THE CO-MODULATION OF APOPTOSIS AND THE CELL CYCLE.

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Dedicated to my Mother Lynda Astley and my Husband Michael Goss for their continued support and constant love throughout this journey.

You believed in me when I doubted myself.
You encouraged me when I was feeling low.
You inspire me every day of my life.
Thank you.

I would also like to acknowledge the following people for all their the help and support through this PhD, my supervisors Professor Mohammed al-Rubeai (Birmingham University) and Dr Andrew Racher (Lonza Biologics) My colleges at Birmingham University.

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Synopsis

In modern times we have become increasingly reliant on mammalian cell culture for the production of biopharmaceuticals; therefore research aimed at improving the characteristics of the cell-lines being used for recombinant protein production is essential. In this study I have examined the hypothesis that the creation of a CHO cell-line in which the expression of $p21^{CIP1}$ and Bcl-2 could be combined would un-couple cell growth from cellular proliferation resulting in a significant increase in both the rate of production and culture viability. Analysis of key metabolites together with changes in cell volume, total protein and mitochondrial activity indicate that following the initiation of $p21^{CIP1}$-expression cells undergo an increase in their protein synthesis machinery and that the energy, previously required for cell division may be diverted towards cell growth and product formation.

In addition to the requirement of cell-lines with high production capacities, the biopharmaceutical industry is under constant pressure to develop growth media able to facilitate high yields without the need for the addition of protein or serum. This means it is often necessary to adapt high producing cell-lines to growth in such a defined chemical environment, a process which has proven to be both extremely long and costly. In this thesis I have successfully developed a method for the swift adaptation of commercially important cell-lines to growth within a chemically defined bio-processing environment. I have shown that the expression of $p21^{CIP1}$ is able to reduce the need for extracellular growth factors and that by combining the expression of $p21^{CIP1}$ and Bcl-2 it is possible to further reduce the time required for successful adaptation, supporting the well established theory that Bcl-2 plays an important role in apoptotic signalling pathways.

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Abbreviations

AIF .....Apoptosis inducing factor

AO..... Acridine Orange

Apaf-1……...Apoptosis protease activating factor 1

APC.....Anaphase promoting complex

BH……..Bcl-2 homology

CARD.....Caspase recruitment domains

CCT..... Cumulative cell time

CDK……..Cyclin-dependent protein kinase

CDS..... Cell dissociation solution

ced-3……...…….. *Caenorhabditis elegans* cell death gene

ciks.....cdk inhibitors

DD.....Death domain

DED.....Death effecter domains

dFCS.....Dialysed foetal calf serum

DISC.....Death inducing signalling complex

ELISA..... Enzyme linked immunoabsorbent assay

EGF……..…… Epidermal growth factor

FAD.....Dehydrogenase-flavin adenine dinucleotide

FADD.....Fas associated death domain

GS.....Glutamine synthetase

ICE..... Interleukin-1 converting enzyme

IPTG..... isopropyl b-D-thiogalactopyranoside

IRF-1..... Interferon-repressive factor

Mabs.....Monoclonal antibodies

MSX.....Methionine sulphoximine

MTT..... 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Mdm2..... Murine double minute oncogene

OPD..... *O-phenylenediamine* di-hydrochloride

PBS .....Phosphate buffered saline

PCA..... Guava Personal Cell Analysis

PCNA.....Proliferating cell nuclear antigen

PCV..... Packed cell volume

PI..... Propidium iodide

Rb..... Retinoblastoma protein

TNF.....Tumour necrosis factor

TGF-b..... Transforming growth factor b

1. Introduction

1.1. Overview

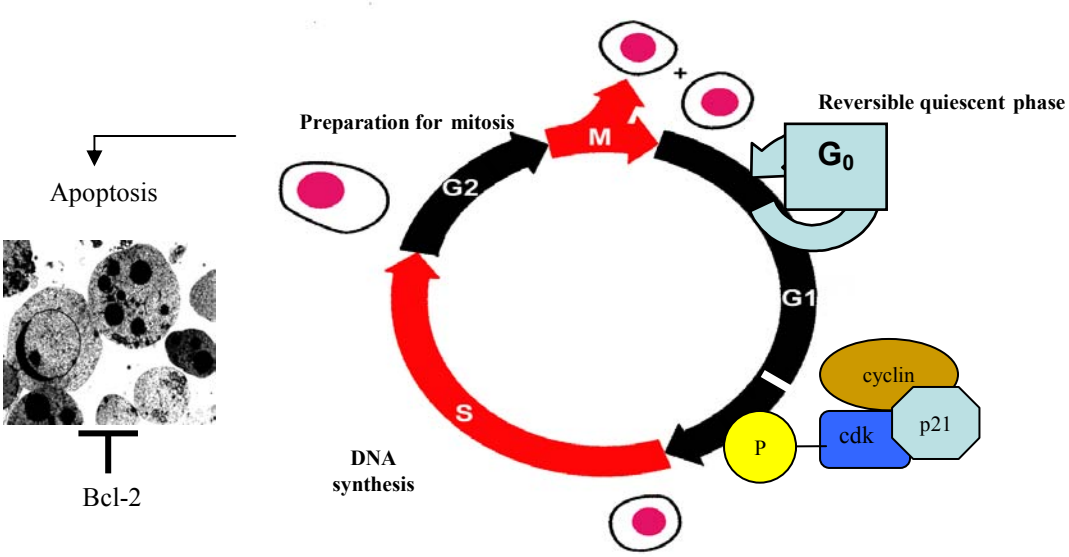
Control over the cell cycle and apoptosis is fundamentally a very important area of research, particularly for industrial purposes. Understanding the mechanisms that drive a cell through the ordered sequence of events that result in DNA replication, cell division and ultimately cell death has given rise to multiple endeavours by research groups worldwide to develop methods of modulating this cycle in order to influence the productivity, viability and sustainability of biologically important cell-lines.

The ability to maintain genomic integrity is vital for cell survival and proliferation. Lack of fidelity in DNA replication and maintenance can result in deleterious mutations leading to cell death or, in multicellular organisms, cancer; therefore the most important events of the cell cycle are those concerned with replication of the genome and segregation of the replicated genomes into the daughter cells formed at division. The processes of cell division, differentiation, survival, and death are all governed by intricate changes in the delicate balance between the multiple regulators of the cell cycle, and as such are responsible for the fundamental means by which all living things are propagated.

The cell cycle itself consists of an ordered set of events, ultimately resulting in cell growth and division to produce daughter cells. The eukaryotic cell cycle can generally be divided into four stages known as G1, S, G2 and M. G1 also known, as gap-phase 1 is the period during which cells prepare for the process of DNA replication. S-phase is defined as the stage in which DNA synthesis occurs. G2 is the second gap-phase

during which the cell prepares for the process of division and M stands for mitosis, the phase in which the replicated chromosomes are segregated into separate nuclei and cytokinesis occurs to form two daughter cells (Figure 1.1). In addition to G1, S, G2, and M, the term G0 is used to describe cells that have exited the cell cycle and become quiescent (*Johnson and Walker 1999*).

Figure 1.1. The mammalian cell cycle



Diagrammatic representation of the mammalian cell cycle phases. Work performed previously by our research group have shown that $p21^{CIP1}$ over-expression is able to halt cell cycle progression in the G1 phase of the cell cycle *(Watanabe et al 2001 and 2002, Ibara et al 2003, Bi et al 2004).* Bcl-2 has also been shown to be able to prevent cell death resulting from apoptosis *(Simpson et al 1998, Perani et al 1999, Tey et al 1999 and 2000).*

The replication of the chromosomes is based on the double helix structure of DNA, which unwinds during S-phase to generate two templates that may then be used for the synthesis of two new complementary DNA strands. During mitosis a bipolar spindle is formed and the two double helix DNA molecules making up each replicated

chromosome become condensed and oriented towards opposite poles of the cell. The DNA molecules attach to microtubules emanating from the spindle poles and moves away from each other toward opposite poles, to be segregated at cell division. Thus the formation of two genomes during the cell cycle occurs at the molecular level during S-phase and at the cellular level during mitosis.

To ensure that each newly formed daughter cell receives a complete genome the onset and progression of S-phase and mitosis are tightly controlled ensuring that several critical criteria are met before the cell is able to progress. These criteria include the progression through the cell cycle in the correct sequence, ensuring each event occurs only once per round of cell division, screening and correction of genetic errors in their execution, and insuring the coordination of cellular growth.

Cell death may be divided into two distinct categories, either apoptosis or necrosis. The former describes a morphological form of cell death which is initiated via an inherent gene directed program that responds to a wide variety of physiological and toxicological stimuli *(Kerr et al 1979).* This forms the basis of an integral part of development and homeostasis within any given organism. Necrosis on the other hand can be considered a passive form of cell death that corresponds to random acts of cellular suicide as a direct result of pathological damage or injury to the cell *(Wyllie 1980).*

The process of apoptosis has been identified as a key feature of many disease pathologies, either through the resulting loss of cells or through its failure to be executed *(Thomson 1995),* for example, tumours arise from the failure of apoptosis to

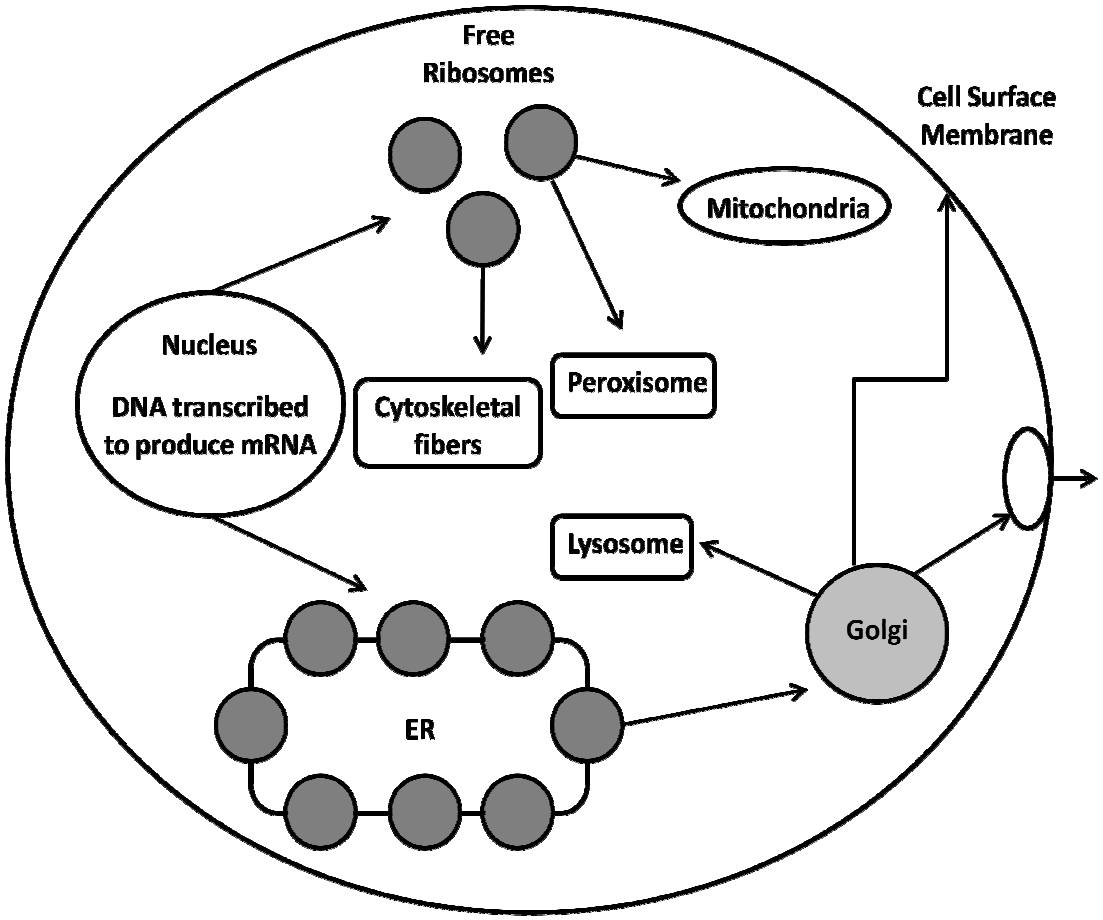
be activated following genetic transformation within the cell, thus leading to the transfer of defective genetic material during the following round of replications. At the other end of the spectrum over activation of apoptosis has been found to be responsible for several neurodegenerative conditions such as Alzheimer's and Parkinson's disease. It has therefore been a long term goal of cellular biologists to be able to determine the exact mechanisms of apoptosis in order to manipulate the outcome of diseases in which it plays a key role.

The use of animal cell technology for the production of biopharmaceuticals has resulted in an increased interest in the process of apoptosis. This is due to the fact that during the process of cell culture, apoptosis plays a huge role in determining the life span and thus productivity of the resulting culture. The need for increased culture viability within bioreactor environments places apoptosis as a primary target for the optimisation of such processes.

1.2. Protein Production

Proteins are essential components of all living cells and as a result make up more than half of the cells dry weight. They play key roles as both structural elements and active functional units responsible for molecular recognition and catalysis. Within the cell DNA serves as the genetic template from which all proteins are produced, however it is the proteins themselves that drive almost all of the processes within the cell. Protein synthesis occurs as a result of the guided polymerization of simple amino acids. In nature protein synthesis is closely entwined with the process of gene expression, in which the information in DNA is first transcribed to mRNA then translated to protein (Figure 1.2).

Figure 1.2. Protein production within a mammalian cell



Simplified diagram of protein production within a mammalian cell. In the nucleus segments of DNA are copied to produce mRNA. These coded instructions for a protein (like a recipe) leave the nucleus and go to either free ribosomes or to ribosomes bound to the endoplasmic reticulum. When released from a free ribosome, a protein can become incorporated into cytoskeletal fibres or into organelles such as a mitochondrion or a peroxisome. Proteins made in the endoplasmic reticulum leave in a vesicle and migrate to the Golgi apparatus. Proteins are sorted in the Golgi before they are then carried in vesicles to lysosomes, secreted or incorporated into the cell's surface membrane.

The specific order of polymerization of a protein's amino acids has to be guided by the particular order of the nucleotide bases of the corresponding DNA. In nature proteins are expressed in living organisms according to tightly regulated mechanisms in order to meet the specific needs of the organism, a process known as homologous gene expression. In contrast the use of recombinant DNA techniques for the expression of genes outside their natural expression environment is described as heterologous gene expression.

Proteins may be heterologously synthesised either *in vivo* or *in vitro*. *In vivo* expression systems include any non-native cellular system that permits the synthesis of the target protein, while *in vitro* expression systems include any non-native, acellular system which permits such synthesis. Over the last 35 years recombinant protein expression technology has developed at an astounding rate giving rise to a variety of *in vivo* and *in vitro* protein synthesis systems allowing for both the production and manipulation of the properties of almost any protein *(Hillebrecht and Chong 2008, Swartz 2006).*

In order to heterologously express a desired protein within a living host, the corresponding sequence of DNA must be known and a suitable method for its transformation and selection has to be available. The ability to clone the corresponding DNA into a suitable vector is vital for its introduction into the selected host. The vector usually consists of an independently replicating DNA or DNA segment which may be integrated into the host genome. In order for the DNA encoding for the protein of interest to be expressed it has to be preceded at the 5` end with a transcription promoter. Depending upon the nature of the target protein either

prokaryotic or eukaryotic hosts may be used for the expression of the required protein. This however is heavily dependent on the type of protein being encoded, as the choice of host can have a significant effect on the nature of the expression as well as the transformation, selection and subsequent purification of the target protein product.

1.2.1. Bacterial production systems

There are many reasons for the popularity of bacteria as host systems including the fact that the genome sequence of the most popular bacteria has been well established and the majority of their biological processes and metabolic pathways are now well understood. There are also many genetic tools readily available that allow for their manipulation. On the down side a bacteria such as *Escherichia coli* will never be completely suitable for every application *(Billman-Jacobe 1996)*, mainly due to limitations in its ability to act as an expression system. Many of the proteins used in modern day industrial applications are highly complex, consisting of multiple subunits, cofactors/prothetic groups, disulphide bonds, and post translational modifications, including glycosylation, that are essential in order to produce a fully functional protein.

Extensive efforts have been made in the direction of proper understanding and improvement of *E. coli* as an expression system for heterologous proteins *(Hannig and Makrides 1998, Hockney 1994)*, however if the protein being produced is to serve a pharmaceutical purpose, post-translation modifications such as protein folding may be critical for its biological activity. Prokaryotic hosts such as *E. coli* are unable to perform such complex modifications and in addition may result in the recombinant

protein product preparation becoming contaminated with endotoxins and pyrogens, both of which are difficult and costly to remove *(Binnie et al 1997).*

In more recent years several species of both Gram-negative and Gram-positive bacteria have been identified as having potential for use in protein synthesis *(Billman-Jacobe 1996)*. Amongst the contenders the Gram Negative species including *Pseudomonas spp*., *Serratia marcescens*, and *Erwenia herbicola* have all demonstrated successful heterologous expression of proteins *(Rangwala et al 1991).* Despite this they are not considered to be strong rivals for the *E. coli* expression system, but do offer the potential of further development. In contrast a number of Gram-positive bacteria have proven to be very good alternatives to *E. coli*. Many are currently used for the production of industrial enzymes through their ability to directly secrete proteins through the cell envelop into the culture medium *(de Vos et al 1997)*. *Lactococcus lactis* which is responsible for the production of lactic acid is one example and has successfully been used in the expression of a large number of heterologous proteins. Due to its classification as a food grade bacterium it, along with others in this category are generally regarded as safe and therefore confer this safety onto the resulting protein produced, thereby making it suitable for human consumption *(Kuipers et al 1997)*. Actinomycetes were previously thought to be fungi due to their filamentous forms however it is now clear that they are not fungi but higher bacteria, closely related to the Mycobacterium. These bacteria surpass most other bacteria in their ability to synthesise protein at significantly higher levels, with titres of more than 300mg/L of heterologous protein being relatively common *(Binnie et al 1997)*.

Other uses for bacterial hosts include the *in situ* production of recombinant protein through the generation of bacterial carriers that are able to produce recombinant proteins within their natural environments. One of the best studied examples of this system is the insecticidal toxin of *Bacillus thuringensis* which has been expressed in other bacteria to facilitate its delivery. For example, the cyanobacteria *Agmenillus quadruplicatus* and *Clavibacter xyli* have both been successfully used to deliver *Bacillus thuringensis* to mosquito larvae and corn borer, respectively, resulting in insecticidal effects *(Billman-Jacobe 1996)*.

An alternative use for bacteria involves the heterologous expression of proteins on the bacteria cell surface. In order to achieve this, the protein must cross the cytoplasmic membrane and the periplasm to the outer membrane of the bacteria, while still retaining the correct conformation and orientation to allow it to function biologically (*Hockney 1994)*. This makes it possible to produce live vaccine vectors able, through the expression of their recombinant proteins to elicit immune responses in higher organisms. Such bacterial strains are usually attenuated and transformed with an expression vector that carries the gene for a specific antigen. When the bacteria are introduced into the host the expression of the antigen elicits immunisation *(Hockney 1994, Billman-Jacobe 1996)*.

1.2.2. Fungal production systems

The wide diversity of fungi species available for the production of recombinant proteins enables the selection of a host specifically to match the target proteins to be produced. In addition many species have the natural capacity to produce large quantities of primary metabolites, such as vitamins, secondary metabolites, such as

antibiotics, as well as extracellular enzymes, all of which can be further optimised by industrial production conditions.

Despite the existence of around 100,000 species of fungi *(Esser and Lemke 1994)* only a handful, mainly those belonging to the yeast and mould species have been sufficiently studied to enable their use as hosts for the heterologous production of protein. Yeast species, like *Saccharomyces, Pichia, and Hansenula*, are well characterised as efficient gene expression hosts with good fermentation properties, for example the yeast species *Pichia pastoris* during fermentation has been shown to be capable of substantial production levels of around 65g/L *(Wolf 1996)*. Similarly mould species like *Trichoderma* and the *Aspergilli*, have been shown to have high enzyme secretion rates that can result in the production of around 20 g/L *(Kruszewska 1999)*.

In most cases the choice of vector used for transforming fungi is a hybrid of both eukaryotic and prokaryotic sequences *(Lemke and Peng 1995, Van, den Hondel et al 1991, Fincham 1989)*. The eukaryotic element is usually one derived from fungal sequences and varies according to the specific requirements of the expression vector. In contrast the prokaryotic section is usually derived from *E. coli*, thereby allowing the processes of replication and selection to occur within the cell. A polylinker containing several restriction enzyme recognition sites allows for the introduction of the foreign DNA sequences. This system allows cloning to be performed within *E. coli* allowing for easy manipulation. On the negative side although fungi are more complex than bacteria, the majority of therapeutic proteins require additional post translational modifications such as *N*-glycosylation. Eukaryotes including yeasts are

able to perform N-glycosylation however the resulting structures and sugar content vary from species to species and therefore therapeutic proteins with non human glycosylation patterns carry a high risk of eliciting an immune response.

Recently much of the research surrounding protein expression in fungi has focused on the re-creation of human glycosylation patterns. In 2003 *Hamilton et al* reported the humanization of the glycosylation pathway of the yeast species *Pichia pastoris* in order to secrete a human glycoprotein with uniform complex N-glycosylation. The process involves the removal of the endogenous yeast glycosylation pathways while properly localizing five active eukaryotic proteins, including mannosidases I and II, *N*-acetylglucosaminyl transferases I and II, and uridine 5-diphosphate (UDP)–*N*-acetylglucosamine transporter. The targeted localization of these enzymes enables the generation of a synthetic in vivo glycosylation pathway, which is then able to produce the complex human *N*-glycan *N*-acetylglucosamine$_2$–mannose$_3$-*N*-acetylglucosamine$_2$ ($GlcNAc_2Man_3GlcNAc_2$).

Alternative studies (*Bobrowicz et al 2004)* have used different methodologies for the engineering of an artificial glycosylation pathway in *Pichia pastoris* blocked in dolichol oligosaccharide assembly. In this study the *PpALG3* gene encoding Dol-P-Man:$Man_5GlcNAc_2$-PP-Dol mannosyltransferase was deleted in a strain that was previously engineered to produce hybrid $GlcNAcMan_5GlcNAc_2$ human N-glycans. Employing this approach, combined with the use of combinatorial genetic libraries, *P. pastoris* strains were engineered that are able to synthesize complex $GlcNAc_2Man_3GlcNAc_2$ N-glycans with a high level of homogeneity.

More recently researchers have also turned their attention towards the production of human antibodies. Presently all of the clinically approved monoclonal antibodies in production are produced within mammalian cell-lines, which are able to secrete monoclonal antibodies with glycosylation structures that are sufficiently similar, though not identical, to their human counterparts. In 2006 *Li et al* reported that it was possible for human antibodies with specific human N-glycan structures to be produced in the glycoengineered yeast *Pichia pastoris* and that antibody-mediated effecter functions can be optimized by generating specific glycoforms.

It is obvious that as our understanding of the glycosylation pathways in yeast develops there will be great potential for their use to increase the variety of recombinant protein products currently on the market. In addition since the scale-up and production potential of yeast is well-established as being significantly higher than its mammalian host counterparts, it is reasonable to expect the high-throughput and cost-effectiveness of glycoengineered yeast strains to make a significant impact on the biopharmaceutical industry.

1.2.3. Plant production systems

Plant expression systems potentially offer an inexpensive and convenient method for the large-scale production of many recombinant protein products. Over the last ten years, a number of successful plant expression systems have been developed, resulting in the production of well over 100 recombinant proteins from a range of different species.

There are several advantages to the use of plant expression systems over other production systems, including the ease of which agricultural production levels can be achieved, cost effectiveness and the existence of established methods for the harvesting, transporting, sorting and processing the derived products. It is estimated that recombinant proteins can be produced in plants at 2–10% of the cost of microbial fermentation systems and at 0.1% of the cost of mammalian cell cultures, although this very much depends on the product yield *(Giddings 2001)*.

A major advantage to plant based expression systems over other systems, comes from the ability to administer several types of recombinant protein in unprocessed or partially processed plant material, resulting in a decrease in the overall downstream processing costs. For example, recombinant vaccines produced in plants may be administered via the consumption of the resulting raw or part-processed fruits and vegetables *(Mason et al 2002)*. Similarly antibodies used for passive immunization may be administered topically as pastes and creams following minimal purification. In contrast the vast majority of pharmaceutical proteins need to be purified before they are suitable for use, which in turn results in a significant increase in the cost and complexity of downstream processing. Several strategies have been developed to reduce downstream processing costs, including the targeted expression of the desired recombinant protein to specific plant parts. For example the oleosin-fusion platform developed by *SemBioSys Genetics,* for use in oil seed crops enables the desired recombinant protein to be fused with oleosin. The fused protein can then be recovered from the plants oil bodies using a simple extraction procedure and the recombinant protein purified via an endoprotease digestion.

It is also possible to utilize a plants natural ability to secrete protein products through its tissues, enabling the continuous production of the desired recombinant protein *(Borisjuk et al 1999, Komarnytsky et al 2000, Drake et al 2003)*. This type of strategy is however only suitable for small-scale production and is limited in its practicality by the need for sealed containment facilities to prevent the resulting recombinant product leaching into the soil or groundwater. Another alternative approach to the use of whole plants is the large-scale cultivation of plant-cell suspensions. Although plant cells are not comparable to microbes in terms of generation time, obtainable cell densities or nutritional requirements they still require similar techniques and equipment. It has been possible to use conventional fermentor equipment, with minor adjustments, to successfully apply modes like batch, fed batch, perfusion and continuous fermentation with scales up to 100,000 litres *(Fischer et al 1999)*. Several other factors can however hinder the use of plants for the production of some recombinant proteins, including the quality, safety and the homogeneity of the final product as well as the previously described inherent difficulty in processing some plant-derived pharmaceutical macromolecules.

There is a great deal of similarity between the protein-synthesis pathways of plants and animals, therefore the expression of human transgenes results in the production of proteins with identical amino-acid sequences to their native counterparts. During post-translational modification there are however some significant differences, including the inability of transgenic plants to correctly modify human collagen unless the gene encoding proline-4-hydroxylase is also expressed *(Merle et al 2002)*. The most significant difference occurs during the synthesis of glycan side chains. Eukaryotes add glycan chains to proteins as they pass through the secretory pathway; however

differences in the expression levels of the enzymes associated with this form of modification results in the production of glycan-chain structures that can vary significantly.

In general plant-derived recombinant proteins lack the terminal galactose and sialic-acid residues, normally found in mammals, but have the carbohydrate group α(1,3)fucose, which has a (1,6) linkage in animal cells, and β(1,2)xylose, which is absent in mammals although present in invertebrates. These differences in the glycan structure have the potential to alter the activity, distribution and longevity of the resulting proteins compared with the native forms. As a result it has been suggested that some plant-specific glycans may be capable of inducing allergic responses in humans *(Lerouge et al 1998)*. Therefore, recent attention has focused on the development of strategies to 'humanize' the glycosylation patterns of recombinant proteins *(Bardor et al 2003)*.

Despite this the last decade has seen the emergence of several plant-based expression systems which are able to compete with existing production systems in the large-scale production of recombinant proteins. As a result several plant based biopharmaceutical products are currently either in the late stages of development or already on the market including antibodies, vaccines human blood products, hormones and growth regulators *(Hood et al 1997, Kusnadi et al 1998 Evangelista et al 1998, Fischer and Emans 2000, Giddings 2001)*.

1.2.4. Animal production systems

The ability to transform animal cells through the introduction of foreign genetic material began in 1910 when Peyton Rous discovered that it was possible to induce the formation of tumours by using a filtered extract of chicken tumour cells, which was later proven to contain an RNA virus *(Rous 1911)*. By the late 1950s the ability of viruses to transform animal cells had been well established and later in 1977 Wigler and associates, developed an efficient method for the introduction of single-copy mammalian genes into cultured cells *(Wigler et al 1977)*.

In the late 1970s interferon was the first drug derived from a cultured human lymphoblastoid cell-line to be licensed. In more recent years, mammalian cells have become the favoured expression system for industrial production of a vast multitude of bio-therapeutics. More than half of the FDA license approvals for biotechnology products from January 1996 to November 2000 were for mammalian cell systems *(Chu and Robinson 2001)*. In addition, monoclonal antibodies produced from mammalian cell-lines are now becoming important drug candidates for many biopharmaceutical companies as there are currently over 20 approved antibodies already in use and about 150 in various stages of clinical studies *(Reichert et al 2005)*.

When a gene is to be expressed in an animal cell it may be either cloned into an expression vector or be transferred directly into the recipient cell where it may independently survive or integrate into the host genome *(Richa and Lo 1997)*. Most mammalian expression vectors contain a copy of the gene of interest which is placed downstream of a promoter sequence and is followed by a transcription termination signal. Some constructs may also incorporate transcription and translation enhancers

as well as selectable markers which enable both manipulation in *E. coli* and selection in the mammalian host. Although eukaryotic mRNA is not generally polycistronic, such constructs have been efficiently made using Internal Ribosome Entry Sites (IRES) that were identified in the single-stranded RNA genome of picorna viruses. These sequences provide secondary ribosome binding sites that have been shown to enable expression from the polycistronic messages *(Ganguly and Shatzman 1999)*.

The major difference between vectors used in the transfection of animal cell-lines and those used to produce transgenic animals is the process of selection. In cultured mammalian cells, selection is generally dependent on the presence of a drug resistance gene included within the expression vector. In contrast the identification of transgenic pre-implantation embryos prior to transfer into the recipient animals is not yet possible. In addition, very little is known about the fate of the transgene following integration into the genome of the transgenic animals *(Bishop 1999)*.

In general there are three methods utilized for the transformation of animal cell-lines including calcium phosphate-DNA co-precipitation which may be applied to cells grown as a monolayer. In this method the DNA is transfected into cells through its incorporation into fine calcium phosphate precipitate. An alternate method is Lipofection in which the DNA is adsorbed to very small spheroid lipid vesicles that fuse to the mammalian cell membrane. The third method is electroporation which is also used for transforming mammalian cells in suspension. In contrast when expression is required within a whole animal the expression construct must be delivered to the pre-implanted embryo, predominantly via the microinjection of the fertilised oocyte. This may then be transplanted into the uterus of a surrogate mother

until delivery. Currently no method exists for the selection of transformed embryos prior to implantation.

It has been claimed that in a number of biopharmaceutical companies more protein species are being produced in CHO cells than in *E. coli* and in *S. cerevisiae (Wurm 1999)*. CHO cells were originally derived from a biopsy of an ovary of an adult Chinese hamster (*Cricetulus griseus*) in 1957. CHO cells posses a number of features which have proven to be advantageous in the process of protein production including the ease of which exogenous DNA may be introduced, together with a high capacity for large scale growth and production. Another well established cell-line is BHK. These cells were originally derived from 1-day-old Syrian hamster (*Mesocricetus auratus)* kidneys back in 1961 and since then they have been utilized for the expression of recombinant proteins such as human factor VIII for therapy of coagulation disorders. They have also demonstrated a susceptibility to viruses, and have therefore also been extensively used for vaccine production.

The third type of cells that are commonly used in industry are hybridoma. Hybridomas are immortalised antibody secreting hybrid cells, produced through the fusion of malignant tumour cells (called myeloma) and spleen lymphocytes of a mouse immunised with a particular antigen *(Kohler and Milstein 1975)*. They are primarily used in the production of monoclonal antibodies (mAb); however they have also proved efficient in producing recombinant mAb by constructing what are called heterohybridoma. The species from which they are derived can contribute to the variant and the constant regions of the secreted mAb in order to produce a bi-specific mAb that can cross react with two antigens. The myeloma cell-lines that are used for the production of hybridomas are themselves also suitable for the production of

recombinant proteins particularly because of their natural secretion capability, their transfection properties and their ability to grow to high cell densities *(Bardouille 1999)*.

In contrast to the production of heterologous proteins in *in vitro* cultured cells, transgenic animal technology enables the targeting of recombinant proteins to specific animal tissues or secretions. The development of transgenic animals began in 1980 when a transgenic mouse was developed through the microinjection of DNA into a fertilised egg *(Gordon et al 1980)*. Currently transgenic animals may be produced either by microinjection of DNA into fertilised eggs or by viral transfection of embryonic stem cells. In both cases when the foreign DNA integrates into the embryo's genome the resulting animal may then be considered transgenic.

The use of tissue-specific regulatory sequences allows the expression of the gene to be targeted to almost into any tissue type. In one study *(Janne et al 1998)* a fusion construct between pig β globulin promoter and human β globulin coding region resulted in the expression of up to 24% completely human haemoglobin A (HbA) within the pigs blood. Interestingly the transgenic animals appeared to tolerate well the high levels of the human HbA in their erythrocytes and structural analysis of the recombinant human HbA showed it to be equivalent to the protein purified from human blood. Despite this the complex chemical composition of blood makes it very difficult to isolate and purify the resulting recombinant product. It is also obvious that circulating blood cannot be used as a source of biologically active recombinant products, such as cytokines and hormones, without creating health problems for the production animals. However, the technique is still valid for producing normal

constituents of blood such as haemoglobin, antibodies and α 1-antitrypsin (*Janne et al 1998)*.

Recombinant proteins have also been engineered to be expressed within the mammary glands of animals such as mice, rabbits, goats, sheep and cows *(Houdebine 1997, Janne et al 1998).* The natural ability of these tissues to secrete the resulting products means that their expression into milk is expected not to create any health problems for the host animal even when highly bioactive peptides and proteins are being synthesized. In addition studies have demonstrated the resulting mammary gland-specific secretion sequences show inter-species compatibility. It has been shown that transgenic sheep are able to express the human α 1-antitrypsin in their milk at 50% of the total proteins resulting in 35 grams of recombinant protein per litre of milk *(Wright et al 1991)*. It was also found that the resulting recombinant protein was correctly glycosylated and therefore fully active.

Despite the potential for transgenic livestock to become a source of recombinant proteins the technology is still relatively new and the process itself is extremely expensive, labour-intensive and technically difficult. The production of a single transgenic calf requires thousands of fertilised oocyte microinjections *(Wall et al 1992)*. In addition to the economic and the technical difficulties there are also ethical issues to consider, particularly those of a religious or culturally sensitive nature.

Table 1.1. A comparison of the key features of host systems used for the production of recombinant protein products.

Host	Overall cost	Production timescale	Scale up capacity	Product quality	Glycosylation	Contamination risks
Bacteria	Low	short	high	low	none	endotoxins
Yeast	Medium	medium	high	medium	incorrect	low risk
Mammalian cell culture	High	long	very low	very high	correct	viruses, prions and oncogenic DNA
Transgenic animals	High	very long	low	very high	correct	viruses, prions and oncogenic DNA
Plant cell cultures	Medium	medium	medium	high	minor differences	low risk
Transgenic plants	Very Low	long	very high	high	minor differences	low risk

Comparison of production costs, timescales, scale-up capacity, product quality, contamination risks and whether correct glycosylation occurs in the most commonly studied expression hosts. This table summarises the key benefits of each system described above as well as providing an insight into the limitations which need to be overcome in order to produce more economically viable production systems.

1.3. The effect of clonal variation on cell-line stability and productivity

The successful development of industrial biopharmaceuticals facilitates the need for the generation of stable clones with high productivity levels. At present most of the work performed in this area has concentrated on complete monoclonal antibodies. This is not surprising considering they constitute the vast majority of products currently in clinical use or in development. During the process of clone development, gene expression platforms are used to achieve high expression levels via

amplification. Two of the most commonly used gene expression and amplification systems are Dihydrofolate reductase (DHFR) and glutamine synthetase (GS) *(Birch and Racher 2006)*. Both of these systems allow for the development of high expressing clones for the purpose of production. The ability to produce clones is vital to the biopharmaceutical industry. As a result strict guidelines have been produced by the ICH for the 'analysis of the expression construct in cells used for production of r-DNA derived protein products' (1996). These guidelines define the use of a single clone for production purposes and limit the amount of time they can safely be in use, thereby reducing the amount of variability within the final product.

Stable clones may be defined in one of two ways *(Barnes et al 2003)*. The first states that in order to be considered stable a clone must be able to successfully pass on the recombinant DNA incorporated into the host cells' genome to the resulting daughter progeny. The second definition of stability is the ability of the cloned cell-line to retain a constant level of recombinant protein production without a decrease in specific productivity during periods of long term culture. Interestingly instability arising due to the loss of the recombinant protein gene and the inherent chromosomal instability may appear as a similar process; however the latter form of instability is particularly significant in two of the most commonly used production cell-lines, Chinese hamster ovary (CHO) and NS0 mouse myeloma cell-lines. Even without heterologous protein production, both cell-lines, which were immortalised artificially, have an inherent genetic instability which cloning may not always resolve *(Barnes and Dickson 2006).*

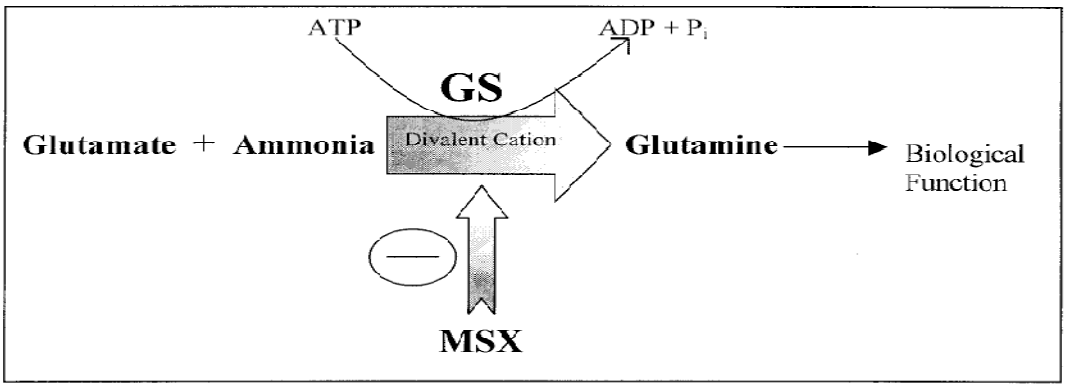
The long term culture of cell-lines, without selective pressure has often been used as a test for production stability of clones. Several studies into recombinant protein production in hybridoma cell-lines have attributed instability to the appearance of a non-producing population of cells, possibly due to environmental factors such as serum *(Couture and Heath 1995, Frame and Hu 1990, Lee and Palsson 1990, Ozturk and Palsson 1990)*. Amplification of clones using chemicals may not always be necessary. It has been suggested *(Barnes et al 2001)* that the use of growth and recombinant protein productivity as selection criteria is not sufficient. The study reported that two of the NS0 clones produced displayed unstable production levels when grown for extended periods of time in a medium lacking the presence of selective pressure. It was theorised *(Kim et al 1998a, Kim et al 1998b)* that the observed fluctuations of productivity arise due to either chromosomal loss, mutation or genetic rearrangement. It is therefore possible to define clonal stability during protein production in terms of cellular recombinant mRNA content. Studies *(Barnes et al 2004)* of monoclonal antibody production in NS0 myeloma cells indicates the existence of a saturation point for the recombinant protein mRNA which defines the stability of protein production in long term culture.

While the concentration of mRNA is an important determinant in stability, the gene copy number has no reflection on the protein production stability. It has been further reported that even in gene amplified clones, instability still occurs and that growing clones without selective pressure is able to give rise to a loss of specific cellular productivity, either due to a loss of recombinant gene or due to mutations within the recombinant gene itself *(Kim et al 1998b)*. The loss of protein production in some recombinant cell-lines can therefore be attributed to the inherent instabilities

discussed above, as well as several other factors such as irregularity in transcriptional and post-transcriptional regulation as described by *Barnes et al (2003)*.

As discussed on page 22, two of the most commonly used gene expression and amplification systems are glutamine synthetase (GS) and Dihydrofolate reductase (DHFR). The first GS is based on the Glutamine synthetase enzyme that allows for the biosynthesis of glutamine from the substrates glutamate and ammonia (Figure 1.3).

Figure 1.3. The GS expression system



The over-expression of GS allows the creation of glutamine within the cell. Methionine sulphoxomine (MSX) inhibits the activity of GS and therefore the addition of MSX to the culture medium amplifies the expression of the GS selection marker.

This enzymatic reaction follows the only existing pathway for glutamine formation within the mammalian cell; this makes the GS gene an ideal candidate for use as a very successful selectable marker. The GS system may be utilised in multiple cell-lines although not all cell-lines respond in the same way. Some mammalian cell-lines,

such as NS0, simply do not express sufficient GS to survive without the addition of glutamine to the growth medium. Therefore when these cells are transfected with the GS gene it is able to function as a selectable marker by only permitting growth of transfected cells within a glutamine-free medium. In cell-lines such as CHO, which express sufficient GS to survive within a growth environment lacking exogenous glutamine the addition of the specific GS inhibitor, methionine sulphoximine (MSX), can be used to inhibit endogenous GS activity and therefore allow the survival of only transfectants expressing the additional GS activity.

In the DHFR expression system dihydrofolate reductase converts dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the synthesis of purines, thymidylic acid, and certain amino acids. Using this system mammalian expression vectors are designed to express the DHFR gene along with the gene of interest. The plasmid is transfected into DHFR negative host cell-line which is unable to synthesize tetrahydrofolate, an important cofactor in the one-carbon metabolism. DHFR negative cells are only able to grow thereby in a media containing thymidine, glycine and hypoxanthine which are required to overcome their inherent deficiency. Cells that have been stably transfected are able to grow within media lacking these components, thereby facilitating their selection. The addition of the cytotoxic drug methotrexate (MTX), a powerful inhibitor of DHFR allows for the selection of cells containing an increased number of copies of the DHFR gene. Increasing the concentration of MTX over time therefore has the potential to not only amplify the DHFR gene, but also the co linked gene of interest within the cell-line. The productivity of any given cell-line can be greatly influenced by the construction of the plasmid vector used. For example in the GS system glutamine synthetase is usually under the control of a weak

promoter, whilst the heterologous protein coding sequence is usually controlled by a powerful promoter *(Brown et al 1992)* meaning that the resulting transfectants are constantly exposed to a positive selection.

As described previously both the GS and DHFR systems rely on the cell-lines used expressing very low levels of endogenous GS or DHFR thereby enabling the selection of the desired transfectant through their growth in a medium deficient of the required elements. The addition of MSX inhibits the expression of endogenous GS whilst also allowing for gene amplification *(Brown et al 1992*) in much the same way as MTX in the DHFR system. The amount of MSX required is very much dependent on the cell-line used, for example the NS0 cell-line generally requires 10-100μM whereas CHO cell-lines require anywhere between 50-500μM of MSX in order to suppress the expression of the cells endogenous glutamine. MSX has a powerful and irreversible effect on GS as a result of its phosphorylation to form MSX phosphate *(Manning et al 1969)* which in turn binds to GS thus blocking its functional activity.

In some cases it has been shown that the use of MTX in the DHFR expression system has resulted in the production of clones resistant to the amplification process that may not undergo any amplification to the DHFR gene copy number *(Assaraf and Schimke 1987)* Such resistance to MTX may be due to the altered affinity of DHFR in these clones *(Flintoff and Essani 1980)* or to a MTX-resistant DHFR enzyme *(Kim et al 2001)*. Recent studies *(Kim et al 2001, Griffiths et al 2002)* have shown that although it is possible to increase gene copy number with successive rounds of amplification, the expression levels of the resulting recombinant protein often does not increase linearly with this increase in copy number. Instead it was found *(Kim et al 2001)* that

during dihydrofolate reductase mediated gene amplification as few as 1 in 23 clones demonstrated any increase in antibody production following amplification, probably as a result of genetic rearrangement or fragmentation occurring either during or prior to integration within the chromosome.

Similar studies have also shown a wide spectrum of productivities resulting from gene copy number amplification, with anything from a 5 to 50 fold increase being observed *(Griffiths 2001, Hayduk and Lee 2005)*. This has lead to the belief that beyond a certain gene copy number productivity levels cannot be related to the gene copy number. One example of this has been demonstrated by Kim et al *(Kim et al 1998b)*. In their study they were able to show that gene amplification within clones resulted in increasing productivity up to a certain methotrexate (MTX) concentration, after which, posttranscriptional factors became limiting. This has meant that process of producing clones can often result in significantly different and random results.

In a study of a chimeric antibody producing CHO cell-line that utilized the DHFR expression system, the productivity and stability of the clones during periods of selection pressure was correlated to changes in growth rate, gene copy number and mRNA levels *(Kim et al 1998a)*. The results once again demonstrated no correlation between productivity and antibody gene copy number or indeed growth rate. It was shown that the loss of copies of the amplified gene resulted in the majority of clonal instability; however reduced transcriptional efficiency and/or reduced mRNA stability also resulted in lower productivity levels per copy of the chimeric antibody gene following a non-selective culture period.

Several interesting observations concerning cell-line productivity and stability have also arisen from studies of the GS expression system, for example analysis of 17 NS0 myeloma cells produced via limited dilution cloning revealed significant variation not only in productivity levels but also in growth rates *(Barnes et al 2007)*. More significantly it was shown that no obvious correlation existed between growth and productivity. This is in agreement with work produced within our group which examined 31 NS0 sub clones and found that slow growth did not always equate to high production levels and vice versa, high growth rate did not necessarily result in low productivity *(Carroll 2005)*. This would seem to contradict other results from our and other groups *(Bi et al 2004, Watanabe et al 2001, Watanabe et al 2002, Fussenegger et al 1998)* which show that productivity may be significantly enhanced by reducing or preventing cell growth. It is worth noting that the majority of the works demonstrating the ability to achieve higher productivities during slower growth were produced by comparing growth in a single clone, thus it is a comparison of dynamic growth rather than actual clonal variation.

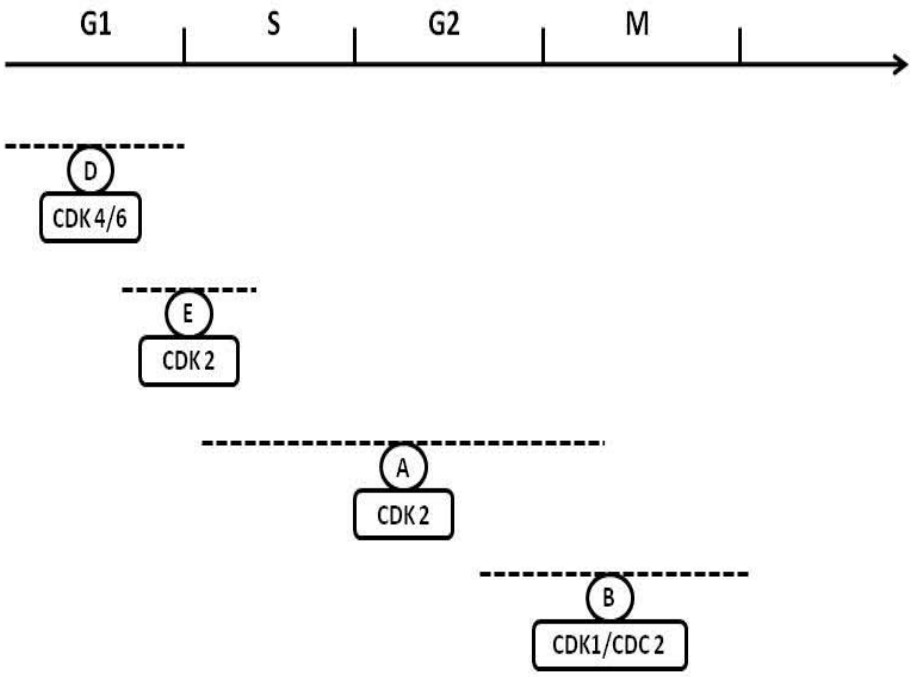
1.4. Regulation of the cell cycle

As previously described the eukaryotic cell cycle may be divided into four stages known as G1, S, G2, and M. During the G1 phase of the cell cycle cells undergo preparation for the process of DNA replication, integrate mitogenic and growth inhibitory signals and therefore make the decision whether to proceed, stop or exit from the cell cycle. Within the G1 phase of both yeast and mammalian cells an important checkpoint exists. If a cell progresses beyond this checkpoint it becomes committed to DNA replication and completion of the cell cycle *(Hartwell et al 1974, Nurse 1975)*. Following the successful completion of the G1 phase the cell passes into

the S phase of the cell cycle, in which DNA synthesis occurs. This is then followed by a second gap phase known as G2, during which the cell prepares for the process of cell division. During M phase the previously replicated chromosomes are segregated and cytokinesis occurs resulting in the production of two daughter cells. An additional phase known as G0 exists in which cells may exit the cell cycle and become quiescent.

The sequential activation and inactivation of cyclin-dependent kinases (cdks), through the periodical synthesis and destruction of cyclins provides the primary mechanism through which the cell cycle is regulated (figure 1.4). To date nine cdks and at least 16 mammalian cyclins have been identified. All cyclins share a common region of homology known as the cyclin box, which is a domain used to bind and activate cdks. Although cyclins and cdks play crucial roles in the progression of the cell cycle, some have alternate functions, including the regulation of transcription, DNA repair, differentiation, and apoptosis. Several studies *(Peng et al 1998, Rickert et al 1996, Roy et al 1994)* have shown cyclin/cdk complexes, such as cyclin C/cdk8, cyclin T/cdk9, and cyclin H/cdk7, to be essential components of the basal transcription machinery.

Figure 1.4. The cyclin/cdk complexes involved in the regulation of the mammalian cell cycle.



A simplified diagram of the cyclin/cdk complexes responsible for mammalian cell cycle progression. The cell cycle is initiated by the binding of cyclin D to cdk4 or cdk6, following which the binding of cyclins E and A to cdk2 results in the progression of the cell cycle. The final stage is catalysed by the binding of cyclin B to cdk1/cdc2. Dashed line represents the time period that the cyclin (circles) and cyclin dependent kinases (squares) are involved.

In addition to the process of cyclin binding, methods for regulating cdk activity also exist. It has been shown that the activity of kinases is able to be both positively and negatively regulated through the phosphorylation of cdk subunits *(Arellano and Moreno 1997).* In has also become increasingly apparent that ubiquitin-mediated proteolysis plays a critical role in the control of the cell cycle. This is made possible through the targeted destruction of cyclins and other regulators at key times during cell cycle progression *(King et al 1996, Pagano 1997)*. The one way directionality of the cell cycle is provided by the irreversibility of proteolysis. Association with two families of proteins, the cdk inhibitors (CKI), is also an important level of cdk regulation. Interestingly, some CKIs appear to positively regulate the cell cycle by functioning as assembly factors for cyclin D/cdk complexes *(LaBaer et al 1997).*

The D-type cyclins are the first to be induced during the G0 phase, stimulating the cell to enter the cell cycle *(Sherr 1994)*. The levels of D-type cyclins do not oscillate during the cell cycle, unlike several of the other cyclins; instead their levels are controlled by the presence of growth factors. D-type cyclins are able to associate with and activate cdk4 and cdk6. It has previously been demonstrated that the D1, D2, and D3 cyclins are, for the most part, functionally redundant, however each has unique tissue specific functions *(Sicinski et al 1995)*. Research has also shown that the primary substrate for D-type cyclin kinases is the retinoblastoma tumour suppressor protein (Rb). Studies of cells lacking Rb have shown that they do not require the activity of D-type cyclin kinases in order to undergo cycle progression (Lukas et al 1995). D-type cyclins, as well as functioning as cdk4 and cdk6 regulatory subunits, also play an additional role in the targeting of Rb and Rb-related proteins for

phosphorylation through direct protein to protein interaction *(Dowdy et al 1993, Ewen et al 1993, Kato et al 1993)*.

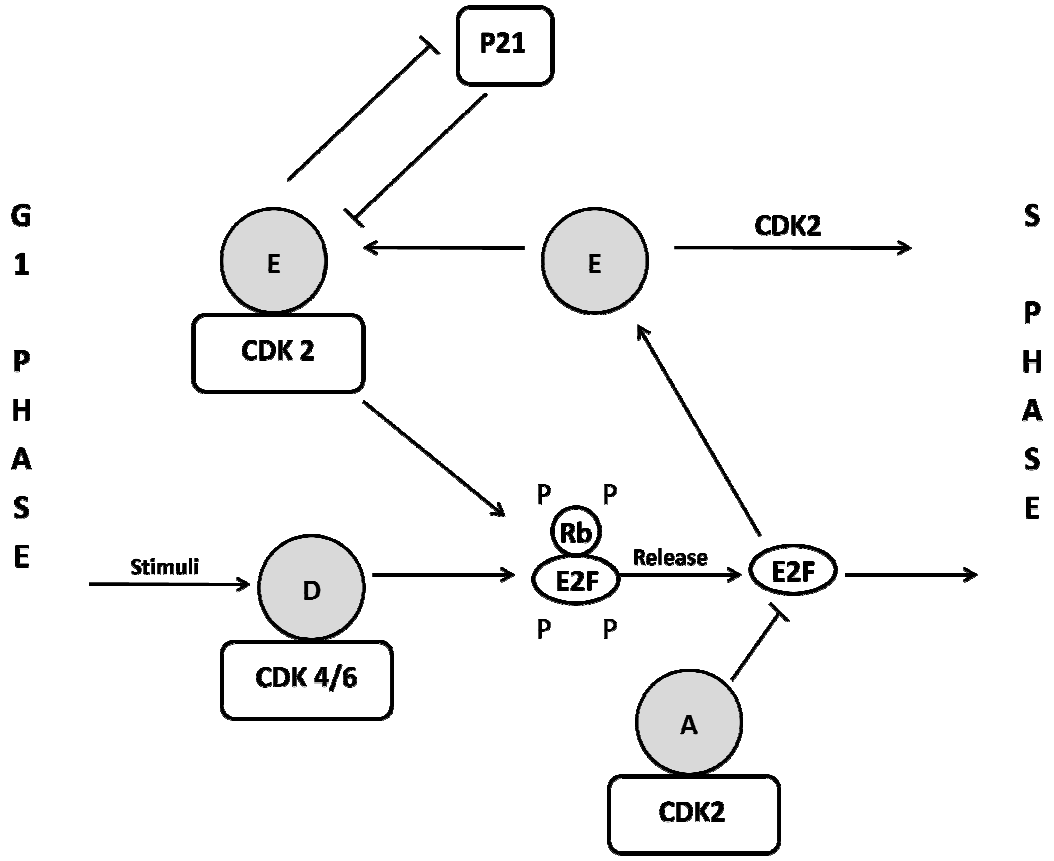
The Rb protein also plays a crucial role in the regulation of G1 phase progression. It is able to bind to and regulate multiple cellular proteins, including members of the E2F family of transcription factors *(Johnson and Schneider-Broussard 1998),* which have been shown to be involved in the regulation of the expression of many genes encoding proteins involved in the cell cycle progression and DNA synthesis, such as cyclins E and A, cdc2 cdk1, B-myb, dihydrofolate reductase, thymidine kinase, and DNA polymerase α. The binding of Rb to E2F results in the inhibition of the transcriptional activation capacity of E2F's and in some cases, changes their functionality from transcriptional activators to transcriptional repressors. Rb may become dissociated from E2F through its phosphorylation by D-type cyclin kinases resulting in the expression of E2F regulated genes (figure 1.5). Once E2F is activated, it is cyclin E that is next to be induced as the cell progresses through the G1 phase of the cell cycle *(Geng et al 1996, Ohtani et al 1995)*.

In order for the cell to progress from the G1 to S phase of the cell cycle cyclin E is required to associate with cdk2, resulting in the formation of a kinase complex *(Ohtsubo et al 1995)*. This complex has been shown to participate in the maintenance of Rb in a hyper-phosphorylated state *(Hinds et al 1992)* thereby providing a positive feedback loop allowing the accumulation of active E2F (figure 1.5). Unlike the D-type cyclins, it has been shown that the activity of cyclin E kinase is still required even in cells lacking Rb. This would seem to indicate the existence of additional substrates for cyclin E/cdk2 *(Ohtsubo et al 1995)*. As with many cyclin/cdk

complexes the cyclin E/cdk2 complex is able to phosphorylate histone H1, which may play an important role in the process of chromatin rearrangement which is required during the replication of the genome.

Another cyclin which is also partially regulated by E2F is cyclin A *(Schulze et al 1995)*. During the G1/S phase transition cyclin A accumulates and then persists throughout S phase. Initially it associates with cdk2 however in late S phase it also associates with cdk1. Cyclin A-associated kinase activity is required for both entry and completion of S phase as well as entry into the M phase of the cell cycle *(Girard et al 1991, Lehner and O'Farrell 1989, Walker and Maller 1991)*. Cyclin A has also been found to co-localize with DNA replication sites, indicating that it may be an active participant in DNA synthesis or have a role in preventing excessive DNA replication. Cyclin A is also able to negatively regulate some members of the E2F family; for example, E2F1, E2F2, and E2F3 all contain domains that are able to bind cyclin A, thereby enabling cyclin A-associated kinases to phosphorylate the E2F heterodimerisation partner DP1. This results in the inhibition of E2F DNA-binding activity (figure 1.5). Unlike cyclin E which as discussed earlier positively regulates E2F activity, cyclin A therefore participates in a negative feedback loop for E2F regulation. The G2 checkpoint responds to DNA damage by inducing a pause in the cell cycle that allows for DNA repair prior to the cell entering mitosis. The M phase of the cell cycle is regulated by cdk1 which is able to associate with the cyclins A, B1, and B2 *(King et al 1994)* to form complexes which are then able to phosphorylate a number of proteins including lamins, histone H1, and possibly components of the mitotic spindle.

Figure 1.5. The G1/S cell cycle checkpoint



Two cell cycle kinases, CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes Rb and E2F are pivotal in controlling this checkpoint. During G1 phase, the Rb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting the downstream transcription. Phosphorylation of Rb by CDK4/6 and CDK2 dissociates the Rb-repressor complex, permitting transcription of S-phase genes encoding for proteins that amplify the G1 to S phase switch and that are required for DNA replication. Many different stimuli exert checkpoint control including TGFb, DNA damage, contact inhibition, replicative senescence, and growth factor withdrawal. Cell cycle progression may be halted in the G1 phase of the cell cycle through the expression of cyclin dependent kinase inhibitors such as $p21^{CIP1}$.

In addition to transcriptional regulation, degradation of the activating cyclins and cdk inhibitors also plays a key role in the regulation of cdks. Cyclins responsible for the G1/S phase transition (D and E) have a relatively short life span in comparison to the cyclins responsible for G2/M progression (A and B), which are far more stable and only specifically targeted for destruction during mitosis. The cyclins A, B and E are degraded via ubiquitination which involves the post-translational modification of a protein by the covalent attachment via an isopeptide bond of one or more ubiquitin monomers. cdk34 is responsible for the expression of an ubiquitin conjugating enzyme that promotes the degradation of G1 phase cyclins and is therefore essential for the initiation of DNA replication *(King et al 1996).* It has previously been shown *(Yew and Kirschner 1997)* that the initiation of DNA replication in *Xenopus* embryos by cdc34 may facilitate the degradation of cyclin E/cdk2 inhibitor via the ubiquitin proteasome pathway. Cyclin E is also degraded via the ubiquitin pathway, but only when it is auto-phosphorylated through its association with cdk2 on Thr-380, thereby providing a specialized mechanism for the regulation of its own half life *(Won and Reed 1996).*

In contrast cyclin B degradation is mediated by a large multi protein complex known as the anaphase promoting complex (APC), or cyclosome that is able to polyunbiqitise cyclin B and other proteins, thereby increasing their sensitivity to proteasome degradation. APC becomes active via phosphorylation in a cell cycle dependent manner and its activity is regulated by the mitotic checkpoint protein MAD2 which when activated blocks the activity of the ubiquitin liagase *(Li et al 1998).* The targets of APC mediated degradation contain a specific nine amino acid sequence known as the destruction box. The above examples indicate that the

proteolysis of specific proteins maybe controlled via cell cycle regulated phosphorylation of the target proteins, by increasing their sensitivity to cdc34 mediated ubiquitination or by the cell cycle regulated activation of ubiquitination machinery, which then enables the degradation of the target proteins.

1.4.1. The p21 family of cdk inhibitors

There are two families of cyclin dependent kinase inhibitors (CKI), known as the INK4 inhibitors and the Cip/Kip inhibitors. The INK4 family specifically inhibits Cdk4 and Cdk6 activity during the G1 phase of the cell cycle, while the Cip/Kip family ($p21^{Waf1/CIP1}$, $p27^{Kip1}$, $p57^{Kip2}$) can inhibit cdk activity during all phases of the cell cycle *(Pietenpol and Stewart 2002)*. p21 was initially identified through its ability to interact with cdk2, however it has since been shown that members of this family are able inhibit a broad range of cyclin/Cdk complexes that include cdk1, 2, 4, 6 and cyclins A, B, D or E, but appear to show a preference for those containing Cdk2 (figure 1.5) *(Nakayama 1998, Dotto 2000)*. In addition p21 is also able to interact with proliferating cell nuclear antigen (PCNA), an elongation factor for DNA polymerase δ, as well as a component of the DNA repair machinery *(Fussenegger and Bailey 1998)*. The binding of p21 is able to inhibit the ability of PCNA to function in DNA replication but not DNA repair.

The activity of this family of inhibitors is controlled at several levels; however the relatively constant levels of p27 mRNA as well as its rate of translation during the cell cycle would seem to suggest that the p27 protein is the main target for regulation. Several studies *(Morisaki et al 1997 and Pagano et al 1995)* have demonstrated the fact that the phosphorylation of Thr-187 in p27 by cyclin E/cdk2 during late G1 phase

sensitizes the protein to proteolysis. The suppression of cyclin D/cdk4, 6 or cyclin E/cdk2 ability to phosphorylate proteins responsible for the establishment of S phase prevents the cell from exiting the G1 phase of the cell cycle. Progression through S phase may also be blocked through the over expression of p21, most likely as a result of suppression of cyclin A,E/cdk mediated phosphorylation of the proteins required for replication of the cell's DNA. p21 and in some cases p27 can also arrest cells in the G2 phase of the cell cycle presumably through the inhibition of cyclin A/cdk2 *(Liu et al 2003)*.

p21 expression is induced by the growth factors that control the Ras- Raf- MEK-MAPK pathway suggesting that p21 may play a positive role in the progression of the cell cycle. It has been suggested *(Liu et al 1996)* that at low concentrations p21 facilitates cellular proliferation by promoting the assembly of p21/cyclin/cdk/PCNA complexes, whereas at high concentrations such as those invoked by cellular stress; it becomes inhibitory as the excess p21 molecules smother the complex. This is in agreement with *(Zhang et al 1994)* who found that p21/cyclin/cdk/PCNA complexes can be found in both catalytically active and inactive complexes in untransformed cells; however inactive complexes were shown to contain significantly more p21 subunits.

Although p21 has been shown to exert both pro and anti-apoptotic effects such effects are not fully understood *(Liu et al 2003)*. It has been suggested that through the binding of p21 to either procaspase 3 or 8, apoptosis signal-regulating kinase 1 (ASK1) or any of the cyclin complexes it is able to inhibit apoptosis. In contrast the binding of p21 to caspase 3 may well induce apoptosis *(Gartel and Tyner 2002)*.

Interleukins -6 and -4 have also been implicated in the prevention of apoptosis through the control of p21 *(Gartel and Tyner 2002)*, resulting in cell cycle arrest instead.

In order to function correctly and prevent degradation p21 requires stabilisation. Some of the stabilization partners include PCNA as mentioned earlier, but also chaperones like WISp39 and Hsp90. Without stabilisation the over-expression of p21 has a limited effect on both cell cycle arrest and the induction of apoptosis *(Liu and Lozano 2005)*. C/EBPα is also reported to be able to stabilise p21 during its interaction with the cdk2 complex in both quiescent hepatocytes and in differentiated adipocytes *(Timchenko et al 1997)* as well as in cultured cells *(Harris et al 2001)*. p21 can also inhibit transcription factors like E2F, thereby blocking the transcription of growth-related genes *(Coqueret 2003, Dotto 2000)*. In contrast the unregulated activity of E2F transcription factors have been shown to induce the over expression of p21, resulting in S phase cell cycle arrest *(Radhakrishnan et al 2004)*. Based on the current literature it would appear that the role the p21 protein takes is very much dependent on its location *(Coqueret 2003)*.

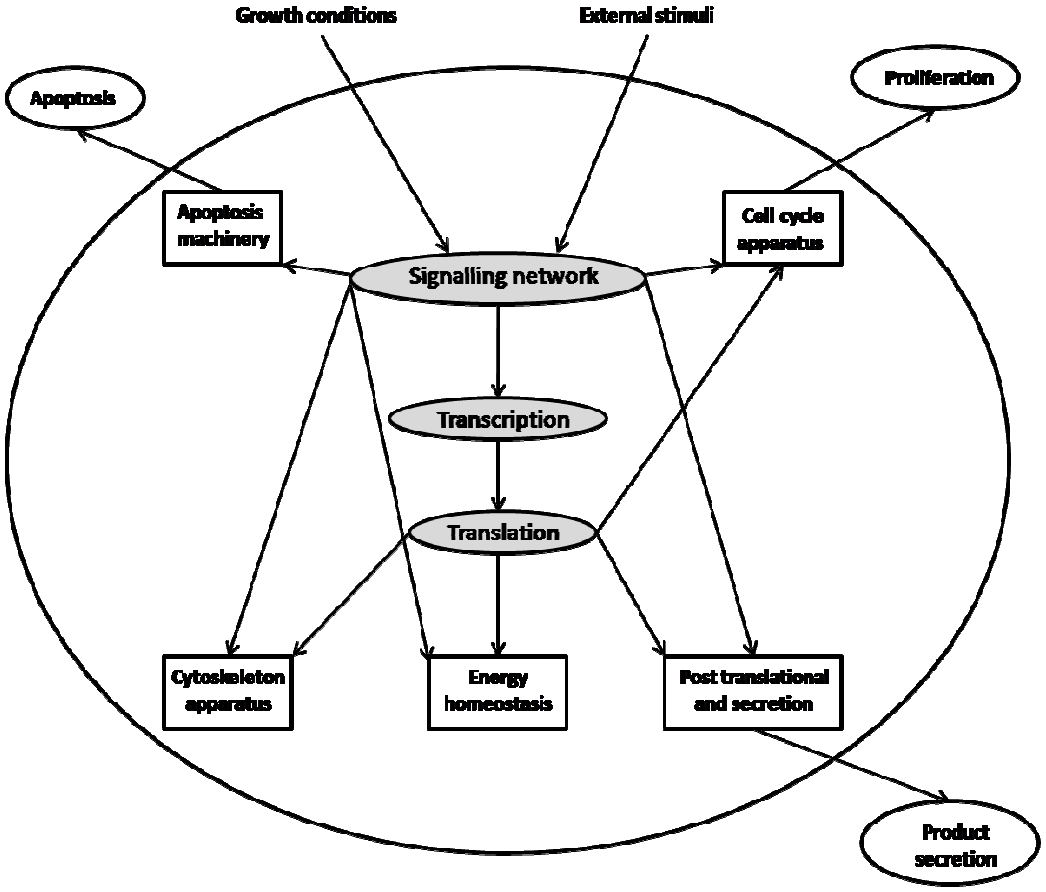
1.5. The control of the cell cycle for enhanced productivity

Control over the cell cycle and thus cell proliferation has long been employed by the biotechnology industry as a method to increase productivity. Unlike the variation in productivity observed between clones, described earlier in this chapter, controlled proliferation methods appear to increase cellular productivity in response to stimuli. The hypothesis behind this concept is based on the idea that slow growing or

proliferation arrested cells are able to devote their metabolic resources and protein synthesis machinery to the production of the desired recombinant protein as opposed to cell division *(Fussenegger and Betenbaugh 2002).* The prevention of cell proliferation in cultures of optimal density has also been shown to reduce nutrient limitation, the accumulation of toxic by-products and cell lysis, as well as potentially reducing product contamination from cellular debris *(Fussenegger et al 1998)*. This has the added effect of improving overall culture viability and duration. To date the debate over which particular phase of the cell cycle is most productive continues although several studies have implicated the G1 and G2 phases as being periods of high productivity *(Carvalhal et al 2003)*.

Several factors can affect productivity, including changes in cell morphology (*Lloyd et al 2000, Mazur et al 1999, Ryu et al 2001)*, clonal variation due to the selection process or gene copy number *(Ryu et al 2000, Kim et al 2000a, Ryu et al 2001, Meents et al 2002),* the cell-line used and the protein being produced *(Yoon et al 2003, Carvalhal et al 2003)* as well as the type of promoter selected *(Fussenegger et al 1997, Carvalhal et al 2003, Lloyd et al 2000, Meents et al 2002, Gu et al 1996, Banik et al 1996)* (figure 1.6). In recent years it has been shown that the prevention of cellular proliferation alone is not directly responsible for the observed increases in productivity. It is possible for proliferation arrest to occur naturally during culture conditions following nutrient and serum depletion. *(Yuk et al 2002, Kim et al 2000b, Sanfeliu et al 2000, Seifert and Phillips 1999, Carvalhal et al 2003)*. During these periods productivity is often adversely affected as media conditions play a vital role in determining productivity levels. *(Lloyd et al 1999, Sanfeliu et al 2000)*.

Figure 1.6. A simplified diagram of the key processes that occur within a production cell-line.



Simplified diagram of the key processes that occur in a production cell-line following stimulation via external stimuli or changes in growth conditions. These signals are processed by the cells signalling network resulting in either cell proliferation or apoptosis as well as product secretion.

In order to improve the productivity of industrially important cell-lines several strategies have been developed, which generally fall into one of three categories, environmental, chemical or genetic. Environmental factors that are able to affect cell proliferation are well documented and include the depletion of essential culture medium nutrients such as glucose, glutamine and serum *(Yuk et al 2002, Lloyd et al 1999, Sanfeliu and Stephanopoulos 1999, Sanfeliu et al 2000)*, the accumulation of toxic by-products such lactate and ammonia *(Yang and Butler 2000)*, changes in temperature *(Jenkins and Hovey 1993)*, and cell-cell contact inhibition *(Modiano et al 1999)*. Although such changes directly affect the rate of proliferation the effect of such conditions on productivity is far from favourable. The analysis of which elements become depleted during periods of high productivity has however helped improve productivity by enabling the development of feed strategies. This enables the depleted nutrients that are essential for improved productivity to be replaced, thereby lengthening the period of production.

More direct environmental methods include changing the growth medium osmolarity. During the culture period the osmolarity of the growth medium naturally increases due to the secretion of by products by the proliferating cells. There has to date been several studies into the effect of increasing or decreasing the osmolarity of the culture media on productivity. One of the most successful of these approaches has been to increase the osmolarity (hyperosmolarity) through the addition of low cost salts such as sodium chloride. *(Kim et al 2000a, Oh et al 1993, Oh et al 1995, Ryu et al 2000, Ryu et al 2001, Takagi et al 2001)*. The exact mechanisms through which these changes are able to increase productivity remains unclear, but has been shown to result in a depression of the rate of cellular proliferation *(Oyaas et al 1994, Lee and*

Park 1995). One possible explanation suggested for this is an increase in recombinant protein mRNA levels *(Lee and Lee 2000, Lee and Lee 2001, Ryu et al 2001)*, but at higher levels of osmolarity (e.g.542mOsm/kg) the enhancement of translation and post-translational processes may also play an important role *(Ryu et al 2001)*. In contrast other studies *(Sun et al 2004)* have shown that increases in mRNA levels may not be responsible for increased productivity and that increased translation rates together with a decrease in the transit time through the endoplasmic reticulum and golgi body may well be the responsible for the observed changes in productivity.

More recently *(McNeeley et al 2005)* examined stained antibody expressing hybridoma cells over a period of 50 hours, in order to examine the effects of osmotic shock on productivity. The results seem to show an initial decrease in intracellular antibody concentration during the exponential phase followed by an increase in comparison to the control culture. This occurred regardless of cell cycle phase and when normalised to the increase in cell size observed in the osmotically shocked cells, was found to be significant. No apparent relationship could be found between the intracellular antibody concentration and the rate of secretion. In another study of osmotically shocked hybridoma cells *(Sun et al 2004)* the analysis was extended to include translational and posttranslational processing elements. It was found that osmotically shocked cells contained both more total RNA and mRNA throughout the culture period than the control culture; however when this increase was normalised with cell number it was determined to be relatively small. It was therefore determined that the levels of mRNA do not significantly contribute to the observed increase in productivity. Examination of the osmotically shocked cells on a per cell basis revealed an increase in translation within 8 hours of induction that continued for 84

hours following the initial increase in osmolarity. This was found to result in a significant increase in both the intracellular protein concentration and productivity. Interestingly this study also found that ER chaperones such as BiP, GRP and PDI did not change significantly during periods of osmotic shock indicating that they do not play a crucial role in improving productivity. It would appear that exposure of the cells to osmotic shock allows them to become more efficient in their utilisation of these chaperones during the process of antibody assembly.

Based on the current literature it would therefore appear that there are at least four possible mechanisms for the observed increase in productivity that results from periods of osmotic shock. These include transcriptional activation due to changes to the chromatin structure *(Lee and Lee 2000, Ryu and Lee 1999)*; induction of chaperone proteins that are directly involved in protein processing and secretion *(Lee 1992)*; enhanced transport of nutrients into the cell *(Oh et al 1993, Oh et al 1995)* and increased ATP production rates *(Lin et al 1999)*. There is also the possibility that cells may accumulate within the G1 phase of the cell cycle. G1 has been previously been observed to be an optimal environment for increased production levels to occur *(Fussenegger et al 1998, Fussenegger and Bailey 1999)* however in this case this final hypothesis may be the least reliable as evidence exists for cells accumulating in the S phase of the cell cycle during hyperosmotic conditions *(Yi et al 2004, Sun et al 2004).*

The effect of medium osmolarity on culture viability and thus productivity has also been extensively studied as a drop in viability would seriously limit the use of hyperosmolarity media in biphasic cultures. The results of these studies vary; for

example *(Carvalhal et al 2003)* demonstrated a decrease in viability that was dose dependent, where as other studies have found no significant decrease *(Sun et al 2004)*. In a similar study on BHK cells, it was found that increasing the osmolarity up to 470 mOsm/kg did not increase the rate of apoptosis *(Yi et al 2004)*, though *(de Zengotita et al 2002*) reported an increase in apoptosis due to elevated osmolarity. This would seem to suggest that effect of medium osmolarity and therefore the potential to increase productivity is very much dependent on the cell-line used *(Ryu et al 2000)* and the gene copy number *(Ryu et al 2001)*.

In another study on several CHO clones with differing recombinant gene copy numbers *(Ryu et al 2001),* it was found that although all clones tested were affected by hyperosmotic conditions, resulting in a reduction in the rate of proliferation and increased productivity, the degree to which proliferation was arrested and productivity increased varied from clone to clone. The results demonstrate that the clone with the highest copy number was most susceptible to the effects of medium osmolarity. In order for osmolarity to be considered as a real strategy for use in the industrial scale production of recombinant proteins, it is essential that its effect on product quality and fidelity is fully understood. To date there have been very few studies in this area however a report published in 1999 *(Cherlet and Marc 1999)* demonstrated that a gradual increase in osmolarity during continuous culture resulted in an increase in the amount of defective antibodies produced. It has also been suggested that the potential exists to utilise hyperosmolarity to reduce the aggregation of proteins such as monoclonal antibodies, to better facilitate their separation and purification *(Franco et al 1999)*. In an alternate study *(Lin et al 1999)* it was found that the adaptation time of hybridoma cells during hyperosmotic conditions was 6 hours and that by increasing

the osmolarity gradually during the period of investigation it was possible to achieve a higher growth rate and thus productivity. In a similar study of Bcl-2 transfected hybridomas it was found that while it was impossible to adapt the control culture to 400mOsm, the Bcl-2 transfected cells were able to withstand the osmotic stress, resulting in long term adaptation to a high salt environment. In addition when grown in a high osmolarity medium, it was found that the productivity of the Bcl-2 transfected cells was 100% higher than that of non adapted Bcl-2 transfected cells grown in normal osmolarity medium.

Another environmental strategy which has been the subject of much investigation is the effect of changes in culture temperature away from the perceived optimal 37^0C. Initial studies of the effects of mild increases in temperature; for the most part have been unsuccessful. Although such changes do result in growth arrest, in a number of instances this may also result in an increase in glucose metabolism as well as a reduction in both viability and productivity *(Fussenegger and Bailey 1999).* In contrast the effect of lowering cell culture temperature has proven to be a quite successful strategy for reducing cell proliferation rates and improving productivity *(Furukawa and Ohsuye 1999, Schatz et al 2003, Bollati-Fogolin et al 2005)*. Evidence exists however which would seem to suggest that these observations may be cell-line, or product` specific as in some studies no effect on either proliferation or productivity was observed *(Yoon et al 2003 and 2006, Barnabé and Butler 1994, Ryll et al 2000)*.

Several studies on CHO cell-lines have demonstrated a positive effect on productivity during periods of growth at colder than usual temperatures. One example of this is a

study of 12 CHO clones subjected to low (32^0C) culture conditions, all the clones tested showed both a reduction in proliferation and an increase in productivity; however following amplification with MTX several clones showed no further enhancement to their productivities *(Yoon et al 2003)*. Other studies have also demonstrated variation in the temperature at which proliferation rate is affected for example analysis of CHO cells grown at temperatures ranging from 30^0C to 37^0C found that only temperatures below 35^0C resulted in a reduction in the rate of proliferation *(Furukawa and Ohsuye 1999)*. In contrast another study on CHO cells found no reduction in proliferation rates at temperatures above 32^0C *(Ducommun et al 2002)* and when hybridoma cells are exposed to similar conditions it has been found that rate of productivity either decreases or remains unchanged *(Al-Fageeh et al 2006)*.

Possible explanations for the ability of mild hypothermia to arrest cell proliferation include the over expression of cold-shock proteins (CSPs) such as cold-inducible RNA-binding protein (CIRP) *(Nishiyama et al 1997, Sonna et al 2002)*, and RBM3 *(Danno et al 2000)*. In addition several studies have also suggested that the observed reduction in cell proliferation occurs as a result of p53-dependent cell cycle arrest, in which cells accumulate within the highly productive G1 phase of the cell cycle *(Fussenegger and Bailey 1999)*. It is interesting to note that over-expression of CIRP at 37^0C results in the accumulation of cells in G1 phase suggesting that arrest at 32^0C is an active process. Although G1 phase has been correlated with enhanced productivity, additional studies have shown other phases of the cell cycle are preferable for recombinant protein production. In fact, *(Fox et al 2005)* have suggested that the S-phase of the cell cycle is preferable for recombinant protein

production in CHO cells. In their study it was demonstrated that when CHO cells were grown at 32^0C, increases in specific productivity directly correlated to the percentage of cells in the S phase of the cell cycle, suggesting that arrest in the G1 phase is not the only factor involved in enhanced productivity during reduced temperature cultivation of CHO cells.

Alternate studies have shown that when the temperature at which CHO cells are cultured is dropped from 37 to 30^0C, up to 87% of cells undergo proliferation arrest at the G1 phase of the cell cycle resulting in a significant delay in the onset of apoptosis *(Moore et al 1997).* Interestingly it has also been reported *(Zhang et al 2001)* that the expression of the anti-apoptotic protein Bcl-2 is enhanced in hippocampal neurons in response to growth at 33^0C. During periods of cultivation at 30^0C it has also been shown *(Moore et al 1997)* that the rate of both oxygen uptake and ATP consumption in CHO cells are reduced by up to 50%. It is therefore quite possible that the observed delay in the onset of apoptosis and its associated increase in productivity during periods of mild hypothermia are partially due to a reduction in cellular metabolism. Despite the emergence of several hypotheses to the mechanisms through which hypothermia is able to induce proliferation arrest, the mechanisms by which productivity is enhanced remain less clear.

There are a number of factors that contribute to increased recombinant protein productivity at reduced temperatures other than the ability to improve culture longevity. Recent studies have reported increases in both total RNA (*Borth et al 1992)* and recombinant mRNA levels *(Furukawa and Ohsuye 1999, Yoon et al 2003)* during growth at reduced temperatures. In a study of CHO cells producing recombinant

human IFN-y (interferon-y), it was found that during periods of growth at lower temperatures productivity increased due to higher recombinant IFN-y mRNA levels *(Fox et al 2005)*. It was also determined that product gene transcription rates increase and that product mRNA stability when normalised with actin mRNA stability, used as a housekeeping gene, were also changed to a similar extent. To date research into the effects of hypothermic growth conditions would seem to suggest that at reduced temperatures either transcription of the recombinant gene of interest is enhanced, or that mRNA stability of these transcripts is increased. This observation could provide, at least partially, an explanation as to why increased recombinant protein production is observed at reduced temperature cultivation.

An alternative to the environmental methods described above is the addition of chemicals or additives to the culture medium that are able to halt cell proliferation. During the early 1990s DNA synthesis inhibitors (thymidine, hydroxyurea, TGF-β and adriamycin) *(Suzuki and Ollis 1990)* selective protein synthesis inhibitors (potassium acetate and cycloheximide) and the RNA synthesis inhibitor actinomycin D *(Suzuki and Oliss 1990)* along with several other chemical additives (aphidicolin, rapamycin, doxorubicin, staurosporine, mimosine and nocodazole) *(Carvalhal et al 2003, Grosjean et al 2002)* underwent trials for their effects on cell proliferation and cell viability. In most cases the addition of these chemicals has been found to result in cell cycle arrest within a specific phase of the cell cycle dependent on the chemical used, but it has also been shown that culture viability can also be adversely affected dependent upon the type and concentration of chemical used *(Fussenegger and Bailey 1999)*. As a result of both their affect on culture viability as well as issues with the

downstream processing and purification process these chemicals are generally considered unsuitable for industrial application.

One chemical which is commonly used in the biopharmaceutical industry is sodium butyrate *(Fussenegger and Bailey 1999)*. It has the ability to not only arrest cell proliferation in a variety of mammalian cell-lines, but also elicits an increase in the production of a wide variety of industrially important proteins including tissue-type plasminogen activator (t-PA) *(Arts and Kooistra 1995, Kooistra et al 1987)*, follicle stimulating hormone (FSH) *(Gebert and Gray 1995)* and monoclonal antibodies *(Chevalot et al 1995, Mimura et al 2001, Oh et al 1993)*. Sodium butyrate has previously been shown to be capable of inducing a number of changes within the nucleus of the cell, including hyperacetylation and DNA methylation *(Fussenegger and Bailey 1999)* increasing transcription and resulting in p53-independent induction of p21 *(Kijima et al 1993, Nakano et al 1997)*. Sodium butyrate itself is known to be cytotoxic at higher concentrations which may well result in a decrease in culture viability. In a recent study of recombinant CHO cells expressing human thrombopoietin (hTPO), grown as serum-free suspension cultures; it was found that productivity could be enhanced through induced proliferation arrest as a result of sodium butyrate addition. During exposure to sodium butyrate it was noted that cells underwent apoptosis in a dose-dependent manner. Through the expression of the anti-apoptotic gene Bcl-2 it was possible to prevent apoptosis and extend culture longevity whilst enabling productivity to be enhanced 10/fold in comparison to non-Bcl-2-expressing cultures *(Sung and Lee 2005)*.

More recently several groups have examined the potential for the use of extracellular nucleotides, nucleosides and bases as proliferation arresting agents. Although natural adenosine derivatives like NADH, NAD, ATP, ADP, AMP and adenosine have previously been shown to inhibit the proliferation of normal and tumour cells *in vitro (Wagner 1997),* in a study of CHO cells by Carvalhal and co-workers *(Carvalhal et al 2003)* they were able to demonstrate the ability of these compounds to enhance productivity. In this study it was found that adenosine 5'-monophosphate (AMP) was able to produce the greatest increase specific productivity as a result of proliferation arrest in the S phase of the cell cycle. Although not clear one possible mechanism for the effect of nucleotides on proliferation and productivity is that during their addition the nucleotides are taken into the cell and phosphorylated by adenosine kinase to AMP which primarily inhibits the 5 Phosphoriboxy-1-diphosphate-synthetase. As a result, the nucleotide pool, with the exception of AMP, ADP and ATP, decreases potentially leading to a reduction in cell growth *(Wagner 1997).* An important consideration when using such natural compounds is that their effect depends on the medium that is used since some mediums include certain amount of the nucleotides, nucleosides and bases *(Carvalhal et al 2003).* The main advantage however of these compounds is that they are naturally synthesized by the cell and are therefore expected to be less toxic than other foreign compounds *(Carvalhal et al 2003)*.

The final approach taken in recent years is the use of recombinant DNA technology in order to produce cell-lines able to express cytostatic genes. The development of expression systems able to coordinated and regulate multiple genes *(Gòdia and Cairo 2002, Fussenegger and Bailey 1999)* has made it possible to in a sense switch on or off the expression of cytostatic genes. One of the best studied of the cytostatic genes

is p21. The biphasic induction of p21 is most commonly established through the use of either tetracycline repressible expression control (TetOFF) *(Fussenegger et al 1997, Sekiguchi and hunter 1998)* or the *lac operon* operator and repressor gene switch (LacSwitch) *(Ibarra et al 2003, Watanabe et al 2002)*. There is the potential for the use of other multicistronic expression systems *(Fussenegger 2001)*; however they are generally not as well documented through the current literature.

Several studies have demonstrated the ability of p21 over-expression to arrest cell proliferation in numerous industrially important cell-lines including BHK, CHO and NS0 *(Sekiguchi and Hunter 1998, Bi et al 2004, Watanabe et al 2002)*. During the over-expression of p21 cell proliferation becomes arrested at the G1 phase of the cell cycle and an increase in cell size is often observed *(Bi et al 2004)*. In order to achieve complete cell cycle arrest the level of p21 expression must be high. This is due to the fact that a single cyclin kinase requires more than one p21 molecule to inhibit its activity *(Sekiguchi and Hunter 1998)*. As a result only the sustained expression of p21 is able to result in long term proliferation arrest *(Watanabe et al 2002)*. When p21 expression is halted it has been shown that cells are quickly able to begin recycling through the cell cycle *(Bi et al 2004)*.

To date almost all of the published work on p21 have shown that during its over-expression the specific productivity of the cell is increased *(Watanabe et al 2002, Fussenegger et al 1997, Fussenegger et al 1998)*, with the exception of one study in which no change in protein production was observed *(Mazur et al 1998)*. The exact mechanisms through which productivity is increased remains unclear however it has

been suggested that an uncoupling of growth and proliferation occurs, resulting in the independent and continued growth of the proliferation-arrested cells. Similarly there is relatively little published material on the metabolic changes and protein quality during p21 induction, however in one study *(Bi et al 2004)* it was noted that metabolic activity, as determined through an MTT assay, increased 1.6/fold during p21-induced proliferation arrest and was in addition accompanied by an increase in mitochondrial mass. Interestingly the study also found an increase in S6 ribosomal protein expression in p21 expressing cultures. Since S6 protein plays a key role in ribosomal biogenesis this may partially explain the observed the increase in protein synthesis.

The effect on culture viability during periods of p21-induced cell cycle arrest seems to vary and may well be cell-line dependent. Several studies have shown that during p21-induced proliferation arrest the viability of the arrested cells does not decrease significantly *(Bi et al 2004)* whilst others have found a slight decrease *(Watanabe et al 2002)*. Despite this there is evidence to suggest that p21 may actually protect certain cell-lines from apoptosis *(Polyak et al 1996, Fussenegger et al 1998)*, although this appears to be limited to certain insults such as mitogen deprivation and prostaglandin A_2 *(Wang and Walsh 1996, Gorospe and Holbrook 1996)*

Like p21, biphasic induction of p27 may be achieved through the use of either the tetracycline repressible expression control (TetOFF) *(Fussenegger et al 1997, Kaufmann et al 2001, Mazur et al 1999)* or a ecdysone-inducible gene regulation system *(Meents et al 2002)*. Expression of p27 has also been shown to result in cell cycle arrest in the G1 phase of the cell cycle phase and as seen with p21 results in

both an increase in cell size *(Meents et al 2002, Mazur et al 1999)* and specific productivity *(Mazur et al 1998, Fussenegger et al 1997)*. In one study in which the ecdysone-inducible system was utilised , the productivity of p27-expressing cultures was observed to increase steadily until it was approximately 5/fold higher *(Meents et al 2002)*. Analysis of product quality has shown no change during periods of p27 expression, with both isoform patterns and sialylation ratios remaining similar to those seen in proliferating cells *(Kaufmann et al 2001)*.

Although viability has been shown to remain high in p27-expressing cultures *(Fussenegger et al 1997, Kaufmann et al 2001, Meents et al 2002, Mazur et al 1998)* the spontaneous formation of mutants that are able to escape from proliferation arrest has been shown to readily occur *(Mazur et al 1999)*. In an alternate study *(Fussenegger et al 1998)* was able to demonstrate that by over-expressing p27 together with bcl-x_L, the productivity of SEAP could be further increased by a factor of 3 from that observed during p27 over-expression alone. It was suggested that tetraploidy and the consequent amplification of genetic information following bcl-x_L over-expression could be responsible however it is clear that genetic methods of controlling proliferation have not been as well understood as other methods.

IRF-1 is a transcription factor that has previously been shown capable of inducing the transcription of several interferon-induced genes including the cell cycle-dependent kinase inhibitor p21, the translation-initiation factor 2 kinase PKR, the p200 family of transcription repressors, and 2.5-oligoadenylate synthetase, whose products activate the mRNA-degrading enzyme RNase L *(Kirchhoff et al 1993, Harada et al 1998)*.

This would seem to suggest that IRF-1-induced growth arrest results from the activity of multiple gene products. Previous studies have shown that the over-expression of recombinant IRF-1 results in the inhibition of cell proliferation, the extent of which is dependent on the intracellular concentration of active IRF-1. In order to achieve this in mammalian cell-lines fusion protein composed of IRF-1 and the hormone binding domain of the human estrogen receptor has been used. This system allows the gradual control of cell proliferation by adjusting the intracellular concentration of active IRF-1 via the addition of estradiol to the culture medium *(Kirchhoff et al 1996)*.

IRF-1-induced proliferation arrest has been shown to increase productivity in cell-lines expressing various monoclonal antibodies *(Geserick et al 2000)* as well as resulting in an increase in cell size and a dramatic change in cell morphology *(Carvalhal et al 2002)*. Unlike p21 and p27 the over expression of IRF-1 has been shown to lengthen all phases of the cell cycle, but decreased cell viability, possibly as a result of apoptosis, but more likely, due to necrosis *(Carvalhal et al 2001, Carvalhal et al 2002)*. The specific productivity of IRF-1 expressing BHK cells has been reported as being either unchanged or lower than the control cultures despite an increase in the total protein content per cell towards the end of the culture. This is likely to be due to an increase in intracellular protein synthesis, including higher proteolytic enzyme activity *(Kirchhoff et al 1996, Kirchhoff et al 1993, Carvalhal et al 2001, Müller et al 1999)*.

It has been reported that some cell-lines may be able to 'adapt' to the presence of estradiol, thereby releasing the cells from proliferation arrest *(Carvalhal et al 2001)*. In an attempt to overcome this problem it has been shown that by cycling the addition

of estradiol rather than continuously adding it to the growth medium it is possible to maintain proliferation arrest without the risk of cells becoming resistant to its effects *(Carvalhal et al 2001)*. This method may serve a dual purpose as long term exposure to estradiol and the subsequent arrest has been reported to result in a reduction in culture viability *(Carvalhal et al 2000)*. Despite this during the long term perfusion culture of IRF-1 inducible BHK cells it has been shown that it is possible to increase the production of antibody by 6/fold upon growth arrest, although the protein expression did decrease gradually over the 7 week culture period. In addition it was also found that these cells could not be induced further in subsequent growth-repression cycles *(Geserick et al 2000)*.

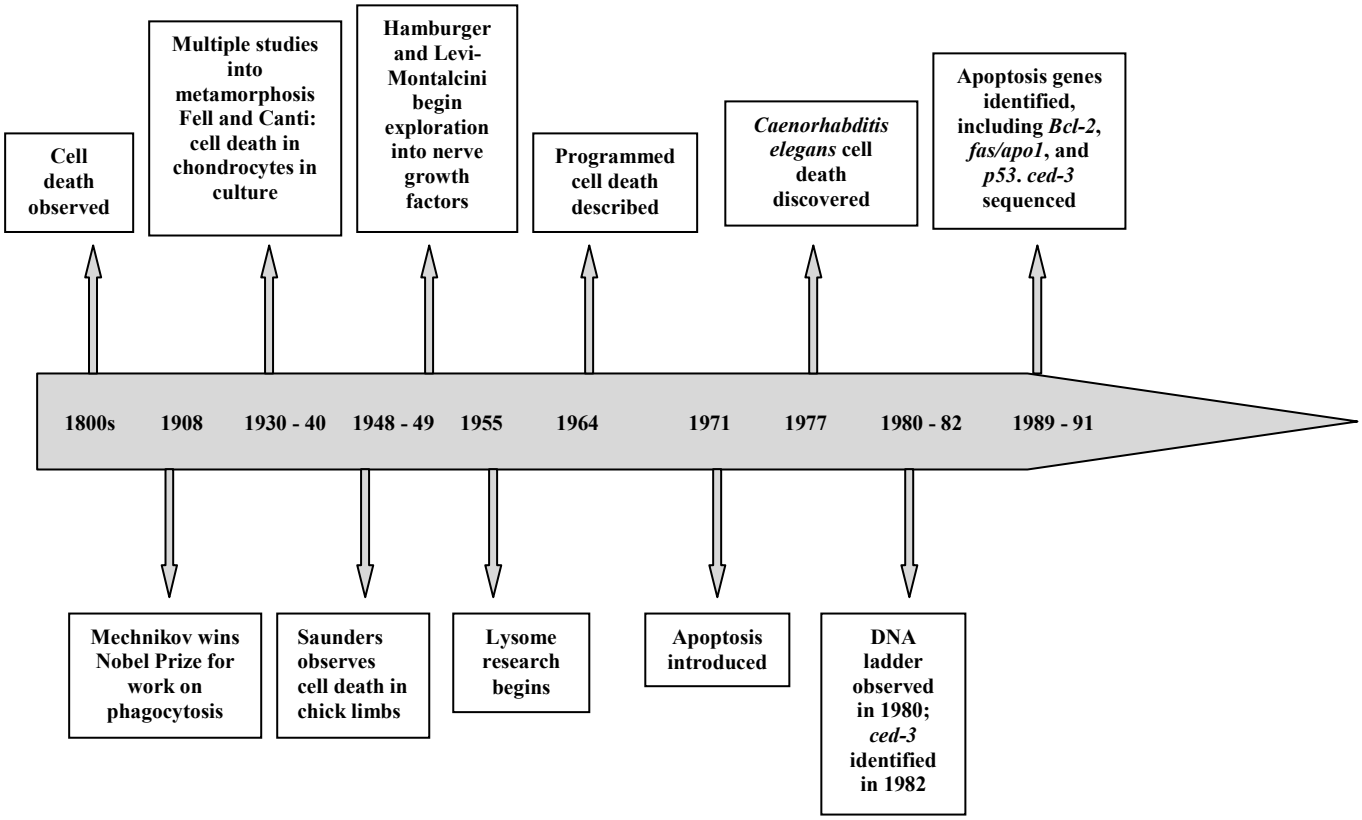
Upon IRF-1 induction there is a significant change in the metabolism of the cell. It has been shown that although the DNA content remains unchanged, the cell undergoes a significant increase in proteolytic enzyme activity, metabolic activity, as determined through a MTT assay, protein synthesis and mitochondrial activity *(Carvalhal et al 2002)*. These changes have been shown to result in an increased catabolic flux of glucose and glutamine with lower yields of ammonia and lactate *(Carvalhal et al 2002)*. In terms of protein quality, IRF-1 induction results in proteins with equivalent glycosylation patterns to that of the control as well as a slight increase in sialylation *(Mueller et al 2000)*. In summary` controlled proliferation either through the addition of chemical additives, environmental changes or genetic engineering, can be achieved in animal cell culture. Using the techniques described above, proliferation can be effectively controlled, and in numerous cases result in higher specific productivity of heterologous protein than the non-arrested controls

1.6. An overview of the development of research in the field of programmed cell death

The subject of cell death has been challenging scientists for almost 200 years (see figure 1.7 for summary). As early as 1842 the physiological existence of cell death had been identified and by the late nineteenth century several publications had began to emerge describing cell death during the development and metamorphosis of insects (*Clark and Clark 1996*). By the 1950s it had also been established that cell death was also able to occur in individual cells during vertebrate embryo development (*Glucksman 1951*). This eventually led to the discovery that cell death played a vital role in the sculpting of tissues during embryonic development.

It was not until the mid-twentieth century that the term programmed cell death (PCD) was really introduced as a term used to describe the observed process of cell death during development *(Lockshin and Zakeri 2001, Clarke and Clark 1996)*. Up until this point the terminology used had been confusing to most scientists and was used to describe different things by different research groups. Lockshins definition described a series of events that was predetermined and occurred as a specific series of events. In 1966 it was determined that PCD was not universal to all cell types and therefore not always necessary, instead it was shown that PCD could be activated by external stimuli such as through exposure from secreted hormones from a different cell type *(Saunders 1966)*. These findings ultimately lead to the discovery that cells are able to utilize the process of PCD to facilitate their own demise in response to unfavourable growth conditions.

Figure 1.7. A schematic diagram of key historic events in the field of cell death.



A basic timeline of the key discoveries in the field of cell death which have lead to the development of our understanding of the process of apoptosis.

As the subject of cell death grew and diversified within the biological fields a multitude of terms were put forward to describe PCD including necrosis, cell shrinkage and necrobiosis. In 1972 Kerr, Wyllie and Currie introduced the term “apoptosis” based on an ancient Greek word for the "falling off" of leaves from trees or petals from flowers, to describe a mechanism of controlled cell death, which appeared to have a complementary but opposite role to mitosis in the regulation of cellular proliferation *(Kerr et al 1972).* It was observed that during the process of apoptosis the cells followed a specific pattern of morphological changes that included

the shrinkage of the cell with little or no damage to organelles, coalescence and margination of chromatin, and the fragmentation of the cell and its nucleus. This led to the belief that apoptosis was the primary mechanism by which all cells die and are replaced within most normal tissues as well as being responsible for the destruction of cells, as necessary during embryonic development.

By the second half of the 1970s the increasing development of molecular genetics techniques made it possible to analyze the mechanisms through which cell death is controlled. The discovery of the complete cell lineage of *Caenorhabditis elegans (Kimble and Hirsh 1979, Sulston et al 1983)* made it possible to analyze cell destruction and formation in the developing *Caenorhabditis elegans* embryo, enabling John Sulston and Robert Horvitz to demonstrate that approximately 13% of somatic cells in the developing embryo die predictably at set time intervals *(Sulston and Horvitz 1977*). This provided the first model to allow the study of the genetic basis of cell death and by 1982 members of the Massachusetts Institute of Technology laboratory had successfully established the existence of genes that controlled essentially all the somatic cell deaths, including *ced-3 (Horvitz et al 1982, Hedgecock et al 1983, Horvitz et al 1983, Trent et al 1983, Ellis and Horvitz 1986, Horvitz 2003).*

During the 1990s interest into the subject of apoptosis surged, leading to several studies by research groups from various backgrounds. The identification of p53s function as a pro-apoptosis regulator by a research group headed by Elisheva Yonish-Rouach (*Yonish-Rouach et al 1991, Lowe et al 1993*) inspired several other groups to take up the search for inducers of apoptosis and eventually lead to the discovery of a similar function for c-Myc (*Buttyan et al 1988, Evan et al 1992*). At the same time

another group lead by David Vaux and his colleagues identified the B-cell lymphoma gene Bcl-2 as an anti-apoptosis gene (*Vaux et al 1988*) thus, bringing the subject of apoptosis into the field of immunology and leading to the aggressive pursuit of the identification of Bcl-2 and its family members by other research groups including that of Stanley Korsemeyer. *(Korsemeyer 1999).* The final key discovery was the identification of Fas/Apo-1 as a death-transducing cell-surface receptor (*Trauth et al 1989).* These discoveries began the avalanche that eventually lead to the unification of immunology, cell biology, embryology and neurobiology in the pursuit for and understanding into the mechanisms and regulation of the process of cell death.

1.7. Morphological features of apoptosis

Apoptotic cells all undergo several significant morphological changes that include the overall shrinkage of the cell, deformation and loss of its ability to make contact with neighbouring cells, chromatin condensation and margination of the nuclear membrane *(Majno and Joris 1995, Kressel and Groscurth 1994, Rao et al 1996, Liu et al 1998).* Blebbing or budding also occurs within the plasma membrane, ultimately leading to the fragmentation of the cell into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, condensed chromatin, and cellular organelles.

In order to promote their phagocytosis apoptotic cells often also undergo several plasma membrane changes that are able to trigger a response from macrophages, including the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Within living tissues the macrophages engulf the apoptotic bodies thus removing them without an inflammatory response. These morphological changes

occur as a direct result of molecular and biochemical events occurring from within the cell. These events include the activation of proteolytic enzymes which facilitate the cleavage of DNA into oligonucleosomal fragments. Proteolytic enzymes are also responsible for well the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles *(Hengartner 2001, Rosenblatt et al 2001, Grimsley and Ravichandran 2003)*.

The alternative to apoptosis is a process known as necrosis in which in response to a major insult there is a loss of membrane integrity, swelling and rupturing of the cellular membranes *(Leist and Jaattela 2001)*. Unlike during apoptosis, the cellular contents of necrotic cells are released in an uncontrolled manner, resulting in significant damage to neighbouring cells and the activation of a strong inflammatory response *(Shimizu et al 1996, Nicotera et al 1998)*.

1.8. The mechanisms of apoptosis

While it is relatively easy to distinguish morphologically apoptosis from necrosis there are some examples where the characterisation as either apoptotic of necrotic is not so clear cut (*Columbano 1995*) therefore an understanding into the biochemical pathways involved in both apoptosis and necrosis is key to understanding fully the processes through which cell death occurs.

Apoptosis is a highly complex and strictly regulated sequence of events that through the process of evolution has resulted in the formation of a strategy in which the components of the apoptotic machinery are compartmentalised within different cellular regions, to be brought together only in response to specific stimuli. Within

mammalian cells it is generally considered that there exist 3 major compartments in which the apoptotic machinery is housed. The first of these compartments include the cell membrane and cytosol. Following the initiation of apoptosis, the contact between neighbouring cells is lost. Experimentation on embryonic epithelia and cultured Madin-Darby canine kidney cells grown as monolayers has shown that dying cells are able to generate signals that activate actin- and myosin-dependent mechanisms in the surrounding cells *(Rosenblatt et al 2001)*. This in turn is able to facilitate the removal of dying cells from the epithelia and monolayer. The loss of microvilli results in the formation of plasma membrane protrusions and the cell shrinks. The plasma membrane protrusions then separate, forming apoptotic bodies densely packed with cellular organelles and nuclear fragments that may then be engulfed through phagocytosis *(Hengartner 2001)*.

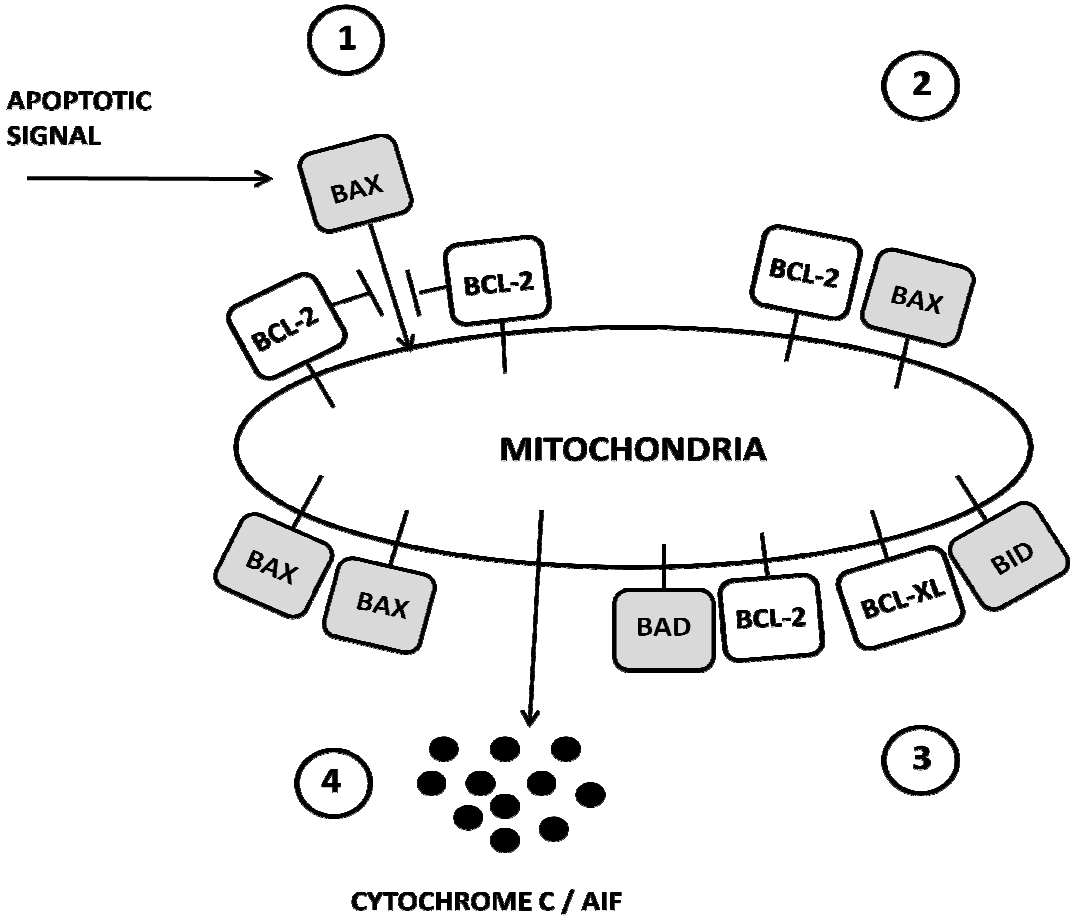
Following the initiation of the apoptotic cascades several structural proteins are processed including actin, *(Mashima et al 1997)* which results in the formation of microfilaments and thereby regulates the cells shape. Other proteins that are cleaved by caspases during apoptosis include spectrin, fodrin, ß-catenin, gelsolin, growth arrest-specific 2 (Gas2) and p21-activated kinase 2 (PAK2). These proteins have been shown to be involved in the maintenance, organization, and attachment of the cytoskeleton as well playing a role in cell-to-cell junctions *(Dejana 2004)*. The over-expression of cleaved gelsolin and Gas 2 has been shown to result in changes in cell shape that resemble those that occur during apoptosis *(Kook et al 2003)*. Similarly Death-associated protein kinase (DAP kinase) and DAP kinase-related kinase (DRP kinase) have recently shown to play a role in membrane blebbing *(Inbal et al 2002)*. Both these kinases function independently of caspase activity, but it has been shown

that activated DRP kinase is able to activate caspase-dependent events. In experiments involving Dominant-negative mutants of DAP and DRP kinase, reduced blebbing and, interestingly, reduced autophagy have both been shown to occur *(Inbal et al 2002)*.

The second compartment in which the apoptotic machinery is housed is the mitochondria. Changes including cellular stress and the cells bio-energetic state can lead to the initiation of apoptosis and are therefore continuously monitored by the mitochondria. Mitochondria are then able to integrate these multiple pro-apoptotic signals into common apoptotic degradation cascades, primarily through the permeabilization of the mitochondrial membrane *(Ferri and Kroemer 2001)*. The initiation of mitochondrial membrane permeabilization has been shown to be regulated through the activity of pro-apoptotic members of the Bcl-2 family *(Kroemer et al 2007, Chipuk and Green 2008)*, in contrast anti-apoptotic members of the Bcl-2 family have been shown to be able to inhibit this process as illustrated in figure 1.8 *(Armstrong 2006*, *Kroemer et al 2007*).

Permeation of the mitochondrial membrane has been shown to result in a large number of proteins being released into the cytosol, including cytochrome c, Apoptosis inducing factor (AIF) and Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO) *(Shi 2002, Ravagnan et al 2002)*. Cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), resulting in the formation of an Apaf-1 oligomer (the apoptosome) which in turn is able to bind and activate caspase-9 *(Adrain et al 2006)*. Smac/DIABLO also facilitates the activation of caspases through its binding to a family of proteins known as inhibitor of apoptosis proteins (IAP) *(Shiozaki and Shi 2004)*.

Figure 1.8. Representation of the possible interactions between anti-apoptotic and pro-apoptotic members of the Bcl-2 family



Representation of possible interactions between anti-apoptotic (white) and pro-apoptotic (grey) members of the Bcl-2 family at the outer mitochondrial membrane. Apoptotic signals relocate Bax from the cytoplasm to the mitochondrion (1). Anti-apoptotic members of the Bcl-2 family, such as Bcl-2 itself and Bcl-X_L can block the pro-apoptotic effects of Bax by binding it and forming heterodimers (2). However, other pro-apoptotic Bcl-2 proteins, e.g. BAD and BID, can interact with Bcl-2 and Bcl- X_L and prevent their anti-apoptotic function (3). Eventually, the relationship between pro-apoptotic and anti-apoptotic factors determines the cells susceptibility to apoptosis. If there are more pro-apoptotic factors, the mitochondria subsequently loses its membrane potential and a number of apoptosis- promoting molecules, such as cytochrome c and apoptosis-inducing factor (AIF) are released into the cytoplasm (4).

In contrast to the caspase-activating proteins AIF is able to translocate in to the nucleus where it is able to induce chromatin condensation and DNA fragmentation *(Sanges and Marigo 2006)*. Despite the significant changes that occur within the mitochondria following the initiation of apoptosis their appearance when viewed with either a light or electron microscope remains relatively unchanged until the later apoptosis, when they swell like other cell organelles.

Morphologically the hallmark features of apoptosis occur in the nucleus and include chromatin condensation and nuclear fragmentation. Microscopically chromatin condensation can be observed, beginning initially along the nuclear membrane, forming a crescent or ring like structure. In the later stages of apoptosis the nucleus condenses further until it finally breaks down *(Majno and Joris 1995)*. As well as chromatin condensation, it is also possible to detect DNA strand breaks through the use of terminal Deoxynucleotidyl Transferase and Nick Translation Assays *(Gorczya et al 1993*). These morphological changes may be initiated through the cleavage of various proteins by the caspases. Nuclear fragmentation is dependent on the disintegration of the nuclear lamina and the collapse of the nuclear envelope. The first of these events involves the proteolysis of lamins A, B and C by caspases *(Rao et al 1996)*. Experimentation involving the expression of mutant forms of lamins, resistant to caspase-mediated proteolysis have shown to be able to prevent the loss of laminar integrity, therefore delaying the onset of DNA fragmentation *(Rao et al 1996)*. Although several other nuclear membrane and laminar proteins have been identified as caspase substrates, none of these proteolytic events has so far been shown to result in nuclear fragmentation *(Lüthi and Martin 2007).*

Another major hallmark of apoptosis that occurs within the nucleus is the internucleosomal fragmentation of double-stranded DNA by a number of caspase substrates that are involved in DNA repair and replication *(Wyllie et al 1980, Kerr et al 1972)*. To date it still remains relatively unclear why the nucleus fragments and disperses throughout the cell body during apoptosis however this process may well contribute towards the efficient removal of potentially immunogenic chromatin, as well as making the process of cell death irreversible.

A cells actin cytoskeleton also plays an important role in nuclear fragmentation. The nuclear lamina is surrounded by a net of actin, which is also associated with the nuclear envelope. Inhibition of either Rho-associated, coiled-coil containing protein kinase 1 (ROCK1), myosin light chain kinase or the disruption of actin filaments have all been shown to prevent nuclear fragmentation *(Croft et al 2005)*. Caspase-mediated proteolysis results in the loss of the C terminus of ROCK1 which in turn activates this kinase resulting in the re-organization of the actin–myosin system *(Coleman et al 2001, Sebbagh et al 2001)*. Due to the fact that the actin cytoskeleton and the nuclear envelope are connected the nucleus becomes torn apart *(Croft et al 2005)*. The cleavage of lamin alone is not enough to cause nuclear fragmentation but is able to weaken the nuclear lamina, possibly resulting in the nuclear envelope tearing *(Croft et al 2005)*. During nuclear fragmentation the microtubule-based cytoskeleton has been shown to play a role in the dispersal of nuclear fragments into the resulting plasma membrane blebs *(Moss et al 2006)*. The endoplasmic reticulum (ER) has also been shown to be involved in apoptosis regulation and has been found to be the location of caspase 12, another apoptotic protease which may be activated in response to stress in

the ER such as the presence of high levels of intracellular Ca^{2+}, brefeldrin A or tunicamycin *(Shiraishi et al 2006)*.

1.8.1. Caspases

Within any given cell there are several forms of apoptotic executors, but by far the most important and well understood are the family of cysteine proteases known as caspases which function via the use of the sulphur atom in cysteine to cleave polypeptide chains. Caspases are not indiscriminate proteases; they have the ability to recognise specific sequences in their target proteins, typically cleaving solely next to aspartate amino acids (thus the name caspase "c" for cysteine protease and "asp" for the strong aspartate preference). All living cells contain caspases however the number and function may vary from species to species. Through the recent development of sequence analysis it has been found that there are probably only seven caspases within the *Drosophilla* genetic code *(Dorstyn et al 2005, Kumar and Doumanis 2000)* compared 14 within mammalian cell-lines, located mainly within the cytosol *(Eckhart et al 2005, Shi 2002).*

The caspases are generally considered to be divided into 2 separate groups based upon their sequence alignment *(Thornberry and Lazebnik 1998*, *Shi 2002)*. The first group consists of those that are related to Ced-3 and the second to those that are related to caspase 1 (Interleukin-1 converting enzyme ICE). Members of the Ced-3 subclass, including caspases 2, 3, 6, 7, 8, 9 and 10 have all been shown to play a critical role in the initiation and propagation of apoptosis whereas members within the ICE subfamily such as caspases 1, 4, and 5 are probably involved in proteolytic activation *(Earnshaw et al 1999)*. Members of the Ced-3 subclass can be additionally divided

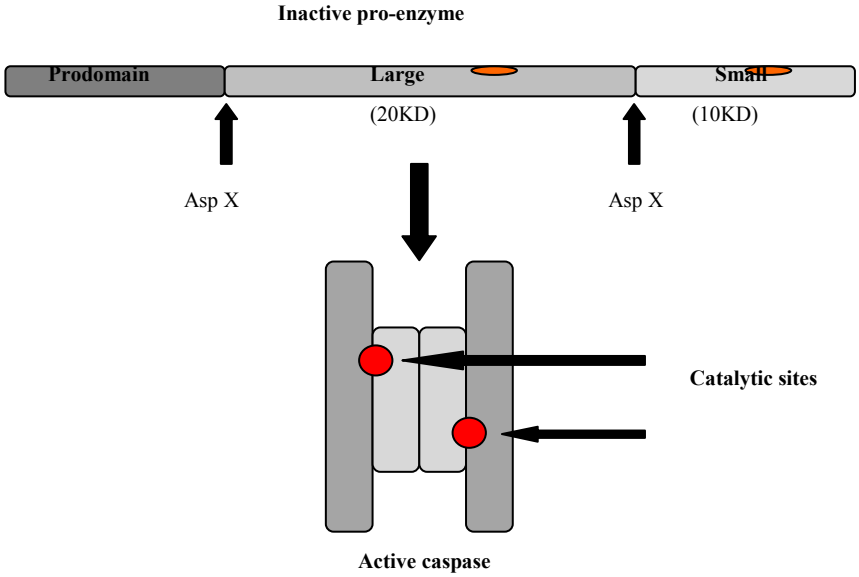
into 2 further sub classes; initiator caspases such as 2, 8, 9 and 10 and executor caspases such as 3, 6 and 7. Initiator caspases function by activating executor caspases leading to the activation or deactivation of the cellular proteins responsible for either the apoptotic pathway or the dismantling of the cell *(Mehmet 2000).*

All caspases are proteolytic enzymes that when inactive lie dormant within the cell in the form of zymogens. These procaspase molecules have 3 basic domains, the prodomain, the large subunit and the small subunit. Caspases involved with the initiation of apoptosis posses a long prodomain and are regulated by proteins other than caspases whereas caspases involved with the execution of apoptosis possess a short prodomain and receive regulation directly from other caspases. As the activation of an initiator caspase in cells inevitably triggers a cascade of downstream caspase activation, it is tightly regulated and often requires the assembly of a multi-component complex under apoptotic conditions *(Adams and Cory 2002)*.

Upon receiving a pro-apoptotic stimulus, the procaspases are sequentially processed into a precursor consisting of a non-catalytic domain and two catalytic subunits a large subunit 17-20 kDa containing the active site Cys, and a smaller 10 kDa subunit *(Muzio et al 1998, Yamin et al 1996)* to produce a dimer of these two subunits. This catalytic tetramer contains two active sites, each of which is characterized by the presence of a deep pocket which binds to the Asp found at the cleavage site together with a second more irregular pocket that binds the preceding three amino acids. The structure of the second pocket results in the formation of a unique binding site, specific for each caspase.

Pro-apoptotic caspases containing long prodomains also contain protein motifs, such as caspase recruitment domains (CARD), like those found in caspase 9 and 2 or death effecter domains (DED) found in caspase 8 and 10). These protein domains contain several charged patches on their surfaces that are able to interact with adaptor proteins that contain a similar structure and a complementary charge. This leads to the formation of a multimeric complex that is required in order to bring two caspase precursors together to activate each other and produce an active tetramer (Figure 1.9).

Figure 1.9. A simplified diagrammatic representation of caspase activation



The procaspase is cleaved at specific caspase cleavage sequences (Asp X). 2 large and 2 small subunits combine to form the active tetrameric enzyme

Initiator caspases can be activated via 2 pathways, either the Death Receptor or extrinsic Pathway which utilizes the receptors found in the plasma membrane or by the mitochondrial or intrinsic pathway which utilizes apoptosis protease activating factor 1 (Apaf-1) and therefore requires the presence of cytochrome c from the mitochondria *(Nagata 1999)*. Following apoptotic stimuli, several proteins are

released into the cytoplasm from the intermembrane space of the mitochondria *(Wang 2001)*.

In the extrinsic pathway there are at least five different cell death receptors, all of which are members of the transmembrane tumour necrosis factor (TNF) receptor super family. The two most well understood members of this family are Fas and the type I TNF receptor *(Nagata 1999)*. Within all death receptors there is a polypeptide domain termed the death domain (DD) located within the cytoplasmic domain that is structurally very similar to that of the caspase recruitment domains (CARD) and death effecter domains (DED). When these death receptors bind with TNF or other homologous ligands they are able to bind additionally to other proteins such as Fas associated death domain (FADD) via the interactions with their own death domain. FADD also contains a death effecter domain making it possible to bind and activate caspase 8 and caspase 10 via their own death effecter domains to produce a complex known as the death inducing signalling complex (DISC) *(Peter and Krammer 2003)*.

In parallel the activation of caspase 2 uses the adaptor protein receptor death domain which contains both a caspase recruitment domain (CARD) that is able to bind to the corresponding CARD domain on caspase 2 and a death domain which appears to bind to a yet unidentified receptor *(Duan and Dixit 1997* and *Ahmad et al 1997)* thus unlike in *C. elegans* which has a single round of caspase activation in mammalian cells there are 2 stages of activation required in order to complete the pro-apoptotic cleavages; simplified as an initial activation of initiator caspases triggered by a variety of stimuli followed by a second stage of executioner caspases activated as a final pathway.

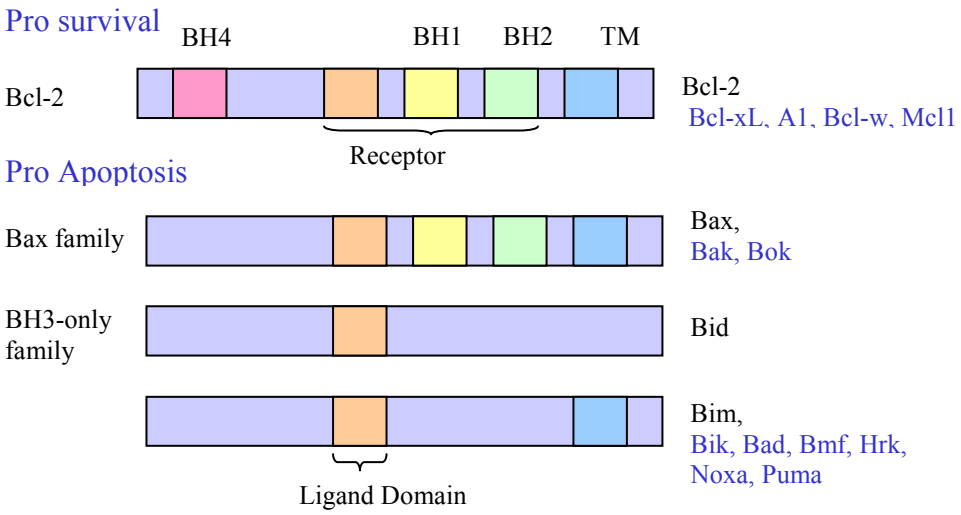
In the mitochondrial or intrinsic pathway several apoptogenic factors such as cytochrome *c*, apoptosis inducing factor (AIF) and procaspases 2, 3 and 9 which are located within the intermembrane space of the mitochondria are released into the cytosol via the opening of a transmembrane potential pore following apoptotic stimuli *(Rodriguez and Lazebnik 1999, Zou et al 1999, Saleh et al 1999)*. Cytochrome *c* released from the mitochondria binds to the cytosolic protein Apaf-1. This interaction results in a conformational change in Apaf-1 which, when stabilised by the binding of ATP, allows molecules of Apaf-1 to associate with each other *(Li et al 1997)*. This results in the formation of a wheel-like structure that contains 7 molecules each of Apaf-1, cytochrome *c* and ATP. This wheel-like structure, known as the apoptosome, permits the recruitment of 7 molecules of procaspase 9 to the complex *(Rodriguez and Lazebnik 1999)*. The exact mechanism of caspase activation is still uncertain although two possibilities have been proposed. In one case the Apaf-1, cytochrome *c* and procaspase 9 complexes can act as a platform for the activation of cytosolic procaspase 9, as it is recruited to the apoptosome. In the other scenario two apoptosome have been proposed to interact with each other and to activate the caspase 9 located on the other apoptosome *(Riedl and Salvesen 2007)*. Activation of caspase 9 or other initiator caspases leads to the activation of other downstream executor caspases thus unleashing the death cascade and the release of the caspases 2, 3 and 9 from the mitochondria. Their subsequent activation in the cytosol is then able to contribute to the amplification of the caspase cascade.

1.9. Regulation of apoptosis

It is as yet unknown what factors promote the release of cytochrome *c* from the mitochondria however since Bax, and other Bcl-2 proteins, show structural similarities with pore-forming proteins it has been suggested that Bax can form a transmembrane pore across the outer mitochondrial membrane *(Jurgensmeier et al 1998, Tsujimoto 2000)*. This may lead to the loss of membrane potential and efflux of cytochrome *c* and AIF (apoptosis inducing factor). Bcl-2 and Bcl-XL may therefore be responsible for preventing this pore formation *(Dlugosz et al 2006)*. Evidence to substantiate this shows that heterodimerisation of Bax or Bad with Bcl-2 or Bcl-XL appears inhibit their protective effects *(Dlugosz et al 2006, Antignani and Youle 2006)*. It is also thought that proteins such as Bax and Bad can promote the formation of the large diameter PT pore, with subsequent loss of cytochrome *c* and initiation of apoptosis *(Tsujimoto 2000)*.

At least 29 Bcl-2 family proteins have been discovered in mammalian cells as well as others in *Drosophila, C elegans* and multiple virus species *(Gross et al 1999, Igaki et al 2000)*. It is generally accepted that members of the Bcl-2 family may be divided into 3 distinct groups all of which share at least one conserved Bcl-2 homology (BH) domain (Figure 1.10). It is the BH domains which are responsible for the functionality of each of the Bcl-2 related proteins by dictating their ability to bind to neighbouring Bcl-2 proteins or other necessary proteins *(Willis et al 2007, Youle 2007)*. Bcl-2 itself comprises of all four domains, and close relatives also possess at least the first three (BH1-BH3) domains.

Figure 1.10. The three subfamilies of Bcl-2 related proteins.



The four receptor domains (BH1–4) are highly conserved among all family members. Most members have a carboxyl-terminal hydrophobic domain that aids association with intracellular membranes, the exceptions being A1 and many of the BH3-only proteins (Bad, Bid, Noxa, Bmf and Puma).

1.9.1 The Bcl-2 family.

Bcl-xL *(Muchmore et al 1996)*, Bcl-2 *(Petros et al 1996)* and Bcl-w all have a similar molecular structures that they share with the viral Bcl-2 homologue (*Huang et al 2002).* All contain five amphipathic α-helices surrounded by two central hydrophobic α-helices. As noted previously they also all posses at least one of the four possible Bcl-2 homology (BH) domains. Members of the Pro-survival family all have the ability to inhibit apoptosis in response to many cytotoxic and physical insults. These proteins contain a unique hydrophobic carboxy-terminal domain that enables them to target the cytoplasmic surface of intracellular membranes such as the outer mitochondrial membrane, the endoplasmic reticulum (ER) and the nuclear envelope *(Thomadaki and Scorilas 2006)*. Bcl-2 exists permanently within these membranes,

whereas Bcl-w and Bcl-xL only become associated with the membrane after a cytotoxic signal is received, indicating that there may be induced conformation changes that must occur before the protein is able to associate with the membrane *(Cory and Adams 2002)*.

In contrast it would appear that the anti-apoptotic Bcl-2 family members function by preventing the release of apoptogenic proteins from the mitochondria *(Adams and Cory 2001).* The exact mechanisms of this form of regulation remains unclear, but it thought that the formation of ion channels by Bcl-2 family proteins on the outer mitochondrial membrane may play a key role *(Youle and Strasser 2008)*. Evidence to support this pore forming theory comes from three-dimensional structure of the Bcl-xL protein that resembles the pore forming bacterial toxins diphtheria and colicin (*Muchmore et al 1996*, *Minn et al 1997, Qian et al 2008, Anderluh and Lakey 2008)*. Additionally it has been shown that Bcl-xL, Bcl-2 and Bax have the ability to form ion channels in synthetic membranes within an in-vitro environment. (*Minn et al 1997, Schendel et al 1997, Antonsson et al 1997*). It would also appear that the ability to integrate within an intracellular membrane is crucial for the regulation of mitochondrial permeability as studies examining mutated Bax which lacked the C-terminal transmembrane domain display no pro-apoptotic activity (*Zha and reed 1997, Shimizu et al 1999, Simonen et al 1997*). All cells require the presence of at least one Bcl-2 homologue in order to survive, and the abundance of these pro-survival proteins is responsible for the regulation of tissue homeostasis. In contrast the inactivation of the Bcl-2 homologous genes results in apoptosis in specific cell types, presumably because the concentrations of other Bcl-2 family members are too low to compensate.

1.10. Aims and Objectives of this thesis

The research in this thesis is divided between the four results chapters that follow, each of which has its own hypothesis, aims and objectives. In the first of these, (chapter 3) it was hypothesised that it may be possible combine the inducible expression of $p21^{CIP1}$ with the continuous expression of Bcl-2 in an industrially relevant CHO cell-line previously engineered to express the monoclonal antibody IgG. The objective for this work was to establish if the combined expression of $p21^{CIP1}$ and Bcl-2 would have a significant impact of the levels of productivity that could be obtained during the culture period. It was also theorised that the expression of anti-apoptotic Bcl-2 would also result in a reduction in the rate of cell death due to apoptosis and therefore the overall viability of the resulting cell-line was expected to remain significantly higher than cell-lines in which Bcl-2 was not expressed.

This chapter was successful in terms of the creation of a several clones capable of expressing $p21^{CIP1}$, Bcl-2 and the chimeric antibody IgG4. Analysis of the clones growth profiles, productivity, ability to undergo $p21^{CIP1}$ induced cell cycle arrest and withstand cellular stress when challenged with staurosporine and serum deprivation, enabled the successful selection of the clone that exhibited the most desirable characteristics. The expression of both $p21^{CIP1}$ and Bcl-2 were additionally confirmed via western blot analysis, resulting in the successful development of a $p21^{CIP1}$/Bcl-2 co-expressing cell-line which could then be used in the remaining chapters

In the second of the results chapters (chapter 4) I examined the hypothesis that a relationship exists between the expression of $p21^{CIP1}$ and the increase in productivity of CHO cells observed in chapter 3. In addition I hypothesised that that cellular

growth and proliferation may be uncoupled in $p21^{CIP1}$-expressing cultures. The aim of this chapter was therefore to identify potential changes occurring in the cells metabolism during periods of $p21^{CIP1}$-induced proliferation arrest, the objective being to determine if such metabolic changes are able to have a direct impact on cellular productivity.

In this chapter an IgG4 expressing CHO cell-line in which $p21^{CIP1}$ expression could be inducibly expressed was successfully created. Using this cell-line it was possible to determine the exact phase of the cell cycle in which cells congregate following the initiation of $p21^{CIP1}$-induced cell cycle arrest, as well as the effect of $p21^{CIP1}$-expression on cell volume during the culture period. Due to limitations in the equipment available at the time of analysis, it was not possible to do an in-depth examination of the changes which occur in the metabolic pathways of $p21^{CIP1}$ expressing cell lines. Despite this I was able to examine the usage of key nutrients including glucose, glutamine, ammonia and lactate during periods of cell cycle arrest, which when combined with information obtained on cell volume, total protein and mitochondrial activity would seem to support my previous hypothesis that cell growth and proliferation may be uncoupled during periods of cell cycle arrest. In addition there would appear to be indications that changes occur within the pathways involved in energy metabolism as a result of the over-expression of $p21^{CIP1}$.

The third results chapter (chapter 5) focuses on the hypothesis that the ability to halt cell cycle progression in the G1 phase of the cell cycle, through the over-expression of $p21^{CIP1}$ would facilitate cell survival within suspension and protein-free growth environments by reducing the requirement for growth factors. In addition I theorised

that the prevention of cell cycle progression may also allow the resulting genome damage, created through the direct withdrawal of serum, to be repaired prior to cell cycle release. The objective of this chapter was therefore to examine the effect of periods of $p21^{CIP1}$-induced cell cycle arrest on the ability of an anchorage-dependent CHO cell-line to survive and adapt to both suspension and protein-free growth environments whilst maintaining productivity.

In the final results chapter (chapter 6), the hypothesis of chapter 5 were further expanded, to include the anti-apoptotic effects of the over-expression of Bcl-2. The objective of this chapter was to determine if combining the expression of Bcl-2 with the over-expression of $p21^{CIP1}$ would further reduce the time required for successful adaptation to both suspension and protein-free culture conditions.

The results of these final two chapters potentially have significant implications for the biopharmaceutical industry, as I was successfully able to demonstrate the ability to substantially reduce the time required for successful adaptation to both suspension and protein free growth environments, through the expression of the cell cycle inhibitor $p21^{CIP1}$. In addition I was able to demonstrate a further reduction in adaptation time is possible when combining periods of $p21^{CIP1}$ induced cell cycle arrest with the anti-apoptotic properties of Bcl-2 expression. These findings may ultimately lead to the swift development of commercial cell-lines, better able to meet the increasing demands for higher productivity and longevity whilst withstanding the stresses imposed by growth within a chemically defined bio-processing environment.

2. Materials and Methods

2.1 Cell culture and maintenance

These methods were employed for the creation and maintenance of the cell-lines used in chapters 3, 4, 5 and 6 of this thesis prior to experimentation.

2.1.1. Transfection and maintenance

The parental cell-line CHO-22H11 was kindly supplied by Lonza Biologics (Slough, UK). CHO-22H11 has previously been transfected with the human-mouse chimeric B72.3 IgG4 antibody gene, using the glutamine synthetase (GS) expression system *(Bebbington et al 1992, Bi et al 2004)*. Cells were maintained in a high glucose (17mM) medium DMEM NUT MIX F-12 without glutamine (GIBCO, Paisely, UK) supplemented with 10% heat inactivated foetal calf serum (PAA laboratories GmH), 50μM methionine-sulphoxomine (MSX; Sigma, Poole, UK) and 2% Glutamine synthetase (GS) supplement (Sigma, Poole, UK). Cells were cultured in vented tissue culture flasks (T-flask) at 37°C in the presence of 5% CO_2.

In order to obtain inducible expression of $p21^{CIP1}$ the Lacswitch system was used. First lac repressor expressing cells were constructed using CMVlacI plasmid DNA (Stratagene), kindly supplied by Dr K Saqib (Institute for Cancer Studies, Birmingham University, UK). DNA was introduced into CHO-22H11 cells by liposome-mediated transfection using Lipofectin reagent (Life Technology, UK) according to manufacturer's instructions. Transfected cells were selected in DMEM NUT MIX F-12 without glutamine medium (GIBCO, Paisely, UK) containing 400μg/ml Hygromycin B (Sigma, Poole, UK). After 2 weeks selection, the whole population of stable transfectants was then transfected with

pOPRSV1/p21^{CIP1} plasmid DNA using Lipofectin reagent (Life Technology, UK) to produce a selection of p21^{CIP1}-expressing clones from which the 3B2 clone was previously selected (*Bebbington et al 1992, Bi et al 2004).* Plasmid pOPRSV1/p21^{CIP1}, which contains the mouse p21^{CIP1} cDNA *(Erhardt and Pittman 1998)*, was kindly provided by Dr. R. Pittman (University of Pennsylvania, Philadelphia, PA). Stable clones were selected in 400µg/ml Geneticin 418 (GIBCO), and isolated by limiting dilution.

To create a Bcl-2-and-p21^{CIP1}-co-expressing cell-line the expression vector pEF*Bcl-2* (carrying the human *Bcl-2* gene) and control vector pEF*neo* (kindly provided by Susan Cory, University of Melbourne, Australia) on a plasmid containing a hygromycin B gene was first introduced into the parental 22H11 line via liposome-mediated transfection, using Lipofectin reagent (Life Technology, UK) according to the manufacturer's instructions. Transfected cells were selected by the addition of 400µg/ml Hygromycin B (Sigma, Pool, UK) to the growth medium for a period of 2 weeks; after which, the whole population of stable transfectants were then transfected with the pOPRSV1/p21^{CIP1} plasmid deoxyribonucleic acid (DNA) containing the mouse p21^{CIP1} complementary DNA *(Erhardt and Pittman 1998)* kindly provided by Dr. R. Pittman (University of Pennsylvania, Philadelphia, PA) using Lipofectin reagent (Life Technologies). Stable clones were selected in the presence of 400µg/ml Geneticin 418 (GIBCO Paisley, UK) and isolated by limiting dilution.

Induction of p21^{CIP1}-expression was achieved via the addition of 5mM isopropyl-β-D-thiogalactoside (IPTG; GIBCO) to the culture medium. Cells were maintained for several passages before experimentation in Dulbecco's modified Eagle's medium (DMEM) F12 minus glutamine supplemented with 10% heat-inactivated foetal calf protein (PAA, Yeovil,

UK), 50μM methionine sulphoxomine (MSX), and 2% GS supplement (Sigma, Poole, UK). Cells were cultured in vented tissue culture flasks with 5% CO_2 at 37°C. All cultures were passaged every 72 hours by washing the cells with warm 1× phosphate-buffered saline (PBS) followed by the addition of non-enzymatic cell dissociation solution (Sigma, Poole, UK). Cells were then washed with warm culture medium before being centrifuged at 90g for 5 minutes and re-suspended in fresh culture medium.

2.1.2. Cell cloning

Cells from mid exponential cultures were diluted to 100, 50, and 10 cells/ml concentrations. 100μl of each of the dilutions were dispensed into each well of a 96 well culture plate containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50μM MSX. Clones expressing the desired vectors were selected by the presence of 400μg/ml Hygromycin B (Sigma, Poole, UK) and 400μg/ml Geneticin 418 (GIBCO Paisley, UK) in the culture medium. The plates were incubated at 37˚C in the presence of 5% CO_2 for 72 hours before they were inspected using inversion microscopy for the presence of single colonies. Wells exhibiting single cell colonies were then marked and had a 50% culture medium change performed every 72 hours until they became confluent. The cultures were then expanded into 24 well plates and the process repeated until sufficient cells were produced for cell banking and experimentation.

2.1.3. Medium preparation and additives

All the cell-lines used in this thesis were grown in either DMEM F12 without glutamine culture medium supplemented with 10% heat inactivated foetal calf serum (PAA laboratories ltd, Yeovil, UK) or ProCHO4 protein-free growth medium (Cambrex, UK), in both cases supplemented with 50μM MSX. Continuous growth of the GS-CHO $p21^{CIP1}$/Bcl-2 cell-lines

in selection media containing both Hygromycin B and Geneticin 418 is known to inhibit growth and cause changes in cellular metabolism. Therefore the cells were cultured without the presence of these antibiotics for 5 passages to enable successful growth. After 5 passages the antibiotics were added to the cell culture medium for 2 passages at a concentration of 400µg/ml of Hygromycin B (Sigma, Poole, UK) and 400ug/ml of Geneticin 418 (Sigma, Poole, UK).

2.1.4. Heat inactivation of foetal calf serum

Serum is commonly used as a supplement to basal growth medium in cell culture. The most common type of serum used for cell growth is foetal bovine serum (FBS), also known as foetal calf serum (FCS). Foetal bovine serum is obtained from foetuses harvested in abattoirs from healthy dams fit for human consumption. Occasionally, there may be use of other bovine sera, such as newborn calf serum or donor bovine serum. In cell culture, serum provides a wide variety of macromolecular proteins, low molecular weight nutrients, carrier proteins for water – insoluble components, and other compounds necessary for in vitro growth of cells, such as hormones and attachment factors. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. Heat inactivation of serum is performed by raising the temperature of the serum to 56^{o}C and maintaining that temperature for 30 minutes. Heat inactivation is the method of choice to destroy complement, and to ensure that the cells will not be lysed by antibody binding.

2.1.5. Cell culture vessels and sub-culturing.

During routine culture conditions all cells were cultured in either sterile static vented tissue culture flasks (T flask) or sterile Erlenmeyer flasks rotated at 125 rpm. During cloning sterile well plates of various capacities were used. The cells were seeded at 1-6x10^{5} cells/ml into a

culture vessel containing 1/3 of the vessels working volume of medium to allow for a sufficient surface / volume ratio for oxygen transfer. All cultures were maintained at 37˚C and 5% CO_2 and passaged during mid-exponential growth at approximately 72 – 96 hours dependent on culture volume.

2.1.6. Cryopreservation

Cells from dividing cultures were harvested during mid exponential phase and centrifuged at 90g for 5 minutes before being re-suspended in a solution of DMEM F12 containing 10% (v/v) heat inactivated foetal calf serum and 10% (v/v) Dimethyl sulfoxide (DMSO) (Sigma, Poole, UK) to give a final cell concentration of 10^7 cells/ml^{-1}. 1ml aliquots were then transferred to 2ml max volume cryovials (Nunc) and labelled with the cell-line name, date, and users name. They were then placed into a flask containing iso-propan-2-ol (Sigma, Poole, UK) which had previously been allowed to warm to room temperature. The flask was then placed into a - 70°C freezer overnight or for at least 4 hours. The frozen vials were then removed and placed into a long-term storage Dewar containing liquid nitrogen.

2.1.7. Cell revival

Cryovials were removed from the liquid nitrogen storage Dewar and warmed to 37°C in a water bath. Once fully thawed the cells were transferred into a suitable vessel and 10ml of pre-warmed culture medium was added drop by drop to expel the DMSO from within the cells without causing cell lysis. The cells were then centrifuged at 90g for 5 minutes and re-suspended in fresh pre-warmed culture medium before incubation at 37°C. Culture viability was assessed and cells cultured for at least 3 passages before use for experimentation

2.2. Experimental methods

The methods described in this section are those which were utilised to fulfil the experimental objectives of chapters 3, 4, 5 and 6.

2.2.1. Cell counting via Trypan blue exclusion method.

Viable and total cell numbers were accessed by counting cells using a Neubauer haemocytometer under a phase contrast microscope. 100µl of cells taken from the cell culture were diluted 1:1 with Trypan blue (Sigma, Poole, UK 0.4% (w/v) in water) in a 96 well plate. The cells were counted with the aid of a microscope (Olympus BH-2, Olympus, Japan) at 10x or 20x magnification. At least 4 grids were counted on each side of the haemocytometer. This was then repeated

Cell density = (number of cells counted) / (number of grids counted) x 2 x10000

The cell density is given in 10^5 cells/ml. For the viability determination, the total number of cells (stained or unstained) is counted in a similar fashion as mentioned above. The calculations for viability based on one side of the haemocytometer were as follows:

Cell viability = (number of unstained cells) / (total number of cells) x100

2.2.2. Determination of apoptosis via acridine orange/ propidium iodide (AOPI) method

Acridine orange is a fluorescent dye which binds to genetic material and can differentiate between deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Propidium iodide is an intercalating agent and a fluorescent molecule that can be used to stain DNA. It can be used to differentiate necrotic, apoptotic and normal cells.

A solution was prepared by mixing 100µl of 1mg/ml^{-1} propidium iodide (PI, Sigma, Poole, UK) and 100µl of 1mg/ml^{-1} acridine orange (AO, Sigma, Poole, UK) to 10mls of 1x PBS. The stain solution was added to the cell sample and 100 cells counted in triplicate to determine the percentage of early/late apoptotic, necrotic and viable cells.

2.2.3. Glucose concentration

Glucose measurements were carried out in triplicate from experimental replicates using samples of cell free supernatants taken at various time points. Samples of the culture supernatant were stored at -20^{0}C and allowed to warm to room temperature before readings were taken. Glucose levels were determined using a blood glucose meter (Ascensia Blood Glucose meter, Bayer Healthcare LLC, IN, USA) with a wide operating range of 0.6-33.3mmol/l. Each test stripe was placed into the meter and drop of well-mixed sample was allowed to react on the strip. The meter produces an electrochemical reaction utilising a glucose dehydrogenase-flavin adenine dinucleotide (FAD) immobilized strip with ferricyanate as a mediator for electron transfer. The meter was calibrated using a glucose control solution to determine the linearity of the measurements.

2.2.4. Lactate concentration

Lactate concentrations were determined from the supernatant of three replicate cultures by enzymatic analysis (r-biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Samples were measured at 340nm using a microplate reader. All samples were done in triplicate. Readings were taken every half an hour until no changes in absorbance could be seen. Samples were compared to the calibration standard that is provided with the kit.

2.2.5. Ammonia concentration

Ammonia concentrations were determined by an ammonia enzymatic kit (r-biopharm, Darmstadt, Germany) using 3 replicate cultures. Samples were diluted 10 times due to the low working range of the assay but maintained an absorbance above 0.1 absorbance units. Measurements were carried out in triplicate at 340nm using a 96-well microplate reader. Readings were taken every half an hour until the absorbance was constant. Subtraction from the initial absorbance could subsequently be done to provide an accurate reading. The supplied assay control solution was used as an internal standard in order to check the assay performance and to determine whether the sample solution was free from interfering substances:

2.2.6. Glutamate concentration

Glutamate concentrations were determined from the supernatant of three replicate cultures by enzymatic analysis (r-biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Samples were measured at 492nm using a microplate reader. All samples were done in triplicate. Readings were taken every half an hour until no changes in absorbance could be seen. Samples were then compared to the calibration standard that is provided with the kit.

2.2.7. Total protein concentration

Total intracellular protein was determined using the BCA protein assay kit. BCA or bicinchoninic acid is a colorimetric assay utilised for the detection of total protein. After counting cells, 5mls of cell culture containing at least $2x10^6$ cells was centrifuged at 500g for 5 minutes. The supernatant was then removed and 2mls of sterile PBS was added to disperse

the cell pellet before the cells were centrifuged again at 500g for 5 minutes. This process was repeated with 2mls of distilled water but this time the cells were centrifuged at 500g for 10 minutes. Care was taken not to disturb the cell pellet when removing the supernatant. 100µl of lysis buffer (Cellytic M, Sigma, Poole, UK) was then added and gently vortexed for 1 minute before freezing at -20^{0}C until analysis. At the time of analysis, the samples were allowed to warm to room temperature before being vortexed gently to mix. Debris was removed by centrifuging at 15000g for 15 minutes. The remaining supernatant was then diluted 60-80x with distilled water before use. The BCA assay was run according to the manufacturer's instructions using a microplate reader and a standard curve with a working range from 5-250µg/ml. Samples were analysed from three cultures in triplicate.

2.2.8. Secreted Monoclonal antibody concentration

An enzyme linked immunoabsorbent assay (ELISA) was used to determine the concentration of MAb excreted into the culture medium. This method was adapted from that used by Dr Margaret Goodall at the Department of Immunology at the Medical school of Birmingham University. Nunc maxisorp plates were prepared by the addition of 10µl of coating antibody (Anti-human IgG fc antibody (does not recognise γ2) (Sigma, Poole, UK) in 10ml coating buffer (Na_2CO_3) (BDH AnalaR 10240 4H) 1.59 g/l, $NaHCO_3$ (Sigma, Poole, UK) 2.93 g/l in distilled water. Adjust to pH 9.6 if necessary) (1/1000 dilution) dispensed at 100µl per well.

Each plate was then covered with a plate sealer and incubated at 4˚C overnight. The next day the plates were washed four times with wash buffer (Tween 20 (polyoxyethylenesorbitan monolaurate) (Sigma, Poole, UK), 0.05% v/v in 1x PBS). The plates were then tapped gently onto a tissue to remove excess wash buffer before 100 µl of blocking buffer (Skimmed milk powder (MARVEL) 10 g/l in 1x PBS) was dispensed into each well. The plate was once

again sealed and incubated at room temperature for 1 hour to block any remaining binding sites. Whilst the plates are incubating serial dilutions of the standards (Supplied by Lonza Biologics, clarified supernatant from NSO 6A1 culture, in which the levels of monoclonal antibody have previously been determined) and culture supernatant samples were prepared. The standard requires a broad range dilution to allow for the production of a straight line on the standard curve likewise the dilution used for the samples must be such as to allow the absorbance to lie within the limitations of the standard curve (figure 2.1). Following the hour's incubation the plates were once again washed four times with wash buffer and tapped to remove excess fluids before 100µl of each dilution of the standard were added in duplicate to each of the plates. Next 100µl of the diluted supernatants was added to the remaining wells, ensuring 2 wells remain empty in order to produce the blank control reading. The plates were the resealed and incubated for one hour at room temperature. Following incubation the plates were then washed four times with wash buffer before being tapped dry. 100µl of the secondary antibody (Anti-human kappa light chain antibody conjugated to horseradish peroxidase (Sigma, Poole, UK). (1:10 000 dilution) diluted in blocking buffer was then added to each of the wells before re-sealing the plates and incubating for a further hour at room temperature.

The plates were then washed four times in wash buffer and 100µl of the substrate solution (0.1M Citric acid 5ml, 0.2M di-Sodium phosphate 5ml, O-phenylenediamine dihydrochloride (OPD) (Sigma, Poole, UK) 4 mg (1 tablet), Hydrogen peroxide (4µl) dispensed into each well. The plate was once again sealed and incubated at room temperature in a dark room until the appearance of a colour change on the standards. 100µl of stop solution (Sulphuric acid (H_2SO_4) 12.5 % v/v) was then added to each well and the absorbency at 492 nm using a 620 nm reference was determined using a SLT spectra ELISA plate reader.

Figure 2.1. Elisa standard curve

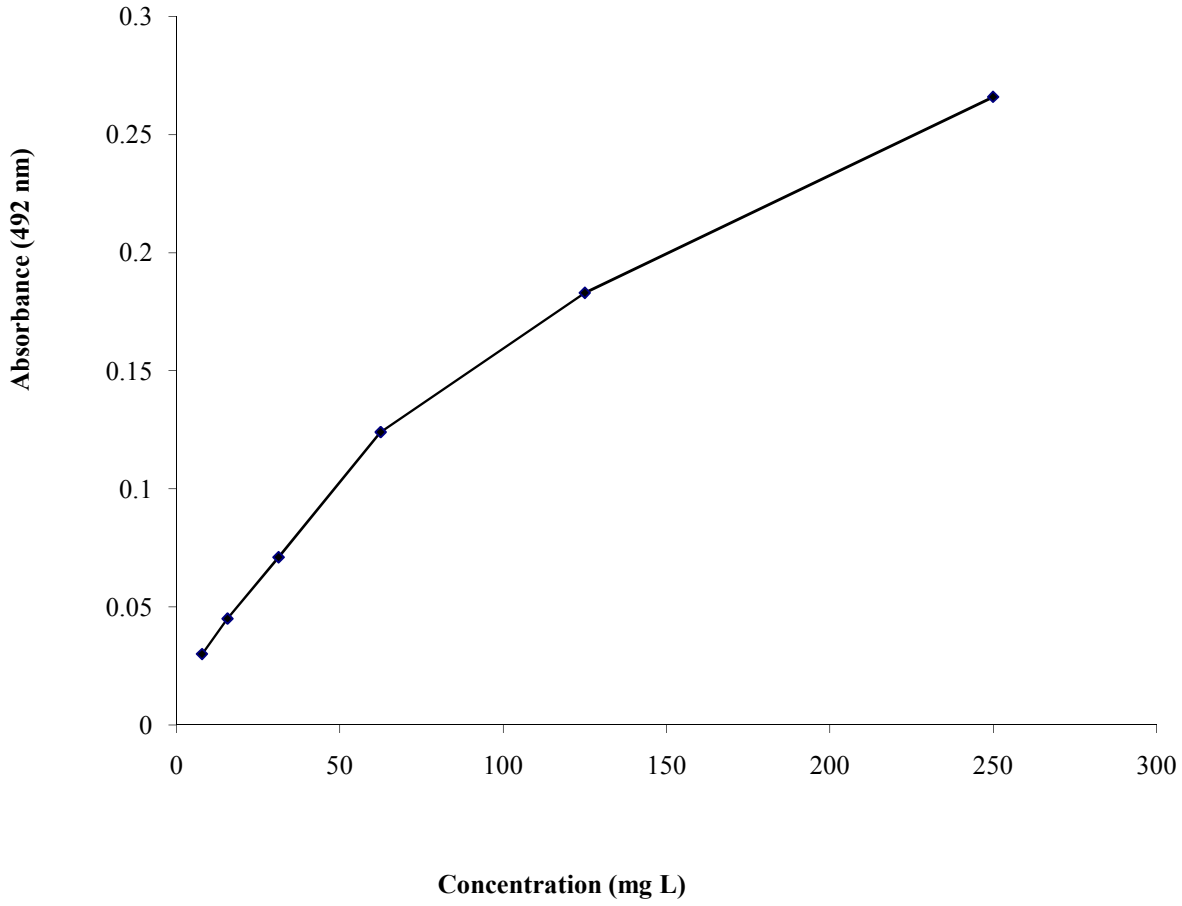


Figure 2.1. ELISA standard curve produced through the serial dilution of the clarified NSO 6A1 supernatant standard (Supplied by Lonza Biologics), in which the levels of monoclonal antibody have previously been determined. The amount of monoclonal antibody present in the experimental samples was determined by comparison of their absorbance to that of the standard.

2.2.9. Western blot analysis

The Western Immunoblotting protocol was adapted from *Sambrook et al (1989)*. $1x10^6$ cells were harvested then centrifuged at 450g for 5 minutes. The supernatant was removed and the remaining pellet re-suspended in warm PBS to wash cells. The cells were then centrifuged at 450g for 5 minutes, the supernatant removed and the pellet re-suspend in to 500µl of Cellytic M lysis buffer. The samples were then stored at -70^0C until SDS-PAGE gels could be run. Before running gels, the samples were thawed and diluted with 2x Lamelli sample buffer (Sigma, Poole, UK) before being denatured at 95^0C on a PCR thermal cycler (Eppendorf UK Limited, Cambridge, UK) to reduce liquid loss.

SDS-PAGE gels were run using Pierce (Pierce Biotechnology, IL, USA) Precise pre-cast gels (8% gels). For all samples the same cell volume was added into each well. The gels were then run at 75V for about 90 minutes in BupH Tris-HEPES-SDS running buffer (Pierce Biotechnology, IL, USA) using a BioRad Mini-protean II gel tank (Bio-Rad Laboratories, CA, USA). The proteins were then transferred to Hybond ECL nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) in BupH Tris-Glycine transfer buffer (Pierce Biotechnology, IL, USA) for 1 1/2hr at 100V (10V per $1cm^2$).

After the transfer, the membranes were washed before blocking took place. Blocking was done by incubation in 10% Marvel milk powder dissolved in PBST for 1 1/2 hours on a rocking plate (30-35 rpm). After blocking, membranes were washed with PBST 3 times before adding 5% milk solution with the primary mAb (see table 2.1) at the dilution: of 1:500 to 1:1000. This was allowed to react on a rocker (25 rpm) for 1 1/2 hours at room temperature.

After primary antibody coupling, the membrane was washed with PBST 3 times for 5 minutes each**.** This was to make sure that the entire primary antibody was removed. The secondary antibody (see table 2.1) was added (1:2000 to 1:10000 in 5% Marvel milk powder dissolved in PBST) and incubated for 1hr at room temperature on the shaker (25 rpm). Detection was achieved via an ECL kit (ECL Plus, GE Healthcare, Buckinghamshire, UK) and visualized on an X-ray film (Hyperfilm, GE Healthcare, Buckinghamshire, UK). Fixing and developing solutions were obtained from Kodak (Kodak Limited, Hempstead, UK).

Table 2.1. Primary and secondary antibodies used for western blot analysis.

Antibody name	Description	Supplier	Catalogue number
Anti p21	Mouse anti-human p21	Santa Cruz Biotechnology	SC-817
Anti-Bcl-2	Mouse anti-human Bcl-2	Santa Cruz Biotechnology	SC-130307
Anti-Rabbit IgG (Whole molecule)-peroxidase conjugate	Goat Anti-rabbit IgG (Whole molecule)	Sigma	A6154

The detection of $p21^{CIP1}$ and Bcl-2 was achieved through western blot analysis using the primary and secondary antibodies listed in this table. Supplier and catalogue numbers are provided for clarity.

2.2.10. Mitochondrial activity via MTT

Cells were seeded at $2.5x10^6$ with/without 5mM IPTG into fresh growth medium then incubated for 72 hours at 37˚C in the presence of 5% CO_2. A 5mg/ml solution of MTT with PBS was made and sterile filtered before use. Cells were then counted and $2x10^6$ cells harvested and re-suspend in 1ml of medium plus 100µl MTT. The cells were then incubated for 2 hours to allow the MTT to diffuse throughout before the cells were centrifuged at 200 g and the pellet re-suspend in 1ml iso-propanol. The samples were then placed on a shaker at

90g for 20 minutes prior to being serial diluted to 1x 2x 4x 8x 16x and 32x concentrations with iso-propanol. 100µl of the sample solutions were placed into a 96 well plate and read at 540 nm with a SLT Spectra Elisa plate reader.

2.2.11. Guava cell cycle analysis

The cell concentration was determined via the Trypan blue exclusion method and the cell volume adjusted to between $5x10^5$ to $1x10^6$ cells/ml. 200µL of each cell sample was then placed into individual tubes (Eppendorf UK Limited, Cambridge, UK) before being centrifuged at 450g for 5 minutes with the brake on low. The supernatant was then removed and the same volume of 1X PBS was added to each tube as was in the original culture (or to get approximately $1x10^6$ cells/ml). The samples were then mixed well by vortexing or pipetting repeatedly to ensure a homogenous suspension before once again being centrifuged at 450g for 5 minutes with the brake on low. The supernatant was once again removed leaving approximately 500µl of 1X PBS. The cells in the residual 1X PBS were then re-suspended and transferred drop-wise while vortexing on medium speed (setting at 5) into a 50ml conical tube containing enough 70% ethanol to make the final concentration approximately 10^6 cells/ml. The samples were then refrigerated for at least 12 hours prior to staining. Fixed cells are stable for several weeks at 4 °C and for two to three months at –20°C.

Following fixation the cell cycle staining reagent (Guava) and 1x PBS were warmed to room temperature. The tubes containing the samples were then centrifuged at 450g for 5 minutes with the brake on low at room temperature and the supernatant was removed carefully as not to touch the pellet. Using a Multi-channel pipette 200µl of 1X PBS was added to each tube and mixed well by pipetting up and down several times. The tubes were then allowed to stand

at room temperature for 1 minute before being centrifuged at 450g for 5 minutes with the brake on low at room temperature. Once again the supernatant was removed and discarded being careful not to touch the pellet. 200µl of Cell Cycle Staining Reagent was then added to each tube and mixed well by pipetting up and down several times. The samples were then incubated at room temperature shielding away from light for 30 minutes before running them on the Guava PCA system.

To further analyze the data for more subtle changes between the phases of the cell cycle Multicycle software (De Novo, Los Angeles, CA) that applies sophisticated curve fitting algorithms to the Guava cell cycle data that more accurately calculates the percentages of the cell populations and their relevant statistics were used

2.2.12. Cell volume via Malvern mastersizer

The cell size and volume was determined using the Mastersizer S instrument with a small volume sample unit (Malvern Instruments, Worcestershire, UK). The Mastersizer uses laser diffraction technology to determine size distribution in a sample. Size range was determined to be between 5um to 30um to remove debris and cellular aggregates. Between 1-2x10^6 cells were used for the analysis and readings were taken over a period of 5 minutes in triplicate.

2.2.13. Determination of cell volume via volumetric analysis

It has previously been reported *(Stettler et al 2006)* that the analysis of packed cell volume may be used as a means of assessing biomass in mammalian cell culture and was therefore used here as a means of gauging the changes in cell volume in this study. A total of 2x10^5 cells from both arrested and non arrested cultures were used for each sample to determine the packed cell volume. The cells were counted in triplicate and a correct volume of cell culture

was transferred into the packed cell volume tubes (PCV, TPP AG, Switzerland). The tubes were then placed in a microcentrifuge (Eppendorf UK Limited, Cambridge, UK) and centrifuged at 2000g for 5 minutes. The tubes were visually inspected to observe the difference in the cell volume and photographic records were made.

2.2.14. Growth profile of clones

All clones were cultured as static in T flasks containing DMEM F12 plus 10% heat inactivated FCS and 50µM MSX. Flasks were set up for each day of the experiment seeded at a density of 2 x 10^5 (cells/ml) at time point 0. Each day cells from one of the flasks were washed with 1 x PBS before being removed from their T-flask using 1x cell dissociation solution. The viable and total cell numbers were determined each day via the Trypan blue exclusion method.

2.2.15. The effect of serum deprivation.

Clones previously grown as static cultures in DMEM F12 containing 10% heat inactivated FCS and 50µM MSX were washed with 1 x PBS before being removed from their T-flasks using 1x cell dissociation solution. A 1x PBS wash was repeated before cells were centrifuged at 90g. Cells were then seeded into fresh T-flasks containing DMEM F12 and 50µM MSX at a density of 1x10^5 cells/ml. Cultures were incubated at 37ºC for 72 hours before being harvested and analysed for cell death using the AOPI staining method, which utilises acrylamide orange and propidium iodine to distinguish between viable, apoptotic and necrotic cells.

2.2.16. The effect of exposure to staurosporine

Each of the cloned cell-lines had previously been grown as monolayer cultures in tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated FCS and 50μM MSX. Cells were washed with 1 x PBS before being removed from their tissue culture flasks using 1x non-enzymatic cell dissociation solution (Sigma, Poole, UK). A 1x PBS wash was repeated before the cells were centrifuged at 90g for 5 minutes. Cells were then seeded into fresh T25 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated FCS and 50μM MSX at a cell density of $1x10^5$ cells/ml. 0.5μM staurosporine (stock) was added to each of the flasks before the cultures were incubated at 37ºC for 24 hours. Cultures were then harvested and analysed for cell death using the AOPI staining method and cell viability via the Trypan blue exclusion method.

2.2.17. The determination of $p21^{CIP1}$-expression

Each of the cloned cell-lines had previously been grown as monolayer cultures tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated FCS and 50μM MSX. Cells were washed with 1x PBS before being removed from their tissue culture flasks using 1x non enzymatic cell dissociation solution (Sigma, Poole, UK). A 1x PBS wash was repeated before cells were centrifuged at 90g for 5 minutes. Cells were then seeded into fresh tissue culture flasks containing DMEM F12 supplemented with 10% heat inactivated FCS and 50μM MSX at a density of $2x\ 10^5$ cells/ml. For each clone 2 flasks were set up as described above. 5mM of isopropyl b-D-thiogalactopyranoside (IPTG) (Sigma, Poole, UK) was added to the culture medium of one of the flasks at the start of experimentation; the other flask was kept as a control (0mM IPTG). Cultures were incubated

at 37ºC for 96 hours before the cells were harvested and the viable cell count determined via the Trypan blue exclusion method.

2.2.18. Analysis of the rate of adaptation of a CHO cell-line to growth in a suspension culture environment through the expression of $p21^{CIP1}$ or $p21^{CIP1}$/Bcl-2

Cultures previously grown in static conditions were detached from the surface of the tissue culture flask using 1x non-enzymatic cell dissociation solution (Sigma, Poole, UK) and re-suspended in fresh culture medium consisting of DMEM F12 growth medium supplemented with 10% heat inactivated foetal calf serum and 50μM MSX. The viable cell number and percentage viability was determined via the Trypan blue exclusion method, before the cells were centrifuged at 90g for 5 minutes and re-suspended in fresh growth medium (prepared as described above) All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks (Corning Ltd, UK). The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture.

For experiments involving the expression of either $p21^{CIP1}$ or $p21^{CIP1}$/Bcl-2 cell-lines, four flasks were set up as described above. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG Sigma-Aldrich) was added to the culture medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$ vector expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs, the cultures were centrifuged at 90g and re-suspended in fresh culture medium (as previously described). Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the

fresh culture medium. Non-Induced cultures were passaged back to 4×10^5 cells/ml. Following 3, 6, and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers. Mean and standard error were calculated from this data and the significance determined via *t*-test analysis.

2.2.19. Analysis of the rate of Adaptation of a CHO cell-line to growth in a protein-free culture medium through the expression of $p21^{CIP1}$ or $p21^{CIP1}$/Bcl-2

Suspension cultures previously grown in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50μM MSX were harvested during mid-exponential phase and the viable cell number and percentage viability were determined via the Trypan blue exclusion method. The cells were then washed in warm 1% PBS before being centrifuged at 90g for 5 minutes. The cells were then re-suspended in fresh protein-free medium (ProCHO4; Cambrex BioScience) supplemented with 50μM MSX. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks (Corning Ltd, UK). The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture.

For experiments involving the expression of either $p21^{CIP1}$ or $p21^{CIP1}$/Bcl-2 cell-lines, four flasks were set up as described above. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG Sigma-Aldrich) was added to the culture medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$-expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs, the cultures were centrifuged at

90g and re-suspended in fresh protein free culture medium (as previously described). Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the fresh culture medium. Non-arrested cultures were passaged back to 4×10^5 cells/ml. Following 3, 6, and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers. Mean and standard error were calculated from this data and the significance determined via *t*-test analysis.

2.2.20. Determination of cell aggregation during adaptation to suspension culture

During the process of adaptation to protein-free media, 1ml samples of culture medium were taken in triplicate from each of the four cultures, set up as described previously, at 72 hour intervals. The samples were stained using the Trypan blue exclusion method and the number and size of cell aggregates determined. The aggregations were classified as single-cell, 2-, 3-, 4-, 5- and more-than-5-cell aggregates.

2.2.21. Determination of aggregation following cryopreservation and growth in static cultures

In order to establish if the $p21^{CIP1}$-adapted cell-lines were better able to return to their state of adaptation following growth as static cultures or after periods of cryopreservation, protein-free suspension cultures that had previously been adapted to these conditions with or without the use of periods of $p21^{CIP1}$-induced cell cycle arrest were seeded into 75ml tissue culture flasks containing protein free-culture medium (ProCHO4 Cambrex) supplemented with 50μM MSX at a concentration of 2×10^5 cells/ml. All cells were allowed to attach to the flasks' surfaces and to divide for 72 hours, after which they were removed from the flasks by washing the cells with warm 1× PBS followed by the addition of 1x non-enzymatic cell

dissociation solution (Sigma, Poole, UK). Cells were then washed with warm protein-free medium before being centrifuged at 90g for 5 minutes and re-suspended at a cell density of 4×10^5 cells/ml back into a protein free suspension environment consisting of 100ml of fresh protein-free growth medium supplemented with 50μM MSX in vented 250ml Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37°C in the presence of 5% CO_2. Samples were taken in triplicate at 72 hour intervals for viability and determination of aggregate formation, as described above, before being passaged back to 4×10^5 cells/ml.

To find out if the characteristics of adapted cells would remain unchanged after cryopreservation, cells previously adapted to growth as protein free suspension cultures were frozen down as described on page 78. Following 6 weeks of storage, vials were removed from the liquid nitrogen storage and thawed according to the normal procedure described previously. The cells were then centrifuged at 90g for 5 minutes and re-suspended into a suspension culture consisting of 100ml of fresh, pre-warmed, protein-free growth medium supplemented with 50μM MSX in vented 250ml Erlenmeyer flasks. The cultures were agitated at a rate of 125 rpm at 37 ◦C in the presence of 5% CO_2. Samples were taken at 72 h intervals for viability and determination of aggregate formation before being passaged back to 4×10^5 cells/ml. The aggregations were classified as single-cell, 2-, 3-, 4-, 5- and more-than-5-cell aggregates.

2.2.22. The effect of $p21^{CIP1}$-expression on anchorage-dependent growth

Monolayer cultures were set up in duplicate in vented T75 flasks seeded at a density of 4×10^5 cells/ml. At the start of experimentation, one flask had 5mM IPTG (Sigma-Aldrich) added in order to initiate cell cycle arrest, and the remaining culture was left un-arrested. Following 24 hours growth as static cultures, cells were washed with a warm 1× PBS solution before the

addition of 10ml of 1:1 dilution of non enzymatic cell dissociation: 1× PBS solution. At 1-minute intervals, samples were taken in triplicate for determination of viable and total cell numbers via Trypan blue exclusion.

2.3 Cell culture calculations

The calculations described in this section are those utilised in chapters 3, 4, 5 and 6 to determine the validity of the results obtained

2.3.1. The specific growth rate.

This provides a measurement of the rate of growth per unit of biomass based on the assumption that all cells within the culture are within the exponential growth phase. In order to determine the growth rate between two points the following calculation was used.

$$\mu = (\mathrm{Ln}\ Xv_1 - \mathrm{Ln}Xv_0) / (t_1 - t_0)$$

Where: Ln = Natural logarithm (log)

Xv_1 = Viable cell concentration at time 1 ($10^5 ml^{-1}$ or $10^6 ml^{-1}$)

$Xv_1 > Xv_0$

Xv_0 = Viable cell concentration at time point 0 ($10^5 ml^{-1}$ or $10^6 ml^{-1}$)

t_1 Elapsed time 1

$t_1 > t_0$

t_0 = Elapsed time 0

2.3.2. The doubling time

Doubling time is another method for providing the growth rate at which a culture is growing and can therefore be related to the specific growth rate as follows

$$t_d = Ln2/\mu$$

Where: $Ln = Log_e 2$

μ = Specific growth rate as calculated previously

2.3.4. Cumulative cell time

The area under the growth profile is estimated as the sum of the individual areas associated with each pair of data points (see figure 2.2 below). The area of each rectangle is calculated as the average viable cell concentration between the two data points multiplied by the time difference between the two data points.

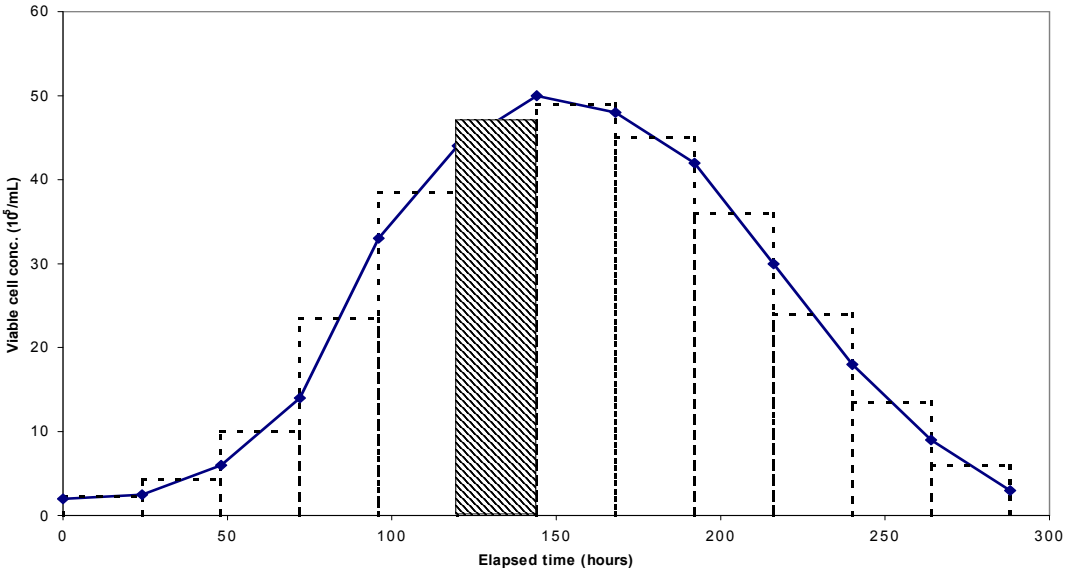
The area associated with an individual pair of data points as shown above is calculated using the following equation. Note that for consistency viable cell concentrations should be expressed in 10^6/ml giving CCT units of 10^9 cell h/l.

$$\text{Cell time} = \frac{Xv_0 + Xv_1}{2} . (t_1 - t_0)$$

Where:

	Xv_0	Viable cell concentration at first sample (10^6/ml)
	Xv_1	Viable cell concentration at second sample (10^6/ml)
	t_0	Elapsed time at first sample (hours)
	t_1	Elapsed time at second sample (hours)

Figure 2.2. Cumulative cell time as calculated during batch culture.



The calculation of cumulative cell time is an estimate of the area under the growth profile. Increasing the number of data points increases the accuracy of this estimation.

Example (highlighted area of figure 2.2)

Xv_0	44.0×10^5/ml = 4.4×10^6/ml
t_o	120 hours
Xv_1	50.0×10^5/ml = 5.0×10^6/ml
t_1	144 hours
Cell time	112.8×10^9 cell h/l

Cumulative cell time (CCT) is then calculated as the sum of the individual areas across the entire growth profile

$$CCT = CT_1 + CT_2 + CT_3 + \ldots\ldots + CT_n$$

2.3.5. Product concentration

The product concentration is obtained by dividing the amount of recombinant protein produced by the volume. Measurement of productivity was obtained with the standard analytical methods ELISA.

2.3.6. Specific Antibody production

The production of antibody is modeled as a function of the viable cell density. In batch culture, assuming there is no degradation of antibody, the equation describing the rate of change in antibody concentration is:

$$\frac{dAb}{dt} = q_P X_V$$

The specific rate of production qp (pg/cell/day) is assumed to be constant during the time interval measured.

Solving for the specific production rate:

$$q_P = \left[\frac{Ab_t - Ab_o}{Xv_t - Xv_o}\right] \ln\left[\frac{Xv_t}{Xv_o}\right]$$

Where

Ab_t = Antibody concentration at time 2

Ab_0 = Antibody concentration at time 0

Xv_t = Cell concentration at time 2

Xv_0 = Cell concentration at time 0

Alternatively specific productivity can be calculated by equating assayed product concentration with culture cumulative cell time. Typically two distinct specific production rates can be calculated.

- **q_p harvest**
- **q_p overall**

q_p harvest is calculated by equating the product concentration at harvest with the cumulative cell time at harvest

$$q_p \text{ harvest} = P_{harv} / CCT$$

Where:	**P_{harv}**	Product concentration at harvest (mg/L)
	CCT	Culture cumulative cell time (10^9 cell h/L)

q_p overall is calculated as the linear regression slope of the plot of product concentration against cumulative cell time. As for q_p harvest, q_p overall provides no information as to the productivity characteristics during culture. Because the calculation is based on multiple data points it is less prone to measurement errors.

2.3.7. Specific utilisation (or accumulation) rate

As per specific production rate; although more useful information is obtained by looking at smaller subsets of the entire data. Calculation of daily rates is the basis of some nutrient feed control strategies akin to the prediction of viable cell concentration detailed above.

2.4. Statistical analysis

The calculations described in this section are those utilised in chapters 3, 4, 5 and 6 to determine the significance of the results obtained

2.4.1. Standard error

The Standard Error, or Standard Error of the Mean, is an estimate of the standard deviation of the sampling distribution of means, based on the data from one or more random samples.

$$SD = \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

Where

SD = Standard deviation

X = Sample

$\bar{X}$ = Sample mean

n = Sample number

From the standard deviation the standard error of the mean is calculated as follows

$$SE = \frac{SD}{\sqrt{n}}$$

Where

SD = Standard deviation

n = Sample number

2.4.2. Students t-test

A t-test is any statistical hypothesis test in which the test statistic has a Student's t distribution if the null hypothesis is true. There are different versions of the *t* test depending on whether the two samples are independent of each other or paired, so that each member of one sample has a unique relationship with a particular member of the other sample.

$$t = \frac{\overline{X}_1 - \overline{X}_2}{s_{\overline{X}_1 - \overline{X}_2}} \text{ where } s_{\overline{X}_1 - \overline{X}_2} = \sqrt{\frac{s_1^2 + s_2^2}{n}}$$

Where s is the grand standard deviation (or pooled sample standard deviation), 1 = group one, 2 = group two. The denominator is the standard error of the difference between two means. For significance testing, the degrees of freedom for this test is 2n − 2 where n = # of participants in each group.

2.4.3. P-value

Once a *t* value is determined, a p-value can be found using a table of values from Students t-distribution. The p-value of a statistical significance test represents the probability of obtaining values of the test statistic that are equal or greater in magnitude than the observed test statistic.

3. Multigenic expression of $p21^{CIP1}$/Bcl-2 and IgG in a CHO cell-line for improved productivity and increased cellular robustness

3.1. Abstract

The ability to extend a period of high productivity at high cell density is extremely desirable for the industrial production of biopharmaceuticals. Previous studies of low density cultures have shown that arresting cell proliferation in the G1 phase of the cell cycle results in an increased productivity. This increase in productivity is often accompanied by a much less desirable increase in the rate of apoptosis as a result of preventing cell cycle progression and thus cell division. In this study we have overcome this problem via the multigenic manipulation of both the cell cycle and apoptosis to significantly enhance the specific production rates of an industrially relevant CHO cell-line, whilst also enhancing cellular robustness through a reduction in apoptosis, thereby increasing the overall time scale for which productivity can be maintained. We examined the growth and productivity profiles of several CHO clones, which have been engineered to constitutively expresses a chimeric IgG4 antibody, co-transfected to inducibly expresses the $p21^{CIP1}$ cyclin-dependent kinase inhibitor and the wild type Bcl-2 anti-apoptotic protein. The resulting clones were analysed for their expression of $p21^{CIP1}$ and Bcl-2 as well as the impact upon cell proliferation, cell viability during batch culture, serum deprivation and exposure to staurosporine. All clones exhibited some levels of reduction in cell proliferation following induction of $p21^{CIP1}$ and all demonstrated some degree of increased cellular robustness when faced with chemical toxicity or serum deprivation in comparison to the control cell-lines. Through the combined expression of $p21^{CIP1}$ and Bcl-2 this study has demonstrated that it is not only possible to substantially improve productivity but that it is also possible to break the link between cell cycle arrest

and the initiation of apoptosis leading to an increase in the overall length of viable cell culture at much higher production rates.

3.2. Introduction

In recent years significant progress has been made in the development of strategies for the optimisation of growth conditions, in order to maximize the production potential of mammalian cell cultures. As our understanding of the mechanisms that regulate cell proliferation, cell death, and the protein synthesis and secretion have increased, new strategies have been developed which have enabled production processes to be intensified. Despite this progress, the scale-up and optimization of cell cultures to meet the ever increasing demands of the biopharmaceutical industry has proved to be extremely challenging. This is particularly true for mammalian cell-line cultures, which in comparison to most other host systems have a tendency to result in low productivity levels and instability in the cell-lines used. Despite this CHO has remained the preferred choice for the large scale production of recombinant protein, due to the fact that they are fast growing, relatively easy to transfect and have the ability to perform complex post-translational modification required for biological activity

In order to optimize the mammalian cell-line production processes active cell growth, without the accumulation of toxic metabolites or limiting conditions that will confer loss of cell viability and productivity are essential. In an ideal situation the cells would be allowed to expand until they have reached their peak productivity phase, at which point they are maintained by the sustained induction of cell cycle arrest and thus cessation of

cell division. This shift away from cell replication would allow for the diversion of energy away from cell division, towards the synthesis of protein products whilst avoiding the problems associated with high cell density cultures (*Al Rubeai et al 1992a, Kaufmann et al 2001, Simpson et al 1999, Watanabe et al 2001 and 2002, Bi et al 2004)*.

Several strategies designed to control cell proliferation have been developed including the transfection of industrially relevant cell-lines with cytostatic genes. One of the earliest attempts involved the expression of the interferon-repressive factor 1 (IRF-1) which was found to inhibit cell growth as well as producing tumour suppression activities within the transfected cell-line *(Kirchhoff et al 1993 and 1996, Geserick et al 2000, Carvalhal et al 2001, Schroeder et al 2002).* Alternate efforts by our laboratory and others have focused on cytostatic genes such as cdk inhibitors, in particular p21, p27 and the tumour suppressor gene p53, all of which have been shown to result in a G1 phase cell cycle arrest following their over expression *(Fussenegger et al 1997 and 1998, Mazur et al 1998, Fussenegger and Bailey 1998, Kaufmann et al 2001, Fussenegger 2001 review, Watanabe et al 2001 and 2002, Bi et al 2004, Lloyd et al 1997).*

As discussed in the introduction of this thesis, $p21^{CIP1}$ is one of the key cyclin-dependent kinases inhibitors which are responsible for the regulation of cell cycle progression and is known to inhibit kinase activity in a wide range of cyclin-cyclin-dependent kinase complexes involved in the G1/S phase transition of the cell cycle *(Gartel et al 1996 review, Harper et al 1993 and 1995, Xiong et al 1993).* Of interest here is the fact that earlier studies by our group have demonstrated that during periods of G1 phase cell cycle

arrest in both CHO and NS0 cell-lines, productivity is substantially enhanced *(Watanabe et al 2001 and 2002, Bi et al 2004, Ibarra et al 2003).* It has been suggested that this improvement in productivity occurs as a direct result of deferring the energy usually required for cell division away from the cell cycle machinery. This energy may then be re-focused towards the process of product formation.

Although the prevention of cell cycle progression potentially offers a solution to low production levels, studies by our laboratory and others have shown that despite the maintenance of low cell densities apoptosis remains a problem during periods of cell cycle arrest and may in fact occur as a direct result of preventing cell cycle progression *(Singh et al 1994, Tey et al 2000, Watanabe et al 2002).* Apoptosis also presents a problem in proliferating cultures and in recent years several studies have demonstrated that during large scale cultivation in the bioreactor environment mammalian cells are prone to undergo apoptotic cell death as a result of either amino acid, glucose, oxygen and serum deprivation, the build up of toxic metabolites or as a result of shear forces within the bioreactor environment itself *(Al-Rubeai et al 1990 and 1992a, Franek and Dolnikova 1991, Mercille and Massie 1994, Singh et al 1994).*

In order to overcome this problem several groups have investigated the possibility of improving cellular robustness through the transfection of cell-lines with genes possessing anti-apoptotic properties. The most commonly used target for this type of research has been the well classified Bcl-2 gene. So far the transfection of several cell-lines including Burkett's Lymphoma *(Singh et al 1994)* insect cells *(Alnemri et al 1992, Mitchell-Logean*

and Murhammer 1997) and hybridoma cell-lines *(Itoh et al 1995, Simpson et al 1998, Fassnacht et al 1998b and 1999)* with anti-apoptotic genes, have all shown a significant improvement in the overall cellular robustness achievable. As a consequence Bcl-2-expressing cultures exhibit an extended lifespan which has been shown to result in 40–400% greater productivity of monoclonal antibody *(Itoh et al 1995, Simpson et al 1997, Suzuki et al 1997, Terada et al 1997)*

I hypothesise that it may be possible to create a CHO cell-line in which the expression of $p21^{CIP1}$ may be combined with the anti-apoptotic properties of Bcl-2 and that in doing so we will be able to create a cell-line capable of producing increased levels of monoclonal antibody for extended periods of time. More over we would expect such a cell-line to respond more favourably to the stresses imposed by the culture environment. To achieve this we have adopted a genetic strategy in which the effect of apoptosis due to cell cycle arrest can be eliminated through the over-expression of the anti-apoptotic Bcl-2 gene. Such a strategy based on the simultaneous control of cell proliferation and apoptosis would obviously offer significant advantages for the improvement of culture productivity.

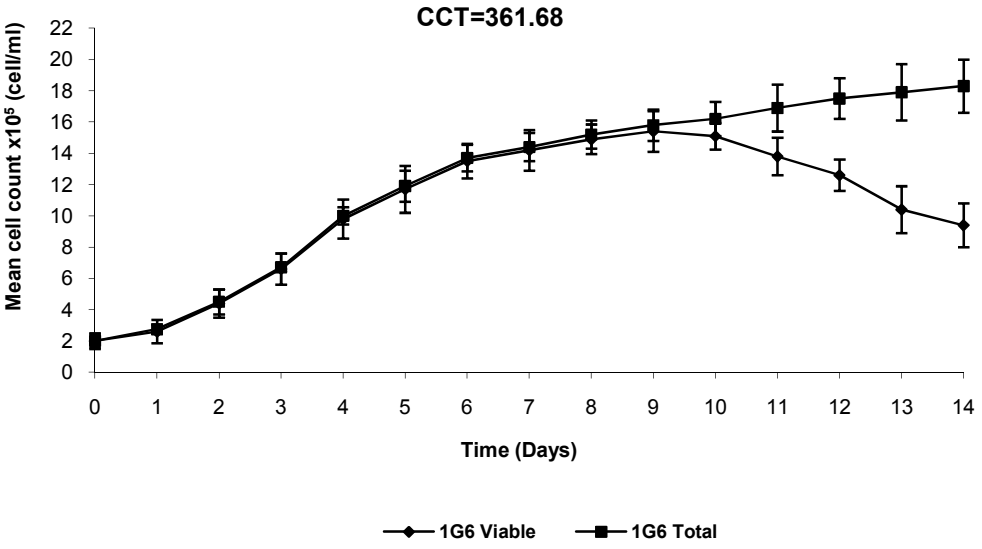
3.3. Results.

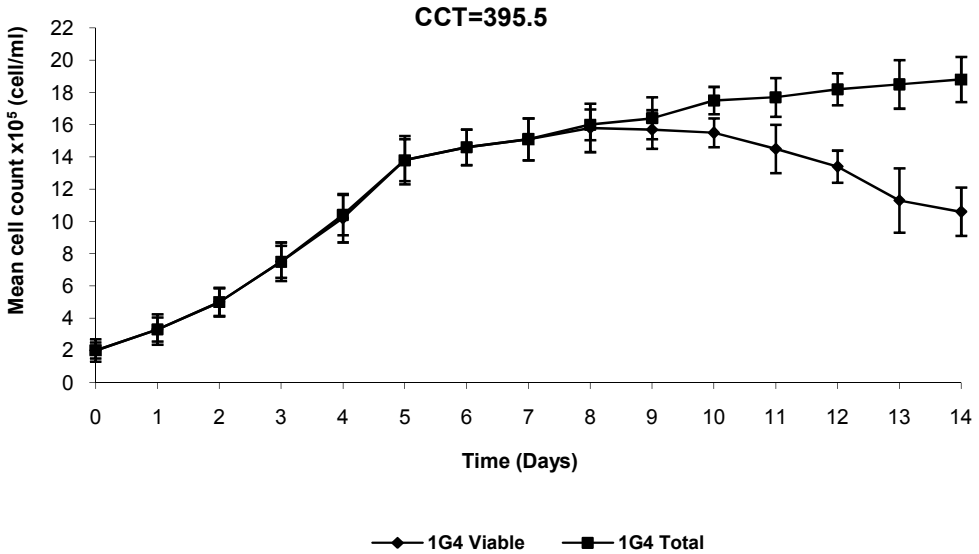
To create a Bcl-2-and-$p21^{CIP1}$-coexpressing cell-line the expression vector pEF*Bcl-2* and control vector pEF*neo* was first introduced into the parental 22H11 line via liposome-mediated transfection as described in the Materials and Methods. The 22H11 cell-line has previously been engineered to express the human-mouse chimeric B72.3 IgG4 antibody

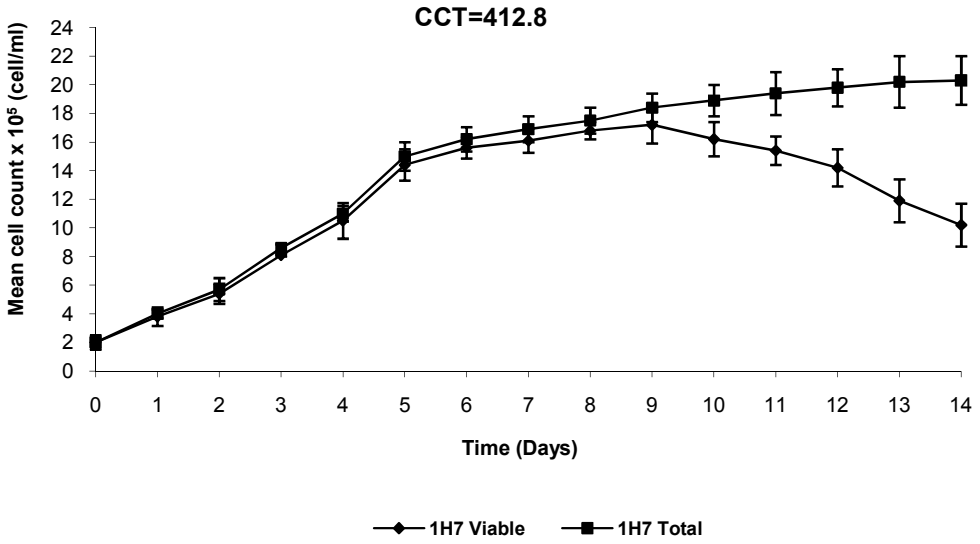
gene, using the glutamine synthetase system and in order to obtain inducible expression of $p21^{CIP1}$ the Lacswitch system was used. Bcl-2 transfected cells were selected by the addition of 400μg/ml Hygromycin B to the growth medium for a period of 2 weeks; after which, the whole population of stable transfectants were then transfected with the pOPRSV1/$p21^{CIP1}$ plasmid DNA containing the mouse $p21^{CIP1}$ complementary DNA using Lipofectin reagent. Stable clones were selected in the presence of 400μg/ml Geneticin 418 and isolated by limiting dilution. The resulting transfectants were then cloned via limiting dilution to produce the clones used for the rest of this study.

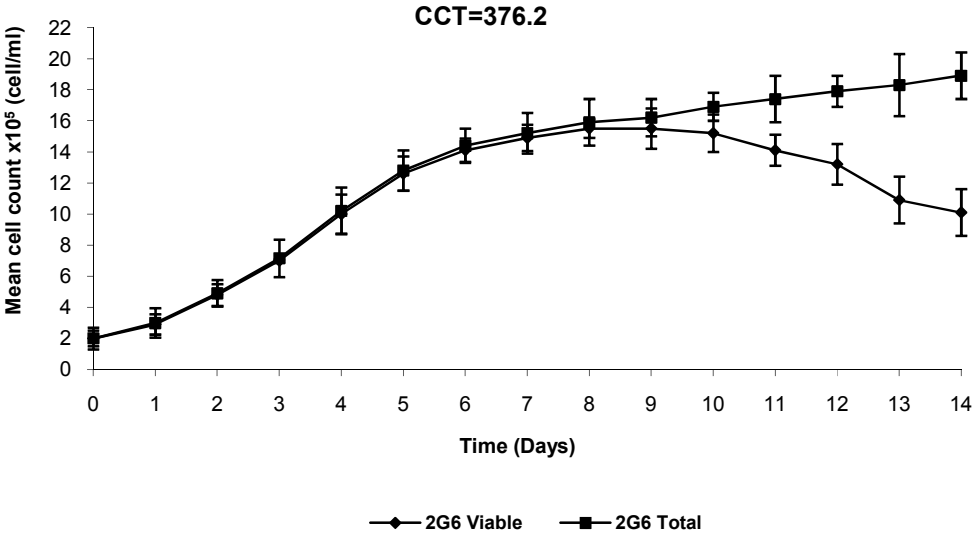
3.3.1. The growth profile of $p21^{CIP1}$/Bcl-2-expressing clones

Before batch analysis of the resulting clones was carried out the stability of the clones, in terms of growth rate and antibody production was carefully monitored in triplicate for a period of 30 days prior to the initiation of experiments. The clones were cultured in tissue culture flasks, seeded at a density of 2×10^5 (cells/ml) and passaged at 72 to 96 hour intervals dependent on cell density. Throughout this period the maximum obtainable viable cell count for each of the clones remained unchanged indicating a constant growth rate. Figure 3.1 shows the typical growth curve obtained for each of the clones and shows a relatively narrow spectrum of maximum obtainable viable cell densities ranging from 17.5×10^5 cells/ml for 1H7 to 15.4×10^5 cells/ml for clone 1G6 a difference of 2.1×10^5 cells/ml.


CCT=361.68
Mean cell count x10⁵ (cell/ml)
Time (Days)
1G6 Viable
1G6 Total


CCT=395.5
Mean cell count x10⁵ (cell/ml)
Time (Days)
1G4 Viable
1G4 Total


CCT=412.8
Mean cell count x 10⁵ (cell/ml)
Time (Days)
1H7 Viable
1H7 Total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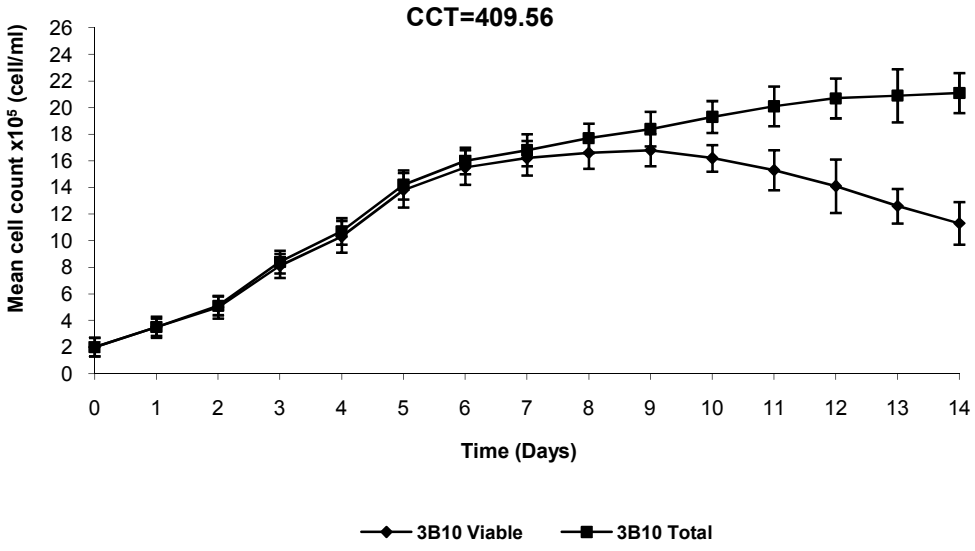
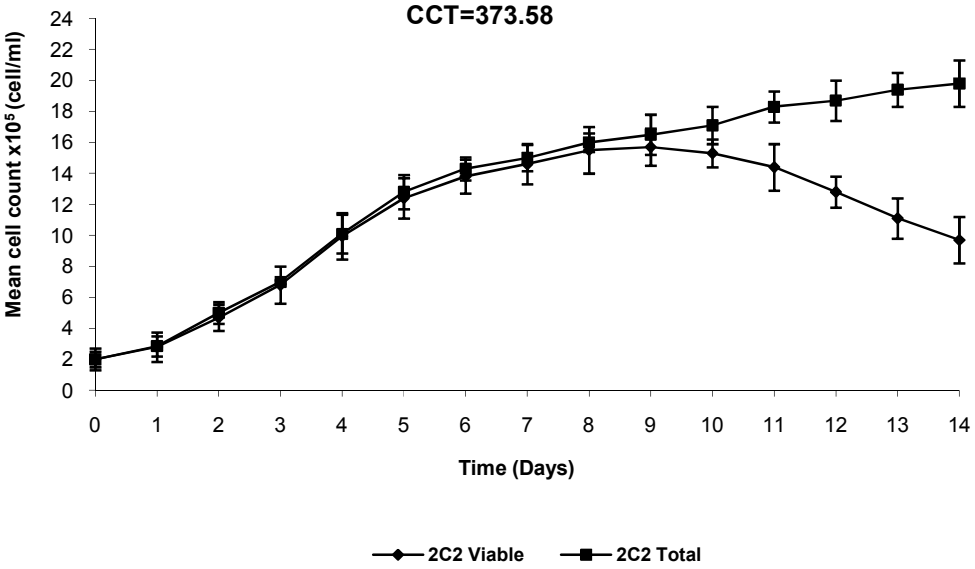
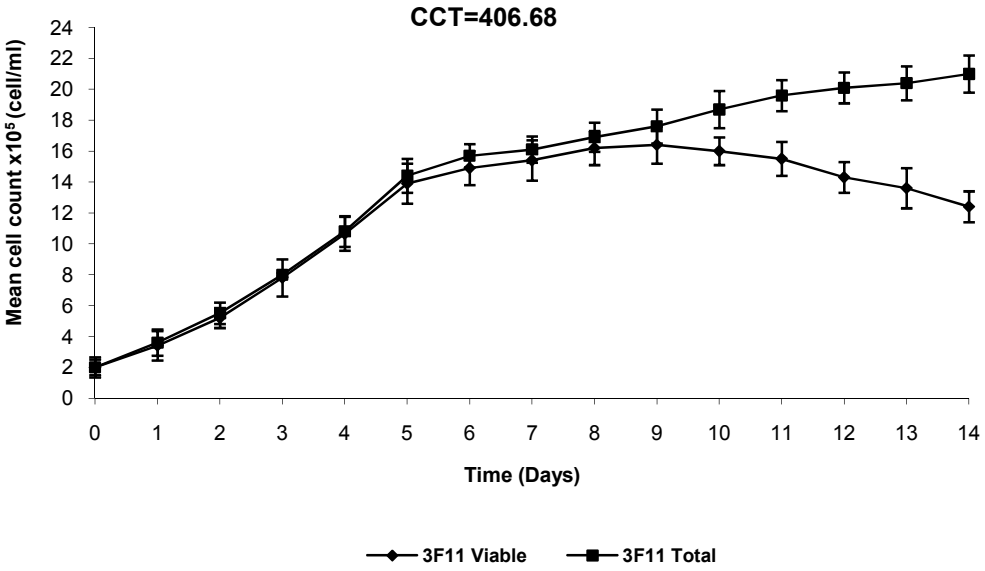
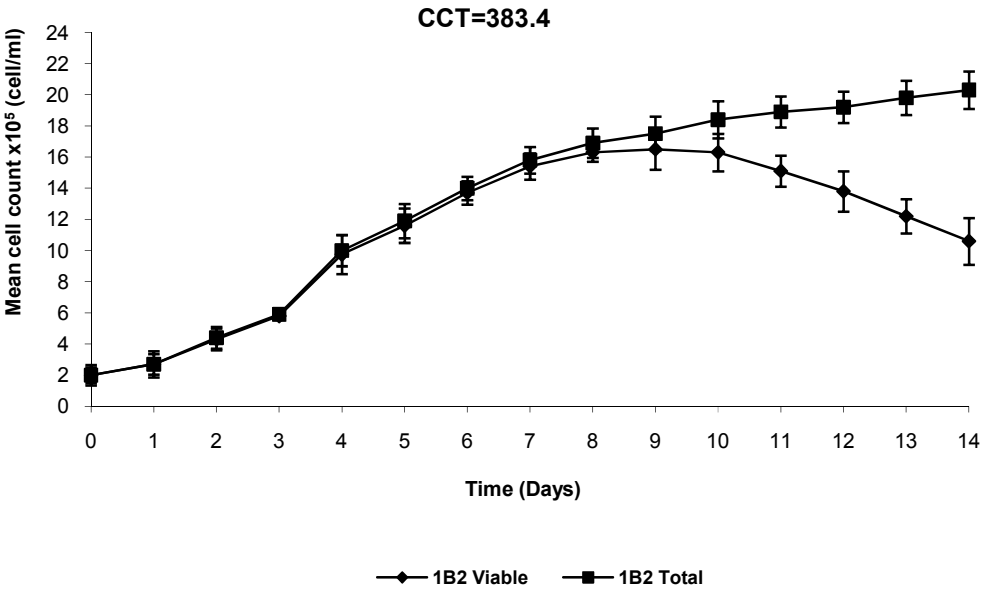
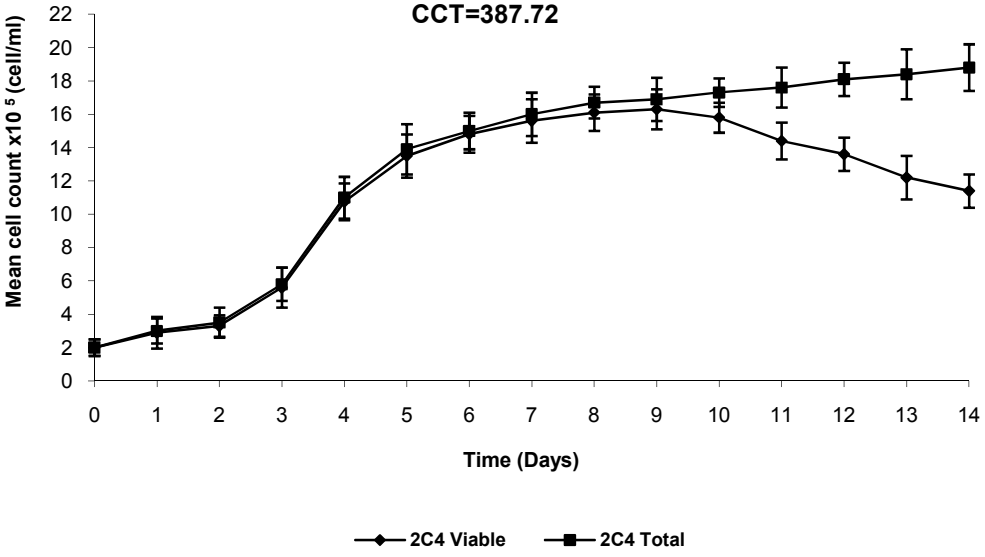
Mean cell count x10⁵ (cell/ml)
Time (Days)
2G6 Viable
2G6 Total

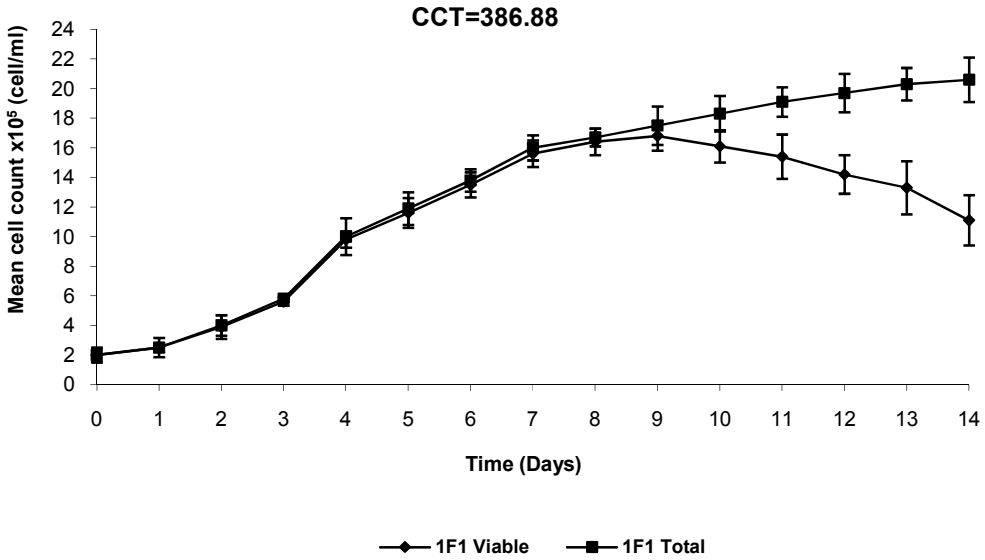

CCT=409.56
Mean cell count x10⁵ (cell/ml)
Time (Days)
3B10 Viable
3B10 Total


CCT=373.58
Mean cell count x10⁵ (cell/ml)
Time (Days)
2C2 Viable
2C2 Total


CCT=406.68
Mean cell count x10⁵ (cell/ml)
Time (Days)
3F11 Viable
3F11 Total


CCT=383.4
Mean cell count x10⁵ (cell/ml)
Time (Days)
1B2 Viable
1B2 Total


CCT=387.72
Mean cell count x10 5 (cell/ml)
Time (Days)
2C4 Viable
2C4 Total


CCT=386.88
Mean cell count x10⁵ (cell/ml)
Time (Days)
1F1 Viable
1F1 Total

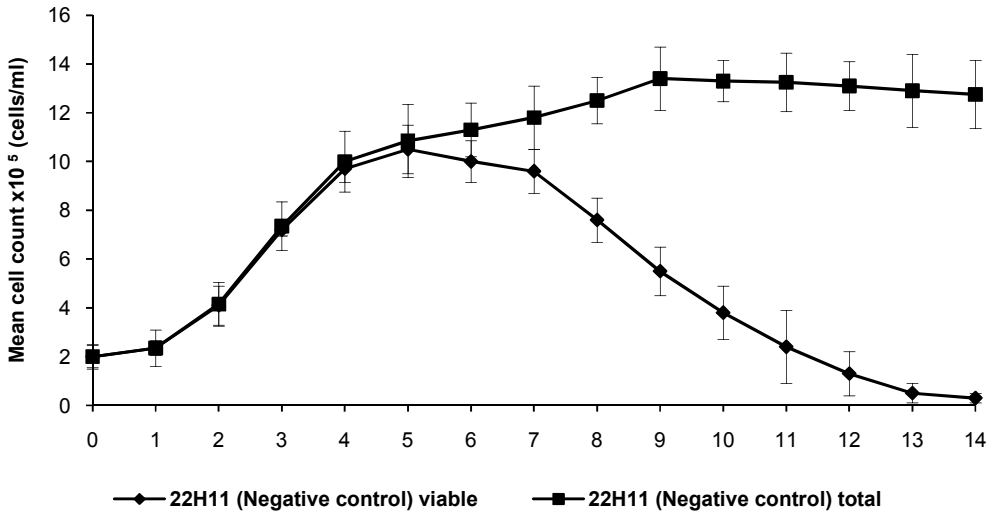


Figure 3.1. Static batch cultures of p21^{CIP1}/ Bcl-2 transfected clones. In order to determine the growth profiles of each of the clones they were cultured in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum. p21^{CIP1}-expression was not induced during the culture period however Bcl-2 was expressed throughout. Cultures were set up in T75 tissue culture flasks and incubated at 37^{o}C. Error bars show the standard error of three replicates. The cumulative cell time (CCT) was calculated in order to give an estimate of the integral of the viable cell concentration against a time curve. The effect of Bcl-2-expression was compared to the non-p21^{CIP1}/Bcl-2-expressing negative control cell-line (22H11) to determine if Bcl-2-expression could have an effect on the culture viability and duration.

The typical growth curve for the 10 resulting clones was accessed over 336 hours (Figure 3.1) the exponential growth phase was found to last between 192 to 216 hours, dependent on the clone, at which point the viability begins to decline. In order to access the number of viable cells produced during the period of batch culture, the cumulative cell time (CCT) was used as this allows for a direct comparison of total cell growth for each of the clones. Clone 1H7 had the highest CCT where as clone 1G6 was found to have the

lowest. All of the clones exhibited a similar overall viability with the onset of cell death occurring between 192 to 216 hours. Figure 3.2 shows the specific growth rates for each of the clones and although there appears to be some variation between the highest (0.01061 hr^{-1}) and the lowest (0.01016 hr^{-1}) the actual difference found to be less than 5% and therefore is not considered significant.

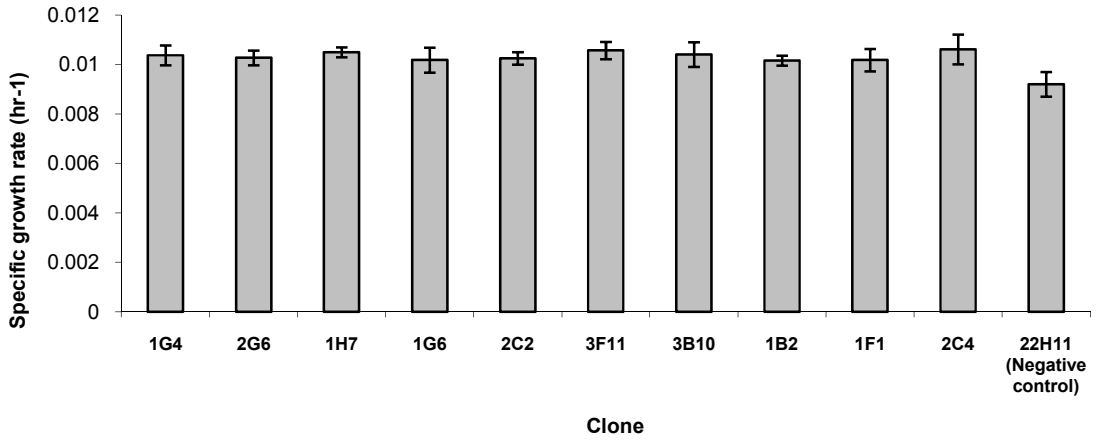


Figure 3.2. Specific growth rate. Results were obtained during the exponential growth phase of non-induced $p21^{CIP1}$/Bcl-2-transfected clones grown as static cultures in T75 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum. $p21^{CIP1}$ was not expressed during the culture period however Bcl-2 was continuously expressed. The specific growth rates of the transfected clones were compared to a negative control cell-line (22H11) which does not over-express either $p21^{CIP1}$ or Bcl-2. Error bars show the standard error of three replicates.

3.3.2. The effect of $p21^{CIP1}$-expression on $p21^{CIP1}$/Bcl-2-expressing clones.

To access the effects of $p21^{CIP1}$-expression on the resulting clones, their growth behaviour was examined during $p21^{CIP1}$-induced and non-induced culture conditions over a period of 96 hours (Figure 3.3) All clones were seeded in duplicate into 75ml tissue

culture flasks at an initial density of 2 x 10^5 cells/ml with or without the addition of 5mM IPTG. Following 96 hours growth all cultures were analysed. This process was then repeated in triplicate to get the standard error of the mean. Positive, $p21^{CIP1}$-expressing (3B2) and negative, non-$p21^{CIP1}$-expressing cell-lines (22H11) were used as controls throughout.

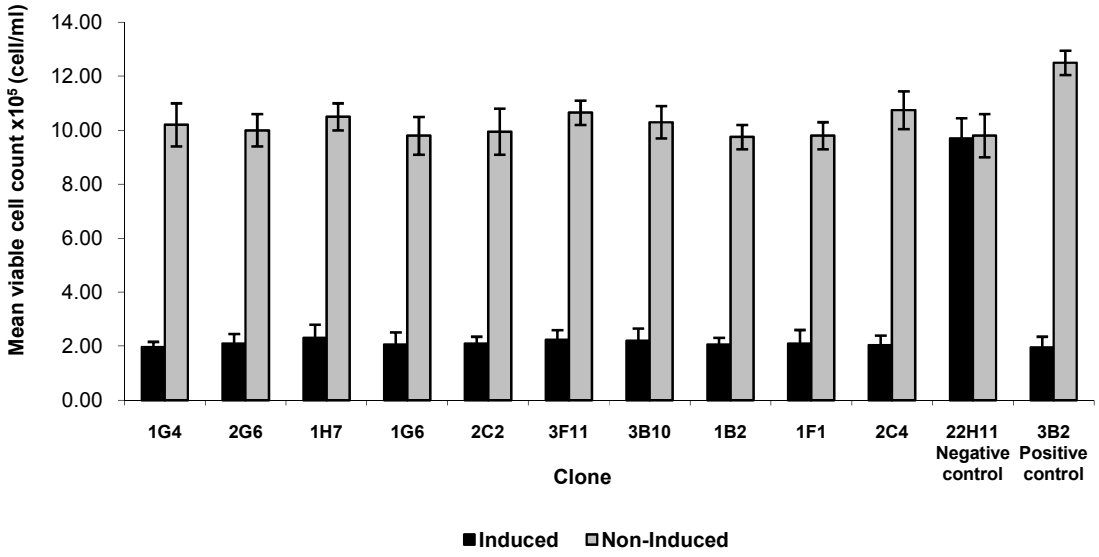


Figure 3.3. The effect of $p21^{CIP1}$-expression on the viable cell count of $p21^{CIP1}$/Bcl-2-transfected cell-lines following 96 hours of static culture in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum. Black bars represent the viable cell count in cultures in which the addition of 5mM IPTG at the start of experimentation has resulted in $p21^{CIP1}$-induced cell cycle arrest. Grey bars represent cultures which have not been exposed to IPTG and therefore are not over-expressing $p21^{CIP1}$. Cultures were set up in triplicate in order to determine the standard error of the mean. Student's *t*-test indicated significant differences between the $p21^{CIP1}$/Bcl-2-expressing clones and the negative control which does not express $p21^{CIP1}$ or Bcl-2 ($p < 0.0001$). A positive control cell-line expressing $p21^{CIP1}$ (previously described in the Materials and Methods) was also used in order to compare the effect of combined $p21^{CIP1}$/Bcl-2-expression to $p21^{CIP1}$-expression alone.

Although there was some variation in the levels of cell cycle arrest amongst the clones, all demonstrated high levels of $p21^{CIP1}$-expression when compared to the non-$p21^{CIP1}$-expressing control cell-line. Clone 2C4 exhibited excellent cell cycle arrest following the addition of IPTG and also showed the highest viable cell count when $p21^{CIP1}$-expression was not induced. Clones 1H7 and 3F11 also demonstrated a good viable cell count when not arrested, however the expression of $p21^{CIP1}$ in these clones failed to result in complete cessation of the cell cycle. All clones exhibited a lower overall cell count than the $p21^{CIP1}$-expressing control cell-line, indicating that the additional expression of Bcl-2 may have a negative impact on the growth rate of the $p21^{CIP1}$/Bcl-2-expressing clones. Expression of $p21^{CIP1}$ was additionally confirmed via the Western blot technique as described in the Materials and Methods. Samples were taken following 96 hours of growth from cultures in which $p21^{CIP1}$-expression had either been induced through the addition of IPTG at the beginning of experimentation or had remained un-induced throughout (Figure 3.4).

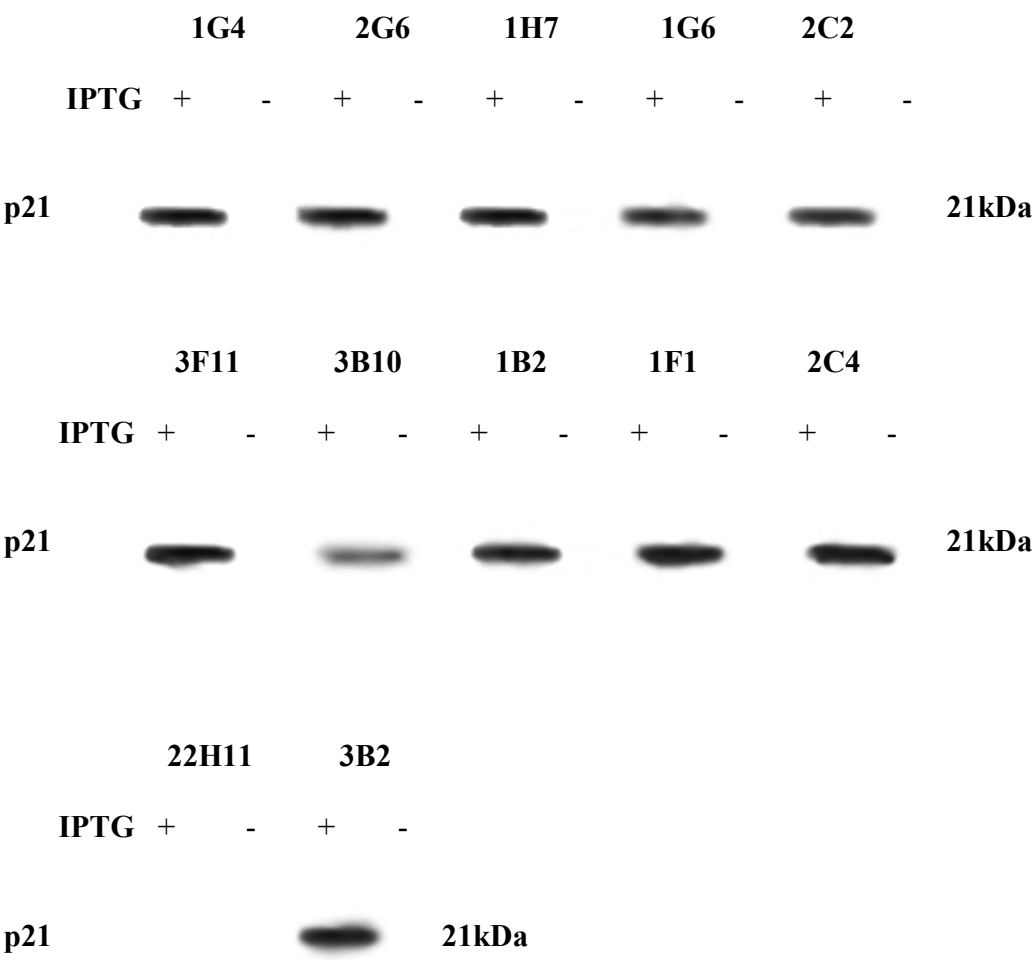


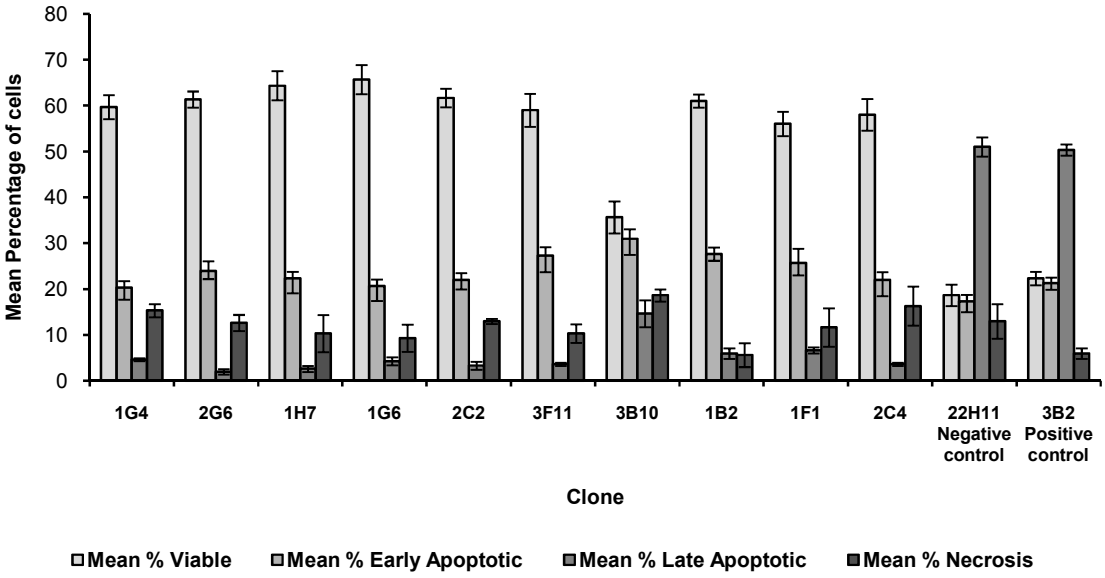
Figure 3.4. Western blot analysis of $p21^{CIP1}$-expression in $p21^{CIP1}$/Bcl-2-expressing clones. Samples were analysed following 96 hours growth as static batch cultures grown in T75 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum. Each clone was cultured either with or without the addition of 5mM IPTG in order to induce $p21^{CIP1}$-expression at the start of experimentation. 22H11 was used as a negative control as this has not been transfected with $p21^{CIP1}$. 3B2 was used as a positive control as it has previously been transfected with $p21^{CIP1}$ (as described in the Materials and Methods). Each well was loaded with 30µl of sample which was previously normalised to the same cell density.

3.3.3. The effect of Bcl-2-expression on $p21^{CIP1}$/Bcl-2-expressing clones

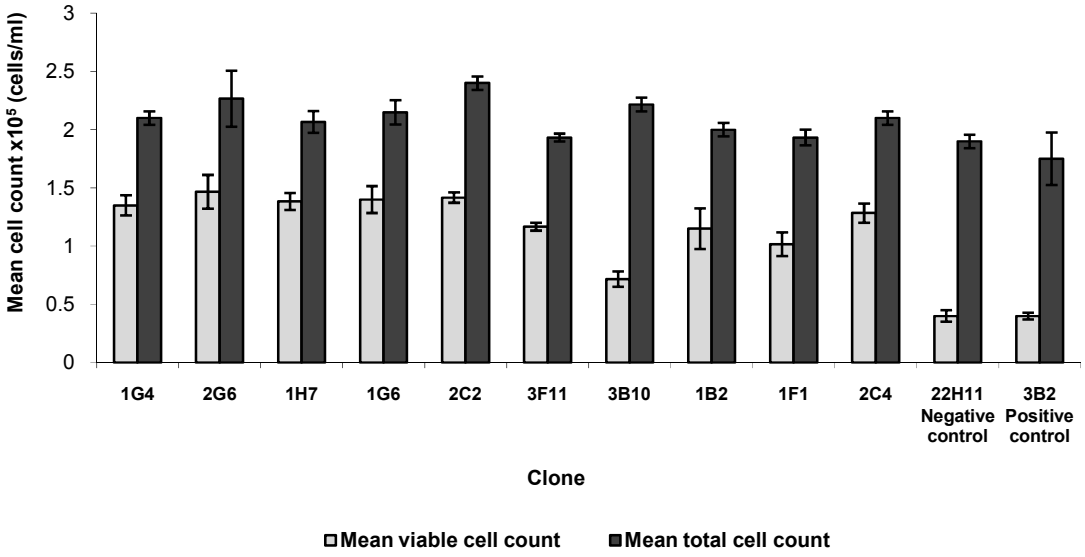
To determine that the expression of the anti-apoptotic Bcl-2 gene would result in enhanced cell survival, the clones were exposed to the toxic effects of the protein kinase inhibitor staurosporine. Cultures were set up in triplicate as described previously in the Materials and Methods. At the start of experimentation staurosporine was added to each of the culture flasks at a concentration of 0.5μM. After 24 hours exposure, cells were harvested from their tissue culture flasks and the viability determined via the Trypan blue exclusion method. Levels of apoptosis and necrosis were also determined for each of the clones using fluorescence microscopy and the AOPI staining method. In order to determine that the transfection of $p21^{CIP1}$ had no positive influence on culture viability and apoptosis, a second control cell-line, which had previously transfected with $p21^{CIP1}$ was used along with a non-$p21^{CIP1}$/Bcl-2-expressing control.

The results shown in (Figure 3.5A) demonstrate that in all of the clones tested, the expression of Bcl-2 is able to produce a significant reduction in the percentage of cells undergoing both early and late apoptosis, however there was also a slight increase in the percentage of cells dying by necrosis when compared to the non-Bcl-2-expressing controls, suggesting that Bcl-2 is able to offer protection against apoptosis but not necrosis.

A


Mean Percentage of cells
80
70
60
50
40
30
20
10
0
1G4
2G6
1H7
1G6
2C2
3F11
3B10
1B2
1F1
2C4
22H11 Negative control
3B2 Positive control
Clone
Mean % Viable
Mean % Early Apoptotic
Mean % Late Apoptotic
Mean % Necrosis

B


Mean cell count x10^5 (cells/ml)
3
2.5
2
1.5
1
0.5
0
1G4
2G6
1H7
1G6
2C2
3F11
3B10
1B2
1F1
2C4
22H11 Negative control
3B2 Positive control
Clone
Mean viable cell count
Mean total cell count

C

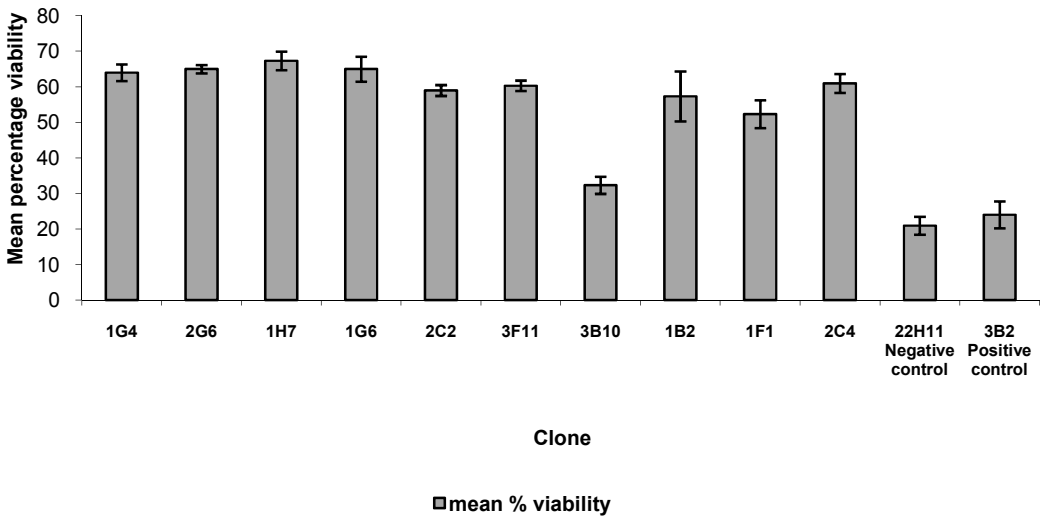


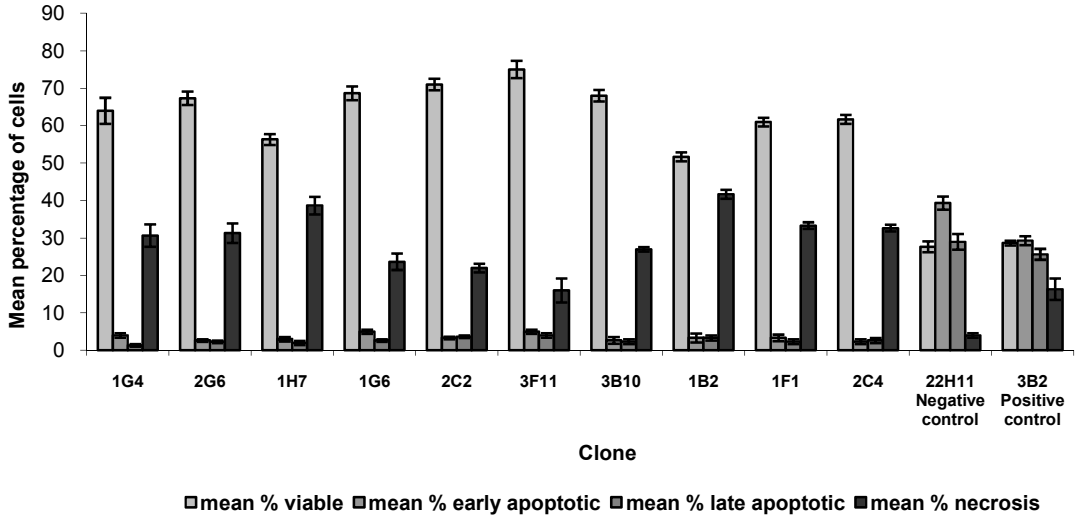
Figure 3.5. The effect of staurosporine on p21^{CIP1}/Bcl-2-expressing clones. A) shows the effect of Bcl-2-expression on apoptosis following exposure to staurosporine as determined by AOPI. B) and C) show the effect on the viable cell count and culture viability as determined via the Trypan blue exclusion method. Clones previously grown as static cultures in DMEM F12 culture medium containing 10% heat inactivated foetal calf serum and 50µM MSX were washed with 1x PBS before being removed from their tissue culture flasks using 1x cell dissociation solution. A 1x PBS wash was repeated before cells were centrifuged at 90g for 5 minutes. Cells were then re-suspended into fresh T25 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX at a cell density of 1x10^5 cells/ml. 0.5µM staurosporine was added to each flask before the cultures were incubated at 37ºC for 24 hours. Cultures were then harvested as described in the Materials and Methods and analysed for cell death using the AOPI staining method. Cell viability was also determined via the Trypan blue exclusion method. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the Bcl-2-expressing clones and the negative control (22H11) which does not express p21^{CIP1} or Bcl-2 ($p < 0.0001$). To determine that the transfection of p21^{CIP1} had no effect on culture viability or apoptosis a control cell-line (3B2) which had previously transfected with p21^{CIP1} (see chapter 2 Materials and Methods) was also used.

Following exposure to staurosporine clone 1G6 maintained the highest viable cell count as determined through AOPI staining (Figure 3.5A) and also demonstrated a substantial reduction in the percentage of early and late apoptotic cells when compared to the non-Bcl-2-expressing control cell-lines. In contrast clone 3B10 showed a relatively poor viable cell count and an increased percentage of both apoptotic and necrotic fractions, this however was still an improvement on the non-expressing controls. When the clones were analysed via Trypan blue exclusion, the results (5B and C) showed that during exposure to staurosporine the Bcl-2 clones still exhibited a higher viable and total cell count than the control cultures and most maintained a viability within the 60 to 70% range with the exception of 3B10 which only showed a 33% viability. This however was still higher than the control cultures which had viabilities ranging from 25 to 30%

To test the effect of Bcl-2-expression on nutrient limitation as a result of serum deprivation experiments were set up as described previously in the Materials and Methods. Cultures were placed DMEM F12 culture medium lacking foetal calf serum, following 72 hours growth cells were removed from their tissue culture flasks and examined via AOPI and Trypan blue exclusion method (Figure 3.6A-C). The results in Figure 3.6A show a clear reduction in the percentage of cells undergoing apoptosis for all of the Bcl-2-expressing clones when compared to the non-Bcl-2-expressing control cell-lines. Clone 3F11 demonstrated both the highest percentage of viable cells and the lowest percentage of necrotic cells; in contrast clone 1B2 exhibited both the lowest percentage of viable cells and the highest proportion of necrotic cells. As seen previously with exposure to staurosporine clones expressing Bcl-2 showed enhanced protection against

apoptosis but not necrosis and in comparison to the control cell-lines the level of necrosis was greatly increased. This would once again suggest a switch in the mode of cell death occurs during Bcl-2-expression.

A



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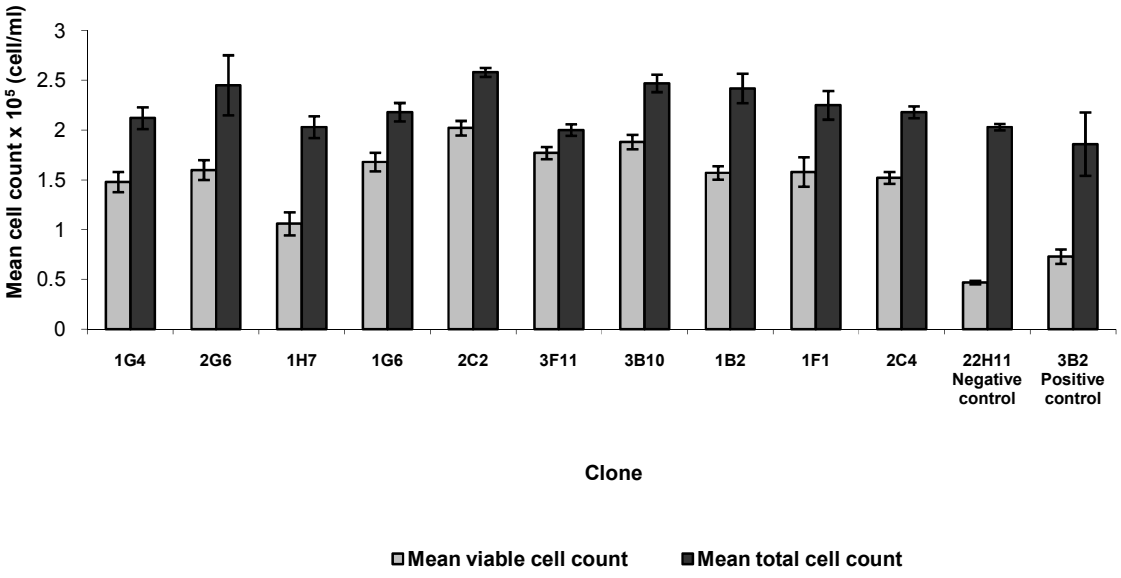

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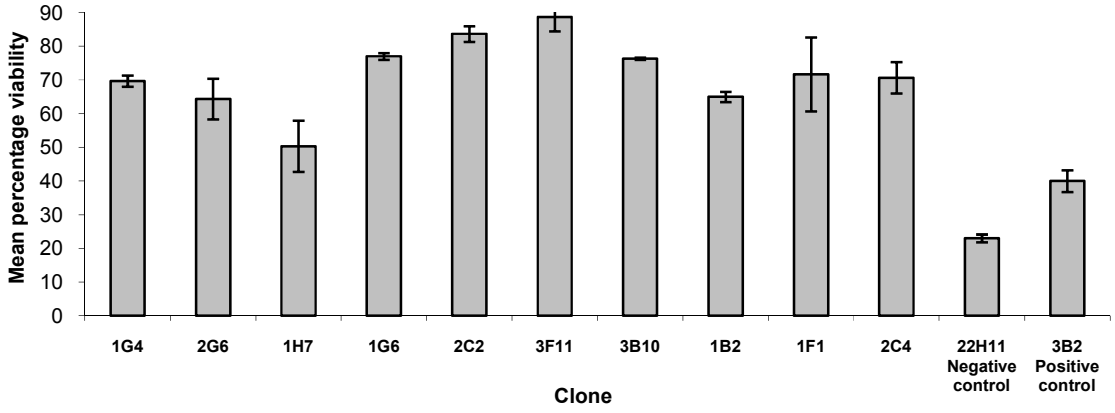


Figure 3.6. The effect of serum deprivation on p21^{CIP1}/Bcl-2-expressing clones. A) shows the effect of Bcl-2 on apoptosis following the withdrawal of serum from the culture medium as determined by AOPI. B) and C) show the effect on the viable cell count and culture viability as determined via the Trypan blue exclusion method. Clones previously grown as static cultures in DMEM F12 culture medium containing 10% heat inactivated foetal calf serum and 50µM MSX were washed with 1 x PBS before being removed from their tissue culture flasks using 1x cell dissociation solution. A 1x PBS wash was repeated before cells were centrifuged at 90g for 5 minutes. Cells were then re-suspended into fresh T25 tissue culture flasks containing DMEM F12 culture medium supplemented with 50µM MSX at a cell density of 1x10^5 cells/ml. The cultures were incubated at 37ºC for 72 hours. Cells were harvested as described in the Materials and Methods and analysed for cell death using the AOPI staining method. Cell viability was also determined via the Trypan blue exclusion method. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the Bcl-2-expressing clones and the negative control (22H11 as described in the chapter 2 Materials and Methods) which does not express p21^{CIP1} or Bcl-2 ($p < 0.0001$). To determine that the transfection of p21^{CIP1} had no effect on culture viability or apoptosis a control cell-line which had previously transfected with p21^{CIP1} (see chapter 2 Materials and Methods) was also used.

The results (Figure 3.6B and C) show the viable and total cell counts and percentage viability following 72 hours of serum deprivation, all clones showed a significantly higher viable cell count compared to the non-$p21^{CIP1}$/Bcl-2-expressing control lines, and most achieved an overall higher cell density. The results also show a marked decrease in cell proliferation during the 72 hour test period indicating the need for serum for cell proliferation.

All of the clones tested showed a significant improvement in the overall viability as determined via t-test analysis, demonstrating the effect of Bcl-2-expression; this was further confirmed via western blot analysis (Figure 3.7). Each of the clones was tested following 96 hours growth as static cultures. All clones appear to express the 26-kDa Bcl-2 protein; this however was not observed in the 22H11 control cell-lines.

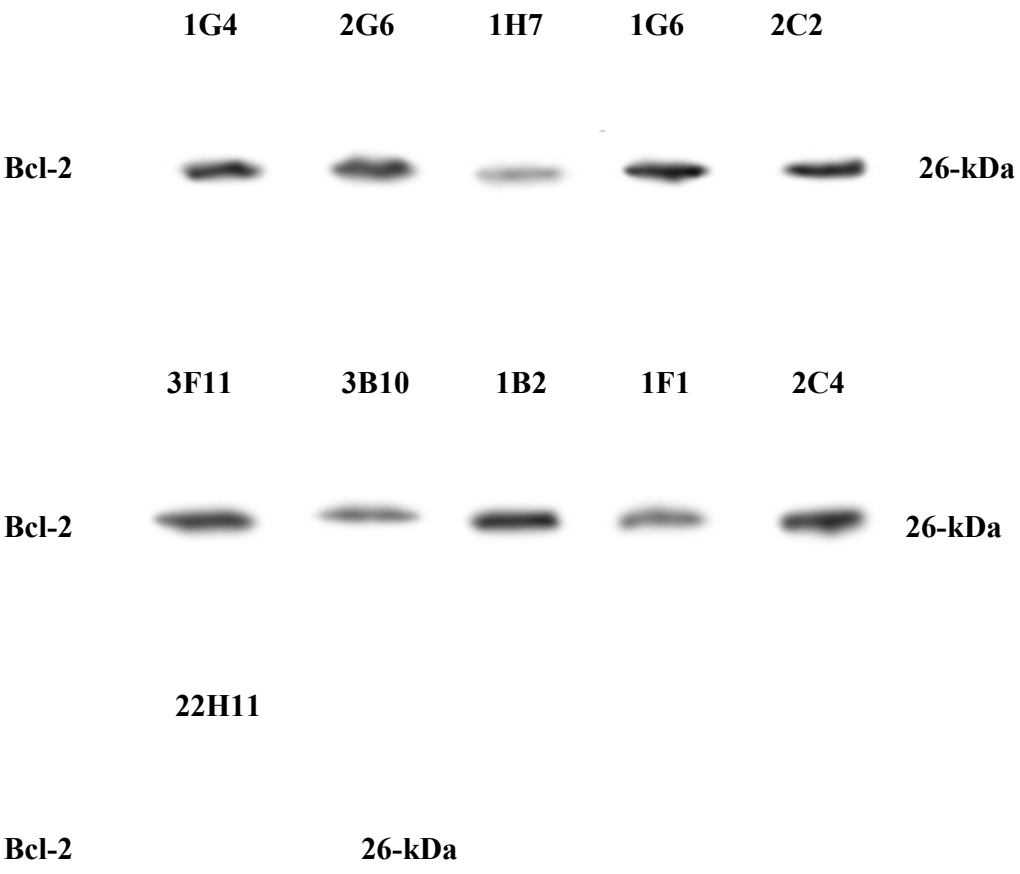


Figure 3.7. Western blot analysis of Bcl-2-expression in $p21^{CIP1}$/Bcl-2 transfected clones. Samples were analysed following 96 hours growth as static batch cultures grown in T75 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum. Each well was loaded with 30µl of sample which was previously normalised to the same cell density. The 22H11 cell-line (previously described in chapter 2 Materials and methods) was used as a negative control

3.3.4. Clone Productivity.

The productivity of each clone was analysed following the initiation of $p21^{CIP1}$ expression at the start of experimentation through the addition of IPTG to the culture medium. The results (Figures 3.8 and 3.9) show a broad range of variation in productivity amongst the clones both during normal growth and following the induction of $p21^{CIP1}$-expression. Due to the nature of the transfection used expression of Bcl-2 was not inducible therefore it was expressed continuously in all of the clones tested. The analysis of clones in which $p21^{CIP1}$-expression was not induced revealed several clones exhibit a slight decrease in productivity when compared to the non-$p21^{CIP1}$/Bcl-2-expressing control cell-line. This would seem to indicate that the expression of Bcl-2 alone affords no enhancement to the productivity of the CHO cell-line used in this study.

During periods of $p21^{CIP1}$-induced cell cycle arrest, all clones did however show a significant increase in their specific production rates; with some clones specific production rates being increased by up to 90% in comparison to the corresponding non-induced $p21^{CIP1}$/Bcl-2-expressing cultures, as determined via t test analysis. Several clones also exhibited up to a 65% higher production rate than that observed in the non $p21^{CIP1}$/Bcl-2-expressing control culture.

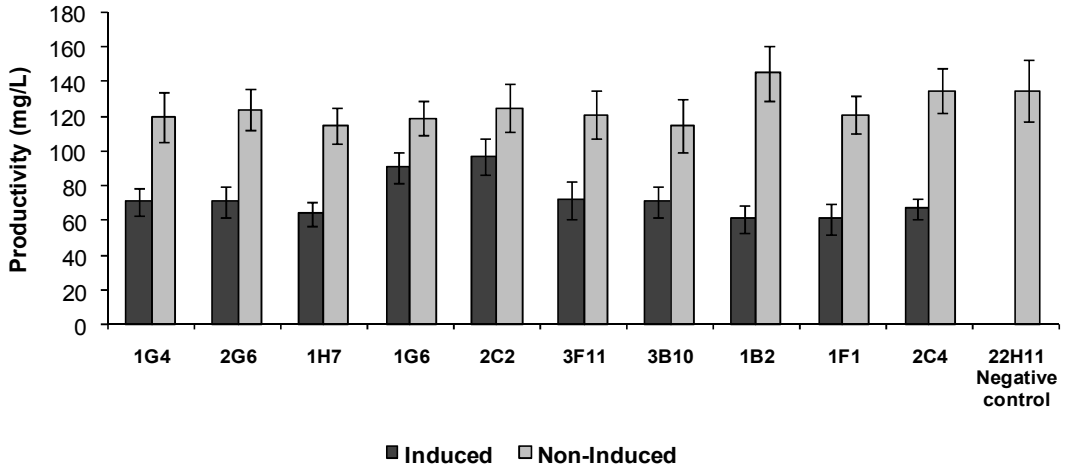


Figure 3.8. The productivity of chimeric antibody in p21^{CIP1}/Bcl-2 expressing cultures. The human-mouse IgG4 chimeric MAb concentration was determined via enzyme linked immunoabsorbent assay (ELISA) as described in the Materials and methods. Results were obtained following 120 hours of growth as monolayer cultures in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50μM MSX. For each cell-line duplicate cultures were set up. One culture had the expression of p21^{CIP1}-induced through the addition of 5mM IPTG at the start of experimentation. The other culture was left non-induced throughout. Dark grey bars represent productivity of chimeric antibody in p21^{CIP1}/Bcl-2-expressing cultures. Light grey bars represent the productivity of the same cell-lines during culture without the induction of p21^{CIP1}-expression. Results were compared to a negative control (22H11) culture (described in chapter 2 Materials and Methods) which does not express p21^{CIP1} or Bcl-2. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of Induced p21^{CIP1}/Bcl-2-expressing cultures and the control. ($p < 0.0003$.

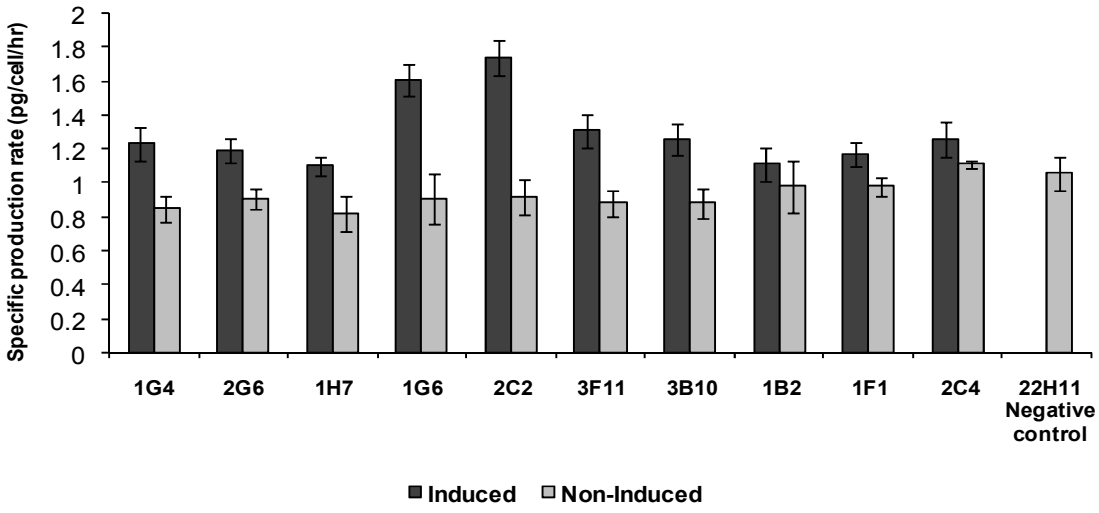


Figure 3.9. The specific production rate of p21^{CIP1}/Bcl-2-expressing clones grown as monolayer cultures in- DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX. Dark grey bars represent productivity of chimeric antibody in p21^{CIP1}/Bcl-2-expressing cultures. Light grey bars represent the productivity of the same cell-lines during culture without the induction of p21^{CIP1}-expression. Results were compared to a negative control (22H11) culture (described in chapter 2 Materials and Methods) which does not express p21^{CIP1} or Bcl-2. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of induced p21^{CIP1}/Bcl-2-expressing cultures and the control. ($p < 0.0003$.)

3.4. Discussion

In this study we have succeeded in demonstrating that through the combined expression of $p21^{CIP1}$ and Bcl-2 it is possible to engineer a CHO cell-line that is able to better withstand the rigors of the bio-processing environment whilst also improving productivity. During the course of experimentation several clones expressing both Bcl-2 and an inducible copy of $p21^{CIP1}$ were produced and although some variation was observed between each of the clones, the expression of Bcl-2 resulted in a significant increase in cellular robustness when challenged via nutrient deprivation and cytotoxic stimuli in all of the clones tested. This in turn led to a vast reduction in the occurrence of apoptotic cell death and an increase in culture duration and viability

This ability to simultaneously control apoptosis and cell proliferation has the potential to significantly improve the overall obtainable product titres of several industrially important mammalian cell-lines. Previously our group *(Watanabe et al 2002)* has reported that G1 phase cell cycle arrest, as a result of inducible $p21^{CIP1}$-expression is able to produce a 4 fold enhancement in cell productivity in NS0 cell-lines. In addition it has also been shown that Bcl-2-expression is able to dramatically improve culture viability *(Bierau et al 1998)* and may also through its expression exert an influence upon the G1 to S phase transition *(Marvel et al 1994, O`Reilly et al 1996, Simpson et al 1999)*. These findings are in agreement with the results demonstrated in this study which showed a significant increase in productivity during cell cycle arrest in the $p21^{CIP1}$/Bcl-2-expressing clones tested, however the enhancement was not as pronounced as those seen in NS0-lines *(Watanabe et al 2002)* possibly as a result of the combined expression of

$p21^{CIP1}$ and Bcl-2 as non-induced clones expressing Bcl-2 alone were found to have a slightly lower specific production rate when compared to the non-Bcl-2-expressing control line. Also we cannot exclude the possibility that the parental cell-line is a high producing clonal cell-line that may have reached its optimal intrinsic productivity level.

This finding is in agreement with other studies from our laboratory (*Tey et al* 2000) and others *(Murray et al* 1996), who have reported no significant increase in productivity as a result of Bcl-2-expression alone. It would appear that although the expression of Bcl-2 offers protection against nutrient limitation the exhaustion of the precursors for gene expression has a limiting effect on productivity. Other studies into murine hybridomas (*Fassnacht et al 1998b*) however have found that a significant improvement in productivity in high cell density perfusion culture. Moreover fed batches of NS0 cell-lines resulted in a significant increase in both the maximum cell number and antibody productivity when compared to the non-Bcl-2-expressing control (*Tey et al 1999*).

The significant increase in productivity during cell cycle arrest in $p21^{CIP1}$/Bcl-2-expressing clones is most likely the result of altering the growth rate, potentially leading to a change in the energy transfer of the cellular biosynthetic machinery away from proliferation and towards product synthesis. However this has proven to be relatively difficult to achieve due to the induction of apoptosis under cell cycle arrest *(Singh et al 1994)*. In this study the combination of $p21^{CIP1}$ and Bcl-2-expression appears to have overcome this problem resulting in a 42.85% and 18.64% increase in the specific production rate when compared to Bcl-2 and $p21^{CIP1}$-expressing cell-lines, respectively.

Interestingly arresting the cells in the G1 phase of the cell cycle may also have an influence on the cells requirement for growth factors/complex nutrients that are present in the growth medium, therefore arresting cells at G1 may also potentially reduce the need for nutrients previously determined as essential for productivity (*Tey et al 2000*).

What is clear from this study is the fact that through the genetic modification of the host cell-line it may be possible to significantly enhance the period of time cells are able to remain highly productive and viable. This may lead to the ability to specifically tailor the host cell-line to the desired culture conditions and production needs rather than matching the culture conditions with the growth requirements of the cell-line used.

4. The effect of $p21^{CIP1}$-expression on cell size, cell cycle, metabolism and productivity in CHO cell-lines

4.1. Abstract

In this chapter we have examined the effect of G1 phase cell cycle arrest through the expression of the cyclin dependent kinase inhibitor $p21^{CIP1}$ on the productivity of a chimeric IgG4 antibody expressing CHO cell-line, whilst also analysing the associated changes in cellular metabolism that occur during periods of high productivity. The results of this study clearly demonstrate that through the induction of cell cycle arrest it is possible to uncouple cell growth from cellular proliferation which in turn increases the overall specific production rate. Analysis of the rate of glucose and lactate metabolism show a 78.4% and 166.6% increase respectively in induced cultures in comparison to cultures in which $p21^{CIP1}$ was not expressed. This was also accompanied by a 614.2% increase in the rate of glutamate metabolism and an 86% increase in ammonia production rates. This clearly demonstrates a significant increase in the activity of the cells metabolic pathways during periods of cell cycle arrest and may well explain the observed increase in productivity. When cell division was halted it was observed that the cells increased in volume by up to 125%. This was also accompanied by a 183% increase in the cells total protein content suggesting that the protein normally synthesised prior to cell division is stored within the arrested cell thus leading to the observed increase in cell size. This study therefore provides several insights into how productivity may be improved in industrially relevant CHO cell-line as well as offering vital information into the metabolic changes that occur during periods of cell cycle arrest, and its effect on productivity.

4.2. Introduction

Intricate metabolic processes control the growth and productivity of all mammalian cell cultures as well as the cells physiology. The accumulation of waste products, the depletion of energy sources, amino acids, lipids, vitamins or trace elements as well as the exhaustion or inactivation of growth factors *(Ljunggren and Haggstrom 1995)* all limit the metabolic potential of cell cultures commonly used by industry. In an attempt to overcome these limitations and thereby improve productivity several methods have been employed, including media optimisation *(Xie and Wang 1994, Zhang and Robinson 2005)* and feed strategies *(Wurm 2004, Sauer et al 2000, deZengotita et al 2000)* both of which are designed to improve process scale-up and development. Using such strategies several groups have shown that it is possible to increase productivity, either as a result of higher cell density, improved viability *(Xie and Wang 1997, Zhou and Thompson 1997)* and/or by increasing the specific production rate *(Takagi et al 2001, Wong et al 2005)*.

The energy required by a cell during the process of protein production may well constitute a significant part of the total cellular energy. Therefore an understanding of the mechanisms through which a cell obtains and uses cellular energy is critical for the optimization of protein production. Several studies *(Bi et al 2004, Carvalhal et al 2001, Carvalhal et al 2003, Fussenegger et al 1998, Ibara et al 2003, Watanabe et al 2001)* have demonstrated the ability to increase the productivity of industrially important cell-lines during periods of cell cycle arrest. In these studies significant changes in the cells metabolism have also been observed, which have resulted in the accumulation of cellular constituents and thus cell death (*Sukhorukov et al 1994, Saunders et al 1999, Carvalhal*

et al 2002). Based on these findings it is clear that the production of a cell-line which is able to devote a significant proportion of its metabolic and energy resources towards the production of a desired protein at high cell density would represent an ideal industrial process. Such a diversion of resources would lead to high product yields and less metabolic wastage.

In the previous chapter I was able to successfully demonstrate that the productivity of CHO cells could be enhanced through the expression of $p21^{CIP1}$, which results in the cessation of the cell cycle and thus cellular proliferation. Understanding the relationship between the cell cycle and productivity is vital in order to determine the mechanisms behind product synthesis and also to enable the optimisation of commercial culturing protocols that will maximize the final product titres. Several studies *(Feder et al 1989, Kromenaker and Srienc 1991, Al-Rubeai and Emery 1990, Fussenegger et al 1997, Gu et al 1993)* have produced conflicting evidence on the relationship between cell cycle and productivity, however it is generally accepted that productivity is higher in larger cells.

It is not possible to assume that the changes in metabolism that occur during periods of cell cycle arrest are universal for all methods used, or that such changes are directly linked with the observed increase in productivity therefore in this chapter aim to confirm the existence of a relationship between $p21^{CIP1}$-induced cell cycle arrest and the productivity of CHO cells. Furthermore I aim to examine the hypothesis that cellular growth and proliferation may be uncoupled in $p21^{CIP1}$-expressing cultures, whilst also identifying potential changes which occur in the cells metabolism during periods of

proliferation arrest, the hope being that I may be able to determine if such metabolic changes have a direct impact on the cellular productivity.

In order to do this I have utilised a Chinese hamster ovary (CHO) cell-line, which through the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium is able to inducibly express the cdk $p21^{CIP1}$. In this chapter I have examined the effect of cell cycle arrest on the metabolism of key nutrients in an attempt to determine if a relationship exists which is able to explain the observed increase in specific production rates during periods of cell cycle arrest.

4.3. Results

In order to produce a CHO cell-line capable of the inducible expression of $p21^{CIP1}$ the Lacswitch system was used. Parental 22H11 cell-line, described previously were transfected with the CMVlacI plasmid DNA as described in the Materials and Methods. Transfected cells were then selected in a medium containing 400μg/ml Hygromycin B. After 2 weeks selection, the whole population of stable transfectants was then transfected with pOPRSV1/$p21^{CIP1}$ plasmid DNA. The resulting transfectants were then cloned via limiting dilution to produce the cell-line used for the rest of this study.

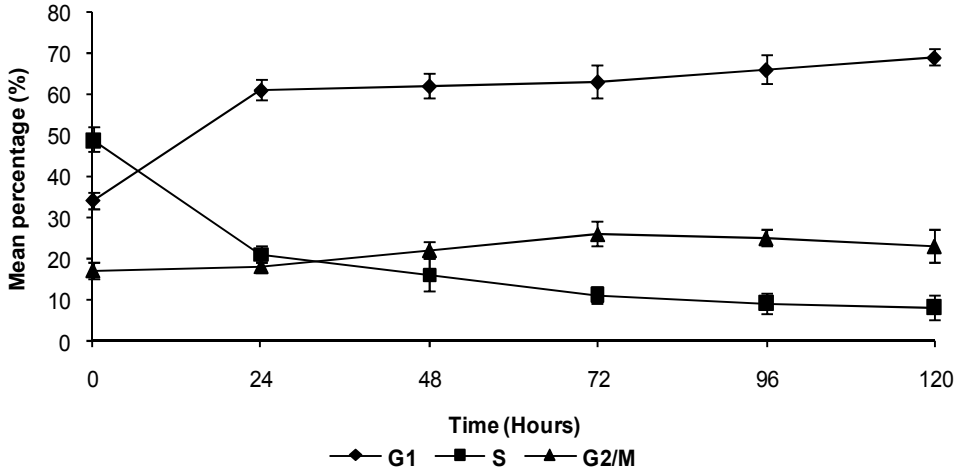
4.3.1. The effect of $p21^{CIP1}$-expression on cell cycle progression

Several studies have previously demonstrated the ability to arrest the progression of the cell cycle of several industrially important cell-lines, through the expression of $p21^{CIP1}$ *(Fussenegger et al 1998, Watanabe et al 2002, Bi et al 2004, Ibara et al, 2003*). In order

to determine the effectiveness of the proliferative arrest caused by $p21^{CIP1}$-over-expression in the CHO cell-line used for this study, cells were cultured for 120 hours in the presence or absence of IPTG. The cultures were then analysed at 24 hour intervals via propidium iodide (PI) staining using Guava personal cell analysis (PCA) over a period of 120 hours.

The results shown in Figure 4.1A demonstrate a sharp increase in the number of cells in the G1 phase of the cell cycle following the initiation of $p21^{CIP1}$-expression. Approximately 61% of cells were observed to congregate in this phase following 24 hours of $p21^{CIP1}$-over-expression, rising to 69% following 120 hours of expression. A gradual decline in the percentage of cells in S phase from 49% at 24 hours to 8% at 120 hours was also observed. During the course of experimentation it was noted that there was a slight increase in the number of cells in the G2 phase of the cell cycle rising from 18% to 23% over the 120 hour period of experimentation.

A



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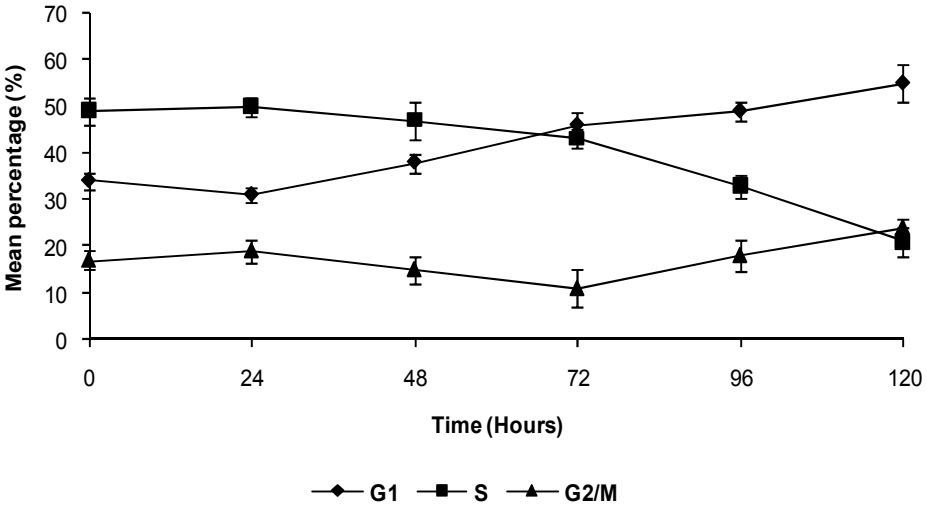


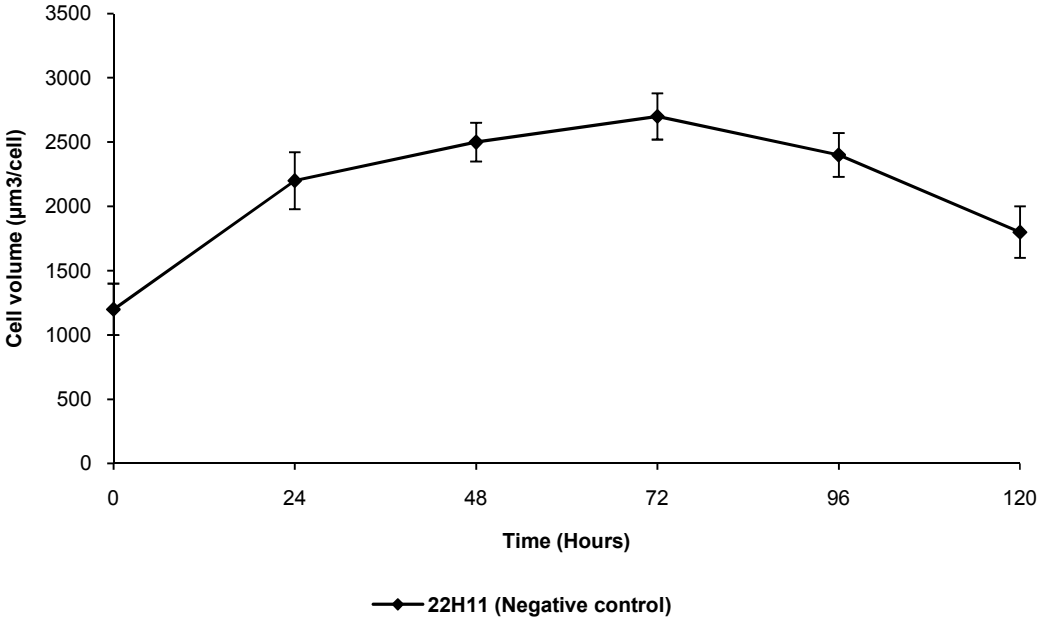
Figure 4.1 Cell cycle of induced (A) and non-induced (B) $p21^{CIP1}$-transfected cell-lines over a 120 hour period determined via Guava cell cycle analysis To further analyze the data for more subtle changes between the phases of the cell cycle Multicycle software that applies sophisticated curve fitting algorithms to the Guava cell cycle data that more accurately calculates the percentages of the cell populations and their relevant statistics were used. Error bars show the standard error of three replicates.

In contrast to the culture expressing $p21^{CIP1}$, the results from the control culture, in which $p21^{CIP1}$-expression was not induced (Figure 4.1B) show the percentage of cells in the G1 phase of the cell cycle to be significantly lower, with only 34% of cells congregating at this phase following 24 hours of culture. During the remaining culture period the percentage of cells entering the G1 phase rose slightly, but still only reached 55% following 120 hours. A progressive decrease in the percentage of cells in S phase from 49% to 21% also resulted as the cells entered stationary phase at around the 120 hour mark.

4.3.2. The effect of p21 ^{CIP1}-expression on cell size and volume

In order to study the effect of p21^{CIP1}-over-expression on cell volume, experiments were set up as described previously in the Materials and Methods. Samples were taken in triplicate on a daily basis from both p21^{CIP1}-expressing and non-expressing cultures. Each sample was analysed via a Malven mastersizer to determine the mean volume of the cells at that particular point in time.

A



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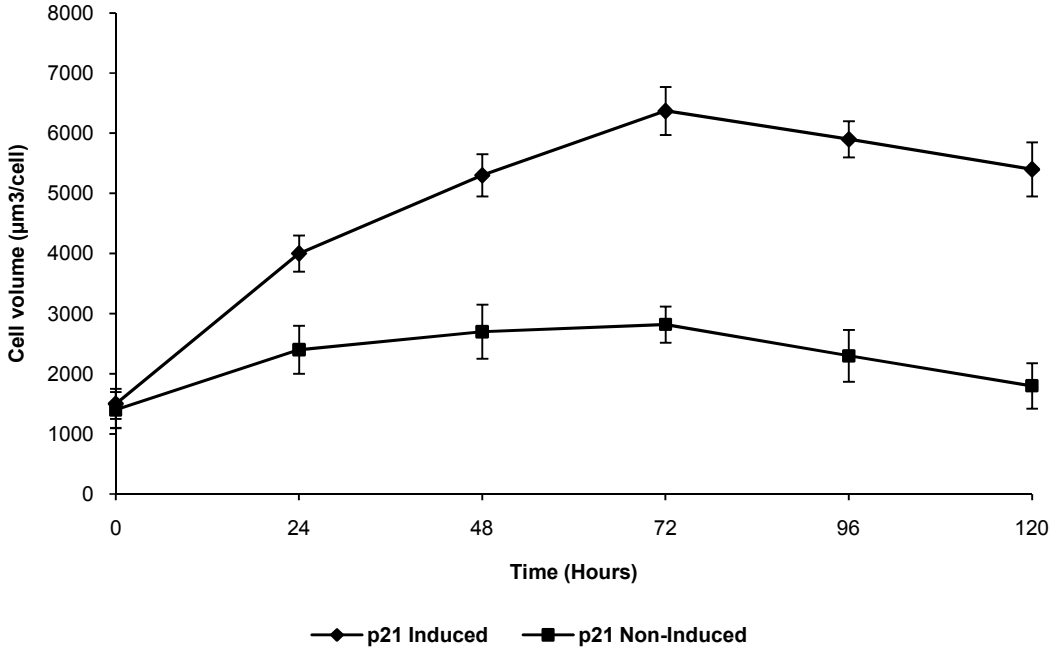


Figure 4.2 Analysis of cell volume in (A) a control non-p21^{CIP1}-expressing cell-line and (B) a p21^{CIP1}-expressing cell-line in which vector expression has been induced (diamonds) or non-induced (squares) at the start of experimentation. The cell volume was determined at 24 hour intervals via a Malven mastersizer as described in Materials and Methods. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between volume of induced and non-induced cultures. ($p < 0.0003$.)

The results shown in Figure 4.2 show a significant increase in the overall cell volume following the initiation of cell cycle arrest at the start of experimentation. p21^{CIP1}-expressing cells were found to be on average up to 125% larger when compared to cultures in which p21^{CIP1}-expression was not induced and up to 135% larger than the non p21^{CIP1}-expressing control cell-line. To confirm this increase in cell volume, samples

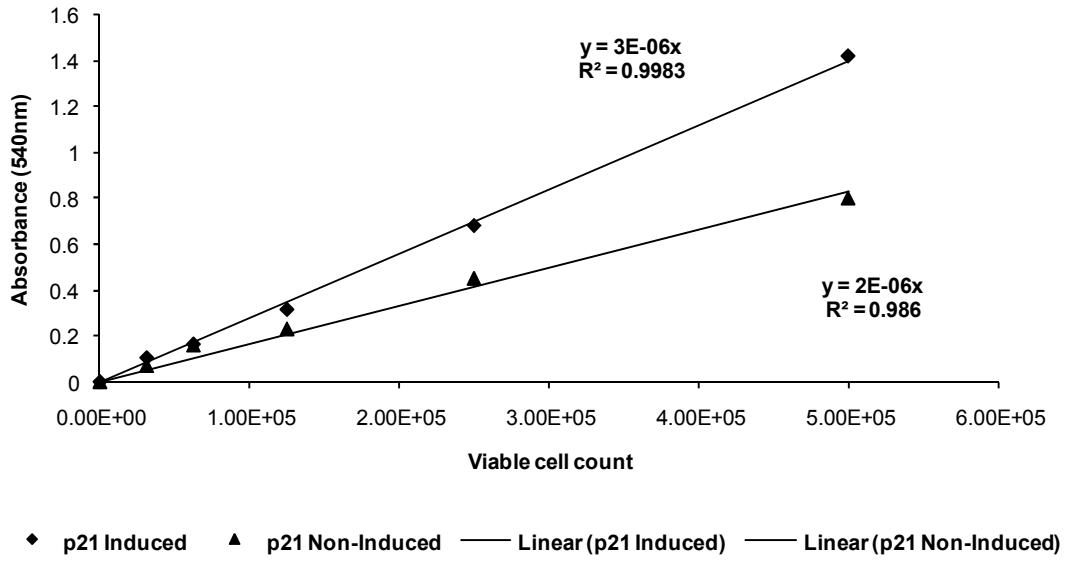
containing an equal number of cells were taken from both induced and non-induced cultures as described in the Materials and Methods. These samples were then centrifuged in order to produce a compact pellet which could be visualised. The results shown in Figure 4.3 demonstrate that after 72 hours of cell cycle arrest there was an increase in cell volume of approximately 100%, which, despite the differences in methods was in relative agreement with the results obtained via the Malven mastersizer previously.

Figure 4.3 Analysis of packed cell volume of cultures in which $p21^{CIP1}$-expression was either induced or non-induced at the start of experimentation. Samples containing $2x10^5$ cells were taken from induced and non-induced cultures before being centrifuged at 2000g to produce a visible pellet. The procedure was repeated in triplicate to ensure the reproducibility of the observed result before photographic evidence was taken.

4.3.3. The effect of p21 ^{CIP1} expression on cell metabolism

In order to understand the physiological mechanisms that operate during periods of cell cycle arrest, firstly the metabolic activity of the cell was determined using a MTT assay as described in the Materials and Methods. This method enables the calculation of mitochondrial dehydrogenase enzyme activity in both induced and non-induced cultures as well as a non p21^{CIP1}-expressing control cell-line based on 2x10^6 cells per sample. The results (figure 4.4A) show the effects of 72 hours of p21^{CIP1}-induced cell cycle arrest on the activity of mitochondrial dehydrogenase enzymes. During p21^{CIP1}-expression a 77.5% increase in activity was observed when compared to non-induced culture and a 118% increase in comparison to the non-p21^{CIP1}-expressing negative control (figure 4.4B).

A



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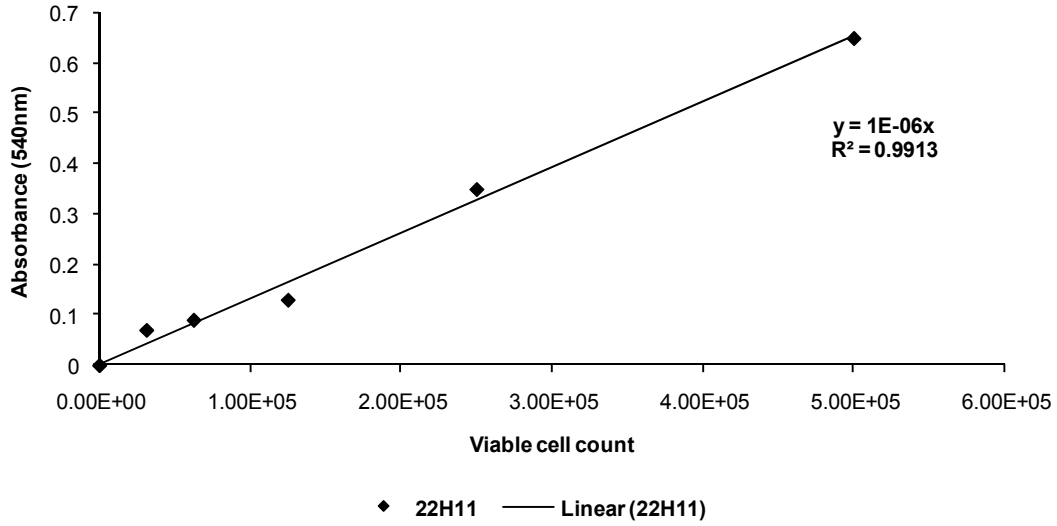

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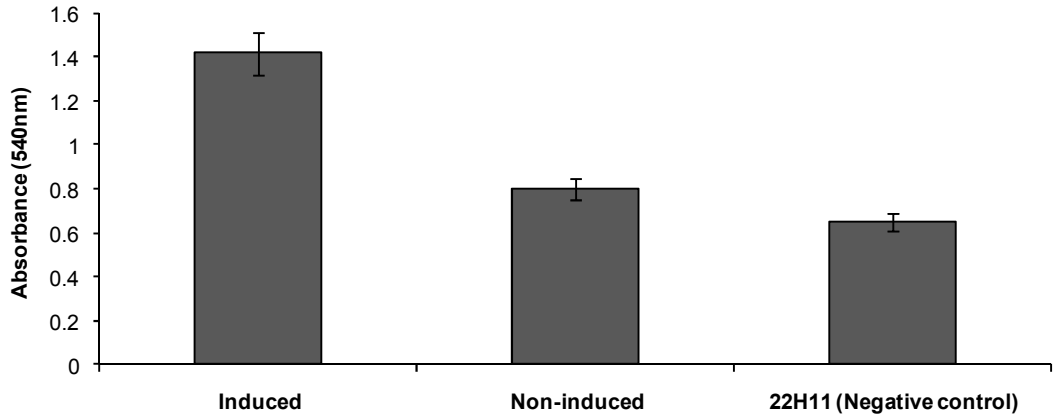


Figure 4.4 Analysis of mitochondrial activity determined via an MTT assay in cultures in which p21^{CIP1}-expression (A) induced (Diamonds) or non-induced (Squares) at the start of experimentation, as well as a (B) control non-p21^{CIP1}-expressing cell-line. (C) summarises the difference between the mitochondrial activity of induced, non-induced and control cultures following 72 hours of culture. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the mitochondrial activity of induced and non-induced cultures. ($p < 0.0003$.)

Analysis of the total protein (Figure 4.5) reveals a significant increase the amount of total protein produced during periods of $p21^{CIP1}$-induced cell cycle arrest, as determined via a BCA total protein kit. The expression of $p21^{CIP1}$ was found to result in approximately a 183% increase in the amount of total protein produced when compared to non-induced cultures and a 214% increase compared to the non-$p21^{CIP1}$-expressing control cell-line. These results would seem to indicate either an increase in overall protein synthesis or that the prevention of cell division results in all of the protein usually synthesized prior to cell division remaining within the cell.

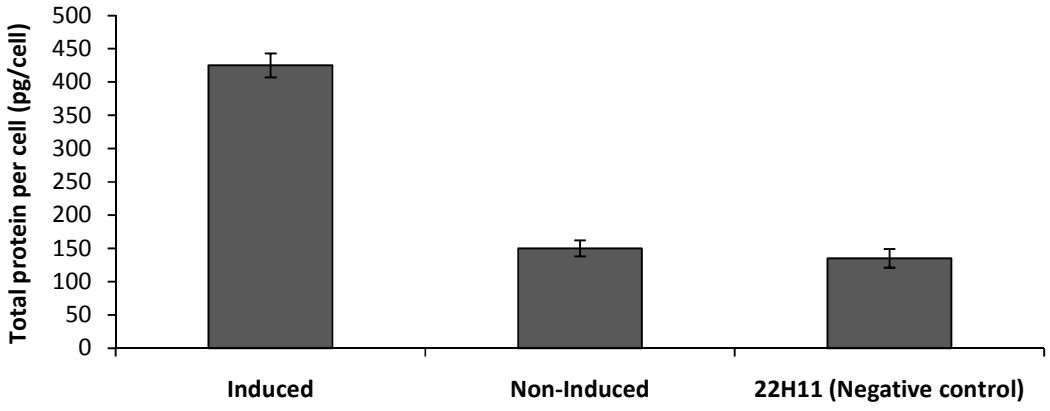
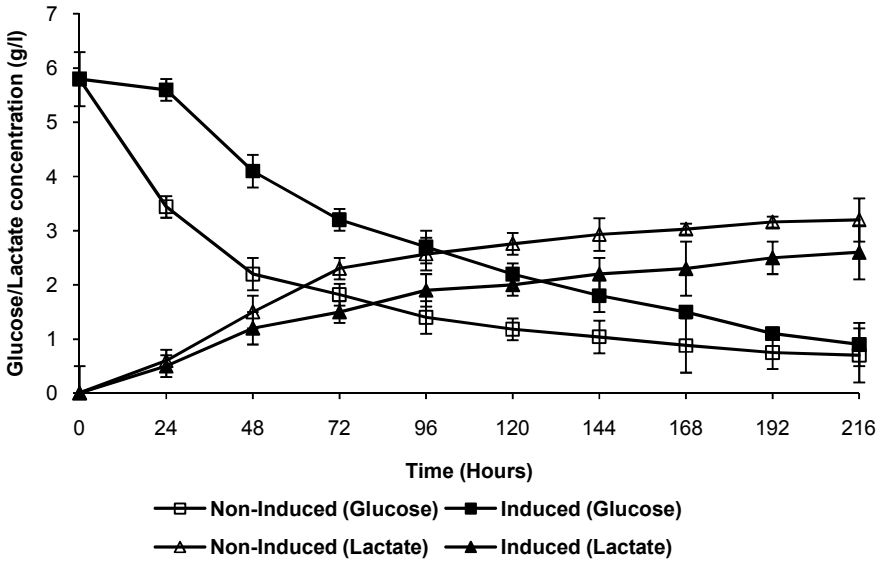


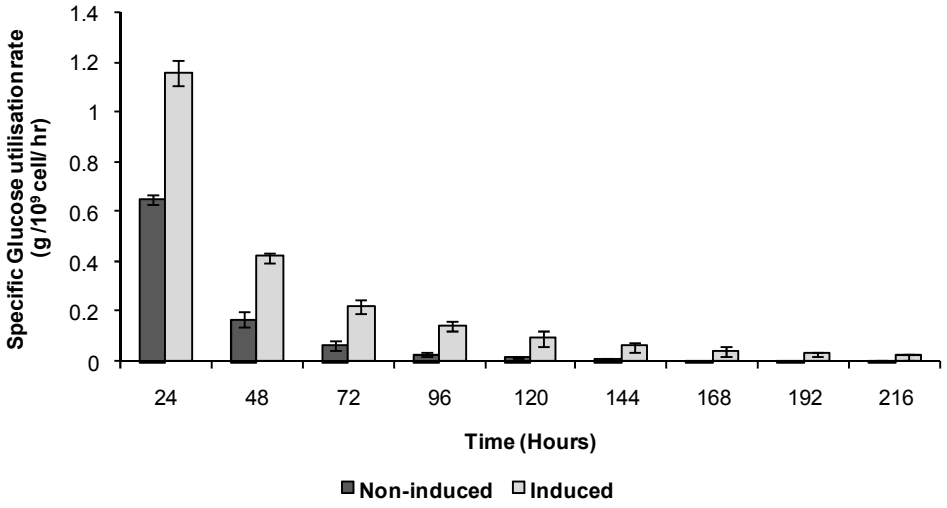
Figure 4.5 Analysis of the total protein content of cultures in which $p21^{CIP1}$-expression was either induced or non-induced at the start of experimentation and a control culture in which $p21^{CIP1}$ was not expressed. The total protein content of the cells was calculated following 72 hours of culture, via a BCA kit as described in Materials and Methods. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the total protein content of induced and non-induced cultures. ($p < 0.0003$.)

Glucose is utilized as the main source of energy and metabolic intermediates of all cultivated mammalian cells *(Altamirano et al 2001)* and its passage through the glycolysis pathway results in the production of waste products such lactate. Figure 4.6A shows the depletion of glucose and the accumulation of lactate in cultures in which $p21^{CIP1}$-expression had been either induced or non-induced, analysed over a 216 hour period. Both cultures display a similar pattern of glucose utilization and lactate accumulation throughout the culture period. In cultures in which $p21^{CIP1}$-expression was induced, the glucose concentration remained significantly higher than that observed in the non-induced culture and as a result the concentration of lactate produced remained lower. This finding however represents the differences in cell density between cultures in which $p21^{CIP1}$-expression had been induced and those in which it had not. To more accurately determine the rate of glucose utilisation and lactate accumulation, these results were analysed on a per cell basis. The results (Figures 4.6B and C) clearly show the specific rate at which glucose is utilized and lactate accumulates to be significantly higher during periods of $p21^{CIP1}$-expression. From this data it was also possible to calculate the yield of lactate from glucose for induced and non-induced cells following 120 hours of culture (YLac/Glc) as 0.454 mol^{-1} and 0.545 mol^{-1} respectivaly. These results clearly indicate that during periods of $p21^{CIP1}$-induced cell cycle arrest, glucose may be utilised more readily than when a cell is undergoing rounds of proliferation.

A


Glucose/Lactate concentration (g/l)
0
1
2
3
4
5
6
7
0
24
48
72
96
120
144
168
192
216
Time (Hours)
Non-Induced (Glucose)
Induced (Glucose)
Non-Induced (Lactate)
Induced (Lactate)

B


Specific Glucose utilisation rate (g /10⁹ cell/ hr)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
24
48
72
96
120
144
168
192
216
Time (Hours)
Non-induced
Induced

C

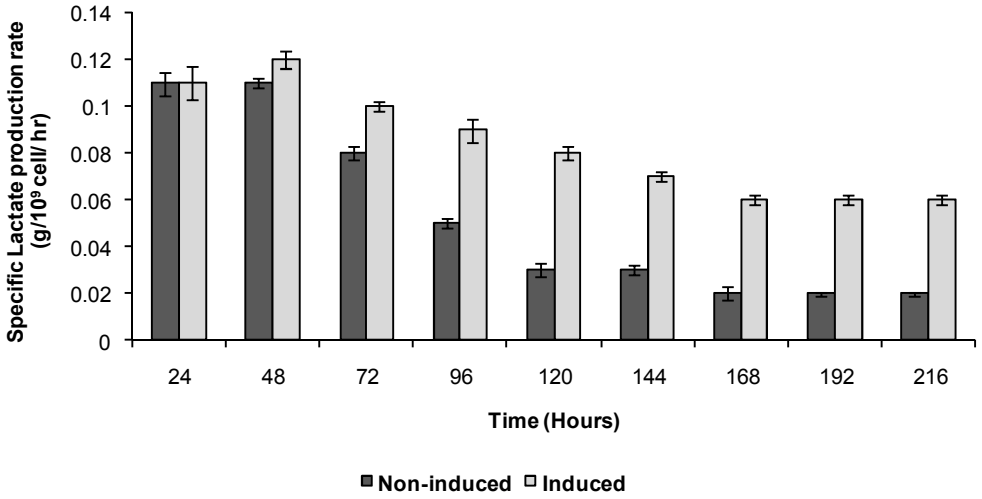


Figure 4.6 Analysis of the concentrations of glucose and lactate (A) in cultures in which p21^{CIP1}-expression had been induced (open squares and triangles) and non-induced (closed squares and triangles) as sampled over 216 hours. Bars demonstrate the changes in specific utilization rates of glucose (B) and (C) the specific production rates of lactate of in cultures in which p21^{CIP1}-expression had been induced (light grey) and non-induced (dark grey) cultures. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the glucose utilization rates and lactate production rates of induced and non-induced cultures. ($p < 0.0003$.)

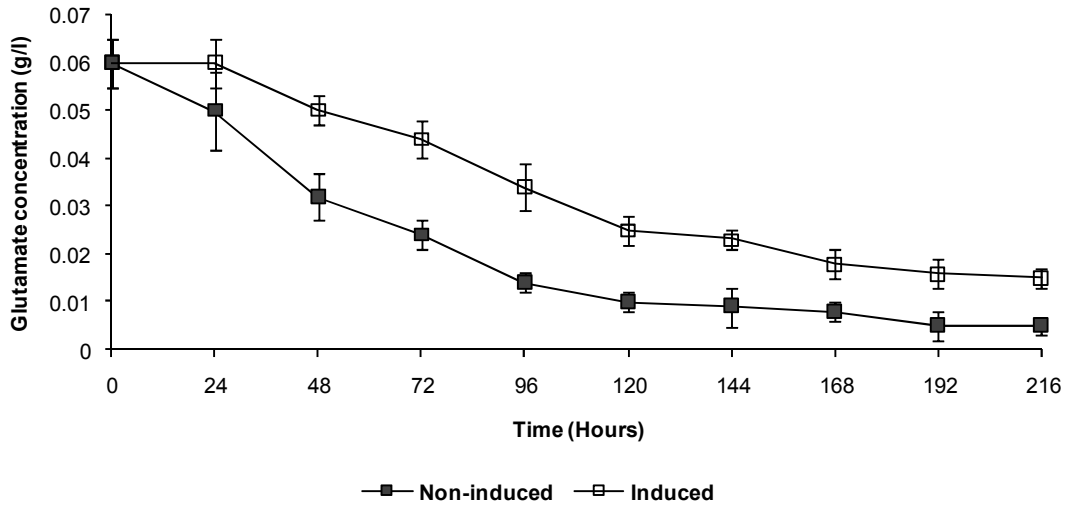
Interestingly it would apear from Figure 4.6B the rate at which glucose is utilised by p21^{CIP1}-expressing cultures decreases following the first 48 hours of culture when compared to the culture in which p21^{CIP1}-expression was not induced. This would sugest that glucose is more readily utilized in the early phases of culture.

The results shown in figure 4.7 demonstrates the changes in glutamate concentration and specific utilization rates over time for both induced and non-induced p21^{CIP1}-expressing cultures. Throughout the culture period the concentration of glutamate decreased in both

cultures, however the overall concentration remained higher in cultures in which $p21^{CIP1}$ was expressed, again reflecting the lower cell density. During the early phases of culture the specific utilization rate of both cultures was found to be relatively similar, but increased at a significantly higher rate in the $p21^{CIP1}$-expressing cell-line during the latter half of the culture period. This was found to result in an approximate 2 fold increase in the specific utilization rate when compared to the non- induced culture.

Levels of ammonia were also monitored during the culture period and the results (Figure 4.8A) show the accumulation of ammonia in cultures in which $p21^{CIP1}$-expression had been induced or non-induced. During the first 48 hours of culture the levels of ammonia similar for both cultures; however from mid exponential phase (72 hours) onwards the concentration of ammonia detected was considerably higher in the non-induced culture, reflecting the increase in cell of the ammonia accumulation rateollowing 48 hours the specific rate of ammonia production decreased in both cultures, the rate of this decrease was much slower in cultures in which $p21^{CIP1}$-expression had been inducedin an 86% specific ammonia production rate .

A



B

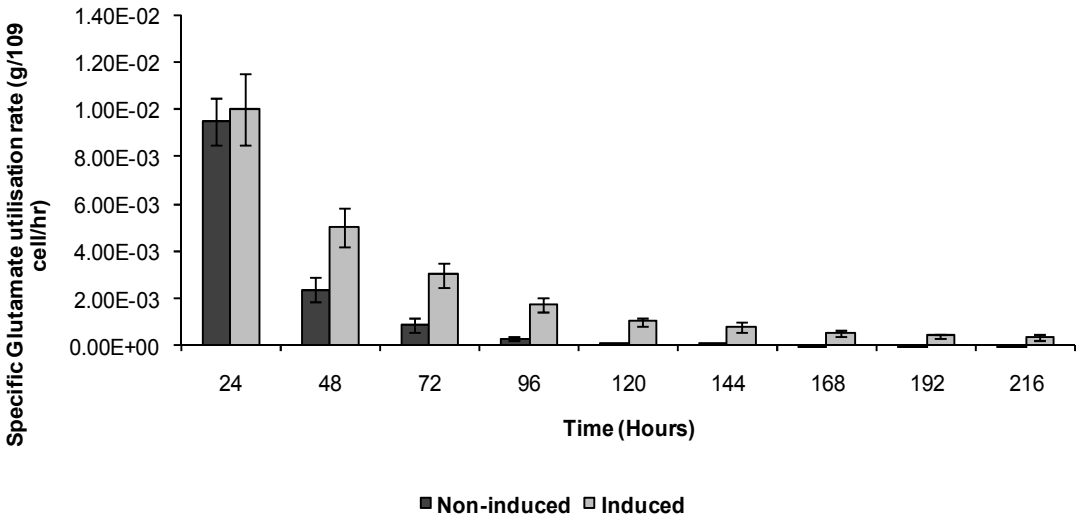
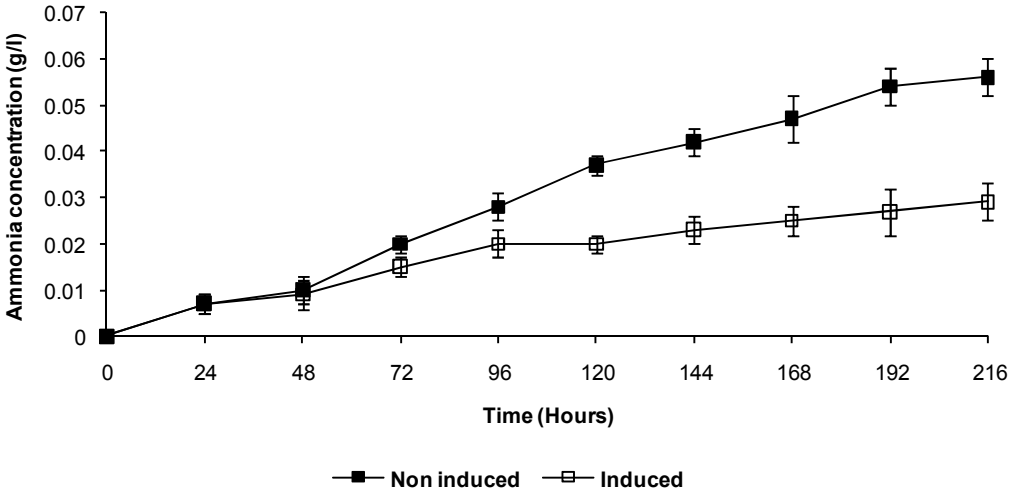


Figure 4.7 Analysis of the concentration of glutamate (A) in cultures in which $p21^{CIP1}$-expression was induced (open squares) and non-induced (closed squares) as sampled over 216 hours. Bars demonstrate the changes in specific utilization rates (B) of induced (light grey) and non-induced (dark grey) cultures. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the glutamate utilization rates of cultures in which $p21^{CIP1}$-expression was induced and non-induced. ($p < 0.0003$.)

A



B

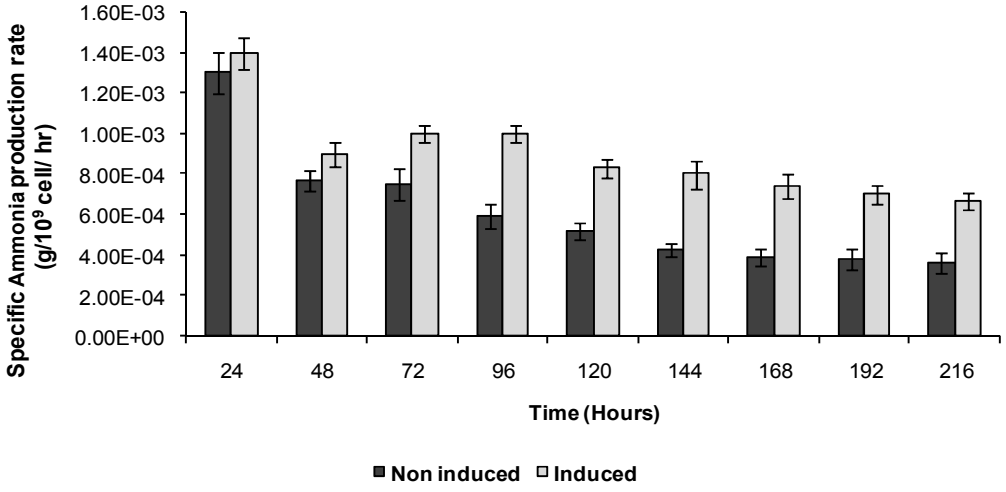


Figure 4.8 Analysis of the concentration of ammonia (A) in cultures in which $p21^{CIP1}$-expression had been induced (open squares) and non-induced (closed squares) as sampled over 216 hours. Bars demonstrate the changes in specific production rates (B) of induced (light grey) and non-induced (dark grey) cultures. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the ammonia production rates of induced and non-induced cultures. ($p < 0.0003$.)

4.3.4. The effect of p21 ^{CIP1}-expression on productivity

The productivity of cultures in which p21^{CIP1}-expression was induced or non-induced was analysed following the initiation of cell cycle arrest via ELISA as described in the Materials and Methods. The results (Figure 4.9) show the specific production rate following 72 hours in culture and demonstrate a 93.44% increase in productivity in p21^{CIP1}-expressing cultures in comparison to the non-p21^{CIP1}-expressing control.

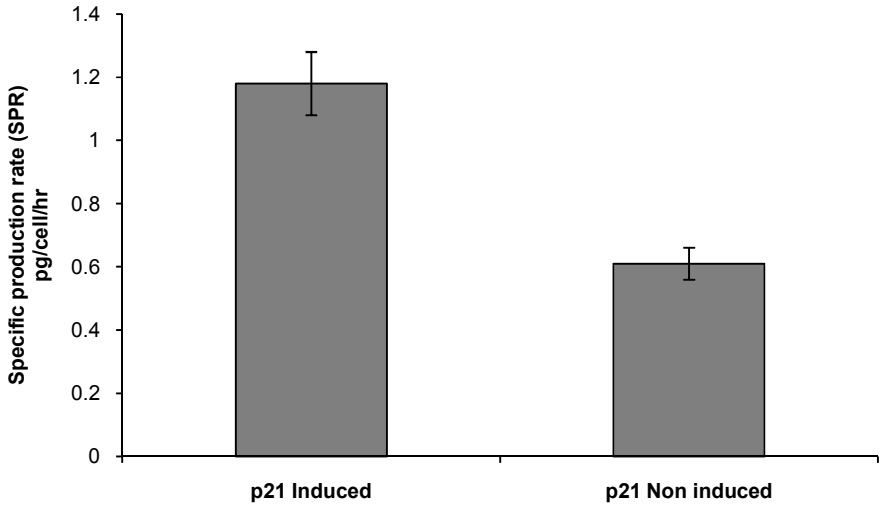


Figure 4.9 Bars show the specific production rate of p21^{CIP1}-expressing cell-lines following 72 hours growth as serum-supplemented static cultures. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of cultures in which p21^{CIP1}-expression had been induced and those in which it was not-induced. ($p < 0.0003$.)

4.4. Discussion

In this study it was confirmed that the induced expression of $p21^{CIP1}$ results in the immediate cessation of cell cycle progression predominantly in the G1 phase. As observed previously *(Bi et al 2004)* during the later stages of culture a small percentage of cells did not accumulate in the G1 phase of the cell cycle and instead appear to be blocked in G2 phase. Despite this cell cycle arrest was shown to be complete and was also accompanied by a significant increase in the overall specific production rate. This is in agreement with our previous findings that during periods of $p21^{CIP1}$-over-expression productivity may be substantially increased *(Watanabe et al 2002 et al 2003).*

During periods of cell cycle arrest an inverse relationship has been shown to exist between the rate of cell proliferation and specific productivity *(Altamirano et al 2001, Carvalhal et al 2003, Fox et al 2005, Kaufmann et al 1999, Lee and Lee 2000)*. Variation in the methods employed to induce proliferation arrest as well as the phase of cell cycle in which the cells congregate have a direct impact on the physical and chemical properties of the cells environment. Such changes have the potential to affect multiple cellular processes and as a result it is clear that the biological basis of how the prevention of cellular proliferation enhances productivity may not be universal to all methods used. This makes its determination extremely complex and as of yet the exact mechanisms remain elusive.

The maintenance of cell size is considered a homeostasis between macromolecular synthesis and degradation. In multicellular organisms, this is linked to the presence of

nutrient and growth factors and hence is also applicable to cell culture. An increase in cell size may well reflect the metabolic potential of a cell, as this parallels its DNA content. It has previously been shown that there is a strong, positive relationship between genome size and cell size *(Saucedo and Edgar 2002)*, similarly it has been reported *(Dolznig et al 2004)* that oncogene-driven cells have a higher rate of proliferation and also show an increase in cell volume.

While the cell number did not increase in the arrested culture a 125% increase in cell volume resulted from the expression of $p21^{CIP1}$ over a 72 hour period, with the average cell having a volume of 6300 μm^3 as opposed to 2800 μm^3 as observed in non-induced cultures. These findings are in agreement with several studies from both our laboratory and others in which proliferation arrested CHO *(Bi et al 2004)* and Rat Schwann cells *(Conlon et al 2001 Conlon and Raff 2003)* show similar size ranges (2000-10000μm^3) and supports the hypothesis that cellular growth is able to occur independently of the cell type and the mode of proliferation arrest. The results presented here therefore indicate that a relationship exists between the specific production rate and cell volume as during experimentation both of these parameters increase simultaneously with time. This results in a peak at around 72 hours into the culture period, which has previously been shown to be the period of maximum productivity in arrested cells *(Bi et al 2004)*.

During the G1 phase of the cell cycle cells replicate their organelles and increase in biomass in preparation for cell division. Once a predetermined threshold is reached, the cell cycle is able to progress into the S phase, at which point it is debatable whether cell

growth continues. During periods of cell cycle arrest it is likely that the accumulation of biomass is able to occur without hindrance. This would result in overall increase in the cells protein assembly machinery, that is the ER and Golgi, which in turn would facilitate the observed increase in specific productivity

Although little is known about the relationship between cellular volume and productivity, in a recent study *(Dinnis et al 2006)* the analysis of four clones that varied in their specific productivities would seem to suggest that a denser cell, that is one having a high protein to cell volume ratio results in higher specific productivity rate. This contradicts previous finding *(Lloyd et al 2000)* that suggest that a larger cell volume results in an increased productivity and would therefore seem to suggest that cell volume and the total protein content are not necessarily correlated. In this study analysis of the cells total protein content reveals a significant increase in this parameter during periods of $p21^{CIP1}$-induced cell cycle arrest. Similar results have previously be obtained in other cell-lines including IRF-1 activated proliferation arrested BHK-21 cells *(Carvalhal et al 2002),* mouse Schwann cells *(Conlon and Raff 2003*) CHO cells *(Bi et al 2004)* and Rat Schwann cells *(Conlon et al 2001, Conlon and Raff 2003)*. This would seem to support the theory that the uncoupling of growth and proliferation observed in this study is not solely restricted to the over-expression of $p21^{CIP1}$ and that alternative mechanisms for increasing specific productivity may well exist. The increase in total protein content would also appear to confirm the theory that during periods of cell cycle arrest an increase in the protein synthesis machinery is more readily able to occur and therefore

that proliferation, that is, the cycling and division of the cell, can be 'uncoupled' from cell growth.

The exact mechanism through which this occurs remains elusive, however it may possible to partially explain this process through two of three models proposed by (*Jorgensen et al 2004)* that originally aimed to explain the relationship between cell growth and cell cycle co-ordination. In the first possible pathway cellular growth and cell cycle progression are viewed as separate events running simultaneously, only combining as a means of overcoming cell cycle inhibition following sufficient cellular growth. It is also possible that as an extension of this parallel pathway, a series of checkpoints exists, that coordinate the two events of cell cycle and cell growth. In contrast the second suggested pathway represents a linear series of events in which the initial cell cycle events themselves result in cellular growth and thus the subsequent events leading up to cell proliferation. Of these pathways, the one in which cell growth and the cell cycle occur in parallel would seem to preferentially fit the findings of this study, as opposed to a linear model and would therefore suggest that cell growth is controlled separately from cell proliferation and hence, only during periods of decreased proliferation could the accumulation of cellular biomass result. These models however only provide an insight into the possible biological pathways at play and do not fully represent the diversity or complexity of the multiple pathways and checkpoints that may well govern cellular growth and proliferation; they do however highlight the possibility for cellular growth to occur independently of cell proliferation.

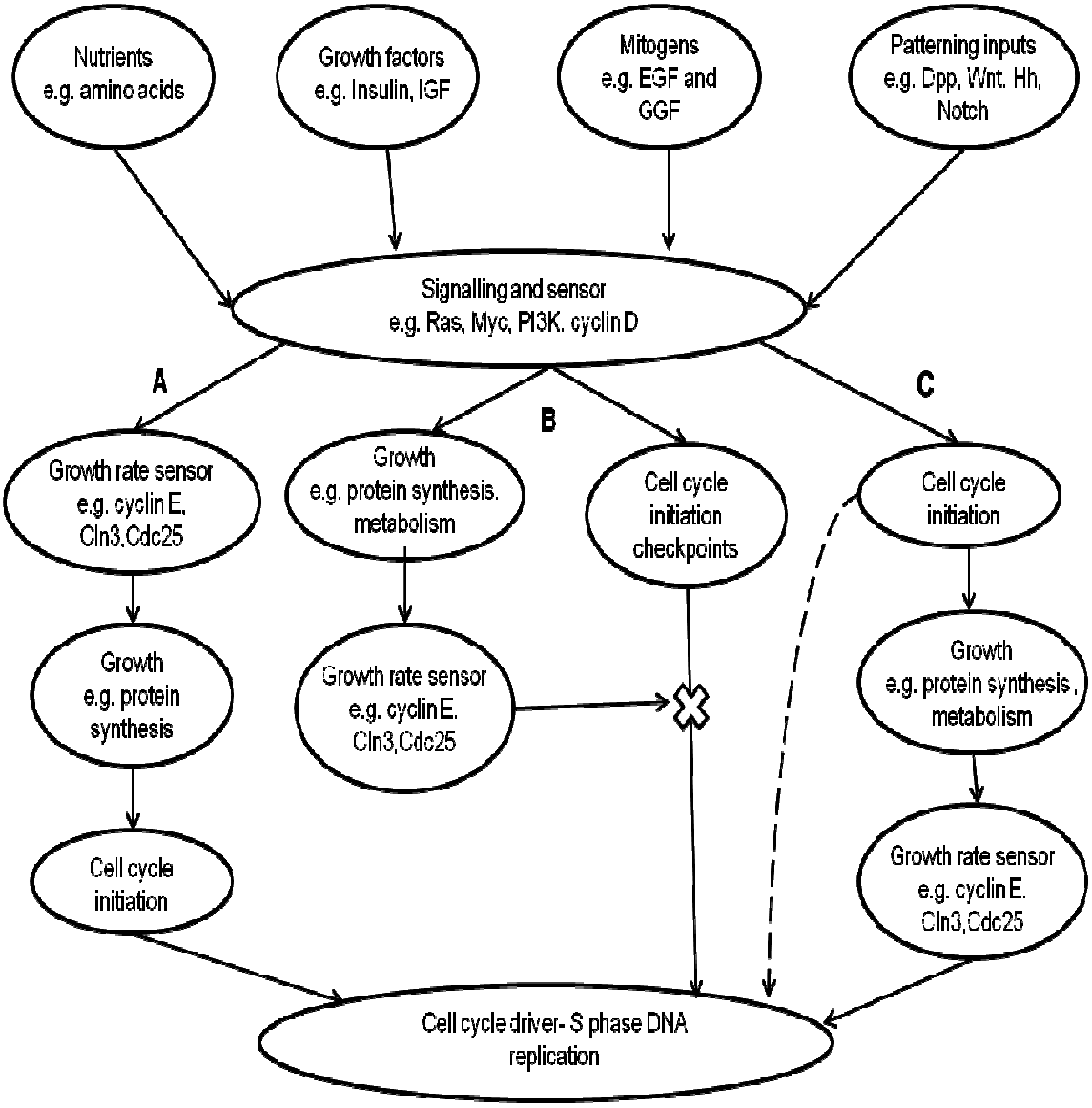


Figure 4.10. Diagramatic representation of the 3 pathways suggested by Jorgensen and Tyers *(Jorgensen et al 2004)*. In pathway A different signals are responsible for the growth of the cells which in turn activates the chain of events leading to cell cycle initiation in a linear fashion. In pathway B growth and cell cycle events are independent of each other up to the point where the inhibition of cell cycle is lifted by the growth rate sensor and in pathway C initial cell cycle events result in cell growth and the subsequent events leading to cell proliferation in a linear fashion.

As discussed previously the finding of a correlation between increased total protein, cell volume and specific productivity in proliferation arrested CHO cells can be attributed to the uncoupling of growth and proliferation. This would additionally suggest that the energy required for cell division would now be available for cell growth and product formation, however in order to determine if such a diversion of cellular resources to recombinant protein production occurs during periods of $p21^{CIP1}$-induced cell cycle arrest, key metabolic processes were also monitored.

Analysis of the mitochondria reveals a significant increase in mitochondrial dehydrogenase enzyme activity during periods of proliferation arrest; this is in agreement with our previous findings *(Bi et al 2004)* where it was observed that during periods of G1 phase cell cycle arrest an up regulation of mitochondrial activity was accompanied by an increase in mitochondrial mass. These results when combined with those presented here would seem to suggest the biogenesis of new mitochondria and not the activation of the existing organelles in arrested cultures. Analysis of the mitochondrial activity also provides an insight into the state of reduction/oxidation (redox), therefore the observed increase in mitochondrial activity in the arrested cultures would also seem to suggest the existence of a higher proliferative potential *(Takahashi and Abe 2002)*.

It would be expected that any increase in productivity would significantly increase the demands on the cell for increased energy metabolism; this was confirmed in this study through the analysis of the rates of glucose and glutamate consumption and lactate and ammonia production. Glucose is utilized by mammalian cells as the main source of

carbon and therefore passes through the glycolysis pathway extremely quickly resulting in the production of waste by-products such as lactate. In this study it was found that the specific utilization rate of glucose was significantly higher in cultures in which $p21^{CIP1-}$ expression had been induced when compared to non induced-cultures. Interestingly the difference in specific utilization rates was highest during the early stages of the batch culture, indicating the possibility of its utilization for the observed increase in cell volume and total protein which also occur during the early stages of culture.

Since the CHO cells used in this study have previously been transfected with the GS expression system the cells are able to synthesize their own glutamine from glutamate when grown in glutamine free media. The conversion of glutamate to glutamine has been previously shown to be utilized within the cell for the metabolism of cellular energy as well as the production of recombinant proteins *(Schneider et al 1996)*. Therefore glutamate becomes a significant factor in determining cell growth during the late exponential phase and its consumption was found to increase in response to diminishing glucose concentrations in the later stages of culture.

Interestingly the results of this study would point to the possibility that a link may exist between the increase in glutamate utilization and productivity; in that the specific production rate increases as the utilization rate of glutamate also increases. This would seem to indicate that glutamate may be utilized by the cells in the process of antibody production and that during periods of cell cycle arrest this process is able to occur more

readily, possibly as a result of decreased cell density and thus a greater abundance of glutamate.

What is clear from this study is the fact that arresting cells in the G1 phase of the cell cycle results in a significant increase in metabolic activity which almost certainly has an effect on production potential. It would also appear that cellular growth is able to be uncoupled from cell division, suggesting that in mammalian cells there is no underlying mechanism to regulate cell growth in the absence of cell division and hence rely on the mechanisms responsible for entry into mitosis and cytokinesis to ensure an appropriate cell size is reached before cell cycle progression. During the early stages of the cell cycle there is a great demand upon the cell to replenish energy supplies as well as the metabolic precursors that will later be required for cell division, ultimately resulting in an increase in cellular mass *(Ko and Prives 1996, Linke et al 1996 and Bi et al 2004).* In conclusion the physiological changes observed during periods of $p21^{CIP1}$-over-expression can be summarized into two categories. The first involves the alteration of metabolic pathways, fuelled by the requirements for intermediates during higher protein synthesis and the second involves changes in energy metabolism that result from the higher demand for cellular energy.

5. The role of $p21^{CIP1}$ in adaptation of CHO cells to suspension and protein-free culture.

5.1. Abstract

The up-regulation of cyclin-dependent kinase inhibitor $p21^{CIP1}$ has been shown to enhance productivity of monoclonal antibodies and has been linked to various regulatory processes. To identify the potential role of $p21^{CIP1}$ in adaptation to suspension and protein-free cultures, I studied the survival and growth of anchorage- and serum-dependent CHO cell-lines that differed only in the period of $p21^{CIP1}$-induced arrest. $p21^{CIP1}$ over-expression led to rapid adaptation of cells to suspension and protein-free cultures. The period taken to achieve adaptation was correlated with the time the cells were arrested after transfer from the monolayer and serum-fed culture. Interestingly, cell aggregation associated with protein-free suspension culture was reduced in $p21^{CIP1}$-expressing cultures in response to the loss of cellular adherence. The processes of adaptation to suspension and arrest did not decrease monoclonal antibody productivity. In contrast, following adaptation to protein-free growth media, an overall increase in specific productivity was observed. The ability of cells to survive in protein-free suspension cultures was due to the requirement of G1 cells to growth factors and to their relatively high resistance to the hydrodynamic forces. This improved process has the advantage of reducing the duration of critical path activity for developing CHO commercial cell-lines from 72 to 36 days.

5.2. Introduction

Animal cell cultures have been widely used to produce therapeutic protein products due to the compatibility of post-translational product modification with the therapeutic target; however, the need for increased yields and the requirement to eliminate animal-derived elements are driving industry forward in the search for suitable methodologies that can sustain high yielding cell-lines and speed up the development and production process.

Anchorage-dependent cells must be adapted to grow in suspension cultures due to the scale-up limitation associated with monolayer cultures. Cells which grow in suspension are readily scaled up to production level bioreactors, which are designed to reduce hydrodynamic forces and heterogeneity *(Al-Rubeai et al 1995, Ludwig 1992, Keane 2003)*. Anchorage-dependent cells can be made to be sufficiently robust in the bioreactor suspension environment by a time-consuming adaptation process that includes the addition of protective agents, such as Pluoronic F68 to the media *(Kilburn and Webb 1968, Al-Rubeai et al 1992b)*. While the effects in suspension culture of hydrodynamic forces and the sensitivity of mammalian cells to hydrodynamic stress have been fully examined, and while the mechanism is well understood *(Hu and Wang 1986, Peterson et al 1988, Handa-Corrigan et al 1989, Oh et al 1989, Croughan et al 1989, Jordan et al 1994, Kunas and Papoutsakis 1990a, 1990b, van der Pol et al 1992)* the adaptation process has remained essentially unchanged.

Interestingly, it would appear that the susceptibility of any given cell to the forces exerted by the bioreactor environment are increased by, for example, decreased serum concentration *(Kunas and Papoutsakis 1990a and b, McQueen and Bailey 1989)* culture medium viscosity *(Croughan et al 1989)* and increased cell size *(Al-Rubeai et al 1995)*. The role of cell size in determining cellular growth, productivity and response to the bioreactor environment may have not been fully appreciated, but cell size regulation is shared with the cell cycle, which, in effect, determines the metabolic potential of a cell and, indeed, its response to the environment.

The ever-increasing need for improved biological safety is pushing industries forward in the development of new serum and protein-free media formulations. Cell-lines will have to be adapted to grow and produce the required product in a defined chemical environment. There have been several approaches for the adaptation of cell-lines to the required environments *(Kovar and Franek 1984, Radford et al 1991, Keen and Steward 1995 Sinacore et al 2000, Ozturk et al 2003)* either by a gradual weaning of cultures via the reduction of serum over a set time period or by direct withdrawal of serum. In both cases, the process of adaptation can be extremely long and costly, depending on the cell-line and medium selected. Therefore, a process that can speed up the rate of adaptation would provide a valuable tool for both industrial and research applications.

As discussed previously the overall control of the cell cycle, and thus cellular proliferation, is based on the activity of cyclin-dependent kinases (cdks), which are activated by their association with cyclin regulatory subunits via phosphorylation and

may, in turn, be inhibited by the alternate binding of cdk inhibitors (ciks), such as $p21^{CIP1}$. The effect of $p21^{CIP1}$ activity in terms of its effect on productivity in cell culture is well documented *(Fussenegger et al 1998, Watanabe et al 2002, Bi et al 2004, Ibara et al, 2003)* however, it is the ability of $p21^{CIP1}$ to induce arrest that is of particular interest in this study, which examines new roles for $p21^{CIP1}$ in process development.

Examination of the proteins and pathways involved in responding to the stresses caused by chemical or physical means has shown that an inbuilt adaptive response exists which is capable of adjusting the levels and/or activity of the genome protecting machinery, usually through the synchronization of cell cycle arrest, DNA repair and apoptosis *(Hofseth 2004)*. By initiating $p21^{CIP1}$ over-expression, cells accumulate in the G1 phase of the cell cycle. This arrest of cell cycle progression may allow additional time for repair of environmentally derived damage before the cell is replicated *(Weinert and Hartwell 1988).*

I therefore hypothesize that an extended period of cell cycle arrest allows cells to survive in a phase where extracellular growth factors are not needed, and/or the accumulation of extracellular autocrine growth factors to a level that can support proliferation. It may also allow the resulting genome damage, created through the direct withdrawal of serum, to be repaired prior to cell cycle release. Interestingly, recent studies also indicate that $p21^{CIP1}$-expression may also provide some protection against apoptosis during the process of adaptation to genotoxic stresses. A recent study *(Roninson 2002)* showed that wild-type $p21^{CIP1}$ has the ability to inhibit apoptosis and stimulate transcription of secreted factors

with mitogenic and antiapoptotic activities. In this chapter, I have examined the effect of $p21^{CIP1}$-induced cell cycle arrest on the ability of an anchorage-dependent CHO cell-line to survive and adapt to a suspension and protein-free growth environment whilst maintaining productivity.

5.4. Results

5.4.1. The effect of $p21^{CIP1}$ over-expression on CHO cell-line adaptation to suspension culture

In order to establish whether a period of cell cycle arrest would have an impact on the ability of industrially relevant CHO cell-lines to adapt to single-cell suspensions in serum-supplemented growth environments, four cultures of the transfected $p21^{CIP1}$-expressing cell-line were set up as described in the Materials and Methods section of this thesis. The results obtained (Fig. 5.1) clearly demonstrate the impact of 3-, 6- or 9-days of $p21^{CIP1}$-induced cell cycle arrest in comparison to the non-induced control culture. Cultures that were maintained in a state of cell cycle arrest clearly demonstrated an increase in the rate of adaptation, as well as an overall increase in culture viability (results not shown). Following passage into fresh growth medium without IPTG, cells began to proliferate.

The results also show a clear relationship between the length of cell cycle arrest and the length of time required for adaptation to single-cell suspension. It would appear that increasing the period of cell cycle arrest at the start of adaptation decreases the overall time required for adaptation to take place. Fig. 5.2 summarizes the overall time taken for

adaptation, using $p21^{CIP1}$-induced cell cycle arrest for 3, 6 and 9 days, whereby it was possible to reduce adaptation time by 12, 23 and 31%, respectively, in comparison to the overall time taken for the unarrested control culture.

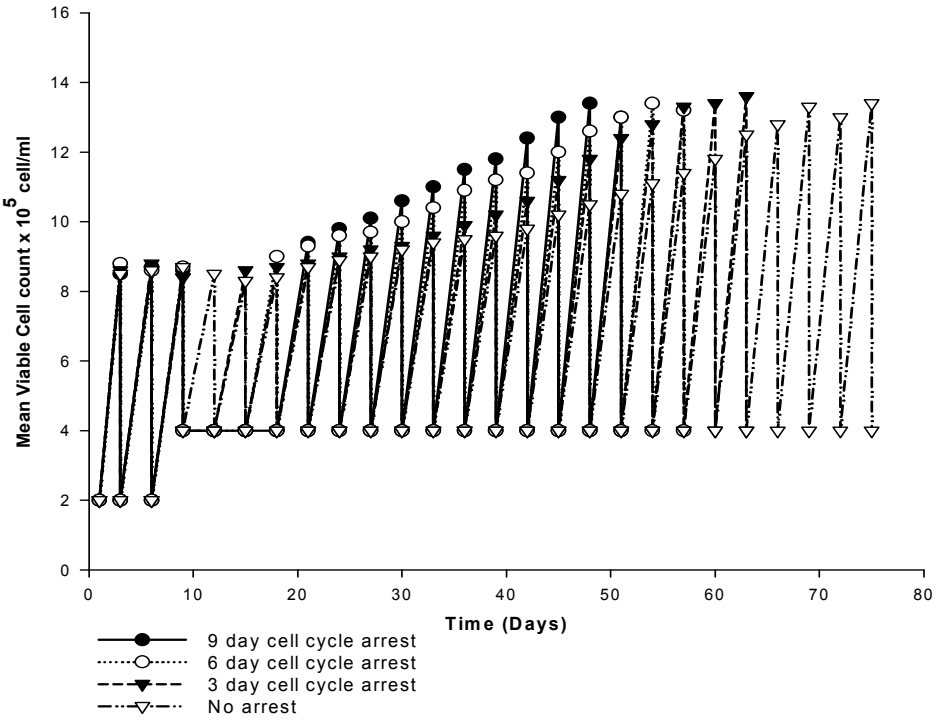


Figure 5.1. Passage cycles during adaptation from monolayer cultures to single-cell suspension through the expression of $p21^{CIP1}$. Cultures previously grown in static conditions were detached from the surface of the tissue culture flask and re-suspended in fresh culture medium consisting of DMEM F12 growth medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX. The viable cell number and percentage viability was determined before the cells were centrifuged at 90g for 5 minutes and re-suspended in fresh growth medium. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed

with the original culture. For each set of experiments, four flasks were set up as described above. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$-expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs, the cultures were centrifuged at 90g and re-suspended in fresh culture medium. Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the fresh culture medium. Non-induced cultures were passaged back to 4×10^5 cells/ml. Following 3, 6, and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers (see chapter 2 Materials and Methods). Mean and standard error were calculated from this data and the significance determined via *t*-test analysis. The first 2 passages represent growth in a static environment prior to the initiation of adaptation to serum-containing suspension. Error bars are not show for clarity but the average standard error of the mean was no more than 0.2.

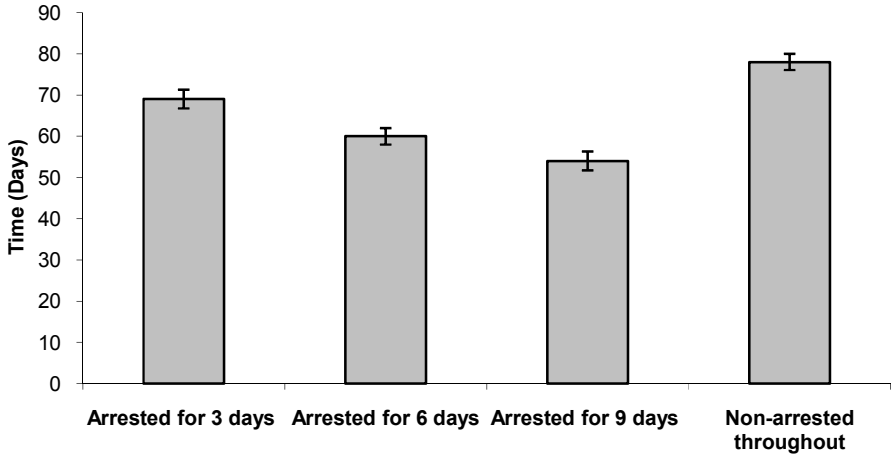


Figure 5.2. Summary of the time taken for adaptation to single-cell (serum containing) suspension using p21^{CIP1}-induced cell cycle arrest as determined from the data presented in figure 5.1. Bars represent the overall time taken for adaptation from static serum-supplemented culture to single-cell serum-supplemented suspension culture following either 3, 6, or 9 days of p21^{CIP1}-induced cell cycle arrest. Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between induced and non-induced cultures ($p < 0.05$).

5.4.2. The effect of p21 ^{CIP1} over-expression on CHO cell-line adaptation to protein-free suspension culture

Cultures that had previously been adapted to single-cell serum-supplemented suspension, as described in previously and shown in Figure 5.1 were used to demonstrate the impact of cell cycle arrest on the process of adaptation to protein-free culture. All cultures underwent a minimum of four passages in the current growth conditions before experimentation into the impact of adaptation to protein-free suspension was initiated. The results in Fig. 5.3 show a similar pattern of behavior to that seen in Fig. 5.1. Similarly, cultures that were initially arrested showed a significant reduction in the time

taken to achieve a successful adaptation to protein-free media, as determined via the *t*-test analysis. The results summarized in Fig. 5.4 show a similar, yet enhanced, profile to those shown in Fig. 5.2. It would therefore appear that the expression of $p21^{CIP1}$ during adaptation to protein-free suspension conditions was able to significantly reduce the time taken for successful adaptation, with arrest periods of 3, 6, and 9 days resulting in 25, 37, and 50% decreases in the time taken for successful adaptation when compared to the non-induced culture.

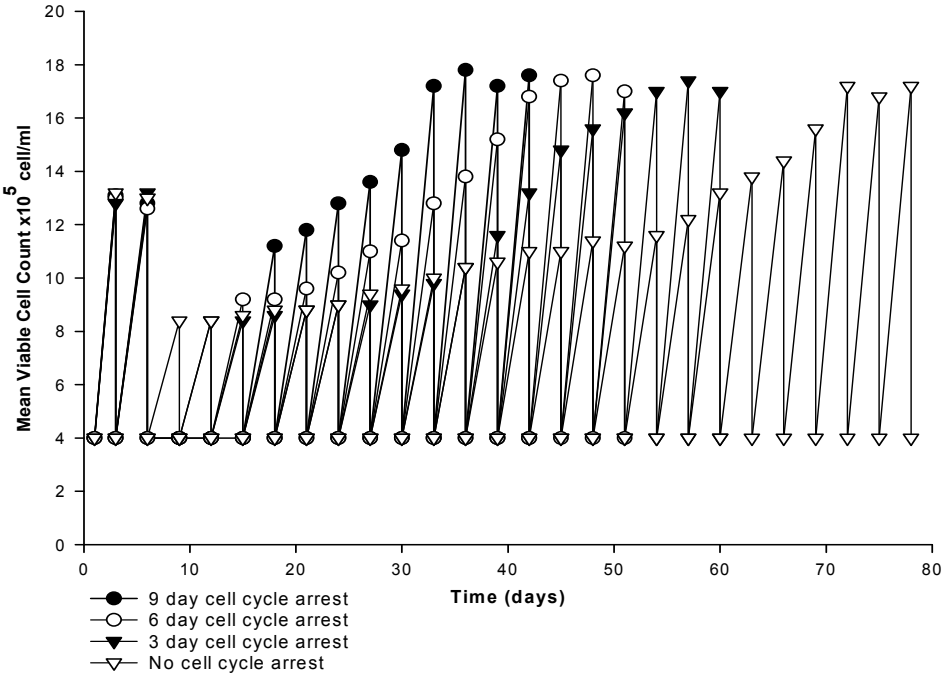


Figure 5.3. Passage cycles during adaptation from serum supplemented culture medium to protein-free culture medium through the expression of $p21^{CIP1}$. Suspension cultures previously grown in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX were harvested during mid-exponential phase and the viable cell number and percentage viability were determined. The cells were then washed in warm 1% PBS before being centrifuged at 90g for 5 minutes. The cells were then re-suspended in fresh protein-free medium supplemented with 50µM MSX. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of

100ml using 250ml vented Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture. For each set of experiments, four flasks were set up as described above. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$-expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs, the cultures were centrifuged at 90g and re-suspended in fresh culture medium. Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the fresh culture medium. Non-induced cultures were passaged back to 4×10^5 cells/ml. Following 3, 6, and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers (see chapter 2 Materials and Methods). Mean and standard error were calculated from this data and the significance determined via *t*-test analysis. The first 2 passages represent growth in a static environment prior to the initiation of adaptation to serum-containing suspension. Error bars are not show for clarity but the average standard error of the mean was no more than 0.2.

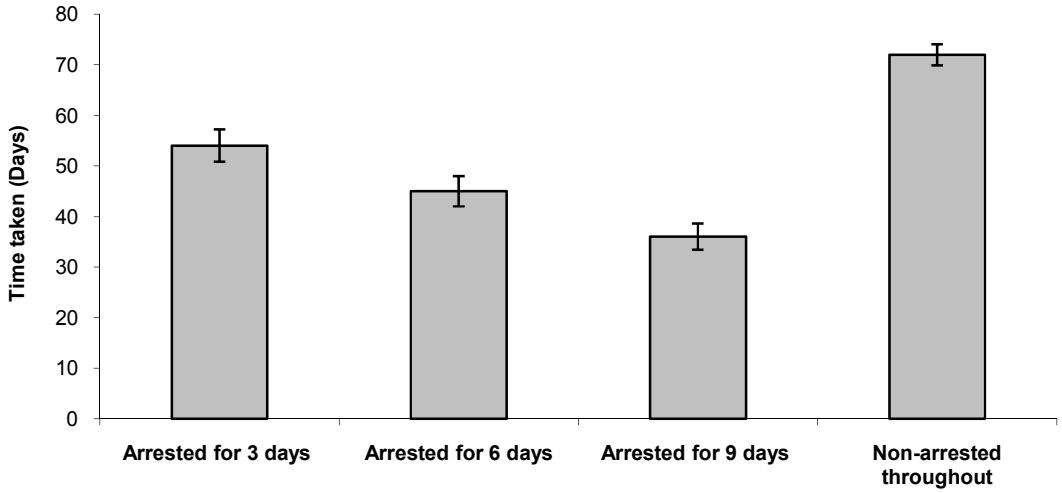


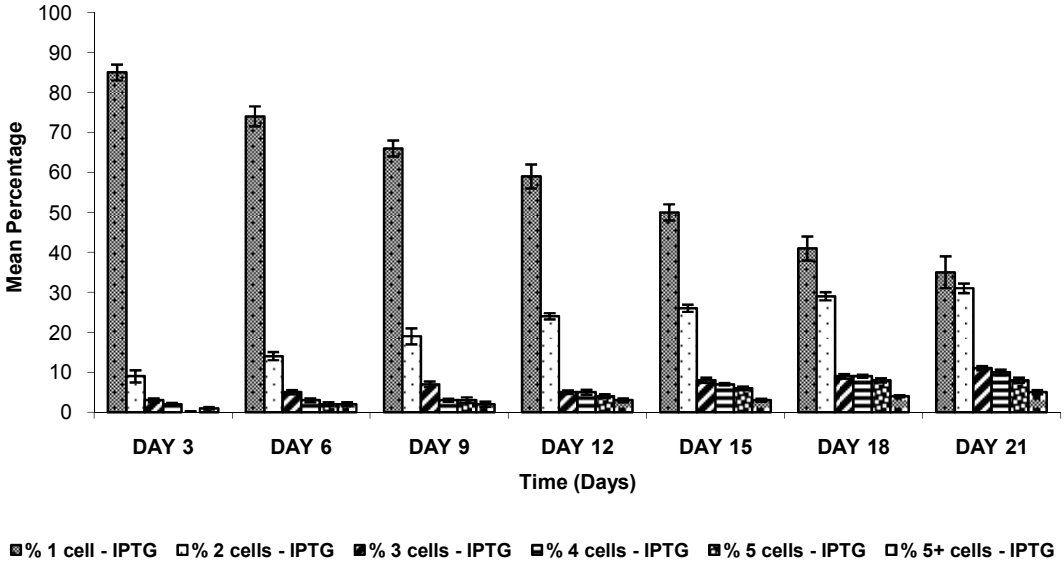
Figure 5.4. Summary of the time taken for adaptation to protein-free growth environments using p21^{CIP1}induced cell cycle arrest as determined from the data presented in figure 5.3. Bars represent the overall time taken for adaptation from single-cell serum-supplemented cultures to protein-free single-cell suspension culture following either 3, 6, or 9 days of p21^{CIP1}-induced cell cycle arrest. Student's *t*-test indicated significant differences between the induced and non-induced cultures ($p < 0.05$).

5.4.3. p21 ^{CIP1}-Dependent cell detachment and disaggregation

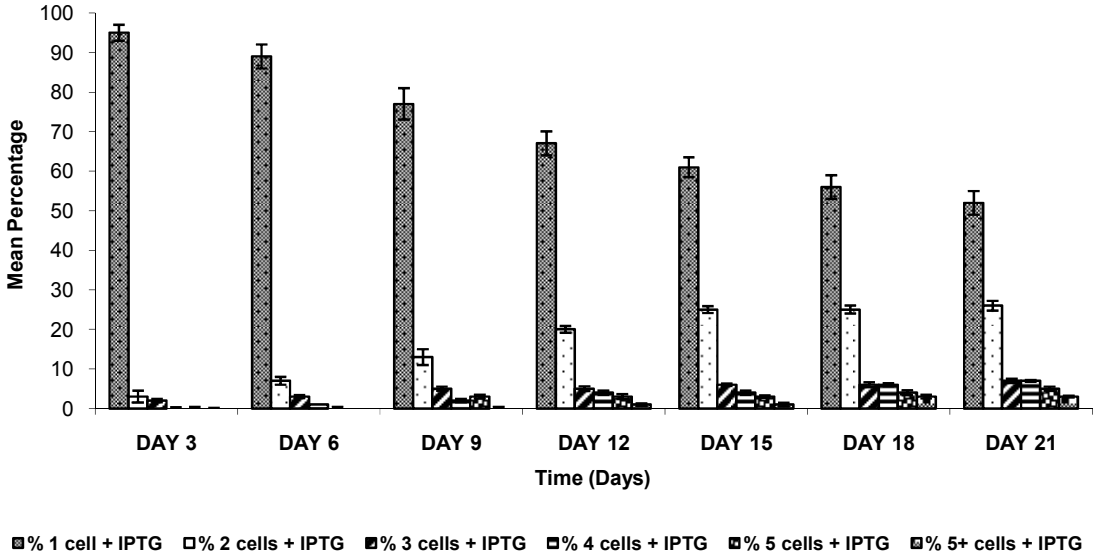
During a preliminary study of adaptation from single-cell serum-supplemented suspension to protein-free suspension, it was observed that a significant amount of cell aggregation occurred, particularly in the non-induced cell-line. In order to determine the effect that p21^{CIP1}-over expression had on the rate of aggregate formation during the process of adaptation, samples were taken from the adapting cultures at 72 h intervals and were analyzed to determine the percentage of single-, 2-, 3-, 4-, 5- and more-than-5-cell aggregates as described in the Materials and Methods. Fig. 5.5 shows the results obtained over the first 21 days of adaptation, which clearly demonstrate an increase in aggregate

formation in the non-induced culture (Fig. 5.5A) in comparison to the induced cultures (Fig. 5.5B–D), where, in the latter, there was a clear reduction in aggregate formation both during the period of cell cycle arrest and subsequently, after the removal of IPTG. The observed reduction in aggregation appears to be directly linked to the length of time cells were maintained in a state of cell cycle arrest, with the most significant result occurring following 9 days of $p21^{CIP1}$-induced cell cycle arrest (Fig. 5.5D).

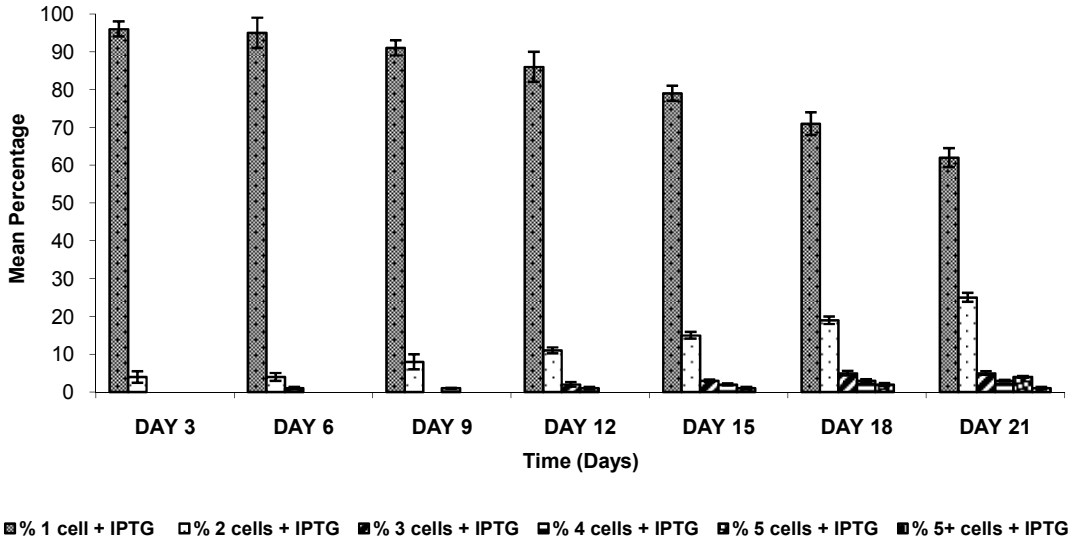
A



B


100
90
80
70
60
50
40
30
20
10
0
Mean Percentage
DAY 3
DAY 6
DAY 9
DAY 12
DAY 15
DAY 18
DAY 21
Time (Days)
% 1 cell + IPTG
% 2 cells + IPTG
% 3 cells + IPTG
% 4 cells + IPTG
% 5 cells + IPTG
% 5+ cells + IPTG

C


100
90
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60
50
40
30
20
10
0
Mean Percentage
DAY 3
DAY 6
DAY 9
DAY 12
DAY 15
DAY 18
DAY 21
Time (Days)
% 1 cell + IPTG
% 2 cells + IPTG
% 3 cells + IPTG
% 4 cells + IPTG
% 5 cells + IPTG
% 5+ cells + IPTG

D

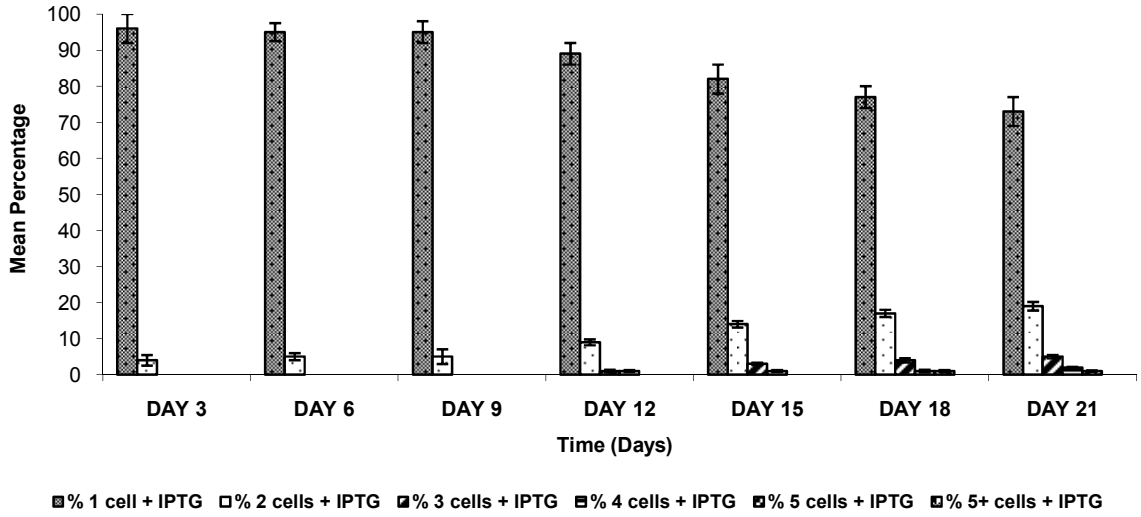


Figure 5.5. Analysis of cell aggregate formation. Cultures were analyzed at 72 hour intervals over 21 days, during adaptation from 10% serum-containing suspension cultures to protein-free suspension (see Fig 5.3). Columns represent the percentage of cells per aggregate in cultures that were (A) left un-induced throughout, (B) induced for 3 days, (C) induced for 6 days, and (D) induced for 9 days (see chapter 2 Materials and Methods). Student's t-test indicated significant differences between the induced cultures in (D) and the corresponding non-induced cultures in (A) ($p < 0.05$)

In order to determine if the reduction in aggregation was a result of loss of anchorage dependence during cell cycle arrest, two static cultures were set up in T75 flasks, where one flask had 5mM IPTG added. The results, in Fig. 5.6, clearly show a reduction in the ability of arrested cultures to attach to the flasks' surfaces, indicating a loss of anchorage dependence. Arrested cells detached at much faster rates than the control cell-line, with 100% increase in cells detached within 5 min of treatment with the dissociation reagent.

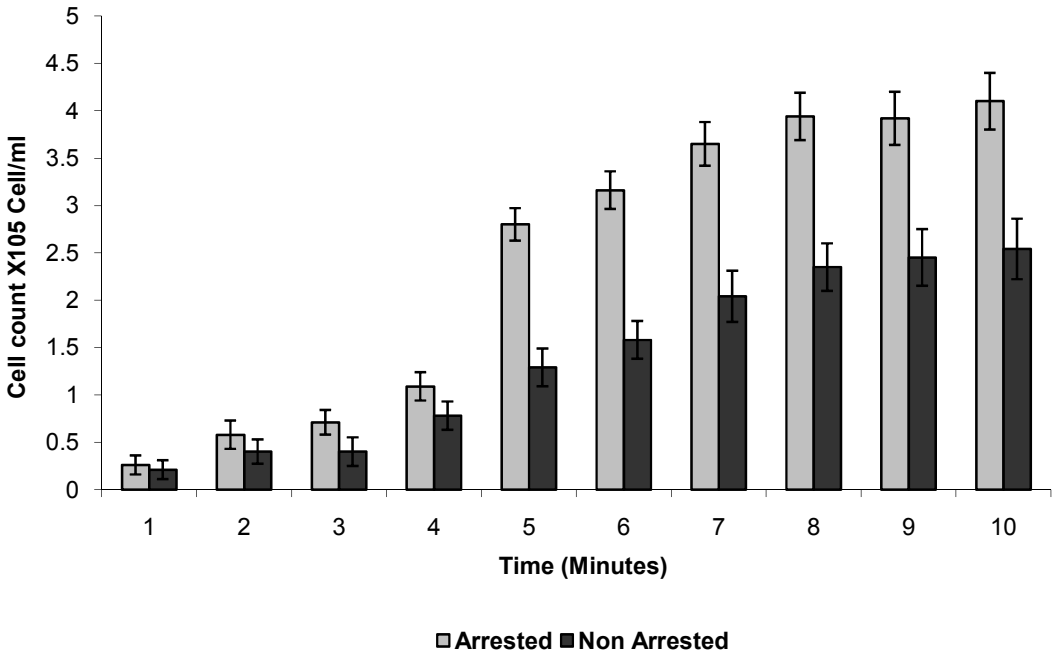
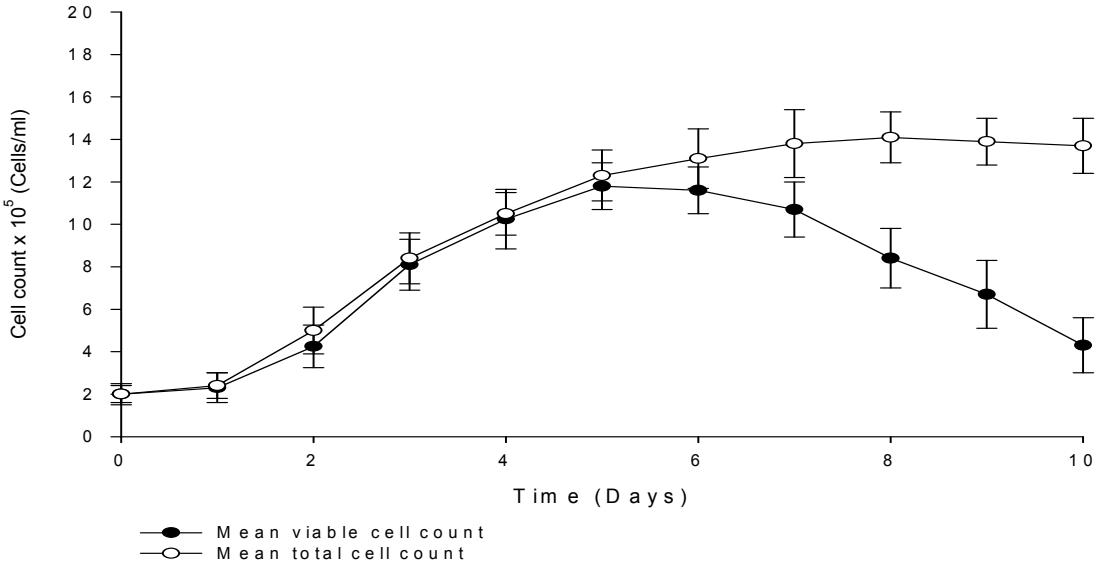


Figure 5.6. The effect of cell cycle arrest on cell adhesion. Cells were grown in static for 24 h with or without the presence of IPTG. Cells were dissociated using 50% dilution of cell dissociation solution (non-enzymatic) and 1× PBS. Samples were taken at 60 second intervals and analyzed via Trypan blue in order to determine the cell concentration. The pale grey bars represented cultures in which $p21^{CIP1}$-expression was induced and the dark grey, non-induced cultures (see chapter 2 Materials and Methods). Error bars represent the standard error of three replicates. Student's *t*-test indicated significant differences between the induced and non-induced cultures at each time point ($p < 0.05$).

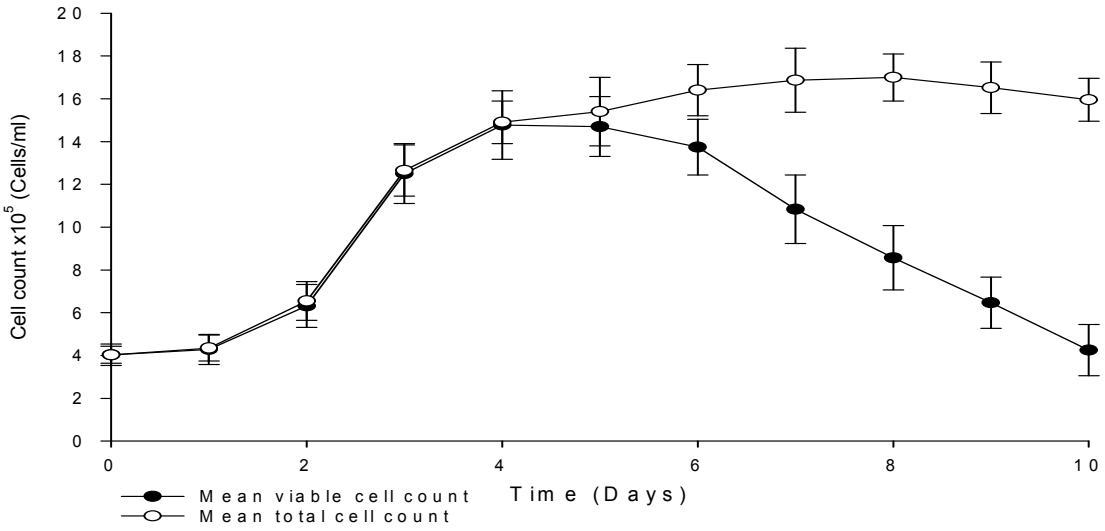
5.4.4. The effect of adaptation using $p21^{CIP1}$-expression on culture growth and productivity

A successful process of adaptation should result in no loss in culture proliferation or productivity. Fig. 5.7 demonstrates this assertion by showing the viable and total cell counts over a 10- day batch culture during three different process conditions.

A


Cell count x 10^5 (Cells/ml)
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (Days)
Mean viable cell count
Mean total cell count

B


Cell count x10^5 (Cells/ml)
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (Days)
Mean viable cell count
Mean total cell count

C

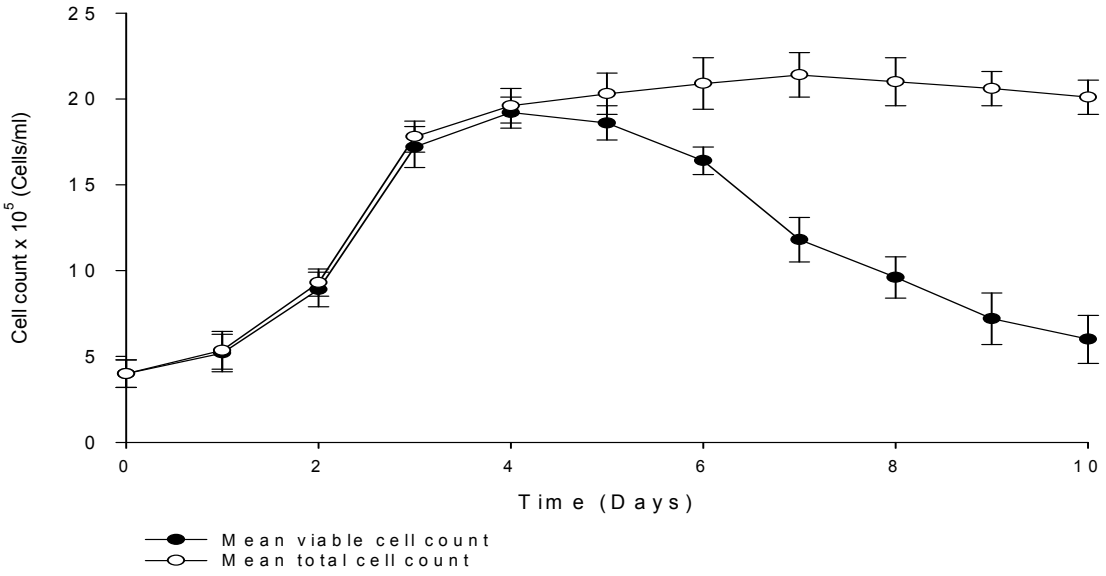


Figure 5.7. Batch cultures of p21^{CIP1}-expressing cell-lines grown as (A) serum-supplemented static (B) serum-supplemented suspension or (C) protein-free suspension cultures. Cultures were set up either in (A) T75 flasks or (B and C) 250ml Erlenmeyer flasks. Samples were taken at 24 hour intervals and viable and total cell counts determined via Trypan blue exclusion (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates.

The results clearly show an increase in attainable viable cell numbers following adaptations to both suspension and protein-free cultures. Moreover, it would appear that the process of adaptation to serum-supplemented suspension culture is able to increase the growth rate significantly in comparison to static culture (Figure 5.8); however, further adaptation to protein-free suspension culture appeared to reduce the growth rate back to that of the static culture. It appears that the protein-free formulation has specifically enhanced volumetric and cell-specific productivity while depressing growth rate (Figs. 5.8–5.10). During growth as static, suspension, serum-supplemented, and protein-free

cultures, the activation of $p21^{CIP1}$ via the addition of IPTG significantly increased the overall cell-specific production rate of antibodies (Fig. 5.10).

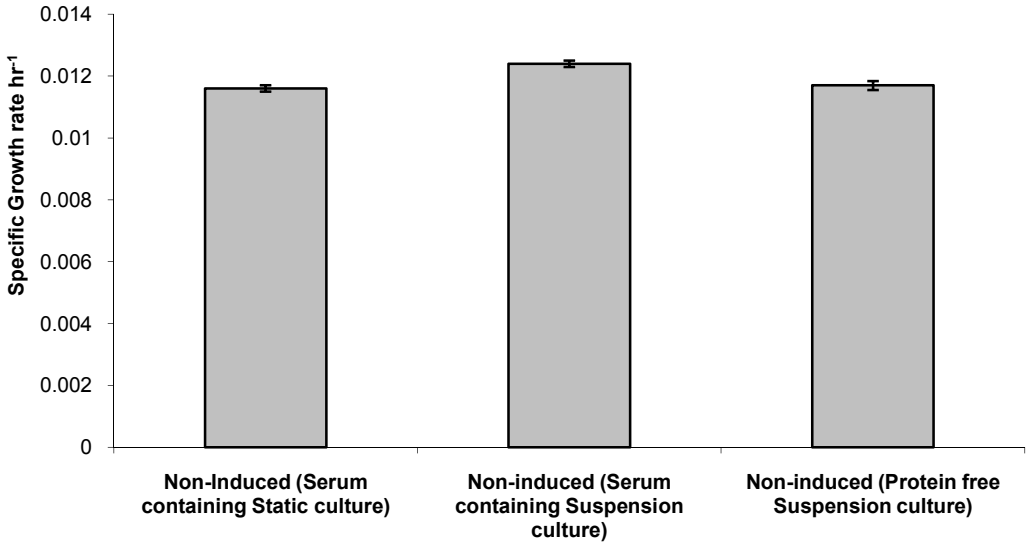


Figure 5.8. Specific growth rate following adaptation to growth in suspension and protein-free growth environments. Results were obtained during the exponential growth phase (see Fig. 5.7) of non-induced cultures grown either as serum supplemented static cultures or following adaptation to serum-supplemented suspension or protein-free suspension (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates.

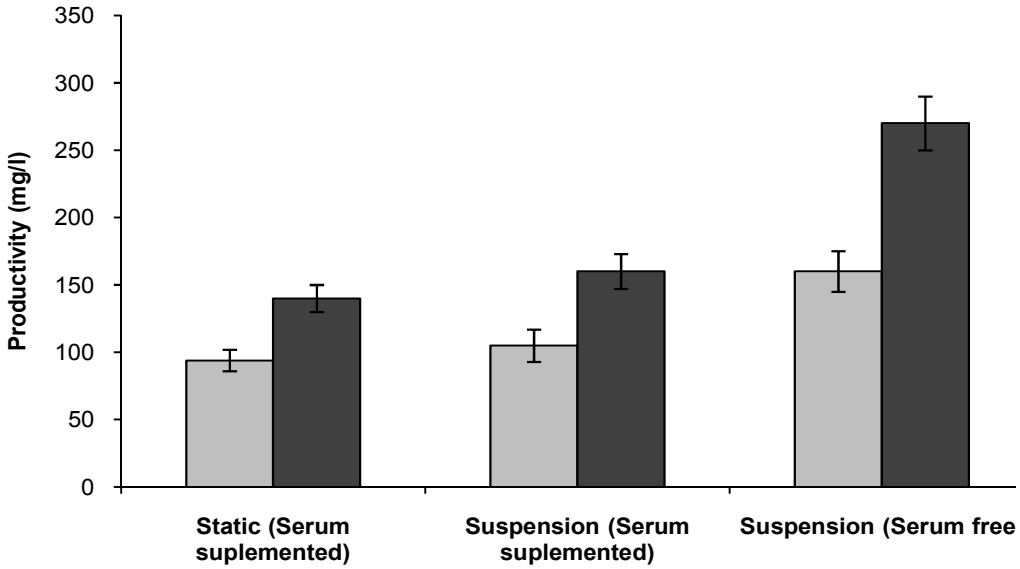


Figure 5.9. Productivity following adaptation to growth in suspension and protein-free media. Results were obtained after 120 h of growth in either, serum supplemented static, suspension or protein-free suspension cultures as determined via ELISA using a standard curve. Dark grey bars represent productivity of chimeric antibody in cultures in which $p21^{CIP1}$-expression had been induced, and light grey bars represent non-induced cultures (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of induced and non-induced cultures at each condition ($p < 0.05$).

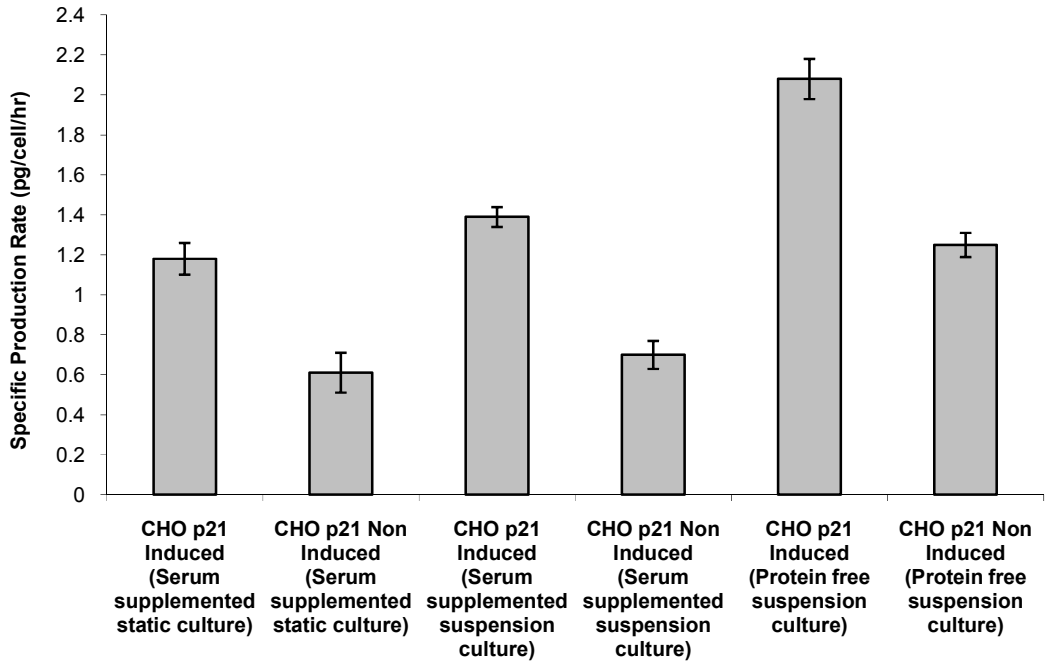
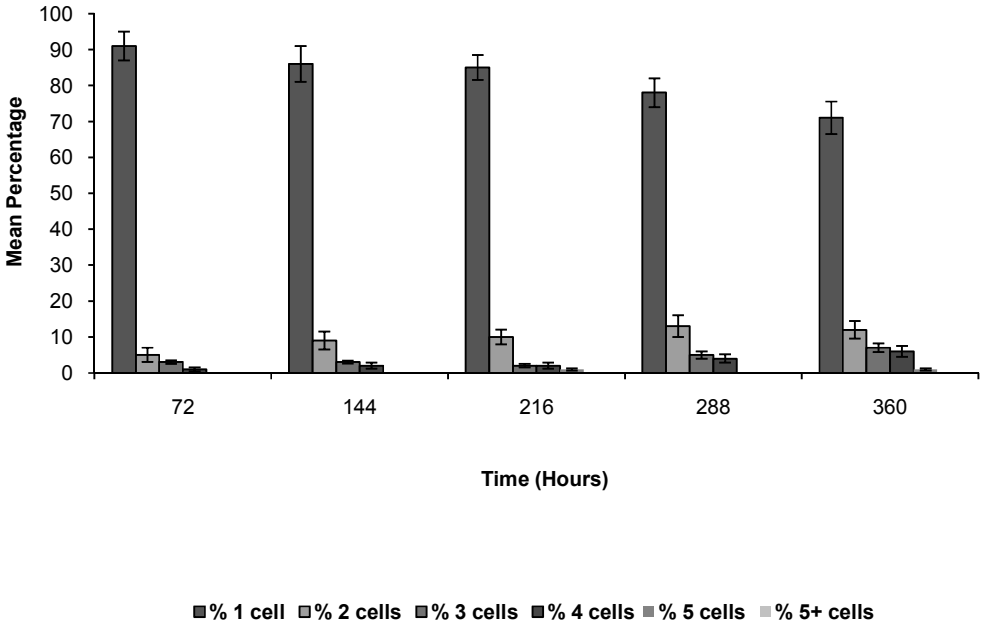


Figure 5.10. Specific production rate following adaptation to growth in suspension and protein-free growth environments. Bars show the specific production rate of cultures in which $p21^{CIP1}$-expression had been induced or non-induced. cells were grown either as serum-supplemented static cultures or following adaptation to serum-supplemented suspension or protein free suspension (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of induced and non-induced cultures at each condition ($p < 0.05$).

5.4.5. Analysis of cell-line stability following adaptation to growth as protein-free suspension cultures

In order to establish the stability of cultures adapted using $p21^{CIP1}$expression, two cultures adapted with and without cell cycle arrest were cryopreserved for a period of 6 weeks, following which they were revived using a standard procedure. Fig. 5.11 shows a decrease in the percentage of aggregates in cultures adapted using $p21^{CIP1}$-induced cell cycle arrest, demonstrating an increased ability to return to a state of single-cell suspension culture through the $p21^{CIP1}$-dependent adaptation in comparison to cultures adapted without the use of $p21^{CIP1}$-induced cell cycle arrest.

A



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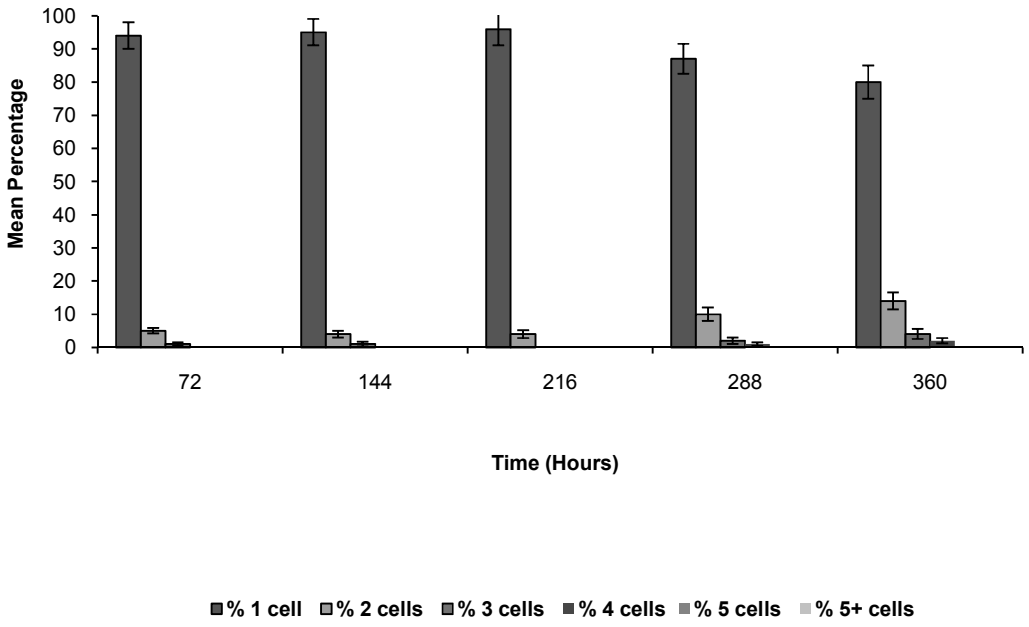

Figure 5.11. Analysis of cell aggregate formation. Bars show the percentage of aggregation observed in cultures previously adapted (A) without or (B) with 9 days of $p21^{CIP1}$-induced cycle arrest (see Figs 5.1 and 5.3) following 6 weeks of storage in cryopreservation (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates.

5.5. Discussion

In this study, I have successfully demonstrated that $p21^{CIP1}$-over-expression can significantly reduce the time necessary for the adaptation of an anchorage-dependent CHO cell-line to suspension and protein-free growth environments without compromising the growth or productivity of the cell-line. A previous study from our laboratory *(Al-Rubeai et al 1995)* has clearly demonstrated that cells in the G2/M phase of the cell cycle are more susceptible to mechanical damage than cells in the G1 phase. The authors have concluded that larger cells, such as those in S and G2, are preferentially destroyed and that the extent of G2 cell destruction is dependent on the intensity of the hydrodynamic forces exerted.

Later, *Abdul Majid and Al-Rubeai (1997)* showed that mitotic cells were more susceptible to mechanical damage than cells in the interphase, thus suggesting that such a response was not merely due to the larger volume of G2/M cells. Therefore, it was proposed that by arresting the cell cycle in the G1 phase of the cell cycle it would be possible to limit the effect of hydrodynamic shear forces on anchorage-dependent cells within an agitated culture during the process of adaptation from a static to a suspension growth environment. Cell cycle arrest may also help cells to survive the lethal forces in an agitated or shaken environment that preferentially destroy dividing cells, thereby giving sufficient time for the cells to display increased robustness and apoptosis inhibitory function and induced expression of the cell surface molecule E-cadherin, which has been demonstrated *(Mueller et al 2000)* to closely correlate with the ability of the cells to grow independently of anchorage.

Interestingly, this study showed that $p21^{CIP1}$-induced cell cycle arrest limited the formation of aggregates during the process of adaptation; this may well be the result of maintaining an increased viable cell number. It has been observed that floating colonies of epithelial cells produce a protein membrane closely resembling those of basement membranes that connect individual cells within and between colonies *(Eckhart et al 2003)*. More importantly, DNA released from dying or dead CHO cells in aggregate culture under suboptimal growth conditions was found to mediate cell–cell adhesion *(Renner et al 1993)*. It appears that, by enhancing viability, $p21^{CIP1}$ prevents the release of degraded DNA and cellular materials into the medium and may also play a role in the organization of the extracellular matrix by reducing the expression of extracellular adhesion factors. The forced expression of $p21^{CIP1}$ resulted in a reduction in cell-to cell adhesion, thereby reducing multicellular aggregation, which can normally cause a reduction in viability due to the nutrient and mass transfer limitations within the spheroid structure of multicellular aggregates.

The arrest of cells in the G1 phase of the cell cycle may also influence the adaptation process to protein-free culture by reducing the cell requirement for growth factors/complex nutrients that are present in the serum. Thus, G1 cells would survive the nutrient-deficient environment until they reach the restriction point, when growth factors are required for further progression toward the S phase. Several studies have demonstrated that cells only require growth factors at the mid-G1 phase to stimulate the formation or activation of cyclin D-cdk4/6 and cyclin E-cdk2, with consequent stimulation of DNA synthesis (for review, see *Assoian 1997*). Therefore, it is expected

that $p21^{CIP1}$-induced cells, arrested in the G1 phase would not require growth factors or complex nutrient for their survival, as these molecules are only essential for the stimulation of cells to enter cell division. Arresting cells prior to adapting them to an environment lacking in serum-derived growth factors would therefore help them to subsequently grow in a protein-free medium.

Finally, the results of this study provide a clear indication of the possible role of $p21^{CIP1}$-induced cell cycle arrest in adaptation of cell-lines to desirable bio-processing environments. We have shown that extended periods of cell cycle arrest can significantly decrease the time required for adaptation of industrially important cell-lines to suspension and protein-free growth environments. Although the precise mechanisms are not fully understood, what is clear is that engineering of cells for induced expression of $p21^{CIP1}$ will enable the rapid development of cell-lines specifically adapted to withstand the rigors and stresses of growth within chemically defined growth media in the bioreactor environment.

6. The role of Bcl-2 and its combined effect with $p21^{CIP1}$ in adaptation of CHO cells to suspension and protein-free culture

6.1. Abstract

The over-expression of the anti-apoptotic gene Bcl-2 has been previously shown to protect cells from undergoing apoptosis during exposure to environmental stress. There is strong evidence that, in addition to its well-known effects on apoptosis, Bcl-2 is involved in antioxidant protection and regulation of cell cycle progression *(Lopez-Diazguerrero et al 2006)*. To determine if the over-expression of Bcl-2 could improve the process of adaptation to suspension and protein-free growth environments I have studied the growth and viability of anchorage-dependent CHO cell-lines that differ only in there expression of Bcl-2. In addition, I examined the effect of combining Bcl-2 and $p21^{CIP1}$-expression during adaptation to suspension and protein–free environments. The results of this study provide evidence of a clear reduction in the overall time required for the process of adaptation to both suspension and protein-free environments in Bcl-2-expressing cultures and that through the combined expression of $p21^{CIP1}$ and Bcl-2 it is possible to further reduce the time. The Bcl-2 results support the well demonstrated concept that this protein plays an important role in apoptotic signalling pathways and suggest that it may also provide more diverse functions beside its death-inhibiting role.

6.2. Introduction

Suspension and protein-free cultures have long been the preferred choice for the industrial production of biopharmaceuticals for several reasons including, the scale-up limitations associated with monolayer cultures; cells which are able to grow in suspension environments are easily scaled up into bioreactors, which have been specifically designed to reduce hydrodynamic forces and heterogeneity within the growth environment (*Al-Rubeai et al 1992b, Al-Rubeai et al 1995, Ludwig 1992, Keane 2003, Kilburn and Webb 1968*). The ever-increasing need for improved biological safety is also driving industries forward in the development of new serum and protein-free media formulations, which in turn require the development of new approaches for the adaptation of the desired cell-lines to the required growth conditions *(Kovar and Farnek 1984, Radford et al 1991, Keen and Steward 1995, Sinacore et al 2000, Ozturk et al 2003)*.

Previous research has shown that mammalian cell-lines are often highly susceptible to apoptosis within the required bioreactor growth conditions (*Singh et al 1994, Perani et al 1999)*; therefore in order to increase productivity within the bioreactor environment several strategies have been employed, including the process of perfusion cultures. This facilitates the development of a high-density culture and thus high product titres can be achieved (*Himmelfarb et al 1969, Tharakan and Chau 1986, Comer et al 1990, Ong et al 1994, Jan et al 1993, Amos et al 1994, Emery et al 1995, Portner et al 1997, Fassnacht et al 1998(a*). However, these systems tend to consist of large numbers of non-viable cells which can have a severe impact on the rate of cell division and thus on productivity (*Al-Rubeai and Emery 1990, Al-Rubeai et al 1992a)*. In addition the build-up of proteases

and glycosidases and other cellular components can lead to the degradation of the desired product while creating additional complications during downstream processing.

As described in chapters 5 of this thesis, several studies (*Al-Rubeai et al 1990, Mercille and Massie 1994, Singh et al 1994, Westlund and Haggstrom 1998, Ishaque and Al-Rubeai 1999, 2002*) have identified a number of factors which are able to affect the viability of cultures within the bioreactor environment including the deprivation of glucose, glutamine, essential amino acids, mineral salts and vitamins as well as the build-up of toxic metabolites such as ammonia. All of these factors are able to initiate apoptotic cell death and thus decrease the duration of culture within the bioreactor environment which in turn limits the overall achievable productivity. One of the most successful strategies to prevent apoptosis has been the over-expression of members of the Bcl-2 family in hybridoma, NS0 and CHO cell-lines.

It has previously been shown (*Perani et al 1999*) that the over-expression of the Bcl-2 protein can protect cells from death resulting from exposure to the stress associated with growth within the bioreactor environment. Under conditions of suboptimal pH, shear stress, hyperosmolarity as well as nutrient deprivation, Bcl-2-expression has been found to increase overall culture viability by at least 20% when compared to non-Bcl-2-expressing cultures. In addition, the specific productivity of Bcl-2 transfected cells adapted to grow in high osmolarity medium has been previously reported to be 100% higher than that produced by cells grown in normal osmolarity medium (*Perani et al 1999*), demonstrating the overall beneficial effect of Bcl-2-expression on the adaptation process and during exposure to a wide range of culture stresses.

In this chapter I examined the possible role for Bcl-2 in the process of adaptation to protein-free suspension cultures. In addition, because previous work (*Astley et al 2007)* has demonstrated the benefit of the cdk inhibitor $p21^{CIP1}$ on adaptation to the same growth conditions, but also found that arresting cells generally results in reduced viability, I have examined the effect of combining the viability enhancer Bcl-2 and $p21^{CIP1}$ on the process of adaptation.

6.4. Results

6.4.1. The effect of Bcl-2/$p21^{CIP1}$ over-expression on the ability of a CHO cell-line to adapt to single-cell suspension culture

In order to establish if the anti-apoptotic effect of Bcl-2 would improve the ability of industrially anchorage dependent CHO cell-lines to adapt to growth as single-cell suspensions, cultures were set up as described in the Materials and Methods section of this thesis. The results shown in Fig 6.1 clearly shows the positive effect of Bcl-2 on adaptation to serum-supplemented suspension compared to the non-Bcl-2-expressing control cell-line leading to a 30% reduction in the overall time taken for adaptation.

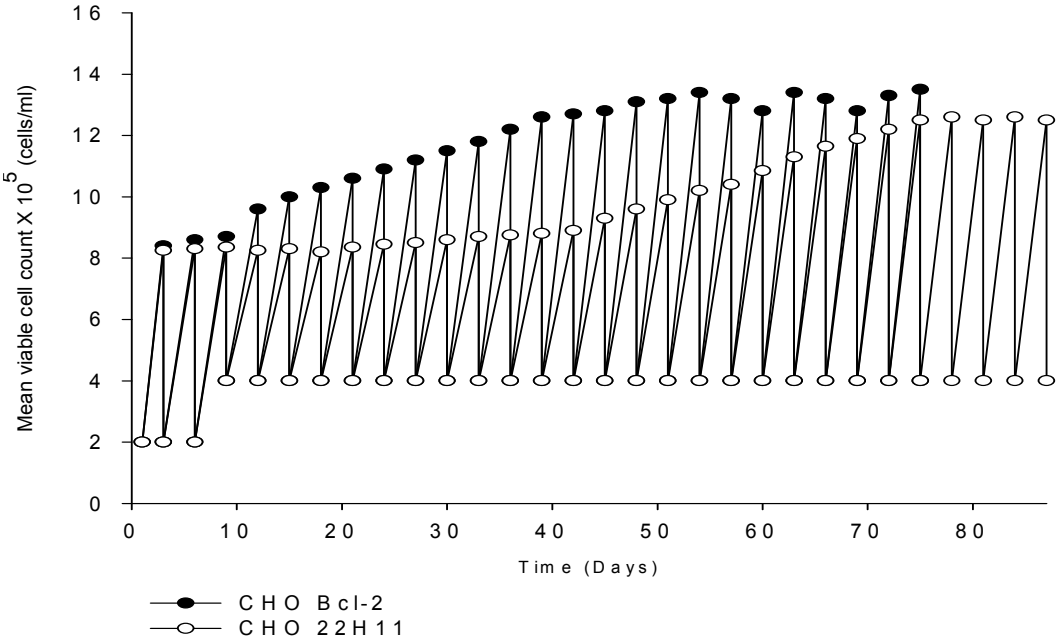

Figure 6.1. Passage cycles during adaptation from a monolayer culture to a single-cell suspension through the expression of Bcl-2. Two cultures consisting of a non-Bcl-2-expressing control culture (22H11) and a cell-line engineered to continuously over-express Bcl-2 (CHO Bcl-2) were set up in triplicate. At the start of experimentation the cells were detached from the surface of the tissue culture flask. The viable cell number and percentage viability were determined before the cells were centrifuged at 90g for 5 minutes and re-suspended in fresh culture medium. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Both cultures were passaged to $4x10^5$ cells/ml every 72 hours and the viable and total cell numbers were recorded (see chapter 2 Materials and Methods). Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture. The first 2 passages represent growth in a static environment prior to the initiation of adaptation. Error bars are not shown for clarity, but the average standard error of the mean was no more than 0.2.

In addition, Bcl-2-expressing cultures also maintained a significantly higher viability during the process of adaptation to either serum supplemented suspension or protein-free suspension culture in comparison to the non-Bcl-2 control culture (data not shown). Fig 6.2 shows the effect of combining Bcl-2-expression with the inducible expression of $p21^{CIP1}$ for either 3, 6, or 9 days at the start of the adaptation process.

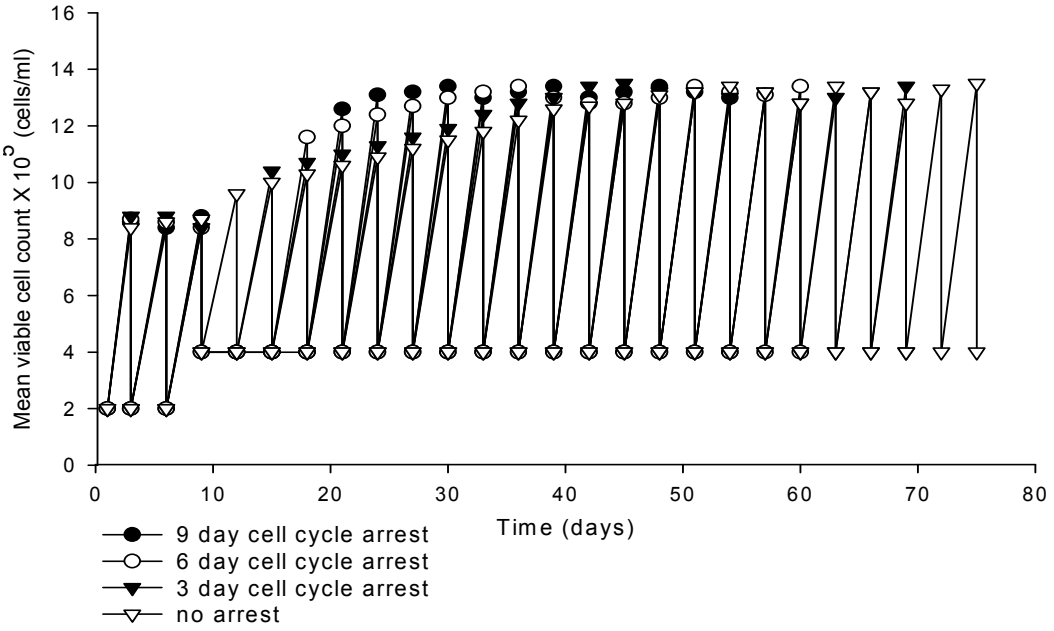

Figure 6.2. Passage cycles during adaptation from monolayer cultures to single-cell suspension through the expression of $p21^{CIP1}$/Bcl-2. Cultures previously grown in static conditions were detached from the surface of the tissue culture flask. The viable cell number and percentage viability was determined before the cells were centrifuged at 90g for 5 minutes and re-suspended in fresh growth medium. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture. For each set of experiments, four flasks were set. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture

medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$-expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs cultures were centrifuged at 90g and re-suspended in fresh culture medium. Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the fresh culture medium. Non induced cultures were passaged back to 4×10^5 cells/ml. Following 3, 6, and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers (see chapter 2 Materials and Methods). Mean and standard error were calculated from this data and the significance determined via *t*-test analysis. The first 2 passages represent growth in a static environment prior to the initiation of adaptation to serum-containing suspension. Error bars are not show for clarity but the average standard error of the mean was no more than 0.2.

In all arrested cultures, cellular viability remained high throughout the entire period of culture which may be explained by over-expression of the anti-apoptotic Bcl-2 which results in a reduction in the rate of cell death normally associated with cell cycle arrest. The expression of $p21^{CIP1}$ following the addition of IPTG was confirmed via western blot (Fig. 6.3), in both a $p21^{CIP1}$ only control and the $p21^{CIP1}$/Bcl-2-expressing cell-line the addition of IPTG resulted in the expression of $p21^{CIP1}$ and lead to cessation of the cell cycle. In contrast the addition of IPTG to the negative non-$p21^{CIP1}$-expressing control cell-line had no effect on the cell cycle.

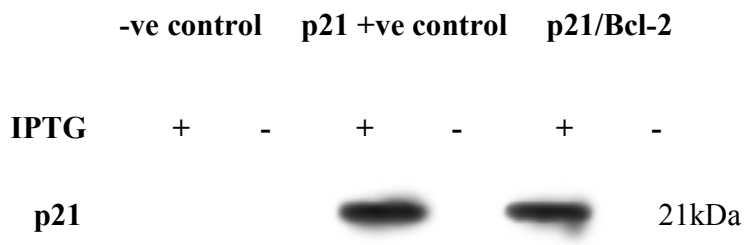

Figure 6.3. Western blot analysis of p21^{CIP1}-expression in p21^{CIP1}/Bcl-2-expressing cell-lines. Samples were analysed following 96 hours growth as static batch cultures grown in T75 tissue culture flasks containing DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX. Each cell-line was cultured either with or without the addition of 5mM IPTG in order to induce p21^{CIP1}-expression at the start of experimentation. 22H11 was used as a negative control as this has not been transfected with p21^{CIP1}. 3B2 was used as a positive control as it has previously been transfected with p21^{CIP1} (see chapter 2 Materials and Methods). Each well was loaded with 30µl of sample which was previously normalised to the same cell density.

The results are in agreement with the findings of the previous chapter, in that increasing the time of arrest before releasing cells can reduce the time to adapt cells to grow as single cells in suspension culture. Additionally, improvement in cellular viability appears to enhance cell adaptation of p21^{CIP1} cells. Previous studies on p21^{CIP1} alone (*Astley et al. 2007*) had found that the expression of p21^{CIP1} for periods of either 3, 6, or 9 days during adaptation to suspension growth resulted in a 12, 23, and 31 percent reductions in adaptation time, respectively. In comparison the combined expression of Bcl-2 and p21^{CIP1} lead to 44, 53 and 63 percent reductions, respectively.

6.4.2. The effect of Bcl-2/p21^{CIP1} over-expression on the ability of a CHO cell-line to adapt to protein-free suspension culture

Adapted serum-supplemented suspension cultures were passaged for a minimum of 4 passages before being set up as described in the Materials and Methods section of this thesis. The results shown in Fig 6.4 demonstrate the effect of Bcl-2-expression on the process of adaptation of serum-supplemented suspension cultures to a protein-free suspension growth environment, resulting in the overall time taken for adaptation being significantly reduced - from 73 days to 46 days (37% reduction).

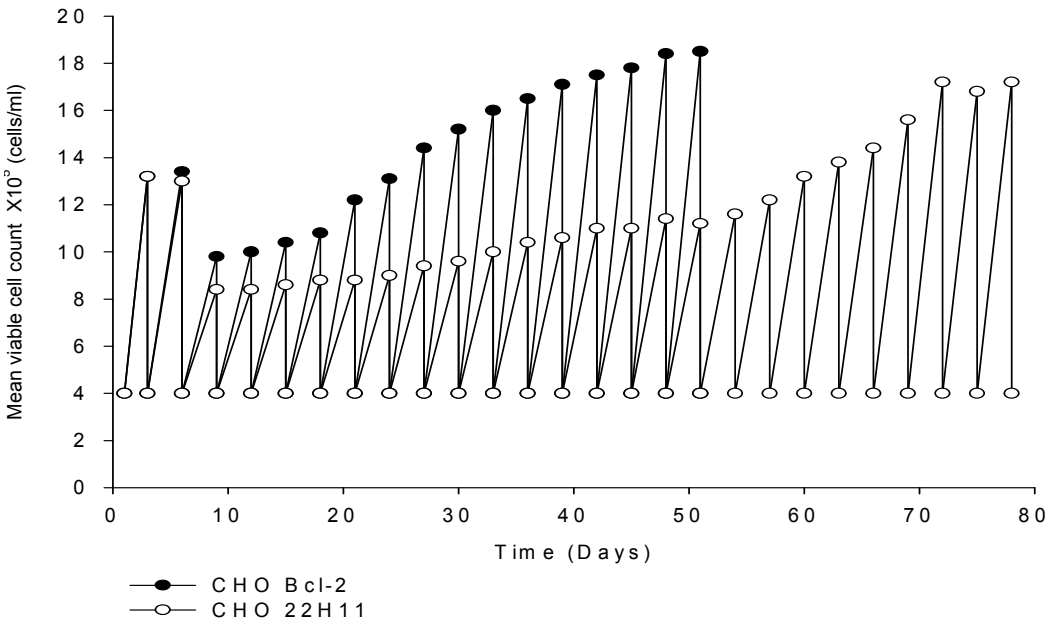

Figure 6.4. Passage cycles during adaptation from serum supplemented culture medium to protein-free culture medium through the expression of Bcl-2. Two cultures consisting of a non-Bcl-2-expressing control culture (22H11) and a cell-line engineered to continuously over-express Bcl-2 (CHO Bcl-2) were set up in triplicate. Both cultures were previously grown as suspension cultures in DMEM F12 growth medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX**. Cells from mid-exponential phase were harvested and the viable cell number and percentage**

viability were determined before the cells were washed in warm 1% PBS and centrifuged at 90g for 5 minutes. The cells were then re-suspended in fresh protein-free medium (ProCHO4) supplemented with 50µM MSX. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks (Corning Ltd, UK). The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Both cultures were passaged to $4x10^5$ cells/ml every 72 hours and the viable and total cell numbers were recorded (see chapter 2 Materials and Methods). Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture. The first 2 passages represent growth in a static environment prior to the initiation of adaptation. Error bars are not shown for clarity, but the average standard error of the mean was no more than 0.2.

Figure 6.5 shows the effect of arresting cells for 0, 3, 6, or 9 days. In this study the combined expression of $p21^{CIP1}$ and Bcl-2 resulted in a 42, 54, and 62 percent decreases, respectively, in the time taken for successful adaptation to a protein-free, suspension culture. In addition, the overall culture viability remained significantly higher throughout the process of adaptation than that observed with the 22H11 control cell-line or in cultures expressing only $p21^{CIP1}$. It was again noted that there was a direct correlation between the length of time spent in cell cycle arrest and the resulting overall culture viability, with cultures experiencing the longest periods of cell cycle arrest exhibiting the highest culture viability throughout the process of adaptation.

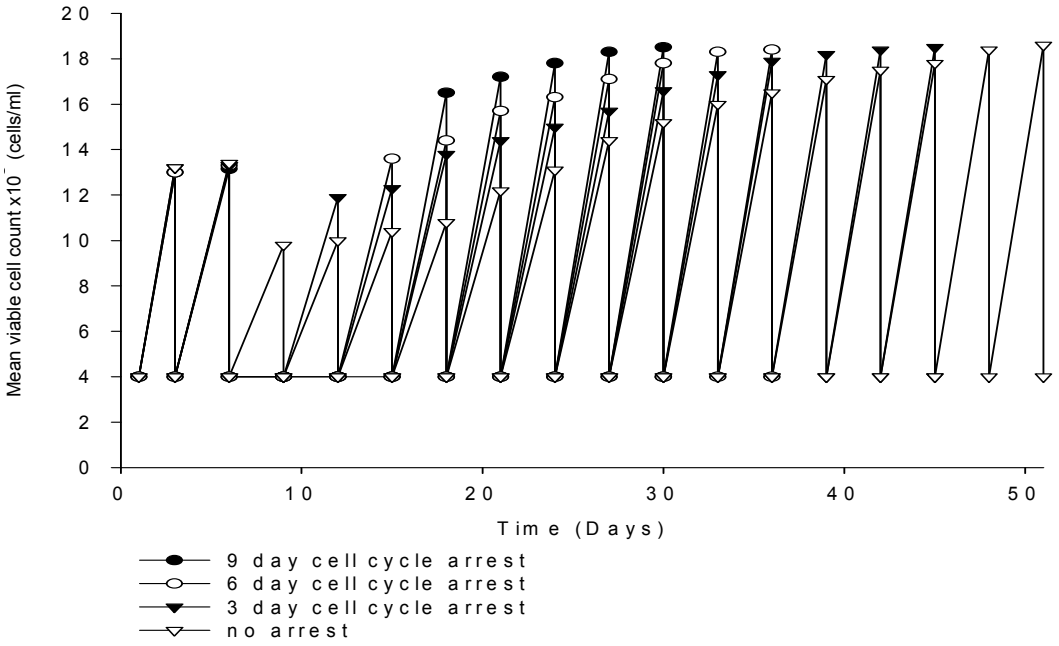

Figure 6.5. Passage cycles during adaptation from serum supplemented culture medium to protein-free culture medium through the combined expression of $p21^{CIP1}$/Bcl-2. Cultures previously grown in DMEM F12 culture medium supplemented with 10% heat inactivated foetal calf serum and 50µM MSX were harvested during mid-exponential phase and the viable cell number and percentage viability were determined. The cells were then washed in warm 1% PBS before being centrifuged at 90g for 5 minutes. The cells were then re-suspension in fresh protein-free medium (ProCHO4) supplemented with 50µM MSX. All cultures were seeded at a cell density of 4×10^5 cells per ml into a final volume of 100ml using 250ml vented Erlenmeyer flasks. The culture was agitated at a rate of 125 rpm at 37◦C in the presence of 5% CO_2. Adaptation was considered successful following the return to an equal or better growth and production rate to that observed with the original culture. For each set of experiments, four flasks were set up. 5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture medium of three of the flasks at the start of experimentation in order to induce $p21^{CIP1}$-expression; the other flask was kept as a control (0mM IPTG). Expression of $p21^{CIP1}$ was maintained in the first flask for a period of 3 days, the second for 6 days and the third for 9 days. Every 72hrs cultures were centrifuged at 90g and re-suspended in fresh culture protein-free medium. Flasks in which $p21^{CIP1}$-expression was to be maintained had 5mM IPTG added back into the fresh culture medium. Non-induced cultures were passaged back to 4×10^5 cells/ml. Following 3, 6,

and 9, days of cell cycle arrest, $p21^{CIP1}$-expression was halted by the removal of IPTG from the culture medium. Each culture was examined daily in triplicate via the Trypan blue exclusion method to determine viable and total cell numbers (see chapter 2 Materials and Methods). Mean and standard error were calculated from this data and the significance determined via *t*-test analysis. The first 2 passages represent growth in a static environment prior to the initiation of adaptation to serum-containing suspension. Error bars are not show for clarity but the average standard error of the mean was no more than 0.2.

6.4.3. The effect of adaptation using Bcl-2 or Bcl-2/p21^{CIP1}-expression on culture growth and productivity.

In order to confirm that the process of adaptation had not resulted in a loss of proliferation or productivity, culture growth rate and viability for the 22H11 control, Bcl-2 only and p21^{CIP1}/Bcl-2 cell-lines was analyzed over 15 days following adaptation to each of the growth conditions. All cultures exhibited an increase in the overall obtainable viable cells (Fig. 6.6) and in the cumulative cell time (CCT) (Fig. 6.7) following adaptation to suspension and protein-free growth environments.

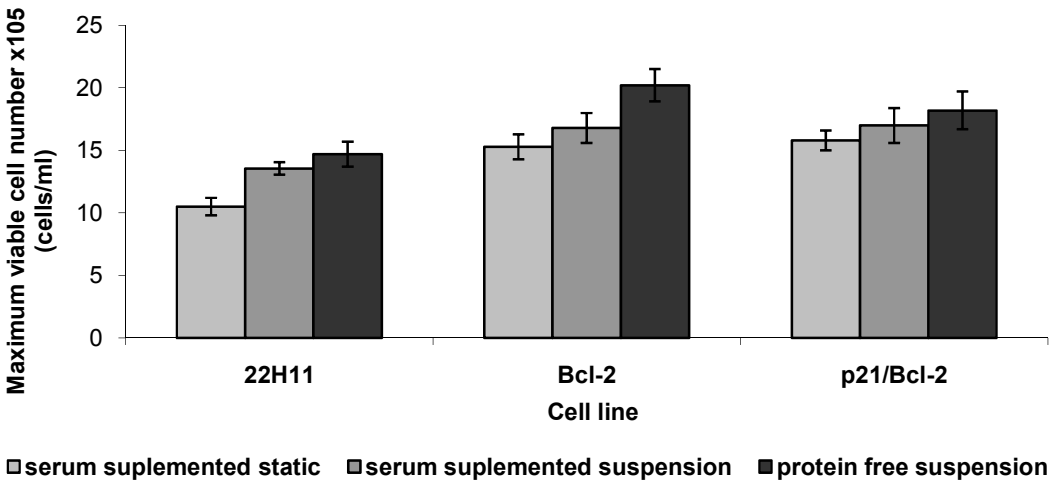

Figure 6.6. Maximum obtainable viable cell number. Results were determined for each of the cell-lines during batch culture, when grown as either serum supplemented static, serum supplemented suspension or protein-free suspension culture (see chapter 2 Materials and Methods). The standard error of the mean was calculated over three replicates and is shown by the error bars. Student's *t*-test indicated significant differences between the maximum viable cell number obtainable through the expression of Bcl-2 or p21^{CIP1}/Bcl-2, grown as either static, suspension or protein-free suspension when compared to the non-p21^{CIP1}/Bcl-2 control (22H11) ($p < 0.05$).

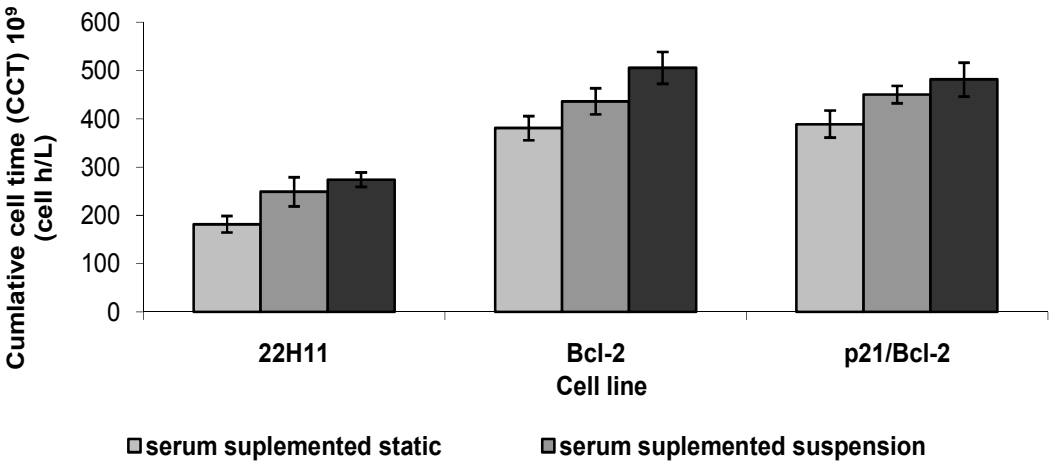

Figure 6.7. Cumulative cell time. Results were plotted to determine the cumulative cell time (CCT) for control, Bcl-2 and p21^{CIP1}/Bcl-2-expressing cell-lines during growth as either serum supplemented static, serum supplemented suspension or protein-free culture conditions (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the cumulative cell times determined for cell-lines expressing Bcl-2 or p21^{CIP1}/Bcl-2, grown as either static, suspension or protein-free suspension when compared to the non-p21^{CIP1}/Bcl-2 control ($p < 0.05$).

In both the Bcl-2 and p21^{CIP1}/Bcl-2 cell-lines the CCT is considerably higher than the control cultures in all growth environments, however the expression of p21^{CIP1} would appear to slightly reduce the CCT in comparison to Bcl-2-expressing cultures, this may be in part due to an increase in the overall genetic information required to be transcribed during each round of replication and may also reflect a slight shift in terms of cellular functionality from growth to productivity. The results also clearly show the positive effect of Bcl-2 on culture viability in both Bcl-2 and non-induced Bcl-2/ p21^{CIP1} cell-lines. This increase is not believed to be due to direct stimulation by Bcl-2 itself but is

more likely due to the suppression of apoptosis during the early stages of the culture, which leads to an increase in the number of dividing cells.

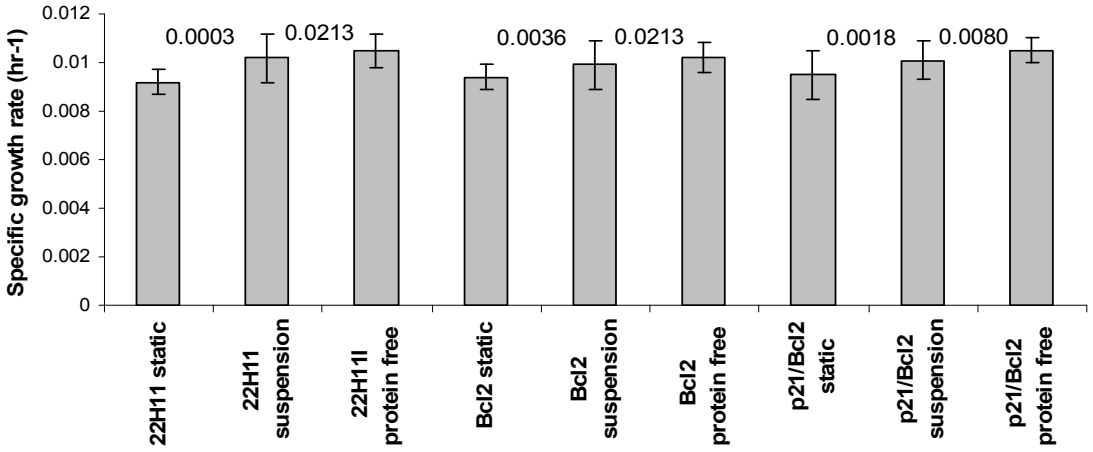

Figure 6.8. Specific growth rate following adaptation to growth in suspension and protein-free growth environments. Results were obtained during the exponential growth phase of non-induced cultures grown either as serum-supplemented static cultures or following adaptation to serum-supplemented suspension or protein-free suspension (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates. Values for P are given between bars demonstrating the significance of the increase in specific growth rate following adaptation to each of the growth environments.

During the process of adaptation to both suspension and protein-free culture environments; Bcl-2 over-expression resulted in increased robustness to the mechanical forces within the culture environment, as well as providing protection against nutrient deprivation and toxic metabolite accumulation. Interestingly, the results of the specific growth rate (Fig. 6.8) shows a significant increase in the specific growth rate of all cell-lines following adaptation from serum supplemented static to serum supplemented

suspension and likewise from serum supplemented suspension to protein-free suspension culture.

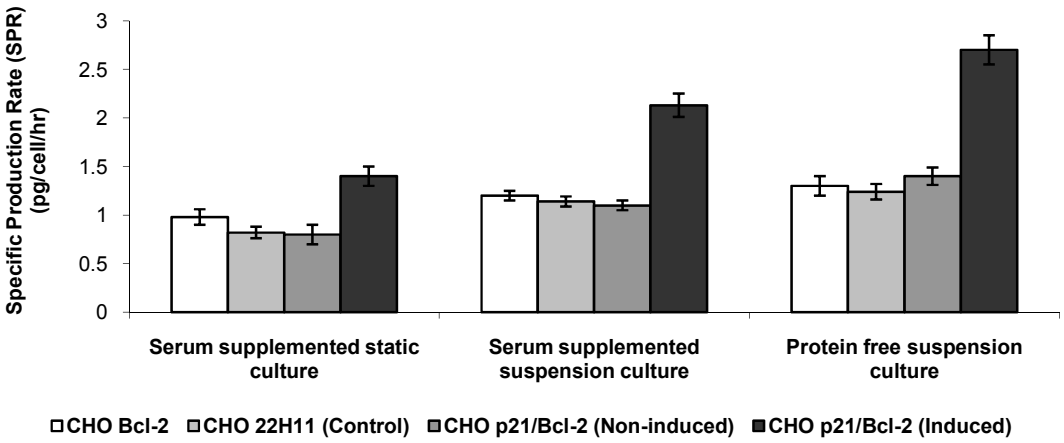

Figure 6.9. Specific production rate following adaptation to growth in suspension and protein-free growth environments. Bars show the specific production rate of cultures in which $p21^{CIP1}$-expression had been induced and non-induced. Cells were grown either as serum-supplemented static cultures or following adaptation to serum-supplemented suspension or protein-free suspension (see chapter 2 Materials and Methods). Error bars show the standard error of three replicates. Student's *t*-test indicated significant differences between the productivity of induced and non-induced cultures at each condition ($p < 0.05$).

All cultures exhibited an increase in the specific productivity rate following adaptation to serum-supplemented suspension (Fig. 6.9); however, a more significant increase was observed following adaptation to the protein-free growth environment. Interestingly, the expression of Bcl-2 alone did appear to have a significant impact on the specific productivity; however, the initiation of cell cycle arrest via the addition of IPTG to the $p21^{CIP1}$/Bcl-2 cell-line resulted in a greater increase in specific productivity in all growth environments.

6.5. Discussion

In this study, I have identified a potential role for Bcl-2 in the process of adaptation of industrially important cell-lines to chemically defined growth environments. I have additionally demonstrated the clear benefit of combining Bcl-2 with periods of cell cycle arrest through the inducible expression of $p21^{CIP1}$.

Several studies have reported various findings to the effect of Bcl-2-expression on productivity. *Itoh et al (1995)* reported a seven fold increase in antibody titre; similarly *Simpson et al (1997)* also reported a 38% increase. This study also found that Bcl-2-expression did result in a slight increase in productivity as compared to the control cell-line. Cultures expressing both $p21^{CIP1}$ and Bcl-2 showed a marked increase in productivity during the period of cell cycle arrest. In contrast, *Fassnacht et al (1998b, Tey et al (2000), Tey and Al-Rubeai (2004)* reported no such increase in obtainable productivity. It is apparent that important factors that contributed to the differences between the two set of findings in productivity were the difference in culture duration, growth rate, maximum cell number, how quickly the cells were dying and when substantial cell death became apparent.

When cultured in large scale for production of biopharmaceuticals mammalian cells can easily become susceptible to hydrodynamic forces that trigger the onset of apoptosis *(Al-Rubeai et al 1995)*, leading to a reduction in product titre and resulting in an increased cost. Apoptosis can be triggered by various factors: mechanical, environmental and chemical. Bcl-2, an integrated component of the mitochondria membrane, is an important

apoptosis regulatory gene, which by maintaining the integrity of the mitochondria membrane, is able to prevent the release of cytochrome C into the cytoplasm, thus blocking the activation of caspases, and thereby preventing the subsequent initiation of apoptosis. It is well established that the over-expression of Bcl-2 can significantly improve the robustness of industrially important cell-lines to conditions found within the bioprocessing environment, including nutrient limitation, growth arrest, oxygen limitation, viral infection and shear stress (*Al- Rubeai and Emery 1990, Franek and Dolnikova 1991, Mercille and Massie 1994, Singh et al 1994, Mastrangelo et al 1996, Mitchell-Logan and Murhammer 1997*). The fact that Bcl-2 allows previously unadapted cells to survive in suspension and serum-free culture supports the postulation that this protein plays an important role in apoptotic signalling pathways and suggest that Bcl-2 may also sub-serve more diverse functions beside its death-inhibiting role. One possible explanation for the reduced period of adaptation is that adaptability to changes in nutritional and physical environment is part of an intracellular defence mechanism that can be stimulated by lack of substratum and serum. The reported cell cycle arrest by shear stress and nutrient deprivation support the notion that Bcl-2 protection may be achieved by prolonging the G1 phase and that this protein plays a key role in optimizing cell numbers to suit the prevailing nutrient and physical environment *(Simpson et al 1999).*

Previously, it has been shown that the expression of the cyclin dependant kinase inhibitor $p21^{CIP1}$ could have a positive impact on the adaptation of CHO cell-lines to suspension and serum-free growth environments by halting cell cycle progression in the G1 phase of

the cell cycle *(Astley et al 2007)*, thereby improving cellular robustness in response to mechanical damage and reducing the requirement for the presence of growth factors/complex nutrients present in serum. Combining the positive effect of $p21^{CIP1}$ on adaptation with that of Bcl-2 ability to reduce cell death and prolong viability in suboptimal condition it is not surprising to observe such a significant decrease in the period of adaptation to suspension and protein-free culture.

Interestingly, in this study Bcl-2-expression also resulted in an increase in the overall maximum viable cell count obtainable in comparison to the control culture. This increase is not believed to be due to direct stimulation by Bcl-2 itself but is more likely due to the suppression of apoptosis during the early stages of the culture, which leads to increased percentage of dividing cells with consequent accumulation of viable cells. During the process of adaptation to both suspension and protein-free culture environments, Bcl-2 over-expression resulted in increased robustness to the mechanical forces within the culture environment, as well as providing protection against nutrient deprivation and toxic metabolite accumulation.

In conclusion, the results of this study provide a clear indication of the possible role for the combined expression of the anti-apoptotic gene Bcl-2 and of $p21^{CIP1}$ -induced cell cycle arrest in the process of adapting of CHO cell-line to industrial applications. I have demonstrated in this study that, through the induction of extended periods of cell cycle arrest combined with the over-expression of Bcl-2, it is possible to significantly decrease the time required for adaptation of CHO cell-line to both suspension and protein-free

growth environments and thereby to enable the swift development of commercial cell-lines able to meet the demands for higher productivity and longevity whilst withstanding the stresses imposed by growth within a chemically defined bio-processing environment.

7. General discussion and future perspectives

7.1. Summary of results

This thesis represents a continuation of our labs previous research into apoptosis and the cell cycle. In chapter 3 I was able to demonstrate that it is possible to create a multicistronic CHO cell line that is able to express the monoclonal antibody IgG through the GS expression system. This cell line was further engineered to express the anti-apoptotic Bcl-2 gene and the cyclin-dependent kinase inhibitor $p21^{CIP1}$ under the control of an inducible expression vector. Following its creation I was then able to successfully characterize the growth and productivity profiles of the resulting clones. Each clone was also examined for its ability to halt cell cycle progression following the induction of $p21^{CIP1}$-expression as well as the anti-apoptotic effect of Bcl-2 when challenged with exposure to staurosporine and serum deprivation. Both $p21^{CIP1}$ and Bcl-2-expression were additionally confirmed for each of the clones through analysis via western blot. Finally the productivity and specific production rates of all clones were determined both during periods of $p21^{CIP1}$-expression and during growth without vector expression. This allowed me to confirm my previous hypothesis, that cell cycle arrest as a result of $p21^{CIP1}$-expression would indeed result in an increase in productivity and that the combined expression of Bcl-2 would not have a negative impact on the overall production rate.

In chapter 4 I examined the hypothesis that a relationship exists that is able to link $p21^{CIP1}$-induced cell cycle arrest to the previously observed changes in cell size and

productivity. In order to investigate this fully a CHO cell line which expresses the monoclonal antibody IgG through the GS expression system was further engineered to allow the inducible expression of $p21^{CIP1}$ through the addition of IPTG to the culture medium. Using this cell line, it was first confirmed that $p21^{CIP1}$-expression does in fact lead to G1 phase cell cycle arrest. A number of key metabolites were analyzed in both induced and non-induced cultures, including glucose and glutamate utilization and lactate and ammonia production rates. To further investigate the effect of $p21^{CIP1}$-induced cell cycle arrest has on the cells metabolism the total protein and mitochondrial activity was also analyzed.

Cell size was examined both via a Malven mastersizer and through the analysis of packed cell volume. The results of this study clearly demonstrated an increase in overall cell volume, confirming the hypothesis that in $p21^{CIP1}$-induced cultures cell growth may be uncoupled from cell division. The increased antibody productivity observed during periods of proliferation arrest could be directly correlated to the increase in cell volume and total intracellular protein. This is likely to reflect the increase in the protein synthesis machinery within the arrested cells. Interestingly it would also appear that a link may exist between the rate of glucose utilization and cell volume as both of these were found to increase simultaneously during the early stages of the culture. Later on in the culture an increase in the rate of glutamate utilization and productivity were also observed in cultures expressing $p21^{CIP1}$, suggesting that glutamate is utilized more readily by arrested cells for the process of antibody production and that during periods of cell cycle arrest

this process occurs more readily, most likely as a result of the decreased cell density and therefore a greater abundance of available glutamate.

In chapter 5 I examined the hypothesis that proliferation arrested CHO cells may be adapted to suspension and protein-free growth environments more successfully than cells that are proliferating. The theory behind this investigation was that cells in the G2/M phases of the cell cycle have previously been shown to be more susceptible to hydrodynamic forces due to the associated increase in cell size and weakening of the cell membrane prior to cell division *(Al-Rubeai et al 1995, Abdul and Al-Rubeai 1997).* I therefore hypothesized that arresting cells in the G1 phase of the cell cycle would result in improved robustness when exposed to the shear forces of a suspension environment. In addition it was theorized that extending the period of cell cycle arrest would reduce their requirement for extracellular growth factors, as well as preventing the accumulation of extracellular autocrine growth factors. It was also suggested that by preventing cell cycle progression cells may be able to utilize the additional time spent in G1 phase arrest to repair the resulting genome damage, created through the direct withdrawal of serum, prior to cell cycle release.

Adaptation to protein-free culture was also found to occur at a greatly enhanced rate during periods of cell cycle arrest, most likely as a result of a reduction in the cells requirement for growth factors/complex nutrients that are usually present in the serum as these molecules are only essential for the stimulation of cells to enter cell division. Interestingly our results also revealed that $p21^{CIP1}$-induced cell cycle arrest was able to

limit the formation of aggregates during the process of adaptation; possibly by enhancing culture viability and thereby preventing the release of degraded DNA and other cellular materials into the medium. It was therefore hypothesized that $p21^{CIP1}$ may play a key role in the organization of the extracellular matrix by reducing the expression of extracellular adhesion factors. This would in turn reduce multicellular aggregation, which can also result in a reduction in viability due to the nutrient and mass transfer limitations within the spheroid structure of multicellular aggregates.

The expression of $p21^{CIP1}$ was also found to reduce apoptosis during the process of adaptation, most likely as a result of preventing the accumulation of toxic metabolites usually associated with cellular overgrowth. To determine if this reduction in apoptosis could be further enhanced I engineered a cell-line in which the expression of $p21^{CIP1}$ and the expression of the anti-apoptotic gene Bcl-2 could be combined. The results clearly demonstrate an improvement in cellular robustness when exposed to mechanical damage as well as a reduced requirement for the presence of growth factors/complex nutrients present in serum. By combining the positive effect of $p21^{CIP1}$ on adaptation with the ability of Bcl-2 to reduce cell death and prolong viability in suboptimal condition it is not surprising that we observed a significant decrease in the period of time required for successful adaptation to both suspension and protein-free culture.

7.2. Recommendations for further work

The work presented in this thesis has the potential to lead to several other studies including:

- Genetic profiling of $p21^{CIP1}$-expressing cultures is vital. Through the use of molecular biology techniques and post-genomic technologies such as gene expression profiling and metabolic profiling, it may be possible to significantly improve our understanding of the physiological state of $p21^{CIP1}$-expressing cells. Such an 'inverse engineering approach' *(Bailey et al 1996)* would allow us to identify alterations in cellular organization that result in the physiological changes and increase in productivity observed in this thesis. There are many factors including increased transcription, post-translational events, metabolic precursor generation and energy generation that may well contribute to the observed increase in productivity during periods of cell cycle arrest. The characterization of changes to the metabolic pathways and the cells physiology may therefore offer additional possibilities for the modulation of the identified pathways to further enhance productivity.

- The effect of combining $p21^{CIP1}$ and Bcl-2 expression on the metabolic pathways observed in this thesis. As described above, it is likely that the physiological changes and increase in productivity observed as a result of the over-expression of $p21^{CIP1}$ occur due to several factors. It has previously been reported that the over-

expression of Bcl-2 may have a positive impact on culture viability and productivity *(Itoh et al 1995)*. In addition its expression has been shown to be associated with the cessation of cell proliferation in the G0/G1 phase as well as effecting the transition through G2/M *(Lopez-Diazguerrero et al 2006)*. It would therefore be interesting to see if the combined expression of $p21^{CIP1}$ and Bcl-2 would have an impact on the cells requirements for key nutrients and/or the cells physiology and production potential as determined through the use of molecular biology techniques.

- The creation of an inducible Bcl-2 or $p21^{CIP1}$/Bcl-2 expressing CHO cell line in which both genes expression may be induced independently. In this study Bcl-2 is expressed permanently, something which has previously been shown to have a negative effect on the growth rate and productivity of the cell line. Therefore in an ideal situation Bcl-2-expression would not be initiated until the cells were in danger of undergoing apoptosis in the latter stages of culture. This may also be beneficial to the cells metabolic needs as the expression of Bcl-2 unnecessarily may well place additional demands on the cell for increased energy metabolism. The controlled expression of Bcl-2 may also be beneficial in the adaptation of cell-lines to new growth conditions following which its expression may not be needed. In this thesis the expression of $p21^{CIP1}$ was under the control of an inducible expression vector, this meant that $p21^{CIP1}$-expression could be switched on on demand enabling cells to reach an optimum cell density before being arrested and thus preventing the associated accumulation of toxic metabolites in

the growth medium, normally associated with cellular over growth. As previously described Bcl-2 was expressed permanently, in a combined strategy $p21^{CIP1}$-expression would be initiated when the cells had reached an optimum density and viability and Bcl-2-expression would not be initiated until there was a risk of loss of viability and thus productivity.

- The combined expression of $p21^{CIP1}$ and Bcl-2 with a fed batch strategy designed to meet the metabolic needs of high producing cell-lines that have been highlighted in this chapter. It is believed that productivity could be further enhanced in $p21^{CIP1}$ and $p21^{CIP1}$/Bcl-2-expressing cell-lines by implementing a strategy that would enable the cells to be grown to a high cell density before the addition of IPTG and the initiation of feeding, in order to supplement the medium with the key nutrients that have been shown to be utilized more readily during periods of high production rates. This would be particularly valuable when both $p21^{CIP1}$ and Bcl-2 are being expressed as $p21^{CIP1}$ has been shown to increase productivity whilst Bcl-2 has been found to reduce apoptosis and therefore extend the culture period. Feeding strategies to meet the demands of the arrested culture may extend the length of the culture thus further and in doing so result in an overall increase in product titre.

7.3. Conclusions

This project has been highly successful in highlighting the possibility for the utilization of genetic methods not only for the enhancement of productivity, but also for improving the adaptability of CHO cell-lines for use in industrial applications. The results of this thesis clearly show that arresting cells in the G1 phase of the cell cycle results in a significant increase in productivity. Analysis of key nutrient utilization and cellular metabolism indicate that when cell proliferation is halted, cellular growth is able to continue independently, resulting in an increase in overall cell volume. This increase in cell volume appears to be associated with increased glucose utilization rates; in contrast the rate of productivity appears to be linked to an increase in glutamate utilization which occurred following the exhaustion of glucose.

Although the exact mechanisms are not clear it is obvious from the observed increase in mitochondrial activity, total protein content and nutrient utilization rates that there are significant changes to the metabolic pathways that occur as a result of $p21^{CIP1}$-induced cell cycle arrest. The identification of these pathways would require genetic analysis techniques beyond the scope of this thesis; however this study does provide a very firm basis for such studies. The determination of changes in these key metabolic pathways will enable the manipulation of existing medium compositions, the formulation of superior growth media and feed strategies that will more precisely meet the increased needs of high producing proliferation arrested cell-lines.

In this thesis I have also successfully developed and tested a novel protocol for the adaptation of CHO cells to both anchorage-independent suspension and protein-free growth environments, through the use of $p21^{CIP1}$-induced cell cycle arrest. I was able to demonstrate that the period of time required for successful adaptation to each of the aforementioned growth conditions could be significantly reduced. This highlights the possibility that $p21^{CIP1}$ may play additional roles other than the prevention of cell division, including reducing the rate of aggregate formation by improving culture viability and thus preventing the release of degraded DNA, as well as down-regulating the expression of extracellular adhesion factors. When the expression of Bcl-2 was combined with periods of cell cycle arrest the results on the rate of adaptation were even more dramatic. It would appear that the ability of Bcl-2 to prevent apoptosis improves the culture viability and prevents cell death usually associated with the accumulation of toxic metabolites.

This thesis has been successful in demonstrating how it is possible to utilize genetic techniques for the manipulation of industrially relevant cell-lines in order to not only enhance their productivity but also to shorten the period of time required for their adaptation to the conditions necessary for the large scale production of biopharmaceuticals. In addition analysis of the mechanisms underpinning the resulting increase in productivity may help in the discovery of the exact mechanisms and pathways through which cellular productivity is achieved. It may also provide identifiable markers which can be used for the production and selection of high producing cell-lines.

In the future it may well be possible to combine environmental and genetic techniques, such as those highlighted by this thesis, in order to produce cell-lines in which the specific production rates are higher than those currently obtainable. In addition the ability to reduce the time required for successful adaptation will better enable the increasing need for protein free production systems to be met. Ultimately this would result in a reduction in the overall production times required as well as substantially reducing the associated cost.

8. Publications and presentations resulting from this thesis.

8.1. Publications

Cell Technology for Cell Products (2007). Proceedings of the 19th ESACT Meeting**,** Harrogate, UK, June 5-8, 2005 Series: ESACT Proceedings , Vol. 3 Smith, Rodney (Ed.) LXXIX, 821 p., Hardcover ISBN: 978-1-4020-5475-4

Astley K, Naciri M, Racher A and Al-Rubeai M (2007). The role of $p21^{CIP1}$ in adaptation of CHO cells to suspension and protein-free culture. Biotechnol. 130: 282–290

Astley K and Al-Rubeai M (2008). The role of Bcl-2 and its combined effect with $p21^{CIP1}$ in adaptation of CHO cells to suspension and protein-free culture. Appl Microbio Biotechnol 78: 391-399

8.2. Conference Presentations

Astley Kelly and Al-Rubeai Mohamed (2005) The effect of preventing cell cycle progression through the expression of $p21^{CIP1}$ on the mechanical strength and cellular robustness of CHO cell-lines during adaptation to suspension and serum-free growth environments. Presented at 15th ESACT Annual UK Scientific Meeting, Gilbert Murray Conference Centre, University of Leicester. 6th-7th January 2005.

Astley Kelly and Al-Rubeai Mohamed (2005) Adaptation without cell division ?
Enhanced adaptation to suspension and serum free culture by the co-expression of $p21^{CIP1}$ and Bcl-2. Presented at 19th ESACT, Harrogate, 5th – 8th June 2005

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