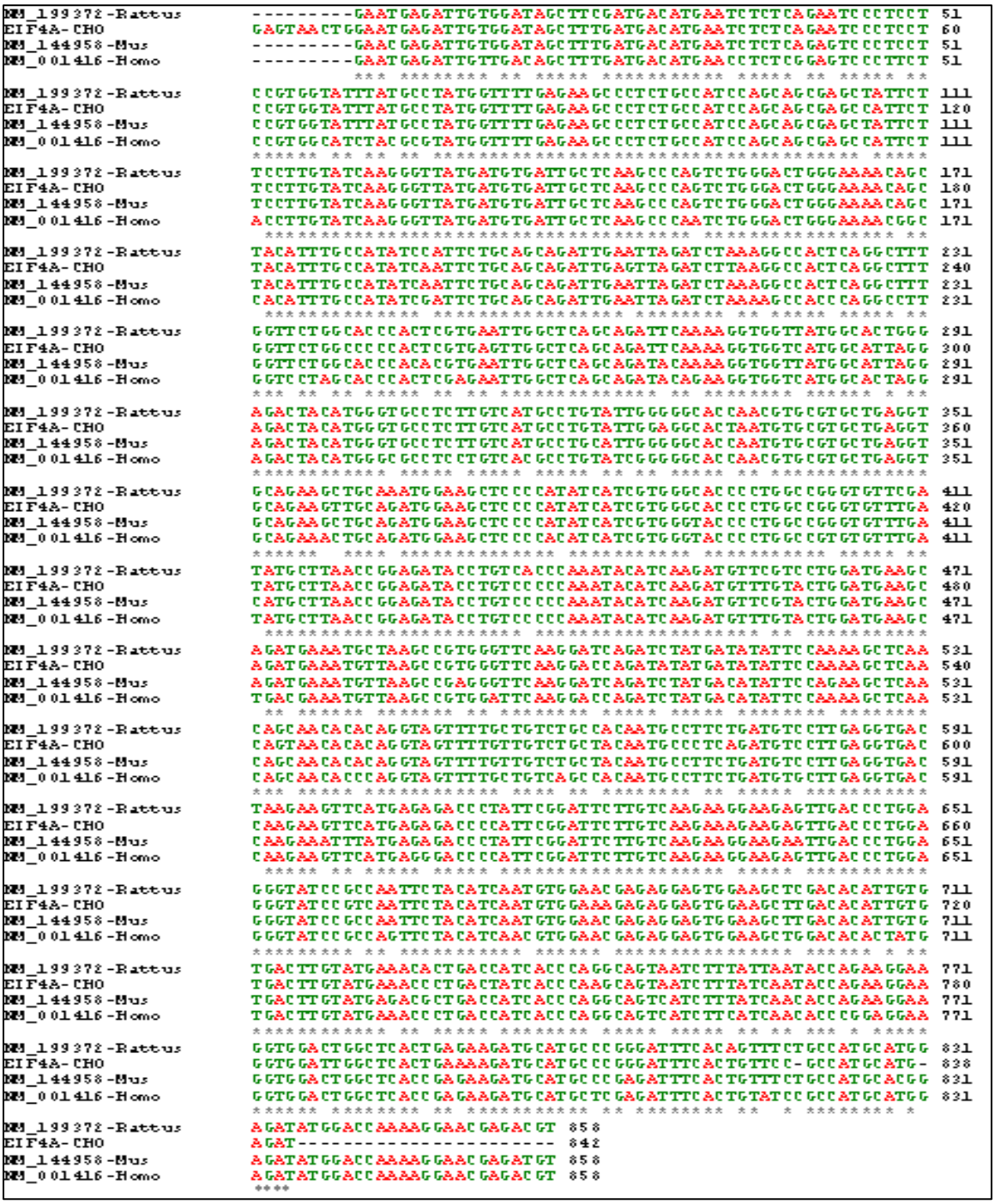

Figure 3.2.6.3. Analysis of multiple orthologous sequence alignment for EIF4A.

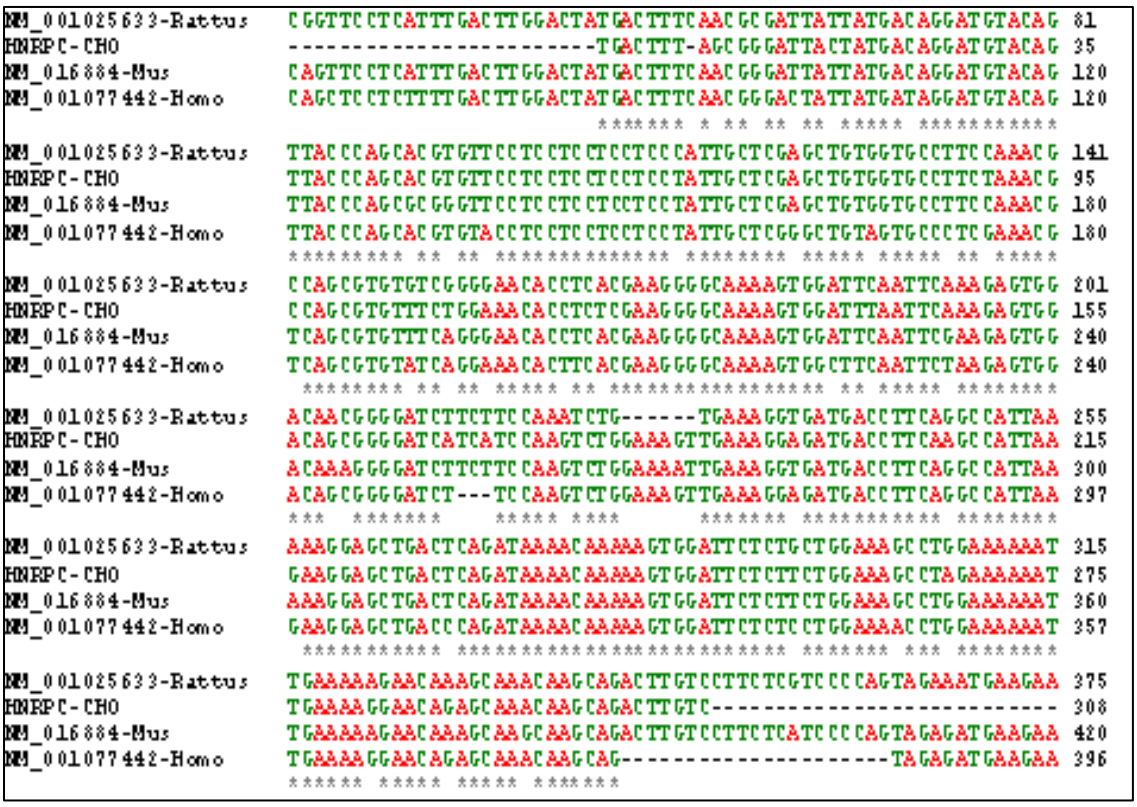

Figure 3.2.6.4. Analysis of multiple orthologous sequence alignment for HNRPC.

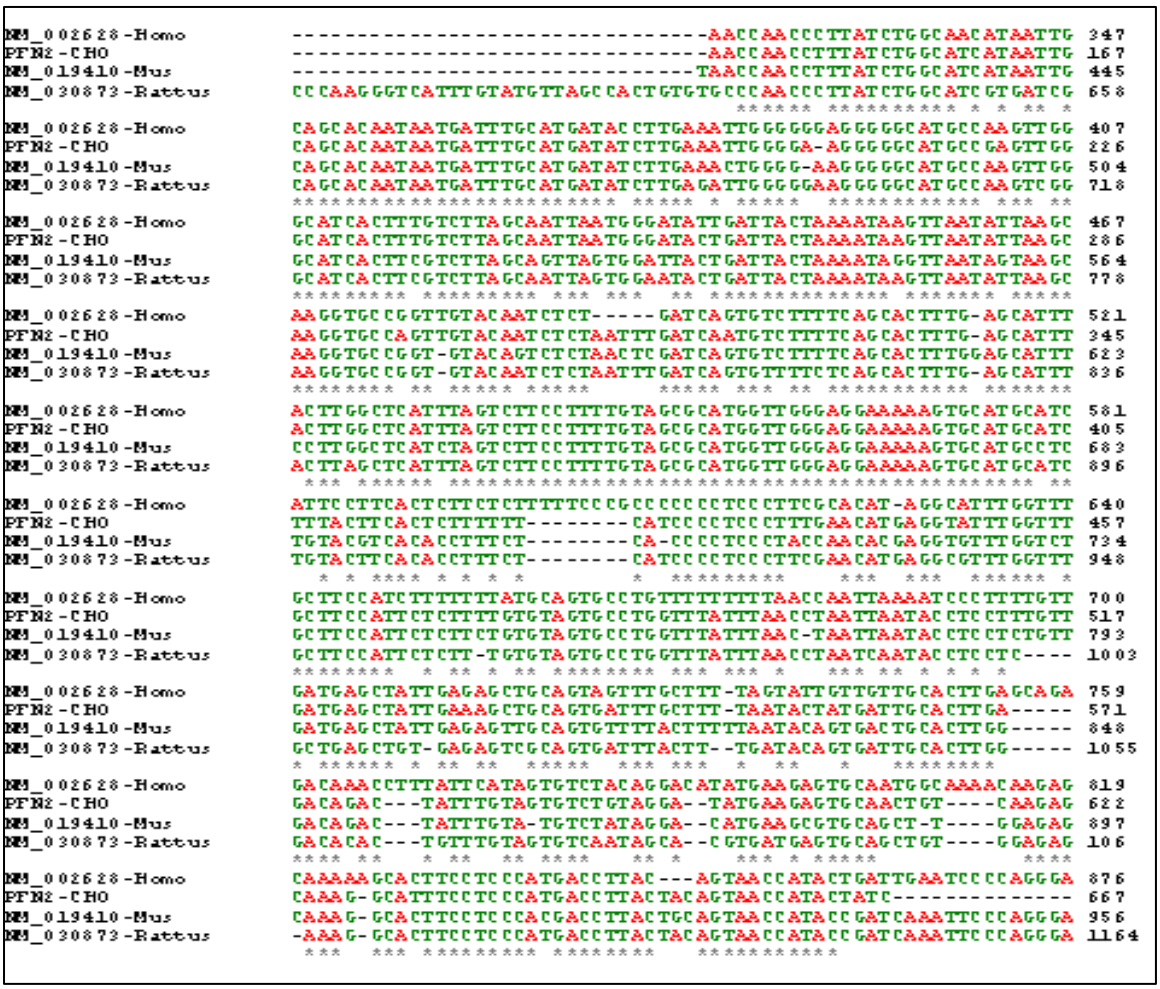

Figure 3.2.6.5. Analysis of multiple orthologous sequence alignment for PFN2.

3.2.7 Effect of silencing protein targets using siRNA knockdown

siRNAs can be transfected to the cells to investigate the effects of selected protein target on desired cellular phenotype by specifically inhibiting the expression of selected target. Therefore siRNAs for EIF4A, HNRPC and PFN2 were transfected into the SEAP-secreting CHO-K1 cells and were evaluated for their effects on cell growth and productivity. Results indicate that the inhibition of these targets can reduce cell growth and improve cell specific productivity in culture.

3.2.7.1 Optimization of siRNA transfection conditions in non-producer suspension-adapted CHO-K1 cells

Optimization of siRNA transfection conditions and initial screening for the effects of knockdown of each target on cell growth and viability was performed in non-producer, suspension and low serum adapted CHO-K1 cells. All transfections were performed in biological triplicates using spin tubes containing $1x10^5$ cells/mL in 2mL final volume. Two independent siRNA molecules for each target were transfected to ensure target-specific effects using neoFX, a lipid-based transfection agent. NeoFX treated cultures were considered as controls for this investigation as no validated siRNA control was available for CHO cells. Un-treated cultures were also monitored to differentiate effects of transfection reagent on these cells. The cultures were monitored at various time points over 144hrs. Figure 3.2.7.1 shows timing of transfection and sampling to measure the impact of siRNA transfection on cell growth. Cell counts and viability measurements were performed by flow cytometry using *Guava ViaCount*® Assays (Section 2.7.8.2). This allowed us to identify the optimal time point to investigate the effects of target knockdown for further studies.

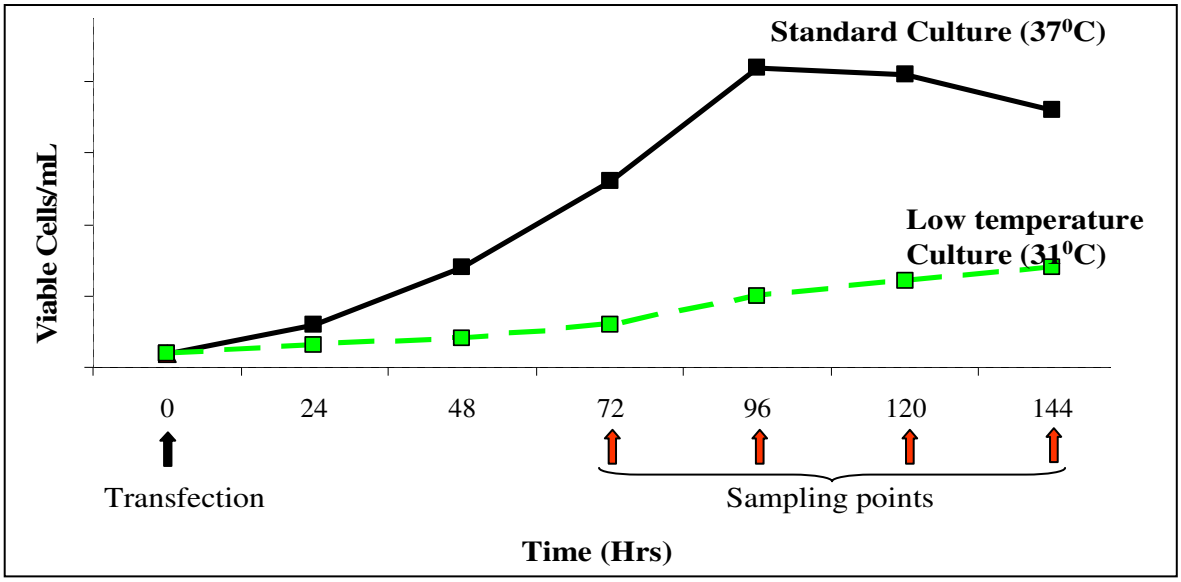

Figure 3.2.7.1 A representative figure of transfected culture showing time of transfection and sampling points for cell growth measurements. All transfection were performed using 2mL of $1x10^5$ cells/mL and cells were subsequently maintained at 37^0C or 31^0C for 144hrs of culture. Samples were collected at every 24hrs after 72hrs of transfection to investigate the effect of transfections on cell growth.

3.2.7.1.1 EIF4A

EIF4A facilitates cap-dependent and independent protein translation and due to this inhibition of EIF4A could be expected to reduce growth and/or viability of culture due to reduced global protein translation. EIF4A is also involved in blocking cell cycle transition and therefore knockdown of EIF4A could be expected to facilate cell-cycle and hence improve cell growth.

EIF4A was 1.81 fold up regulated in cells at 144hrs at 31^0C following temperature-shift in comparison to cells at 72hrs at 37^0C in the proteomics study and therefore siRNA transfection was performed at low temperature (LT, 31^0C). The inhibition of eIF4A at LT was expected to mimic the growth behaviour of cells maintained at standard culture.

Transfections were performed using 2mL of $1x10^5$ cells/mL. Following transfections, cells were maintained at 31^0C for 144hrs of culture. NeoFX-treated cultures were used as controls in this investigation, however un-treated cultures were also monitored. Impact of siRNA transfection on cell growth were measured at every 24hrs time interval after 72hrs of transfection over the period of 144hrs of culture (Figure 3.2.7.1). The cell growth was reduced by up to 75% in comparison to the neoFX-treated control cultures, whereas the cell viability of cultures remained unchanged following siRNA transfection (Figure 3.2.7.2C&D and Table 3.2.7.1). This indicates that inhibition of EIF4A might have reduced the global protein translation in culture.

3.2.7.1.2 HNRPC

HNRPC, a pre-mRNA processing protein, can facilitate cell proliferation and hence inhibition of HNRPC was expected to reduce growth of culture.

HNRPC was 1.52 fold down regulated at 144hrs of culture at 31^0C compared to 72hrs of culture at 37^0C in the 2D-DIGE experiment and therefore to see if we could mimic the effect of temperature-shift on growth, cells were maintained at 37^0C after transfection. Transfections were performed using 2mL of $1x10^5$ cells/mL and cells were subsequently maintained at 37^0C for 144hrs of culture. NeoFX-treated cultures were used as controls in this investigation. Un-treated cultures were also monitored. Cell growth was monitored at every 24hrs time interval after 72hrs of transfection over a period of 144hrs (Figure 3.2.7.1). Following knockdown of HNRPC, cell growth was reduced (Figure 3.2.7.2A and Table 3.2.7.1). The maximum growth inhibition (48%) was observed at 96hrs of culture, however cells recovered at the end of culture (at 144hrs). The viability of culture was

comparable to neoFX treated control cultures through out the testing period (Figure 3.2.7.2B and Table 3.2.7.1). Results indicate that down regulation of HNRPC can reduce cell growth.

3.2.7.1.3 PFN2

PFN2 is involved in the regulation of cytoskeleton in response to various stress conditions including thermal stresses and therefore inhibition of PFN2 was expected to reduce growth and viability of culture due to distortion in the cytoskeleton of cells.

PFN2 were 1.69 fold up regulated in cells at 144hrs at 31^0C following temperature-shift compared to cells maintained at 72hrs at 37^0C in the proteomics study following temperature-shift and therefore transfection was performed at LT. The inhibition of PFN2 expression at LT was expected to mimic the growth behaviour of cells maintained at standard culture. Transfections were performed using 2mL of $1x10^5$ cells/mL. Following transfections, cells were maintained at 31^0C for 144hrs of culture. NeoFX-treated cultures were used as controls in this investigation. Un-treated cultures were also monitored. Impact of siRNA transfection on cell growth were measured at every 24hrs time interval after 72hrs of transfection over the period of 144hrs of culture (Figure 3.2.7.1). Cell growth was reduced up to 74% for siRNA 1 following knockdown of PFN2 (Figure 3.2.7.2C and Table 3.2.7.1). A pattern of reduced cell growth was also observed for siRNA 2, however it was not significant. A drop in the viability was also observed for the siRNA 1 treated culture (Figure 3.2.7.2D and Table 3.2.7.1). Results confirmed that down regulation of PFN2 reduced the growth and viability of culture.

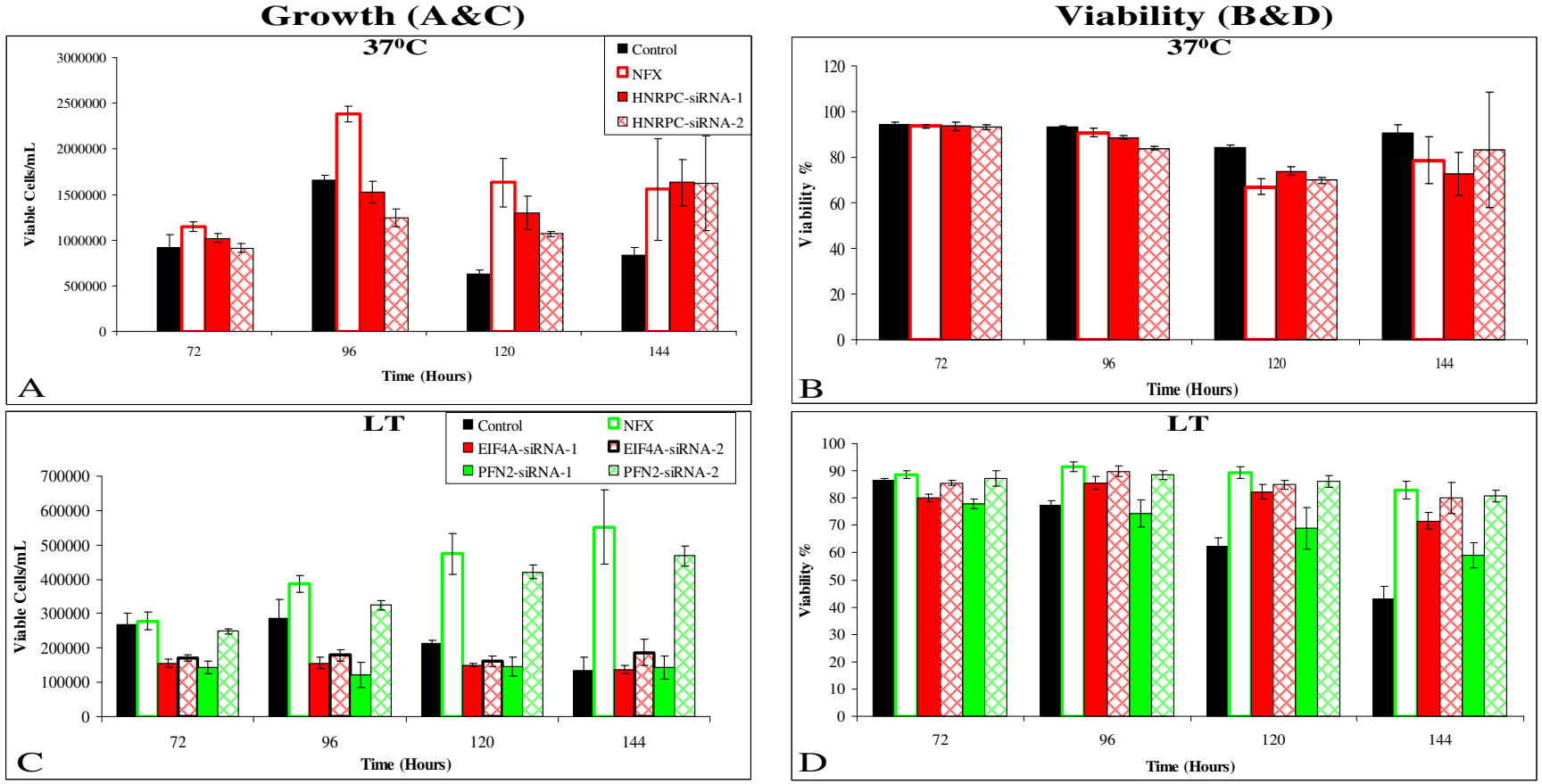

Figure 3.2.7.2. Investigation in to the effects of siRNA transfection targeting HNRPC in standard culture (37^0C) and EIF4A and PFN2 at low temperature 31^0C (LT) on growth and viability of low serum-adapted CHO-K1 cells. A & C represent growth and B & D represent viability of CHO-K1 cells during 144hrs of culture after siRNA transfection. Control denotes un-treated cultures. NFX denotes transfection reagent only treated culture.

Table 3.2.7.1. Comparative analysis of effects of siRNA transfection for protein targets on growth and viability of low serum adapted CHO-K1 cells at standard and low temperature cultures. This data was calculated (in percentage) from Figure 3.2.7.2. by comparing neoFX-treated control cultures (NFX) with siRNA treated cultures. Shaded columns represent the optimized conditions selected for inhibition of expression of target proteins in SEAP-secreting CHO-K1 cells.

Protein target	**Culture Condition**	**Time (Hrs)**	**Cell growth**				**Viability**			
			siRNA 1 (%)	**p-value**	**siRNA 2** (%)	**p-value**	**siRNA 1** (%)	**p-value**	**siRNA 2** (%)	**p-value**
EIF4A	Low temperature culture (31^0C)	72	-44	0.002	-39	0.002	-10	0.002	-4	0.032
		96	-60	0.0002	-54	0.0003	-6	0.02	-2	0.37
		120	-68	0.0007	-66	0.0009	-8	0.02	-5	0.044
		144	-75	0.003	-66	0.005	-14	0.01	-3	0.5
HNRPC	Standard culture (37^0C)	72	-11	0.04	-21	0.004	0	0.96	0	0.63
		96	-36	0.0006	-48	0.0001	-2	0.13	-8	0.004
		120	-20	0.15	-34	0.02	+10	0.04	+4	0.25
		144	+5	0.84	+4	0.89	-7	0.52	+6	0.79

PFN2	Low temperature culture (31^0C)	72	-49	0.002	-11	0.12	-12	0.001	-2	0.44
		96	-69	0.0005	-16	0.02	-19	0.005	-3	0.10
		120	-69	0.001	-11	0.22	-23	0.01	-4	0.12
		144	-74	0.003	-15	0.26	-29	0.0017	-3	0.39

In general, the cultures at 96hrs after transfection represented the most efficient (or comparable to other tested time points) effects on growth and viability of culture and therefore was considered as the optimal time point for the investigation of the effects of knockdown of all protein targets in SEAP secreting CHO-K1 cells (Table 3.2.7.1). A significant difference in growth and vibility of neoFX treated culture compared to untreated culture was also observed highlighting the requirement for an appropriate scambled control in future siRNA transfection studies.

3.2.7.2 Effect of knockdown of protein targets on SEAP-secreting suspension-adapted CHO-K1 cells

Suspension and low serum-adapted SEAP-secreting CHO-K1 cells were transfected with siRNAs against EIF4A, HNRPC and PFN2. After transfection cells were maintained in two culture conditions, standard culture for 96hrs at 37^0C or shifted to 31^0C for a further 72hrs after 24hrs of culture at 37^0C (Figure 3.2.7.3). Cells were analysed for the effects of siRNAs on growth, viability and SEAP productivity after 96hrs of culture (Sections 2.5.7 and 2.8.2). Human scrambled (non-sense) siRNA was included as a control to ensure that the effects observed following transfection are only due to the effect of inhibition of protein targets and are not due to RNA delivery in the cells. The use of a scrambled siRNA designed for human cells is an imperfect control but in the absence of a hamster genome sequence, it was an appropriate choice. NeoFX-treated cultures were also monitored as negative controls. The observations that scrambled siRNAs had similar effects on cellular phenotypes compared to neoFX-only suggests that human scrambled siRNA could be used as negative control in these experiments, however further studies needed to confirm it.

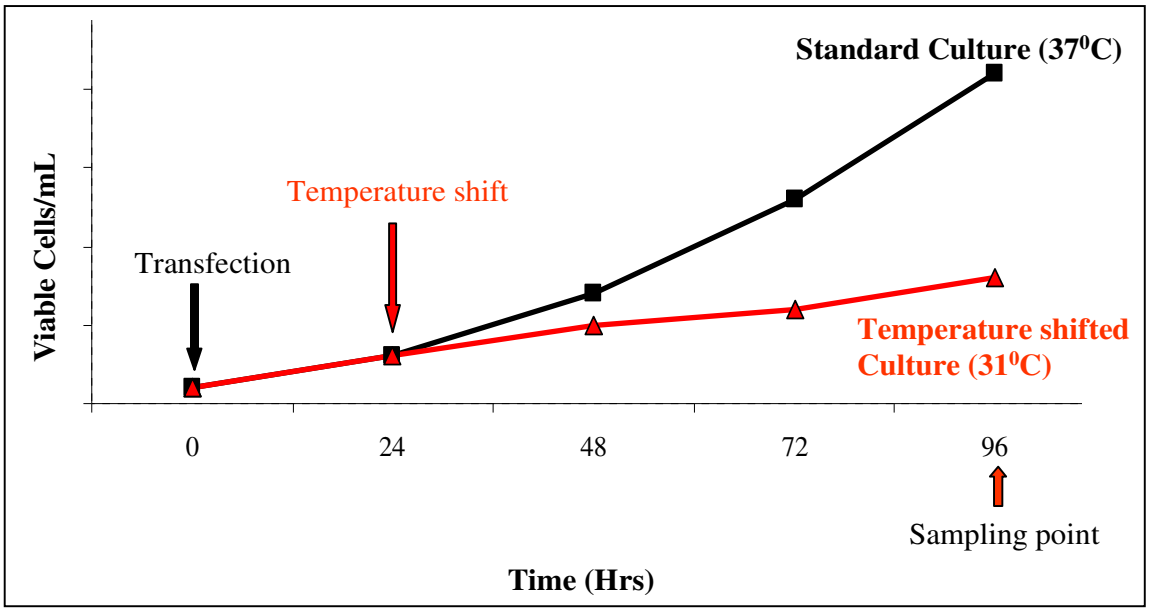

Figure 3.2.7.3. A representative figure of transfected culture showing time of transfection, temperature-shift and sampling points for cell growth measurements. All transfections were performed using 2mL of $1x10^5$ cells/mL and subsequently cells were maintained in standard culture for 96 hrs at 37^0C or shifted to 31^0C for 72hrs after 24hrs of transfection at 37^0C. Samples were collected at 96hrs post-transfection to investigate the effect on growth and productivity of cells.

3.2.7.2.1 EIF4A

The siRNA transfections were performed using 2mL final volume containing $1x10^5$ cells/mL and cells were subsequently maintained in two culture conditions, standard culture for 96hrs at 37^0C or shifted to 31^0C for a further 72hrs after 24hrs of culture at 37^0C (Figure 3.2.7.3). The srambled siRNA-treated cultures were used as a control in this investigation. Un-treated and neoFX-treated cultures were also monitored. The impact of siRNA transfection were measured after 96hrs of transfection. Cell growth was reduced by up to 55% following the inhibition of expression of EIF4A whereas the viability of cultures did not change significantly for either standard or biphasic cultures (Figure 3.2.7.4 and Table 3.2.7.2). This is consistant with the effects observed with the non-producer CHO-K1 cells (Figure 3.2.7.2 and Table 3.2.7.1). The total productivity was also significantly reduced in temperature-shifted and non-shifted cultures (Figure 3.2.7.5 and Table 3.2.7.2). EIF4A is a translation initiation factor and knockdown of EIF4A, apart from reducing cell growth, could be expected to inhibit specific productivity of cells. A pattern of improved cell specific productivity (Qp) was observed, however it was significant only for siRNA1 in temperature-shifted culture and for siRNA 2 at standard culture.

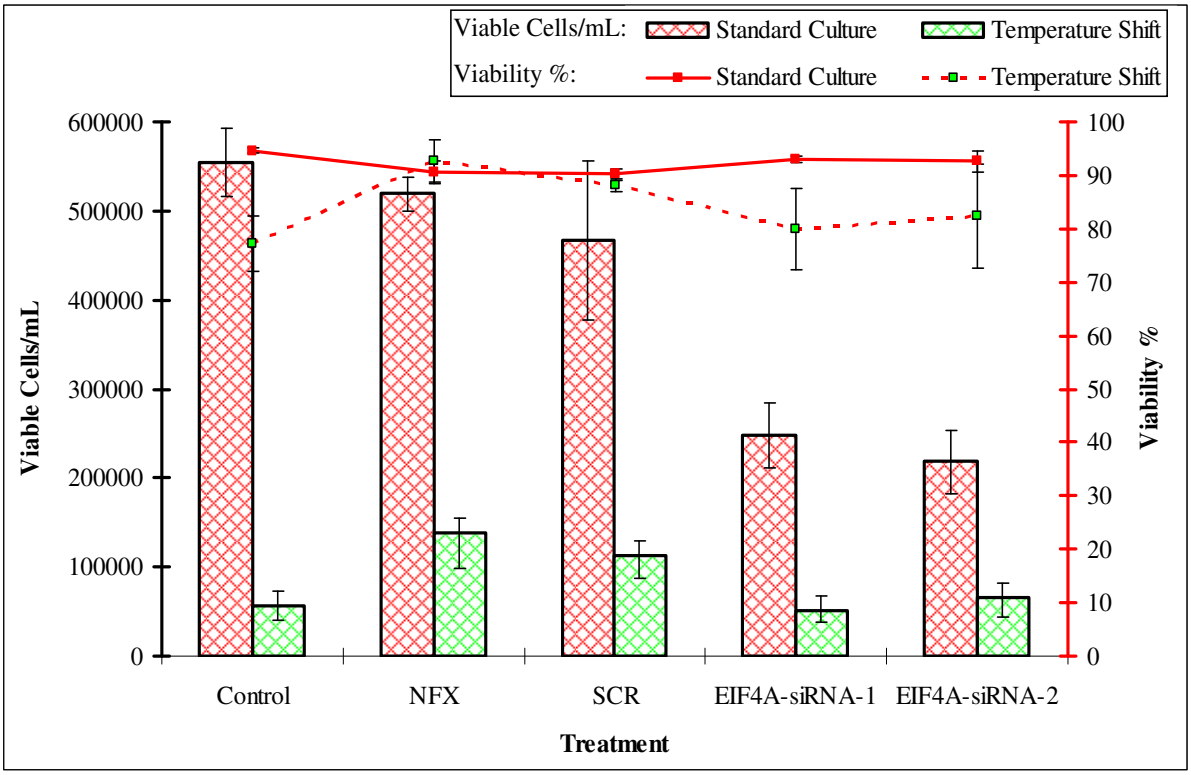

Figure 3.2.7.4. Investigation of the effects of siRNA knockdown of EIF4A on growth and viability of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of EIF4A were measured after 96hrs of transfection. Columns denote viable cells and superimposed red lines show viability of culture. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

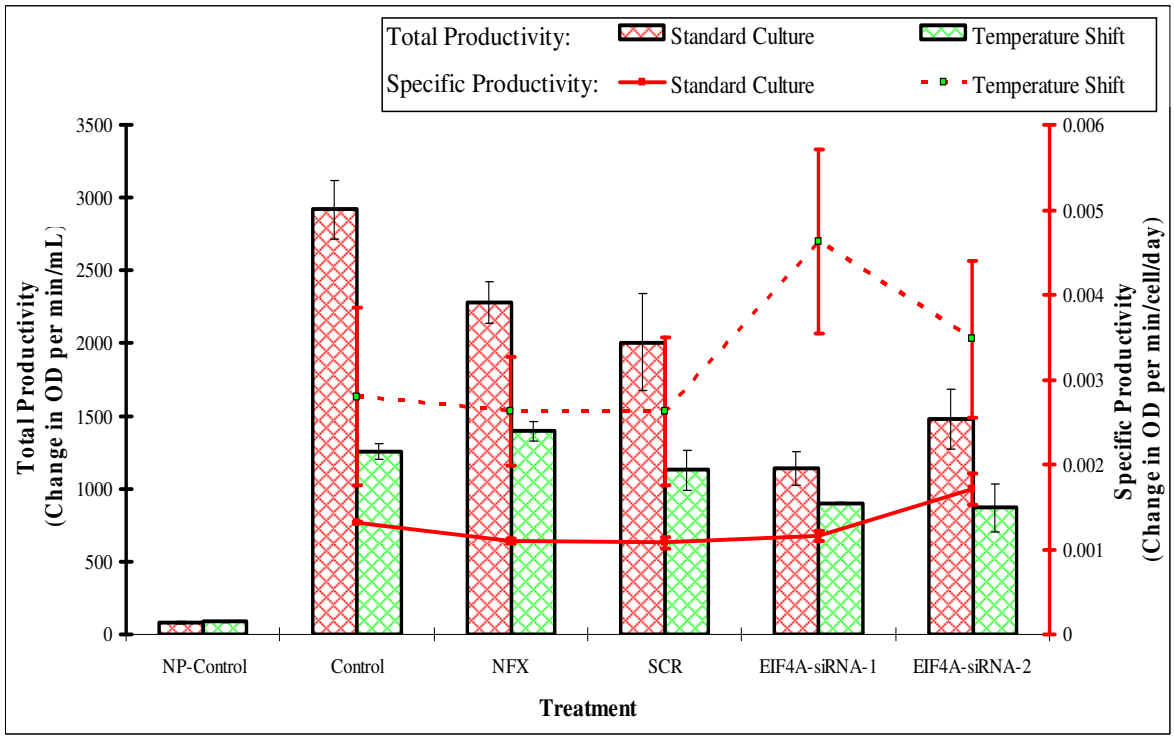

Figure 3.2.7.5. Investigation of the effects of siRNA knockdown of EIF4A on total and specific productivity of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of EIF4A were measured after 96hrs of transfection. Columns denote total productivity and superimposed red lines show specific productivity. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. NP Control represents samples collected from non-producer CHO-K1 cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

Table 3.2.7.2. Comparative analysis of the effects of knockdown of EIF4A on the growth, viability, and total and specific productivity of SEAP secreting CHO-K1 cells in standard and temperature-shifted cultures after 96hrs of transfection. This data was calculated (in percentage) from Figures 3.2.7.4 and 3.2.7.5 by comparing human scrambled treated control cultures (SCR) with siRNA treated cultures. '+' represents increase and '-' represents decrease in effect. Shaded columns represent the significant effects (20% decrease or increase in effect with p-value ≤ 0.05).

Phenotype	siRNA Molecule	Standard Culture		Temperature-shifted Culture	
		Effect (%)	p-value	Effect (%)	p-value
Growth	1	-47	0.005	-55	0.007
	2	-53	0.003	-42	0.035
Viability	1	+3	0.013	-9	0.13
	2	+3	0.13	-7	0.36
Total Productivity	1	-43	0.004	-20	0.02
	2	-26	0.038	-23	0.05
Specific Productivity	1	+7	0.14	+76	0.03
	2	+58	0.001	+32	0.2

3.2.7.2.2 HNRPC

Cells ($1x10^5$ cells/mL) were transfected using 2mL final volume and were maintained in standard culture for 96hrs at 37^0C or shifted to 31^0C for a further 72hrs after 24hrs of culture at 37^0C (Figure 3.2.7.3). The srambled siRNA-treated cultures were used as a control. Un-treated and neoFX-treated cultures were also monitored. The growth and productivity of culture were measured after 96hrs of transfection. As expected a pattern of reduced cell growth in standard culture was observed following the inhibition of expression of HNRPC (Figure 3.2.7.6 and Table 3.2.7.3). It was significant only in the case of siRNA 2 treated cultures. On the other hand, growth in temperature-shifted culture remained unaffected following the inhibition of HNRPC and the reason for this could be that temperature-shifted cultures did not grow enough to show the effects of HNRPC knockdown. The viability of cultures was also comparable to control cultures (SCR as well as NFX) after transfection in both standard and temperature-shifted cultures. HNRPC, which was 1.52 fold down regulated following temperature-shift, was also expected to improve SEAP productivity of CHO cells. The total production of SEAP was not affected significantly in standard culture but was improved in temperature-shifted cultures, 54% for siRNA 1 and 83% for siRNA 2 (Figure 3.2.7.7 and Table 3.2.7.3). As expected, a pattern of improved specific productivity was observed following knockdown of HNRPC. It was improved by 48% for siRNA 1 treated cultures at 37^0C and by 124% and 97% for siRNA 2 treated cultures at 37^0C and in biphasic cultures (Table 3.2.7.3).

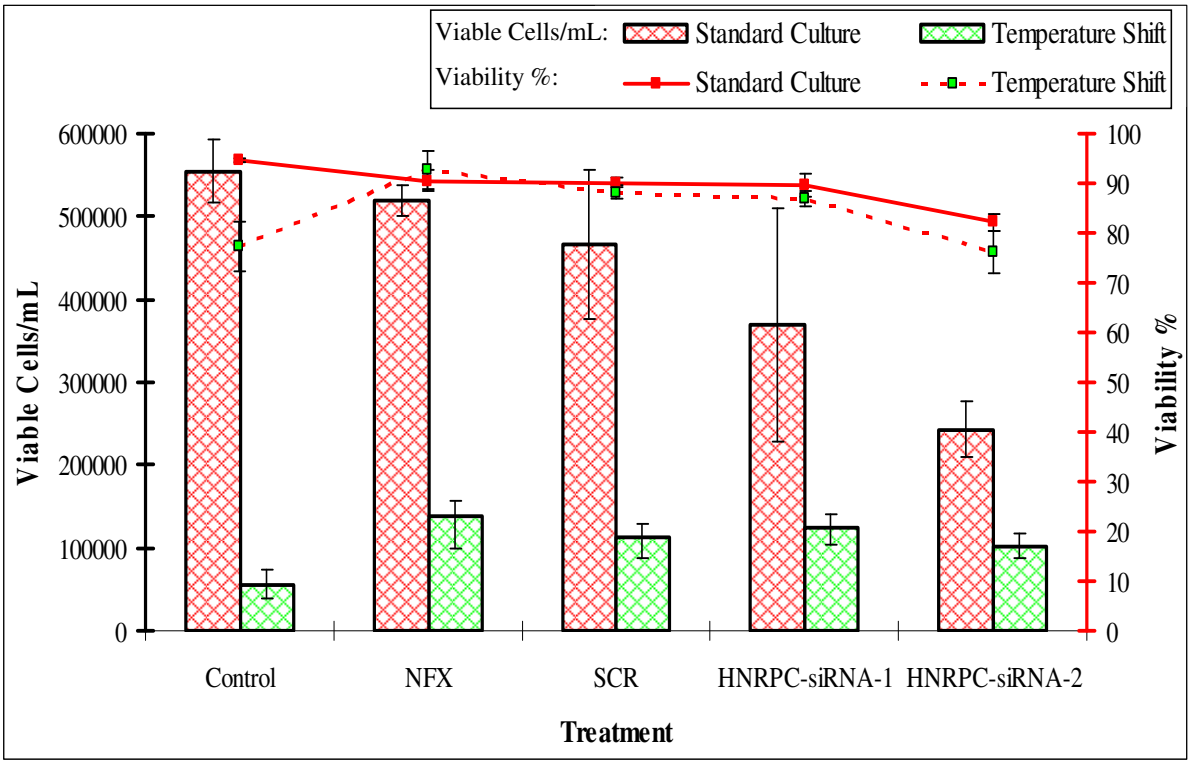

Figure 3.2.7.6. Investigation of the effects of siRNA knockdown of HNRPC on the growth and viability of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of HNRPC were measured after 96hrs of transfection. Columns denote viable cells and superimposed red lines show viability of culture. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

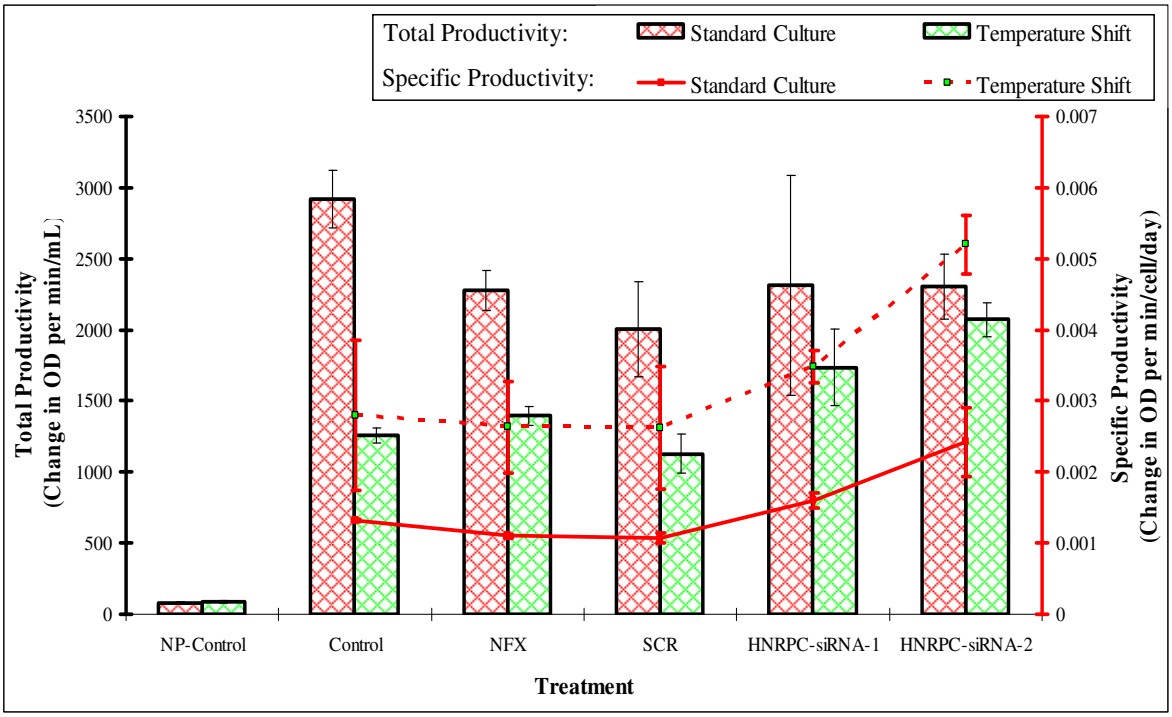

Figure 3.2.7.7. Investigation of the effects of siRNA knockdown of HNRPC on the total and specific productivity of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of HNRPC were measured after 96hrs of transfection. Columns denote total productivity and superimposed red lines show specific productivity. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. NP Control represents samples collected from non-producer CHO-K1 cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

Table 3.2.7.3. Comparative analysis of the effects of knockdown of HNRPC on the growth, viability, and total and specific productivity of SEAP secreting CHO-K1 cells in standard and temperature-shifted cultures after 96hrs of transfection. This data was calculated (in percentage) from Figures 3.2.7.6 and 3.2.7.7 by comparing human scrambled treated control cultures (SCR) with siRNA treated cultures. '+' represents increase and '-' represents decrease in effect. Shaded columns represent the significant effects (20% decrease or increase in effect with p-value ≤ 0.05).

Phenotype	siRNA Molecule	Standard Culture		Temperature-shifted Culture	
		Effect (%)	p-value	Effect (%)	p-value
Growth	1	-21	0.28	+11	0.5
	2	-48	0.0048	-11	0.4
Viability	1	-1	0.69	-2	0.34
	2	-9	0.002	-14	0.009
Total Productivity	1	+15	0.48	+54	0.009
	2	+15	0.18	+83	0.0001
Specific Productivity	1	+48	0.0004	+33	0.1
	2	+124	0.002	+97	0.003

3.3.8.2.3 PFN2

The siRNA transfections were performed using 2mL of $1x10^5$ cells/mL and following transfection, cells were maintained in standard culture for 96hrs at 37^0C or shifted to 31^0C for a further 72hrs after 24hrs of culture at 37^0C (Figure 3.2.7.3). The srambled siRNA-treated cultures were used as a control. Un-treated and neoFX-treated cultures were also monitored. The growth and productivity of culture were measured after 96hrs of transfection. A significant reduction in cell growth in siRNA 1 treated cultures compared to control cultures was observed for cells maintained at 37^0C (Figure 3.2.7.8 and Table 3.2.7.4). The effects of siRNA 1 on cell growth were more pronounced in the case of standard cultures (86%) in comparison to temperature-shifted cultures (54%). The viability of culture was also reduced following transfection with siRNA 1. On the other hand, transfection with siRNA 2 significantly reduced cell growth by 28% in only the standard culture. Although total productivity was reduced by 47% by siRNA 1 in standard culture, no effect was observed in cultures incorporating a temperature-shift (Figure 3.2.7.9 and Table 3.2.7.4). siRNA 2 improved the total productivity in both temperature-shifted and non-shifted cultures. It was improved by 40% in standard culture and by 94% in temperature-shifted cultures. The increased level of PFN2 at low temperature as observed in the proteomics study could be possibly expected to facilitate secretion of recombinant protein product from cells. A pattern of an improvement in Qp was observed following the inhibition of PFN2 in standard and temperature-shifted cultures.

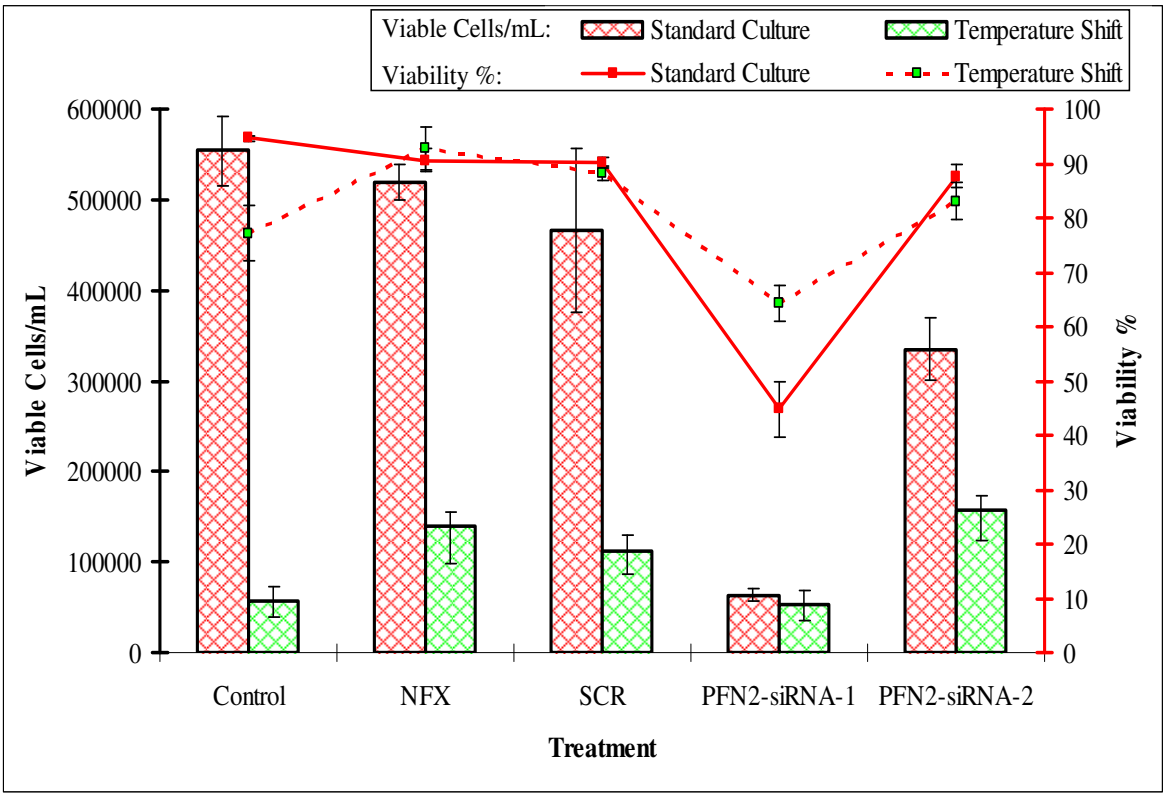

Figure 3.2.7.8. Investigation of the effects of siRNA knockdown of PFN2 on the growth and viability of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of PFN2 were measured after 96hrs of transfection. Columns denote viable cells and superimposed red lines show viability of culture. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

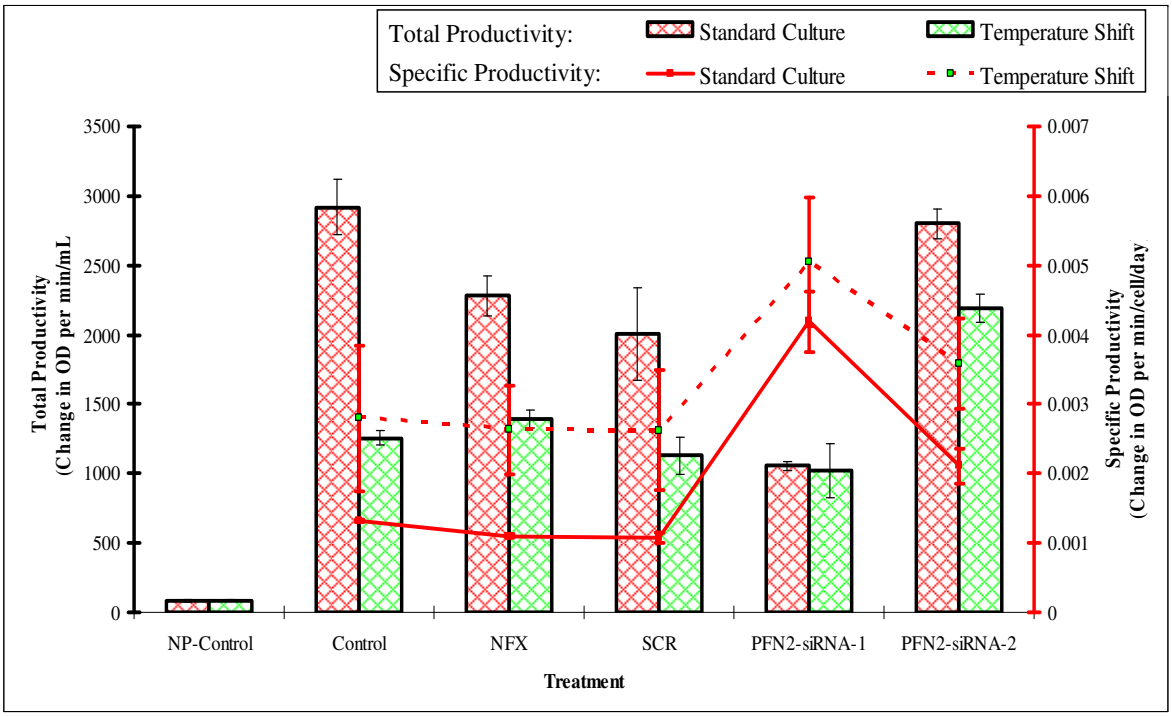

Figure 3.2.7.9. Investigation of the effects of siRNA knockdown of PFN2 on the total and specific productivity of SEAP-secreting CHO-K1 cells in standard and temperature-shifted cultures. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for a further 72hrs after 24hrs of transfection. The effects for inhibition of PFN2 were measured after 96hrs of transfection. Columns denote total productivity and superimposed red lines show specific productivity. Control denotes un-treated cultures. NFX is transfection reagent only and SCR denotes scrambled siRNA treated cells. NP Control represents samples collected from non-producer CHO-K1 cells. Error bars represent standard deviation calculated using 3 biological replicate samples.

Table 3.2.7.4. Comparative analysis of the effects of knockdown of PFN2 on the growth, viability and total and specific productivity of SEAP secreting CHO-K1 cells in standard and temperature-shifted cultures after 96hrs of transfection. This data was calculated (in percentage) from Figures 3.2.7.8 and 3.2.7.9 by comparing human scrambled treated control cultures (SCR) with siRNA treated cultures. '+' represents increase and '-' represents decrease in effect. Shaded columns represent the significant effects (20% decrease or increase in effect at p-value ≤ 0.05).

Phenotype	siRNA Molecule	Standard Culture		Temperature-shifted Culture	
		Effect (%)	p-value	Effect (%)	p-value
Growth	1	-86	0.0002	-54	0.009
	2	-28	0.04	+39	0.08
Viability	1	-50	0.0001	-27	0.0003
	2	-3	0.13	-6	0.08
Total Productivity	1	-47	0.002	-10	0.4
	2	+40	0.005	+94	5.37E-05
Specific Productivity	1	+288	2.77E-05	+92	0.01
	2	+95	0.0005	+36	0.13

3.2.7.3 Validation of siRNA induced inhibition of target protein expression

Proteins from biological duplicate samples were collected after 96hrs of transfection at standard culture and were separated on a 10% SDS gel (section 2.6.10). Western blots were developed using appropriate antibodies. All control samples (untreated, neoFX- and scrambled-treated controls) showed comparable expression levels in Western blots. The expression of PFN2 was however reduced in the scrambled siRNA-treated samples in comparison to the other controls, untreated and neoFX and the reason for this is unclear. The expression of all protein targets was reduced to undetectable levels after siRNA transfection in comparison to their respective control samples (SCR) (Figure 3.2.7.4). Therefore the effects observed on cellular phenotypes, i.e. cell growth, viability and recombinant protein productivity, are likely due to the specific inhibition of the expression of the protein targets.

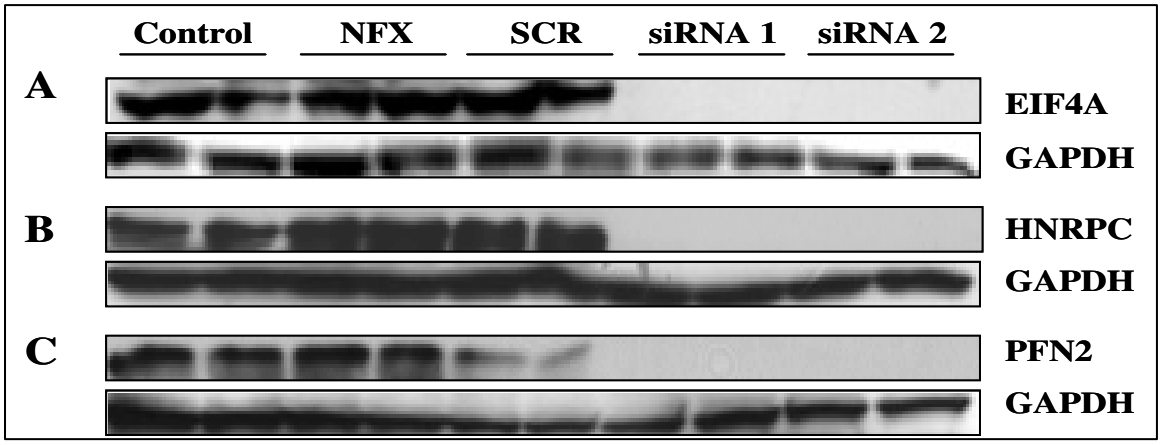

Figure 3.2.7.4. Investigation of inhibition of expression of target proteins due to siRNA transfection at standard culture using Western blotting. **A** represents expression of eIF4A, **B** represents expression of hnRPC and **C** represents expression of PFN2 after 96hrs of siRNA transfection. Expression of GAPDH was used as a loading control in all cases.

3.3 Investigation of low temperature induced differential expression of miRNA using miRNA array

As described in section 1.6, miRNAs are small endogenous single-stranded non-coding RNA molecules. Recently miRNAs have been reported to be involved in regulation of various cellular phenotypes such as cell growth, apoptosis, metabolism and protein secretion, all of which are very important for production of recombinant protein in mammalian cells. Therefore suspension-adapted CHO-K1 cells were cultured in a low temperature based biphasic culture process and analysed for differential expression of miRNAs following temperature-shift using a hybridization-array (bioarray). This allowed us to identify 2 potential miRNAs, miR-7 and miR-24, that could regulate cell growth and productivity of cells.

3.3.1 miRNA-bioarrays

A miRNA-bioarray (Ambion) containing 640 probes representing a comprehensive panel of human, mouse and rat microRNAs was used in this investigation. The miRNA expression profiles of cells maintained at 37^0C for 72 hrs were compared with profiles of cells shifted to low temperature (31^0C) for further 72hrs to identify temperature-dependent changes. Two miRNA, miR-21 and miR-24, were found to be up regulated following temperature-shift and could be associated with regulation of cell growth and productivity of cells at low temperature.

3.3.1.1 Cell culture

CHO-K1 cells were seeded at $1x10^5$ cells/mL in spinner flasks and cultured at either 37^0C for 144hrs or for 72hrs at 37^0C followed by a temperature-shift to 31^0C for a further 72hrs.

As can be seen in Figure 3.3.1.1 the cells that were temperature-shifted immediately ceased logarithmic growth and did not exceed a peak viable cell density of 1.67 $x10^6 \pm 0.15$ cells/mL, whereas the cells cultured at 37^0C continued in logarithmic growth for a further 24hrs and achieved average peak viable density of 2.02 x $10^6 \pm 0.11$ cells/mL. Cells were sampled at 72hrs and 144hrs for RNA extraction reflecting the conditions used for 2D-DIGE experiment. Total RNA quality was checked using Agilent Bioanalyzer and the presence and integrity of small RNA species was confirmed by visualisation on a 15% denaturing polyacrylamide gel (Figure 3.4.1.2).

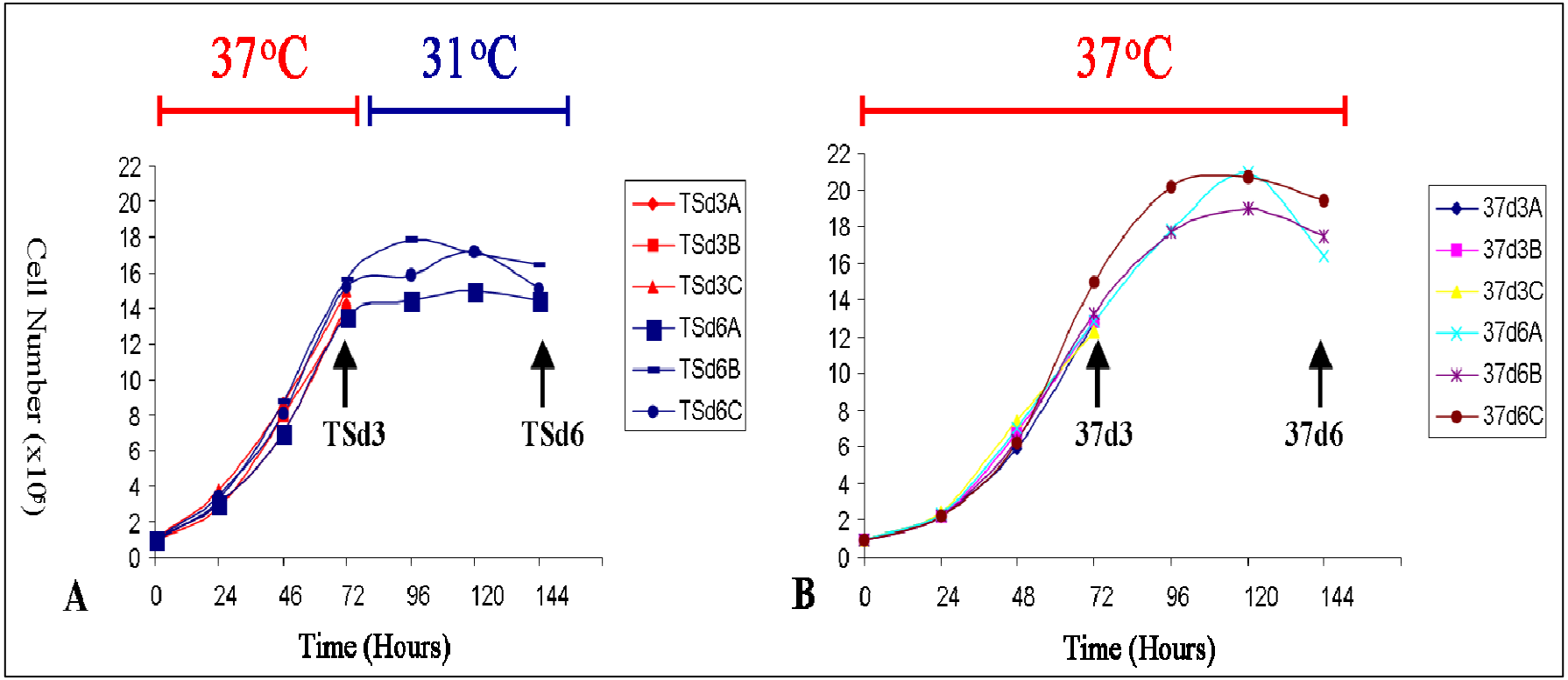

Figure 3.3.1.1. Viable cell counts for CHO-K1 batch culture following seeding at $1x10^5$ cells/mL for cultures incorporating a temperature-shift (A) and cells cultured at a constant temperature of 37 ^{0}C. In each case, samples were taken from 3 independent spinner flasks at 72 and 144hrs post seeding (indicated by arrows).

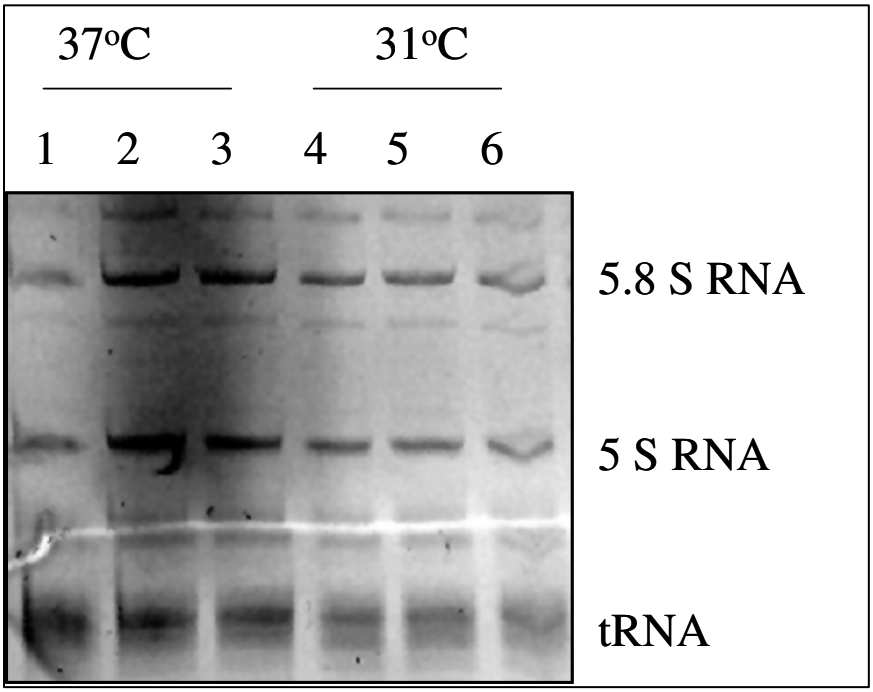

Figure 3.3.1.2**.** A 15% denaturing acrylamide gel analysis of RNA extracted from the temperature-shifted samples demonstrating the yield and integrity of the small RNA species.

3.3.1.2 miRNA differential expression analysis

Biological triplicate samples of total RNA isolated at 72hrs (TSd3) and 144hrs (TSd6) were extracted from cells that were shifted to 31^0C at 72hrs and subsequently used for miRNA bioarray analysis.

The miRNA-bioarrays are designed to be used with human samples and due to this implication of these human miRNA bioarray to investigate the differential expression of miRNAs in CHO cells was an uncertain task. We observed that when human miRNA bioarrays were probed with labelled *Cricetus griseus* RNA, the average percent present call was in the region of 27.3% (± 4.8) which compares favourably with human cell line RNA

which had an average present call of 26.9% (± 5.7). The average fluorescence signal from arrays probed with CHO-K1 RNA was 306.4 ± 55.2 Fluorescence Units which was comparable to human (296.6 ± 71.5). Unsupervised hierarchial cluster analysis of the expression data revealed that CHO-K1 samples clustered as a discrete sub-cluster separate to six human cell lines included in the analysis as non-hamster controls (Figure 3.3.1.3). Unsupervised hierarchial clustering within the CHO-K1 samples also resulted in separation of the exponential samples (72hrs at 37^0C, TSd3) from those in stationary phase (144hrs at 31^0C, TSd6) (Figure 3.3.1.4). Within the sub-clusters, the samples TSd3A & TSd6B were outliers. Since cultures grew similarly and had similar viabilities, it is likely that this is an artefact of labelling and/or hybridisation based on overall lower median foreground readings and lower percent present calls associated with these arrays. This is an important quality control metric for the subsequent analysis steps. The samples TSd3A & TSd6B were therefore omitted during re-analysis of data.

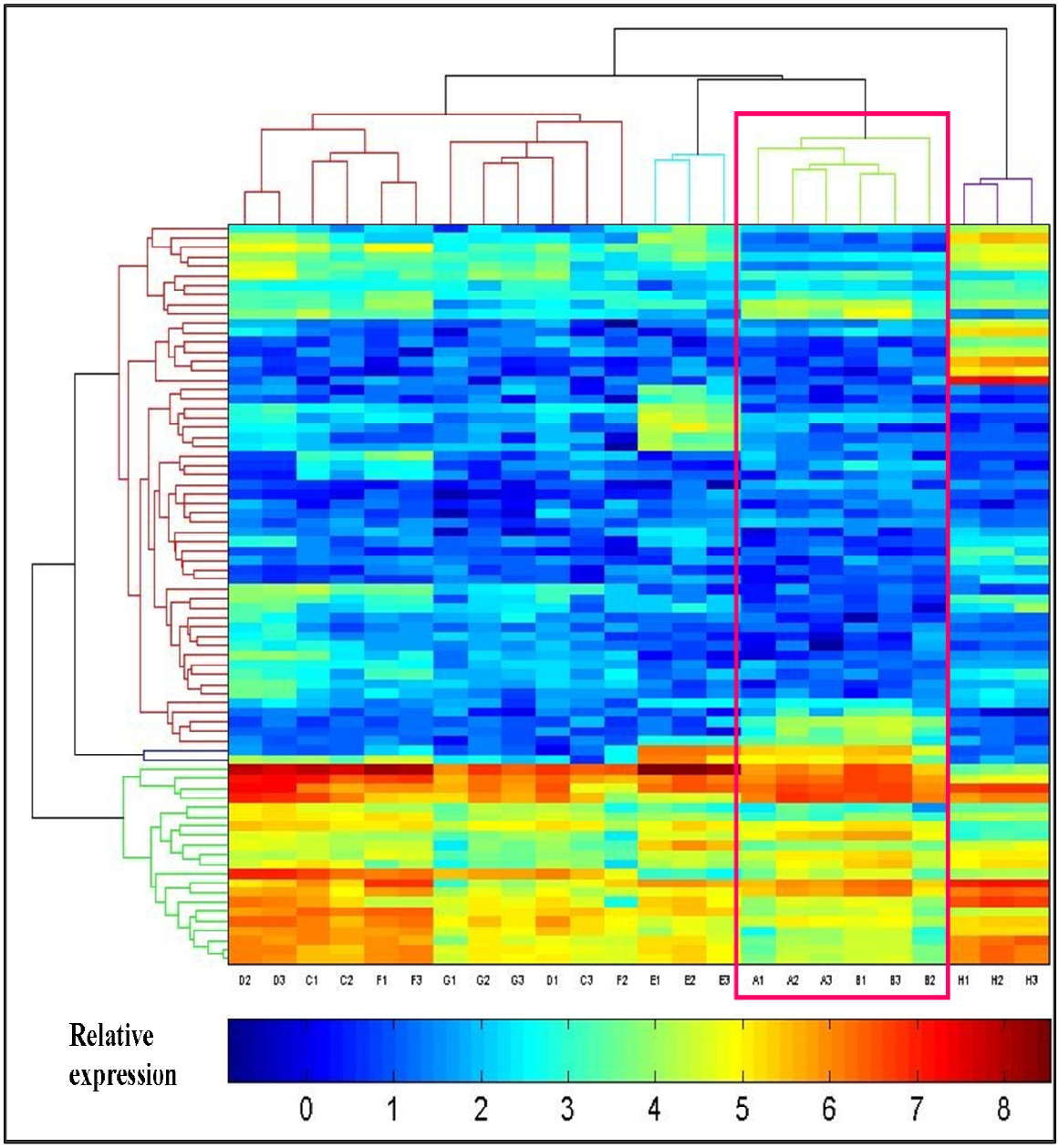

Figure 3.3.1.3. Unsupervised hierarchial clustering analysis of CHO-K1 samples and 6 human samples. CHO samples clustered together and were distinctly separate from human samples based on miRNA expression profiles. CHO samples are denoted by red box.

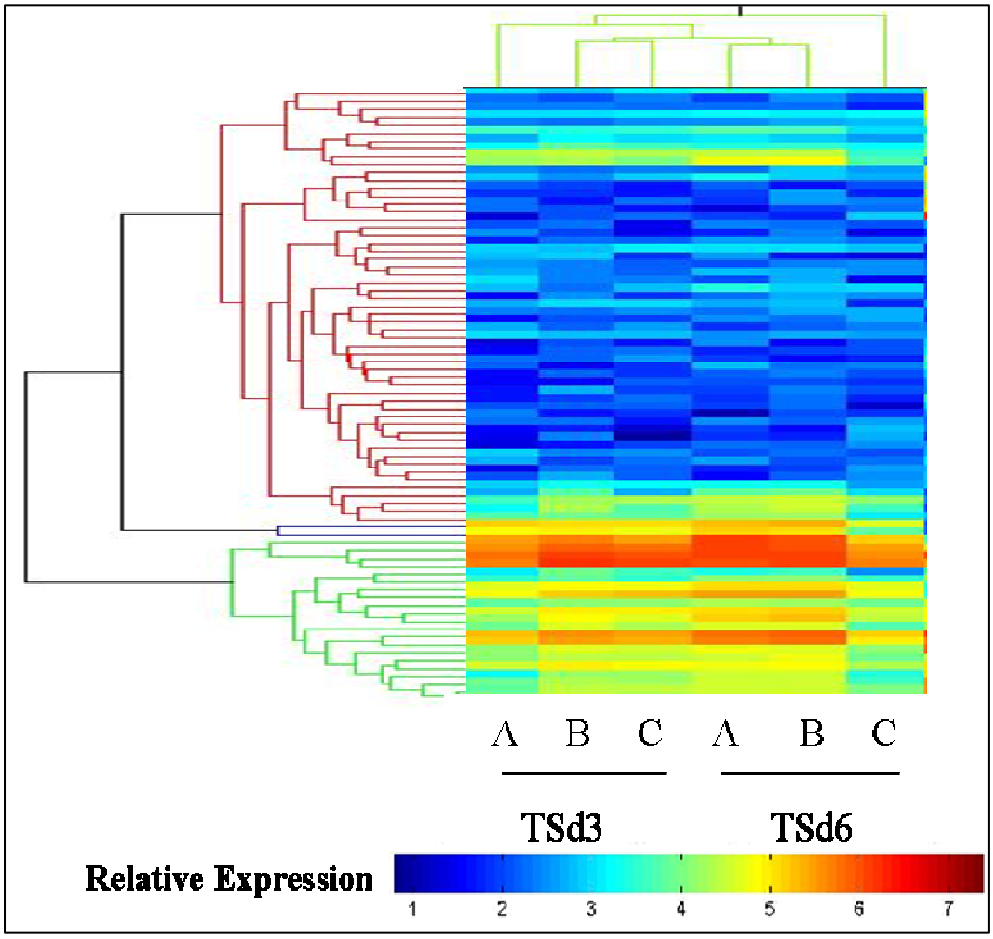

Figure 3.3.1.4. Unsupervised hierarchial clustering analysis of all 6 CHO-K1 samples resulted in 2 main clusters of samples which separate the exponential (72hrs at 37^0C, TSd3) samples from the quiescent (144hrs at 31^0C, TSd6) samples. From the dendrogram at the top it is clear that TSd3A and TSd6B samples are outliers. The relative expression of each miRNA is represented by colour ranging from low (Blue) to high (Red) expression. The range bar of relative expression is given below the cluster.

Using the statistical methods outlined in Section 2.7.4.2 to analyse all samples, it was found that 6 miRNAs were considered statistically different ($p \leq 0.05$) between the 72hrs at 37^0C (TSd3) and 144hrs at 31^0C (TSd6) samples (Table 3.3.1.1). However, removal of the outlier samples identified by clustering (TSd3A & TSd6B) resulted in a total of 23 miRNAs passing the 95% confidence level (Table 3.3.1.2). Overall, 26 different miRNAs were identified as differentially expressed in this system. Two miRNAs, miR-21 and 24, that are known to regulate cell growth were found to be differentially regulated in response to hypothermic conditions at 144hrs at 31^0C compared to 72hrs at 37^0C.

- miR-21 was 2.23 fold increased in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in temperature-shifted culture. miR-21 is known to regulate growth and apoptosis in culture (Chan *et al.* 2005, Schetter *et al.* 2008, Chan *et al.* 2008). For example, the inhibition of miR-21 has been reported to increase growth of HeLa cells (Cheng *et al.* 2005).
- miR-24 was 1.73 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in temperature-shifted cultures. miR-24 could have cell line specific effects as inhibition of miR-24 increased growth of HeLa cells while reduced growth of A549 cells (Chan *et al.* 2005).

This suggests that these miRNAs could be involved in regulation of cell growth and recombinant productivity of culture at low temperature.

Table 3.3.1.1**.** miRNAs that are statistically differentially expressed between samples at 72hrs at 37^0C (TSd3) and 144hrs at 31^0C (TSd6) when all samples are included in the statistical analysis. Fold change was calculated by dividing average raw fluorescence (RF) value at TSd6 by average RF value at TSd3. Negative RF value was considered no-signal-detected.

No.	**miRNA ID**	**p- value**	**TSd3 (37^0C)**			**TSd6 (31^0C)**			Fold Change
			TSd3A	**TSd3B**	**TSd3C**	**TSd6A**	**TSd6B**	**TSd6C**	
1	hsa_miR_27a	0.011	150	244	223.5	471.5	115.5	353	1.52
2	hsa_miR_126_AS	0.018	2.5	1	1	-2.5	-1	-2.5	-1.5
3	hsa_miR_30d_MM1	0.027	9	2.5	9.5	-0.5	0	-2.5	-10.5
4	hsa_miR_516_3p	0.030	-2	-0.5	-3.5	7.5	1.5	7	5.33
5	hsa_miR_191	0.034	140.5	288	237	295.5	83	245	-1.07
6	hsa_miR_495	0.037	2.5	2	8	1.5	-0.5	-4	-3.57

Table 3.3.1.2**.** Statistically differentially expressed miRNAs observed using miRNA-bioarray between samples at 72hrs at 37^0C (TSd3) and 144hrs at 31^0C (TSd6) after removal of outlier samples from the statistical analysis. Fold change was calculated by dividing average raw fluorescence (RF) value at TSd6 by average RF value at TSd3. Negative RF value was considered no-signal-detected.

No.	miRNA ID	p-value	**TSd3 (37^0C)**		**TSd6 (31^0C)**		Fold Change
			TSd3B	**TSd3C**	**TSd6A**	**TSd6C**	
1	hsa_miR_320	0.001	113.5	103	220.5	179.5	1.85
2	hsa_miR_10a	0.009	66.5	54	137.5	111	2.06
3	hsa_miR_126_AS	0.009	1	1	-2.5	-2.5	1
4	hsa_miR_30c	0.010	111.5	105	202.5	178.5	1.76
5	hsa_miR_181a	0.013	239	199.5	488	453	2.15
6	hsa_miR_21	0.015	808	650.5	1844.5	1404.5	2.23
7	hsa_miR_30d	0.017	143	130	213.5	186.5	1.47
8	hsa_miR_29a	0.017	716.5	579.5	1317.5	1154	1.91
9	hsa_miR_125b	0.022	1743	1492	1795.5	1458.5	1.01
10	hsa_miR_513	0.022	2	2	8.5	5.5	3.5
11	hsa_miR_107	0.024	150	131	212	174	1.37
12	hsa_miR_27a	0.025	244	223.5	471.5	353	1.76
13	hsa_miR_449	0.026	1	1.5	8.5	8	6.6
14	mmu_miR_298	0.027	16.5	16	13.5	12	-1.28
15	hsa_miR_24	0.027	705.5	583.5	1254.5	977	1.73
16	hsa_let_7f	0.035	52	43	129	147	2.90

17	hsa_miR_221	0.035	378.5	316	572	502	1.54
18	hsa_miR_516_3p	0.036	-0.5	-3.5	7.5	7	7.25
19	mmu_miR_7b_MM1	0.036	-0.5	-0.5	3	3	3
20	hsa_miR_197	0.038	3.5	2.5	-1	0	-3
21	hsa_miR_19b	0.038	292.5	279	443.5	383	1.45
22	mmu_miR_346	0.049	4.5	4	3.5	3	-1.31
23	hsa_miR_10b	0.049	131.5	81.5	304	262.5	2.66

3.3.1.3 Validation of miRNA-bioarrays results with qRT-PCR

Total RNA from CHO-K1 cells cultured at 37^0C for 144hrs was sampled at 72hrs at 37^0C (37d3), and 144hrs at 37^0C (37d6) (Figure 3.3.1.1B). The RNA from cells incorporating a temperature-shift at 72hrs (TSd3 & TSd6) and cells maintained at 37^0C (37d3 & 37d6) were used for qRT-PCR analysis of selected targets from the bioarray analysis. The reaction is based on a proprietary RT-primer specific for the 3' end of a target miRNA which is then extended into a micro-cDNA by an ArrayScript™ enzyme during the RT-reaction step. The qPCR step is carried out in-situ and uses a 5' miRNA specific primer and a 3' universal primer targeting the universal 3' end of the original RT-primer (Figure 3.3.1.5). Hence this is a highly specific means of amplifying individual mature miRNAs.

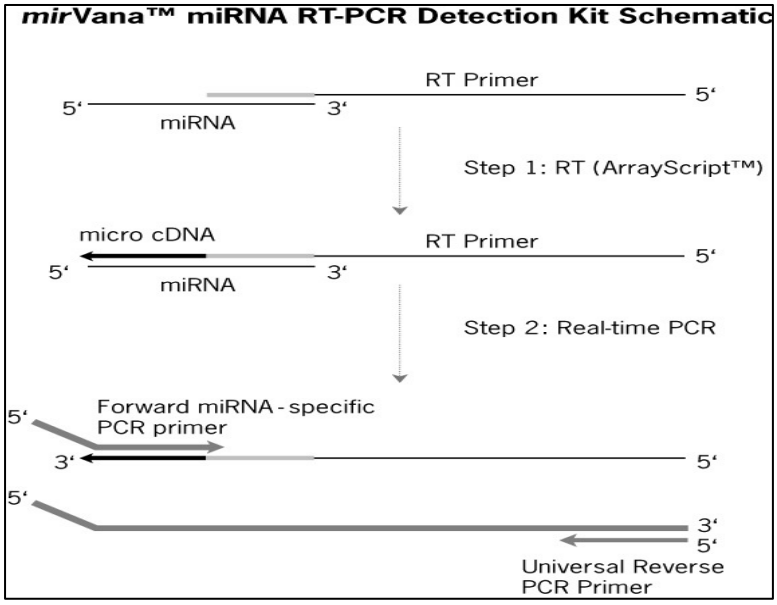

Figure 3.3.1.5**.** An outline of the Ambion qRT-PCR process for the detection and quantification of mature miRNAs. This image has been used courtesy of Ambion Inc.

Five miRNAs were selected for further analysis and the data is presented in Table 3.3.1.3. Of the 5 miRNAs selected, mmu-miR-290 was selected to test the limits of the system as this miRNA was found to have a very low expression level on the bioarray. The expression of mmu-miR-290 was confirmed as being low by qRT-PCR with an average cycle threshold (CT) of 26.2. This is very low expression compared to miR-21 which had an average CT of 17.4. This demonstrates that the relative quantities indicated by the bioarray were reproducible by qRT-PCR. The 1.51 fold increase seen in mmu-miR-290 at TSd6 was not significant ($p = 0.07$) and may be an artefact of the noisy data associated with low-level expression of this miRNA. miR-21 and miR-24 were confirmed as being highly expressed in CHO-K1 cells and to be significantly increased ($p \leq 0.05$) at 144hrs compared to 72hrs regardless of culture temperature (Table 3.4.1.3). The apparently higher level of miR-24 in TSd6 (144hrs at 31^0C) samples compared to 37d6 (144hrs at 37^0C) samples was interesting although not statistically significant ($p = 0.14$). The other miRNAs assayed by qRT-PCR were not found to be significantly differentially expressed.

Table 3.3.1.3. qRT-PCR data for selected miRNAs identified as differentially expressed in miRNA bioarray analysis. The data is presented as fold change increase (+) or decrease (-) compared to the 72hrs (d3) samples for the temperature-shifted culture (TS) and the culture maintained at 37^{o}C. Statistically significant changes as identified by students t-test (P≤0.05), are in bold.

miRNA	**Temperature-shift**			**Standard Culture**		
	TSd3	**TSd6**	**P Value**	**37d3**	**37d6**	**P Value**
let-7f	1	-1.04	0.43	1	-1.01	0.49
miR-21	1	**+1.67**	**0.04**	1	**+1.76**	**0.01**
miR-24	1	**+1.54**	**0.03**	1	**+1.29**	**0.0031**
miR-27a	1	+1.06	0.39	1	+1.23	0.16
mir-290	1	-1.51	0.07	1	-1.03	0.46

3.3.2 Analysis of low temperature induced differential expression of miRNAs using Taqman Low Density Arrays (TLDA)

miRNA-TLDAs are PCR based highly advanced arrays that contain primers for >350 human miRNA targets. PCR is a highly sensitive and reproducible technique and therefore miRNA-TLDAs could be more informative and reliable in comparison to miRNA bioarrays. The miRNA expression profiles for cells cultured at 37^0C for 72 hrs were compared with cells maintained at 31^0C for further 24hrs using miRNA-TLDAs as preliminary experiment to ensure the applicability of miRNA-TLDAs in CHO cells. This investigation has enabled us to identify early-mid differential expression of miRNAs in response to low temperature induction.

3.3.2.1 Cell culture

CHO-K1 cells were seeded at 1×10^5 cells/mL in spinner flasks and maintained at 37°C for 96hrs or for 72hrs at 37°C followed by a temperature-shift to 31°C for another 24hrs. The average viable cell density of both cultures at 72hrs was 1.47 $x10^6$ ± 0.08 cells/mL. The cells that were temperature-shifted were immediately growth arrested and reached only 1.6 $x10^6$ ± 0.16 cells/mL after 24hrs of shift to 31^0C whereas the cells in standard culture reached $1.9x10^6$ ± 0.13 cells/mL at 96hrs of culture. Biphasic cultures were sampled at 72hrs and 96hrs for total RNA extraction from 2 independent experiments.

3.3.2.2 miRNA differential expression analysis

The quality of total RNA was checked using Agilent Bioanalyzer and the presence and integrity of small RNA species including 5S RNA. The total RNA samples were used for

cDNA synthesis with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The cDNA samples were then used for miRNA TaqMan Low Density Array (TLDA) analysis. miR-let-7c (human) was used as an endogenous control in this investigation since the expression of usual endogenous controls, RNU6B, RNU44 and RNU48 was very low. This could be due to various reasons including the fact that the PCR primers on TLDA and the reverse-transcription (RT) step using a primer megaplex are designed specifically for human miRNAs. Primer megaplex is a pool of RT primers that transcribes all miRNA species in the sample in a single RT reaction. Expression of miRNAs in cells grown at 37^0C for 72hrs was compared (using Real-Time *StatMiner*™ software) with the expression of miRNAs after a further 24hrs growth at 31^0C. A total of 237 miRNA species were detected on TLDA cards ($C_T \leq 35$), of which 17 miRNAs were differentially expressed (DE) in the comparison of cells at 96hrs at 31^0C to the cells at 72hrs at 37^0C. Of the 17 DE miRNAs, 9 were up regulated in cells at 96hrs at 31^0C following temperature-shift in comparison to cells maintained at 37^0C at 72hrs (Table 3.3.2.1). Among them, two miRNAs, miR-126 and miR-7, that are known to be involved in regulation of cell growth were observed to be differentially regulated in cells at 96hrs at 31^0C compared to cells at 72hrs at 37^0C.

- miR-126 was 3.06 fold up regulated at 96hrs of culture at 31^0C in comparison to 72hrs at 37^0C in temperature-shifted culture. miR-126 has been reported to inhibit cell cycle progression and regulate cell growth in breast cancer cells (Zhang *et al.* 2008, Guo *et al.* 2008).

- miR-7, was 8.43 fold down regulated in cells at 96hrs at 31^0C compared to cells at 37^0C in biphasic culture. The inhibition of miR-7 has been reported to reduce the cell growth of lung carcinoma cell line, A549 (Cheng *et al.* 2005).

This indicates that these both miRNAs could serve as potential targets to regulate cell growth and recombinant producitivity in culture.

Table 3.3.2.1. miRNAs observed differentially expressed using miRNA TaqMan Low Density Array. Fold change was calculated by dividing expression at 96hrs at 31^0C by expression at 72hrs at 37^0C. miR-200c, a non-significant hit, was included in this list as it was selected for singleplex qRT-PCR validation to test the sensitivity limits of miRNA-TLDA. '+' denotes up regulation and '-' down regulation of miRNA following shift.

No.	miRNA ID	p-value	Fold change
1	Has-miR-101	4.52E-02	-1.69
2	Has-miR-126	8.22E-03	3.06
3	Has-miR-134	4.61E-03	-4.01
4	Has-miR-199b	9.98E-03	-2.58
5	Has-miR-219	1.50E-03	5.42
6	Has-miR-30e-5p	1.46E-02	9.04
7	hsa-miR-320	2.75E-02	-2.84
8	hsa-miR-345	4.70E-02	3.05
9	hsa-miR-374	2.49E-02	2.26
10	hsa-miR-376a	5.32E-03	-4.58
11	hsa-miR-449	3.37E-02	-1.68
12	hsa-miR-489	1.98E-02	2.69
13	hsa-miR-517	1.24E-03	-4.49
14	hsa-miR-518d	2.11E-03	3.5
15	hsa-miR-520d	2.18E-03	-4.23
16	hsa-miR-578	9.36E-03	2.58
17	hsa-miR-7	0.02	-8.43
18	hsa-miR-200c	0.4	1.50

3.3.2.3 Validation of miRNA-TLDA results with singleplex qRT-PCR

The processing of miRNA-TLDAs require all miRNA species present in the samples to be reverse-transcribed using a primer megaplex (a pool of primers) which is designed specifically for human samples. Moreover, the miRNA profiling using TLDA cards was performed only using biological duplicate samples from biphasic culture. Due to these reasons, the validation of miRNA-TLDAs data using singleplex qRT-PCR was an important aspect of this investigation.

The singleplex qRT-PCR was performed to validate the miRNA expression data using biological triplicate samples taken every 24hrs during 144hrs of biphasic and standard cultures. For this, low serum adapted CHO-K1 cells were seeded at 1×10^5 cells/mL in filter-capped spin tubes and maintained at 37°C for 144hrs or for 72hrs at 37°C followed by a temperature-shift to 31°C for a further 72hrs. The cells that were temperature-shifted achieved maximum viable cell density of $1.37x10^6 \pm 0.18$ cells/mL whereas the cells in standard culture achieved $1.82x10^6$ cells/mL on 144hrs of culture (Figure 3.3.2.1A). The growth of temperature-shifted cultures was reduced following the shift. In comparison to temperature-shifted cultures, cells maintained at 37^0C were actively growing throughout the duration of cell culture. The viability of temperature-shifted cultures was slightly better than the standard culture through out (Figure 3.3.2.1B).

Two miRNA targets, miR-7 (significant) and miR-200c (non-significant) were selected for validation using singleplex qRT-PCR based on cycle threshold value ($C_T \leq 35$), expression pattern observed using miRNA-TLDA and evidence of a role in regulation of cell growth in the literature. miR-7 was selected from the list of differentially expressed miRNAs. It was 8.43 fold down regulated in cells at 96hrs at 31^0C compared to 72hrs at 37^0C in

temperature-shifted culture. miR-7 was selected because it has been reported to be associated with cell growth in literature (Zhang *et al.* 2008, Kefas *et al.* 2008). miRNA profiling was done only for biological duplicate samples and miRNA-TLDAs are specifically designed for human cells and therefore one non-significant change (miR-200c) from the comparison of cells at 72hrs at 37^0C with cells at 144hrs at 31^0C was also included for singleplex qRT-PCR validation to confirm the expression pattern observed and the applicability of human miRNA-TLDA in CHO cells. qRT-PCR analysis revealed that

- the expression of miR-7 was gradually decreasing as the culture progressed from lag phase to log and stationary phase of culture. The decrease in miR-7 expression was more pronounced in biphasic cultures at every time point after temperature-shift in comparison to the standard culture. This indicates its growth phase specific and dose dependent expression in culture. The down regulation of miR-7 observed in qRT-PCR is in agreement with the expression pattern observed using miRNA-TLDA.
- miR-200c was not significantly differentially expressed in biphasic or constant cultures at 37^0C. This is in keeping with the expression pattern as observed using miRNA-TLDA.

Taken together results indicated that the human TLDA is a reliable way of profiling miRNA expression in CHO cells.

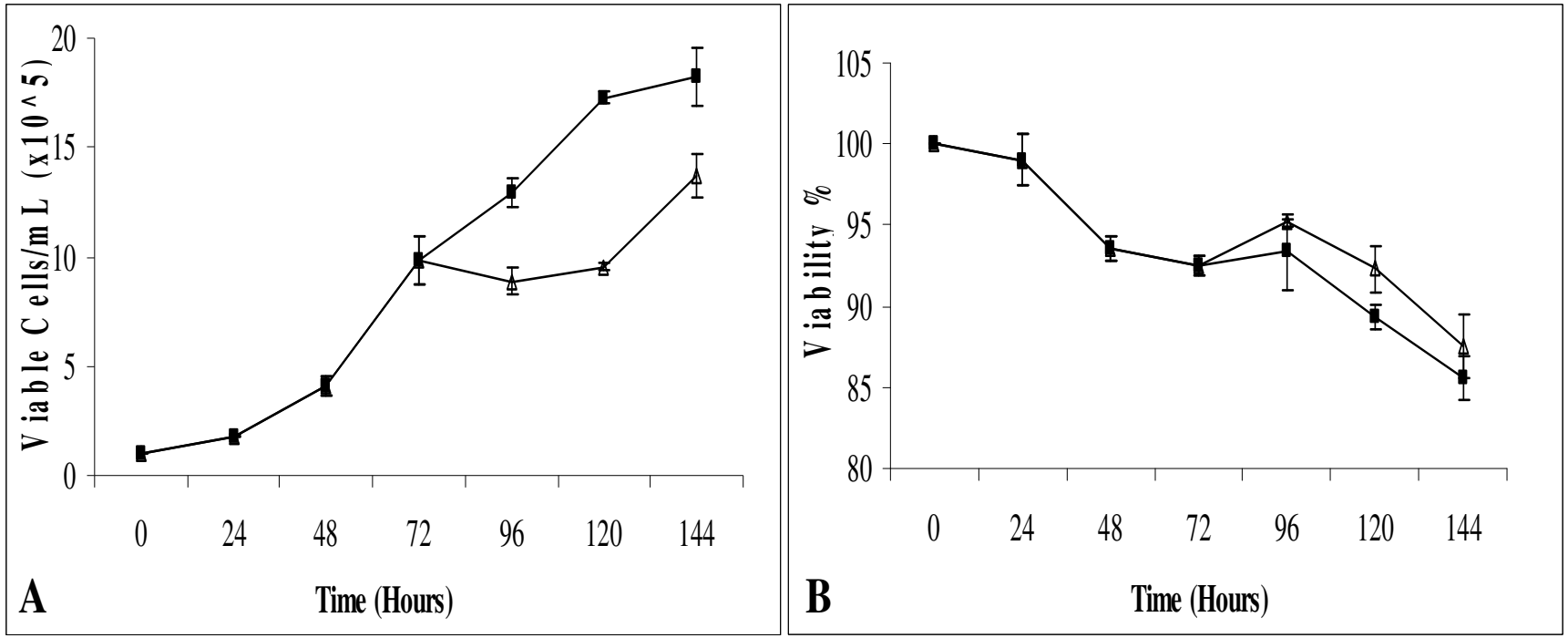

Figure 3.3.2.1. Comparison of viable cell number (A) and percentage viability (B) of low serum adapted CHO-K1 cells grown in either a biphasic temperature-shifted culture (--Δ--) or a constant culture at 37°C (--■--). Error bars represent the standard deviation calculated from three biological replicate cultures.

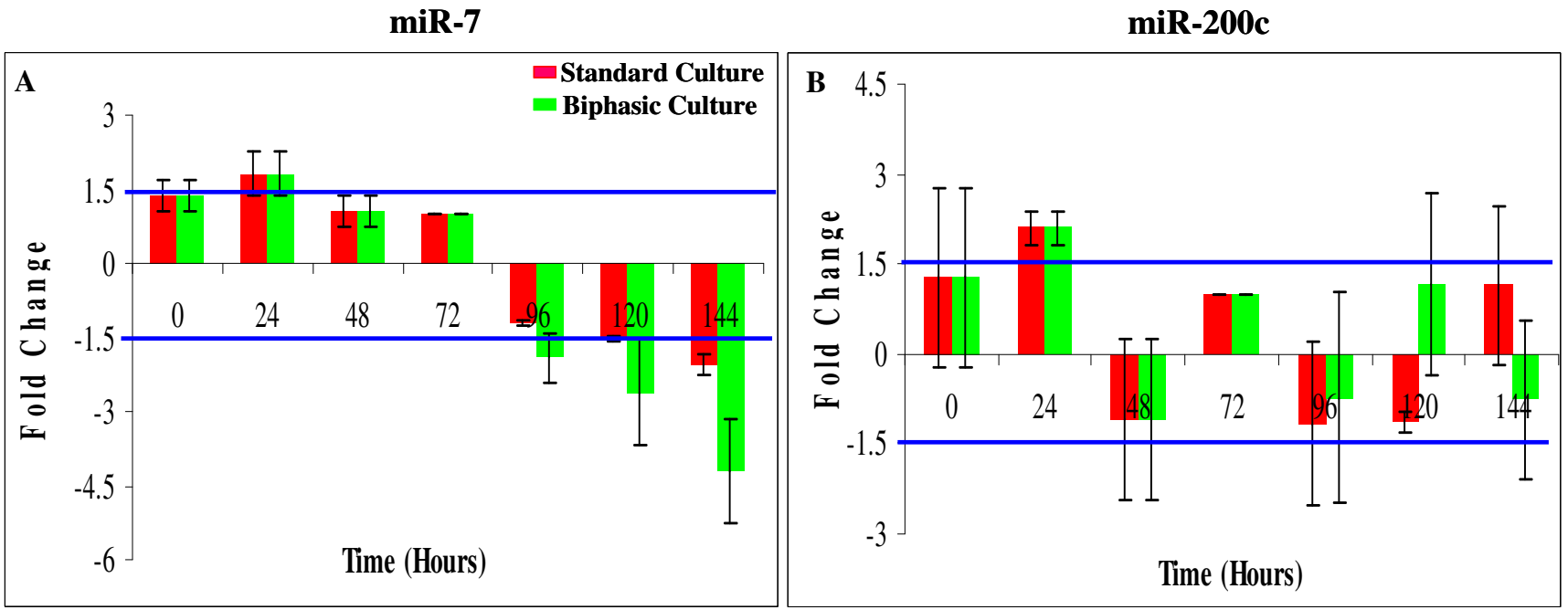

Figure 3.3.2.2 Validation of miRNA-TLDA expression data. Two miRNA targets were selected for validation using qRT-PCR analysis. Samples were taken at every 24hrs from the cells cultered in standard culture at 37^0C for 144hrs and in biphasic culture, where cells were initially cultured at 37^0C for 72hrs and then shifted to 31^0C for further a 72hrs. Red bars represent standard cultures while green columns represent temperature-shifted cultures. The blue line indicates cut-off limits of 1.5 fold up or down regulation of miRNA levels compared to expression at 72hrs. **A** represent miR-7; **B** miR-200c. Error bars represent the standard deviation calculated from three biological replicate cultures.

3.3.2.4 Isolation and cloning of the *Cricetulus griseus* miRNA cgr-miR-21

miRNAs are typically more conserved during evolution in comparison to other genes. The sequences of miRNAs in CHO cells have not been reported yet. The sequencing of miRNAs from CHO cells could help to understand the conservation of miRNAs through various specieses during evolution. miR-21, a well known growth regulator, was selected as an model miRNA to clone and sequence to compare the conservation of miRNAs across the species. Primers were designed for cloning *C. griseus* miR-21 based on alignment of the corresponding genomic regions flanking the pre-miR-21 sequence from *Mus musculus*, *Rattus norvigicus* and *Homo sapiens*. This region was amplified from CHO genomic DNA and was sequenced. The sequence similarity between the *C. griseus* miRNA and other mammalian sequences confirmed a novel *C. griseus* miRNA orthologue of a known human and mouse miRNA, miR-21 (Figure 3.3.2.4). In keeping with the Sanger registry-naming conventions, this miRNA has the prefix cgr- to represent *C. griseus*. This new miRNA cgr-miR-21 has been confirmed by the miRBase registry. The sequence has also been lodged with EMBL and given the accession no. AM600961.

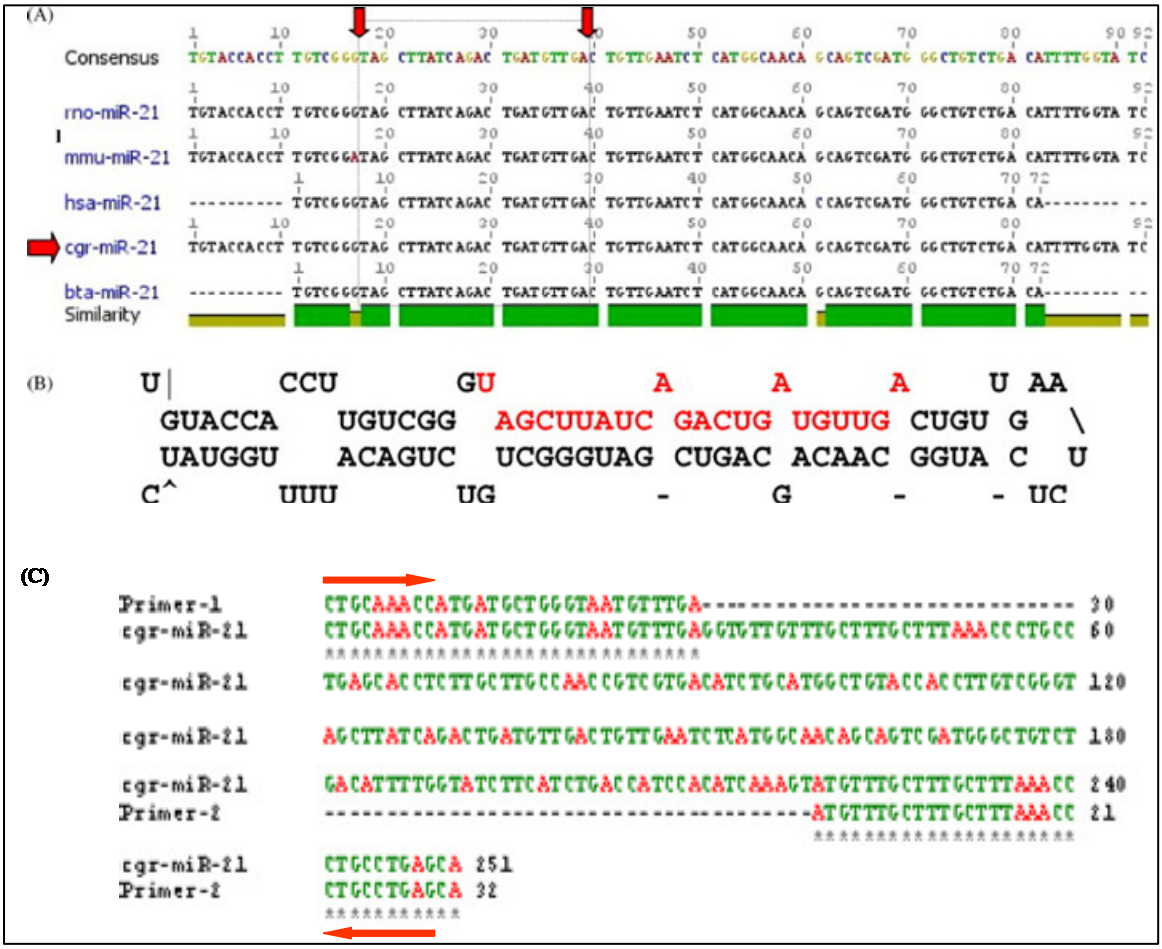

Figure 3.3.2.4. (A) Alignment of CHO-K1 cgr-miR-21 sequence with the sequences of mouse (mmu-), rat (rno-), human (hsa-) and bovine (bta-)miR-21. The CHO sequence is identical to that of the murine sequence (rno-miR-21) published in the Sanger miRNA repository (http://microrna.sanger.ac.uk/sequences/). Red arrow indicates the position of mature cgr-miR-21 (B) Predicted stem loop structure of cgr-miR-21 with the mature miRNA highlighted in red. (C) Alignment of forward and reverse primers which were used for RT-PCR with the sequence of cgr-miR-21. The red arrows indicate direction of PCR amplification.

3.3.2.5 miRNA Target selection

miR-7 was observed to be reduced by 8.43 fold in cells at 96hrs at 31^0C compared to cells at 72hrs at 37^0C in miRNA-TLDAs. The expression of miR-7 reflected a growth phase specific pattern when measured by qRT-PCR (Figure 3.3.2.2) suggesting that regulation of miR-7 expression in a dose dependent manner could provide a valuable means to regulate bioprocess related cellular phenotypes, i.e. growth, viability and productivity in culture. miR-24 was observed to be up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 31^0C using a miRNA-bioarray. miR-24 has been shown to modulate the growth of mammalian cells in culture (Table 3.3.1.2). Therefore, miR-7 and miR-24 were selected for further functional studies in CHO cells.

3.3.3 Effects of inhibition or over expression of miRNA targets in CHO cells

3.3.3.1 Optimization of transfection conditions in non-producer suspension-adapted CHO-K1 cells

Optimization of transfection conditions and initial screening for the effects of both miRNA targets on cell growth and viability were performed in non-producer, suspension and low serum adapted CHO-K1 cells (Figure 3.3.3.1). All transfections were performed in biological triplicates using spin tubes containing $1x10^5$ cells/mL in 2mL final volume. A human scrambled anti-mir miRNA was used as a control for inhibition studies and a human scrambled pre-mir miRNA was used as a control for over expression studies in all transfection experiments. Figure 3.2.7.1 shows timing of transfection and sampling to measure the impact of miRNA transfection on cell growth. Cell counts and viability assays were performed by flow cytometry using *Guava ViaCount*® Assays (section 2.82). This

enabled us to identify the optimal time-point for investigating the effects of miRNA transfection.

3.3.3.1.1 miR-7

In the TLDA experiments, the expression of miR-7 was found to be 8.43 fold down regulated in cells at 96hrs at 31^0C compared to cells at 72hrs at 37^0C in biphasic culture. Therefore in an attempt to mimic effects of biphasic culture, we down regulated the expression of endogenous miR-7 using specific inhibitors (Anti-mirs, AM) in cells growing at 37^0C and artificially over expressed it using Pre-mirs (PM) at low temperature (31^0C).

The miRNA transfections were performed using 2mL of $1x10^5$ cells/mL and cells were maintained over 144hrs of culture (Figure 3.2.7.1). A scrambled anti-mir miRNA for inhibition studies and a scrambled pre-mir miRNA for over expression studies were used as controls. Un-treated and neoFX-treated cultures were also monitored. The impacts of miRNA transfection on cell growth were measured at every 24hrs after 72hrs of transfection over the period of 144hrs (Figure 3.2.7.1). The cell growth was significantly reduced compared to PM control by the over expression of miR-7 at 31^0C (Figure 3.3.3.1C and Table 3.3.3.1). Reduced cell growth compared to AM control was also observed following inhibition of miR-7 at 37^0C, however it was not significant. The viability of both standard and low temperature cultures following inhibition or over expression was comparable to their respective controls (AM or PM controls) (Figure 3.3.3.1 and Table 3.3.3.2).

3.3.3.1.2 miR-24

The expression of miR-24 was observed to be 1.73 fold up regulated in cells maintained at 144hrs at 31^0C compared to cells at 72hrs at 37^0C and therefore we artificially up regulated

its expression in CHO cells at 37^0C and inhibited at 31^0C in order to investigate whether this would induce temperature-shift-like effects. The miRNA transfections were performed using 2mL final volume containing $1x10^5$ cells/mL and cells were maintained over 144hrs of culture (Figure 3.2.7.1). A scrambled anti-mir miRNA for inhibition studies and a scrambled pre-mir miRNA for over expression studies were used as controls. Un-treated and neoFX-treated cultures were also monitored. The impacts of miRNA transfection on cell growth were measured at every 24hrs after 72hrs of transfection over the period of 144hrs (Figure 3.2.7.1). The over expression of miR-24 in the standard culture at 37^0C resulted in reduced cell growth, however it was not significant (Figure 3.3.3.1A and Table 3.3.3.1). The inhibition of miR-24 at 31^0C did not have any effect on cell growth. The viability of cultures remained unaffected by inhibition or over expression of miR-24 at 31^0C or 37^0C respectively (Figure 3.3.3.1 and Table 3.3.3.2).

(Note: it is recognized that this was an incomplete experiment which did not look at all possible combinations of AM and PM miRNAs at temperatures. This was essentially a 'range-finding' exercise leading on to more detailed experiments described below).

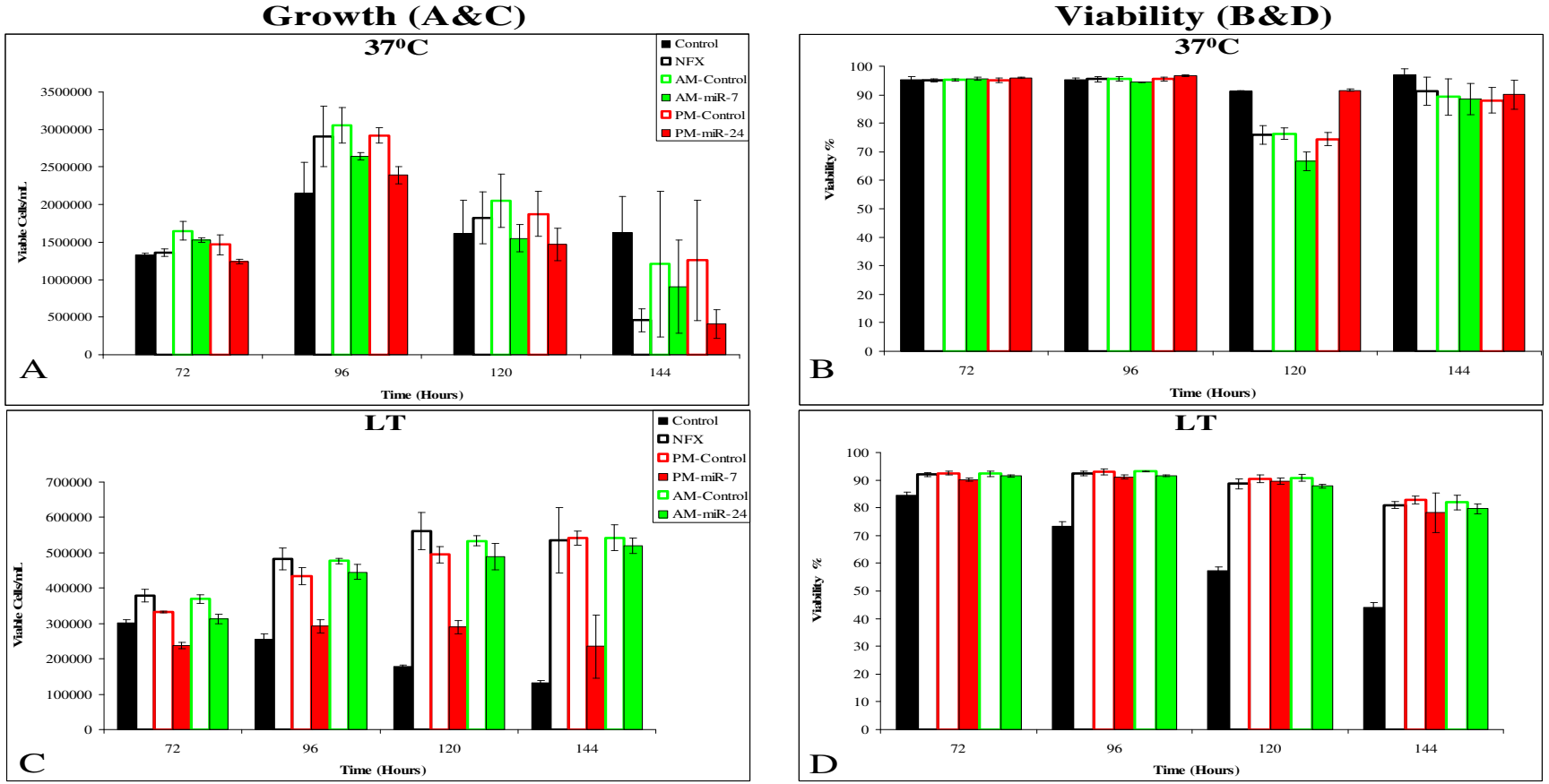

Figure 3.3.3.1. Inhibition and over expression analysis of miR-7 and miR-24 on the cell growth and viability of low serum adapted CHO-K1 cells in standard (37^0C) and low-temperature (31^0C) culture (LT). A & C represent growth and B & D represent the viability of CHO-K1 cells over 144hrs of culture after miRNA transfection. Control denotes un-treated cultures. NFX denoted transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir.

Table 3.3.3.1. Comparative analysis of effects of inhibition and over expression on growth of low serum adapted CHO-K1 cells at standard and temperature-shifted cultures. This data was calculated (in percentage) from Figure 3.3.3.1A&C by comparing AM Control with AM treated cultures and PM Control with PM treated cultures. Shaded columns represent promising observations considered for miRNA transfection in SEAP secreting CHO-K1 cells.

miRNA target	**Culture Condition**	**Time (Hrs)**	**Inhibition**		**Culture Condition**	**Time (Hrs)**	**Over expression**	
			Growth (%)	**p-value**			**Growth (%)**	**p-value**
miR-7	Standard Culture	72	-7	0.17	Low temperature culture	72	-28	5.7E-05
		96	-13	0.04		96	-33	0.001
		120	-24	0.1		120	-42	0.0003
		144	-25	0.67		144	-57	0.004
miR-24	Low temperature culture	72	-11	0.005	Standard Culture	72	-10	0.05
		96	-7	0.07		96	-14	0.004
		120	-8	0.13		120	-22	0.13
		144	-4	0.40		144	-67	0.15

Table 3.3.3.2. Comparative analysis of effects of inhibition and over expression on viability of low serum adapted CHO-K1 cells at standard and temperature-shifted cultures. This data was calculated (in percentage) from Figure 3.3.3.1B&D by comparing AM Control with AM treated cultures and PM Control with PM treated cultures. Shaded columns represent the promising observations considered for miRNA transfection in SEAP secreting CHO-K1 cells.

miRNA target	**Culture Condition**	**Time (Hrs)**	**Inhibition**		**Culture Condition**	**Time (Hrs)**	**Over expression**	
			Viability (%)	**p-value**			**Viability (%)**	**p-value**
miR-7	Standard Culture	72	0	0.36	Temperature-shifted culture	72	-2	0.01
		96	-1	0.09		96	-2	0.08
		120	-13	0.01		120	-1	0.44
		144	-1	0.88		144	-5	0.34
miR-24	Temperature-shifted culture	72	-1	0.23	Standard Culture	72	+1	0.14
		96	-2	0.001		96	+1	0.07
		120	-3	0.02		120	+18	0.0002
		144	-3	0.29		144	+2	0.64

As for siRNA transfection, 96hrs was chosen as an appropriate timepoint subsequent to transfection to assay cell cultures for effects on growth and viability. This time point was used in the further miRNA transfection experiments in SEAP secreting CHO-K1 cells.

3.3.3.2 Effect of miRNAs on SEAP-secreting suspension-adapted CHO-K1 cells

After optimizing transfection conditions in suspension and low-serum adapted CHO-K1 cells, SEAP secreting CHO-K1 cells were transfected with AMs and PMs miRNAs for miR-7 and miR-24 and were maintained in standard culture at 37^0C for 96hrs and biphasic culture at 31^0C for a further 72hrs after 24hrs of transfection at 37^0C (Figure 3.2.7.3). The inhibition and over expression of these miRNAs at both standard and temperature-shifted culture enabled us to investigate the effects of these miRNAs more extensively. Cells were analysed for effects of miRNA transfection on cell growth, viability and SEAP productivity after 96hrs of transfection (section 2.5.3.7, 2.8.2).

3.3.3.2.1 miR-7

The miRNA transfections were performed using 2mL of $1x10^5$ cells/mL and cells were maintained for 96hrs at 37^0C in standard culture and for a further 72hrs at 31^0C after 24hrs of culture at 37^0C in biphasic culture (Figure 3.2.7.3). A scrambled anti-mir miRNA for inhibition studies and a scrambled pre-mir miRNA for over expression studies were used as controls. Un-treated and neoFX-treated cultures were also monitored. The impact of siRNA transfection were measured after 96hrs of transfection.

The over expression of miR-7 at standard culture resulted in almost complete growth arrest as it reduced cell growth by 88% (Figure 3.3.3.2 and Table 3.3.3.3). However, there was no significant effect of over expression on growth in temperature-shifted culture (where growth was already arrested). The viability of cultures was maintained following over expression of miR-7 in standard and temperature-shifted cultures. Inhibition of miR-7 did not result in any significant effect on cell growth or viability in either either culture format. The total yield of SEAP was reduced by 74% following over expression of miR-7 reflecting the reduced cell growth whereas the Qp was improved by 116% in standard cultures (Figure 3.3.3.3 and Table 3.3.3.3). No significant alterations were observed in the total and specific productivity compared to the control treatments following the inhibition or over expression of miR-7 in temperature-shifted culture.

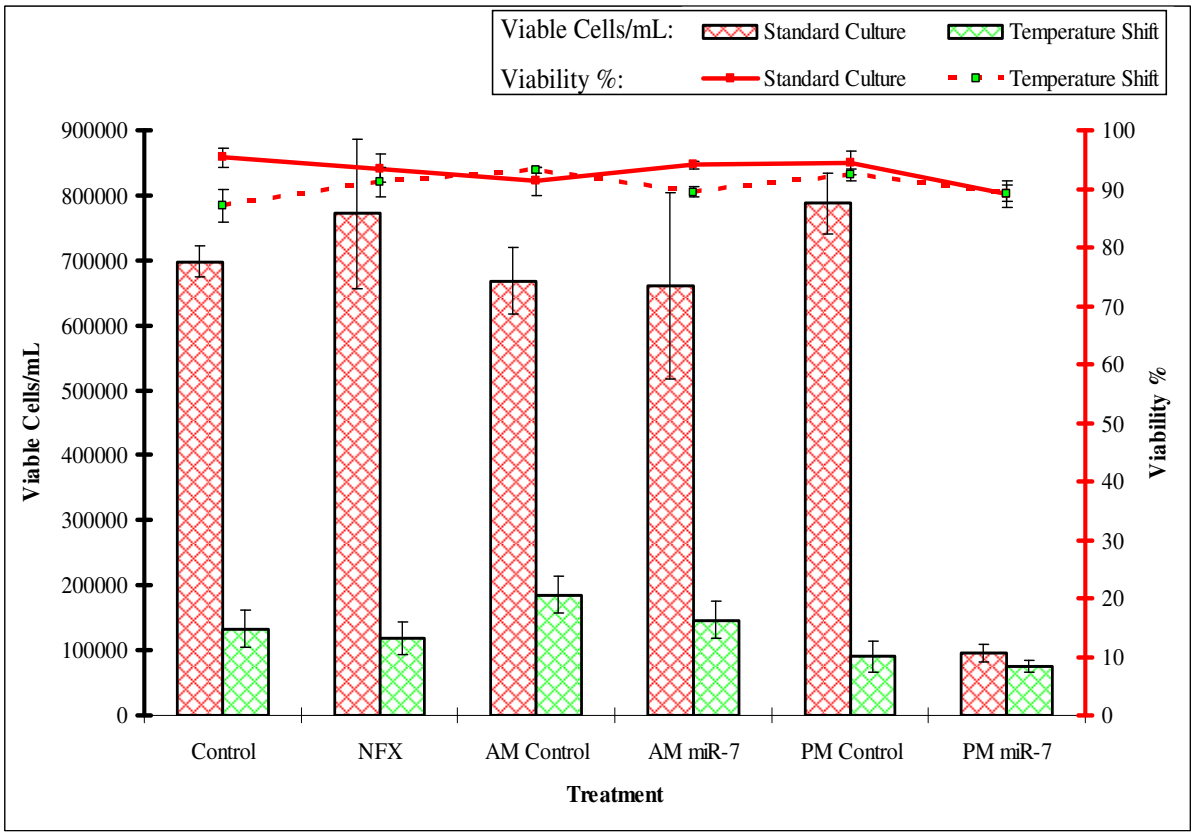

Figure 3.3.3.2. Effects of inhibition and over expression of miR-7 on growth and viability of SEAP secreting CHO-K1 cells at standard and temperature-shifted culture. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for 72hrs after 24hrs of transfection. The effects of miR-7 were measured after 96hrs of transfection. Columns denote viable cells and superimposed red lines show viability of culture. Control denotes un-treated cultures. NFX denoted transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir. Error bars represent standard deviation calculated using 3 biological replicate samples.

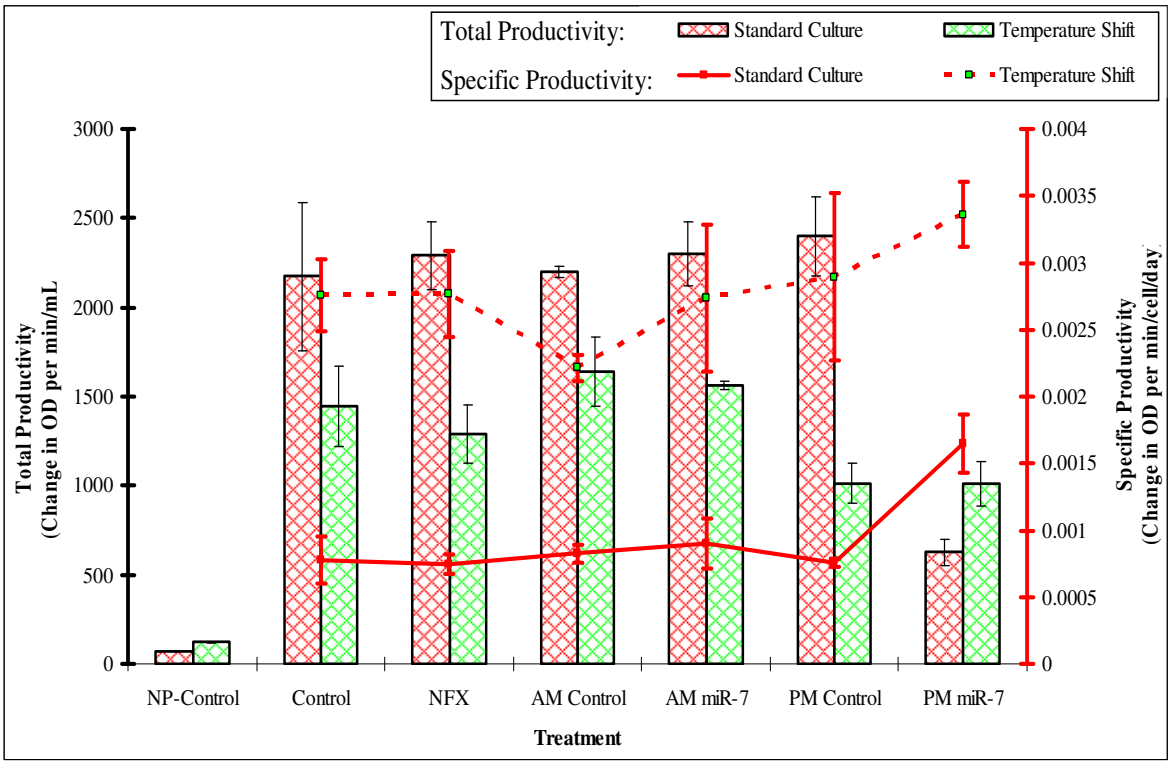

Figure 3.3.3.3. Effects of inhibition and over expression of miR-7 on the total and specific productivity of SEAP secreting CHO-K1 cells in standard and temperature-shifted culture. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for 72hrs after 24hrs of transfection. The effects of miR-7 were measured after 96hrs of transfection. Columns denote total productivity and superimposed red lines show specific productivity. Control denotes un-treated cultures. NP Control denotes non-producer CHO-K1 cells. NFX denoted transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir. Error bars represent standard deviation calculated using 3 biological replicate samples.

Table 3.3.3.3. Comparative analysis of effects of inhibition and over expression of miR-7 on growth, viability, total productivity and specific productivity of low serum-adapted SEAP secreting CHO-K1 cells in standard and temperature-shifted cultures after 96hrs of transfection. These data were calculated (in percentage) from Figure 3.3.3.2 and 3.3.3.3 by comparing AM Control with AM treated cultures and PM Control with PM treated cultures. '+' represent increase and '-' represent decrease in effect on cell growth or viability of culture. Shaded columns represent the significant effects (20% decrease or increase in effect at p-value ≤ 0.05).

Phenotype	**Culture condition**	**Inhibition**		**Over expression**	
		Effect (%)	**p-value**	**Effect (%)**	**p-value**
Growth	Standard culture	-1	0.93	-88	1.5E-05
	Temperature-shifted culture	-21	0.17	-17	0.36
Viability	Standard culture	+3	0.11	-6	0.02
	Temperature-shifted culture	-4	0.003	-4	0.09
Total productivity	Standard culture	+5	0.37	-74	0.0002
	Temperature-shifted culture	-5	0.53	0	0.10
Specific productivity	Standard culture	+7	0.56	116	0.002
	Temperature-shifted culture	+23	0.18	+16	0.3

3.3.3.2.2 miR-24

The miRNA transfections were performed using 2mL of $1x10^5$ cells/mL and cells were maintained for 96hrs at 37^0C in standard culture and for a further 72hrs at 31^0C after 24hrs of culture at 37^0C in biphasic culture (Figure 3.2.7.3). A scrambled anti-mir miRNA for inhibition studies and a scrambled pre-mir miRNA for over expression studies were used as controls. Un-treated and neoFX-treated cultures were also monitored. The impact of siRNA transfection were measured after 96hrs of transfection.

Cell growth was reduced by 56% following over expression of miR-24 at 37^0C, whereas the inhibition of miR-24 expression had an insignificant effect (Figure 3.3.3.4 and Table 3.3.3.4). There was no significant effect of over expression of miR-24 in temperature-shifted cultures. Cell viability was also unchanged after over expression in standard and temperature-shifted cultures. Total yield was reduced due to over expression of miR-24 at 37^0C which was a reflection of reduced cell number in culture, whereas no significant effect was observed in biphasic cultures. The Qp was observed to be improved by either inhibition (25%) or over expression (68%) of miR-24 at standard culture, while it was unchanged in temperature-shifted cultures (Figure 3.3.3.5 and Table 3.3.3.4).

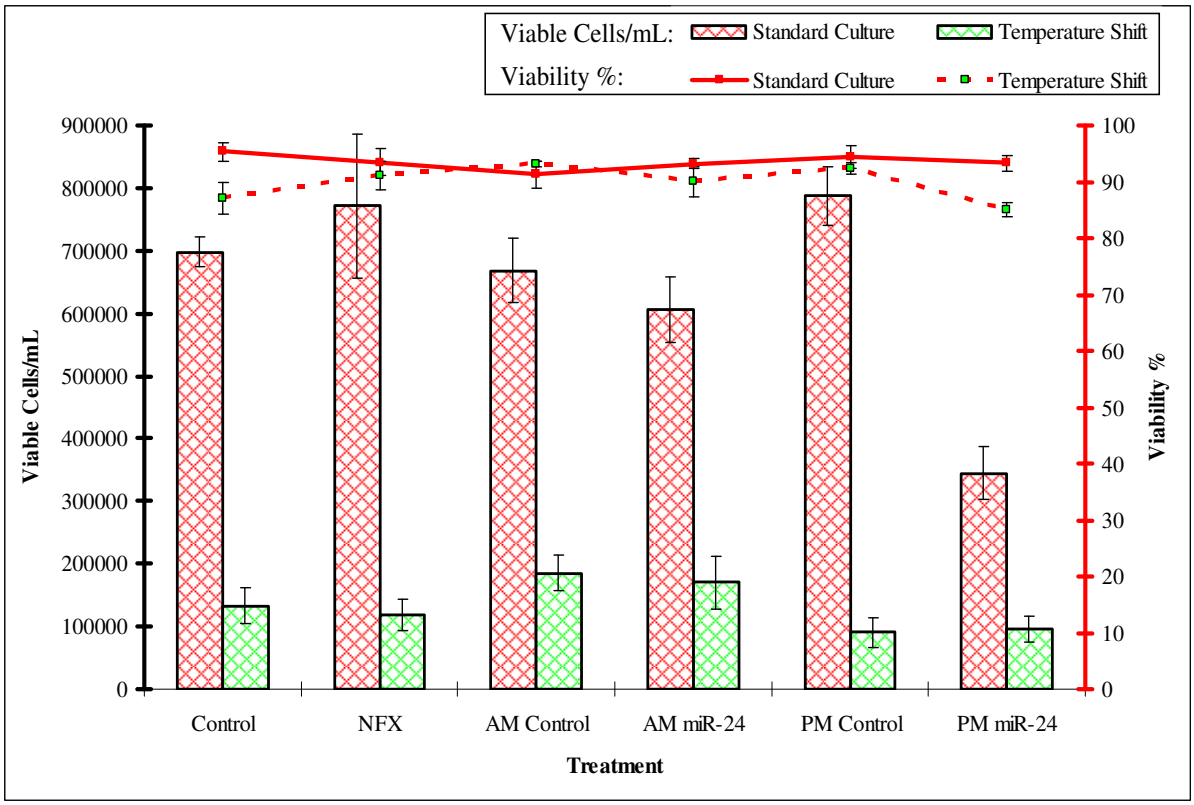

Figure 3.3.3.4. Effects of inhibition and over expression of miR-24 on growth and viability of SEAP secreting CHO-K1 cells in standard and temperature-shifted culture. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for 72hrs after 24hrs of transfection. The effects of miR-24 were measured after 96hrs of transfection. Control denotes un-treated cultures. NFX denoted transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir. Error bars represent standard deviation calculated using 3 biological replicate samples.

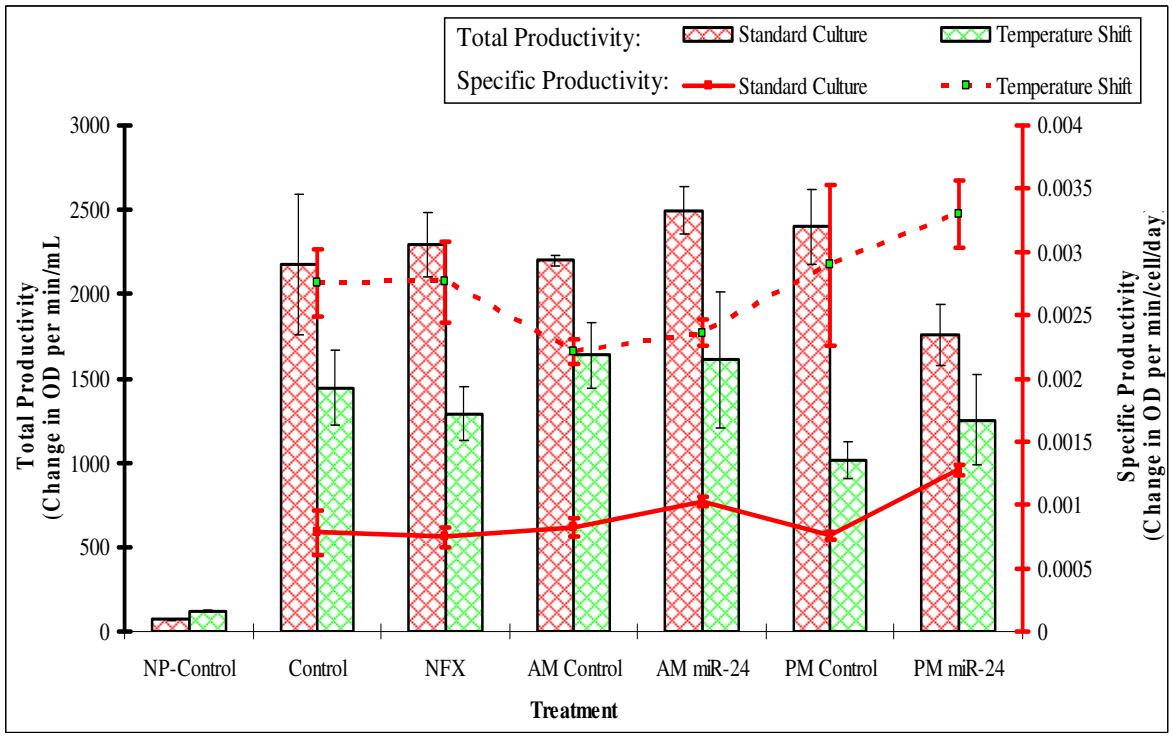

Figure 3.3.3.5. Effects of inhibition and over expression of miR-24 on the total and specific productivity of SEAP secreting CHO-K1 cells in standard and temperature-shifted culture. After transfection (0hrs), the cells were maintained at 37^0C for 96hrs in standard culture and in biphasic culture where cells were shifted to 31^0C for 72hrs after 24hrs of transfection. The effects of miR-24 were measured after 96hrs of transfection. Columns denote total productivity and superimposed red lines show specific productivity. Control denotes un-treated cultures. NP Control denotes non-producer CHO-K1 cells. NFX denoted transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir. Error bars represent standard deviation calculated using 3 biological replicate samples.

Table 3.3.3.4. Comparative analysis of effects of inhibition and over expression of miR-24 on growth, viability, total productivity and specific productivity of low serum-adapted SEAP secreting CHO-K1 cells in standard and temperature-shifted cultures after 96hrs of transfection. These data were calculated (in percentage) from Figure 3.3.3.4 and 3.3.3.5 by comparing AM Control with AM treated cultures and PM Control with PM treated cultures. '+' represent increase and '-' represent decrease in effect on cell growth or viability of culture. Shaded columns represent the significant effects (20% decrease or increase in effect at p-value ≤ 0.05).

Phenotype	**Culture condition**	**Inhibition**		**Over expression**	
		Effect (%)	**p-value**	**Effect (%)**	**p-value**
Growth	Standard culture	-9	0.22	-56	0.0002
	Temperature-shifted culture	-8	0.64	+5	0.82
Viability	Standard culture	+2	0.23	-1	0.47
	Temperature-shifted culture	-3	0.10	-8	0.001
Total productivity	Standard culture	+13	0.02	-27	0.02
	Temperature-shifted culture	-2	0.92	+24	0.22
Specific productivity	Standard culture	+25	0.01	+68	9.69E-05
	Temperature-shifted culture	+6	0.15	+14	0.37

3.3.4 Confirmation of inhibition or over expression of miRNA targets

To ensure that the effects observed on growth, viability or recombinant productivity of CHO-K1 cells following transfection were due to specific inhibition or over expression of miR-7 or miR-24, biological triplicate samples for each transfection conditions were analysed using singleplex qRT-PCR analysis. Results indicated that the expression of miRNA targets was increased following transfection of pre-miR-7 and pre-miR-24 at both standard and temperature-shifted culture conditions (Figure 3.3.4).

The anti-mir molecules bind specifically to thier miRNA target to inhibit its effects in the cell. As such the target miRNA is not degraded and during RNA extraction and reverse transcription, the bound anti-mer is released resulting in no detectable differences in miRNA levels during subsequent qPCR. Therefore, as expected, the inhibition miR-7 and miR-24 was not detected in singleplex qRT-PCR.

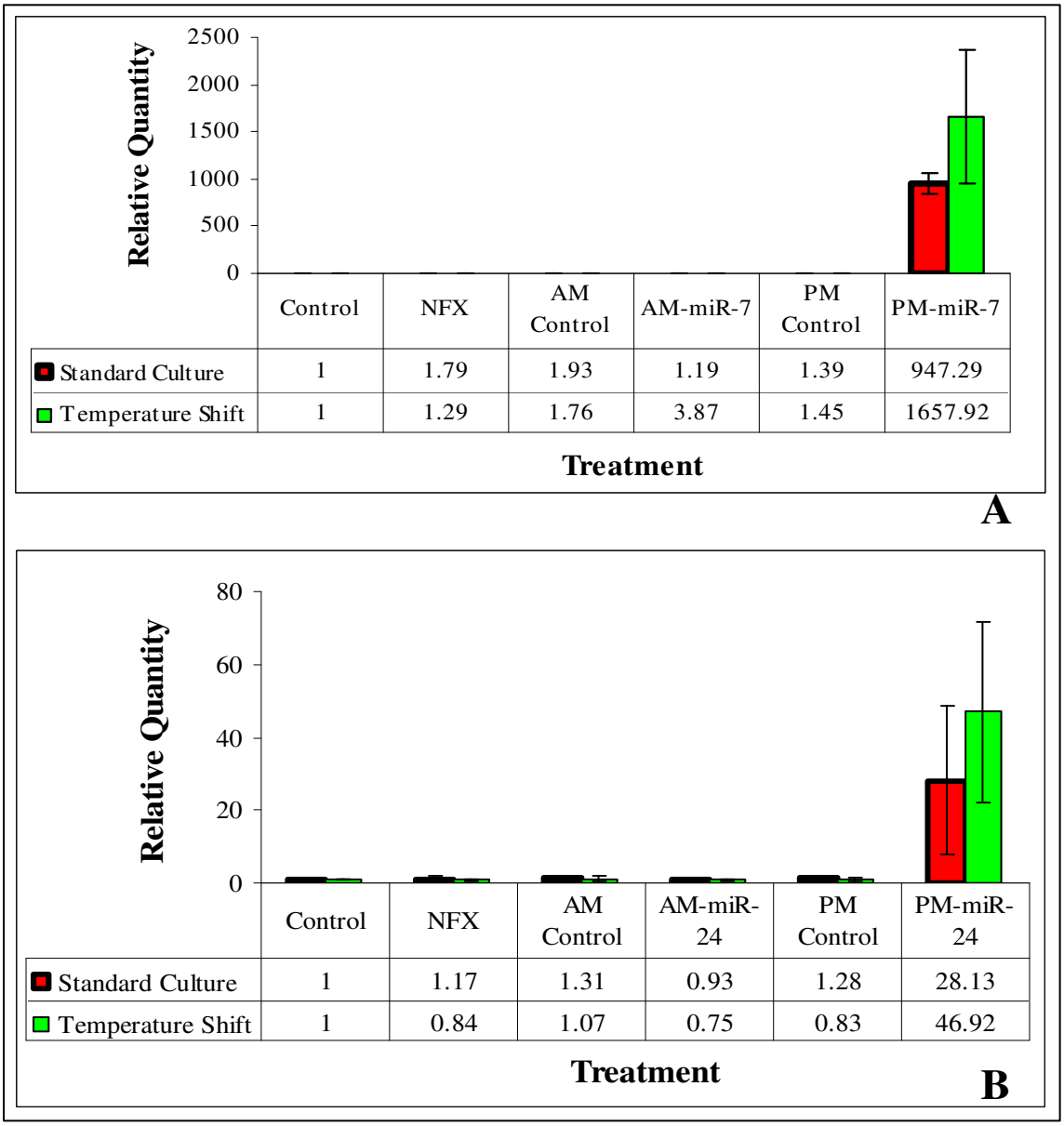

Figure 3.3.4. Investigation of expression of miR-7 and miR-24 following AM and PM transfection using singleplex qRT-PCR assays specific to mature miR-7 and miR-24. Data represents the relative quantity of miRNAs for biological triplicate samples harvested at 96hrs after transfection. **A** expression of miR-7 and **B** expression of miR-24. NFX denotes transfection reagent only, AM-Control represents scrambled Anti-mir and PM-Control represents scrambled Pre-mir. Error bars represent standard deviation calculated using 3 biological replicate samples.

Conclusion

Data indicate that low temperature cultivation of cells altered expression of miRNAs and this contributed to reduced growth and improved viability and recombinant protein productivity in culture. miRNA-bioarrays and TLDAs were used to identify these temperature-dependent changes in expression of miRNAs in this investigation. Among the differentially expressed miRNAs, two known growth regulatory miRNAs, miR-7 and miR-24, were found to be differentially expressed. miR-7 was down regulated and miR-24 was up regulated in biphasic cultures in comparison to standard cultures. Functional studies revealed that exogenous over expression of miR-7 and miR-24 can significantly reduce cell growth and increase recombinant protein productivity and therefore are potential targets to be implemented in cell engineering based approaches to improve efficiency of industrial production processes.

3.4 Investigation of secreted low molecular weight proteins by CHO-K1 cells in culture using SELDI-ToF MS

As described in section 1.7, secreted proteins can regulate cell growth, viability and recombinant protein productivity in culture. The culture environment can modulate post translational modifications (PTMs) and hence the quality of the protein product. There is a possibility that secreted proteins can also contribute towards the regulation of PTMs in protein products. Moreover, knowledge of secreted host cell proteins can help in designing efficient product recovery processes from production culture. Therefore conditioned media from different phases of the growth cycle of low-serum adapted CHO-K1 cells were investigated using SELDI-ToF MS in collaboration with Priyanka Maurya from the Proteomics Group at NICB, Dublin City University Dublin, Ireland.

3.4.1 Cell culture

CHO-K1 cells were grown in low serum medium (0.5%) over 144hrs of culture. Cells were seeded at $3x10^5$ cells/mL in T-75cm^2 flasks. Four biological replicate flasks were set up at each time point. Cell counts were performed and conditioned media samples were collected every day. Control flasks containing cell free media were also set up at each time point to account for serum proteins that may be consumed by the cells over time in culture. Figure 3.4.1 shows the growth of the cells over 144hrs of culture and clearly represents the different stages of growth (lag, logarithmic and stationary phases). Cells were in lag phase at 24hrs, mid-log phase at 72hrs and stationary phase of growth cycle at 120hrs of culture (Figure 3.4.1A). The viability of the culture also remained high throughout the testing period (Figure 3.4.1B).

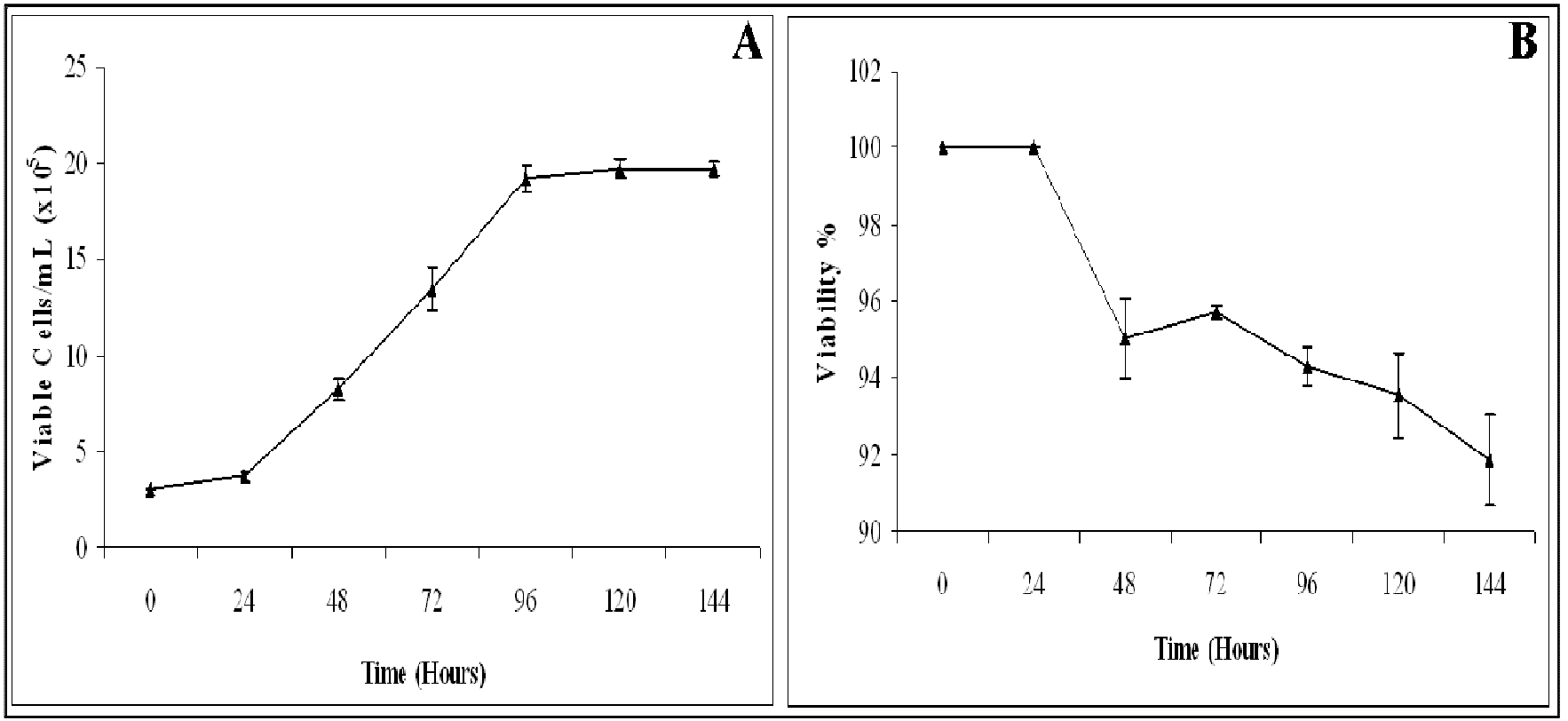

Figure 3.4.1. Growth profiles of CHO-K1 cells adapted to growth in low serum (0.5%) medium in monolayer culture. **A.** Viable cell counts. **B.** Percentage viability percentage. Error bars represents the standard deviation calculated from data obtained from the experiment (n=4).

3.4.2 Selection of chip surface

Conditioned media samples were concentrated using 5000 MW cut-off centricons (Millipore). Prior to full profiling analysis, optimization studies were performed using 10 μg and 50 μg of extracted protein from conditioned media samples representing 96hrs of culture on four different chromatographic surfaces to see which protein concentration and Protein Chip array gave optimal results. The following Protein Chip arrays were selected, an IMAC30 (immobilized metal affinity capture) activated with copper ions, CM10 (weak cation-exchange) with 20 mM Tris pH 4.5, Q10 (strong anion-exchange) with 50 mM Tris pH 8.0 and H50 (reverse-phase hydrophobic surface) with 10% acetonitrile/0.1% trifluoroacetic acid. The IMAC30 and Q10 ProteinChip 8-spot arrays provided better profiles under these conditions in terms of number and resolution of peaks and therefore were considered for the investigations (Table 3.4.2). The conditioned media from biological duplicate samples of CHO-K1 cells representing 96hrs of culture with and without media change after 48hrs of culture were profiled using IMAC surface to investigate if this alters the profiles on the chromatographic surface due to the removal of proteins accumulated in culture. Similar profiles for both culture conditions (with and without a change of culture medium) were observed in terms of resolution and detected peaks in both the low and high molecular weight range (Figure 3.4.2). The numbers of peaks detected for the low molecular weight range are greater in comparison to the high molecular weight range.

Table 3.4.2. Comparative analysis of the number of the peaks detected using SELDI-ToF MS with different chromatographic surfaces.

Array type	**Total no of peaks with conc. of 10 µg**	**Total no of peaks with conc. of 50 µg**
Cation exchange surface CM10	25	29
Anion exchange surface Q10	70	75
Reversed-phase surface H50	26	34
Metal affinity chip surface IMAC30	29	50

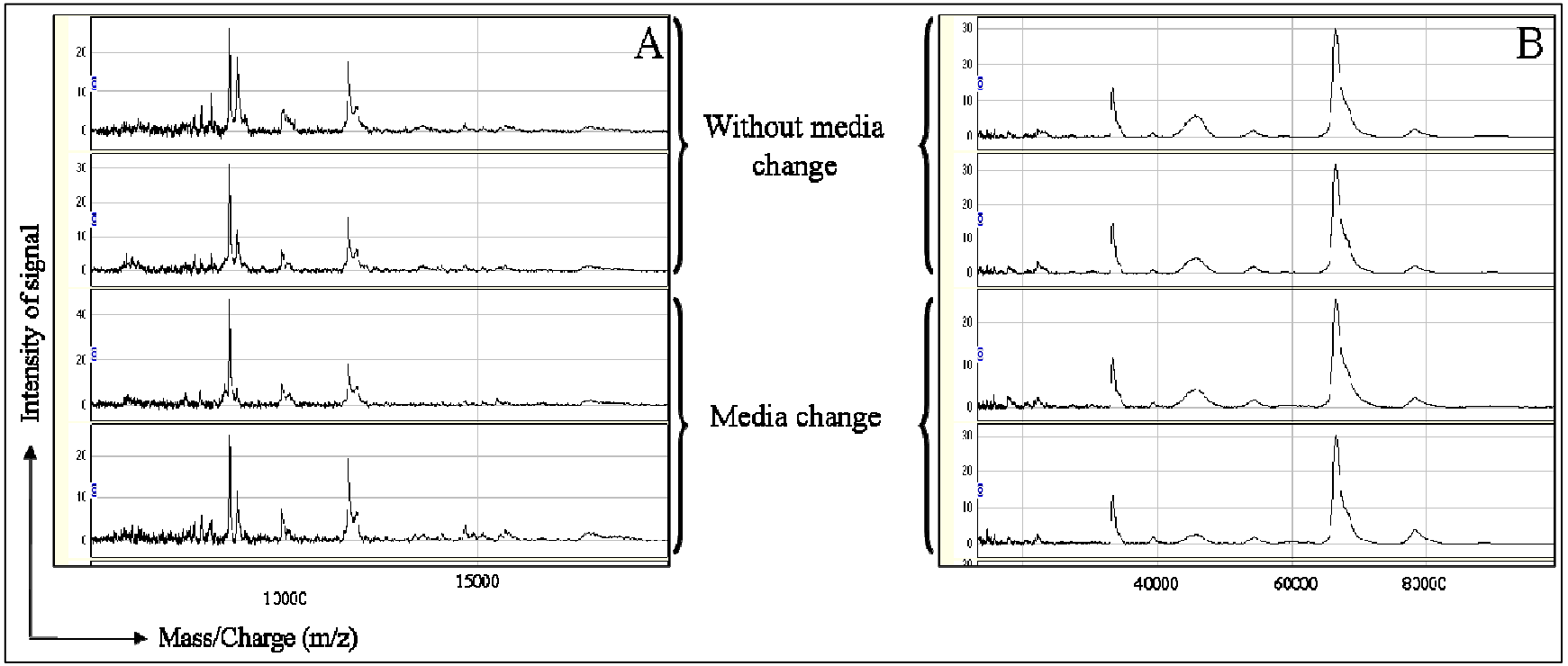

Figure 3.4.2. Investigation of the effects of proteins accumulated in late-exponential phase of culture on the binding of proteins with the IMAC surface. This experiment was carried out using biological duplicate samples. **A** represents the profile at low molecular weight range (5-20000Da) and **B** represents the profile at high molecular weight range (20-100000Da).

3.4.3 Analysis of differential expression of proteins/peptides on selected surfaces

Three biological replicate samples from each time point were profiled on each of the two surfaces, IMAC30 and Q10 ProteinChip arrays. Twenty four proteins/peptides were found to be differentially expressed over the 144 hrs of culture with a p-value < 0.05. The results for these 24 peaks are shown in Table 3.4.3. Figure 3.4.3.1A shows the SELDI-TOF MS profiles from two of these differentially expressed peaks at *m/z* 8118 (8.1 kDa) from the IMAC30 chip. Figure 3.4.3.1B clearly shows that the 8.1 kDa protein is increased until 72hrs of culture and then its expression is decreased over the next 72hrs. The 14.7 kDa protein is accumulated at later stages in culture, only appearing during the stationary phase, at 120 and 144hrs of growth (Figure 3.4.3.2). Figure 3.4.3.3 and 3.4.3.4 show the SELDI-TOF MS profile of two differentially expressed peaks from the Q10 array at *m/z* 7346 (7.3 kDa) and 11347 (11.3 kDa). The 7.3 kDa protein is increased over time in culture from 24hrs to 144hrs (Figure 3.4.3.3) while the 11.3 kDa protein is only increasing over time 96hrs onwards (Figure 3.4.3.4). The majority of the proteins differentially expressed on the two chromatographic surfaces used in this study are increased over time in culture, such as the 7.3 kDa and the 11.3 kDa proteins detected on the Q10 array.

Table 3.4.3. Protein/peptide peaks differentially expressed over time in culture of CHO-K1 cells on Q10 and IMAC30 ProteinChip arrays.

No.	**Q10**		**IMAC30**		
	***m/z* of peak**	**p-value**		***m/z* of peak**	**p-value**
1	5753.43	0.042	1	5109.49	0.037
2	5913.81	0.029	2	5261.48	0.045
3	6191.46	0.032	3	5864.21	0.026
4	7346.27	0.022	4	6065.27	0.043
5	7542.91	0.048	5	8118.21	0.032
6	8492.30	0.027	6	8591.44	0.034
7	9746.85	0.034	7	14735.28	0.029
8	9882.51	0.031	8	17975.62	0.022
9	10883.34	0.044	9	20343.55	0.023
10	11347.01	0.046	10	20853.77	0.021
11	12168.91	0.047	11	25798.46	0.037
12	13593.09	0.038			
13	15215.28	0.024			

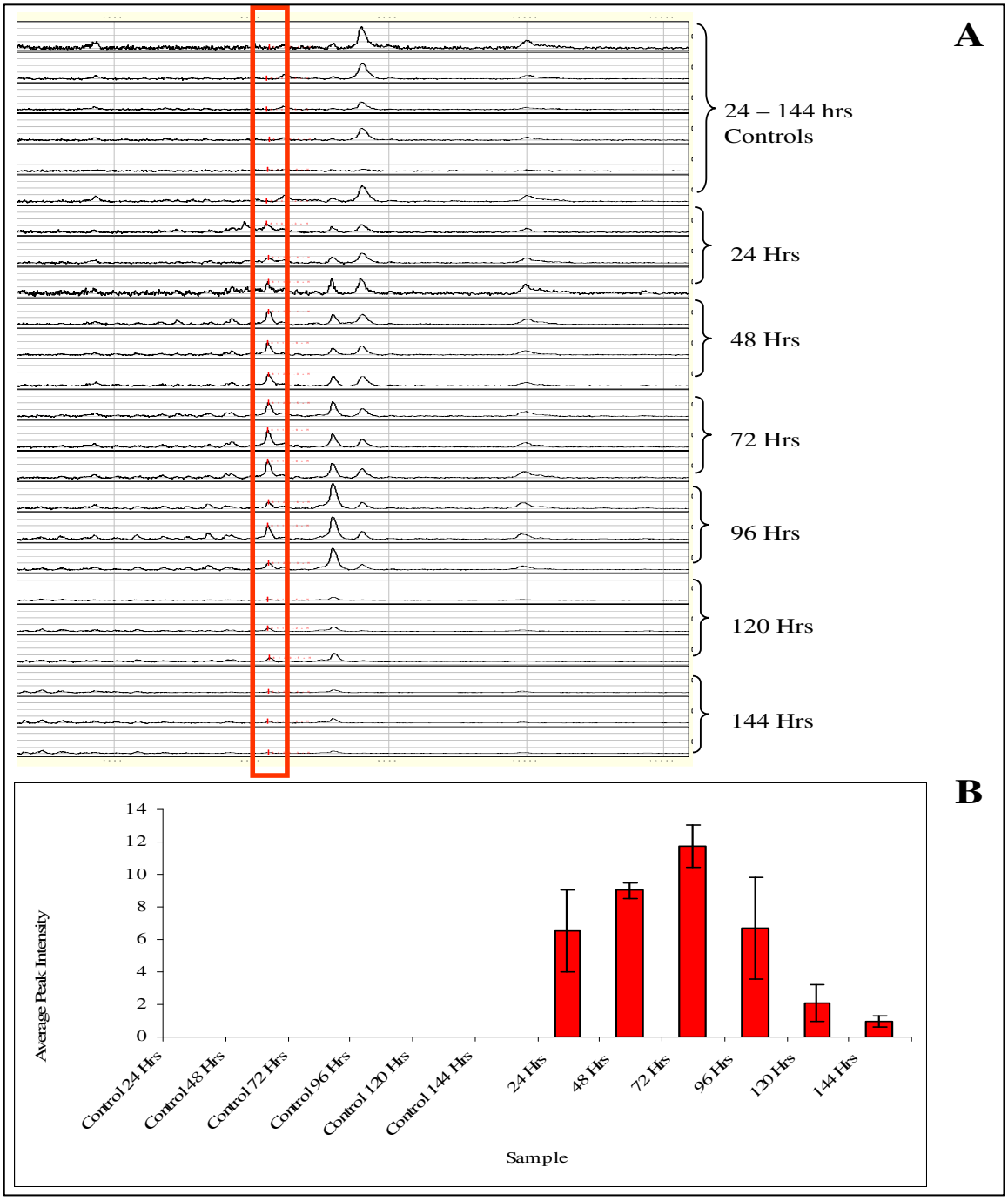

Figure 3.4.3.1. Differentially expressed proteins of CHO-K1 cells observed at 8.1 kDa over time in culture using IMAC-Cu^{2+} array. **A** represents the profile of three biological replicate samples from each time point from 24-144hrs. Profiles from control, cell-free flasks at each time point are also shown, i.e. one profile from each time point. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities over time in culture.

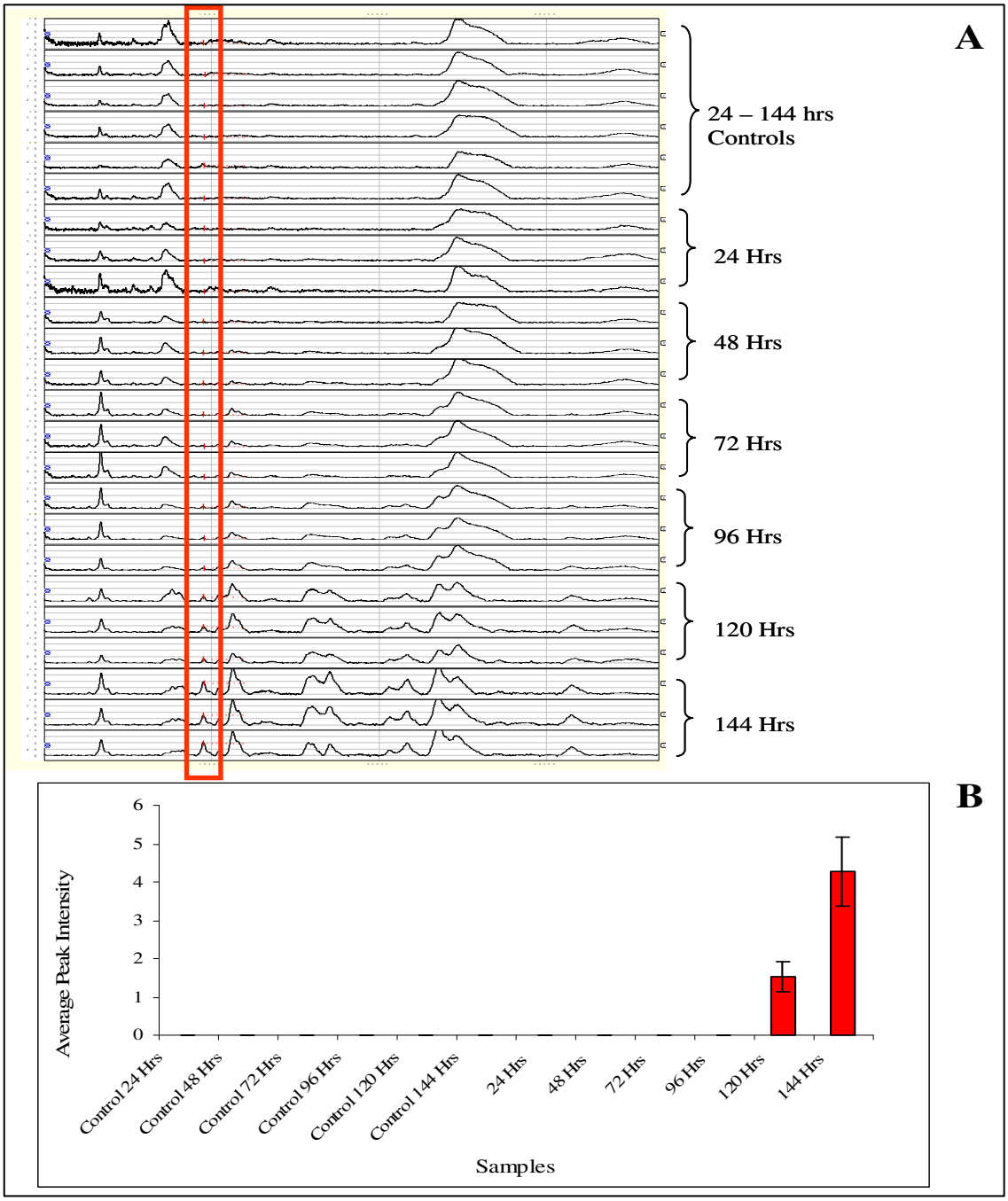

Figure 3.4.3.2. Differentially expressed proteins of CHO-K1 cells observed at 14.7 kDa protein/peptides over time in culture using IMAC-Cu^{2+} array. **A** represents the profile of three biological replicate samples from each time point from 24-144hrs. Profiles from control, cell-free flasks at each time point are also shown, i.e. one profile from each time point. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities over time in culture.

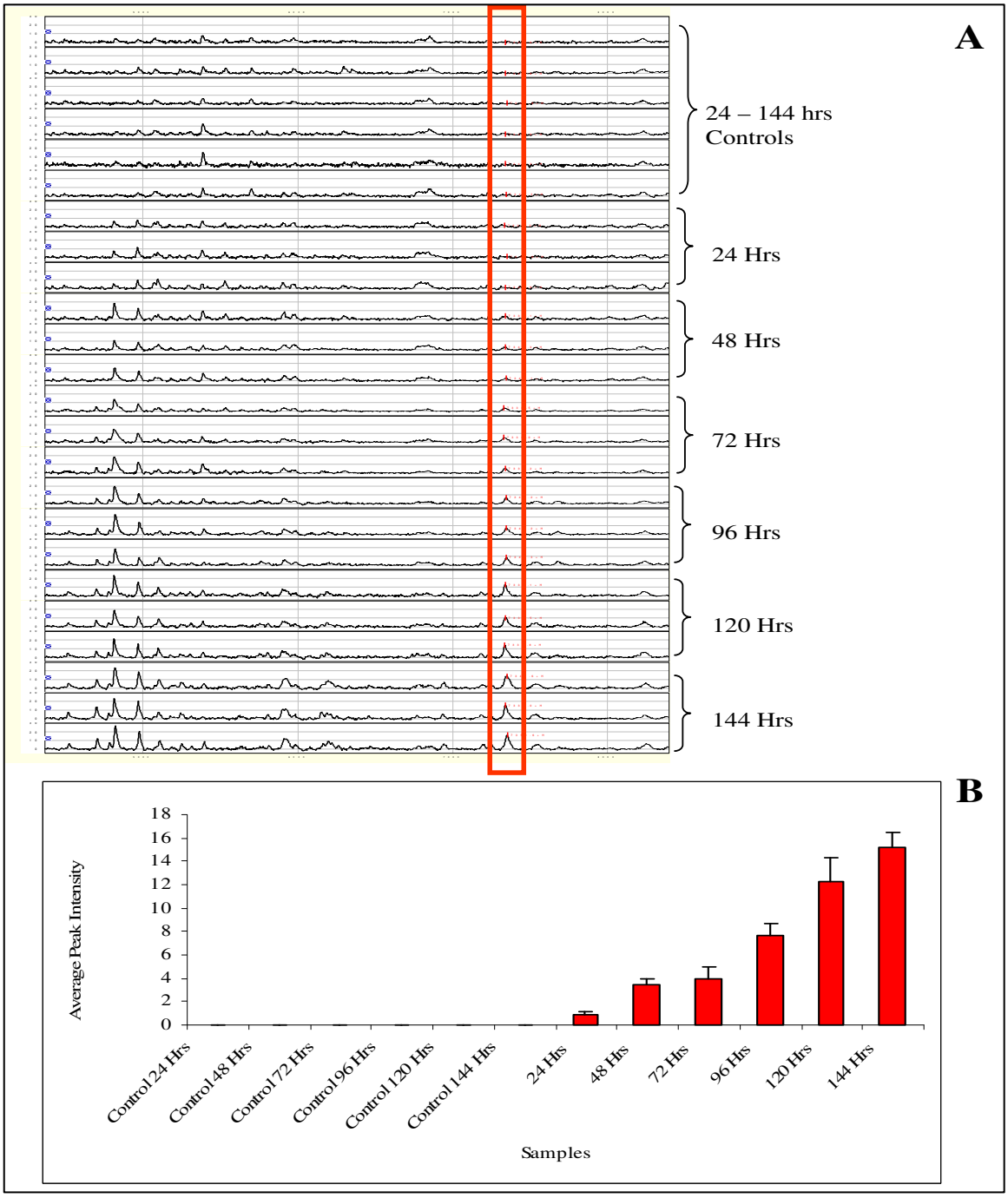

Figure 3.4.3.3. Differentially expressed proteins of CHO-K1 cells observed at 7.3 kDa protein/peptides over time in culture using Q10 array. **A** represents the profile of three biological replicate samples from each time point from 24-144hrs. Profiles from control, cell-free flasks at each time point are also shown, i.e. one profile from each time point. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities over time in culture.

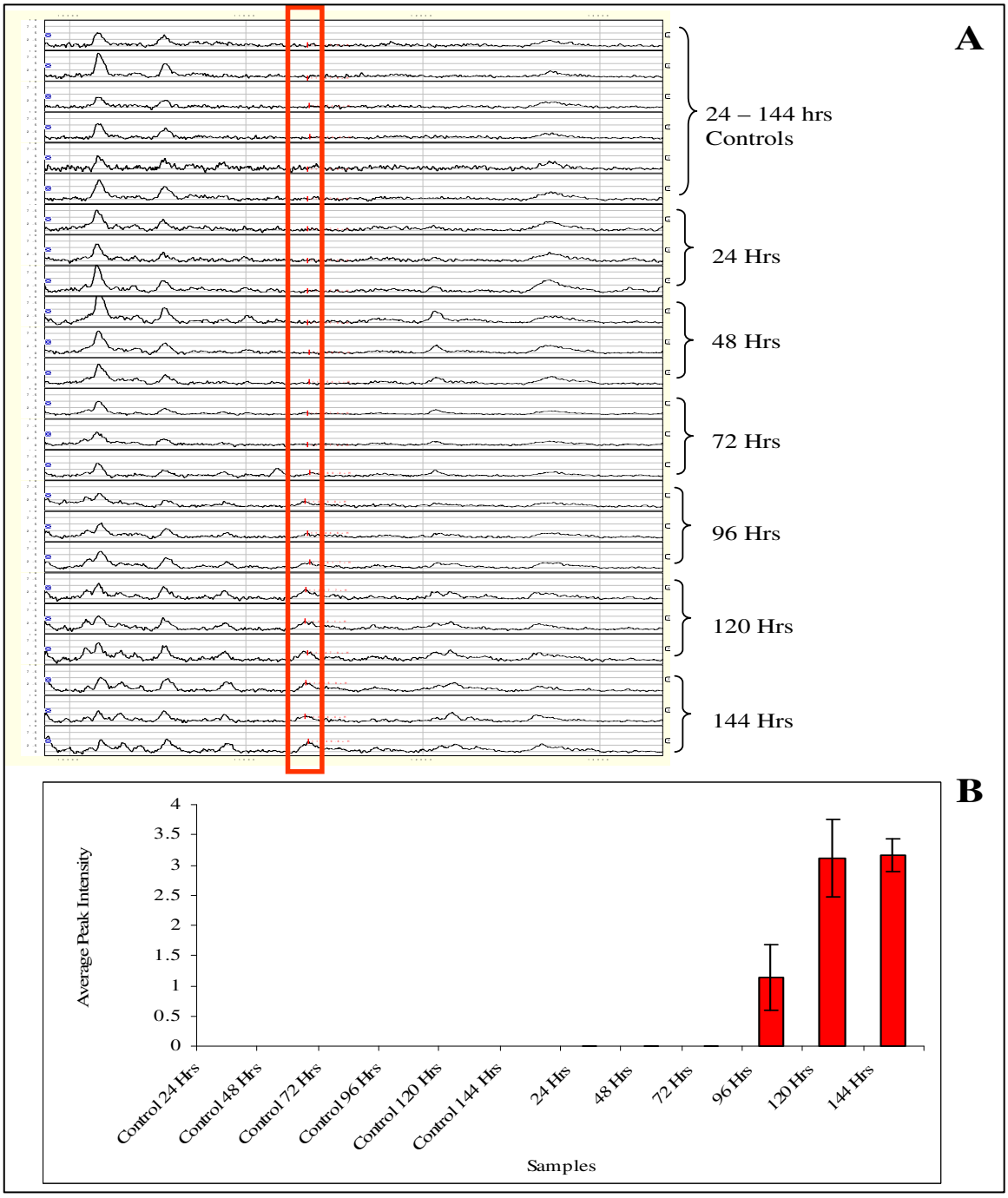

Figure 3.4.3.4. Differentially expressed proteins of CHO-K1 cells observed at 11.3 kDa protein/peptides over time in culture using Q10 array. **A** represents the profile of three biological replicate samples from each time point from 24-144hrs. Profiles from control, cell-free flasks at each time point are also shown, i.e. one profile from each time point. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities over time in culture.

3.4.4 Analysis of reproducibility of results

Three biological replicate samples of conditioned media representing lag, log and stationary phases of growth in culture were profiled, along with a technical duplicate of each sample, to assess the reproducibility of SELDI-TOF MS for profiling conditioned media samples from CHO-K1 cells. One time point was selected from each of the growth phases; 24 hrs from lag phase, 72 hrs from logarithmic phase, and 120 hrs from the stationary phase of growth. Figure 14A shows the SELDI-TOF MS profiles for each of these 6 samples at the three selected time points from different phases of the growth cycle. Figure 3.4.4.1 and 3.4.4.2 shows the increased expression of two peaks; one at *m/z* 11347 (11.3 kDa protein) on the Q10 array and a second peak at *m/z* 14735 (14.7 kDa protein) on the IMAC30 array. From this experiment, coefficients of variance (CV) values were calculated from the intensity readings from five randomly selected peaks from each conditioned media sample from each of the ProteinChip array surfaces. The CV values ranged from 11.42% to 20.7% for the Q10 array, and from 8.05% to 17.4% on the IMAC30 array, demonstrating the reproducibility of the technique. We also looked at inter-experimental variability, and found that the average mean CV from the intensity readings from 5 randomly selected peaks across all experiments in the study was 22.68%.

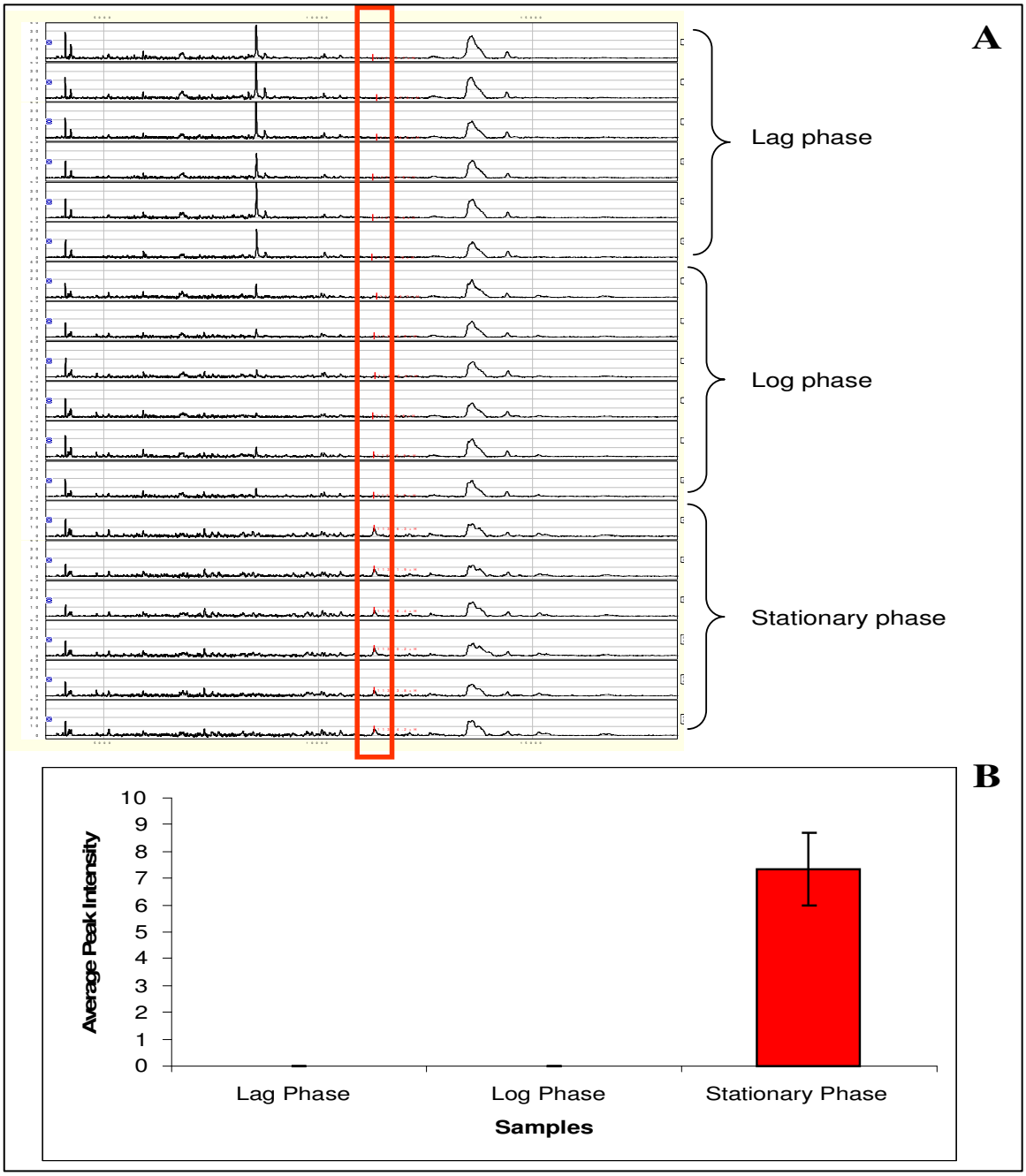

Figure 3.4.4.1. SELDI-TOF MS profiling of 24hrs (from lag phase), 72hrs (from log phase) and 120hrs (from stationary phase) conditioned media from CHO-K1 cells for the 11.3 kDa species on the Q10 array. **A** represents the profile of three biological replicate samples from each time point along with a technical duplicate (total of 6 samples per time point) to demonstrate the reproducibility of the method. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities.

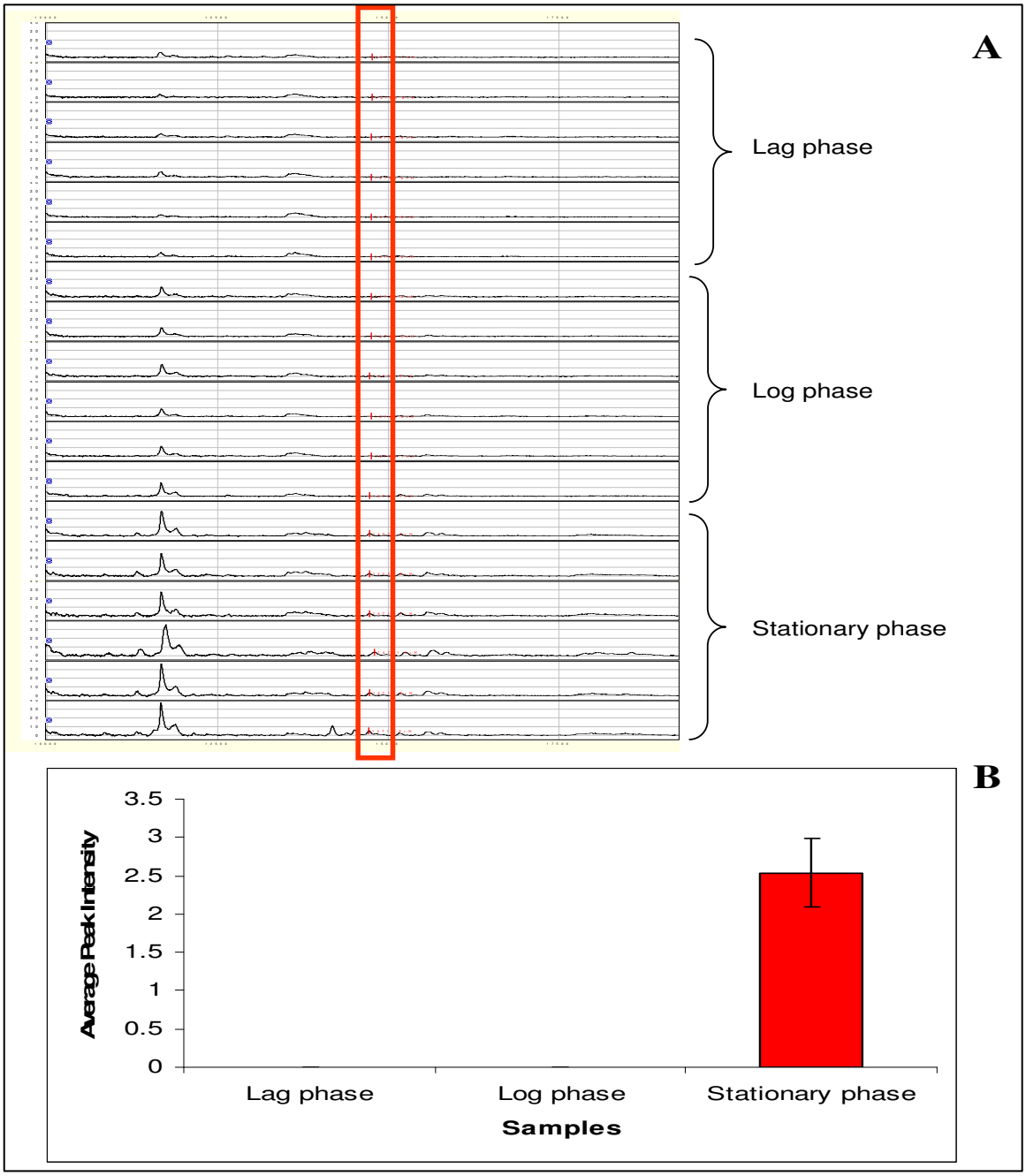

Figure 3.4.4.2. SELDI-TOF MS profiling of 24hrs (from lag phase), 72hrs (from log phase) and 120hrs (from stationary phase) conditioned media from CHO-K1 cells for the 14.7 kDa species on the IMAC30 array. **A** represents the profile of three biological replicate samples from each time point along with a technical duplicate (total of 6 samples per time point) to demonstrate the reproducibility of the method. The red box highlights the location of the differentially expressed peak. **B** represents the graph of the comparison of the average peak intensities.

Section 4.0

Discussion

Mammalian cells, specially Chinese Hamster Ovary (CHO) cells, are being extensively used for the production of recombinant proteins for therapeutic use. The prevalence of mammalian cells is largely attributable to their ability to impart appropriate protein folding and post-translational modification (glycosylation, phosphorylation, etc.) in the recombinant protein product, such modifications are required for the biological activity and quality of many products (Wurm 2004, Bollati-Fogolin *et al.* 2005, Schriebl *et al.* 2006). Currently, about 70% of all mammalian-cell-derived protein pharmaceuticals licensed in the USA were produced in CHO cells (Birch and Racher 2006). Approximatly $70 billion of sales revenue has been estimated to be generated using CHO cell factories in the biopharmaceutical market by the end of 2010 (Walsh 2006). These products include recombinant human growth hormone (rhGH) which is used to treat growth hormone deficiencies and erythropoietin (rEPO) which promotes the formation of red blood cells in the bone marrow.

The demand for protein therapeutics is increasing and this necessitates improving productivity from CHO cells. The methods currently applied to improve productivity include optimization of culture conditions (temperature, pH, aeration rate, etc.), improved reactor designs, medium formulation and cell engineering. Low temperature cultivation of mammalian cells has been observed to arrest cell growth while maintaining cell viability and this inhibition has been positively correlated with increased productivity and therefore has been used in industrial settings (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Kaufmann *et al.* 1999). However, the mechanisms regulating these phenotypes remain poorly understood. Therefore, low temperature-induced differential expression of proteins and miRNAs were investigated in CHO cells in this study to improve our understanding of the low temperature response in mammalian cells and help to improve recombinant protein productivity. The

secretome of CHO cells over distinct phases of culture, i.e lag, log and stationary phase, was also analysed to identify differently expressed secreted proteins that may affect growth and productivity of CHO cells.

4.1 Establishment of a recombinant protein-secreting CHO-K1 cell lines

The majority of industrial recombinant protein (rP) production processes are performed in suspension culture. Removal of serum from culture medium minimizes the risk of contamination from microbial species (viruses or mycoplasma etc), batch-to-batch variation and cost of down stream processing. Therefore a recombinant protein product expression CHO-K1 cells were established that were able to grow in reduced serum supplemented medium.

4.1.1 Cell Adaptation

Adherent CHO-K1 cells growing in 10% serum supplemented medium were adapted to grow in suspension by culturing them in spinner flasks. The growth behaviour of the cells gradually improved with time as cells at P-42 had shorter lag phase and achieved higher maximum cell densities in comparison to the cells at early stage of adaptation (P-1) (Figure 3.1.1). The cultures were also able to maintain high viability (≥94%) through out the adaptation which is in keeping with previous studies (Sinacore *et al.* 1996, Sinacore *et al.* 2000, Astley and Al-Rubeai 2008).

These suspension-adapted CHO-K1 cells were further adapted to grow in low serum (0.5%) or serum-free culture conditions (Figure 3.1.2). The cells took only 4 days to adapt to each reduced serum level from 5 to 1% in culture medium, may be because even 1% serum in the medium could be providing a sufficient nourishment and protection for the growth of CHO cells. When serum was reduced to 0.5%, the cells started to take more time to meet set criteria of adaptation (≥ 1x 10^6 cells/mL within 4

days of culture) in new culture environment. For example, cells took 10 days in 0.5 % FCS and 34 days in SFM (ExCell). To summarise, CHO-K1 cells were successfully adapted to grow in adherent as well as suspension culture in 0.5% FCS supplemented ATCC medium and ExCell serum-free medium.

4.1.2 Establishment of SEAP secreting CHO-K1 cells

Suspension-adapted CHO-K1 cells, exponentially growing in 0.5% FCS supplemented ATCC culture medium, were grown in adherent culture and transfected with a plasmid pcDNA-SEAP2 containing a gene of interest, secreted alkaline phosphatase (SEAP) (SEAP) (Figure 3.3.2.3). SEAP, a glycosylated protein, is a well known reporter protein used to investigate the recombinant protein production by cells (Carvalhal *et al.* 2003, Fussenegger *et al.* 1998, Kaufmann *et al.* 2001). The use of low serum adapted CHO-K1 cells minimized the risk of loosing the SEAP gene due to genetic re-arrangements that may occur during the process of cell adaptation (Brill *et al.* 1979, Sobecky *et al.* 1992, Prozorov 2001), whereas use of adherent culture facilitated clone isolation through limiting dilution. Only cells that have incorporated the neomycin resistant gene, with or without the SEAP, would survive in presence of G418 in the culture and therefore use of G418 allowed us to select transfectant cells (Figure 3.1.2.3). The transfectants were tested for SEAP productivity by measuring the enzymatic activity of SEAP in culture media. Only 1 clone (Clone 19) and 2 subclones from clone 19 were isolated as SEAP-producing clones. The comparison of productivity among these clones revealed that 12s was the highest producer clone followed by 3s and clone-19 respectively. Clones 3s and 12s were found to be stable in SEAP productivity over testing period (27days). However, longer testing-periods including cell-freezing and thawing cycles would be advantageous to further confirm the stability in expression of SEAP in both clones and suitability as model cell lines. Therefore, both subclones were

adapted to grow in suspension (Figure 3.1.2.9) and since 12s was the highest producer, it was selected for further studies.

4.2 Analysis of low temperature induced differential expression of proteins in CHO-K1 cells

The incorporation of a temperature reduction step is commonly employed during production cell culture in the biopharmaceutical industry. This temperature-shift is used as a means of simultaneously inducing growth arrest and extending long term culture viability thus increasing recombinant protein productivity and yield (Al-Fageeh *et al.* 2006, Kaufmann *et al.* 1999, Marchant *et al.* 2008, Yee *et al.* 2008). The mechanism allowing improved productivity are poorly understood and therefore a greater understanding of these mechanisms would enables us to manipulate cellular machinery to further increase yield.

Proteomics is the large-scale study of structures and functions of the proteins (section 1.9.1). Two dimentional gel electrophoresis has been used to investigate the molecular mechanism of various cellular processes, such as growth, apoptosis or secretion in mammalian expression systems (Baik *et al.* 2006, Underhill and Smales 2007, Dinnis *et al.* 2006). While 2D- gel electrophoresis is very good for looking at a large number of changes in protein expression, it has limitation in investigating very acidic, basic, hydrophobic, small and large proteins as well as low or overly abundant proteins. Moreover, only one sample can be separated on a single gel using 2D-gel electrophoresis and therefore this technique suffers from low sensitivity and reproducibility (Bunai and Yamane 2005). A modified 2D-gel electrophoresis, 2D-DIGE, enables separation of 2-3 fluorescently labelled protein samples on a singe gel and this minimizes gel-to-gel variation and improves reproducibility and sensitivity.

Although 2D-DIGE has been extensively used to investigate the changes in protein expression associated with various diseases such as cancer (Kondo 2008, Maurya *et al.* 2007), its use in bioprocess-related research is still limited. Here, we used 2D-DIGE and MALDI-ToF mass spectrometry to investigate the temperature-dependent differential expression of proteins to understand the mechanisms regulating the effects of low temperature in CHO-K1 cells.

4.2.1 Cell Culture

CHO-K1 cells were cultured in a biphasic process where cells were maintained for 72hrs at 37^0C and were then shifted to reduced temperature (31^0C) for a further 72hrs. Cells were also maintained in standard culture (37^0C) paralally. Following the temperature-shift, cell growth was immediately arrested compared to cells maintained in standard cultures (Figure 3.2.1A). Cells in temperature-shifted culture were observed to maintain a steady viable cell density throughout the culture whereas the cells at 37^0C began entering the death phase by 120hrs of culture. While the viabilities of both cultures were comparable throughout the 144hrs of culture, it was slightly reduced towards the end of the experiment for the cells maintained at 37^0C (Figure 3.2.1B). It is possible that the high percentage of serum (10%) in the culture medium protected both cultures from apoptotic cell death (Zanghi *et al.* 1999), therefore maintaining the high viabilities recorded. The CHO cells cultured in 10% serum supplemented culture medium were used in this early stage investigation as it was carried out before adapting cells to grow in reduced serum supplemented medium.

4.2.2 Protein identification

Following 2D-DIGE analysis to compare the differences in global protein expression at 72hrs and 144hrs of culture, 201 differentially expressed (DE) spots were observed in the temperature-shifted cells whereas 404 differentially expressed spots were observed for the cells maintained at 37^0C. A total of 247 spots comprising of 127 up and 120 down regulated at 144hrs were picked for identification using MALDI-ToF MS, of which, 53 proteins were successfully identified (Table 3.2.4). A number of spots resulted in high quality spectra but were not successfully identified. This could be due to various reasons including that the number of protein sequences for CHO cells are still very low in the protein database, i.e. Swiss-Prot+TrEMBL database contains only ~600 CHO proteins. The CHO proteins being investigated could have post-translational modifications and may differ in their peptide mass fingerprints (PMFs) compared to orthologous proteins from other mammalian species. Additionally the amino acids in the CHO proteins may also be different to orthologous sequences resulting in differing PMF.

4.2.3 Analysis of differential expression of proteins

The identified differentially expressed proteins were mainly analyzed in three groups, 72hrs at 37^0C vs. 144hrs at 31^0C (temperature-shifted culture), 72hrs at 37^0C vs. 144hrs at 37^0C (standard culture) and 144hrs at 31^0C vs. 144hrs at 37^0C (stationary culture). These comparisons enabled us to identify temperature-dependent and independent changes in protein expression.

4.2.3.1 Temperature-shifted culture (72hrs at 37^0C vs. 144hrs at 31^0C)

A total of 53 differentially expressed proteins were identified in this investigation, of which 35 proteins were in the temperature-shifted culture (72hrs at 37^0C vs. 144hrs at 31^0C) (Figure 3.2.4.3). Of these 35 proteins, 22 were up and 13 were down regulated in temperature-shifted cells. A number of proteins have previously been identified as differentially expressed following temperature-shift in CHO cells, including up regulation of vimentin and GAPDH (Baik *et al.* 2006, Yee *et al.* 2008). These proteins were up regulated in our study also (Table 4.2.1). RNA binding motif protein 3 (RBM3), a well known up regulated cold inducible protein in a variety of cell lines including CHO cells (Yee *et al.* 2008, Danno *et al.* 2000), was observed to be up regulated following temperature-shift using Western blotting in our study, although it was not identified to be differentially regulated using 2D-DIGE possibly due to its low molecular weight (~17kDa) (Table 4.2.1). β-actin, prohibitin and triosephosphate isomerise were observed to be differentially regulated in previous studies in response to low temperature culture (Baik *et al.* 2006) but they were found to be unchanged in our investigation possibly due to a different temperature (33^0C instead of 31^0C) or clonal heterogeneity. Apart from this, the proteins identified as differentially expressed in this study have not been previously reported to be associated with low temperature response in CHO cells and may help to improve the understanding of low temperature response in mammalian cells.

Table 4.2.1. Comparison of differentially expressed proteins identified in this study with previous studies on the effect of low temperature.

Protein	**This Study**	**Previous Study**			**Function**
		Protein/Transcript	**Author**	**Effect**	
Vimentin	Up regulated	Vimentin	Baik et al, 2006	Up regulated	Cell size, proliferation, cell growth rate and apoptosis.
			Yee et al, 2008	Up regulated	
GAPDH	Up regulated	GAPDH	Baik et al, 2006	Up regulated	Apoptosis, nuclear tRNA export, DNA replication, DNA repair and transcription
ACTB	No Change	β-actin (identified at multiple spots)	Baik et al, 2006	Up regulated at 2 spots and down regulated at 1 spot	Cell size, shape and proliferation, transcription and apoptosis
PHB	No Change	Prohibitin	Baik et al, 2006	Up regulated	Proliferation, differentiation and apoptosis
TPI1	No Change	Triosephosphate isomerise	Baik et al, 2006	No change	Stress conditions and cell death
			Yee et al, 2008	Down regulated	
RBM3	Up regulated	RNA binding motif protein 3	Yee et al, 2008	Up regulated	Transcription, translation, proliferation, differentiation and apoptosis

Comparing proteins differentially expressed in temperature-shifted cultures with those in standard cultures revealed that 23 proteins to be specifically altered in response to low temperature (Table 3.2.4), whereas 12 proteins were differentially regulated in both temperature-shifted and standard culture representing the temperature-independent changes in culture.

4.2.3.1.1 Analysis of temperature-specific differential expression of proteins

Of the 23 temperature-dependent differentially expressed proteins, 18 were up regulated and 5 were down regulated at 144hrs at 31^0C. These temperature-specific differentially expressed proteins were associated with the regulation of a variety of cellular functions, including protein translation, cell growth, apoptosis, structure and metabolism. Analysis of these proteins may identify targets critical for the regulation of low-temperature induced cellular phenotypes and to improved recombinant protein productivity.

4.2.3.1.1.1 Protein Translation

It has been observed that protein synthesis rates are reduced at low temperature due to a general inhibition of cap-dependent protein synthesis (Roobol *et al.* 2009, Phadtare *et al.* 1999). However some proteins, *e.g.* RBM3, are translated under cold stress via an IRES-mediated cap-independent mechanism (Al-Fageeh *et al.* 2006, Al-Fageeh and Smales 2006, Chappell *et al.* 2001). This type of protein translation initiation requires both eukaryotic initiation factors (EIF2, EIF3, EIF4A, EIF4B and EIF4F) and message-specific cellular IRES *trans*-acting factors (ITAFs) such as upstream of N-ras (Unr) and polypyrimidine tract binding protein (PTB) (Pilipenko *et al.* 2000). A number of proteins that are involved

in regulation of both cap-dependent and independent protein translation, such as EIF4A and HNRPC, were observed to be differentially regulated following temperature-shift in this investigation (Table 3.2.4).

EIF4A is an RNA helicase enzyme that plays an important part in translation of mRNAs and was found to be increased by 1.81 fold following temperature-shift. EIF4A exhibits RNA-dependent ATPase activity and ATP-stimulated RNA binding activity and unwinds local secondary structures in the 5′ UTR of mRNAs to facilitate cap-dependent translation. EIF4A is also one of the critical translational initiation factors that is required in IRES mediated cap-independent translation, however the molecular mechanism is unknown (Lin *et al.* 2009, Pilipenko *et al.* 2000, Pause *et al.* 1994). Thus, the over expression of EIF4A following temperature-shift may facilitate translation for proteins required by cells to cope with hypothermic conditions.

HNRPC is a nuclear pre-mRNA binding protein and was observed to be 1.52 fold down regulated following temperature-shift. HNRPC can interact with the IRESs to regulate expression of proteins involved in various cellular phenotypes such as cell growth (c-myc) and stress management (Upstream of N-Ras, Unr). HNRPC has been shown to interact with IRESs in the 5'UTR of Unr and facilitates cell proliferation through IRESs, including the PITSLRE IRES, which is activated at mitosis (Schepens *et al.* 2007) and known to be essential for eukaryotic cell cycle control (Tinton *et al.* 2005). This is important because during mitosis, similar to low temperature culture inductions, protein synthesis is rapidly and severely repressed possibly due to the inhibition of global cap-dependent translation (Fan and Penman 1970).

The fact that EIF4A and HNRPC are both differentially expressed in this experiment following temperature-shift clearly indicates the importance of IRES-based regulation of the translational machinery under hypothermic conditions. This also establishes EIF4A and HNRPC as potential protein targets that need to be investigated for their roles in the regulation of low-temperature induced phenotypes in CHO cells. EIF4A and HNRPC are our novel finding as they have not been previously reported to be associated with recombinant protein production in CHO cells.

4.2.3.1.1.2 Cell growth

The most obvious effect following reduction of culture temperature is the immediate decrease in growth. A number of growth-related proteins were identified as being differentially expressed in a temperature-specific manner, including EIF4A, HNRPC, RRN3 and LGALS1 (Table 3.2.4 and 3.2.6).

EIF4A is also involved in the regulation of cell growth being up regulated in G2 arrested CHO cells (Sasaki *et al.* 2000). The reduced expression of EIF4A has been observed to inhibit translation resulting in inhibition of cell cycle and growth (Daga and Jimenez 1999). Therefore, increased expression of EIF4A in this study as a cause or consequence of cell cycle arrest due to temperature-shift remains unclear.

HNRPC may also have a role in regulating cell growth as well as translation. HNRPC can enhance the translation of c-myc mRNA and thus increase proliferation in human embryonic kidney cells (HEK293/T) (Kim *et al.* 2003). Silencing of HNRP C1/C2 protein expression by siRNA has also been shown to result in the inhibition of cell proliferation

(Schepens *et al.* 2007). These results are in keeping with our finding that HNRPC is down regulated following the reduction of the culture temperature.

RRN3 (RRN3 RNA polymerase I transcription factor) level was found to be 1.53 fold down regulated at the end of the temperature-shifted culture compared to exponentially growing cells. RRN3, also known as TIF-IA, is an essential polymerase-associated protein required for transcription. However it is unclear whether RRN3 is required for initiation or elongation by RNA polymerase I (Pol I). Growth arrest can result in reduced RRN3 is reduced in stationary phase cells following drug-treatments or nutrient deprivation or in an inactive dephosphorylated forms (Zhao *et al.* 2003). The growth-dependent control of Pol I transcription through RRN3 suggests that this factor is targeted by signal transduction pathways which are important for cell proliferation. Therefore, down regulation of RRN3 following temperature-shift is consistent with its activity in stationary cells.

LGALS1 (lectin, galactoside-binding, soluble, 1 or galectin 1) was 1.62 fold up regulated on temperature-shift. LGALS1 has an affinity for β-galactoside sugars which upon binding to the appropriate glycoligands elicits a multitude of biological activities. LGALS1 has been shown to have effects on cell adhesion (Mahanthappa *et al.* 1994), regulation of cell proliferation (Wells and Mallucci 1991, Allione *et al.* 1998) and apoptosis (Perillo *et al.* 1995). LGALS1 acts as a negative cell growth regulator in fibroblasts (Wells and Mallucci 1991, Allione *et al.* 1998) and reduces proliferation of mouse thymocytes (Vespa *et al.* 1999). LGALS1 blocks the mitogenic MAP kinase cascade that is normally activated by binding of growth factor ligand to the appropriate receptor tyrosine kinase (Vespa *et al.* 1999). LGALS1 induces apoptosis of activated human T cells (Perillo *et al.* 1995) and human T leukaemia cell lines (Allione *et al.* 1998). Conversely, LGALS1 can act in a dose-

dependent manner, at low concentrations, significantly promoting cell proliferation without inducing cell death in the mouse leydig tumour cells (MA-10) (Biron *et al.* 2006). Moreover, LGALS1 has been reported to be down regulated in high producer CHO cells in comparison to low producers, indicating its potential involvement in the regulation of recombinant protein productivity in CHO cells (Meleady *et al.* 2008). LGALS1 was up regulated in the temperature-shifted cells in our investigation and therefore correlates with the reduced cell growth.

4.2.3.1.1.3 Apoptosis

Another common feature of cells that are cultured at reduced temperatures is delayed apoptosis compared to cells cultured at 37^0C (Al-Fageeh and Smales 2006, Moore *et al.* 1997). It has been proposed that the reduction in apoptosis is a result of reduced cellular metabolism at low temperatures (Moore *et al.* 1997). However, others have reported the induction of anti-apoptotic proteins (e.g. BCL-2) following culture at reduced temperature (Zhang *et al.* 2001). BCL-2 (a low molecular weight protein, which are often under represented on 2D-gels) was not identified as differentially expressed in this study but a few apoptosis-associated proteins were identified including importin-α (Table 3.2.4 and 3.2.6).

Importin-α, a nuclear import receptor, was 1.8 fold down regulated in temperature-shifted cultures. It facilitates trafficking of the growth promoting Ras effector, RASSF5, into the nucleus (Kumari *et al.* 2007). Over expression of importin-α results in significant increases in p21(waf1/cip1) transcript levels and apoptosis, implicating it in the nuclear import of p53 (Kim *et al.* 2000).

4.2.3.1.1.4 Structure

A number of cytoskeletal proteins including vimentin, profilin and LASP1 were differentially expressed following temperature-shift (Table 3.2.4). Vimentin and profilin were also observed to be differentially expressed by Baik et. al. (Baik *et al.* 2006) and support their potential involvement in low temperature response in CHO cells.

Vimentin, a major cytoskeletal proteins, was 2.05 fold up regulated in temperature-shifted cells and is consistant with recent observations in CHO cells (Baik *et al.* 2006, Yee *et al.* 2008, Underhill and Smales 2007). Its role in the regulation of cell growth and recombinant protein productivity is complex as increased levels of vimentin have also been shown in the heat-resistant phenotypes of CHO cells (Lee *et al.* 1992), whereas reduced expression has been observed in high producer CHO cells (Meleady *et al.* 2008). The investigation of expression of vimentin in low, medium and high producer clones could possibly improve our understanding of role of vimentin in regulation of growth and productivity in CHO cells.

Profilin (PFN2) is a small ubiquitous actin monomer sequestering protein and it is found to be 1.69 fold up regulated in temperature-shifted culture. PFN2 is important for spatial and temporal growth control of actin microfilaments, an essential process in the regulation of shape, structure and integrity of cells. PFN2 has been shown to be critical for normal actin polymerization in response to thermal stress (Yeh and Haarer 1996a) and therefore increased level of profilin 2 following low temperature induction may have roles in the regulation of cytoskeleton of cells in response to hypothermic conditions.

The LIM and SH3 domain protein, LASP1, was observed to be 2.06 fold up regulated following temperature-shift. This protein functions as an actin-binding protein and possibly

in cytoskeletal organization, however the exact cellular function is not known yet. LASP1 has been observed to be down regulated in high producer CHO cells (Meleady *et al.* 2008) suggesting that the up regulation of LASP1 in response to low temperature could be associated with regulation of cytoskeleton at hypothermic conditions. Similar to vimentin, investigation of LASP1 level in a range of producer clones could improve our understanding of its role in regulation of growth and productivity.

4.2.3.1.1.5 Metabolism

Cellular metabolism is generally reduced in low culture temperature (Moore *et al.* 1997) but this does not apply to every enzyme and pathway. GAPDH, a multi-functional glycolytic enzyme, was almost 2 fold up regulated following temperature-shifted cells, similar to other published data (Table 4.2.1) (Baik *et al.* 2006). GAPDH has also been observed to be abundant in high producer NS0 cells (Dinnis *et al.* 2006) indicating that increased expression could be associated with increased productivity.

Another enzyme alpha glucosidase 2 (alpha neutral subunit) (GANAB) is a component of the glycan-processing enzyme, glucosidase II and is involved in carbohydrate metabolism. GANAB was 2.19 fold up regulated in temperature-shifted cells. It trims two α-1, 3-linked Glc residues from the glycoprotein oligosaccharide Glc2Man9GlcNAc2 to give Glc1Man9GlcNAc2 and Man9GlcNAc2 in the endoplasmic reticulum (Totani *et al.* 2006). Monoglucosylated glycans generated by this process play a key role in glycoprotein quality control in the ER, as they are primary ligands for the lectin chaperones calnexin (CNX) and calreticulin (CRT) that are involved in ensuring glycoprotein quality prior to secretion (Totani *et al.* 2006). The increased levels of GANAB may contribute to the improved

product quality observed following low culture temperature. Further work including knockdown of GANAB expression followed by glycoprotein quality analysis could clear the role of GANAB in cell growth and quantitative and qualitative productivity.

The presented data indicate that the expressions of a number of proteins were specifically altered in response to low temperature. These proteins were involved in the regulation of variety of functions, such as translational machinery (EIF4A and HNRPC), cell growth (EIF4A, HNRPC, RRN3 and LGALS1), apoptosis (Importin-α), cell structure (vimentin, LASP1 and profilin 2) and cellular metabolism (GAPDH and GANAB). Investigation of these proteins could enable us to improve the growth behaviour of cells and recombinant protein production in culture.

4.2.3.1.2 Analysis of temperature-independent differential expression of proteins

Of 53 identified proteins, 12 proteins were commonly differentially expressed in both the temperature-shifted and standard culture (Table 3.2.5). These 12 proteins (4 up and 8 down regulated at 144hrs at 37^0C) represented the process-dependent changes in expression of proteins in culture. The majority of proteins were similarly regulated between temperature-shifted and standard cultures indicating time-dependent changes in culture. For example, levels of AHSA1, TXNL2, FKBP4 and 1HLUA were similar in both culture conditions and therefore could be involved in the regulation of house keeping proteins in cells. Only calponin 3 (CNN3) was up regulated in temperature-shifted culture. CNN3 is ubiquitously distributed in intermediate microfilaments (Fujii *et al.* 2000). Besides interating with actin, CNN3 can interact with gelsolin, one of the abundant cytoskeleton proteins required to

sever actin filaments coming from damaged compartments. Calponin by reducing the actin-binding activity of gelsolin during actin filament formation could regulate the cellular morphology (Ferjani *et al.* 2006). The increased level of CNN3 might strengthen the cytoskeleton due to increased gelsolin activity and this could contribute to the improved viability of cells maintained at low temperature.

4.2.3.1.3 Analysis of possible low temperature-induced post-translational modifications of proteins

Two proteins, ALDH and DPYSL2, were identified at two different pIs on gels from the temperature-shifted culture suggesting potential low temperature-induced post-transcriptional or translational modifications (PTMs). Identification of these modifications could improve our understanding of the signalling pathways and other associated proteins in response to low temperature.

ALDH is a $NAD(P)^+$-dependent enzyme that can protect against hyperoxia-induced cell death through reduction of ROS, activation of ERK/MAPK and PI3K-Akt cell survival signalling pathways (Xu *et al.* 2006). Analysis of the ALDH spots revealed 1.63 and 1.98 fold up regulation in temperature-shifted culture.

DPYSL2 promotes microtubule formation by binding to tubulin heterodimers (Fukata *et al.* 2002). Different variants, possibly phosphorylated, of DPYSL2 have been reported previously (Franzen *et al.* 2003). In this study, at one spot, DPYSL2 was 2.58 fold up regulated following temperature-shift, whereas it was 1.97 fold up regulated in standard culture. However, at second spot, DPYSL2 was 2.51 fold down regulated only in temperature-shifted cultures.

The functional association of ALDH and DPYSL2 with low temperature response is unknown. In-silico phosphoproteome analysis predicted that both ALDH and DPYSL2 contain phosphorylation sites and hence the multiple spots may indicate that they are phosphorylated. A previous study also observed two proteins (80 and 180 kDa) as being phosphorylated at tyrosine residues in CHO cells cultured at low temperature (Kaufmann *et al.* 1999) suggesting that active cell signalling occurs in response to temperature-shift.

4.2.3.2 Standard culture (72hrs at 37^0C vs. 144hrs at 37^0C)

The identification of differential protein expression between cells in exponential phase at 72hrs of culture at 37^0C and cells in stationary phase of growth at 144hrs of culture at 37^0C may reveal proteins involved in regulating cellular phenotypes, such as cell growth and apoptosis. A total of 30 differentially expressed proteins were identified comparing exponential and stationary phase of standard culture in this investigation (Figure 3.2.4.3 and Table 3.2.4). Of these, 18 proteins were specifically differentially regulated between exponential and stationary phase of standard culture.

The cells at 144hrs in standard culture were in the early death phase as reflected by the slight reduction in viability (Figure 3.2.1B). This is likely due to a variety of reasons including nutrient limitation, oxygen depletion and waste accumulation. As such, metabolic enzymes PYP and ATP5B, involved in energy generation, were 1.58 and 2.28 fold down regulated respectively in the cells at 144hrs in comparison to exponentially growing cells at 72hrs (Table 3.2.4).

A number of apoptosis and growth-related proteins were found to be differentially regulated in cells at stationary phase at 144hrs in comparison to cells growing exponentially at 72hrs of culture (Table 3.3.4).

4.2.3.2.1 Apoptosis-related metabolic proteins

Cells generate energy mainly from carbohydrate metabolism and usually store it temporarily in the form of ATP using it to drive activities, such as DNA synthesis and cell growth. Defects in energy generation therefore may lead to the cell death in culture. A number of apoptosis-related metabolic proteins, including prohibitin (PHB), Aldehyde dehydrogenase family 1, subfamily A1 (ALDH1A1) and alpha-enolase (ENO1), were observed to be differentially regulated in standard culture.

PHB is an evolutionarily conserved and ubiquitously expressed membrane protein. PHB was observed to be 1.66 fold down regulated in the cells in stationary phase at 144hrs in comparison to the cells exponentially growing at 72hrs. Prohibitins can be localized to different cellular compartments, the plasma membrane, the nucleus and mitochondria. A diverse array of cellular functions have been attributed to prohibitin, however the mechanism by which it regulates such a diverse range of functions remains poorly understood. PHB was observed to block DNA synthesis in normal fibroblasts and HeLa cells thereby arresting cell cycle progression into the S-phase with antisense inhibition of PHB recovering the cell growth (Nuell *et al.* 1991). Similarly, over expression of PHB inhibits cell proliferation by arresting cells in G0/G1 phase and has also been shown to increase survival of T47D cells (Wang *et al.* 2002). Besides anti-proliferative effects, PHB has been implicated in transcriptional regulation (Fusaro *et al.* 2003), cellular signalling

(Rajalingam *et al.* 2005), mitochondrial biogenesis (Nijtmans *et al.* 2000) and apoptosis (Fusaro *et al.* 2002). PHB is capable of physically interacting with p53 *in vivo* and *in vitro* promoting p53-mediated transcriptional activity by enhancing its recruitment to promoters (Fusaro *et al.* 2003). Although the localization of PHB which can affect the cellular function was not investigated in the present study, the down regulation of the expression of PHB suggests that it may protect cells from stationary/apoptotic conditions in culture.

ALDH1A1 is a cytosolic enzyme belonging to the aldehyde dehydrogenase family of proteins which are involved in the conversion of wide variety of aldehydes (retinaldehyde, acetaldehyde, etc.) to their corresponding acids (retinoic acid, acetic acid, etc.) by $NAD(P)^+$ dependent reactions (Yoshida *et al.* 1998). It was 1.64 fold down regulated in cells at 144hrs compared to cells at 72hrs in standard culture and could be contributing to stationary phase or apoptosis in culture. Down regulation of ALDH1A1 results in increased generation of toxic reactive-oxygen-species (ROS) that include highly reactive superoxide molecules and hydrogen peroxides which could have proliferative as well as apoptotic effects on cells (Gyamfi *et al.* 2006). ALDH1A1 has been observed to be down regulated in lens epithelial cells from human, mouse and rat that have increased susceptibility towards apoptosis (Choudhary *et al.* 2005). Down regulation of ALDH1A1 in this investigation is therefore in agreement with the published literature and suggests its negative correlation with cell death in culture. Up regulation of ALDH1A1 levels in culture could help to maintain high viability for longer periods of time.

ENO1 is an essential cytosolic enzyme for glycolysis and gluconeogenesis. ENO1 was observed to be 1.51 fold up regulated in cells at 144hrs compared to 72hrs in standard culture. ENO1 catalyses the conversion of 2-phosphoglycerate (2-PG) into

phosphoenolpyruvate (PEP). The over expression of ENO1 has been shown to inhibit cell growth and induce apoptosis in neuroblastoma cells (Ejeskar *et al.* 2005). Recently ENO1 has been reported to be over expressed in high producer CHO cells implicating the possibility that it could be involved in the regulation of recombinant protein productivity in cells (Meleady *et al.* 2008). ENO1 was observed to be unchanged in temperature-shifted cultures which are known to improve productivity and this complicates the elucidation of its role in improving recombinant productivity of CHO cells. Therefore increased level of ENO1 in cells in stationary phase (144hrs) in comparison to cells exponentially growing (72hrs) in standard culture suggests that ENO1 may have potential role in regulation of apoptosis in culture, although no evaluation of the rate of apoptosis in the culture for any of these apoptosis-related proteins has been made. Further, it would be necessary to look at the rate of apoptosis in the stationary and exponentially growing cells.

4.2.3.2 Growth and apoptosis-related structural proteins

The cytoskeleton acts as the mechanical supporting framework to maintain the shape of cells. It is generally accepted that cells under going apoptosis show a dramatic change in their cytoskeleton. The alteration of structural proteins may dysregulate mechanical coupling between cytoskeletal microtubules and actin microfilaments, and this could play a key role in control of proliferation as well as apoptosis (Rodolfo and Piacentini 2002). A number of structural proteins including ACTB and CAPZB were observed to be down regulated in cells at stationary phase indicating their potential role in regulation of cell death in culture.

ACTB, was identified at two different pIs and molecular weights on the gel containing samples from standard culture indicating that different variants of ACTB may exists in CHO cells. However ACTB was down regulated by 1.97 and 1.72 fold at both positions in cells at 144hrs in comparison to cells at 72hrs in standard culture. ACTB is involved in many biological functions in the cells including regulation of cell shape and growth. Increased expression of ACTB has been shown to provide resistance against apoptosis in HL-60 myeloid leukemia cells by supporting morphological changes associated with the development of cell death (Veselska *et al.* 2003). ACTB has been observed to be abundant in NS0 cells with increased productivity of Mab (Dinnis *et al.* 2006) indicating that cytoskeleton proteins also important for efficient protein production by cells. The down regulation of ACTB at 144hrs in comparison to 72hrs of culture possibly indicate that ACTB should be up regulated to maintain growth, viability and productivity in culture.

CAPZB is an actin-binding protein and was 1.5 fold down regulated in cells at 144hrs in comparison to cells at 72hrs in standard culture. CAPZB binds to one end of actin filaments, regulates actin polymerization including the number and length of actin filaments, and strengthens the actin cytoskeleton in the cytoplasm (Pappas *et al.* 2008, Xu *et al.* 1999). Therefore, reduced expression of CAPZB, as well as ACTB, is likely to be associated with dysregulation of cytoskeleton and possibly increased apoptosis in stationary phase of standard culture.

4.2.3.3 Growth-promoting protein

GRB2, involve in the regulation of cell growth, was observed to be 1.72 fold down regulated in cells at 144hrs compared to cells at 72hrs in standard culture. GRB2 contains

SH2 and SH3 domains and is involved in the signal transduction pathways involving epidermal growth factor receptor to regulate cell proliferation in culture (Yu *et al.* 2008a & b). Increased GRB2 expression in colorectal carcinoma correlates to increased tumour growth (Yu *et al.* 2008a). The down regulation of GRB2 at the stationary phase of culture reflects the reduced growth.

Therefore, data presented here indicates that altered levels of PHB, ALDH1A1, ENO1, ACTB and CAPZB could be associated with development of stationary phase of culture whereas GRB2 contributes to the reduced growth of cells. This suggests that these proteins may possibly be implemented in to the production culture to regulate growth and productivity of culture.

4.2.3.3 Stationary culture (144hrs at 31^0C vs. 144hrs at 37^0C)

Identification of differentially expressed proteins between stationary phases at temperature-shifted and standard cultures could further help us to identify proteins that may be involved in the regulation of cell death and various other stress conditions such as nutrient or oxygen depletion. A total of 21 proteins were observed to be differentially expressed, of which, 17 proteins were up regulated and 4 were down regulated (Table 3.2.6). A number of proteins that were involved in the regulation of growth and/or apoptosis were observed to be differentially expressed. These include vimentin, importin-α, ENO1, CNN3, ACTB, CAPBZ and ALDH1A1 etc. As discussed in sections 4.2.3.1 and 4.2.3.2, investigation of these proteins could enable us to regulate growth and hence recombinant protein productivity of mammalian cultures.

4.2.4 Selection of protein target selection to investigate their effects on cell growth and productivity using siRNA knockdown technique

Based on the expression pattern observed using 2D-DIGE and information in published literature, 5 protein targets were selected for knockdown of their expression in CHO-K1 cells in suspension. The targets are described in Table 4.2.4. As discussed in section 4.2.3, these protein targets were specifically differentially regulated in temperature-shifted culture and were in agreement with the published literature in respect of various cellular phenotypes such as cell growth and apoptosis, that are typically observed in low temperature culture. Briefly,

- EIF4A, a critical factor for cap-independent translation pathways, was up regulated in temperature-shifted cultures. EIF4A has also been shown to block cell cycle progression. Therefore the up regulation of EIF4A could be associated with the reduced cell growth due to its role in cell cycle transition and/or improved recombinant protein production due to role in the regulation of cap-independent translation pathways at low temperature.
- HNRPC was down regulated following temperature-shift. HNRPC can induce translation of c-myc which is known to regulate cell growth in culture. HNRPC is also involved in regulation of cap-independent translation. Down regulation of HNRPC following temperature-shift could potentially be contributing towards reduced cell growth and increased recombinant protein productivity at low temperature.
- Importin-α was down regulated in temperature-shifted cells. Importin-α is involved in transporting cell proliferation and apoptosis related factors into the nucleus, so down

regulation of importin-α at low temperature could be associated with the regulation of cell growth and resistance to apoptosis in culture.

- LGALS1 was up regulated following temperature-shift. LGALS1 is known to inhibit cell growth in culture. Due to this, it may have potential involvement in reducing the cell growth at low temperature.
- PFN2 was over expressed in cells at low temperature. PFN2 has been shown to regulate normal actin polymerization in response to thermal stresses. This suggests that PFN2 may be involved in regulation of cell structure in response to hypothermic conditions and hence could regulate growth and apoptosis in culture.

Therefore, it was hoped that inhibiting the expression of these protein targets could be expected to give a greater insight into the regulation of growth in culture and to improve recombinant protein productivity in culture.

Table 4.2.4 Differential expression of selected protein targets observed using 2D-DIGE in temperature-shifted and standard culture at 144hrs of culture in comparison to cultures maintained at 72hrs. NDE denotes proteins that are Not Differentially Expressed at the set criteria (±1.5 fold up/down regulated with t-test score ≤0.05) in the comparison of cells at 72hrs with cells at 144hrs of culture.

Target	Temperature-shifted Culture	Standard Culture	Cellular Function
EIF4A	1.81	NDE	IRES-mediated cap-independent protein translation, cell cycle arrest
HNRPC	-1.52	NDE	Cell proliferation and IRES mediated cap-independent protein translation
Importin-α	-1.8	NDE	trafficking of the growth promoting and apoptotic factors to nucleus
LGALS1	1.62	NDE	cell adhesion and negative regulation of the cell cycle/proliferation
PFN2	1.69	NDE	normal actin polymerisation in response to thermal stress

The CHO genome has still not been fully elucidated and as a result of this, siRNAs for selected protein targets are currently not commercially available for CHO-K1 cells. Sufficient sequence information was obtained by amplifying part of the coding sequences using PCR primers designed against conserved regions based on orthologous sequence

alignment enabling design of siRNAs against 3 protein targets using the *Silencer Select*™ *Algorithm*® by Applied Biosystems. Two siRNAs were generated for each target to ensure at least effective siRNA was identified by the siRNA design algorithym.

4.2.5 Effect of silencing protein targets

Small interfering RNA molecules (siRNA) were used to investigate the effects of knocking down selected protein targets on growth and recombinant protein productivity in CHO cells. siRNAs can inhibit the expression of target proteins by directing the degradation of their mRNA transcript. However there is evidence that siRNAs can exert off-target effects by inhibiting the expression of other genes non-specifically following transfection (Jackson *et al.* 2003). The transfection reagent used (neoFX) or siRNA delivery can also cause temporary changes in the cells, for example, by affecting growth or viability. Therefore, each set of siRNA experiments at both culture conditions (temperature-shifted and non-shifted) included as many appropriate controls (neoFX only and scrambled siRNA controls) as possible. There is no definitive scrambled siRNA control available for CHO cells as its genome is not fully sequenced. Therefore scrambled siRNAs based on human sequence information were used in this investigation. NeoFX-only and scrambled siRNA-treated cultures resulted in nearly similar effects on cellular phenotypes suggesting that human scrambled siRNA control could be used as a negative control, however, this is a suitable control remains unclear. NeoFX only and scrambled siRNA controls used for transfection in this study were same for temperature-shifted and standard culture. The effects of target knockdown on the cellular phenotypes were calculated by comparing the observed effects for target-siRNA-treated cultures with scrambled siRNA-treated cultures. Both of these controls ensured that the effects observed in CHO cells after transfection were not an

artefact of the RNA delivery process. In each experiment, the protein target was inhibited using 2 independent siRNA molecules so that the target could be silenced at least by any of these siRNA.

Although 2D-DIGE experiment was performed using suspension-adapted CHO-K1 cells growing in 10% FCS supplemented medium, siRNA knockdown experiments were performed using suspension and low serum-adapted SEAP-secreting CHO-K1 cells (Figure 3.2.7.3). The reason for this is that low serum culture condition is more closer to industrial requirements compared to serum supplemented conditions. Secondly, the effects of low temperature on cells were observed to be more intense for HNRPC and RBM3 using Western blotting in low serum-adapted cells compared high serum-adapted in this laboratory suggesting the suitability of low serum-adapted cells to investigate the effect of target knockdown. The cells were maintained in standard culture (37^0C) for 96hrs and in temperature-shifted culture where cells were maintained at 31^0C for 72hrs after 24hrs of transfection at 37^0C (Figure 3.2.7.3). The effects of the target knockdown on cell growth and recombinant protein productivity were evaluated after 96hrs of siRNA transfection. Western blot analysis of samples from cells transfected at standard culture confirmed the successful knockdown of all 3 protein targets (Figure 3.2.7.10), however knockdown efficiency for temperature-shifted cultures need to be validated. After 96hrs at 37^0C, the expression of all target proteins was observed to be reduced to undetectable levels in the transfected cultures in comparison to controls (neoFX-only and scrambled-treated), which is unusual. This could possibly be due to the partial sequence information used for designing siRNAs for all target proteins were generated in this laboratory using genomic DNA from the same CHO cells in which knockdown experiments were performed. This

would have improved the efficiency of siRNA designing algorithm. Moreover, the *Silencer Select™ Algorithm®* itself has been developed to achieve ~99% knockdown which might have further improved the efficiency of siRNA designing. Crucially, Western blot analysis of samples from siRNA-transfected cells maintained in biphasic culture would further confirm the efficiency of target knockdown following transfection. Therefore, the effects observed on cellular phenotypes are most likely to be due to knockdown of specific protein targets. However, it is also possible that these siRNAs may be causing off-target effects in the cells. Moreover, it will be necessary to confirm the extent of target knockdown and its effects on cellular phenotypes at different time-points in temperature-shifted and standard culture conditions.

4.2.5.1 EIF4A

EIF4A was observed to be 1.81 fold up regulated following temperature-shift in proteomics study. EIF4A has previously been shown to be up regulated in G2-arrested CHO cells (Sasaki *et al.* 2000) suggesting that its inhibition should improve cell growth. In contrast, cell growth was reduced following siRNA-mediated inhibition of EIF4A in temperature-shifted and non-shifted cultures in both non-producer and SEAP-secreting CHO-K1 cells without any significant impact on viability (Figure 3.2.7.2 & 3.2.7.4 and Table 3.3.7.2). The expression of EIF4A was reduced to non-detectable levels following its knockdown (Figure 3.2.7.10). Thus, inhibition of EIF4A could have compromised global cap-dependent and independent protein translation pathways extensively (Pilipenko *et al.* 2000, Pause *et al.* 1994). Indeed, the knockdown of EIF4A in biphasic cultures would need to be confirm by Western blotting. This might be expected to reduce the translation of house

keeping proteins, as reflected in Figure 3.2.7.10 where expression of GAPDH was observed to be slightly reduced in siRNA-treated cultures compared to control cultures, resulting in reduced cell growth. Similar results have been observed by other groups previously for the translation factor EIF5A where its inhibition was observed to reduce the growth of CHO without affecting viability for a period of time depending on the level of EIF5A inhibition (Park *et al.* 1994). The inhibition of other translation factors has also been observed to impair proliferation of mammalian cells (Kang and Hershey 1994, Jasiulionis *et al.* 2007, Lei *et al.* 2002, Cnop *et al.* 2007). Therefore, the titration of siRNAs used to inhibit the EIF4A to see if effects on growth could be modulated and would further improve our understanding of the role of EIF4A in regulation of low temperature response in CHO cells. Given the 6-fold difference in cell numbers, there would also be off-target effects of siRNAs in temperature-shifted cultures.

The reduced cell numbers accounted for reduced total SEAP yield. In general, a pattern of improved cell specific productivity (Qp) was observed, however it was statistically significant only for siRNA 1 in temperature-shifted culture and siRNA 2 in standard culture (Figure 3.2.7.5 and Table 3.2.7.2). The expression of EIF4A was observed to be reduced to undetectable levels using Western blot analysis for both siRNAs (siRNA 1 & 2) following transfection at 37^0C (Figure 3.2.7.10). However, Western blot analysis of samples from siRNA-transfected cells maintained in biphasic culture would be advantageous to confirm the effects of EIF4A knockdown on Qp. Therefore, discrepancies for the effect of inhibition of EIF4A on Qp between both siRNA could possibly a reflection of off-target effects by any of these siRNA. Taken together, this supports the hypothesis that EIF4A needs to be up regulated to improve productivity of culture as it was up regulated in cells following

temperature-shift. However, reduced cell growth and improved cell specific productivity due to inhibition of EIF4A establishes it as a potential candidate to be employed to regulate growth and productivity in culture. For instance, the inhibition of EIF4A, once a production culture has achieved a desired biomass concentration (~60-70% confluency), could arrest growth and improve the cell specific productivity given that it improves viability over 144hrs (Figure 3.2.7.2). This may increase the yield of culture compared to the standard cultures after ~5-7th day of inhibiting EIF4A expression due to increased Qp (7-58%, depending on the siRNA used (Table 3.2.7.2)) and culture longetivity, while the standard culture would enter into the decline phase.

4.2.5.2 HNRPC

HNRPC is a nuclear pre-mRNA binding protein. It was 1.52 fold down regulated following temperature-shift in proteomics study. Cell growth of non-producer CHO-K1 cells was significantly reduced following inhibition of HNRPC in standard culture (Figure 3.2.7.2 and Table 3.2.7.1). HNRPC can enhance cell proliferation by inducing IRES-mediated translation of c-myc mRNA (Kim *et al.* 2003). Silencing of HNRP C1/C2 protein expression by siRNA has also been shown to inhibit cell proliferation due to defects in G2/M transition phase of cell cycle (Schepens *et al.* 2007). These findings are in keeping with our results and suggest that reduced levels of HNRPC could be one of the factors responsible for reduced cell growth at low temperature. Reduced cell growth in SEAP-secreting CHO-K1 cells following knockdown of HNRPC was also observed; however this was not evident in the temperature-shifted culture (Figure 3.2.7.6 and Table 3.2.7.3). This could possibly be due to that the cells in temperature-shifted cultures had only 24hrs to

grow (at 37^0C) before shift to low temperature (31^0C) and therefore cultures may not have enough growth to show any effect of HNRPC inhibition. The viability of culture was comparable to controls throughout the testing period in all cases.

The total productivity of SEAP was also not affected significantly in standard culture but was improved in temperature-shifted cultures following inhibition of HNRPC (Figure 3.2.7.7 and Table 3.2.7.3). Improved Qp was observed in both standard and temperature-shifted cultures presumably due to reduced levels of HNRPC which was reflected in an improvement in the total productivity value. The improvement in Qp was not significant for siRNA 1 for temperature-shifted cultures but when siRNA 1-treated cultures were compared with neoFX-only-treated controls cultures (instead of scrambled-treated), this improvement turned into a significant change (33% with p-value 0.048). This suggests that the use of neoFX-only-treated controls cultures could help to reveal the effects of knockdown experiments in the absence of designated scrambled control for CHO cells. It should be noted that while there was complete knockdown of the HNRPC at 37^0C as determined by Western Blotting, the extent of knockdown in temperature-shifted cultures has not been evaluated. Further given the almost 10-fold reduction in cell number in temperature-shifted controls compared to the standard culture, it is highly possible that there are non-specific effects occurring at 31^0C. HNRPC can regulate the cap-independent translation by inducing IRES-mediated expression of the number of specific proteins including 'upstream of N-Ras' (Unr) which is also involved in translation of specific proteins using cap-independent translation pathways during mitosis (Schepens *et al.* 2007). This is important because during mitosis, similar to low temperature exposure, protein synthesis is rapidly and severely repressed possibly due to the inhibition of global cap-

dependent translation (Fan and Penman 1970). The cap-independent translation is thought be increased at these hypothermic conditions. Since the inhibition of HNRPC has been reported to abrogate the cap-independent translation during mitosis (Schepens *et al.* 2007), the role of reduced HNRPC as observed in this investigation to improve recombinant protein production remains unclear. The improvement in the Qp due to inhibition of HNRPC was more pronounced in standard cultures in comparison to biphasic cultures and this suggests that the effects of HNRPC on Qp could be temperature-independent. However, it should be considered that the extent of knockdown was not confirmed using Western blotting in temperature-shifted cultures in this study and therefore, differential effects of HNRPC on Qp could be due to differences in extent of its knockdown between temperature-shifted and standard cultures. Over expression of HNRPC in CHO cells and the investigation of HNRPC-associated proteins may help to clarify the role of HNRPC in the regulation of cell growth and recombinant protein productivity at low temperature. The inhibition of growth and the increase in Qp after knockdown of HNRPC in CHO cells at 37^0C establishes it as an attractive target for cell engineering-based approaches to regulate cell growth and recombinant protein production in culture providing the previously mentioned issues (such as optimised knockdown conditions) can be overcome. Based on these findings, the temporal inhibition of HNRPC, once the culture has reached the desirable cell density, would induce growth arrest as well as improving Qp of the culture contributing to improved overall recombinant protein productivity. This could increase the yield of culture compared to the standard cultures after 1-5 day of inhibiting HNRPC expression due to increased Qp (48-124%) and culture longetivity and as standard culture would be in the decline phase.

4.2.5.3 PFN2

PFN2 (Profilin 2), a small ubiquitous actin monomer sequestering cytoskeletal protein, was observed to be 1.69 fold up regulated following temperature-shift in proteomics study. PFN2 is typically required for normal actin polymerisation in response to thermal stress (Yeh and Haarer 1996b). The inhibition of PFN2 was expected to reduce viability and growth since dysregulation of cytoskeleton has been associated with increased cell death in culture (Rodolfo and Piacentini 2002, Kulms *et al.* 2002) and this was shown to be the case for siRNA 1 (more striking in standard cultures than temperature-shifted cultures) but not for siRNA 2 (Figure 3.2.7.8 and Table 3.2.7.4). siRNA 1 was more effective in reducing the viable cell counts in both culture conditions with opposing effects of siRNA 2. The expression of PFN2 was observed using Western blotting to be completely knockdown after 96hrs of transfection at 37^0C (Figure 3.2.7.10) but the extent of PFN2 knockdown at 31^0C was not evaluated. Moreover, there appears to be some process effects as the PFN2 in scrambled-treated controls is also quit strongly reduced compared to neoFX-treated control and is different from the observations with EIF4A and HNRPC (Figure 3.2.7.10). Opposing effects for both siRNAs were observed for total productivity in standard culture (siRNA 1 reduced while siRNA 2 increased) (Table 3.2.7.4). In the temperature-shifted cultures, the effects of siRNA 1 was insignificant but for siRNA 2, the effect was significant. However, compared to the satandard culture controls, siRNA 2 improved total productivity.

The effects of siRNA 1 and 2 on viability and total and specific productivity of culture were also varying extensively among themselves. This suggests that the titration of siRNAs concentration for inhibiting PFN2 levels or use of other independent siRNAs (i.e. 3rd siRNA) could help to further clarify its specific effects in CHO cells at low temperature.

Investigation of PFN2 knockdown in temperature-shifted cultures using Western blotting would help to understand the reason for phenotypic differences observed between 2 siRNAs. However, cell specific productivity was observed to be improved for both temperature-shifted and non-shifted cultures. Therefore, the role of PFN2 in the regulation of growth and recombinant protein productivity at low temperature remains unclear. The Western blot analysis of the samples from siRNA-transfected cells maintained in biphasic culture could help to understand the effects of PFN2 inhibition of these cellular phenotypes. Moreover, investigation of actin level following siRNA transfection using Western blotting or other quantitative technique would further confirm the role of PFN2 in regulation of actin cytoskeleton as well as efficiency of PFN2 knockdown in cells.

As an engineering target, the most effective approach would be to switch-off expression of endogenous PFN2 using an inducible shRNA system, once the required cell density (~60-70% confluency) has been reached. This would have dual effects of inhibiting further cell growth and increasing specific productivity during the production phase of a batch or fed-batch culture. This may increase the yield of culture compared to the standard cultures within further 24hrs of inhibiting PFN2 expression due to increased Qp (95-288%). However the main drawback would be the detrimental effects on viability and significant variation between batches in biphasic cultures. Alternatively PFN2 could be over expressed in early stage of culture where it might increase growth early in the process and then switch-off its expression to improve Qp.

4.3 Analysis of low temperature induced differential expression of miRNAs in CHO-K1 cells

An understanding of the molecular events regulating cell growth, protein production and death in CHO suspension culture is a crucial initial step in developing ways to increase the longevity and productivity of recombinant CHO cell lines. A number of transcriptional and proteomic profiling studies have been recently published describing the genes and pathways involved in CHO growth, death and temperature response (Marchant *et al.* 2008, Baik *et al.* 2006, Wong *et al.* 2006, Swiderek and Al-Rubeai 2008). However there are apparent discrepancies between microarray and proteomic data in the literature, for example PDI, phosphoglycerate kinase and heat shock cognate 71 kDa protein were up regulated upon reduction of culture temperature, although the transcripts encoding these proteins were not significantly altered (Baik *et al.* 2006). It is possible that the increases in these proteins may be through the change in expression of one or more miRNAs or other levels of translational controls. miRNAs are small (~22nt) non-coding RNAs (ncRNAs) that regulate gene expression at the level of translation and each miRNA apparently can regulate multiple genes (Lim *et al.* 2003, Miska 2005). Moreover miRNAs have been found to regulate cell proliferation (O'Donnell *et al.* 2005), apoptosis (Cheng *et al.* 2005, Cimmino *et al.* 2005), cell stress (Hua *et al.* 2006), metabolism (Gauthier and Wollheim 2006) and secretion (Poy *et al.* 2007), all of which are important characteristics to consider for production cell lines in bioreactors. miRNAs have been suggested recently to impact on global protein translation by interacting with RBM3 (Dresios *et al.* 2005).

One of the challenges in profiling gene expression in CHO cells is the lack of sequence data. Although both rat and mouse cDNA arrays have been employed to profile CHO

systems (Baik *et al.* 2006) with some success, mouse has been identified as likely to be the most appropriate for CHO study based on mitochondrial genome comparisons (Wlaschin *et al.* 2005). In comparison with mRNA and proteins, sequence conservation at miRNA level has been observed to be much higher between species (Berezikov *et al.* 2005). Therefore miRNA profiling technologies that are developed for human and/or mouse to date may be a useful route to gain a greater understanding of regulation of the gene/protein expression in CHO cells. In this work, samples from CHO-K1 cells growing exponentially at 37^0C were compared with the cells growth arrested at 31^0C in a biphasic culture process using miRNA-bioarrays and highly sensitive and reproducible miRNA Taqman Low Density Array (TLDA) approaches to identify low temperature-induced differential expression of miRNAs. This has enabled us to identify miRNAs that have been found to regulate growth and productivity in CHO culture and have potential to be implemented in industrial production process to improve product yield.

4.3.1 Analysis of low temperature induced differential expression of miRNAs using miRNA-bioarrays.

In a preliminary experiment, temperature-shifted cells at 144hrs at 31^0C were compared to the exponentially growing cells at 72hrs at 37^0C using miRNA-bioarrays. miRNA-bioarrays are microarrays comprised of primers for a comprehensive selection of human, mouse and rat miRNAs. Unsupervised hierarchical clustering analysis of CHO samples with 6 human cells lines indicated that CHO cells are unique in profile of miRNAs that they express (Figure 3.3.1.3). In this investigation, a total of 26 miRNAs were identified as being significantly differently expressed between 72hrs at 37^0C (TSd3) and 144hrs at 31^0C

(TSd6) samples (Table 3.3.1.1 and 3.3.1.2). These include two well known growth regulatory miRNAs, miR-21 and miR-24.

- miR-21 was 2.23 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in biphasic culture. miR-21 has been observed to be elevated in various cancers, such as breast, ovarian and lung cancer, as well as glioblastomas (Chan *et al.* 2005, Schetter *et al.* 2008, Chan *et al.* 2008). The effect of miR-21 can be cell line specific; inhibition of miR-21 induced growth in the HeLa cells and reduced growth in HCC cells but had no effect on A549 cells (Connolly *et al.* 2008). Although, the role of miR-21 in regulation of growth in CHO cells is not clear, the increased expression of miR-21 following temperature-shift could be expected to facilitate reduced cell growth at low temperature.
- miR-24 was 1.73 fold up regulated at 144hrs at 31^0C compared to 72hrs at 37^0C in temperature-shifted cultures. The inhibition of miR-24 induced the growth of HeLa cells while reducing growth of A549 cells (Chan *et al.* 2005). miR-24 suppresses $p16^{INK4a}$ translation in cultured human cells (Lal *et al.* 2008). $p16^{INK4a}$ inhibits the cyclin-dependent kinases CDK4 and CDK6, thereby keeping cells arrested (Shapiro *et al.* 2000). miR-24 can also exert its regulatory effects on cell proliferation by activin-associated activities (Wang *et al.* 2008). Activin is a member of TGF-β superfamily and has been observed to inhibit growth of hematopoietic progenitor cells (HPCs). The ectopic expression of miR-24 has been shown to attenuate the anti-proliferative effect of activin resulting into increased proliferation of HPCs (Wang *et al.* 2008).

This suggests that these miRNAs could be involved in regulation of cell growth and recombinant productivity of culture at low temperature.

Of these 26, 4 miRNAs were chosen for qRT-PCR validation using temperature-shifted and non-shifted samples and this enabled us to identify temperature-dependent changes. miR-21 and miR-24 were observed to be significantly up regulated at 144hrs in a temperature-independent manner in qRT-PCR analysis (Table 3.3.1.3). Of the two miRNAs that were not found to be significantly changed by qRT-PCR, miR27a displayed a 1.76 fold increase in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C using the bioarrays which was not reflected by the qRT-PCR. Therefore it may be that 2 fold or higher may be a more appropriate cut-off when selecting targets from array profiling. Let-7f was expressed at intensities less than 150 units and this has been used by other researchers as a cut-off for analysis (Gu and Iyer 2006). Let-7f was used as an endogenous control in qRT-PCR quantification in this investigation. This analysis suggests that miRNA-bioarray could be used to identify potential bioprocess-related miRNA targets, however it is important to confirm with another alternative technique.

4.3.2 Analysis of low temperature induced differential expression of miRNAs using Taqman Low Density Arrays (TLDA).

In the first miRNA profiling study, we analysed the cells maintained for 72hrs at 37^0C with the cells shifted for a further 72hrs at 31^0C using miRNA bioarrays. To gain a greater insight into the role of miRNAs in regulation of cellular phenotypes, i.e. growth and viability, at low temperature, we also investigated cells that were temperature-shifted to 31^0C for only 24hrs after growing at 37^0C for 72hrs (96hrs) in comparison to the cells

continuously maintained at 37^0C for 72hrs (72hrs) using PCR-based Taqman Low Density miRNA Arrays (miRNA-TLDA). miRNA-TLDAs are more sensitive and reproducible in comparison to miRNA bioarrays. Duplicate samples were used for each condition. The aim of this investigation was to identify early-mid stage alterations in the expression of miRNAs in response to cold-shock. Seventeen miRNAs were differentially regulated in cells at 96hrs at 31^0C compared to cells at 72hrs at 37^0C in biphasic culture, of which 9 were down and 8 were up regulated. From the list of differentially expressed miRNAs, miR-7 (8.43 fold down regulated) and miR-126 (3.08 fold up regulated) have been recently reported to play a role in the regulation of cell growth (Zhang *et al.* 2008, Kefas *et al.* 2008).

- Inhibition of miR-7 has been reported to reduce the cell growth of lung carcinoma cell line, A549 (Cheng *et al.* 2005). miR-7 has been reported to exert differing effects on apoptosis. Increased apoptosis in culture were observed in A549 cells following inhibition of miR-7 (Cheng *et al.* 2005), whereas over expression of miR-7 also increased apoptosis glioma lines (U87MG and T98G) (Kefas *et al.* 2008). Therefore the reduced expression of miR-7 following temperature-shift could be facilitating reduced cell growth or apoptosis in culture.
- miR-126 has been reported to be down regulated in breast cancer cells, MDA-MB 231 and MCF-7 and inhibits cell cycle progression from G1/G0 to S (Zhang *et al.* 2008, Guo 2008). Over expression of mir-126 decreased the expression of insulin receptor substrate 1 (IRS-1) at the translation level and drastically suppressed cell growth in HEK293 and MCF-7 (Zhang *et al.* 2008). Therefore up regulation of miR-126

following temperature-shift could be associated with the reduced cell growth at low temperature.

Therefore miR-7 and miR-126 could be playing a critical role in regulation of growth and productivity at low temperature.

In the miRNA-TLDA protocol, a pool of primers is used to reverse transcribe all species of miRNAs in the sample whereas singleplex qRT-PCR uses primers specific only for 1 miRNA target. The confirmation of a non-significant change in expression observed using miRNA-TLDA with singleplex qRT-PCR could help to establish the sensitivity as well as applicability of miRNA-TLDA in CHO cells. Two miRNAs, miR-7 and miR-200c, were analysed for their expression using singleplex qRT-PCR over 144hrs of culture in both temperature-shifted and non-shifted culture. miR-200c was selected as an example of miRNA that was identified as being differentially expressed (+1.5 fold) on miRNA-TLDA but with a p-value $\geq$0.05 (non-significant). The differential expression of miR-200c was also found to be non-significant using singleplex qRT-PCR, confirming the data from miRNA-TLDA. Differential expression of miR-7 observed using qRT-PCR confirmed the change observed using miRNA-TLDA. The expression of miR-7 was gradually reduced in stationary phase of culture in comparison to lag and log phase of cultures maintained at 37^0C but this inhibition was more pronounced in temperature-shifted cells (Figure 3.3.3.2). Therefore miR-7 might be expected to exert its effect on cells in a **dose-dependent** manner. This data demonstrate that miRNA-TLDA can be used to identify CHO miRNAs that could be involved in regulation of particular phenotypes, however it is important that results are further confirmed using alternative techniques.

4.3.3 Isolation and cloning of *Cricetus griseus* miRNAs

At the time of writing (31 October 2008), there were no *Cricetus griseus* miRNA sequences listed in the miRNA registry (http://microrna.sanger.ac.uk/) apart from the miRNA sequence which we submitted. The sequence for miR-21 has been generated successfully in this investigation. The sequence of miR-21 from *C. griseus* was observed to be similar to the orthologous miRNA sequences from human and mouse (Figure 3.3.1.5). This was in agreement with the fact that miRNAs are more conserved during evolution in comparison to other genes. This also ensured the applicability of miRNA probes from orthologous species to investigate the expression of miRNA in CHO cells. The sequence for miR-21 has been submitted in Sanger database. In keeping with the Sanger registry-naming conventions, this miRNA has the prefix cgr- to represent *C. griseus*. This new miRNA cgr-miR-21 has been confirmed by the miRBase registry. The sequence has also been lodged with EMBL and given the accession no. AM600961.

4.3.4 Selection of miRNA targets for functional validation

miR-24 and miR-7 expression levels were consistently altered in both the miRNA-array study as well as in singleplex qRT-PCR analysis in both temperature-shifted and standard culture. These were selected for further studies to investigate their role in the regulation of cell growth and recombinant protein productivity in CHO cells by exogenous inhibition or over expression.

4.3.5 Effects of inhibition or over expression of miRNA targets

Chemically modified RNA molecules can be transfected into the cells to inhibit or enhance miRNA levels. This enables us to investigate the effects of artificial inhibition or over expression of selected miRNA targets on cellular phenotypes. The cells were maintained in standard culture at 37^0C for 96hrs and in temperature-shifted culture where cells were maintained at 31^0C for 72hrs after 24hrs of transfection (Figure 3.2.7.3). The impact of miRNA transfections were measured after 96hrs of culture. Each set of experiments included, as much as possible, appropriate controls for miRNA transfection. Since no validated non-specific control for miRNA inhibition and over expression in CHO cells is available, scrambled anti-mir (AM, for inhibition) and pre-mir (PM, for over expression) controls, which are designed to be nonsense miRNAs in human cells, were used in this investigation. In addition, neoFX (lipid based transfection reagent) only treated cultures were also closely monitored to ensure that the effects observed were due to specific inhibition or over expression of miRNAs and not an artefact of the delivery. qRT-PCR analysis was used to confirm the efficiency of miRNA knockdown or over expression in CHO cells.

4.3.5.1 miR-7

The expression of miR-7 was observed to be 8.43 fold down regulated in temperature-shifted cells at 96hrs at 31^0C compared to cells maintained at 72hrs at 37^0C using miRNA-TLDAs (Table 3.3.2.1). Growth of CHO-K1 cells was significantly inhibited following over expression of miR-7 in standard culture (Figure 3.3.3.2 and Table 3.3.3.3). miR-7 levels were found to be gradually reduced in stationary phase of culture in comparison to cells maintained in lag and log phase of culture at 37^0C in qRT-PCR (Figure 3.3.2.2). This

reduction was more pronounced in temperature-shifted cells compared to the cells maintained at 37^0C. Growth phase specific expression of miR-7 suggests that it could regulate cell growth in dose-dependent manner. Compared to the PM- or neoFX-controls, over expression of miR-7 reduced cell growth significantly across the time frame without affecting viability at standard culture, reflecting the expression profile observed using qRT-PCR during the validation of miR-7 (Figure 3.3.2.2A) suggesting some other mechanisms controling miR-7 following temperature-shifts. miR-7 has been previously reported to reduce growth of the lung carcinoma cell line, A549 due to increased apoptosis (Cheng *et al.* 2005). In contrast, we did not observe any affect on growth and viability of CHO cells following inhibition of miR-7 expression in any culture conditions, i.e. standard or temperature-shifted cultures. It could be that either the effect of miR-7 on growth might be cell line-dependent or reduction in expression of miR-7 is a consequence of low temperature induction and not a cause for low-temperature-induced cellular phenotypes.

miR-7 has been shown to regulate the expression of the transcription factor Yan by a reciprocal negative feedback loop. The 3'-UTR of human EGFR contains binding sites for miR-7 and recently has been demonstrated as being potently and directly regulated the miR-7 (Kefas *et al.* 2008). Apart from EGFR, miR-7 can inhibit the Akt pathway that is critical for cell growth. A marked down regulation of miR-7 has been observed in glioblastoma (Kefas *et al.* 2008). miR-7 negatively regulates the expression of lymphoid-specific helicase (LSH) that is essential for correct DNA methylation patterning (Ilnytskyy *et al.* 2008). Aberrant methylation of DNA has been associated with cancer. The loss-of-function mutations in the LSH gene result in dramatic hypomethylation of the murine genome and reactivation of transposable elements possibly leading to cancer (Huang *et al.* 2004). Over

expression of LSH, contrarily leads to re-establishment of methylation pattern and is viewed as a genome stabilizing event (Myant and Stancheva 2008).

The total productivity of SEAP was reduced following over expression of miR-7 at 37^0C, which was expected as growth was completely arrested and volumetric yield is dependent on the number of producer cells (Figure 3.3.3.3 and Table 3.3.3.3). Conversely, the specific productivity (Qp) was improved by 2 fold at 37^0C due to over expression of miR-7. miR-7 has been found to be highly expressed in pancreatic islets and the neurosecretory cells of the annelid *Platynereis dumerilii* and of the zebrafish (Bravo-Egana *et al.* 2008, Tessmar-Raible *et al.* 2007). Pancreatic islets and neurosecretory cells are specialized in secretion of specific proteins and therefore miR-7 has been proposed as an endocrine miRNA. Published data is in agreement with our observations that over expression of miR-7 can improve protein production. The Qp was un-affected in biphasic culture following over expression of miR-7. This indicates that over expression of miR-7 utilizes a temperature-independent route to improve Qp. The nearly complete growth-arrest and improved Qp of CHO cells due to over expression of miR-7 can offer potential for its implementation in cell engineering based approaches to improve recombinant protein production providing the total yield can be improved over standard culture conditions. The over expression of miR-7 using a inducible expression system, once the culture reached a desirable biomass (~60-70% confluency) should arrest growth and be able to maintain viability for a longer period of time while simultaneously improving Qp in the culture. This could be expected to increase the yield of culture compared to the standard cultures within further 24hrs of temporal expression due to increased Qp (116%) following miR-7 over expression assuming that over expression of miR-7 exerts its effects immediately.

4.3.5.2 miR-24

mir-24 was observed to be 1.73 fold up regulated in cells at 144hrs at 31^0C compared to cells at 72hrs at 37^0C in temperature-shifted culture using miRNA-bioarrays. A marked reduction in cell growth was observed following over expression of miR-24 at 37^0C (Figure 3.3.3.4 and Table 3.3.3.4) but no significant effect was observed on the temperature-shifted cultures. Inhibition of miR-24 has been shown to induce growth in HeLa cells, whereas it down regulates growth of A549 cells (Chan *et al.* 2005). Our data demonstrate that over expression of miR-24 inhibits growth of CHO cells supporting miR-24 to be cell line specific in exerting its effects. The lack of effects in biphasic culture could be due to the short period of time (24 hrs) at 37^0C to grow before being shifted to low temperature (31^0C). Inhibition of miR-24 had no effects on either culture. Taken together, miR-24 might affect cell growth only when it is expressed above a certain level but does not have any effect on CHO cell growth below this concentration or it could have curtailed effects at low temperature. The viability of cultures were unaffected by inhibition of miR-24 which is in agreement with published literature (Chan *et al.* 2005). Over expression also did not affect the culture viability.

The total yield of SEAP in standard culture was reduced following over expression of miR-24 compared to the over expression control (PM-Control) but was not significantly different from untreated cultures (neoFX-Controls), whereas it was unchanged in temperature-shifted culture (Figure 3.3.3.5 and Table 3.3.3.4). The Qp was improved significantly due to both over expression and inhibition of miR-24 in standard culture, whereas no significant effect was observed in temperature-shifted cultures. There is no published information available to date on the role of miR-24 in recombinant protein

production. This is the first demonstration of miR-24 improving cell specific protein productivity in recombinant cell lines. It is proposed that over expression of miR-24 in production culture, once the desired cell density (~60-70% confluency) has been reached, could attenuate cell growth, increase culture longevity and improve Qp hence contributing to improved yield of recombinant protein providing that the effects of miR-24 over expression is not hampered by temperature-shift. This could increase the yield of culture compared to the standard cultures after 3rd day of temporal expression due to increased Qp (68%) following miR-24 over expression and because the standard culture would enter into the decline phase by next 2-3 days of culture assuming that over expression of miR-24 exerts its effects immediately.

miRNAs are endogenous, small, usually single-stranded and non-coding RNAs (ncRNAs) that can play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression (Bartel 2004). Recently a number of miRNAs that are involved in regulation of various cellular phenotypes such as cell proliferation (Gammell 2007, Brennecke *et al.* 2003), apoptosis (Xu *et al.* 2007) and metabolism (Gauthier and Wollheim 2006) have been reported. All these phenotypes are very important for the bioprocess industry.

Low temperature based biphasic culture strategy is commonly employed in pharmaceutical industry to reduce cell growth and hold culture viability for a longer period of time and this contributes to improved recombinant protein production in culture. We have investigated the changes in miRNA expression in response to temperature-shift to 31^{0}C in suspension-adapted CHO-K1 cells in this study and identified 2 miRNAs, miR-7 and miR-24, that

were observed to regulate cell growth and recombinant protein productivity in CHO cells. The over expression of miR-7 and miR-24 was found to significantly arrest growth and improved specific productivity of CHO-K1 cells. Although effects of miR-7 were more pronounced in comparison to miR-24, both miRNA targets have potential to be used in cell-engineering based approaches to improve recombinant protein production in mammalian cells, providing the total productivity can be improved over standard culture conditions.

Employing temperature-shift in culture is a costly, cell line specific and inconsistent process. Low temperature improves cell specific productivity for only a short period of time as the cells have tendency to adapt to grow in hypothermic culture conditions. This has limited the implication of low temperature cultivation of producer cells in the pharmaceutical industry. A number of chemical compounds have also been tried to improve recombinant protein production of mammalian cells. For example, sodium butyrate (NaBu) can reduce growth and improve productivity of recombinant proteins in CHO cells. However, increased apoptosis due to NaBu treatment limit the beneficial effects of improved productivity. Implementation of either miR-7, miR-24 or both and their temporal expression would potentially eliminate the need for performing such costly treatment, i.e. temperature-shift or NaBu treatments, to improve production. Engineering of miR-7 or miR-24 might also be used in conjunction with temperature-shift or NaBu treatment to achieve synergistic effects on yield.

A more in-depth investigation of the molecular mechanism by which these miRNAs exert their effects on cell growth and recombinant protein productivity could also help to improve the efficiency of production process. Differential expression profiling of proteins

in cells stably expressing miR-7, miR-24 or both using proteomics tools, i.e. 2D-DIGE or SILAC coupled with mass-spectrometry, could enable the identification of proteins or biological pathways that are being regulated specifically by these miRNAs. These differentially expressed proteins (following over expression of miR-7 and miR-24) could enable us to identify downstream targets for miR-7 and miR-24 and to identify other potential targets which may be more critical in regulation of growth and productivity in culture. This information could then be applied to improve efficiency of the production process. The expression of miR-7 and miR-24 could also be investigated for their potential involvement in regulation of metabolism, i.e. nutrient consumption or waste production in culture etc, or post-translational modification in recombinant protein product expressed by the cells. This will further clear the role for miR-7 and miR-24 in regulation of low temperature response in mammalian cells.

Apart from biopharmaceutical research, this knowledge could be used in other biomedical research areas such as efficient storage of organs and in cancer research. Organs, such as heart and kidney etc, are typically stored for a short period of time at low temperature. The improved understanding of low temperature response in mammalian cells could lead to identify targets that might help to store organs for longer period of time while maintaining stability and bioactivity of organs. The over expression of miR-7 and miR-24 were observed to reduce cell growth. Reduction of cancerous cell growth is one of the major goals of the cancer research and therefore both miRNAs could be screened for their effects in various type of cancer, for example, breast cancer and lung cancer etc. This knowledge would be critical to regulate growth of mammalian cells and offers more potential to be implemented in cancer research.

4.4 Investigation of secreted proteins by CHO-K1 cells in culture

Protein profiles generated for both total intracellular and extracellular samples from bioreactors will hopefully allow the identification of markers that may be exploited to improve bioprocess cultures, with the ultimate aim to improve productivity of protein therapeutics. Most of the studies to date have concentrated on profiling cell lysates from high producer CHO cells and other producer cell lines such as NS0 to gain an understanding of the biology of these cells to improve productivity (Marchant *et al.* 2008, Baik *et al.* 2006, Smales *et al.* 2004, Swiderek and Al-Rubeai 2008, Seth *et al.* 2007, Krampe *et al.* 2008, Meleady 2007), however few, if any, studies have explored the proteome of extracellularly secreted proteins from mammalian cell lines. In this investigation, the conditioned medium from low serum adapted adherent CHO-K1 cells was profiled using different chromatographic surfaces, i.e. anionic and cationic, coupled with SELDI-ToF MS to monitor protein/peptide expression over time in culture.

4.4.1. Cell culture

Low serum-adapted CHO-K1 cells (0.5% FBS) were used in this investigation. SELDI-ToF MS is a highly sensitive technique and therefore the presence of serum proteins in conditioned medium could potentially mask the proteins secreted from cells. The cells represented well demarcated distinct phases of the growth cycle over 144hrs of culture (Figure 3.4.1). The use of cell-free conditioned medium as a control in this investigation enabled us to separate CHO-induced changes in expression of proteins from the changes originating from culture medium.

4.4.2 Analysis of differentially regulated secreted low molecular weight proteins using SELDI-ToF MS

The analysis of SELDI-ToF MS profiles from the conditioned media from CHO-K1 cells using IMAC30 and Q10 chip surfaces indicated that 24 low molecular weight proteins/peptides were differentially expressed in the different phases of the growth cycle over 144hrs of culture. These proteins/peptides appear to have a number of distinct profiles over the course of the culture. A peak at *m/z* 8118 (8.1kDa) was observed at the early stage of culture (24hrs) since no peak at 8.1kDa was observed for cell-free medium. The 8.1kDa protein/peptide was increasing until 72hrs of culture and then decreased over the next 72hrs (Figure 3.4.3.1). This is a very similar situation to the production of lactate in CHO cells, where cells initially secrete lactate into the medium and later start to consume it as a carbon and energy source (Tsao *et al.* 2005). The 8.1kDa protein/peptide could possibly be an exogenous growth factor or a cytokine produced by the cell and secreted into the media, and its expression increased as the biomass increased over time. During early stages of growth, there are possibly many proteins that could be secreted into the media such as exogenous growth factors and cytokines, and knowledge of these may be useful in designing and improving serum-free and protein-free media formulations. This 8.1 kDa protein/peptide is then possibly consumed by the cell at/after 96hrs of culture as the biomass levels increase, and then decreased over 120 and 144hrs of culture as the viable cell numbers begin to plateau and decline. Recently truncated transferrin fragment has been reported in the conditioned medium from melanoma cells and is possibly due to the degradation of transferrin protein by proteolytic enzymes secreted by the cells in to the culture medium (Vandewalle *et al.* 1989, Dowling *et al.* 2007, Gronborg *et al.* 2006).

Therefore the decrease in the expression of the 8.1kDa protein/peptide could also be due to the protein being degraded as a result of proteolysis. A number of other studies have also reported increased proteolytic activity todate in the production culture. For example, an MMP pro-enzyme has been found to be released from CHO cells during the production phase of recombinant factor VIII, and as a result of auto-proteolysis, a number of smaller, less specific MMPs were also detected (Sandberg *et al.* 2006). In another study, pro-MMP9 (gelatinase B) was identified from conditioned media of CHO-K1 cells grown in serum-free conditions (Elliott *et al.* 2003). These studies demonstrate that knowledge of proteolytic enzymes secreted by CHO cells is important for optimal bioprocess conditions. In manufacturing recombinant proteins, the use of serum-free media for cell cultivation is preferred in order to meet quality and regulatory requirements but this could increase the exposure of the expressed product to proteolytic attacks that can result in product heterogeneity, denaturation and loss of protein function. This further necessitates investigations targeting the identification of proteins/peptides secreted by cells into the production culture so that the quality and productivity of cells can be improved.

The majority of the proteins differentially expressed on the two chromatographic surfaces used in this study are increased over time in culture and are therefore most likely associated with increasing numbers of cells within the cultures; examples of these are the *m/z* 14735 peak (14.7kDa) at IMAC30 chip surface and the *m/z* 7346 peak (7.3 kDa) and the *m/z* 11347 peak (11.3 kDa) detected on the Q10 chip (Figure 3.4.3.1, 3.4.3.2 & 3.4.3.3). These proteins are accumulating in the media over time and are potential contaminants that need to be purified from recombinant protein. The accumulation of these contaminants could be increasing the osmolality of culture medium which can induce apoptosis in culture and

result in decreased cell growth (Zhu *et al.* 2005). Additionally the accumulation of proteins/peptides over time can alter the composition of host cell proteins (HCP) in culture. This could potentially affect the efficiency of the purification process of recombinant protein product since the change in HCP composition could reduce the affinity of HCP to the chromatographic surface that are typically used for purification. The product quality assessment assays may also be affected with the change of HCP composition since the antibody used in these assays could have compromised specificity for such changes in HCP composition (Denizli and Piskin 1995, Ouyang *et al.* 2007). Therefore knowledge of these potential contaminant proteins could be useful for designing strategies for monitoring and removing host cell proteins from the recombinant product and to improve the quality of product.

The 14.7 kDa protein/peptide is first detected at day 120hrs of culture and then increased on 144hrs of culture (Figure 3.4.3.2), even though the total cell number is similar for 96, 120 and 144hrs of cultures but viability is beginning to decline from 96hrs onwards (Figure 3.4.1). The 11.3kDa protein/peptide also appeared in the culture on Q10 chip surface after 96hrs of culture and was increased over time. These proteins/peptides could be host cell proteins that are perhaps linked to apoptosis and are therefore released by the cells as the viability begins to drop and waste products begin to accumulate in the media. The 7.3kDa protein/peptide was increased over time in culture from 24hrs to 144hrs of culture as the biomass increases and therefore it can be expected that 7.3kDa protein could be associated with cell number. This knowledge may be useful in monitoring cell-engineering strategies using anti-apoptotic mechanisms to increase viability over time, especially for suspension cultures which are subjected to harsher conditions compared to cells grown in monolayer.

4.4.3 Reproducibility of Results

Recently SELDI-TOF MS-based serum profiling has been subject to criticism, with results published by some groups not being reproduced by others (Diamandis 2004, Baggerly *et al.* 2004, Liotta *et al.* 2005). However, the same level of criticism may not apply to cell culture-based analyses since multiple biological replicate samples from the same cell line are easily obtainable. Therefore reproducibility of SELDI-TOF MS for profiling of conditioned media from CHO-K1 cells was also estimated for the samples representing lag, log and stationary phases of culture. The coefficients of variance (CV) values for intra-experiment was ≤17.4% and for five randomly selected peaks inter-experiments for IMAC30 and Q10 arrays were ≤22.7% and therefore confirmed the reproducibility and applicability of the technique in similar type of systems with SELDI. This study suggest that SELDI-TOF MS profiling of conditioned media from cell culture processes may be a useful method to monitor the secretion of low molecular weight host cell proteins into the media.

Future work will identify these 24 proteins/peptides using various enrichment techniques followed by mass spectrometry, and also to apply the technique to more bioprocess-relevant conditions such as serum-free suspension culture. Identification of protein markers by SELDI-ToF MS remains challenging due to the fact that it is difficult to elute proteins/peptides of interest from ProteinChip arrays for direct mass spectrometry identification, as compared to 2D gel-based methods. Identification can involve fractionation and enrichment of proteins/peptides of interest using spin columns with the

same chemistry as the ProteinChip array surfaces, followed by separation on 1D gels and identification by mass spectrometry (Butler 2005, Paradis *et al.* 2005).

However, despite these challenges, the use of SELDI-ToF MS to monitor culture progress for process optimization is attractive because of the small sample size and the rapid assay time, compared to other possible monitoring methods. Therefore we are interested to profile the conditioned media from a low temperature based biphasic culture using SELDI-ToF MS to identify secreted proteins that may have impact on the cellular phenotypes observed at low temperature. The proteomic patterns (or protein 'fingerprints') may also be very useful for distinguishing or predicting a 'good' or 'bad' culture with high sensitivity and specificity, and this approach is being used in discovering protein profiles that distinguish disease and disease-free states (Ciordia *et al.* 2006). The comparison of proteomic profiles of good or bad cultures or time points could enable to identify proteins/peptides that are driving the development of those phenotypes. This could also have potential to identify biomarkers for apoptosis in culture. The proteomic profiles could also enable us to identify the best time point for product harvesting.

Conclusions

The increasing demand for therapeutically important protein products is leading to improve the recombinant protein productivity of mammalian cells. Low culture temperature has been shown to reduce the growth rate, metabolism and shear sensitivity and increase culture longevity and productivity in CHO cells but the mechanisms are still poorly understood. Understanding cellular response to low temperature at the molecular level could help in designing more efficient production processes. In this study, we have investigated alterations in the expression of proteins and miRNAs associated with the low temperature response or different phases of culture in CHO-K1 cells to identify potential proteins and miRNAs. Based on the findings described in this thesis, the following conclusions can be made.

- Adherent CHO-K1 cells were maintained continuously in suspension culture and were adapted to grow in suspension. These suspension-adapted CHO-K1 cells were further adapted to grow in low serum-supplemented or serum-free culture medium. In this investigation, we have successfully established CHO-K1 cells in low serum-supplemented and serum-free culture medium (ExCell, Sigma) and SEAP-secreting CHO-K1 cells in low serum-supplemented culture medium.

- Suspension-adapted CHO-K1 cells cultured in a temperature-shifted biphasic culture (grown at 37^0C for 72hrs and shifted to 31^0C for a further 72hrs) and standard culture (37^0C for 144hrs) were analyzed using 2D-DIGE to identify temperature-dependent and independent differential expression of proteins. Twenty-three proteins were specifically

differentially regulated in the temperature-shifted cultures (72hrs at 37^0C vs. 144hrs at 31^0C) and were involved in the regulation of variety of cellular functions such as cell growth (HNRPC), cap-independent translation (EIF4A) and the cytoskeleton (PFN2) of cells. Two proteins, DPYSL2 and ALDH, were identified at more than one location on the temperature-shifted 2D-gel indicating a possible low temperature-induced post-translational modification of these proteins.

- A total of 21 proteins (17 up regulated and 4 down regulated) were specifically differentially regulated in standard culture at 144hrs at 37^0C compared to 72hrs at 37^0C. The majority of the identified differentially expressed proteins in this group were involved in the regulation of cell growth (GRB2), structure (ACTB) and apoptosis (ENO1) in culture.

- Twelve proteins were commonly differentially regulated in both temperature-shifted and standard culture indicating the process-dependent changes in expression, except CNN3.

- EIF4A was up regulated in temperature-shifted cultures. EIF4A is a critical factor for both cap-dependent and independent translation pathways. Inhibition of EIF4A with siRNAs reduced growth of CHO-K1 cells and total SEAP productivity but increased cell specific productivity (Qp) for one of the two siRNAs. By inhibiting EIF4A at the desired biomass, it may be possible to increase total productivity of production culture.

- HNRPC, down regulated in temperature-shifted biphasic culture, is involved in IRES-mediated cap-independent protein translation and can regulate cell proliferation. Following knockdown of HNRPC at 37^0C, growth was reduced and Qp and the total productivity was improved. The improvement in Qp in temperature-shifted culture was more pronounced in comparison to standard culture suggesting that HNRPC may improve recombinant protein productivity in a temperature-independent manner. As with EIF4A, HNRPC may be an excellent protein target to inhibit at the desired biomass. However the effect of the siRNA knockdown at the reduced temperature have yet to be established.

- PFN2, up regulated in temperature-shifted culture, is a cytoskeletal protein and required for normal actin polymerisation in response to thermal stress. One of the two siRNA inhibited PFN2 resulting in reduced cell growth, viability and total productivity. Qp was improved due to the inhibition of PFN2 in both temperature-shifted and standard culture and as with EIF4A and HNRPC, PFN2 may be a suitable target to inhibit at the desired biomass.

- Differential expression profiles of miRNAs for temperature-shifted cultures sampled after 24hrs or 72hrs compared to 72hrs at 37^0C revealed up regulation of miR-24 and down regulation of miR-7 which was confirmed using qRT-PCR. qRT-PCR analysis further showed that expression of miR-7 was gradually reduced from lag and log phase to stationary phase. This inhibition was more pronounced in temperature-shifted cells. This

indicates that miR-7 could be involved in the regulation of cell growth and productivity in a dose-dependent manner.

- Over expression of miR-7 in standard culture resulted in nearly complete growth arrest (88%) in SEAP-secreting CHO-K1 cells. The Qp was improved by 116% following over expression of miR-7 at 37^0C but was unchanged in temperature-shifted cultures. This suggested that miR-7 may improve recombinant productivity of cells in temperature-dependent manner. Thus, miR-7 may be an excellent target to regulate the growth and improve recombinant protein production in culture once the appropriate biomass is achieved in standard cultures. The cell growth arrest, improved culture longevity and Qp could result in a significant improvement in the total yield of recombinant protein product.

- Over expression of miR-24 reduced growth by 56% in standard culture without affecting the viability of culture. It also improved Qp at standard but not in temperature-shifted cultures and may be a useful target to over express in standard culture once desired biomass is achieved.

- The conditioned medium representing different phases of growth cycle (i.e. lag, log and stationary phase) from low serum-adapted CHO-K1 cells was profiled using IMAC30 and Q10 chromatographic surfaces with SELDI-ToF MS. A total of 24 proteins/peptides were observed to be differentially expressed in this investigation. These proteins showed differing patterns of expression over culture period. For example, 8.1 kDa protein/peptide

was initially secreted into the conditioned media and later was either consumed by the cells or degraded by proteolysis. The 11.3 kDa and 14.7 kDa proteins/peptides were observed in the stationary phase of culture which possibly could be due to either the accumulation of cellular waste arising from cell death or increased biomass in culture. The 7.3 kDa protein/peptide was increased over time and could be associated with cell number. Identification of these proteins could enable us to identify proteins/peptides that may be critical for regulating growth and apoptosis in culture.

Future Plans

- SEAP-secreting CHO-K1 cells were adapted to grow in low serum supplemented culture medium but will need to be adapted to grow in serum-free culture conditions as serum-free culture is more relevant to the industrial production process.

- Western blot analysis was performed only for cells siRNA-transfected at standard culture. It would be necessary to investigate the expression of protein targets in temperature-shifted cultures following siRNA transfection to further confirm the efficiency of knockdown at reduced temperature using Western blotting.

- The inhibition of expression of EIF4A reduced growth and total productivity of SEAP-secreting CHO-K1 cells. A pattern of improvement in the cell specific productivity (Qp) was also observed, however it was not significant in all cases. Therefore over expression of EIF4A in CHO cells could reveal its role in improving recombinant protein production at low temperature.

- The inhibition of HNRPC was found to improve the Qp of SEAP-secreting CHO-K1 cells in a temperature-independent manner. The development of CHO cells stably expressing reduced levels of HNRPC would be ideal to further investigate the role of HNRPC towards improved productivity at low temperature.

- siRNA 2 for PFN2 exerted opposite effects on growth in temperature-shifted culture compared to standard culture. The use of higher concentrations of siRNA 2 or other independent siRNA molecules (i.e. 3rd siRNA) for PFN2 should further clarify its impact on cell growth at low temperature.

- DPYSL2 and ALDH proteins were identified at more than 1 location on the temperature-shifted 2D-gels suggesting the possible low temperature-induced post-translational modification (PTMs) of these proteins. Analysis of these proteins with LC-MS/MS and immunoprecipitation studies could potentially identify PTMs and associated proteins involved in the cell signalling pathways induced by low temperature.

- A number of miRNAs (miR-7, miR-21 and miR-24) associated with regulation of cell growth were observed to be differentially expressed following temperature-shift. However the genomic sequences of these miRNAs for CHO cells have not been reported previously. We have published the sequence CHO miRNA, cgr-miR-21. This is the first known miRNA from CHO cells to date. Future work includes cloning and sequencing miR-7 and miR-24.

- The over expression of miR-7 and miR-24 reduced growth and recombinant protein productivity of CHO cells. This proves that controlling the expression of miRNAs holds the prospect of modulating several cellular pathways and functions simultaneously and temporal expression of these miRNAs could improve efficiency of recombinant protein production once suitable biomass has been achieved. Further 2d-DIGE in miR-7 and/or

miR-24 over expressing clones could identify protein targets and biological pathways modulated by these miRNAs, leading to greater understanding of the regulation of cell growth and productivity in culture. In addition, it would be interesting to investigate the effects of the over expression or inhibition of these miRNAs in other mammalian cells lines including NS0 to see if they have cell-line dependent effects. This might open the possibility of implementing these miRNAs in other expression systems (i.e. NS0) or medico-biological fields such as tissue storage etc.

- Twenty-four secreted proteins/peptides were observed to be differentially regulated in the conditioned media of CHO-K1 cells in different phases of growth cycle (i.e. lag, log and stationary phase). Of these proteins, the level of 8.1 kDa protein/peptide was initially increased and latter reduced in culture either due to consumption by cells or by proteolysis. Identification of this protein could lead to understand the role of 8.1kDa protein/peptide in regulation of cell growth or increased proteolytic activity in culture. The expression of 7.3 kDa protein/peptide was increased over time and could be associated with cell number. This protein/peptide may be used as a highly sensitive indicator of cell growth in culture. Apart from these proteins/peptides, a numbers of proteins were observed to accumulate in medium in the late-stage of culture, possibly due to either cellular waste accumulation arising from cell death or increased biomass in culture over time. Identification of these proteins could enable us to identify apoptosis-related or stress-related proteins.

- Differential profiling of secreted proteins for culture incorporating temperature-shift using SELDI-ToF MS followed by identification of differentially expressed protein/peptides using LC-MS/MS could enable us to identify temperature-dependent secreted proteins that may have role in regulation of growth and recombinant protein productivity in culture. This information would be useful to design better strategy to improve efficiency of production process.

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