A thesis submitted to

Thesis submitted in partial fulfilment for the requirements for the degree of Doctor of Philosophy

Department of Chemical and Biological Engineering

The University of Sheffield

The role of intracellular signalling pathways in CHO cell growth in a synthetic environment

Robert Whitfield

January 2014

To my mum and dad,

Sandra and Dave

Declaration of authenticity

I, Robert Whitfield declare that I am the sole author of this thesis and that the work contained within it is the product of my own efforts and achievements.

Robert Whitfield
January 2014

Acknowledgements

Firstly, thanks go to my supervisor Professor David James for his guidance and most of all patience throughout the course of my PhD. I would also like to thank my industrial supervisor, Dr Mark Leonard and all the people at Pfizer who have been a great help in numerous ways.

I would like to thank the rest of the James lab, who have been a pleasure to work with during my time in Sheffield. These thanks are extended to the rest of the academic department in Chemical and Biological Engineering.

A big thank you to all my friends for remaining patient with me and for always being there for me whenever I needed someone to go for a beer with – you know who you are!

Finally, to all my family and Lucy, I could not have got to this stage without all your love and support. You're the best.

Abstract

Industrial batch and fed-batch culture of Chinese hamster ovary (CHO) cell lines for the production of recombinant proteins, utilises the ability of engineered CHO cells to grow and proliferate in suspension in a synthetic environment that is free of many of the extracellular signals (e.g. exogenous growth factors, cell to matrix and cell to cell contacts) that control growth in vivo. This functional capability derives from extensive cell culture experimentation and lengthy adaptation processes to generate production cell lines. Many different approaches have been used in this study to develop an understanding of the underlying signalling pathway changes that facilitate CHO cell adaptation to suspension growth in a synthetic environment.

Initial characterisation experiments compared the growth of a parental adherent CHO cell line (CCL-61) and a directly adapted suspension CHO cell line (CHO-SA) in different growth environments. Results from this characterisation showed that in this model system, unadapted CCL-61 cells are unable to grow and proliferate in suspension conditions, while suspension CHO-SA cells are able to grow and proliferate in suspension conditions but only when an exogenous growth factor, in this case Insulin, is present in the culture media. Differences in the PI3K/Akt and MAPK signalling pathway activation status between the unadapted CCL-61 and suspension adapted CHO-SA cell lines growing in adherent and suspension conditions were comparatively mapped by western blotting analysis and immunoprecipitation/immunoblot analysis. Results indicate that a shift in signalling flux occurs when cells are taken from an adherent growth environment to a suspension growth environment. In adherent cells, integrin-mediated attachment to the extracellular matrix stimulates an up-regulation in signalling flux via the MAPK pathway. When integrin-mediated attachment is abolished, as in suspension growth, a shift in signalling flux is seen towards the PI3K/Akt pathway. Further experimental data using chemical inhibitors against specific signalling intermediates such as PI3K, Akt and MEK 1/2 suggests that activation of these signalling intermediates is vital for cell survival and proliferation in differing synthetic environments and therefore they

are viable targets for genetic engineering strategies used to functionally substitute for extracellular signals.

Confocal microscopy and flow cytometry were used to deduce how the PI3K/Akt and MAPK signalling pathways interact with specific cell surface membrane transducers such as integrins and specific receptor tyrosine kinases (RTKs) such as the insulin receptor. Analysis of integrin expression levels between adherent and suspension cell lines shows that despite the lack of integrin-mediated adhesion utilized by suspension cells, the expression levels of various integrins, inclucing $\beta 1$, $\alpha 5$ and $\alpha 1$, are not down-regulated in suspension adapted CHO cells. Further analysis of the activation status of key adaptor proteins such as Focal Adhesion Kinase (FAK) demonstrates how changes at the cell surface are transmitted to downstream signaling networks.

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Abbreviations

4EBP1   4E-binding protein 1

Ab   Antibody

APSM   Antibody Production Suspension Media

ATCC   American Tissue Culture Collection

Bcl-2   B-cell lymphoma 2

BHK   Baby Hamster Kidney

BM   Basement Membrane

BSA   Bovine Serum Albumin

CaMk IV   Calcium/calmodulin-dependent protein kinase IV

Cdc42   Cell division control protein 42 homolog

CDK   Cyclin-dependent kinase

CHO   Chinese Hamster Ovary

CREB   cAMP response element binding

DHFR   Dihydrofolate reductase

DMSO   Dimethyl sulfoxide

DNA   Deoxyribonucleic acid

eIF4B   Eukaryotic translation initiation factor 4B

ECM   Extracellular matrix

EGF   Epidermal growth factor

ERα   Oestrogen receptor-α

ERK   Extracellular signal-related kinase

FAK   Focal adhesion kinase

FBS   Foetal bovine serum

FKHR   Forkhead transcriptional regulator

GAP	GTPase-activating protein
Grb2	Growth factor receptor-bound protein 2
HEK	Human embryo kidney
HRP	Horseradish peroxidise
IE	Immediate-early
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IRS-1	Insulin receptor substrate 1
kDa	Kilodalton
Mabs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MK	MAPK-activated protein kinase
MNK	MAPK-interacting kinases
MOPS	3-(N-morpholino)propanesulfonic acid
MSK	Mitogen and stress activated protein kinase
mTOR	Mammalian target of rapamycin
MTX	Methotrexate
NF-κB	Nuclear factor κB
PAK	p21-activating kinases
PBS	Phosphate buffered saline
PBS-CMF	Phosphate buffered saline – Calcium and Magnesium free
PDK1	3'-phosphoinositide-dependent kinase-1
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
PIP$_3$	Phosphatidylinositol (3,4,5)-trisphosphate

PTEN	Phospholipid phosphatase
Rpm	Revolutions per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
S6K 1/2	70 kDa ribosomal S6 kinase-1/2
SGK	Serum and glucocorticoid-inducible kinase
SH2	Src homology 2
SIE	Sis inducible element
siRNA	Small interfering RNA
Sos	Son of sevenless
SRF	Serum response factor
t_d	Doubling time
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline tween-20
TM	Trademark
TSC1	Tuberous sclerosis complex protein 1 (also known as Hamartin)
TSC2	Tuberous sclerosis complex protein 2 (also known as Tuberin)
V	Volts
VCD	Viable cell density

Chapter 1

Introduction

1.1 The Biopharmaceutical Industry

Since the discovery of recombinant DNA and monoclonal antibody technologies in the 1970s, the biopharmaceutical industry has seen a vast increase in size. The first "biosynthetic" product approved for therapeutic use was "human" insulin, which first appeared on the market in 1982. By the year 2000, 84 biopharmaceuticals had been approved for marketing and were in general medical use (Walsh, 2000). By 2005, biopharmaceuticals represented approximately 38% of all new pharmaceuticals entering the global market, generating in excess of US$40 billion in sales annually (Walsh, 2005).

Despite the approval rate of new biopharmaceuticals slowing over the past five years, the biopharmaceutical industry still represents a significant and expanding part of the pharmaceutical market (Walsh, 2010). With a recent market value of over $US99 billion in global sales and an average yearly growth rate of >35% since 2001, it is of little surprise that the biopharmaceutical industry is predicted to grow by 7-15% over the next decade (Aggarwal, 2007; Walsh, 2010).

As well as growing significantly in size, the biopharmaceutical industry has also evolved over the past 30 years. Many of the early market approved recombinant therapeutic products were replacement proteins that had amino acid sequences that were identical to the native human protein or molecule, such as human insulin, Factor VIII and human growth hormone (Walsh, 2000). In the past 15 years a greater proportion of engineered proteins have been market approved for use in clinical diagnostics and therapeutics. The major targets of these engineered biopharmaceuticals that have been recently undergoing clinical

trials, include various cancers, such as Metastatic Colorectal Cancer and Non-Hodgkins Lymphoma, Cardiovascular Disease and various infectious diseases (Walsh, 2000; Walsh, 2005).

1.2 The production of monoclonal antibodies

Monoclonal antibody technology has evolved considerably since the first generation of monoclonal antibodies (Mabs) were unveiled in 1975 (Pavlou and Belsey, 2005; Reichert, 2001). The original procedure for Mab production which involved fusing mouse lymphocytes and myeloma cells to generate murine hybridomas was extremely unreliable (Reichert et al., 2005). Murine Mabs entered clinical studies in the early 1980's and it soon became apparent that they would be very limited as potential therapeutics (Pavlou and Belsey, 2005; Reichert, 2001). Lack of efficacy and the production of HAMAs (human anti-mouse antibodies) by the human immune system led to the rapid elimination of murine Mabs from the body.

The advance in genetic engineering techniques in the late 1980's and early 1990's produced chimeric and humanized Mabs that provided a viable alternative as potential therapeutics. These chimeric and humanized Mabs have smaller regions of mouse derived sequence and therefore have reduced levels of immunogenicity, a greater ability to recruit immune effector functions and an increased half-life (Reichert, 2008). Humanization has become a well-established technique for reducing the immunogenicity of monoclonal antibodies from rodent sources and for improving their activation of the human immune system (Jones et al., 2003). One of the first methods used was a Mab "chimerisation approach" where the whole of the variable region of a mouse Mab was expressed along with the human constant region. Following this another approach was used termed "CDR grafting", where the murine complementarity determining regions (CDRs) were grafted onto the framework of a human antibody (Wu and Dall'Acqua, 2005). The result was antibodies that had even higher levels of homology to the human sequences than chimeric Mabs. One of the drawbacks of "CDR grafting" was that this technique often lead to a significant reduction in

Mab antigen binding ability. Since then the emergence of many different Mab humanisation strategies have resolved these problems (Wu and Dall'Acqua, 2005).

1.3 Mammalian cell lines and expression systems

Mammalian cells have become the most widely used system for the production of monoclonal antibodies and recombinant proteins for clinical applications (Wurm, 2004). This is because of their capacity to facilitate correct protein folding, assembly and post-translational modifications thus providing greater levels of protein quality and efficacy than other production host cell types such as bacteria, plants and yeast (Wurm, 2004). Approximately 70% of all recombinant therapeutic protein pharmaceuticals are produced by mammalian cell lines (O'Callaghan and James, 2008). The most widely used mammalian cell expression system for the production of recombinant proteins is the Chinese Hamster Ovary (CHO) cell line. Other mammalian expression systems include; murine myeloma NS0 and Sp2/0 cell lines, Baby Hamster Kidney (BHK) cells and Human Embryo Kidney (HEK-293) cells (Birch and Racher, 2006; Wurm, 2004).

The use of CHO cell lines as the main mammalian cell line for the production of recombinant proteins is due to a number of factors. The choice of CHO cells as a production vehicle allows the utlilisation of selection markers such as dihydrofolate reductase (DHFR) for relatively high levels of protein expression (Chusainow et al., 2009). CHO cells have the ability to efficiently post-translationally process complex proteins and recombinant proteins produced by CHO cells possess similar glycosylation profiles to native proteins (Sinacore et al., 1996; Sinacore et al., 2000). From an industrial perspective, the fact that CHO cells have the ability to adapt and grow in suspension as well as an adherent monolayer is valuable as this allows scalability of culture volume and the use of large scale bioreactors (Jayapal, 2007). CHO cells also have the ability to grow in serum-free media which therefore reduces the potential for the introduction of viruses and other transmissible agents (Sinacore et al., 1996).

The adaptability and the ease of genetic manipulation of CHO cells has lead to it becoming the industry's "workhorse" for recombinant protein production (Walsh, 2010). CHO cells can reach extremely high densities as suspension cultures and are readily grown in large scale bioreactors that can hold upwards of 10,000 litres. Improvements in productivity have seen mammalian cells cultivated in bioreactors regularly reach titres of 10 g/L with a specific productivity of approximately 90pg/cell/day, which is more than a 100-fold yield improvement from titres seen in the mid to late 1980's (Birch and Racher, 2006; Wurm, 2004).

1.4 CHO cell survival in a synthetic environment

The batch and fed-batch culture of CHO cells for the production of recombinant proteins is based on the premise of using cell lines that have the capacity to proliferate as single cells existing in a high density, serum-free, suspension, chemically-defined synthetic environment (O'Callaghan and James, 2008; Sinacore et al., 2000). This chemically-defined synthetic environment is one that growth factors, cell to cell contacts and cell-substratum. CHO cells are a fibroblastic cell type and therefore they usually require an environment that is rich in growth factors and cell-substratum to be able to proliferate and survive (Juliano et al., 2004; O'Callaghan and James, 2008; Schwartz and Assoian, 2001). So exactly what allows some cells to adapt and survive in an environment that they are not used to growing in, is an important question in understanding the basis of using mammalian cells for recombinant protein production.

Many recombinant protein manufacturing platforms that utilise CHO cells as production vehicles have been developed using adaptation processes that allow a shift towards single cell autonomy (O'Callaghan and James, 2008; Prentice et al., 2007). These adaptation processes are time and resource consuming and usually require a number of steps. An example of such an adaptation process is given in the paper by Sinacore et al, from 2000. In this paper, a three step protocol is described that allows the selection of clonal derivatives that are able to proliferate as single cells in high density serum-free suspension culture

(Sinacore et al., 2000). Even though these adaptation protocols are widely used and clearly defined, very little is known of the underyling biological changes that allow particular clonal derivatives to survive in such an environment. There are likely to be numerous alterations of cellular processes that allow this, such as changes in the cell surface proteome (adhesion-linked proteins, receptors, transporters) and the signalling cascades that are linked to such receptors and that regulate cell growth and death (Schwartz and Assoian, 2001).

CHO-K1 (CCL-61) cells are an epithelial cell type and usually grow in culture as an adherent monolayer in a growth media that contains serum and exogenous growth factors. The composition of this growth media with the added growth factors allows CHO cells to form integrin mediated contacts with the extracellular matrix and other cells (O'Callaghan and James, 2008; Reddig and Juliano, 2005). This cell to matrix adhesion regulates homeostasis by stimulating signal transduction cascades that influence the regulation of cell growth, differentiation and cell death (Reddig and Juliano, 2005). Loss of cell anchorage via disruption of integrin mediated attachment triggers a type of apoptosis known as "anoikis" (Grossman, 2002). "Anoikis" is defined as "apoptosis that is induced by inadequate or inappropriate cell-matrix interactions" (Frisch and Screaton, 2001). In CHO cells "anoikis" has been shown to occur when disruption of the $\alpha5\beta1$ integrin-induced signalling pathways leads to a down-regulation in the expression of the anti-apoptotic protein Bcl-2 (Figure 1.1) (Lee and Ruoslahti, 2005; Matter and Ruoslahti, 2001; Zhang et al., 1995).

The inhibition of this $\alpha5\beta1$ integrin-induced signalling survival pathway due to withdrawal of serum from the growth media can be avoided by the overexpression of various activated downstream signalling intermediates (Matter and Ruoslahti, 2001; O'Callaghan and James, 2008). Activated or overexpressed focal adhesion kinase (FAK) has been shown to protect MDCK cells from anoikis (Matter and Ruoslahti, 2001). Constitutively activated forms of FAK that have been shown to rescue established epithelial cell lines, require both the major autophosphorylation site (Y397) and a critical kinase activity site (K454) to be phosphorylated for this effect to be seen (Frisch et al., 1996).
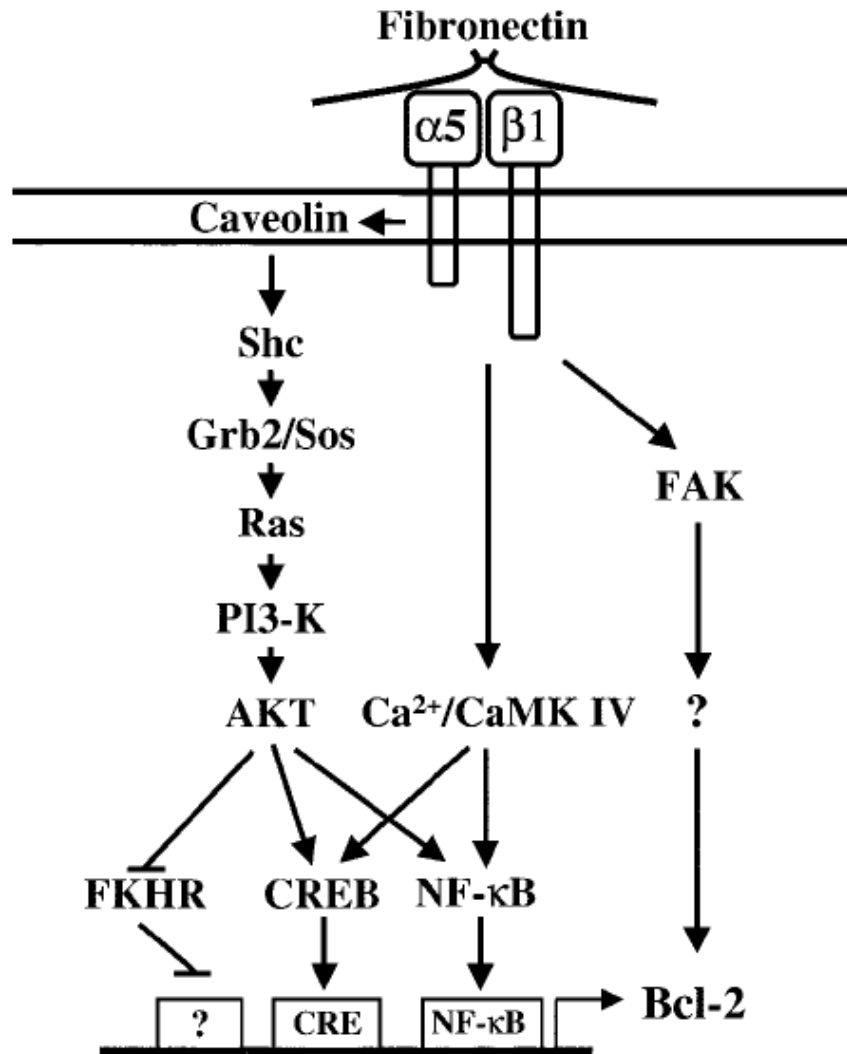
Figure 1.1: Schematic diagram showing the α5β1 integrin-regulated signalling pathways that lead to the up-regulation of Bcl-2 (Lee and Ruoslahti, 2005). The α5β1 integrin enhances the expression of Bcl-2 and consequently cell survival through the interaction of the Akt, FAK and CaMK IV signalling networks. Akt and CaMK IV are known to activate NF-κB and CREB which are positive transcription factors, while Akt has been shown to inhibit the negative transcriptional regulator forkhead (Brunet et al., 1999; Lee and Ruoslahti, 2005). Akt can be activated by the α5β1 integrin cluster recruiting caveolin and Shc, which in turn recruit Grb2/Sos (Wary et al., 1996).

PI3K is another signalling intermediate that can also render cells resistant to anoikis when it is constitutively activated (Khwaja et al., 1997). In other types of epithelial cells, such as mammary epithelial cells, the α6β1 integrin complex in conjunction with activation of the insulin signalling cascade activates PI3K and its downstream signalling effector Akt (also known as protein kinase B (PKB)) to promote cell survival. This indicates that the basement membrane (BM) and soluble factors such as insulin or insulin like growth factors co-operate in survival signalling (Farrelly et al., 1999; Matter and Ruoslahti, 2001).

Cell cycle progression in mammalian cells has been shown to be regulated by integrin-mediated adhesion as well as by growth factor binding to their specific receptor (Schwartz and Assoian, 2001). This potentiating of growth factor receptor tyrosine kinase signalling cascades by integrins is demonstrated in figure 1.2. As well as anchoring the cell to the extracellular matrix (ECM), the binding of some ECM proteins to integrin receptors can trigger outside-in signal transduction cascades. This integrin signalling is similar to that of ligand-dependent activation of growth factor receptor tyrosine kinases (RTKs) (Walker et al., 2005). This regulation of cell cycle progression is controlled by G_1 phase cyclin-dependent kinases (CDKs), which are downstream signalling intermediates that are under the control of integrins and growth factor receptors (Schwartz and Assoian, 2001).

Integrins also fulfil many other functions which are vitally important to maintaining cell performance *in vitro*. As well as potentiating cell signalling they also interact extensively with the cytoskeleton and have been shown to mediate cell to cell cohesion in CHO cells (O'Callaghan and James, 2008). In terms of cell to cell cohesion it was originally hypothesised that integrins and cadherins have distinct and opposing functions, in that integrins are primarily responsible for cell to ECM adhesion while cadherins regulate cell to cell cohesion (Robinson et al., 2003). However, work done by Robinson et al, showed that integrins also have the ability to mediate strong intercellular cohesion particularly when cells are grown as 3 dimensional aggregates. CHO cells transfected to express the
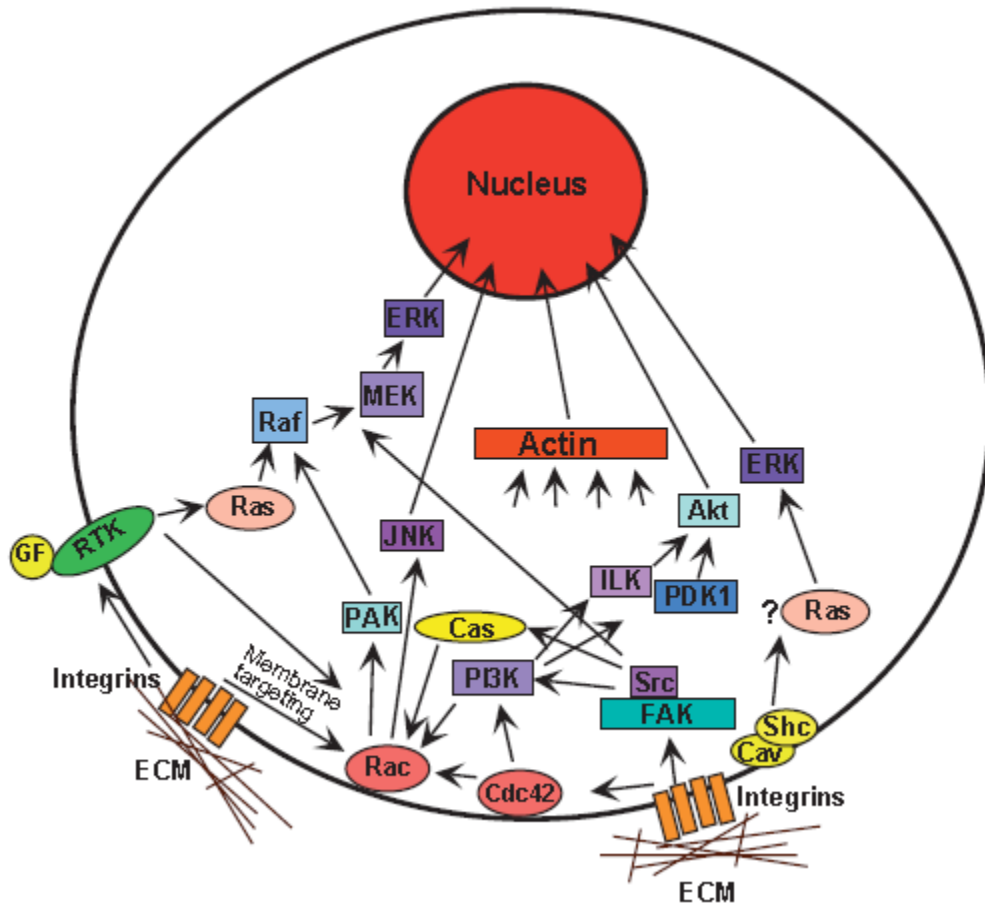
Figure 1.2: Integrin and receptor tyrosine kinase synergies that regulate cell cycle progression (Schwartz and Assoian, 2001). Integrins have been shown to directly activate cell cycle progression and enhance growth factor activation. Activation of RTKs by their specific growth factor ligands is enhanced by the binding of integrins to the ECM, which in turn enhances the signal transmission to various ERKs. Integrins also play a vital role in the direct activation and growth factor activation of Rac and Cdc42. The activation of PI3K is also contributed to by integrins, via the signalling intermediate FAK and Cdc42 (Schwartz and Assoian, 2001).

α5β1 integrin were shown to form compact spherical aggregates with a greater cohesivity than CHO cells transfected with N-cadherin (Robinson et al., 2003).

The cell surface proteome is also important to consider in terms of cell to cell contacts. It is known that clumped CHO cells form cell to cell contacts (or junctions) that involve cytoskeletal components. Two proteins are known to be key for the formation of intercellular junctions, spectrin and vinculin, which are found localised at regions of cell to cell contact (Coppen et al., 1995). Other junction proteins such as cadherins and catenins, that are found on the cell surface, may also have an important role to play in the regulation of signalling pathways that affect a cells survival capabilities (O'Callaghan and James, 2008). Cadherin adhesion proteins have been shown to be key determinants of tissue organisation and are involved in close co-operation with the actin cytoskeleton. In terms of cell signalling it has been demonstrated that they can activate survival signalling via interaction with Rho family GTPases and PI3K (Goodwin and Yap, 2004).

As well as requiring surface attachment, parental CHO-K1 (CCL-61) cells also typically require a supply of growth factors such as insulin, insulin-like growth factor and fibroblast growth factor (O'Callaghan and James, 2008). The effect of insulin binding and the consequent stimulation of many intracellular signalling pathways is vital for CHO cell survival in a synthetic environment. Insulin is the most frequently used growth factor for sustaining cell growth and viability in serum-free mammalian cell cultures (Morris and Schmid, 2000). Insulin exerts a number of physiological effects on a cell such as the stimulation of cell growth and cell cycle progression and the regulation of glucose and lipid metabolism (Saltiel and Kahn, 2001; Wong et al., 2004). Insulin and other growth factors interact with receptor tyrosine kinases that are found on the cell surface and consequently trigger downstream signalling cascades that stimulate a wide variety of cellular processes.

Insulin binds to the insulin receptor which is a receptor tyrosine kinase. This leads to autophosphorylation of the receptor which in turn leads to the further

phosphorylation of cellular proteins such as the relay protein IRS-1, Shc and Cbl (Saltiel and Kahn, 2001). The phosphotyrosines found on IRS-1 interact with the SH2 domains of various signalling proteins such as the adaptor protein Grb2. This leads to the activation of an array of signalling proteins and pathways including, the activation of PI3K, Sos, Ras, the MAPK and Akt signalling cascades as well as CAP/Cbl and the consequent activation of TC10. These pathways converge and diverge at various signalling intermediates and help regulate vital cellular functions such as general gene expression (by altering the rates of transcription and translation), cell growth and differentiation, glucose metabolism and glucose transport (Figure 1.3) (Saltiel and Kahn, 2001).

The elimination of serum and other animal derived growth factor proteins from mammalian cell culture media formulations poses many questions as to how mammalian production cells can survive in culture by assuming independence of these added growth factors (O'Callaghan and James, 2008; Renner et al., 1995; Sinacore et al., 2000). It has been suggested that cell cycle control elements such as cyclins and cyclin-dependent kinases that may be mutated or overexpressed can compensate for the absence of exogenous growth factors. In a paper by Renner et al, it was shown that overexpression of cyclin E or elevated cyclin E levels leads to increased proliferation of CHO cells in media without mitogenic supplements and that cyclin E is a major determinant of growth factor responses and also the contact behaviour CHO-K1 cells (Renner et al., 1995).

It has also been postulated that other components of the growth media such as insulin-mimetic trace metals may compensate for the absence of growth factors such as insulin. Several trace metals (such as nickel and cadmium) have been shown to inhibit insulin-like effects in other mammalian cell types (Wong et al., 2004). The most widely investigated trace-metal is zinc. Zinc supplementation of insulin free growth media has been shown to restore cell growth in CHO-K1, hybridoma and NS0 cell lines (Wong et al., 2006).
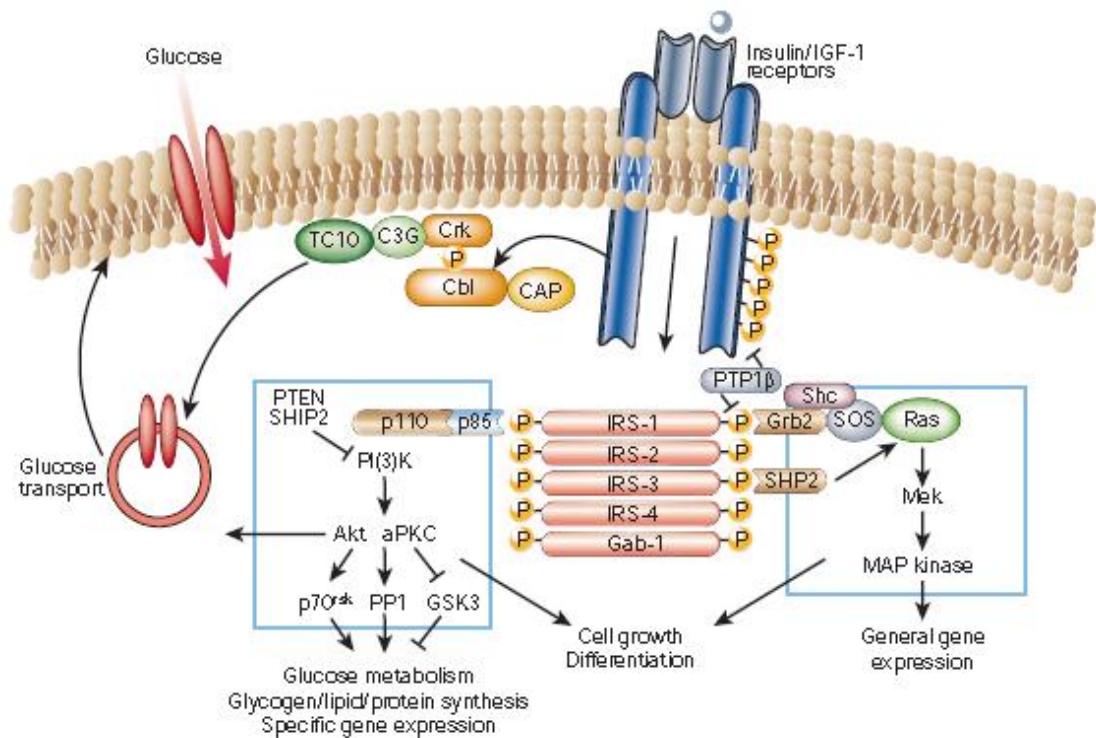
Figure 1.3: Signal transduction events involved with insulin binding (Saltiel and Kahn, 2001). Insulin binds to the insulin receptor which is a receptor tyrosine kinase. This binding event leads to the autophosphorylation of the receptor which in turn leads to the further phosphorylation of cellular proteins, such as the relay protein IRS-1, Shc and Cbl. The phosphotyrosine residues found on IRS-1 interact with the SH2 domains of various signalling proteins such as the adaptor protein GRB2. This leads to the activation of an array of signalling proteins and pathways including the activation of PI3K, Sos, Ras and the MAP kinase cascade as well as CAP/Cbl and the consequent activation of TC10. These pathways converge and diverge at various signalling intermediates and help regulate vital cellular functions such as general gene expression (by altering the rates of transcription and translation), cell growth, glucose metabolism and glucose transport (Saltiel and Kahn, 2001).

Research conducted on NS0 myeloma cells has also suggested that cells grown in a chemically defined, growth factor and serum free media can acquire the ability to produce autocrine growth factors that can enhance cell growth and proliferation (Spens and Haggstrom, 2005). This may also be the case for adapted CHO cells.

The understanding of how mammalian cells are able to adapt to differing synthetic environments in culture whilst still maintaining their growth and productivity capabilities is very limited. The underlying cellular mechanisms, that allow certain subsets of cells to survive as both an adherent monolayer growing in the presence of serum and other growth factors and also as a suspension cell line growing in serum and growth factor free media, are not well characterised and this limits the capability of being able to engineer cells with desired characteristics (O'Callaghan and James, 2008).

1.5 The activation of intracellular signalling pathways

Intracellular signalling pathways play a vital role in linking changes that occur cell environment to the control of cell survival and proliferation. To gain a better understanding of how these pathways function and interact it is possible to map the activation and deactivation of various signalling networks and how they provoke a cellular response to changes in the environment. It is also important to deduce how these pathways interact with proteins at the cell-environment interface.

It is possible to map the activation of a particular pathway by analysing the phosphorylation status of important pathway intermediates. Many signalling intermediates in these pathways are serine/threonine kinases or tyrosine kinases. Therefore using certain techniques it is possible to see whether a particular signalling node is phosphorylated or unphosphorylated when a population of cells are growing in a certain environment.

Two of the key signalling pathways that are responsible for regulating cellular processes such as cell growth, proliferation, survival and metabolism in response to changes in the cellular environment, are the Ras/MAPK and PI3K/Akt pathways (Mendoza et al., 2011). These two pathways are discussed in detail in section 4.1.

1.6 Signalling intermediates as targets for genetic engineering

As section 4.1 demonstrates there are numerous signalling pathway intermediates that are key nodes of signal transduction. However, it is also important to determine which activated signalling intermediates are viable targets for genetic engineering strategies and as such can provide a functional substitute for extracellular signalling.

Possible engineering strategies include over-expression of key intermediates and siRNA knockdown of activation inhibitors. Examples of viable targets include p21-activated kinases (PAKs) which play an important role in the activation of Raf-1 and MEK in integrin mediated cell anchorage signalling pathways. It has been shown that overexpression of a constitutively activated form of PAK can rescue signalling in cells adapted to grow in suspension (Juliano et al., 2004). PI3K has been demonstrated to be another viable target. Over-expression of activated PI3K has been shown to up-regulate bcl-2 transcription levels whilst in cell cultured with the PI3K inhibitor, LY294002, bcl-2 transcription was totally inhibited (Matter and Ruoslahti, 2001). Finally Akt/PKB has been shown to be constitutively activated in many types of human cancer, promoting proliferatrion and increased cell survival (Nicholson and Anderson, 2002). Therefore Akt could provide a viable target for genetic engineering in order to overcome anoikis and cell death due to growth factor withdrawal.

1.7 Project Overview

The overall aim of this research project was to decipher the role that intracellular signalling pathways play in mediating CHO cell growth in a synthetic environment. In order to reach this aim a number of objectives were designed and performed, which are described in this thesis.

1. The first part of the research project involved identifying the model system being investigating and the key signalling network(s) that link to this model system. Two key aspects of the model system involved looking at how CHO cells are adapted to growing in suspension without integrin-mediated attachment and how they are able to utilise added growth factors in the culture media to stimulate cell growth, proliferation and survival. From the literature search conducted, two key signalling pathways emerged that were key in transmitting these events at the cell surface into various cellular responses, the Ras/MAPK and PI3K/Akt pathways.

2. Following the identification of the model system being investigated, extensive cell characterisation experiments were performed in order to define the growth characteristics of the model cell lines used in this study. The growth characteristics of the model cell lines were analysed in both adherent and suspension conditions, as this is one of the key transitions that occurs when CHO cells are adapted to grow in synthetic conditions.

3. Building on the results obtained from the cell characterisation experiments, the pathway activation status of the Ras/MAPK and PI3K/Akt pathways was analysed in the model cell lines growing in both adherent and suspension conditions. The activation status of key intermediates in both pathways such as PI3K, Akt, mTOR, c-Raf, MEK and Erk, was analysed via western immunoblotting using specific phospho-antibodies against those specific intermediates. The results from the western blot experiments were then used to map the pathway activation status of the Ras/MAPK and PI3K/Akt pathways when cells are growing in adherent conditions compared to their growth in suspension

conditions. Further western blotting analysis was conducted in order to analyse the activation status of key intermediates in both the Ras/MAPK and PI3K/Akt pathways, when the three model cell lines (that have undergone different levels of suspension adaptation) are growing in suspension conditions.

4. Additional experimental work was conducted in order to decipher how the activation of the Ras/MAPK and PI3K/Akt pathways is linked to events that occur at the cell surface. Extensive flow cytometry and western blotting experiments were carried out, in order to analyse the expression levels of key integrins on cells cultured in both suspension and adherent conditions. While, confocal microscopy was used to look at the conformation and distribution of key integrins on the cell surface of adherent and suspension cells.

5. The final set of experiments conducted, focused on dissecting the activation status of both the Ras/MAPK and PI3K/Akt pathways, in both adherent and suspension conditions, by using specific chemical inhibitors that inhibit particular signalling nodes in each pathway. The experimental data generated, suggested that activation of certain signalling intermediates is vital for cell growth and survival in different synthetic environments, while activation of other intermediates, such as PI3K, is required in both adherent and suspension synthetic conditions.

Chapter 2

Materials and Methods

Chapter 2 Aims

In this chapter the methods and materials that were used this study are described.

2.1 Cell Culture

2.1.1 Cell lines

A number of different cell lines cultured in various cell culture environments were used throughout this project, including:

- CHO-K1 clone CCL-61, adherent cell line, from the ATCC, received from Pfizer.
- CHO-SA, suspension cell line, Pfizer proprietary cell line, received from Pfizer.
- Cell Line 1 + Ins, mAb producing suspension cell line, Pfizer proprietary cell line, received from Pfizer.
- Cell Line 1 – Ins, mAb producing suspension cell line, Pfizer proprietary cell line, received from Pfizer.
- Cell Line 2 + Ins, mAb producing suspension cell line, Pfizer proprietary cell line, received from Pfizer.
- Cell Line 2 – Ins, mAb producing suspension cell line, Pfizer proprietary cell line, received from Pfizer.
- CHO-S, suspension cell line, received from Invitrogen.

The mAb producer cell lines 1 and 2 stably produce an FC fusion protein and were generated using the DHFR/MTX expression system.

The cell lines listed were specifically chosen for analysis as they have all undergone various levels of adaptation from the parental adherent cell line CHO-K1. This adaptation process is described in detail in section 3.2.

2.1.2 Handling of the CHO-K1 (CCL-61) adherent cell line

2.1.2.1 Revival of CHO-K1 cells from cryopreservation

CHO-K1 cells were thawed rapidly by gently agitating a vial of cells in a water bath set to 37°C. The vial contents were transferred to a centrifuge tube containing 9 mL of pre-warmed complete medium and centrifuged at 1000 rpm (revolutions per minute) for 5 to 7 minutes. The cell pellet was resuspended in 5 mL of pre-warmed complete medium and added to a T75 culture flask already containing 10 mL of pre-warmed complete medium.

2.1.2.2 Cell culture maintenance of adherent CHO-K1 cell line

The adherent CHO-K1 cell line was cultured in ATCC-formulated F-12K media (ATCC, Manassas, USA) with 10% (v/v) fetal bovine serum (Biosera, Ringmer, UK) and 1% (v/v) Penicillin/Streptomycin solution (Sigma-Aldrich, Poole, UK). CHO-K1 cells were routinely subcultured every 3 to 4 days. The conditioned culture media was removed and the flask rinsed with 5-10 mL sterile 1 x phosphate buffered saline without calcium and magnesium (PBS-CMF; Invitrogen, Paisley, UK) before incubation with 1-2 mL 0.05% (w/v) Trypsin-EDTA (1x) (Invitrogen) at 37°C for 10 min or until all the cells detached from the flask surface. Flasks were inspected under an inverted microscope to check for cell layer dispersion, before 6-8 mL of complete growth medium was added to stop the trypsin reaction. The cell suspension was removed from the flask and a 0.6 mL aliquot was taken to calculate the viable cell concentration using the Vi-Cell XR Series Cell Viability Analyser (Beckman Coulter, High Wycombe, UK). The

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Vi-Cell XR uses the trypan blue dye exclusion method to determine cell concentration and viability. From the Vi-Cell results the volume of cells needed to inoculate the new culture was calculated. T-75 flasks (Fisher Scientific, Loughborough, UK) containing approximately 15 mL of pre-warmed growth medium were seeded at a concentration of 5,000 cells cm^2 for a 4 day passage and 10,000 cells cm^2 for a 3 day passage. The flask was placed on a level surface in a Heraeus HeraCell CO_2 static incubator (ThermoFisher Scientific, MA, USA) set to 37°C, 80% humidity, 5% CO_2. Cells were maintained up to a maximum of 20 passages.

2.1.2.3 Adherent cell culture vessels

Three different sizes of culture vessel were used when handling the adherent CHO-K1 cell line. T25 flasks (25 cm^2 growth area) containing 5 mL of culture medium were used for small-scale experimental work. T75 flasks (75 cm^2 growth area) containing 15 mL of culture medium were used for normal subculture routines and for some experimental work. T225 flasks (225 cm^2 growth area) containing 30 mL of culture medium were used for larger-scale experimental work. All flasks used were from Fisher-Scientific.

2.1.3 Handling of suspension cell lines

2.1.3.1 Revival of suspension cells from cryopreservation

All suspension cell lines were thawed rapidly by gently agitating a vial of cells in a water bath set to 37°C. The vial contents were transferred to a centrifuge tube containing 9 mL of pre-warmed complete medium and centrifuged at 1000 rpm for 5 to 7 minutes. The cell pellet was then resuspended in 5 mL of pre-warmed complete medium and added to a 125 mL Erlenmeyer flask (Corning, Surrey, UK) already containing 25 mL of pre-warmed complete medium.

2.1.3.2 Cell culture maintenance of suspension cell lines

The suspension cell line CHO-SA was routinely subcultured in proprietary suspension media 1 (PSM1) received from Pfizer (Andover, USA). The mAb producing suspension cell lines, Cell Line 1 and Cell Line 2, were routinely subcultured in proprietary suspension media 2 (PSM2) received from Pfizer. The CHO-S cell line was cultured in CD-CHO medium (Invitrogen) that was supplemented with 8 mM l-glutamine (Sigma-Aldrich). All suspension cell lines were subcultured after 3 or 4 days (alternating). For a 3 day passage cells were seeded at a viable cell concentration of 0.4 x 10^6 cells mL^{-1}, while for a 4 day passage cells were seeded at a viable cell concentration of 0.2 x 10^6 cells mL^{-1}. For routine subculturing a 0.6 mL aliquot was taken to calculate the viable cell concentration using the Vi-Cell XR Series Cell Viability Analyser. From the Vi-Cell results the volume of cells needed to inoculate the new culture was calculated. The appropriate volume of viable cells was then seeded into a pre-equilibrated Erlenmeyer shake flask. All suspension cells lines were routinely subcultured in vented 125 mL Erlenmeyer flasks with a working volume of 30 mL. Flasks were incubated in a Multitron shaking incubator with an orbital throw of 25 mm. The standard incubator conditions were set to 37°C, 140 rpm, 5% CO_2. Cells were maintained up to a maximum of 20 passages.

2.1.3.3 Suspension cell culture vessels

The suspension cell lines were cultured in Erlenmeyer shake flasks of various different sizes. For routine subculturing cells were cultured in 125 mL Erlenmeyer flasks with a working volume of 25 to 30 mL. 250 mL Erlenmeyer flasks with a working volume of 60 mL were used for some experimental work, while larger scale batch characterisation experiments were conducted in 1 L Erlenmeyer flasks with a working volume of 240 mL. All flasks used were from Corning.

2.1.4 Maintaining frozen cell banks of all CHO cell lines

Master and working cell banks for all cell lines were prepared by harvesting cells on day 3 (mid-exponential phase) of culture. Cells were centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cell pellet was re-suspended in a freezing solution consisting of the cells particular growth media with 10% (v/v) DMSO at a concentration of 1×10^7 cells/ mL. 1.5 mL aliquots of the cell suspension were placed into separate Nunc cryovials (ThermoFisher Scientific, MA, USA), which were then placed into a "Mr. Frosty" container (Nalgene, Roskilde, Denmark) for slow freezing and stored at -80°C for a minimum of four hours. The cryovials were transferred to liquid nitrogen (-196°C) for long term storage.

2.2 Protein extraction from cultured cells

2.2.1 Protein extraction from cultured cells using RIPA Buffer

RIPA buffer was purchased from ThermoFisher Scientific or was prepared by adding 50 mM Tris-Base, 150 mM NaCl, 0.5% Deoxycholate, 1% Triton-X-100 and 0.1% SDS to Milli-Q H_20 and mixing thoroughly with a magnetic stirrer. The basic RIPA buffer solution did not contain any protease or phosphatase inhibitors, therefore a HALT Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) was added prior to use. The inhibitor cocktail protects proteins from potential degradation by endogenous phosphatases and proteases that can be released during the protein extraction and isolation process. Immediately before starting the protein extraction, the inhibitor cocktail was added at 10 μl/ mL directly to the RIPA buffer stock solution to produce a 1X final concentration. Additionally, to inhibit metalloproteases, EDTA was added to the RIPA stock at 10 μL/ mL of lysis buffer to achieve a 1X working concentration.

2.2.2 RIPA lysis of monolayer-cultured CHO cells

To extract proteins from cells cultured as an adherent monolayer, the cell culture medium was carefully decanted and the adherent cells were washed twice with cold PBS. Cold RIPA buffer was added to the cells, using 0.5 mL of buffer per T75 flask containing approximately 5×10^6 cells or 1 mL of buffer per T175 flask containing approximately 1×10^7 cells. The flask was placed on ice for 15 minutes and was swirled occasionally to ensure complete coverage. The cell lysate was collected using a cell scraper and transferred to a microcentrifuge tube. Samples were centrifuged at 15000 x g for 15 minutes using a Heraeus Pico 17 Microcentrifuge (ThermoFisher) to pellet the cell debris. Post centrifugation, the sample pellets were sonicated using a sonicating waterbath for 30 seconds with 50% pulse. The supernatants were transferred to a new tube for further analysis or for storage at 2-8°C for up to 2 weeks or -20°C for longer term storage.

2.2.3 RIPA lysis of suspension-cultured CHO cells

To extract proteins from cells cultured in suspension conditions, samples containing 5×10^6 cells or 1×10^7 cells were taken and the cells pelleted by centrifugation at 2500 x g for 5 minutes (4K15 centrifuge, Sigma, Poole, UK). After the supernatant was discarded the cells were washed twice with cold PBS and again centrifuged at 2500 x g for 5 minutes. Cold RIPA buffer was added to the cell pellet, using 0.5 mL of buffer per 5×10^6 cells or 1 mL of buffer per 1×10^7 cells. To increase yields, the sample pellets were sonicated using a sonicating waterbath for 30 seconds with 50% pulse. The samples were placed on ice for 15 minutes with gentle shaking and were then centrifuged at 15000 x g for 15 minutes using a Heraeus Pico 17 Microcentrifuge (Thermofisher) to pellet the cell debris. The supernatants were transferred to a new tube for further analysis or for storage at 2-8°C for up to two weeks or -20°C for longer term storage.

2.2.4 BCA protein quantification assay

Following protein extraction using RIPA buffer, protein concentration was calculated using the Pierce BCA Protein Assay Kit (Thermo Scientific). The BCA assay was performed in a 96-well microplate and the procedure was conducted as per the manufacturer's instructions. Briefly, a sample to working reagent ratio of 1:8 was used and 10 µL of RIPA extract was used per well. Samples were run in triplicate on each plate. The plate was then placed in a static incubator at 37°C for 30 minutes before the sample concentrations were then determined using a BioTek Power Wave XS microplate reader (BioTek, Bedfordshire, UK)reading at 570 nm.

2.3 SDS-PAGE and western immunoblotting

2.3.1 SDS-PAGE sample preparation

After calculating the total protein in each sample, an appropriate amount of LDS Sample Buffer (4x), Reducing Agent (10x) and Deionised Water were added to the protein extract, giving a loading concentration of 25 µg protein per well. The samples were heated at 70°C for 10 minutes in a heating block and allowed to cool for a period of time. After heating, the samples were micro-centrifuged for 5 minutes at 1,000 rpm.

2.3.2 SDS-PAGE/Protein Blotting

An electrophoresis system was set up for 1 NuPAGE 10% Bis-Tris gel (Invitrogen) using (1x) MOPS buffer (Invitrogen) to be run under reduced conditions. The ladder, standards and samples were loaded onto the gel in the same gel layout each time. The Bis-Tris gel was then run in MOPS buffer between 175-200 volts for approximately 90 minutes. The protein blot was then electro-transferred to a nitrocellulose/PVDF membrane using an iBlot dry blotting system (Invitrogen).

2.3.3 Membrane blocking and antibody incubations

Post transfer to a nitrocellulose/PVDF membrane, the membrane was washed with 25 mL TBS for 5 minutes at room temperature. The membrane was then incubated in 25 mL of blocking buffer for 1 hour at room temperature and then washed 3 times for 5 minutes each with 15 mL of TBS/T wash buffer. The membrane was then incubated with the primary antibody (at the appropriate dilution) in 10 mL of primary antibody dilution buffer with gentle agitation overnight at 4°C. After overnight incubation, the membrane was again washed 3 times for 5 minutes each with 15 mL of TBS/T. The membrane was then incubated with the species appropriate HRP-conjugated secondary antibody (1:2000 dilution) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 mL of blocking buffer for 1 hour at room temperature. Again, the membrane was washed 3 times for 5 minutes each with 15 mL of TBS/T.

2.3.4 Detection of proteins and image analysis

In order to visualise the blot using chemiluminescent detection, the membrane was incubated in 10 mL of Immobilon™ Western Chemiluminescent HRP substrate (Millipore). To prepare the HRP substrate, equal volumes of Luminol Reagent and Peroxide Solution were mixed in a clean Falcon tube to give a final volume of 10 mL. The membrane was placed protein side up in a clean container and the HRP substrate was carefully applied to the membrane and left to incubate for 5 minutes at room temperature. After 5 minutes the excess substrate was drained off and the membrane was imaged using an ImageQuant™ RT ECL™ system (GE Healthcare) via the chemiluminescent imaging mode. GE Healthcare's proprietary imaging software, ImageQuant TL 1D gel analysis software was used to quantify the bands of interest by densitometry.

2.4 Flow cytometry analysis

2.4.1 Preparation and fixation of adherent and suspension cells for flow cytometry

In order to prepare cells for analysis via flow cytometry, the adherent CCL-61 cells were first harvested from their culture vessel via treatment with phosphate buffered saline (PBS) (Invitrogen) with 2 mM EDTA (Fisher Scientific) (PBS-EDTA). The cells were incubated for approximately 10 minutes in PBS-EDTA to ensure as many cells as possible were detached from the surface of the culture vessel. Both adherent and suspension cells were then micro-centrifuged for 5 minutes at 1,000 rpm and then washed with PBS. The cells were then fixed using 1% para-formaldheyde (PFA) solution. The 1% PFA solution was prepared by diluting a 4% PFA stock solution (Sigma Aldrich) 1 in 4 with PBS. 1 mL of 1% PFA was then added to 5×10^6 of either adherent or suspension cells and incubated for 20 minutes at 4°C. The cells were then again washed with PBS and then stored in PBS solution at a concentration of 1×10^7 cells/ mL for up to 1 week.

2.4.2 Flow cytometry set-up and analysis

All flow cytometry analysis was performed using a FacsCalibur flow cytometer (BD Biosciences, Oxford, UK). Fluorescence excitation was generated using a 488 nm Argon laser while the fluorescence emission was detected by the FL1 (525 nm) filter. For each independent experiment the instrument settings were selected depending on the results given from the autofluorescence of unstained cells from each cell line. All samples that were analysed were run until the maximum cell count was reached and all samples were run in triplicate. The Median Fluorescence Intensity (MFI) value for each sample run was calculated from the gated region of positively stained cells. All flow cytometry data was analysed using FlowJo version 7.6.5 software (Freestar Inc., Ashland, USA)

2.5 Confocal microscopy analysis

2.5.1 Preparation and fixation of adherent and suspension cells for confocal microscopy

Prior to confocal microscope analysis of adherent cells, sterile cover slips were placed in 6 well plates (Nunc) and were incubated overnight in 1 mL of PBS in standard static incubator conditions (5% CO_2, 37°C). For confocal analysis, adherent CCL-61 cells were cultured in standard adherent conditions in these 6 well plates at an original seeding density of 5,000 cells/ cm^2. For each experiment, cells were stained on day 3 of culture. Prior to fixation, cells cultured on the cover-slips were washed twice with PBS before 1 mL of 1% para-formaldehyde was added to each well. The cells in PFA were then incubated at room temperature for 20 minutes and then washed twice with PBS.

For suspension cells, cells were cultured in standard suspension conditions, before 2.5×10^6 cells were harvested on day 3 of culture. Cells were washed twice with PBS before being transferred to an ibidi 35 mm imaging µ-Dish (ibidi GmbH, Martinsried, Germany). 1ml of 1% paraformaldehyde was added to each dish and the cells were fixed at room temperature for 20 minutes. The cells were then washed twice with PBS and analysed that day.

2.5.2 Confocal microscopy set-up and analysis

All confocal microscopy analysis for both adherent and suspension cells was performed on an inverted Zeiss LSM510 Meta Confocal Microscope (Zeiss, Oberkochen, Germany). Samples were analysed with a Plan-Neofluar 40x/1.3 Oil DIC objective via excitation with an argon laser. In terms of excitation and emission, for Alexa Fluor 488 and FITC the excitation wavelength used was 488 nm and the emission was collected using a Band Pass 500-550 nm IR filter. For Alexa Fluor 546 the excitation wavelength used was 514 nm and the emission was collected using a Long Pass 560 nm filter. When cells were co-stained with Alexa Fluor 488/FITC and Alexa Fluor 546, the excitation wavelength for Alexa

Fluor 546 was changed to 543 nm. All images obtained from confocal microscopy experiments were analysed using Zeiss LSM Image Browser Version 4,2,0, 121.

2.6 Monoclonal antibody titre analysis

2.6.1 Calculating Mab titre using FastELISA

Cell culture supernatants from the Mab producing cell line Cell Line 1 were taken every 24 hours and were measured for Mab concentration using the FastELISA human IgG quantification kit (2B Scientific, Upper Heyford, UK). The Mab assay was performed as per the manufacturer's instructions. In a brief explanation, cell culture supernatant samples were diluted 1:40 in the dilution buffer provided and then added to the pre-coated assay plate. The HRP-conjugate was then added to the plate wells and was then incubated for 15 minutes. Next, the HRP-substrate was added and was also incubated for 15 minutes, before the assay stop solution was finally added. The sample Mab concentrations were then determined using a BioTek Power Wave XS microplate reader (BioTek, Bedfordshire, UK) reading at 450 nm and 620 nm.

2.7 Equations used throughout thesis:

Cell specific growth rate (μ) was calculated using the equation given below:

$$\mu\ (h^{-1}) = \frac{\ln(\text{VCC}_1) - \ln(\text{VCC}_2)}{t_2 - t_1}$$

In the equation VCC_1 is the viable cell concentration at time point 1, VCC_2 is the viable cell concentration at time point 2, while t is time.

The integral of viable cell concentration (IVCC) was calculated using the equation below which is based on the trapezoid rule (Balcarcel and Stephanopoulos, 2001):

$$ICT = \frac{VCC_1 + VCC_2}{2} \; x \; (t_2 - t_1)$$

In the equation, ICT is the integral of cell time, VCC_1 and VCC_2 are the viable cell concentrations at the start (t_1) and end (t_2) of each time interval. The IVCC is the total of all the values for each time point, which is represented below:

$$IVCC \; (cell \; h^{-1}) = \sum \frac{VCC_1 + VCC_2}{2} \; x \; (t_2 - t_1)$$

Cell volume was calculated using the equation for the volume of a sphere, which is given below:

$$Cell \; Volume \; (\mu m^3) = \frac{4}{3} \pi \; x \; r^3$$

The cell radius is obtained from the average diameter reading given by the Vi-Cell.

The specific Mab production rate (qMab) was calculated using the equation:

$$qMab \; (pg \; cell \; h^{-1}) = \left(\frac{T_2 - T_1}{\frac{VCC_2 + VCC_1}{2}} \right) / t_2 - t_1$$

In the equation T_1 is the Mab titre at time point 1 and T_2 is Mab titre at time point 2. VCC_1 is the viable cell concentration at time point 1 and VCC_2 is the viable cell concentration at time point 2.

Chapter 3

Growth characterisation studies of model system cell lines

Acknowledgements

The data, figures and text in the following chapter are the work of the candidate.

Chapter 3 Aims

In this chapter the growth characteristics of the CCL-61 and CHO-SA model system cell lines growing in both adherent and suspension conditions are investigated. The relationship between the cell lines is explained in detail.

3.1 Introduction

The underlying biological changes which take place when cells are placed into serum-free, suspension conditions are not well defined (O'Callaghan and James, 2008). In order to gain an insight into the changes seen in key intracellular signalling pathways in response to this change in growth environment, two related cell lines were selected as model system cell lines and their growth characteristics in both adherent and suspension conditions were investigated.

In terms of cell line development strategies, the starting point for many cell lines is the parental adherent cell line CHO-K1, clone CCL-61. The CHO-K1 (CCL-61) cell line was derived as a sub-clone from the original parental CHO cell line, which was isolated from a biopsy of an ovary of an adult Chinese hamster by T.T. Puck in 1957 (Kao and Puck, 1967; Puck et al., 1958). In order to generate some of their production cell lines Pfizer used the CHO-K1 cell line (from the ATCC) as a parental cell line.

The focus of this chapter is the relationship between the adherent cell line CHO-K1 (CCL-61) and the directly adapted CHO-SA (Suspension Adapted) cell line. The CHO-SA cell line, received from Pfizer, was generated by taking a sub-set of CHO-K1 (CCL-61) cells and adapting them over a period of time to grow and proliferate in serum-free, suspension conditions in chemically-defined proprietary suspension media (PSM). The type of adaptation procedure used in this process is detailed in section 3.1.1. From this CHO-SA cell line, Pfizer have then generated further suspension cell lines that are antibody producing. This chapter will concentrate on cell lines that are not antibody producing, as cell lines that have been screened for their production capabilities, rather than just

for their ability to grow in suspension, will have been placed under additional selective pressure(s) (Browne and Al-Rubeai, 2007).

When considering the model system it is important to note that in this particular investigation the proprietary media used in each case is considered to be an integral part of the model system. Work done in further chapters shows that when cells are taken off a surface and placed into suspension conditions the changes seen in signalling pathway activation, growth characteristics and cell surface properties is not solely down to the effect of the change of growth media. The aim of the model system design was to characterise the model cell lines with regard to their growth phenotypes. In order to investigate the ability of the model cell lines to grow in both adherent and suspension growth modes, each cell line was placed into standard adherent conditions and standard suspension conditions (table 3.1).

For each cell line the standard growth conditions are different. The CCL-61 cell line typically grows as an adherent monolayer in mF12 media supplemented with 10% FBS. However, the CHO-SA cell line usually grows in serum-free, suspension conditions in PSM media supplemented with 10 mg/L insulin. The inclusion of insulin in the proprietary suspension media adds another dimension to the model-system analysis. As mentioned in section 1.4, insulin is the most frequently used growth factor for sustaining cell growth and viability in serum-free mammalian cell cultures (Morris and Schmid, 2000). Therefore, in this particular model system, the inclusion of a growth factor in the culture media such as insulin may be vital for the ability of a cell line to grow and proliferate in suspension conditions. So as well as investigating how the two cells lines respond to growing on and off a surface, this study is also looking at how the two cell lines respond to the inclusion of a growth factor, such as insulin, in the growth media. An outline of the model system and the different growth conditions investigated is shown in figure 3.1.
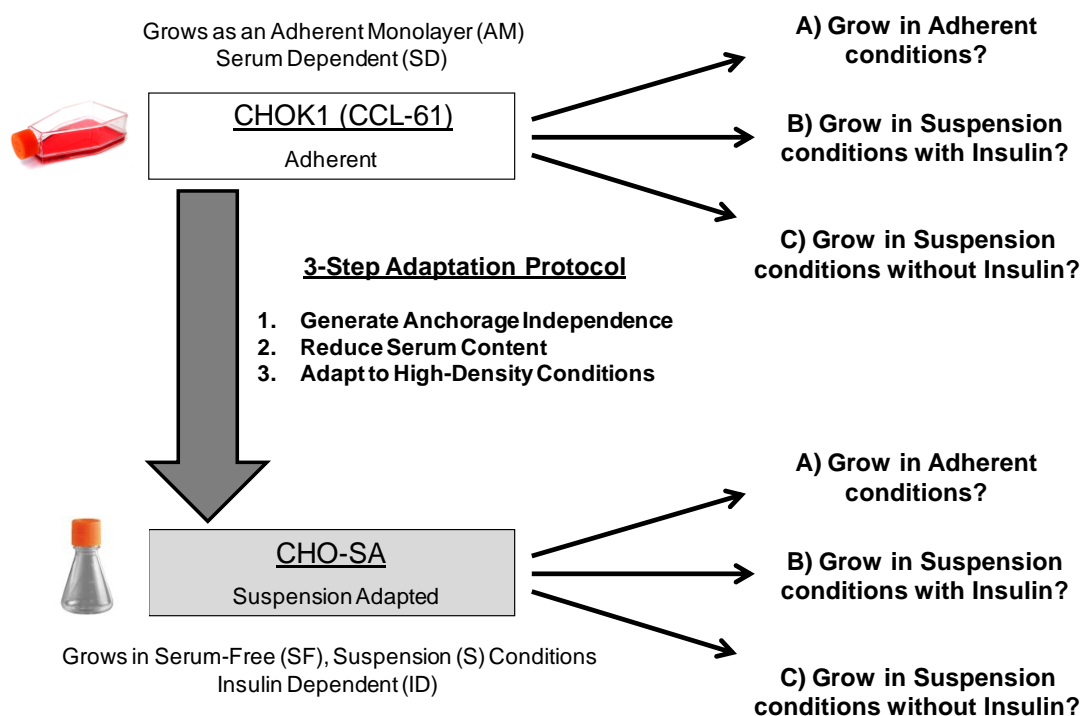
Figure 3.1: Outline of model system. The suspension cell line CHO-SA is directly adapted from the parental adherent cell line CHO-K1 (CCL-61) using a three-step adaptation protocol, as discussed in detail in section 3.1. The CCL-61 cell line is cultured as an adherent monolayer in F12 media supplemented with 10% FBS. The cells are cultured in standard static conditions (37°C, 5% CO_2). The CHO-SA cell line is cultured in serum-free, suspension conditions in proprietary SA media received from Pfizer. The SA media contains insulin at a concentration of 10mg/L. The cells are cultured in standard suspension conditions (37°C, 140 rpm, 5% CO_2). As shown in the figure above, both of the cell lines were characterised growing in three different growth environments A) In adherent conditions B) In suspension conditions with insulin in the growth media and C) In suspension conditions without insulin in the growth media.

Culture Mode	Media	Culture Conditions
Adherent	ATCC formulated F-12K media +10% FBS	Static culture (37°C, 5% CO_2)
Suspension + Insulin	Proprietary Suspension Media (PSM) + 10 mg/L insulin	Suspension culture (37°C, 140 rpm, 5% CO_2)
Suspension - Insulin	Proprietary Suspension Media (PSM) -10 mg/L insulin	Suspension culture (37°C, 140 rpm, 5% CO_2)

Table 3.1: Detailed explanation of the different conditions used to characterise the growth performance of the model cell lines. The three different culture modes (Adherent, Suspension + Insulin and Suspension – Insulin) with their relevant media and culture conditions are given. The proprietary suspension media, which is formulated by Pfizer, is usually made containing 10 mg/L insulin, as the CHO-SA cell line when growing in standard conditions requires this. For the purposes of this study, to investigate whether cells cultured in suspension without an exogenous growth factor (such as insulin) are able to grow and proliferate, Pfizer provided PSM that was exactly the same formulation but without insulin.

3.1.1 Adaptation strategies from adherent to suspension growth

One of the key considerations when adapting cells from adherent to serum-free suspension culture, is that such a strategy can cause a change in the growth characteristics (growth rate, viability, cellular productivity) of cells as well as the structure and function of the recombinant protein product. Previous work, conducted by many different groups, has shown that a decrease in cell viability and cell specific growth rate is seen when cells are transferred to serum-free conditions (Griffiths and Racher, 1994; Sinacore et al., 2000; Zang et al., 1995). This effect is thought to be caused by a disruption in the signalling pathways that control G_1 phase cyclin-dependent kinases and subsequent entry into S-phase as well as the initiation and activation of apoptotic pathways (Assoian, 1997; di Jeso et al., 1995; Ruoslahti and Reed, 1994; Sinacore et al., 2000).

There are two main types of adaptation strategies that are used to adapt CHO cell lines to serum-free suspension culture; direct adaptation and sequential adaptation. A direct adaptation strategy is where cells are "dumped" directly into suspension conditions usually in specially formulated, chemically defined, protein and serum-free media. Where as a sequential adaptation, involves numerous steps to gradually generate a fully adapted serum-independent suspension cell line.

The adaptation strategy used in the paper by Sinacore et al, 2000, is a three-phase adaptation strategy. The first stage of this adaptation protocol involves generating anchorage independence, by trypsin treating the adherent cells and transferring them to a suspension culture vessel in suspension growth media supplemented with FBS. The second stage of the process involves weaning the cells off the added FBS by gradually lowering the concentration added to the suspension culture media. The third and final step involves adapting the cells to high density culture conditions by adding growth inhibiting substances such as lactic acid and ammonia to the suspension culture, in order for the cells to develop tolerance to such substances.

3.2. Methodology

3.2.1 Adherent characterisation

To analyse the growth characteristics of the two cell lines in adherent conditions, both the CCL-61 and CHO-SA cell lines were harvested at day 3 of culture (growing in their standard conditions). Both cell lines were washed in PBS and then seeded into standard adherent conditions and batch cultured for 8 days. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser.

3.2.2 Suspension characterisation

To analyse the growth characteristics of the two cell lines in suspension conditions, both the CCL-61 and CHO-SA cell lines were harvested at day 3 of culture (growing in their standard conditions). Both cell lines were washed in PBS and then seeded into standard suspension conditions and batch cultured for 8 days. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser.

3.2.3 Cell morphology studies

For the cell morphology studies the two cell lines were seeded at a concentration of 5,000 cells/ cm^2 in T75 flasks in standard adherent conditions for approximately 4 days (or until the flasks became fully confluent). For the adherence development studies the two cell lines were seeded at a concentration of 5,000 cells/ cm^2 in 6 well plates in standard adherent conditions and images taken at different time intervals up to 8 hours in culture. For the confluence studies the two cell lines were seeded at a concentration of 5,000 cells/ cm^2 in T75 flasks in standard adherent conditions. The confluency of each flask was monitored until one of the flasks reached 100% confluent.

3.3 Results

3.3.1 Characterisation results of the CHO-K1 (CCL-61) and CHO-SA cell lines growing in adherent conditions

The viability and the viable cell density of the two cell lines growing in adherent conditions was measured every 24 hours using a Vi-Cell Cell Viability Analyser. In adherent conditions both cell lines maintain a high viability throughout the duration of culture. The CCL-61 cell line remains at a viability of >95% through culture, while the CHO-SA cell only drops below 95% viability after the 192 hour time-point (Figure 3.2a). The viable cell density of the two cell lines growing in adherent conditions is shown in figure 3.2b. The two cell lines display a typical exponential growth phase between 48 and 96 hours in culture. Both cell lines reach a maximum viable cell density at 96 hours, with the CCL-61 cell line reaching a higher maximum density of 0.43 x 10^6 cells/ cm^2 compared to 0.35 x 10^6 cells/ cm^2 for the CHO-SA cell line (Table 3.2). Post 96 hours in culture a short stationary phase is observed before both cell lines enter death phase.

The cell specific growth rate of the cell lines growing in adherent conditions was calculated from the viable cell density. Both the cell lines display an early peak in cell specific growth rate (μ) at 48 hours, with a μ_{max} of 0.057 h^{-1} for the CCL-61 cell line and a μ_{max} of 0.055 h^{-1} for the CHO-SA cell line (Figure 3.3a). Between 48 and 72 hours the CCL-61 cell line maintains a μ of >0.05 h^{-1}, compared to the CHO-SA cell line which shows a decrease in μ from 48 hours onwards. Between 48 and 144 hours in culture the CCL-61 cell line displays an average decline in proliferation rate of 0.015 h^{-1} and the CHO-SA cell line an average decline in proliferation rate of 0.014 h^{-1}.

The integral of viable cell concentration (IVCC) of the two cell lines growing in adherent conditions is shown in figure 3.3b. The IVCC is calculated using the area under the curve of the viable cell density using the trapezoid rule as described in chapter 2. The CCL-61 cell line reached an IVCC of 0.047 x 10^9 (cell h) cm^2 at 192 hours in culture compared to the CHO-SA cell line which reached an IVCC 0.041 x 10^9 (cell h) cm^2 at the same time point.
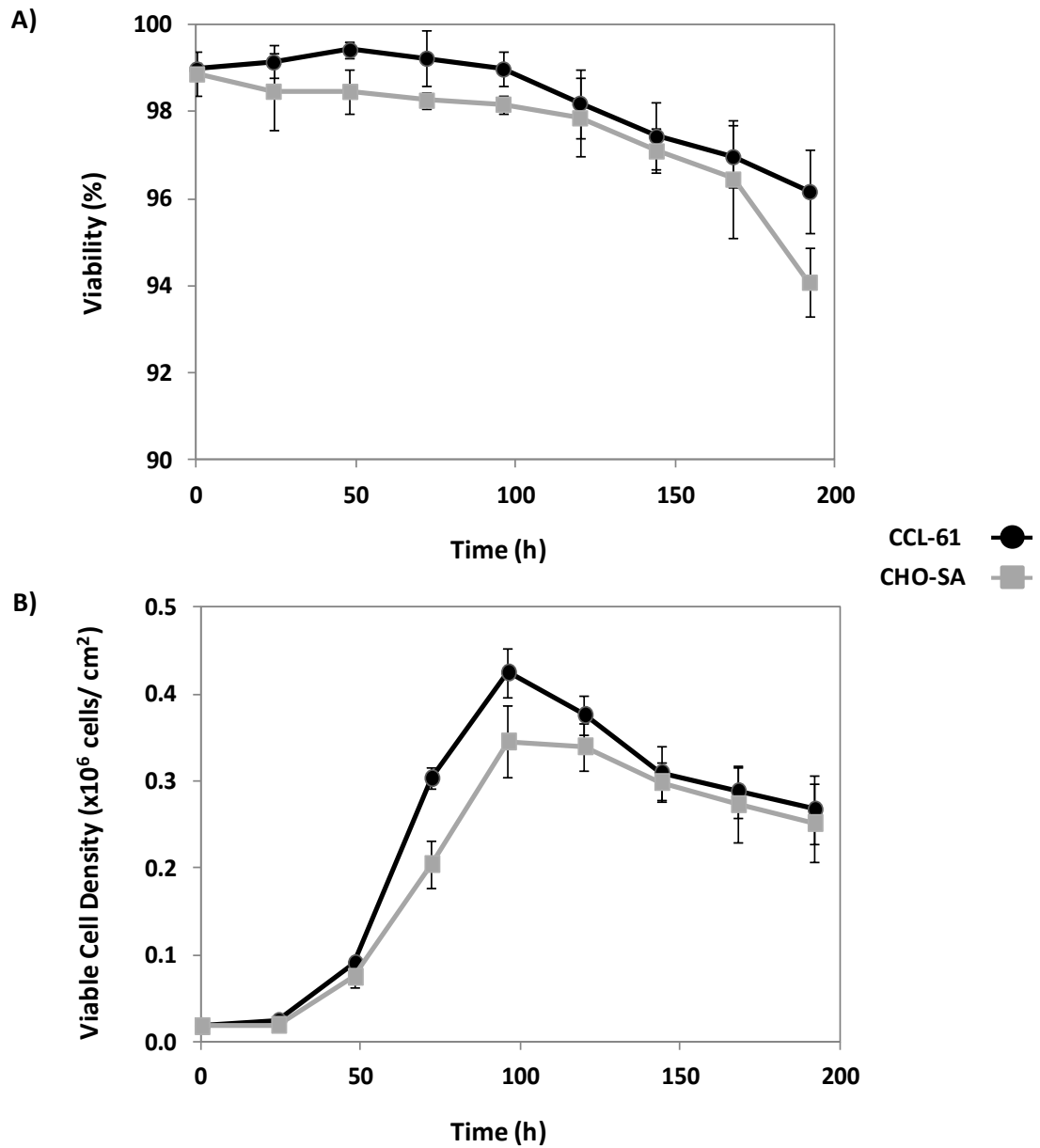
Figure 3.2: Growth characterisation results of the CHO-K1 (CCL-61) and CHO-SA cell lines in adherent culture conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in T75 flasks for 196 hours in adherent conditions using F12 media with 10% FBS. A) The viability of the CCL-61 and CHO-SA cell lines through culture. B) The viable cell density of the CCL-61 and CHO-SA cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.
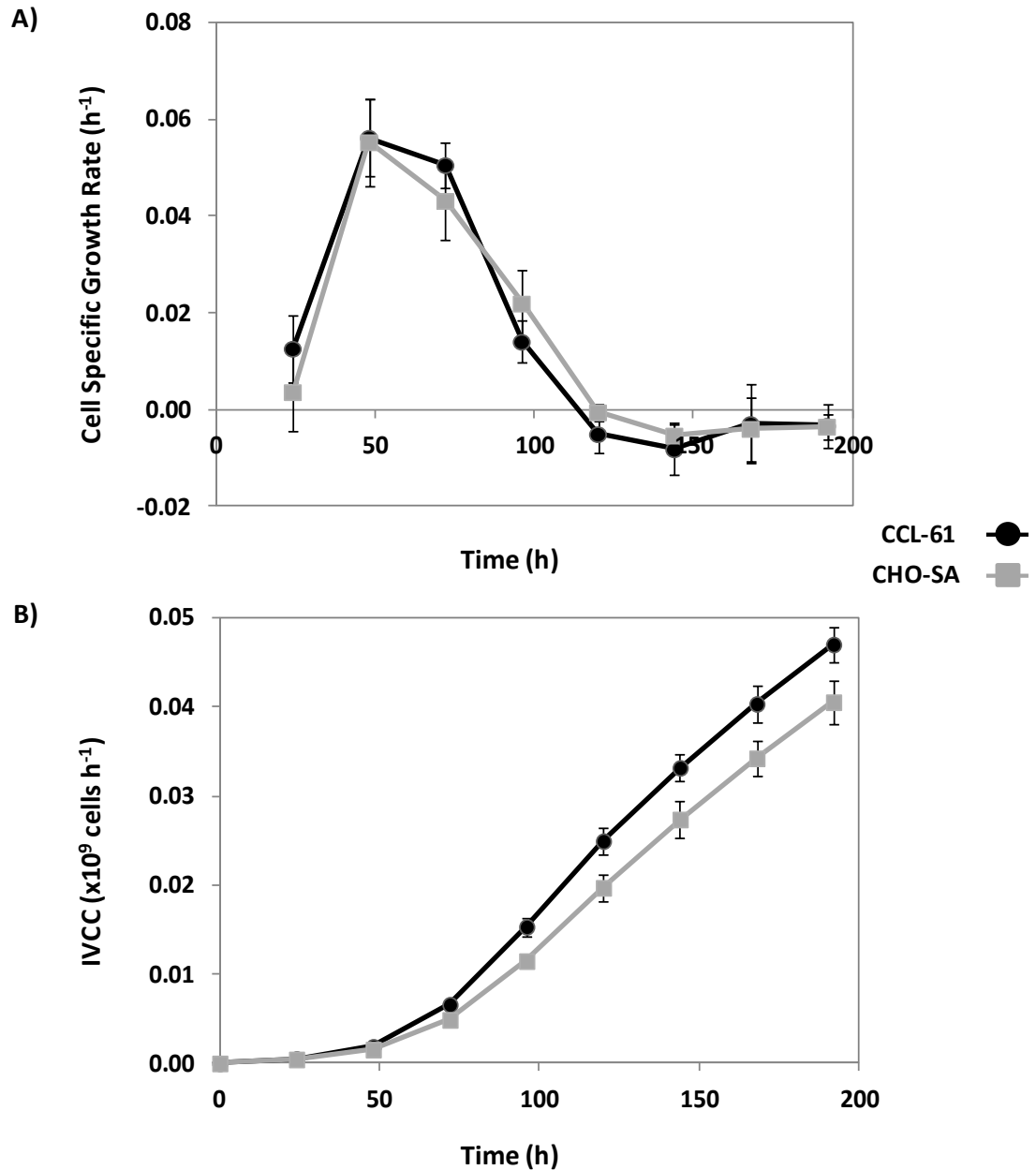
Figure 3.3: CHO-K1 (CCL-61) and CHO-SA growth rates in adherent culture conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in T75 flasks for 196 hours in adherent conditions using F12 media with 10% FBS. A) The cell specific growth rate (μ) of the CCL-61 and CHO-SA cell lines through culture. B) The integral of viable cell concentration (IVCC) of the CCL-61 and CHO-SA cell lines through culture. The cell specific growth rate and IVCC were calculated using the viable cell density. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

The volumes of the two lines through adherent culture were calculated using the diameter readings given from the Vi-Cell Cell Viability Analyser. Post-seeding both cell lines show an initial increase in volume up to 24 hours in culture with the CCL-61 cell line reaching a higher volume of 1703 μm^3 compared to 1497 μm^3 for the CHO-SA cell line. Between 24 hours and 72 hours in culture the CCL-61 cell line volume plateaus before entering a decline phase after 96 hours in culture. Post 24 hours the CHO-SA cell line shows a brief decline between 24 and 48 hours before reaching a maximum volume of 1624 μm^3 at 96 hours in culture. Post 96 hours the CCL-61 cell line displays a similar rate of decline as that of the CHO-SA cell line as both cell lines enter stationary growth phase. The average volume through culture for the CHO-SA cell line of 1339 μm^3 is lower than that of the CCL-61 cell line, 1427 μm^3.

The cellular protein content of the two cell lines growing in adherent culture conditions is shown in figure 3.4b. Both the CCL-61 and CHO-SA cell display a similar trend in protein content in that a slight initial increase is seen between 0 and 48 hours in culture. Post 48 hours in culture a downward trend in protein content is observed in both cell lines. In batch culture conditions, this may be a consequence of the decreased growth that is observed after 48 hours in culture and the depletion of nutrients in the growth media.
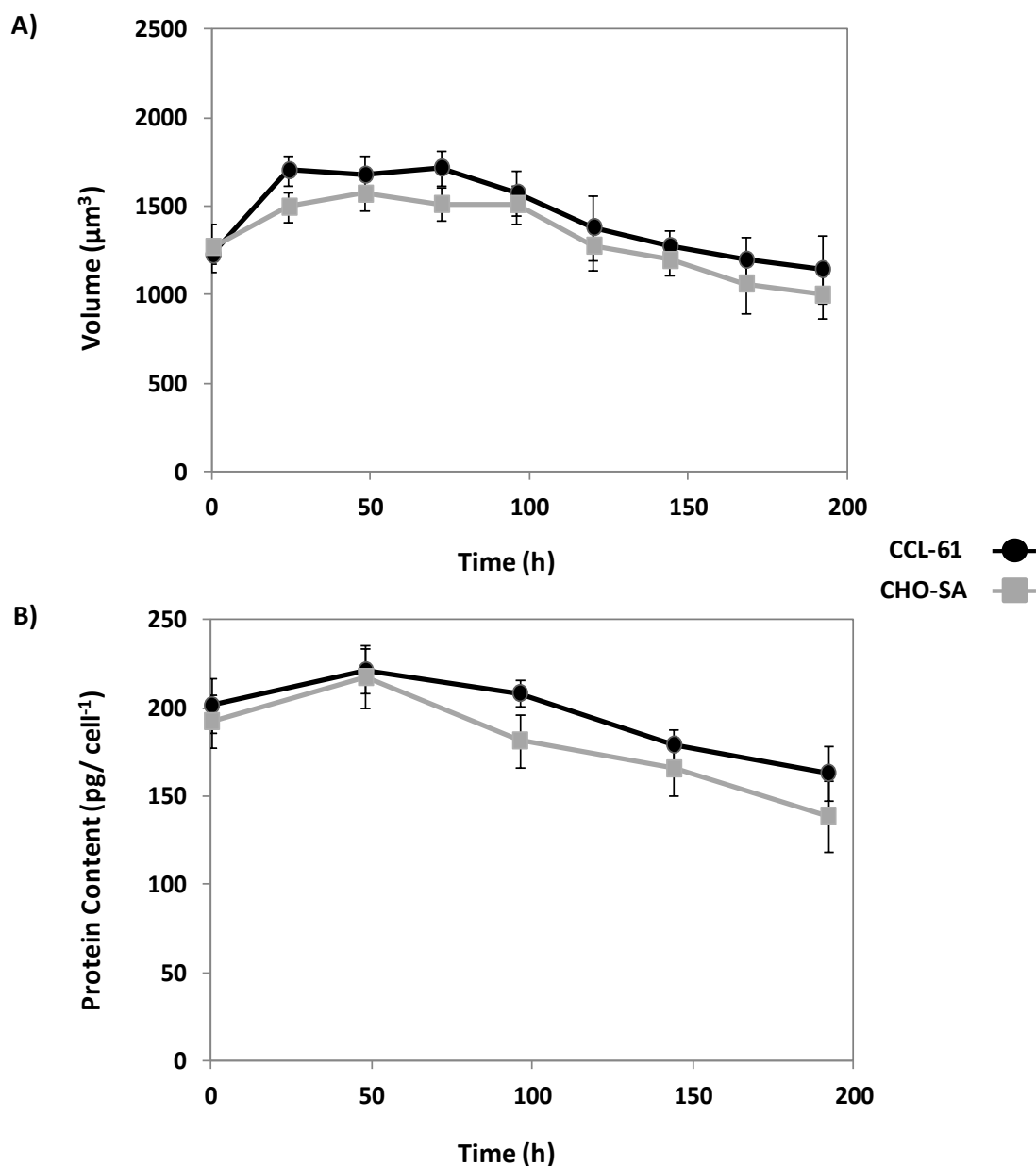
Figure 3.4: Cell volume and protein content results of the CHO-K1 (CCL-61) and CHO-SA cell lines in adherent culture conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in T75 flasks for 196 hours in adherent conditions using F12 media with 10% FBS. A) Cell volume of the CCL-61 and CHO-SA cell lines through culture. B) Cellular protein content of the CCL-61 and CHO-SA cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

3.3.2 Characterisation results of the CHO-K1 (CCL-61) and CHO-SA cell lines growing in suspension conditions

The viability and the viable cell density of the CCL-61 and CHO-SA cell lines growing in suspension conditions in PSM with or without 10 mg/L insulin in the cell culture medium was measured every 24 hours using a Vi-Cell Cell Viability Analyser. The viability of the CCL-61 and CHO-SA cell lines growing in suspension conditions is shown in figure 3.5a. There is significant variation in cell viability between the CCL-61 + Ins cells and CCL-61 – Ins cells through batch culture. The CCL-61 + Ins cells maintain a viability of >90% throughout the duration of culture. The CCL-61 – Ins cells maintain a viability of >90% until 72 hours in culture. Between 72 and 192 hours in culture a sharp decline in viability is observed, from 91% viability at 72 hours to 51% at 192 hours. The CHO-SA + Ins cells maintain a viability of >95% up to 120 hours in culture. Between 120 and 192 hours in culture a significant decline in viability is observed from 95% at 120 hours in culture to 64% viability at 192 hours. The CHO-SA – Ins cells maintain a viability of >90% up to 144 hours in culture before a slight decline in viability is observed down to 74% at 192 hours in culture.

In terms of viable cell density (figure 3.5b) there is significant variation between the CCL-61 and CHO-SA cell lines growing in suspension conditions with and without insulin in the growth media. Neither the CCL-61 + Ins or CCL-61 – Ins cells exhibit a "typical" growth profile. The CCL-61 – Ins cells do not display an exponential growth phase, with the maximum viable cell density obtained of 0.24 x 10^6 cells mL^{-1} at 24 hours in culture. Post 24 hours in culture, a slow decline in viable cell density is observed until the end of culture. In a similar manner the CCL-61 + Ins cells do not exhibit a typical exponential growth phase, but rather an extended lag phase with a slow increase in viable cell density up to 96 hours in culture, where a maximum viable cell density of 0.86 x 10^6 cells mL^{-1} is reached. Post 96 hours a slow decline in viable cell density is observed until the end of culture. Neither of the CCL-61 cell lines growing in suspension exhibit the typical growth pattern of exponential phase, stationary phase, death phase. By comparison, the CHO-SA + Ins cells display a more "typical" growth profile.

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Between 24 and 48 hours in culture the CHO-SA + Ins cells enter exponential growth phase which continues until 120 hours in culture, where a maximum viable cell density of 5.30 x 10^6 cells mL^{-1} is reached. After 120 hours in culture the CHO-SA + Ins cell line begins to enter death phase with a significant decline viable cell density observed until the end of culture. In a similar manner to the CCL-61 + Ins cells, the CHO-SA - Ins cells do not exhibit a typical exponential growth phase. An extended lag-phase with a slow increase in viable cell density is observed up until 144 hours in culture, where a maximum viable cell density of 1.70 x 10^6 cells mL^{-1} is reached. After 144 hours in culture a slight decline is viable cell density is observed until the end of culture.
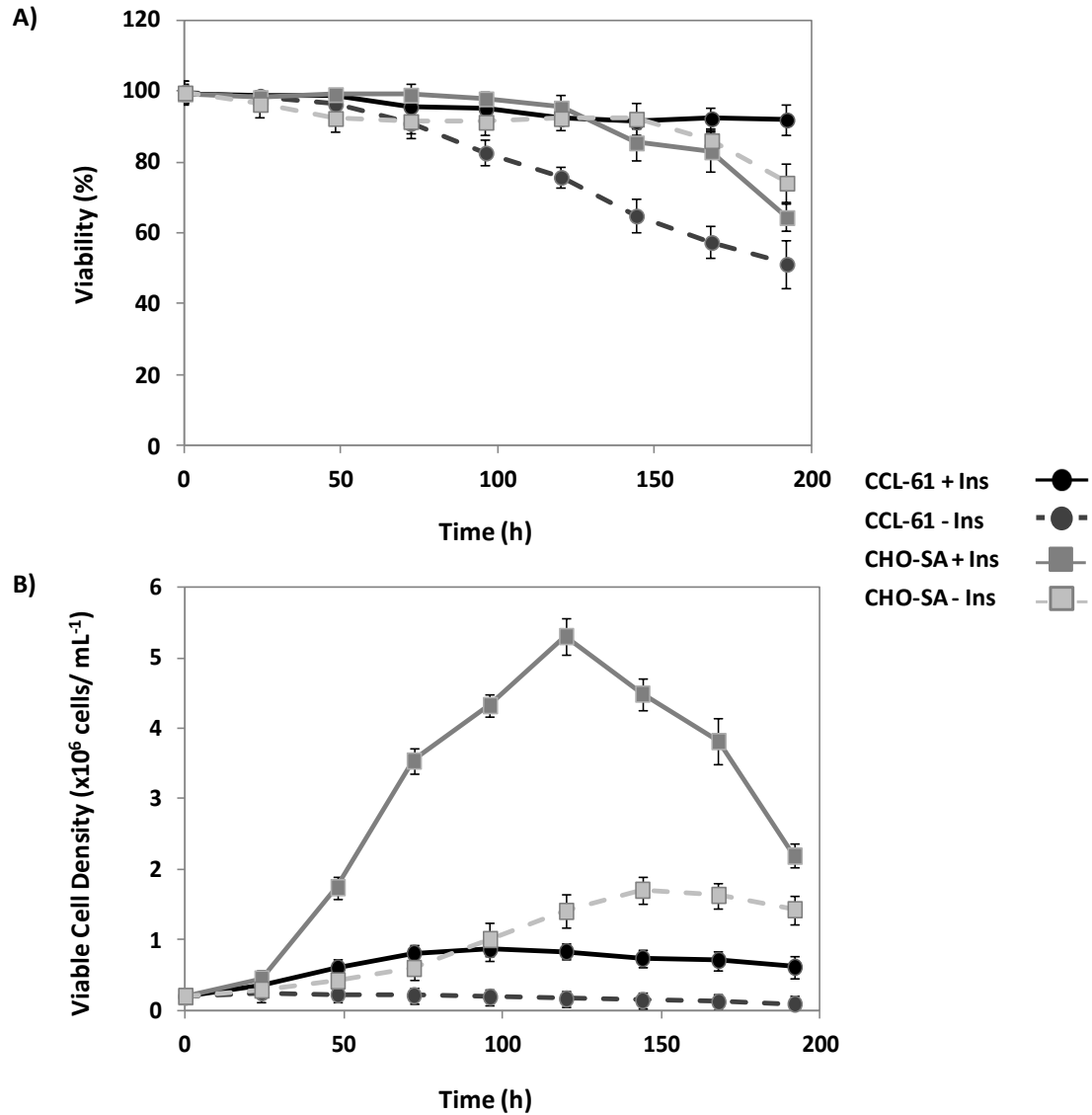
Figure 3.5: Growth characterisation results of the CHO-K1 (CCL-61) and CHO-SA cell lines in suspension culture conditions with and without insulin in the growth media. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in 500 mL Erlenmeyer flasks for 196 hours in suspension conditions using PSM + or – 10 mg/L insulin. A) The viability of the CCL-61 and CHO-SA cell lines through culture. B) The viable cell density of the CCL-61 and CHO-SA cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

The cell specific growth rate of the cell lines growing in suspension conditions was calculated from the viable cell density, which is shown in figure 3.6a. The proliferation rate of the CCL-61 + Ins cells reached a μ_{max} of 0.024 h^{-1} at 24 hours in culture. Post 24 hours, a subsequent decline in proliferation rate was observed until the end of culture. The CCL-61 – Ins cells reached a μ_{max} of 0.011 h^{-1}, also at 24 hours in culture. Post 24 hours in culture a negative proliferation was observed until the end of culture. The CHO-SA + Ins cells displayed an early peak in proliferation rate at 48 hours in culture, with a μ_{max} of 0.057 h^{-1}. After 48 hours in culture a subsequent decline in μ was observed until the end of culture with an average decline in proliferation rate of 0.013 h^{-1}. The CHO-SA – Ins cells maintained a steady proliferation rate of approximately 0.015 h^{-1} between 24 and 72 hours in culture. A slight increase in proliferation rate was observed when the CHO-SA – Ins cells reaches a μ_{max} of 0.022 h^{-1} at 96 hours in culture. After 96 hours an average decline in proliferation rate of 0.007 h^{-1} was observed until the end of culture.

The integral of viable cell concentration (IVCC) of the CCL-61 and CHO-SA cell lines growing in suspension conditions is shown in figure 3.6b. The IVCC is calculated using the area under the curve of the viable cell density using the trapezoid rule as described in chapter 2. The CCL-61 + Ins cells reaches a maximum IVCC of 0.13 x 10^9 cells h^{-1} at 192 hours in culture compared to the CCL-61 – Ins cells which only reached a maximum IVCC of 0.04 x 10^9 cells h^{-1} at the same time point. The CHO-SA + Ins cells reached a maximum IVCC of 0.59 x 10^9 cells h^{-1} at 192 hours in culture which is significantly higher than the IVCC of the CHO-SA – Ins cells at the same time point of 0.18 x 10^9 cells h^{-1}.
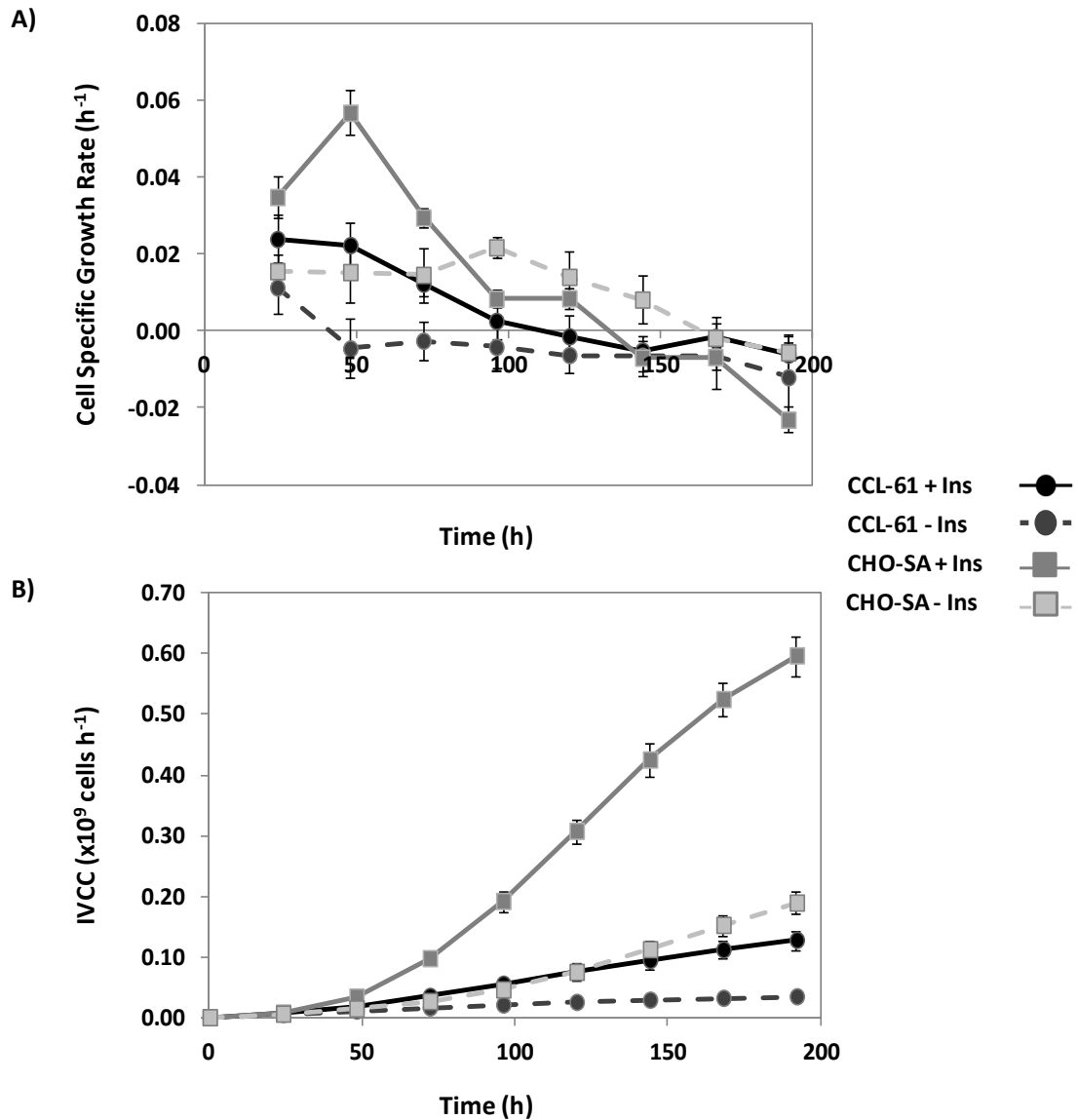
Figure 3.6: CHO-K1 (CCL-61) and CHO-SA growth rates in suspension culture conditions with and without insulin in the growth media. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in 500 mL Erlenmeyer flasks for 196 hours in suspension conditions using PSM + or – 10 mg/L insulin. A) The cell specific growth rate (μ) of the CCL-61 and CHO-SA cell lines through culture. B) The integral of viable cell concentration (IVCC) of the CCL-61 and CHO-SA cell lines through culture. The cell specific growth rate and IVCC were calculated using the viable cell density. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

The cell volumes of the CCL-61 and CHO-SA cell lines growing in suspension with and without insulin in the growth media are shown in figure 3.7a. The volumes were calculated using the diameter readings given from the Vi-Cell Cell Viability Analyser. There is significant variation between the cell lines in terms of cell volume through culture. Both the CCL-61 + Ins and CHO-SA + Ins cell lines show a significant increase in cell volume up to 24 hours in culture, with the CCL-61 + Ins reaching a maximum volume of 2003 μm^3 and the CHO-SA + Ins cell line a maximum volume of 2150 μm^3. Post 24 hours in culture the CHO-SA + Ins cell line displays a significant decline in volume up until 144 hours in culture with a final cell volume of 648 μm^3 at 192 hours. The CCL-61 + Ins cell line also show a decline in cell volume from 24 to 144 hours in culture before the cell volume plateaus at approximately 1250 μm^3. By comparison the CCL-61 – Ins cell line declines in cell volume immediately post-seeding. Over the duration of culture the CCL-61 – Ins volume decreases from 1564 μm^3 post-seeding to 649 μm^3 at the end of culture. The CHO-SA - Ins cell line shows a slight increase in volume up to 24 hours in culture, reaching a maximum volume of 1492 μm^3. After 24 hours in culture the volume of the CHO-SA declines to about 1100 μm^3 at 72 hours and remains approximately around that volume until the end of culture.

Figure 3.7b shows the cellular protein content of the CCL-61 and CHO-SA cell lines growing in suspension. In a similar manner to the cell volume both the CCL-61 + Ins and CHO-SA + Ins cell lines show an initial increase in protein content between 0 and 48 hours in culture before declining in protein content post 48 hours until the end of culture. The maximum protein content of the CCL-61 + Ins cell line was 271.31 pg cell[-1] at 48 hours, which was similar to that of the CHO-SA + Ins cell line of 263.62 pg cell[-1]. The CHO-SA – Ins cell line also shows a similar trend to the two + Ins cell lines with an initial increase in protein content up to a maximum of 219.20 pg cell[-1] at 48 hours in culture, followed by a decline up until the end of culture. The CCL-61 – Ins cell line shows a decline in protein content from initial culture seeding until the very end of culture. At 0 hours the CCL-61 – Ins cell line has a maximum protein content of 199.29 pg cell[-1] which declines throughout culture to a final protein content of 133.04 pg cell[-1] at 192 hours.
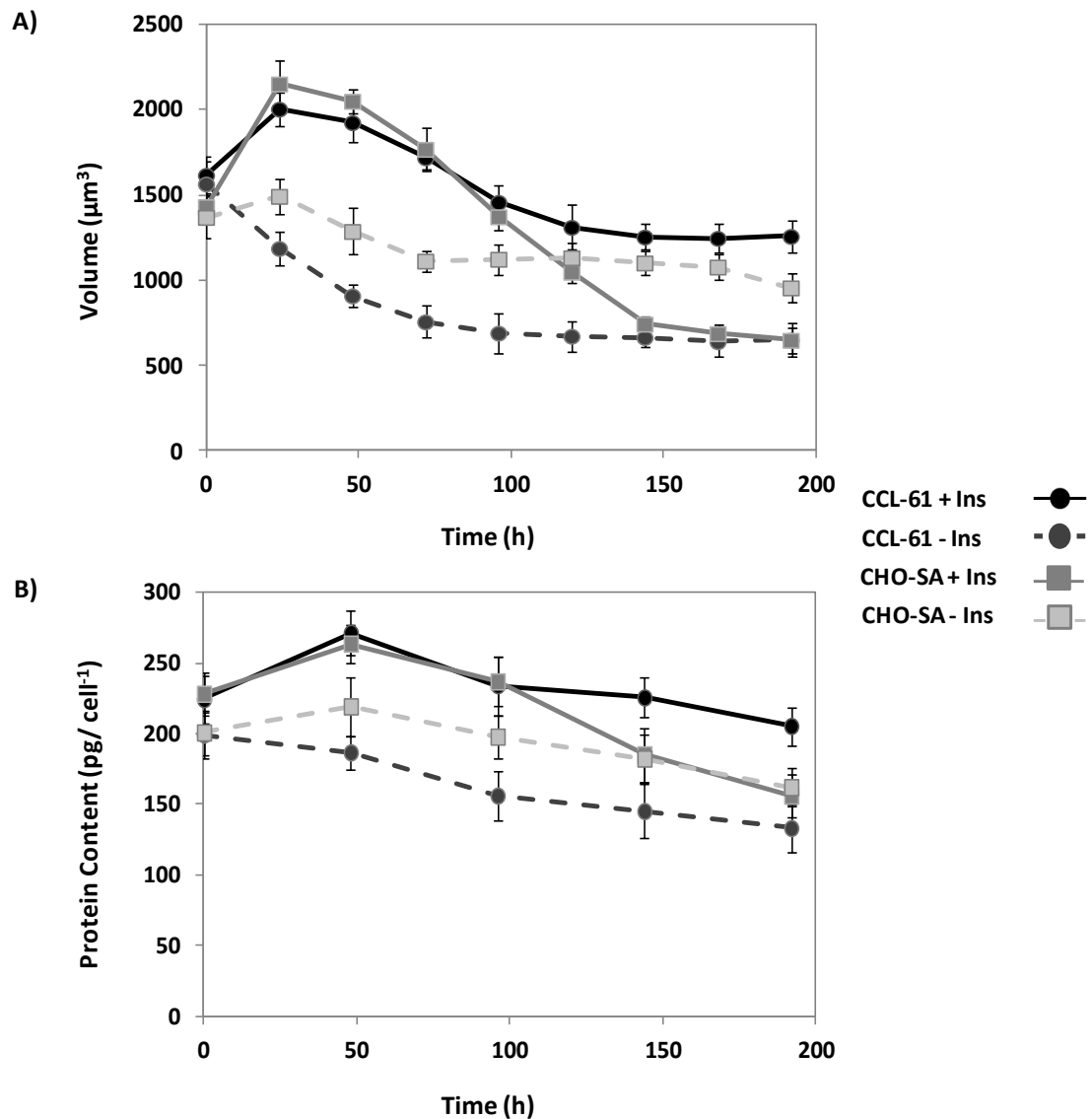
Figure 3.7: Cell volume and protein content results of the CHO-K1 (CCL-61) and CHO-SA cell lines in suspension culture conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in 500 mL Erlenmeyer flasks for 196 hours in suspension conditions using PSM + or – 10 mg/L insulin. A) Cell volume of the CCL-61 and CHO-SA cell lines through culture. B) Cellular protein content of the CCL-61 and CHO-SA cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

A)

Cell Line	Max. viable cell density (x 10^6 cells cm²) (±S.D.)	Max. µ (±S.D.)	IVCC (x10^9 cells h^{-1}/ cm²) (±S.D.)
CCL-61	0.43 (0.03)	0.057 (0.008)	0.047 (0.002)
CHO-SA	0.35 (0.04)	0.055 (0.009)	0.041 (0.003)

B)

Cell Line	Max. viable cell density (x 10^6 cells mL^{-1}) (±S.D.)	Max. µ (h^{-1}) (±S.D.)	IVCC (x10^9 cells h^{-1}) (±S.D.)
CCL-61 + Ins	0.86 (0.09)	0.024 (0.005)	0.13 (0.013)
CCL-61 - Ins	0.24 (0.02)	0.011 (0.005)	0.04 (0.001)
CHO-SA + Ins	5.31 (0.19)	0.057 (0.004)	0.59 (0.023)
CHO-SA - Ins	1.71 (0.16)	0.022 (0.001)	0.18 (0.009)

Table 3.2: Maximum viable cell density, maximum µ and IVCC of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. A) Results of the CCL-61 and CHO-SA cell lines in adherent conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in T75 flasks for 196 hours in adherent conditions using F12 media with 10% FBS. B) Results of the CCL-61 and CHO-SA cell lines in suspension conditions. The parental adherent cell line CHO-K1 (CCL-61) and the directly adapted suspension cell line CHO-SA were batch cultured in 500 mL Erlenmeyer flasks for 196 hours in suspension conditions using PSM + or – 10 mg/L insulin. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

3.3.3 Analysis of the cell morphology, development of adherence and confluence of the CHO-K1 (CCL-61) and CHO-SA cell lines growing in adherent conditions

Further analysis of the CCL-61 and CHO-SA cell lines growing in adherent conditions was conducted, focusing on the visual appearance of cells in culture and how quickly they adapt/ re-adapt to growing in adherent conditions. The CCL-61 is typically an adherent cell line that is used to growing in standard adherent conditions compared to the CHO-SA cell line that has been adapted to grow in suspension conditions. The aim of this experiment was to investigate whether the CHO-SA cell line behaved like the CCL-61 cell line (displayed a reversion phenotype) when it was placed back into standard adherent conditions.

The first parameter that was analysed was the morphology of the two cell lines when placed into adherent conditions. The second was the time duration that it took for each cell line to become adherent. Finally the confluence (the coverage of the well or flask by the cells) was analysed at day four in culture.

Figure 3.8 shows the difference in cell morphology between the CCL-61 and CHO-SA cell lines in standard adherent conditions. In the image in figure 3.8a a zoomed in section of the cells in culture shows how the CCL-61 cells display a typical elongated fibroblast morphology with the presence of definite pseudopodia (Kaesberg et al., 1989). Figure 3.8b shows how a proportion of the CHO-SA cells in adherent culture take on a typically adherent morphology but a small group of cells (as circled in the zoomed image) remain spheroid in shape, as though they are still in suspension culture conditions.

In terms of adherence development, the images in figure 3.9 demonstrate how the CCL-61 cells become attached and begin to take on an adherent morphology after 1 hour in culture. The beginning of pseudopodia formation in the CCL-61 cells can be seen after 30 minutes in culture even though at this stage the majority of cells remain spheroid in shape. By 1 hour in culture, the cells are

beginning to take on more of a stretched-out adherent shape. After 4 hours in culture, the majority of the cells have taken on a fully stretched-out adherent morphology and are fully adhered to the extracellular matrix. By contrast, only a small proportion of the CHO-SA cells had become adherent by 1 hour in culture and displayed the beginnings of pseudopodia formation. By 4 hours in culture, a greater proportion of cells had become adherent and taken on a more stretched-out adherent morphology, although some cells at this stage had still retained a more spheroid suspension morphology. After 8 hours post seeding the majority of the CHO-SA cells had definite pseudopodia formation and had taken a more "spread-out" adherent morphology.

Figure 3.10 shows the levels of confluence reached by the respective cell lines after 4 days in culture. After 4 days in culture the CCL-61 cells had become 100% fully confluent. By comparison the CHO-SA cells growing in adherent conditions were not fully confluent by day 4 in culture. As shown in figure 3.10b there are some small gaps present where the CHO-SA cells have not yet covered the well surface. This difference in well confluence is reflected in the lower maximum viable cell density of the CHO-SA cell in adherent conditions compared to the CCL-61 cell line at day 4 in culture.

A) CCL-61
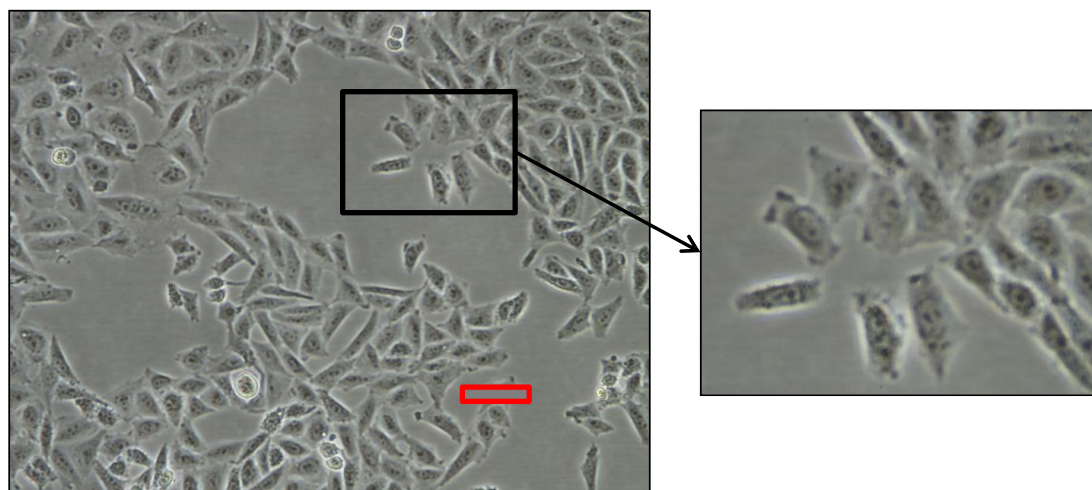
B) CHO-SA
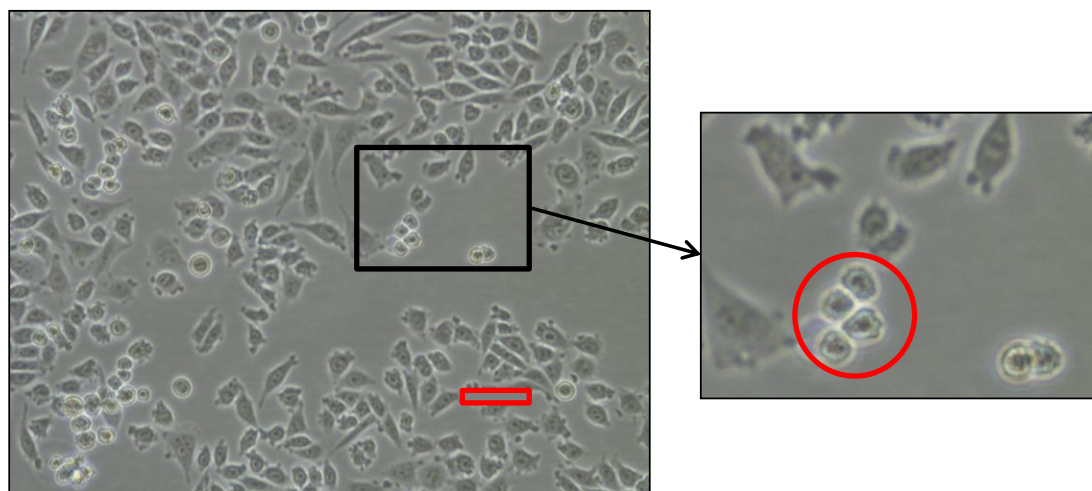
Figure 3.8: Cell morphology of the CCL-61 and CHO-SA cell lines growing in adherent conditions after 3 days in culture. The CCL-61 and CHO-SA cell lines were cultured in T75 flasks in standard adherent conditions. Every 24 hours post-seeding, images were taken using a Nikon Eclipse TS100 microscope with a 40x objective using the NIS Elements F2.30 software. A) The CCL-61 cell line growing in standard adherent conditions. B) The CHO-SA cell line growing in standard adherent conditions. Scale bars in both images are equal to 20 μm.
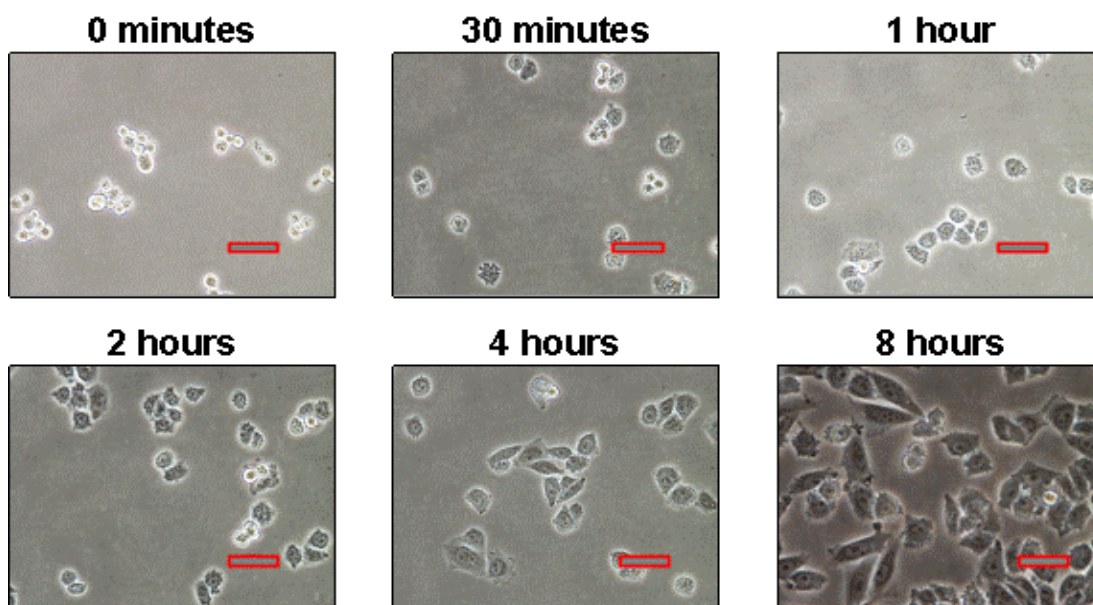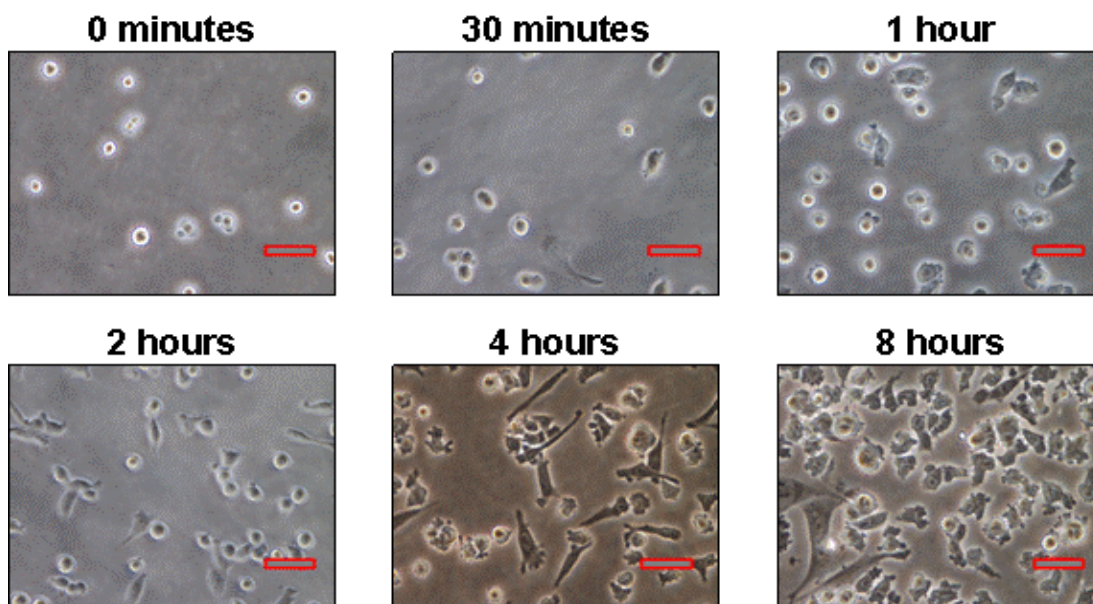
A) CCL-61

B) CHO-SA

Figure 3.9: Analysis of the difference in adherence development between the CCL-61 and CHO-SA cell lines growing in adherent conditions. The CCL-61 and CHO-SA cell lines were cultured in 6 well plates in standard adherent conditions. At the time-points shown above images were taken using a Nikon Eclipse TS100 microscope with a 80x objective using the NIS Elements F2.30 software. A) Adherence development images of the CCL-61 cell line growing in standard adherent conditions. B) Adherence development images of the CHO-SA cell line growing in standard adherent conditions. Scale bars in both images are equal to 20 µm.

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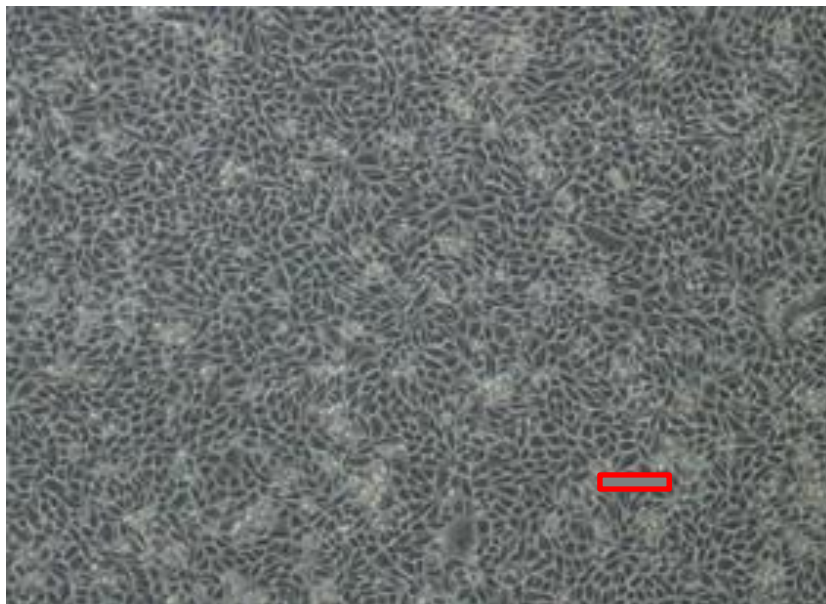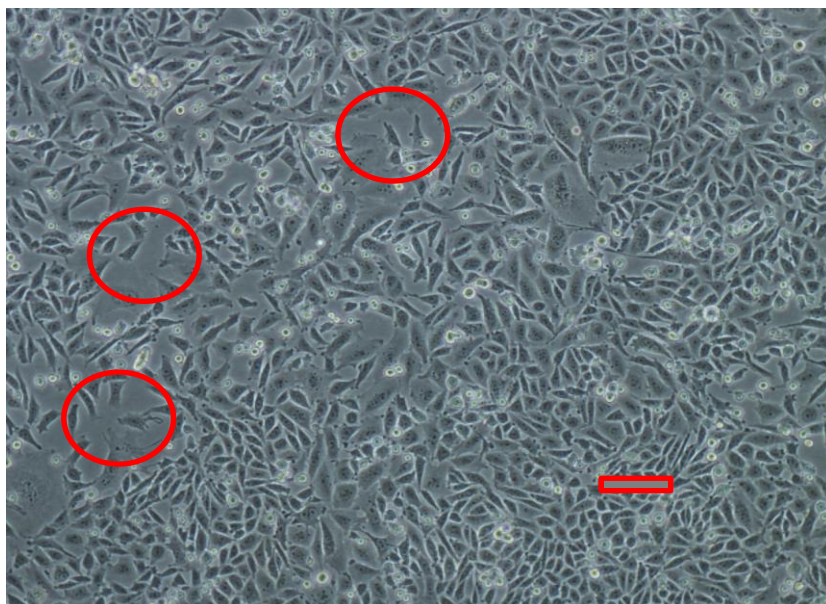
A) CCL-61

B) CHO-SA

Figure 3.10: Analysis of the difference in confluence levels between the CCL-61 and CHO-SA cell lines growing in adherent conditions after 4 days in culture. The CCL-61 and CHO-SA cell lines were cultured in T75 flasks in standard adherent conditions. Every 24 hours post-seeding images were taken using a Nikon Eclipse TS100 microscope with a 40x objective using the NIS Elements F2.30 software. A) The CCL-61 cell line growing in adherent conditions. B) The CHO-SA cell line growing in adherent conditions. Scale bars in both images are equal to 40 μm.

3.4 Discussion

The growth characterisation data of the CHO-K1 (CCL-61) and CHO-SA cell lines cultured in both adherent and suspension conditions shows how each cell line behaves in the specific culture conditions.

As shown in section 3.3.1, the suspension adapted CHO-SA cell line is able to revert and grow and proliferate as an adherent monolayer in adherent conditions in a similar manner to the non-adapted CHO-K1 (CCL-61) cell line. This would indicate that despite going through extensive rounds of suspension adaptation procedures, the CHO-SA cells maintain certain cell surface properties that enable the cells to re-adapt to grow in adherent conditions. The similarity in terms of the viable cell density and cell specific growth rate profiles between the CCL-61 and CHO-SA cells in adherent conditions, would also indicate that the various cellular responses triggered by cellular adhesion to the ECM are similar between the two cell lines. Over the years work by many groups has shown that a number of classical signalling pathways are known to be activated by the interaction of cells with ECM proteins in an integrin-mediated manner (Kumar, 1998). Therefore it would be sensible to hypothesise that when CHO-SA cells are put back into adherent growth conditions, they display a similar signalling activation profile to that of the CCL-61 cells.

In terms of suspension growth conditions, the cell characterisation results in section 3.3.2 highlights the differences between the CHO-K1 (CCL-61) and CHO-SA cell lines in terms of their ability to grow and proliferate in suspension conditions with and without an added growth factor. Both the CCL-61 and CHO-SA cell lines display a level of response to the inclusion of insulin in the growth medium. However only the CHO-SA cells appear able to utilise the insulin in the growth medium to reach a maxiumum viable cell density in batch culture, where the cells could be sub-cultured on a routine basis.

When non-suspension adapted CCL-61 cells are transferred to suspension growth conditions without insulin, the cells remain in an almost quiescent state, in that they do not appear to grow or proliferate. This could be due to the fact that in these conditions integrin-mediated binding to the ECM is abolished, the CCL-61 cells have no added growth factor to stimulate various signalling networks and therefore G1 phase cell cycle arrest occurs (Schwartz and Assoian, 2001). The drop in viability observed immediately after seeding the CCL-61 cells into suspension conditions would indicate that anoikis is occurring due to this surface detachment (Frisch and Francis, 1994; Frisch and Screaton, 2001; Grossman, 2002). CCL-61 cells transferred to suspension conditions with insulin included do display an increase in their maximum viable cell density compared to without insulin conditions, however this maximum VCD is still very low compared to normal suspension cultured cells. This would indicate that CCL-61 cells when growing in suspension are sensitive to insulin but are perhaps not able to utilise to as higher degree as suspension adapted CHO-SA cells. This desensitisation to insulin stimulation of un-adapted cells growing in suspension is similar to what Guilherme et al, observed in their studies. In their paper, when CHO-T cells were attached to a fibronectin matrix, this significantly increased the ability of insulin to enhance tyrosine phosphorylation of the insulin receptor as well as the p85 subunit of PI3K, compared to CHO-T cells in suspension (Guilherme et al., 1998).

By comparison when the suspension-adapted CHO-SA cells are cultured in suspension conditions with insulin included, a significant increase in the maximum VCD and IVCC is observed. This would indicate that these suspension cells are able to utilise the effects of insulin, such as the stimulation of cell proliferation and survival, to a greater degree than the un-adapted CCL-61 cells (Desbois-Mouthon et al., 2000). This ability to respond to growth factor stimulation without surface attachment, is perhaps one of the key underlying changes that occurs when CCL-61 cells are adapted over time to suspension conditions to give the suspension-adapted cell line CHO-SA.

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The importance of the ability to respond to insulin in the growth media, in the case of CHO-SA cells, is highlighted by the fact that when insulin is withdrawn from the culture media CHO-SA cells reach a significantly lower maximum VCD and IVVC.

Section 3.3.3 illustrated how the CHO-SA when put back into standard adherent conditions appeared to take on a "reversion phenotype" in that they become attached to the ECM and take on more spread-out adherent morphology, in a similar manner to the parental CCL-61 cells growing adherently.

In summary, the work done in this characterisation shows that both CCL-61 and CHO-SA cells are able to grow in standard adherent conditions. However, in suspension conditions, only suspension adapted CHO-SA cells cultured with insulin are able to grow and proliferate like a normal functioning suspension cell line would be expected to.

Chapter 4

Analysis of the activation status of the PI3K/Akt and Ras/MAPK signalling pathways in adherent and suspension conditions

Acknowledgements

The data, figures and text in the following chapter are the work of the candidate.

- I would like to thank Dr Robin Heller-Harrison and Pfizer research and development for providing the cell lines and cell culture media that were used in this chapter.

Chapter 4 Aims

In this chapter the activation status of the PI3K/Akt and Ras/MAPK signalling networks is analysed when both the CCL-61 and CHO-SA cells are growing in adherent and suspension conditions. This pathway activation data is then related to the growth characteristics of the model cell lines as shown in chapter 3.

4.1 Introduction

The PI3K/Akt and Ras/MAPK are two key signalling networks that link changes in the cell environment to the control of cell growth, proliferation and survival. When considering the adaptation of CHO cells to serum-free suspension conditions, it is likely that any changes that occur in signalling activation will be observed in at least one of these pathways as both are pathways are closely linked to the mechanisms of integrin-mediated adhesion signalling and insulin stimulated growth factor signalling.

4.1.1   The Ras/MAPK signalling pathway

The Ras-extracellular signal-related kinase/mitogen-activated protein kinase (MAPK) signalling cascade is a key signalling pathway that regulates various cellular processes such as cell growth, cell motility, cell proliferation and cell survival (Anjum and Blenis, 2008). The ERK/MAPK pathway consists of a family of proteins known as the 90kDa ribosomal S6 kinase proteins, which are a group of serine/threonine (Ser/Thr) kinases that act as downstream effectors. These downstream RSKs are directly activated by extracellular signal-related kinase-1 and -2 (ERK1/2) (Figure 4.1).

Cell surface receptor activation by a ligand (such as growth factors or serum) leads to receptor dimerisation and an increase in receptor Tyr-kinase autophosphorylation which in turn creates binding sites for the SH2 domains of the adaptor molecule growth factor receptor-bound protein-2 (GRB2). GRB2 is found associated with SOS (Son of Sevenless), a guanine nucleotide releasing
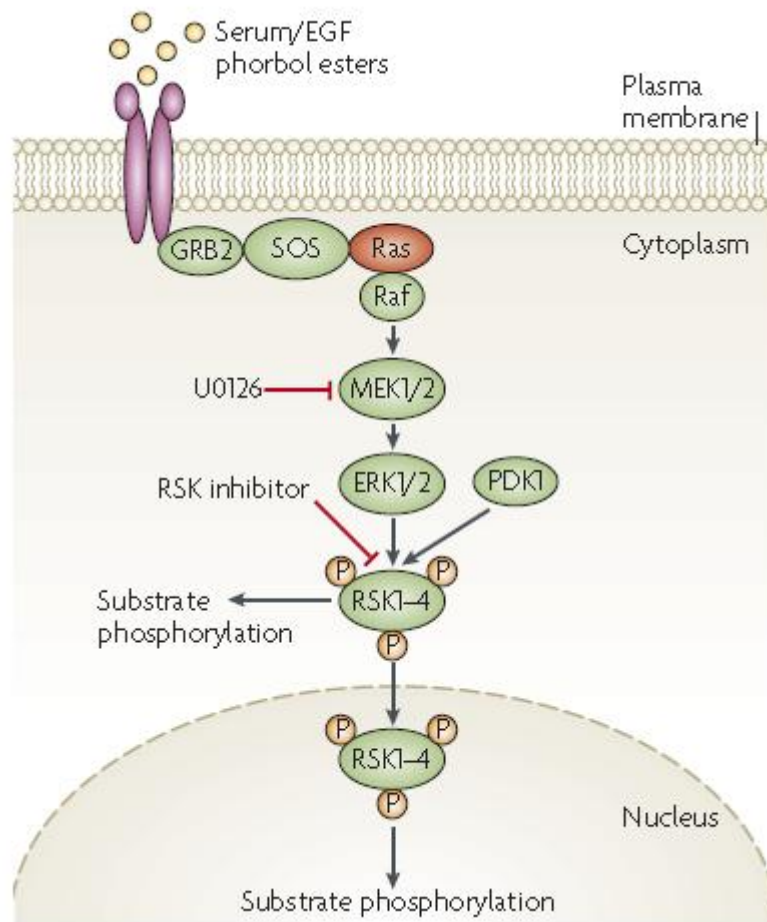
Figure 4.1: A schematic representation of RSK activation in a signalling pathway (Anjum and Blenis, 2008). As shown in the diagram above, the 90 kDa ribosomal S6 kinase (RSK) 1-4 becomes activated due to the upstream activation of the Ras/MAPK signalling cascade. The Ras/MAPK signalling cascade is activated by growth factors, polypeptide hormones, phorbol esters and a variety of chemokines and neurotransmitters.

protein through SH3 domains. This receptor-GRB2-SOS complex causes activation of the G protein Ras. Once Ras has been converted to the GTP-bound and active form it is able to cause the activation of the kinase Raf. Raf kinase activates by phosphorylation MAPK and then the extracellular signal-regulated kinase-1 (MEK1) and MEK2 (Anjum and Blenis, 2008). Activated MEK1 and MEK2 then phosphorylates ERK1 and ERK2 at specific Tyrosine and Threonine residues. RSKs 1-4 are then phosphorylated directly by ERK1/2 and also by 3"-phosphoinositide-dependent kinase-1 (PDK1). PDK1 is a constitutively active Serine/Threonine kinase. These activated RSKs function in various areas of the cell, remaining associated with the membrane, staying in the cytosol or being transported to the nucleus. This allows them to phosphorylate a number of substrates throughout the cell (Anjum and Blenis, 2008).

As well as RSKs, MAPKS also phosphorylate and activate other types of MAPK – activated protein kinases (MKs) such as stress activated (MSKs), the MAPK-interacting kinases (MNKs), MAPK-activated protein kinases 2 and 3 (MK-2 and3) and MAPK-activated protein kinase 5 (MK5) (Roux and Blenis, 2004) (Figure 4.2). RSKs have been shown to regulate gene expression by direct phosphorylation of multiple transcription factors that are involved in immediate-early (IE) gene expression. Examples of these transcription factors include serum response factor (SRF), cAMP response element-binding protein (CREB), Sis-inducible element (SIE), ER81, oestrogen receptor-β (ERβ), nuclear factor-κβ (NF-κβ), NFATc4 and TIF1A – a transcription initiation factor (Anjum and Blenis, 2008). As well as phosphorylating a number of transcription factors, RSKs also phosphorylate many immediate-early gene products such as c-Fos, c-Jun and Nur77. For the posttranslational modification of such immediate-early proteins, sustained RSK activation is required and this provides a dual mechanism of immediate-early gene control (Roux and Blenis, 2004).
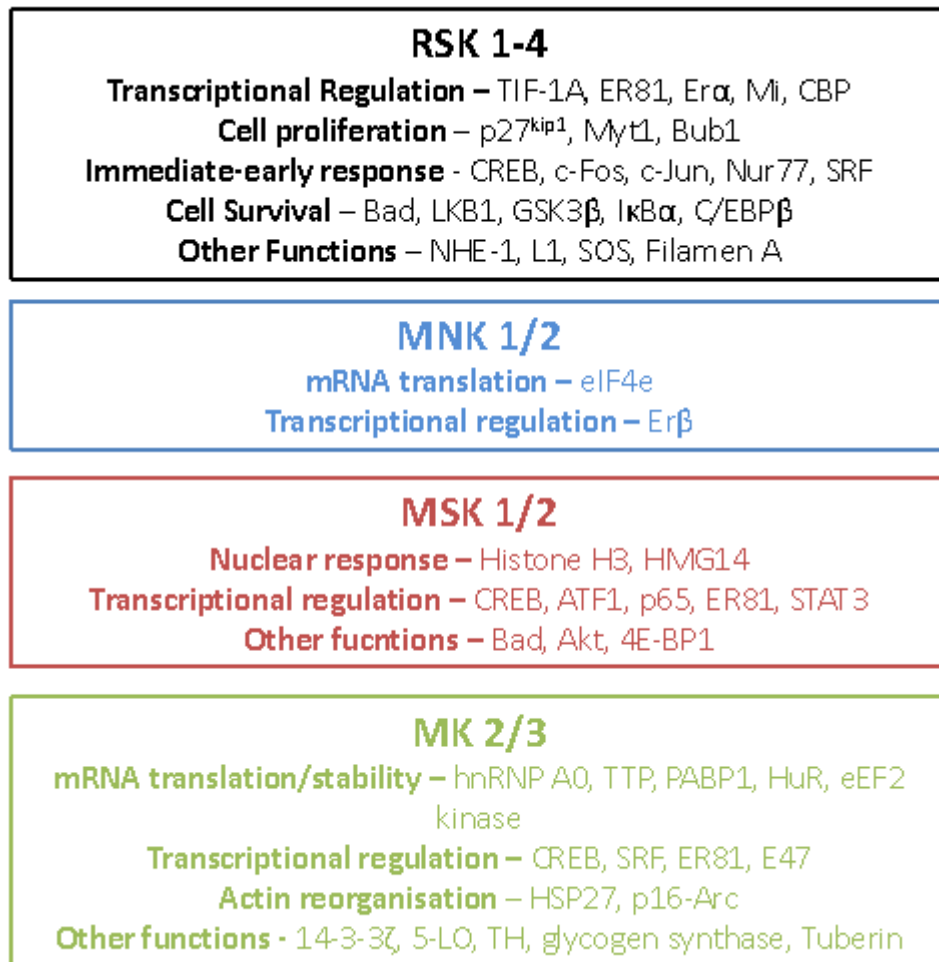
Figure 4.2: A list of downstream subtrates of the MAPK-activated protein kinases. When activated RSKs, MSKs, MNKs and MK2/3 phosphorylate many downstream effectors and therefore are involved in the regulation of numerous cellular processes such as the regulation of transcription, mRNA translation, cell survival and cell proliferation. Figure adapted from: (Roux and Blenis, 2004).

4.1.2 The PI3K/Akt pathway

The PI3K/Akt pathway has been shown to be vital for the control of a wide array of cellular processes such as cell proliferation and survival, apoptosis and cytoskeletal rearrangement (Vivanco and Sawyers, 2002). The PI3K/Akt pathway is also the main pathway that regulates translation. PI3Ks are heterodimeric lipid kinases that have two main subunits, one regulatory (p85) and one catalytic (p110). The PI3K cascade is activated by numerous hormones and growth factors such as insulin, insulin-like growth factors (IGFs), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), by integrin related signals, by different forms of cellular stress signal such as oxidative stress or cell swelling and by activation of Ras (Vivanco and Sawyers, 2002). Of particular interest in this case is the mechanism behind insulin and IGF stimulation of the PI3K signalling pathway.

Localisation of the receptor-PI3K complex at the plasma membrane allows the p110 subunit of PI3K to phosphorylate PIP_2 (PtdIns 4,5) to form PIP_3 (PtdIns 3,4,5). PIP_3 then recruits Akt and PDK1 to the plasma emebrane which bind to PIP_3 via their PH-(pleckstrin homology-domains. PKB/Akt is primarily activated at Ser473 by PDK2 and at Thr308 by PDK1. PDK1 is involved in the further activation of serum and glucocorticoid inducible kinase (SGK) and atypical protein kinase C (aPKC). PKB/Akt, aPKC and SGK further phosphorylate a wide variety of downstream signalling intermediates that are involved in the regulation of cellular growth, survival and proliferation such as FKHR, GSK3β, mTOR and p70S6K and the regulation of apoptosis including Bad, caspase 9, Iκβ, FKHR and Mdm2 (Alessi, 2001; Toker and Newton, 2000). PKB/Akt is involved in the further activation of the mammalian target of rapamycin (mTOR) signalling intermediate. mTOR is a kinase that stimulates the uptake of nutrients such as glucose, amino acids, cholesterol and iron. It plays a vital role in the regulation of the phosphorylation of p70S6K which is also activated by PDK1. mTOR further activates eIF4E-binding protein-1 and is therefore involved in the regulation of translation (Figure 4.3) (Vivanco and Sawyers, 2002).
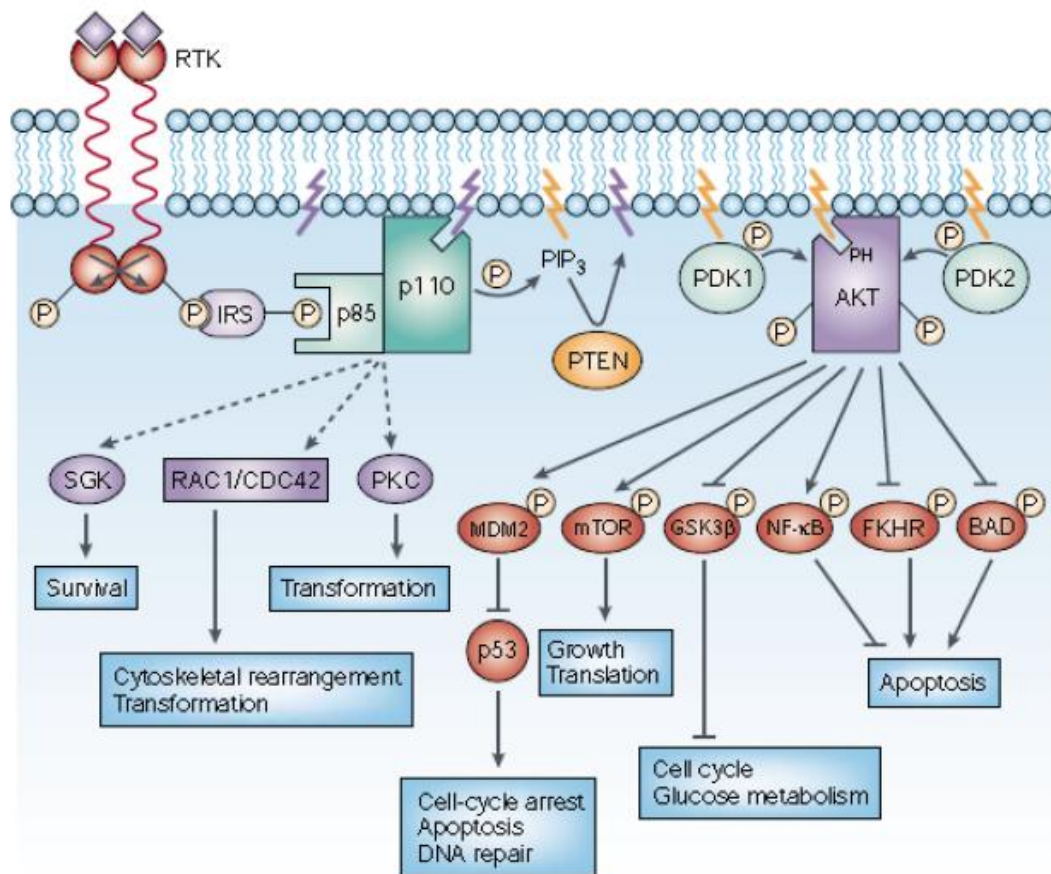
Figure 4.3: Regulation of the PI3K/Akt signalling pathway (Vivanco and Sawyers, 2002). Activation of PI3K occurs through the action of receptor tyrosine kinases and the assembly of the receptor-PI3K complex. PI3K activates the downstream signalling intermediate PDK1 which in turn activates Akt. Akt has a wide range of substrates as illustrated above. One of the substrates is the signalling intermediate mTOR which is a key regulator of cell growth and protein translation (Vivanco and Sawyers, 2002).

As well as activation of various intermediates of the PI3K pathway, there are also various negative effectors that can inhibit the activation of such intermediates. Examples include phospholipid phosphatase (PTEN) – which negatively regulates intracellular levels of PIP_3 and the Tuberous Sclerosis Complex proteins TSC1 (hamartin) and TSC2 (tuberin) which are inhibitors of the initiation factor 4E (4EBP1) (Kozma and Thomas, 2002).

4.1.3 Signalling pathways involved in translational control

In order to properly control cell growth and proliferation it is vital that the process of translation is tightly regulated. An increase in the rate of translation is essential for entry into and through G_1 phase of the cell cycle, while translation deregulation has been shown to cause aberrant growth and apoptosis (Gingras et al., 1999). Translation is stimulated by the MAPK and PI3K pathways by increasing activation of the rates of translation initiation and elongation as well as the stimulation of ribosome biogenesis (Anjum and Blenis, 2008). It has been shown in recent studies that the Ras-MAPK pathway via certain RSKs impinges on the PI3K-mTOR pathway at varying stages, also regulating translation (Figure 4.4).

Akt plays a vital role in regulating translation by phosphorylating and therefore inactivating tuberous sclerosis complex -1/2 (TSC1/2), which in turn negatively regulates the protein Rheb (Anjum and Blenis, 2008). The TSC1-TSC2 complex blocks the ability of TSC2 to act as a GTPase-activating protein (GAP) for Rheb. Rheb-GTP associates with mammalian target of rapamycin complex-1 (mTORC1) and then activates mTORC1 (Manning and Cantley, 2007). mTORC1 phosphorylates downstream targets such as the downstream translational effectors 70kDa ribosomal S6 kinase-1/2 (S6K 1/2) and 4E-binding protein-1 (4EBP1) (Anjum and Blenis, 2008; Fingar et al., 2004). The 90 kDa S6 kinase phosphorylates rpS6 which is a protein of the 40S ribosomal subunit and also eIF4B, eukaryotic translation initiation factor-4B, therefore promoting cap-dependent translation (Anjum and Blenis, 2008).
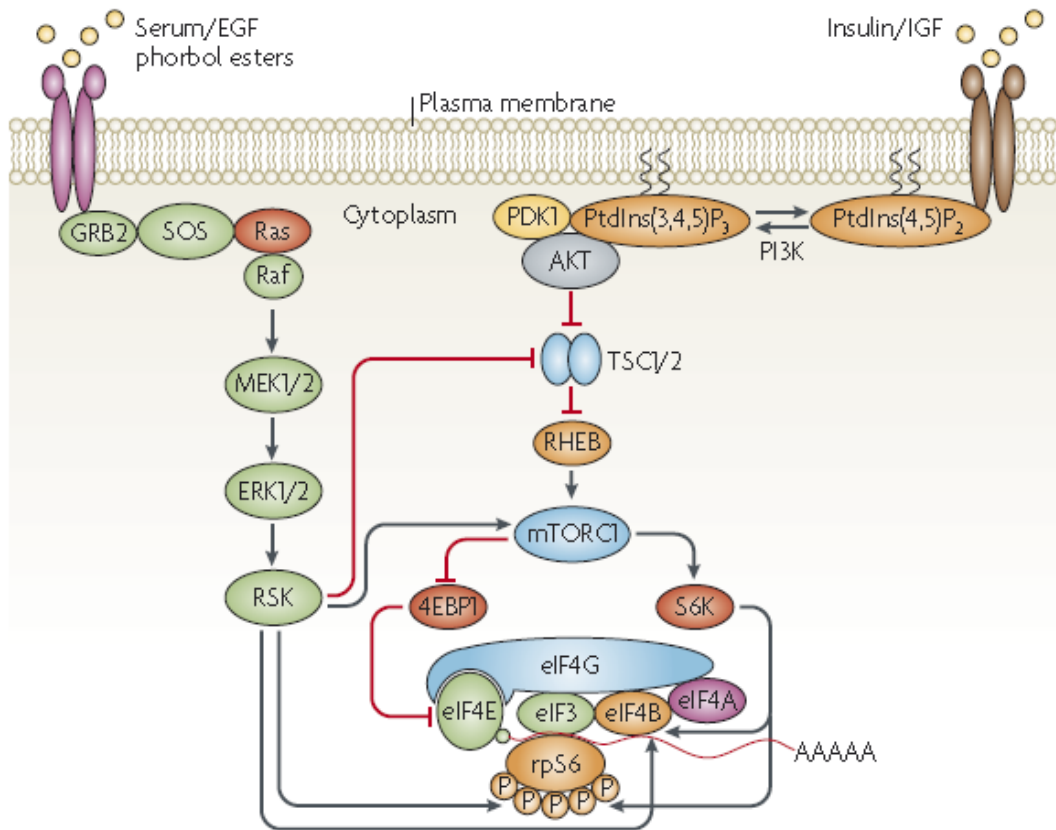
Figure 4.4: Regulation of translation by the Ras/MAPK and PI3K/Akt pathyways (Anjum and Blenis, 2008). The PI3K/Akt pathway is the main pathway that is involved in the regulation of translation. However, the Ras/MAPK pathway impinges on the PI3K/Akt at several points to provide another mechanism of activation. One of the main proteins involved in this pathway cross-talk is RSK which promotes mTOR signalling by inactivating TSC1/2.

The Ras-MAPK pathway impinges on the PI3K pathway at various stages to provide another route of translation regulation. Activated RSK phosphorylates TSC2, which results in the inhibition of the tumour suppressor function of the TSC 1/2 complex, thus promoting mTOR signalling and translation (Anjum and Blenis, 2008; Fingar et al., 2004). RSK also activates the eukaryotic translation initiation factor-4B (eIF4B) which in turn stimulates heterotrimeric eIF4F. eIF4F is formed by a complex of the cap binding protein eIF4E, eIF4G (which is a scaffolding protein) and a helicase protein eIF4A. eIF4B stimulates the activity of eIF4F by promoting the RNA-helicase activity of eIF4A (Rozen et al., 1990). The phosphorylation of eIF4B increases the interaction between eIF4B and eIF3 which increases the rate of translation (Holz et al., 2005).

4.2 Methodology

4.2.1 Harvesting adherent and suspension cells

For the western immunoblotting analysis CCL-61 and CHO-SA cells that were cultured in both adherent and suspension conditions were harvested at exactly 72 hours in culture (day 3). As described in detail in materials and methods section 2.2, approximately 5×10^6 cells were harvested from each flask and cells were lysed and proteins extracted using RIPA buffer.

4.2.2 Calculating protein content from cell extracts

As described in materials and methods section 2.2.4 the BCA total protein assay was used to calculate the total protein from every protein extraction performed. The results from the BCA assay were then used to calculate the amount of each extract to load into each well of the SDS-PAGE gel to give a loaded concentration of 25 μg of protein per well.

4.2.3 SDS-PAGE and western immunoblotting

All the western blots shown in this chapter were performed using the method given in materials and methods section 2.3. All western immunoblots that are shown in this chapter are representative of three repeated blots for each protein/phospho-protein of interest. As shown in section 4.3.1, all western immunoblots performed included a biotinylated protein ladder to confirm that the bands observed are approximately at the correct molecular weight for the target protein.

4.2.4 Primary antibodies used for western immunoblot analysis

The following antibodies were used in these experiments:

Antibodies used to analyse signalling intermediates in the PI3K/Akt pathway:

- PI3 Kinase p85 (19H8), Rabbit mAb, Cell Signaling Technology
- PI3 Kinase p110α (C73F8), Rabbit mAb, Cell Signaling Technology
- Phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199), Rabbit mAb, Cell Signaling Technology

- PTEN (138G6), Rabbit mAb, Cell Signaling Technology
- Phospho-PTEN (Ser380), Rabbit mAb, Cell Signaling Technology
- PDK1 (D37A7), Rabbit mAb, Cell Signaling Technology
- Phospho-PDK1 (Ser241) (C49H2), Rabbit mAb, Cell Signaling Technology
- Akt, (pan) (C67E7), Rabbit mAb, Cell Signaling Technology
- Phospho-Akt (Thr308) (C31E5E) Rabbit mAb, Cell Signaling Technology
- Phospho-Akt (Ser473) (193H12) Rabbit mAb, Cell Signaling Technology
- mTOR (7C10), Rabbit mAb, Cell Signaling Technology
- Phospho-mTOR (Ser2481), Rabbit Antibody, Cell Signaling Technology
- Phospho-mTOR (Ser2448) (D9C2), Rabbit mAb, Cell Signaling Technology

Antibodies used to analyse signalling intermediates in the Ras/MAPK pathway:

- c –Raf, Rabbit Antibody, Cell Signaling Technology
- Phospho-c-Raf (Ser338) (56A6), Rabbit mAb, Cell Signaling Technology
- Phospho-c-Raf (Ser259), Rabbit Antibody, Cell Signaling Technology
- Phospho-c-Raf (Ser289/296/301), Rabbit Antibody, Cell Signaling Technology
- MEK 1/2 (D1A5), Rabbit Antibody, Cell Signaling Technology
- Phospho-MEK 1/2 (Ser217/221) (41G9), Rabbit mAb, Cell Signaling Technology
- p44/42 MAPK (Erk1/2) (137F5), Rabbit mAb, Cell Signaling Technology
- Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP, Rabbit mAb, Cell Signaling Technology
- Phospho-MSK (Thr581), Rabbit Antibody, Cell Signaling Technology
- Phospho-p90RSK (Ser380) (9D9), Rabbit mAb, Cell Signaling Technology

Other antibodies used:

- FAK, Rabbit Antibody, Cell Signaling Technology
- Phospho-FAK (Tyr397) (D20B1), Rabbit mAb, Cell Signaling Technology
- Phospho-p70 S6 Kinase (Thr389) (108D2), Rabbit mAb, Cell Signaling Technology

- Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP, Rabbit mAb, Cell Signaling Technology
- Phospho-S6 Ribosomal Protein (Ser240/244) (D68F8) XP, Rabbit mAb, Cell Signaling Technology

Unfortunately the antibodies used to analyse the activation of Ras did not give an appropriate level of signal to be used in this analysis.

4.2.5 Analysis and statistics used from western blotting analysis

All the western blots shown in this chapter were repeated in triplicate and were laid out as shown in figure 4.6. In order to analyse the relative activation of each intermediate for the two cell lines growing in the different environments, the relative activation of each intermediate was normalised against the value observed for that cell line growing in adherent conditions. For example, looking at the relative activation of phospho-FAK (Tyr397) for CCL-61 cells, the densitometry readings obtained for CCL-61 cells growing adherently (CCL-61 (Adh)) were given an arbitrary value of 1. The densitometry readings for CCL-61 cells growing in suspension conditions were then normalised to the value given for CCL-61 cells growing adherently to give a relative activation level in arbitrary units. This was repeated for CHO-SA growing in different environments.

For the statistical analysis, Student's t-test was used to determine levels of significance between the CCL-61 or CHO-SA cells growing in adherent conditions compared to suspension conditions. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4.2 Overview of the PI3K/Akt and Ras/MAPK signalling pathways
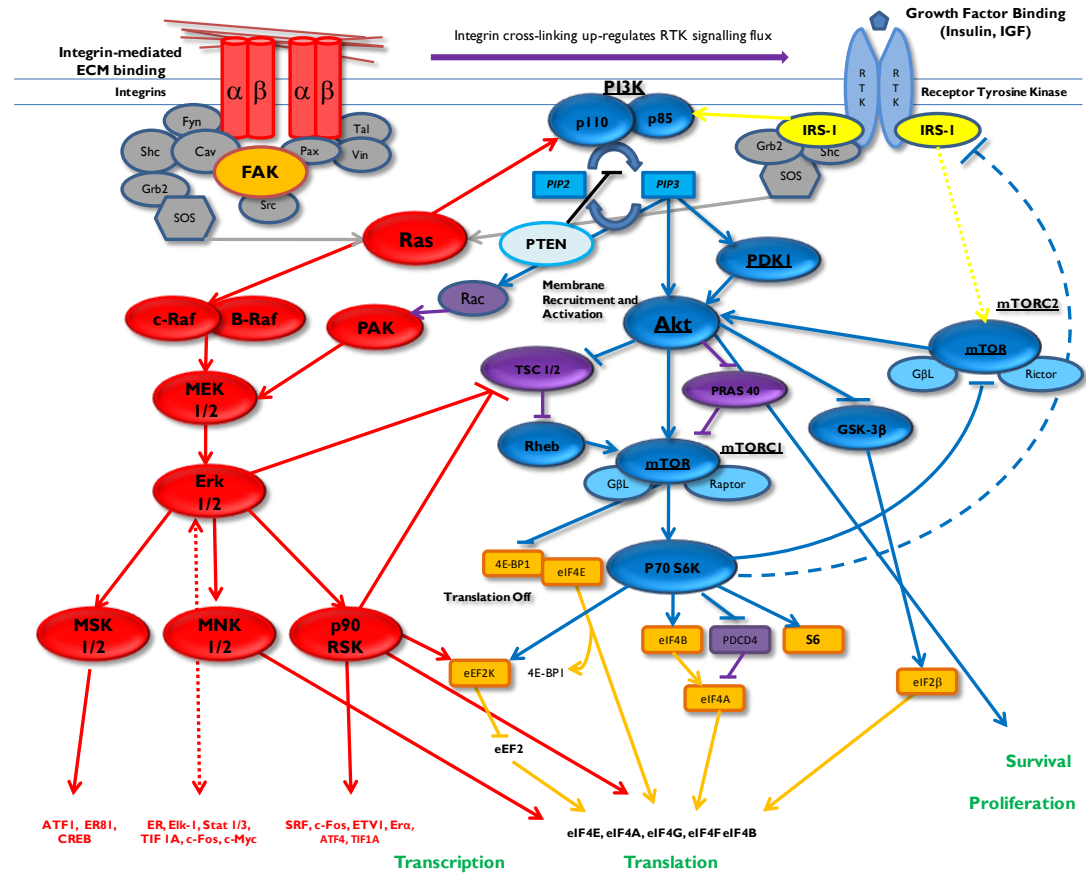
Figure 4.5: Schematic overview of the PI3K/Akt and Ras/MAPK signalling networks. The key signalling intermediates of the PI3K/Akt pathway are shown in blue, the key intermediates of the Ras/MAPK are shown in red. The above schematic shows how the two pathways link with the cell surface. Integrin-mediated binding to the extracellular matrix (ECM) and growth factors (such as insulin, IGF-1) binding to their respective receptor tyrosine kinases, triggers signalling cascades such as the ones shown. Signalling network diagram generated from sources including: cellsignal.com, (Anjum and Blenis, 2008; Schwartz and Assoian, 2001; Vivanco and Sawyers, 2002).

4.3 Results

4.3.1 Validation of western blotting technique

A)

Lane 1: Biotinylated Ladder

Lane 2: -

Lane 3: CCL-61 (Adh)

Lane 4: CCL-61 (Sus) + Ins

Lane 5: CCL-61 (Sus) - Ins

Lane 6: CHO-SA (Adh)

Lane 7: CHO-SA (Sus) + Ins

Lane 8: CHO-SA (Sus) - Ins

B)

Lane 1: Biotinylated Ladder

Lane 2: Erk 1/2 Positive Control Cell Extract

Lane 3: CHO-SA (10 µM U0126)

Lane 4: CHO-SA (10 µM U0126)

Lane 5: CHO-SA (5 µM U0126)

Lane 6: CHO-SA (5 µM U0126)

Lane 7: CHO-SA (1 µM U0126)

Lane 8: CHO-SA (1 µM U0126)

Lane 9: CHO-SA (Untreated)

Lane 10: CHO-SA (Untreated)

Figure 4.6: Exemplar western immunoblots showing different method validation measures. The blot shown in figure 4.6.A) is an example of one complete western immunoblot for the loading control protein GAPDH. The figure shows that for every single blot performed a biotinylated protein ladder was run to confirm that the bands observed are approximately at the correct molecular weight for the target protein. Figure 4.6.B) is an example of one complete western immunoblot showing the relative levels of phospho-Erk 1/2 (Thr202/Tyr204) of CHO-SA cells treated with various concentrations of the inhibitor U0126. The figure shows that as well as running a biotinylated ladder, an Erk 1/2 positive control cell extract was run as a positive control. Where possible, the appropriate positive control cell extracts as well as negative control cell extracts were run on each western immunoblot.

4.3.2 Analysis of the pathway activation status of the PI3K/Akt and Ras/MAPK pathways in CCL-61 and CHO-SA cells growing in both adherent and suspension conditions

The cell line characterisation work done in chapter highlighted the difference in cell growth and proliferation rate between the non-adapted CCL-61 cells and the adapted CHO-SA when cultured in suspension conditions. The CHO-SA cell is able to reach significantly higher viable cell densities when growing in suspension with insulin added as a growth factor, compared to the CCL-61 cell line. In order to analyse the underlying changes in key signalling networks that enable the CHO-SA cell line to achieve this higher rate of growth, extensive western immunoblotting studies were conducted focusing on key intermediates in both the PI3K/Akt and Ras/MAPK pathways as well as important adaptor proteins and key cell surface molecules such as integrins and specific RTKs.

The first set of western blot experiments focused on the levels of pathway activation of the PI3K/Akt and Ras/MAPK pathways at specific time points through culture when both the CCL-61 and CHO-SA cell lines are growing in A) standard adherent conditions B) suspension conditions with insulin in the growth media C) suspension conditions without insulin in the growth media. The major time point in culture that was investigated was day 3 in culture, as this was identified to be approximately the middle of the exponential phase of growth when CHO-SA cells are growing in suspension with insulin. Therefore at this time point we would expect to see some differences in the activation state of key signalling intermediates.

By directly comparing on the same western blot, the activation status of important signalling intermediates when the CCL-61 and CHO-SA cells are grown in adherent and suspension conditions with and without an added growth factor, it is possible to address to two key questions in the adaptation from adherent to suspension growth: 1) Is there a significant shift in signalling activation when cell are removed from a cell surface? 2) How do added exogenous growth factors

affect the ability of un-adapted and adapted cell lines to grow in suspension conditions?

4.3.3 Analysis of the activation status of the PI3K/Akt signalling pathway

The results from the analysis of the activation status of the key PI3K/Akt signalling pathway intermediates PI3K, PTEN, PDK1, Akt, and mTOR are shown in figures 4.7 to 4.13.

Western blot analysis of the total expression levels of the PI3K p85 regulatory subunit content and the p110α catalytic subunit content shows that when CCL-61 and CHO-SA cells are transferred from adherent to suspension conditions that there is not a significant increase or decrease in the total levels of these two particular subunits (figure 4.7). In suspension conditions there is not a significant difference in total p85 and p110α content when both cell lines are cultured with or without insulin. In terms of PI3K activation the bottom immunoblot, in both figures 4.7 and 4.8, shows the phosphorylation levels of the p85 regulatory subunit at the specific site (Tyr458) and the p55 regulatory subunit at the specific site (Tyr199). Figure 4.8 shows that when CCL-61 cells are transferred from adherent to suspension conditions there is a significant decrease in the level of phosphorylation at these two key regulatory sites. Figure 4.8.B shows that at phospho-PI3K p85 (Tyr458), CCL-61 cells cultured in suspension conditions without insulin show significantly decreased levels of phosphorylation compared to CCL-61 cells growing in adherent conditions (Student's t-test, $p < 0.01$). CCL-61 cells cultured in suspension conditions with insulin, show higher levels of phosphorylation compared to without insulin conditions but they are still significantly lower than that of CCL-61 cells growing adherently (Student's t-test, $p < 0.05$). In terms of the CHO-SA cell line, when CHO-SA cells are transferred to suspension conditions without added insulin a significant decrease in phosphorylation is observed compared to CHO-SA in adherent conditions ($p < 0.05$). However, when CHO-SA cells are transferred to suspension conditions with added insulin in the growth media a significant increase in phosphorylation at p85 (Tyr458) is observed ($p < 0.05$).

This relative activation of PI3K across the cell lines cultured in different environments, is mirrored in the activation profile of PI3K at the site phospho-PI3K p55 (Tyr199) (Figure 4.8.C). CCL-61 cells cultured in suspension conditions with or without added insulin, both display a significant decrease in the relative phosphorylation level at this site compared to CCL-61 cells growing in adherent conditions (CCL-61 (Sus) + Ins, p<0.01), (CCL-61 (Sus) – Ins, p<0.001). CHO-SA cells cultured in suspension conditions without insulin also show a significant decrease in phosphorylation (p<0.01) compared to adherent conditions. In contrast, when CHO-SA cells are cultured in suspension with added insulin, a significant increase in phosphorylation is observed (p<0.01) compared to adherent conditions.

Figure 4.9 shows the total PI3K p85 subunit expression levels as well as the phospho-tyrosine content of the p85 subunit after immunoprecipitation with a total p85 antibody followed by immunoblotting with a 4G10 anti-phosphotyrosine antibody as described earlier. In a similar manner to the phosphorylation profile of the phospho-PI3K p85 (Tyr458)/p55 (Tyr199) immunblot, when CCL-61 cells are transferred to suspension culture conditions a significant decrease in phospho-tyrosine content is observed compared to when the CCL-61 cells are cultured in adherent conditions (CCL-61 (Sus) + Ins, p<0.05), (CCL-61 (Sus) – Ins, p<0.05) . Inclusion of insulin in the media in suspension culture leads to a slight increase in the phospho-tyrosine content of p85 but not a significant increase. By contrast when the CHO-SA cell line is transferred to suspension culture conditions with insulin in the growth media a slight increase in the total phospho-tyrosine content of p85 is observed compared to when the CHO-SA cells are growing in adherent conditions. When CHO-SA cells are growing in suspension culture without insulin a slight decrease in the phospho-tyrosine content of p85 is observed compared to CHO-SA cells in adherent conditions and CHO-SA in suspension + Insulin conditions.

This indicates that when both cell lines are transferred to suspension conditions the adapted CHO-SA cell line is able to respond to the inclusion of a growth in the culture media, via the phosphorylation of these two key phospho-tyrosine sites

A)
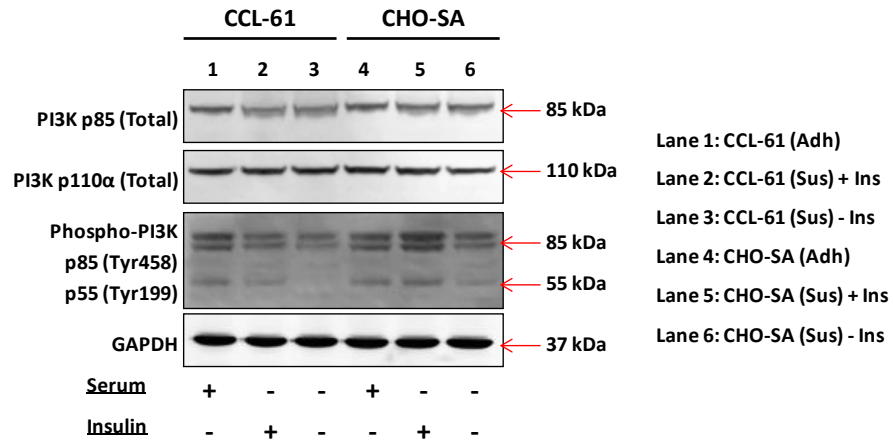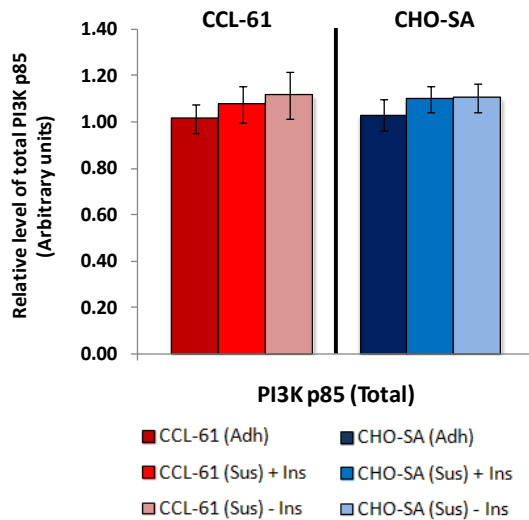
B)   **C)**
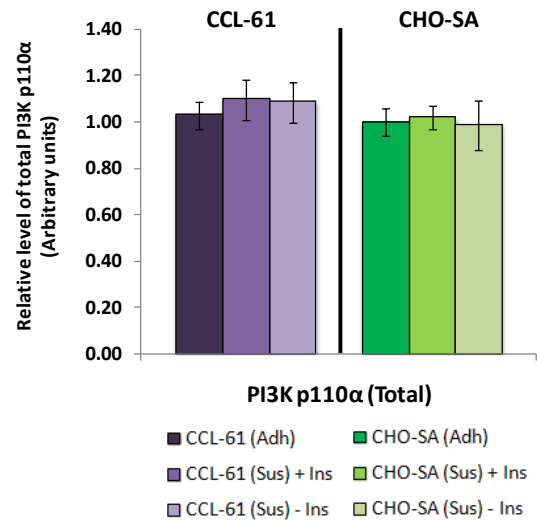
Figure 4.7: Western blotting analysis of the total PI3 Kinase p85 and p110α content and phospho-PI3K p85 (Tyr458) p55 (Tyr199) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total PI3K p85 levels and C) Total PI3K p110α levels were calculated relative to the level of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
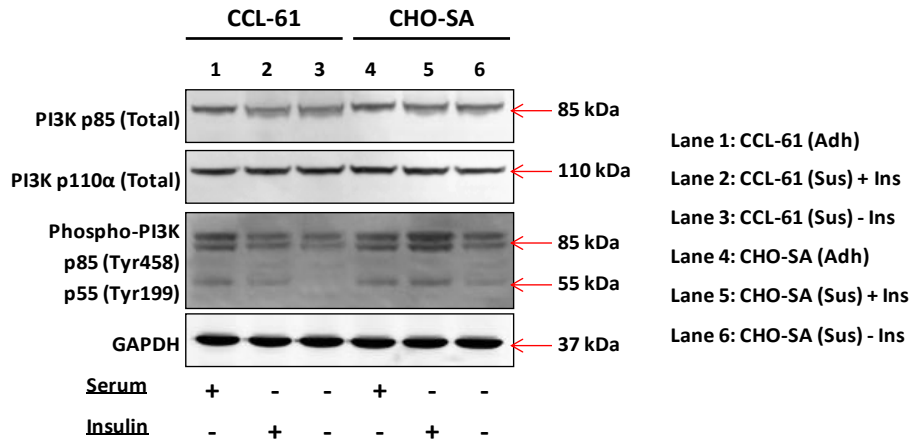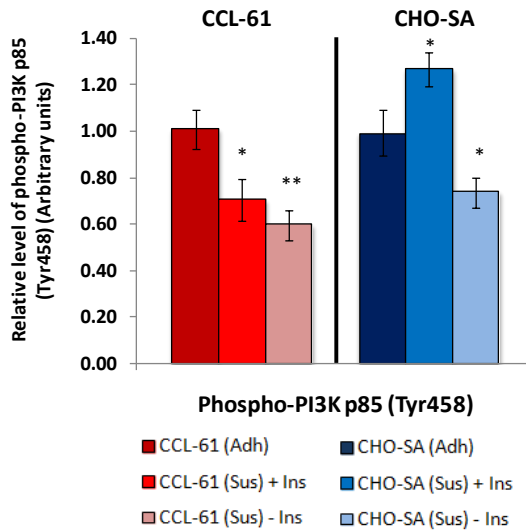
B) **C)**

Figure 4.8: Western blotting analysis of the total PI3 Kinase p85 and p110α content and phospho-PI3K p85 (Tyr458) p55 (Tyr199) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-PI3K p85 (Tyr458) levels and C) Phospho-PI3K p55 (Tyr199) levels were calculated relative to the level of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are ± S.D. (n=3).

A)

B) **C)**

Figure 4.9: Western blotting analysis of the total PI3 Kinase p85 content and phospho-PI3K p85 (Phospho-Tyr) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total PI3K p85 levels and C) Phospho-PI3K p85 (Phospho-Tyr) levels were calculated relative to the level of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

on PI3K, whereas the CCL-61 cell line does not possess the ability to phosphorylate PI3K at these two key sites to as greater level as the CHO-SA cell line in response to growth factor activation.

Figure 4.10 shows the total levels, of the negative regulator of the PI3K/Akt pathway, PTEN and the phospho-PTEN (Ser380) levels of the CCL-61 and CHO-SA cell lines growing in both adherent and suspension conditions. Ser380 is one of three key phosphorylation sites located within the C-terminus that regulates the stability and biological activity of PTEN (Vazquez et al., 2000). In terms of total PTEN expression levels, CCL-61 cells show a slight increase in total PTEN levels when cultured in suspension conditions compared to adherent conditions. CHO-SA cells also exhibit a slight but not significant increase in PTEN content when transferred to suspension conditions. In terms of phospho-PTEN (Ser380) levels, only CCL-61 cells, when transferred to suspension growth conditions without insulin, display a significant increase in phosphorylation when compared to adherent conditions ($p < 0.05$). There is not a significant change in phosphorylation when CCL-61 cells are cultured in suspension with insulin. When CHO-SA cells are transferred to suspension culture conditions, there is not a significant change in the level of phosphorylation compared to CHO-SA cells in adherent conditions.

As PTEN is a negative regulator of the PI3K/Akt pathway, it is clear to see that when cells have a relatively high growth rate (as in the case of CCL-61 and CHO-SA in adherent conditions and CHO-SA in suspension + Insulin) lower levels of phospho-PTEN (Ser380) are observed.
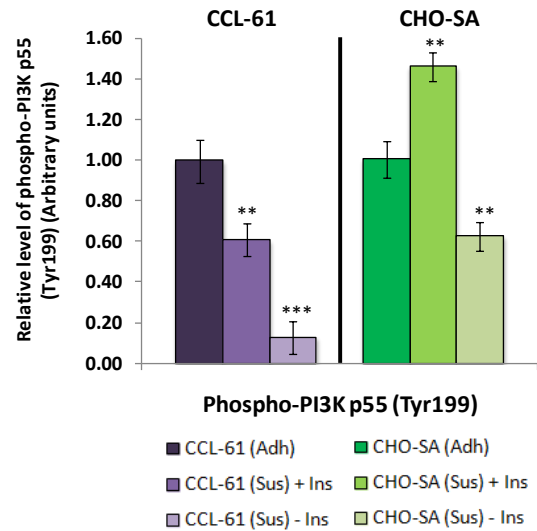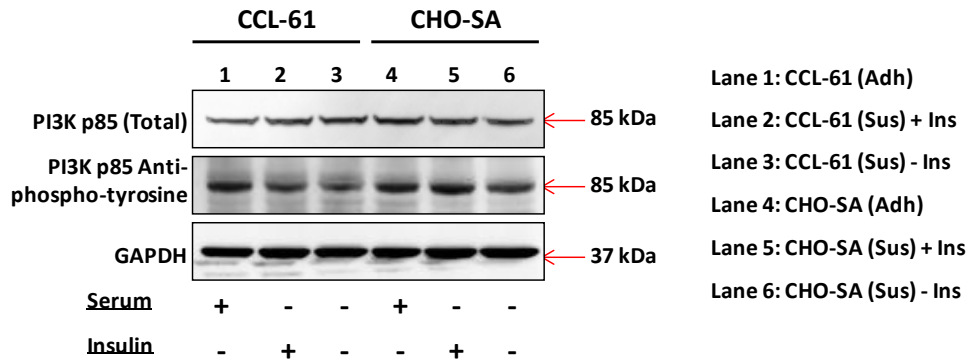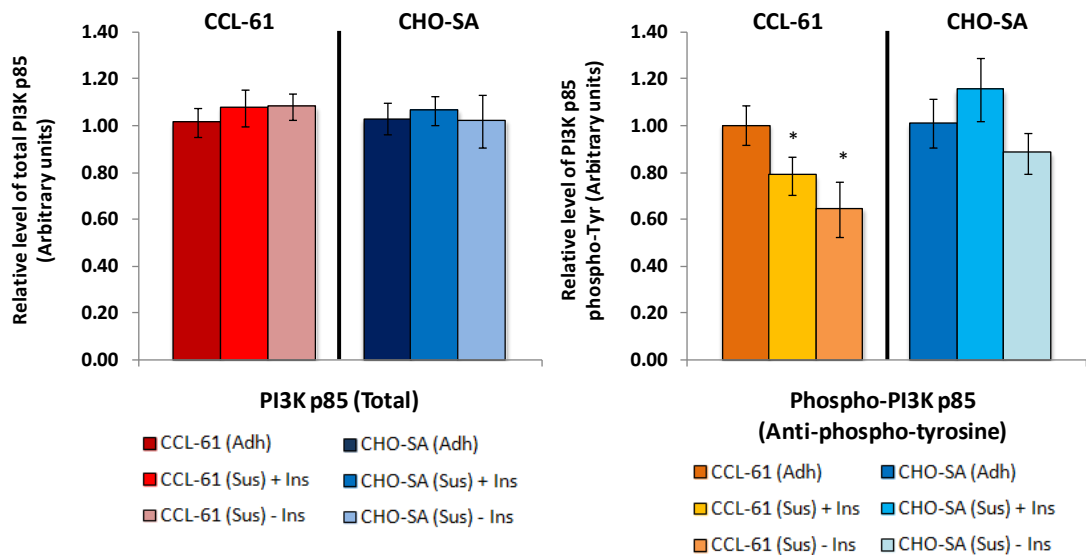
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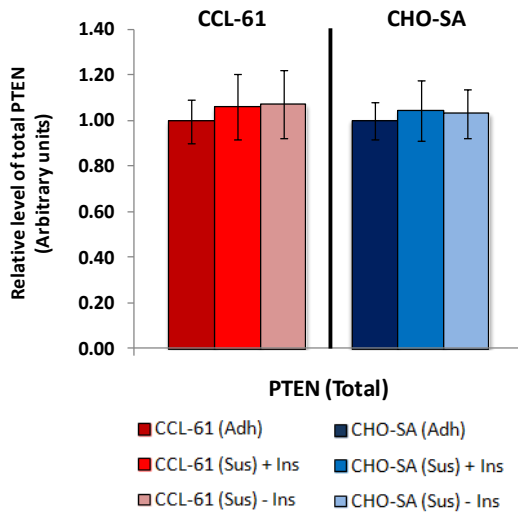
C)

Figure 4.10: Western blotting analysis of the total PTEN content and the phospho-PTEN (Ser380) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total PTEN levels and C) Phospho-PTEN (Ser380) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).
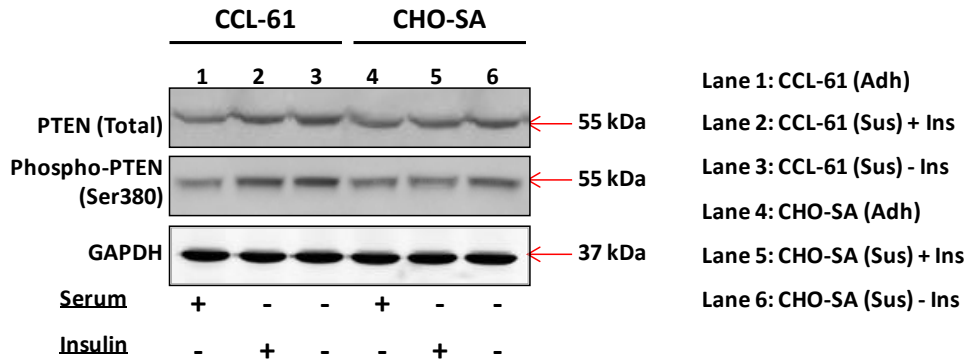
The total PDK1 content and phospho-PDK1 (Ser241) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions is shown in figure 4.11. There is no significant difference in the total levels of PDK1 between the cell lines growing in different conditions at 72 hours despite the significant differences seen in growth rate at this time point. However, when looking at the activation status of PDK1, there is a change in phosphorylation levels when both the CCL-61 and CHO-SA cell lines are cultured in suspension conditions. Several serine sites including Ser25, Ser241, Ser393/396 and Ser410 are phosphorylated in response to various stimuli in PDK1 (Riojas et al., 2006). Phosphorylation on the activation loop site Ser241 by autophosphorylation has been shown to be necessary for PDK1 activity (Casamayor et al., 1999). Focusing on the Ser241 phosphorylation site, in a similar manner to PI3K, when CCL-61 cells are transferred into suspension conditions without insulin a significant decrease in phosphorylation at this site is seen ($p < 0.05$). There is a slight but not significant decrease in phosphorylation observed when CCL-61 cells are cultured with insulin in suspension. In comparison, when the CHO-SA cell line is cultured in suspension conditions with insulin, there is a significant increase in relative phosphorylation levels at Ser241 compared to when the CHO-SA cells are cultured in adherent conditions ($p < 0.05$). However when the CHO-SA cells are cultured in suspension conditions without insulin, there is not a significant increase in phosphorylation at site Ser241 compared to adherent conditions.

Further downstream from PI3K and PDK1 in the PI3K/Akt pathway is Akt, which is also referred to as protein kinase B (PKB). The total levels of Akt and the relative levels of two key phosphorylation sites, phospho-Akt (Ser473) and phospho-Akt (Thr308) are shown in figure 4.12. The activation of Akt is controlled by two key phosphorylation sites. Akt becomes activated in response to phospholipid binding and phosphorylation of site Thr308 in the activation loop of Akt by PDK1 (Alessi et al., 1996). Akt activation is also regulated by phosphorylation at site Ser473 within the carboxy terminus of Akt. The phosphorylation site Ser473 in the hydrophobic motif of Akt is regulated by the rapamycin insensitive TORC2 complex, which consists of mTOR in complex with rictor and Sin1 (Jacinto et al., 2006; Sarbassov et al., 2005).
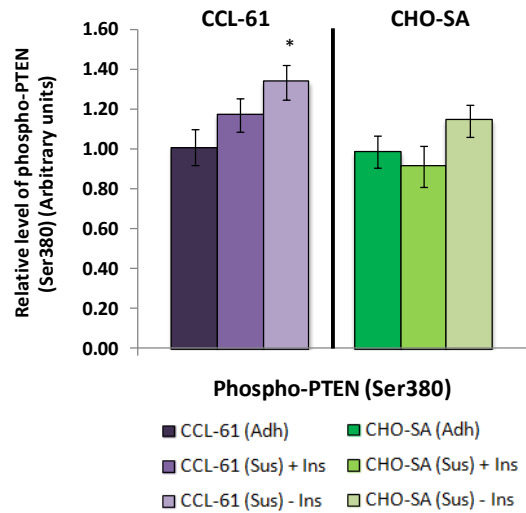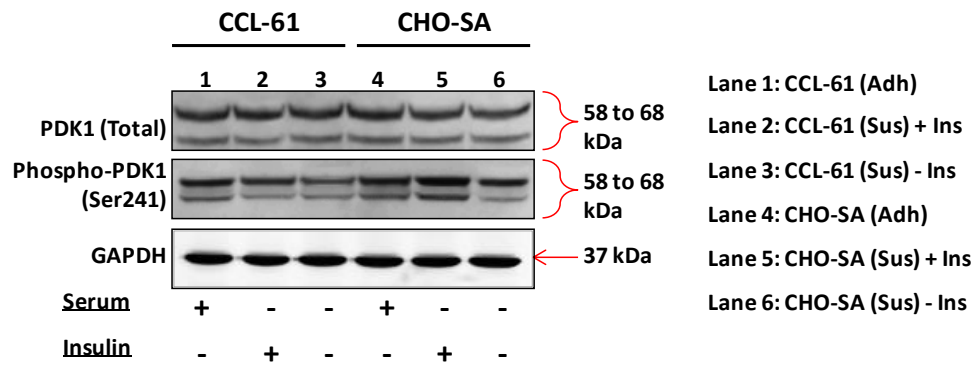
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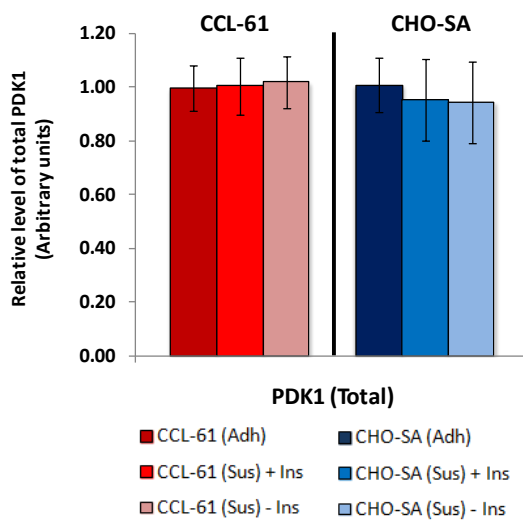
C)
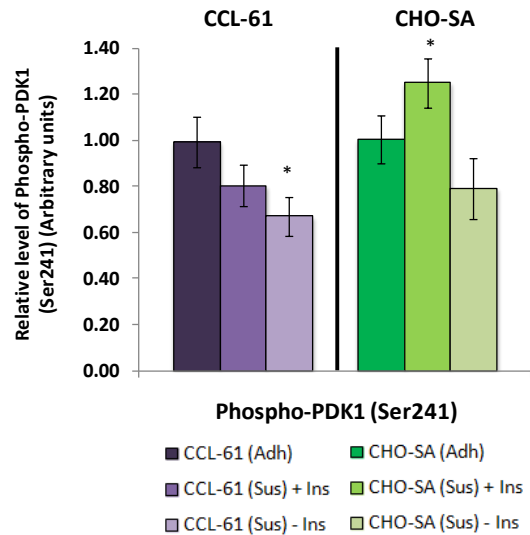
Figure 4.11: Western blotting analysis of the total PDK1 content and the phospho-PDK1 (Ser241) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total PDK1 levels and C) Phospho-PDK1 (Ser241) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
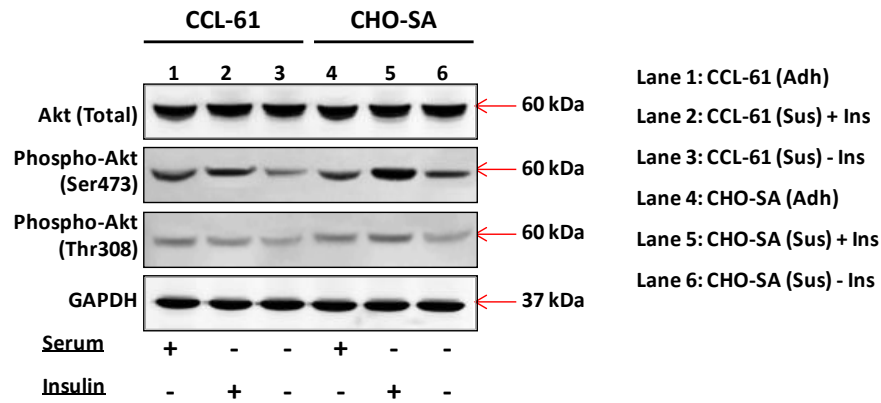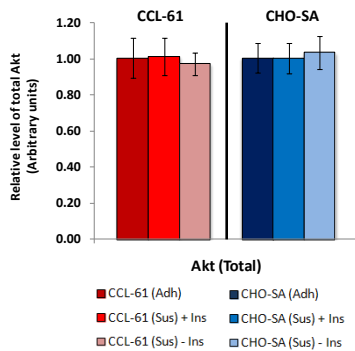
B) **C)** **D)**

Figure 4.12: Western blotting analysis of the total Akt content and the phospho-Akt (Ser473) and phospho-Akt (Thr308) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total Akt levels, C) Phospho-Akt (Ser473) levels and D) Phospho-Akt (Thr308) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Data points are $\pm$ S.D. (n=3).

From the immunoblot analysis shown in figure 4.12, there is no significant increase or decrease in the total Akt content when both CCL-61 and CHO-SA cells are grown in adherent or suspension culture conditions. In terms of Akt activation, there is a significant change in the phosphorylation levels at sites Ser473 and Thr308 when the CCL-61 and CHO-SA cell lines are cultured in different environments.

Focusing on phosphorylation site Ser473, the CCL-61 cells, when transferred to suspension growth conditions in the presence of insulin, show a similar level of phosphorylation as the CCL-61 cells in adherent conditions. In contrast when CCL-61 cells are cultured in suspension conditions without insulin there is a significant decrease in phosphorylation at Ser473 ($p<0.01$). When the CHO-SA cell line is cultured in suspension with insulin a significant increase in phosphorylation at site Ser473 is observed compared to when the CHO-SA cell line is cultured in adherent conditions ($p<0.001$). In comparison, when the CHO-SA cells are cultured in suspension conditions without insulin there is no significant increase or decrease in phosphorylation at Ser473 compared to CHO-SA cultured in adherent conditions.

At the Akt phosphorylation site Thr308, when CCL-61 cells are transferred to suspension culture with insulin, a significant increase in phosphorylation level at this phospho-site is observed compared to CCL-61 cells growing adherently ($p<0.05$). The removal of insulin from the growth media, as in CCL-61 (Sus) – Insulin conditions, leads to no significant change in the phosphorylation level when compared to CCL-61 growing in adherent conditions. In terms of the CHO-SA cell line, when CHO-SA cells are transferred to suspension culture conditions with insulin, a significant increase in the phosphorylation level at Thr308 is observed compared to the CHO-SA cells growing adherently ($p<0.01$). In a similar manner to the CCL-61 cells, when insulin is removed from the suspension culture media, as in CHO-SA (Sus) – Insulin, there is no significant difference in phosphorylation compared to the CHO-SA (Adh) conditions.

Figure 4.13 shows the total mTOR content and phospho-mTOR (Ser2448) and phospho-mTOR (Ser2481) levels of the CCL-61 and CHO-SA cell lines growing in both adherent and suspension conditions. mTOR activation is regulated by two key phosphorylation sites, Ser2448, which is phosphorylated by the upstream PI3K/Akt pathway and Ser2481 which is an autophosphorylation site (Nave et al., 1999; Peterson et al., 2000).

As shown in figure 4.13 the total mTOR content between the cell lines and in different growth conditions is not significantly different at 72 hours in culture. In terms of mTOR activation, there is a significant change in the phosphorylation levels at site Ser2448 when the CCL-61 and CHO-SA cells are cultured in adherent and suspension conditions. When the CCL-61 cells are transferred to suspension culture conditions, both with and without insulin, there is a significant decrease in the levels of phosphorylation at Ser2448 compared to when the cells are cultured in adherent conditions (CCL-61 (Sus) + Insulin, $p<0.01$, CCL-61 (Sus) – Insulin, $p<0.01$). In comparison, the CHO-SA cell line when cultured in suspension conditions with insulin shows a significant increase in phosphorylation at Ser2448 when compared to the CHO-SA cell line growing in adherent conditions ($p<0.05$). In suspension conditions without added insulin there is a significant decrease in phosphorylation at Ser2448 compared to CHO-SA cells in adherent conditions ($p<0.01$).

In a similar manner to the Ser2448 phosphoryaltion site, there is significant variation between the CCL-61 and CHO-SA cell lines growing in different conditions in terms of phosphorylation at site Ser2481. The CCL-61 cells when cultured in suspension conditions, with and without insulin, show significantly reduced levels of phosphorylation, at the key phosphorylation site Ser 2448, compared to CCL-61 cells growing adherently (CCL-61 (Sus) + Insulin, $p<0.01$, CCL-61 (Sus) – Insulin, $p<0.01$). By comparison a significant increase in phosphorylation is seen when the CHO-SA cells are transferred to suspension conditions with added insulin ($p<0.01$). When insulin is removed from the suspension media a significant decrease in phosphorylation at Ser2448 in the CHO-SA cells is observed ($p<0.05$).
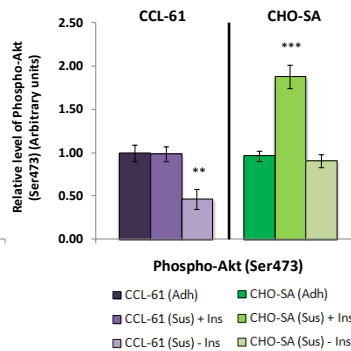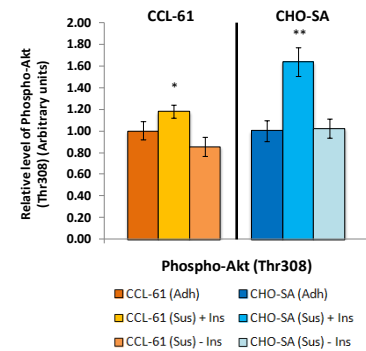
A)
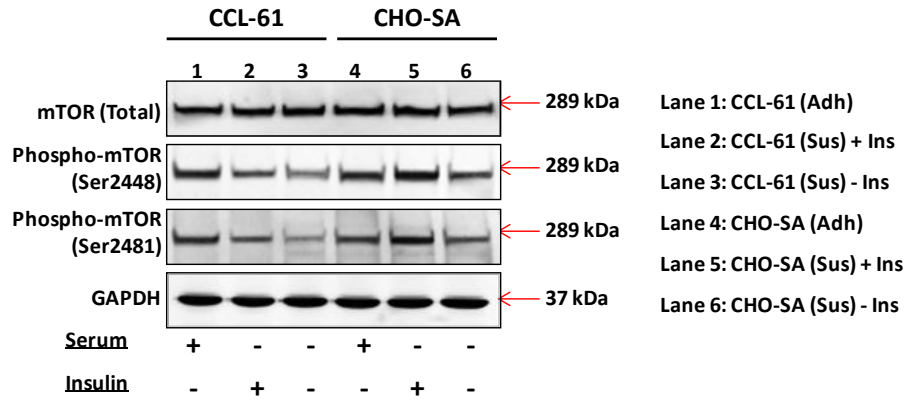
B)   **C)**   **D)**
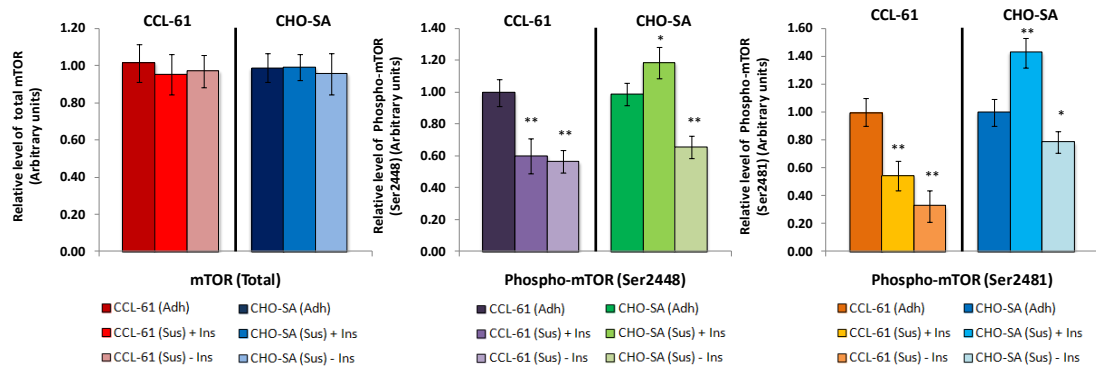
Figure 4.13: Western blotting analysis of the total mTOR content and the phospho-mTOR (Ser2448) and phospho-mTOR (Ser2481) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total mTOR levels, C) Phospho-mTOR (Ser2448) levels and D) Phospho-mTOR (Ser2481) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).
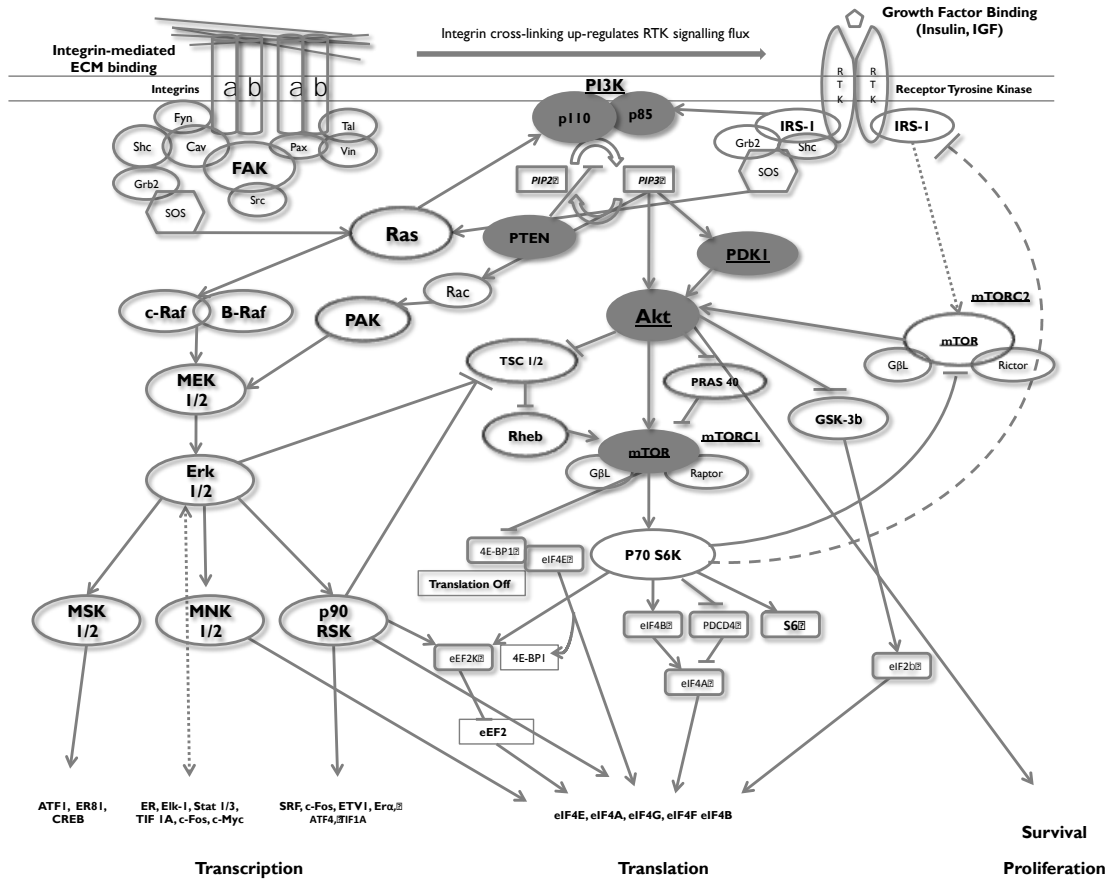
Figure 4.14: Schematic overview of the PI3K/Akt and Ras/MAPK signalling networks. The key signalling intermediates in the PI3K/Akt pathway that have been analysed by western blotting are shown in grey. Signalling network diagram generated from sources including: cellsignal.com, (Anjum and Blenis, 2008; Schwartz and Assoian, 2001; Vivanco and Sawyers, 2002).

4.3.4 Analysis of the activation status of the Ras/MAPK signalling pathway

The results from the analysis of the activation status of the key Ras/MAPK signalling pathway intermediates c-Raf, MEK 1/2, Erk 1/2 and the downstream intermediates p90RSK and MSK1 are shown in figures 4.15 to 4.20.

The MAPK/Erk pathway is activated upstream by the recruitment of the Raf effector proteins (A-Raf, B-Raf and c-Raf) to GTP-bound Ras (Avruch et al., 1994). The mechanism of activation of c-Raf is the most widely understood. c-Raf is phosphorylated at multiple activating sites by various protein kinases.

Figure 4.15 shows the total c-Raf content and the levels of phosphorylation at various regulatory sites. The top immunoblot of figure 4.15.A shows the total c-Raf content of the CCL-61 and CHO-SA cell lines growing in both adherent and suspension conditions. Between the two cell lines growing in the different culture environments there is no significant difference in total c-Raf levels. At activation site Ser338 both the CCL-61 and CHO-SA cell lines growing in adherent culture conditions show significantly higher levels of phosphorylation compared to when both of the cell lines are cultured in suspension conditions (CCL-61 (Sus) + Insulin, $p<0.01$, CCL-61 (Sus) – Insulin, $p<0.001$), (CHO-SA (Sus) + Insulin, $p<0.01$, CHO-SA (Sus) – Insulin, $p<0.001$). In suspension conditions both the CCL-61 and CHO-SA cell lines show a low level response to the inclusion of insulin in the growth media in terms of Ser338 phosphorylation, although there are slightly higher levels of phosphorylation when CHO-SA cells are cultured in suspension with insulin.

In terms of phosphorylation levels at the inhibitory binding site Ser259, there are significantly reduced levels of phosphorylation when the CCL-61 and CHO-SA cell lines are cultured in adherent conditions compared to when the two cell lines are in suspension conditions (CCL-61 (Sus) + Insulin, $p<0.001$, CCL-61 (Sus) – Insulin, $p<0.001$), (CHO-SA (Sus) + Insulin, $p<0.001$, CHO-SA (Sus) – Insulin, $p<0.01$). This increase in phosphorylation at site Ser259 in cells cultured in

A)
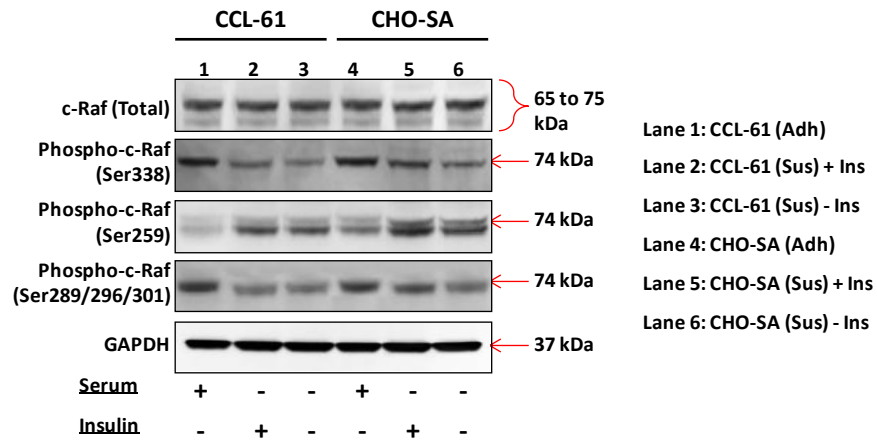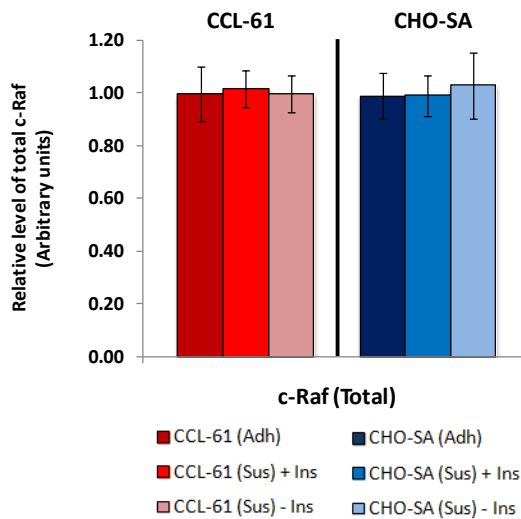
B)   **C)**
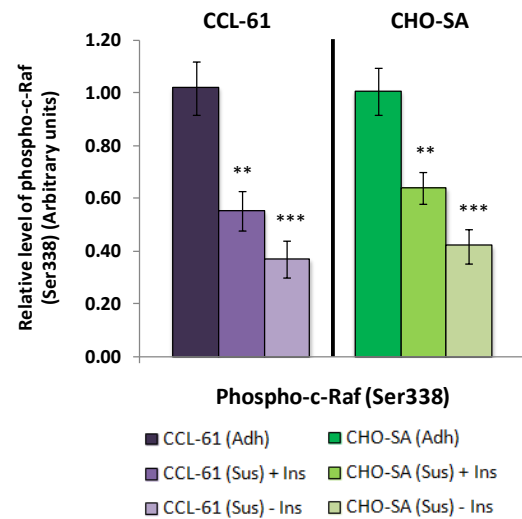
Figure 4.15: Western blotting analysis of the total c-Raf content and the phospho-c-Raf (Ser338), phospho-c-Raf (Ser259) and phospho-c-Raf (Ser289/296/301) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total c-Raf levels and C) Phospho-c-Raf (Ser338) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
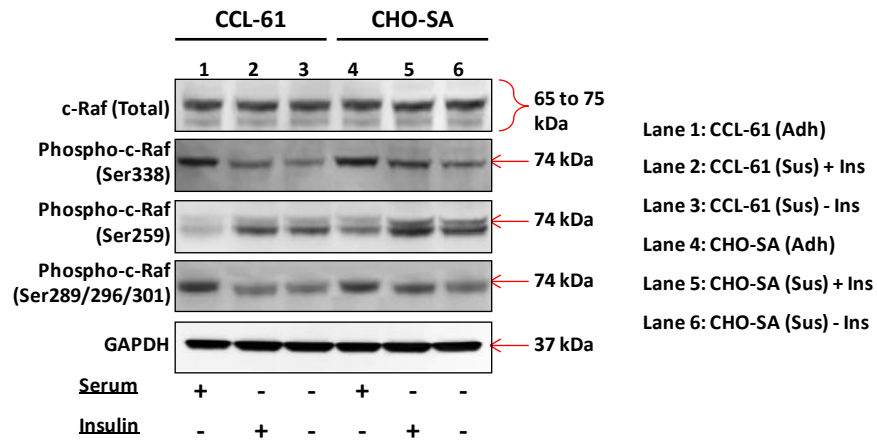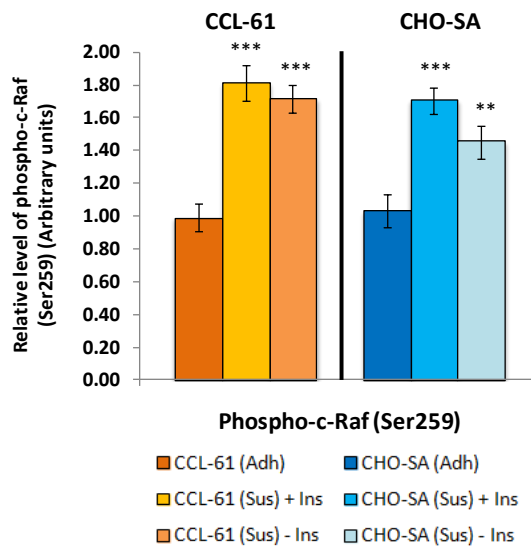
B) **C)**
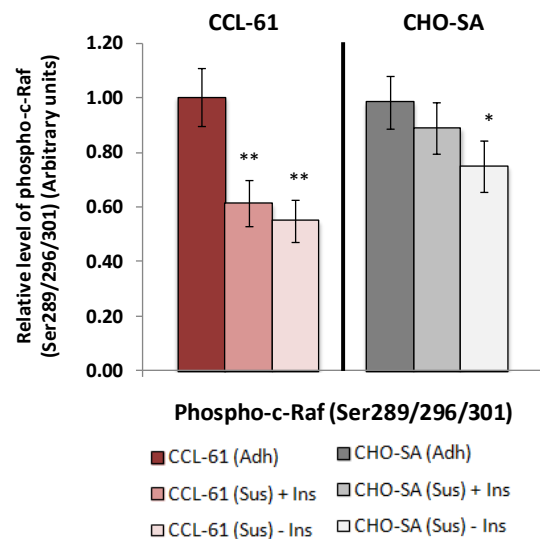
Figure 4.16: Western blotting analysis of the total c-Raf content and the phospho-c-Raf (Ser338), phospho-c-Raf (Ser259) and phospho-c-Raf (Ser289/296/301) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-c-Raf (Ser259) levels and C) Phospho-c-Raf (Ser289/296/301) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

suspension conditions indicates that when cells are cultured in suspension conditions signalling flux via c-Raf is reduced.

At the hyperphosphorylation site Ser289/296/301 there are increased levels of phosphorylation when both the CCL-61 and CHO-SA are growing adherently compared to when the two cell lines are growing in suspension (apart from CHO-SA + Insulin), (CCL-61 (Sus) + Insulin, $p < 0.01$, CCL-61 (Sus) – Insulin, $p < 0.01$), (CHO-SA (Sus) – Insulin, $p < 0.05$). In a similar manner to the phosphorylation profile at Ser338, there is a low level response to the inclusion of insulin in the growth media when both CCL-61 and CHO-SA cells are transferred to suspension conditions. Hyperphosphorylation at this specific site is dependent on downstream MEK activation, therefore this indicates that downstream MEK signalling should be higher in cells growing adherently compared to in suspension (Dougherty et al., 2005).

The levels of MEK1/2 activation in CCL-61 and CHO-SA cells growing adherently and in suspension are shown in figure 4.17. In terms of total MEK1/2 content between the CCL-61 and CHO-SA cell lines growing in both adherent and suspension conditions, there is not a significant difference. By contrast the activation of MEK1/2 changes significantly when CCL-61 and CHO-SA cells are cultured in adherent conditions. The phosphorylation site at Ser217/221 in the activation loop, regulates the activation status of MEK1/2. In adherent conditions both the CCL-61 and CHO-SA cell lines show a significant increase in terms of phosphorylation at Ser217/221 compared to when either cell line is growing in suspension conditions with or without insulin (CCL-61 (Sus) + Insulin, $p < 0.05$, CCL-61 (Sus) – Insulin, $p < 0.01$), (CHO-SA (Sus) + Insulin, $p < 0.01$, CHO-SA (Sus) – Insulin, $p < 0.001$). In a similar manner to the key phosphorylation sites in c-Raf, when CCL-61 and CHO-SA cells are cultured in suspension conditions there is a low level response to insulin in terms of phosphorylation at Ser217/221.

A)
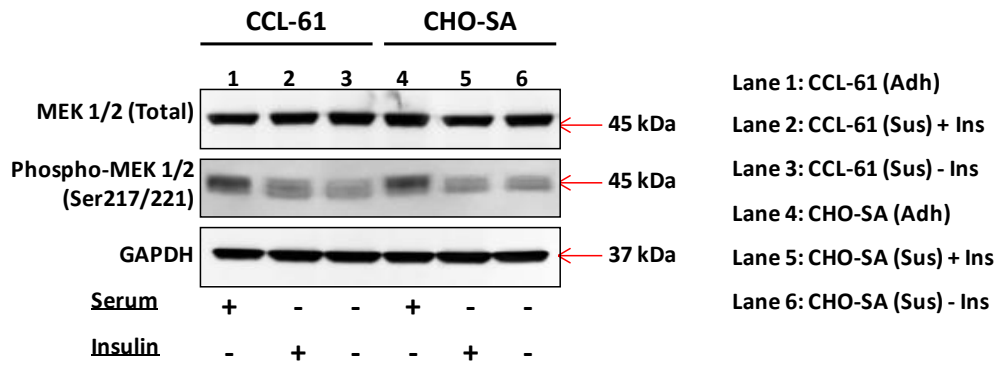
B)
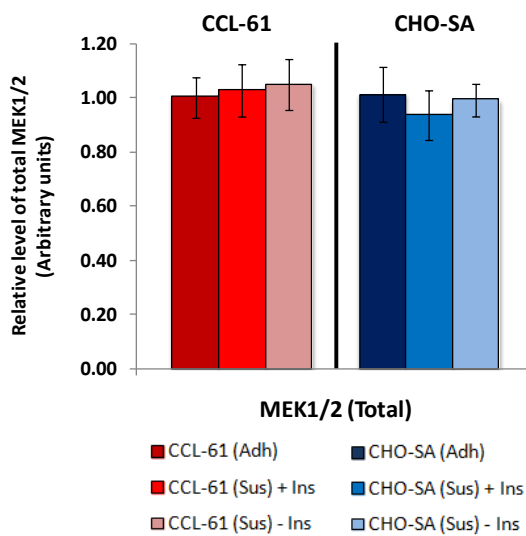
C)
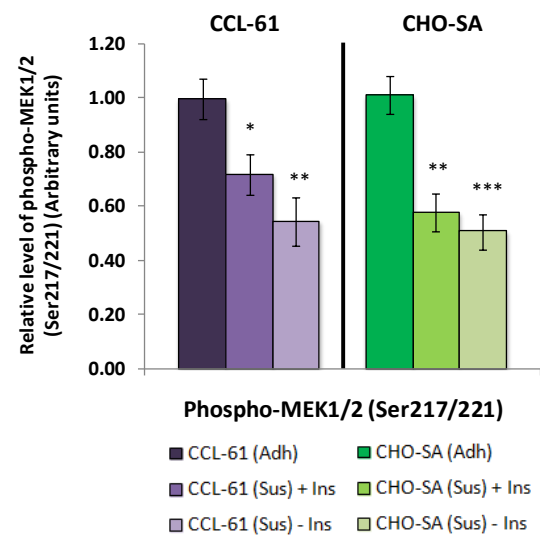
Figure 4.17: Western blotting analysis of the total MEK 1/2 content and the phospho-MEK 1/2 (Ser217/221) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total MEK 1/2 levels and C) Phospho-MEK 1/2 (Ser217/221) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Data points are ± S.D. (n=3).

Further downstream of MEK1/2 lies the MAP kinase Erk1/2. p44/42 MAPK or Erk1/2 is activated by MEK1/2 mediated phosphorylation at the activation loop residues Thr202/Tyr204 of Erk1 and Thr185/Tyr187 of Erk2 respectively. Figures 4.18 and 4.19 show the total content and relative activation levels of Erk1/2 in CCL-61 and CHO-SA cells growing adherently and in suspension. In terms of total Erk1/2 levels, when CCL-61 and CHO-SA cells are cultured in adherent conditions there is a slight increase in the total level of Erk1/2 protein compared to when each cell line is cultured in suspension conditions. The level of activation of Erk1/2 at the phosphorylation sites Thr202/Tyr204 changes significantly when CCL-61 and CHO-SA cells are cultured in adherent conditions compared to suspension conditions. There is a significant increase in phosphorylation in both Erk1 (p44) and Erk2 (p42) at the key phosphorylation sites Thr202/Tyr204 and Thr185/Tyr187. When both CCL-61 and CHO-SA cells are cultured in suspension conditions there is a significant reduction in the phosphorylation levels of Erk1/2 (Erk1 - (CCL-61 (Sus) + Insulin, $p<0.001$, CCL-61 (Sus) – Insulin, $p<0.001$), (CHO-SA (Sus) + Insulin, $p<0.001$, CHO-SA (Sus) – Insulin, $p<0.001$)), (Erk2 - (CCL-61 (Sus) + Insulin, $p<0.01$, CCL-61 (Sus) – Insulin, $p<0.01$), (CHO-SA (Sus) + Insulin, $p<0.05$, CHO-SA (Sus) – Insulin, $p<0.01$)). In a similar manner to the upstream intermediates c-Raf and MEK1/2, when CCL-61 cells are cultured in suspension there is a slight response to the inclusion of insulin in the growth media in terms of Erk1/2 phosphorylation. This is also the case for CHO-SA cells, as CHO-SA cells growing in suspension also display low levels of insulin responsiveness in terms of activation of Erk1/2. This is shown by the increase in Erk1/2 phosphorylation levels when CHO-SA cells are growing in suspension with insulin conditions compared to without insulin conditions.

A)
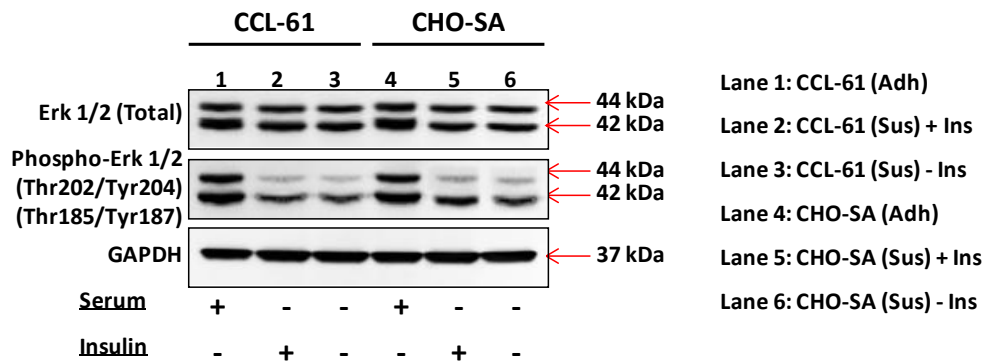
B)
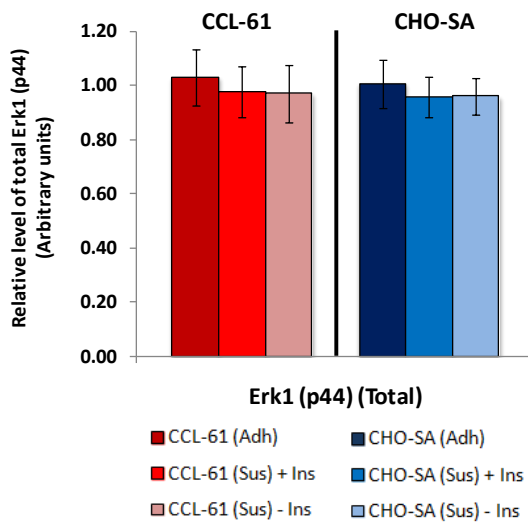
C)
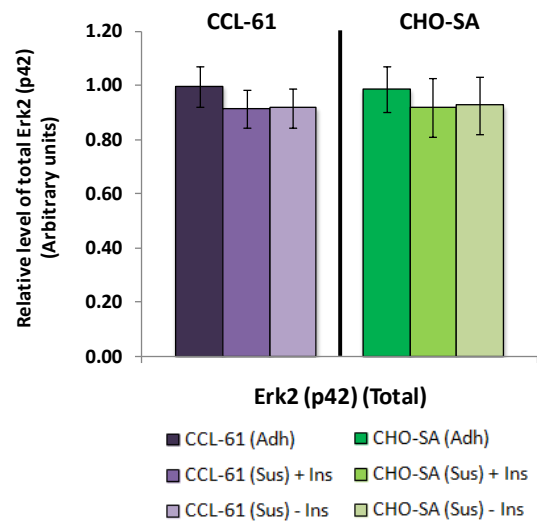
Figure 4.18: Western blotting analysis of the total Erk 1/2 (p44/42) content and the phospho-Erk 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total Erk1 (p44) levels and C) Total Erk2 (p42) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).
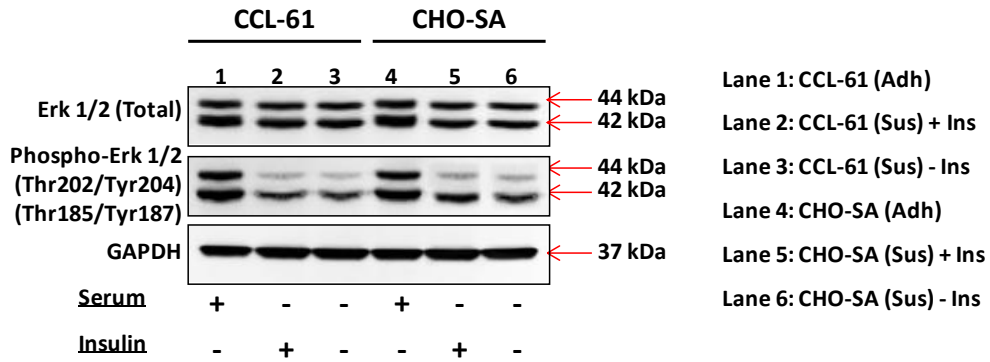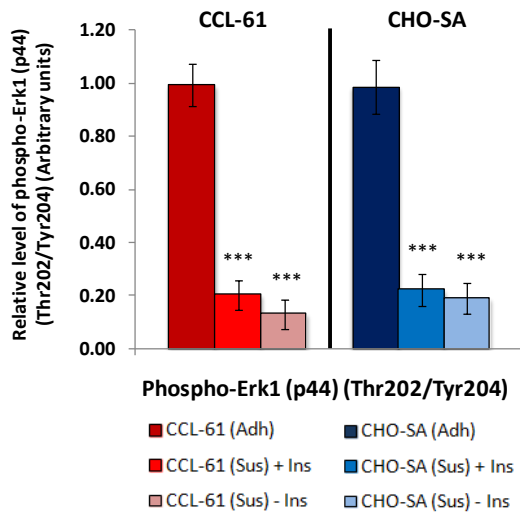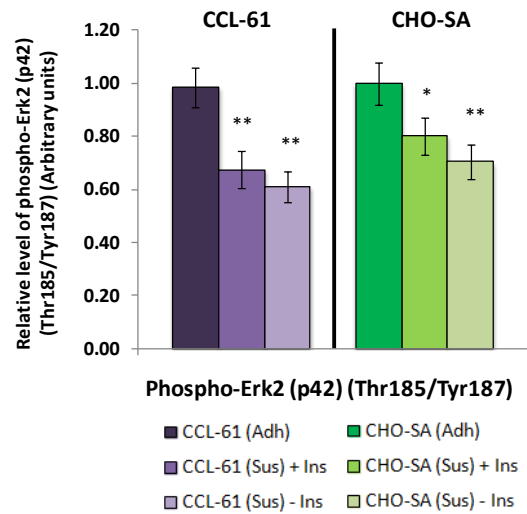
Figure 4.19: Western blotting analysis of the total Erk 1/2 (p44/42) content and the phospho-Erk 1/2 (Thr202/Tyr204)/(Thr185/Tyr187) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-Erk1 (p44) (Thr202/Tyr204) levels and C) Phospho-Erk2 (p42) (Thr185/Tyr187) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

Two downstream activation targets of Erk1/2 are MSK1, a mitogen and stress activated protein kinase and p90RSK which is a member of the ribosomal S6 kinases. MSK1 is activated via phosphorylation at the key regulatory sites Thr581 and Ser360 by Erk1/2 and p38 MAPK. Figure 4.20 shows the relative phospho-MSK1 (Thr581) levels in CCL-61 and CHO-SA cells growing in both adherent and suspension conditions. In a similar manner to its upstream activator Erk1/2, MSK1 is phosphorylated at site Thr581 to a significantly higher level when both the CCL-61 and CHO-SA cell lines are growing in adherent culture conditions (CCL-61 (Sus) + Insulin, $p < 0.001$, CCL-61 (Sus) – Insulin, $p < 0.0001$), (CHO-SA (Sus) + Insulin, $p < 0.001$, CHO-SA (Sus) – Insulin, $p < 0.0001$). This pattern of activation is mirrored in the activation profile of p90RSK. p90RSK is activated by Erk1/2 and has several critical phosphorylation sites including Ser380, Thr359, Ser363 and Thr573. In terms of p90RSK activation at site Ser380, both the CCL-61 and CHO-SA cells show much higher levels of phosphorylation when cultured in adherent conditions (CCL-61 (Sus) + Insulin, $p < 0.001$, CCL-61 (Sus) – Insulin, $p < 0.001$), (CHO-SA (Sus) + Insulin, $p < 0.001$, CHO-SA (Sus) – Insulin, $p < 0.001$).

A)
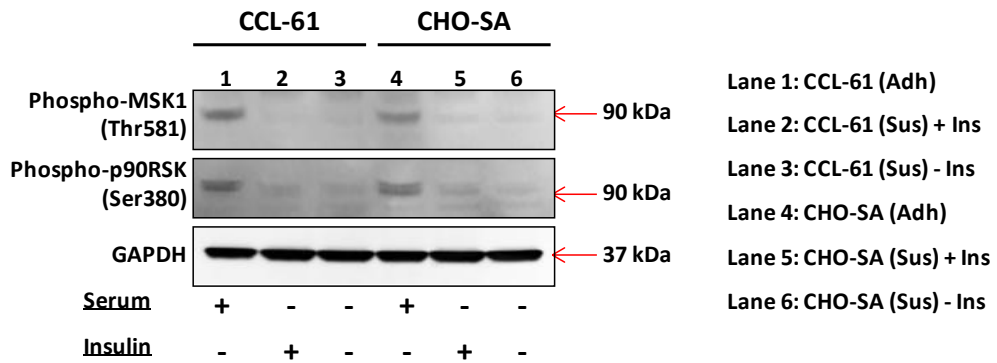
B)
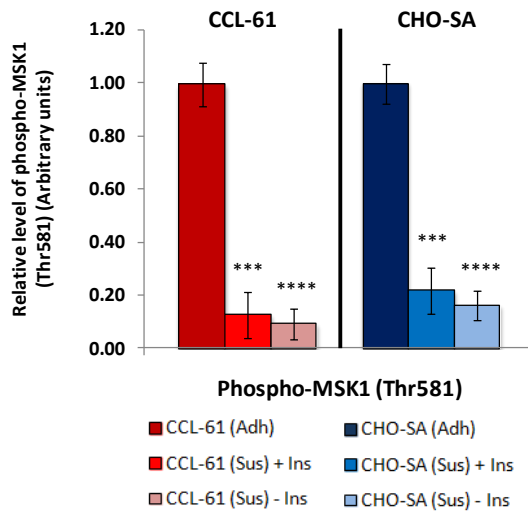
C)
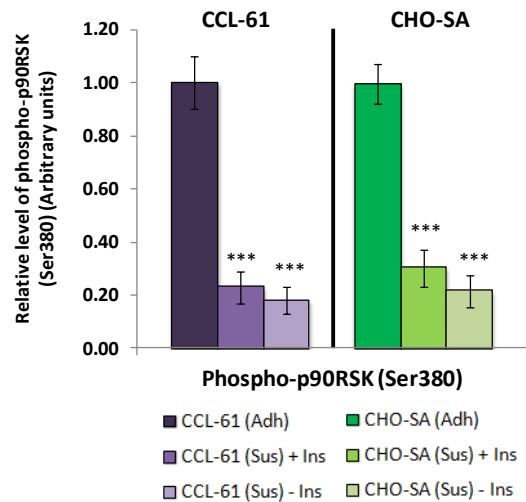
Figure 4.20: Western blotting analysis of the phospho-MSK1 (Thr581) levels and the phospho-p90RSK (Ser380) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-MSK1 (Thr581) levels and C) Phospho-p90RSK (Ser380) levels were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).
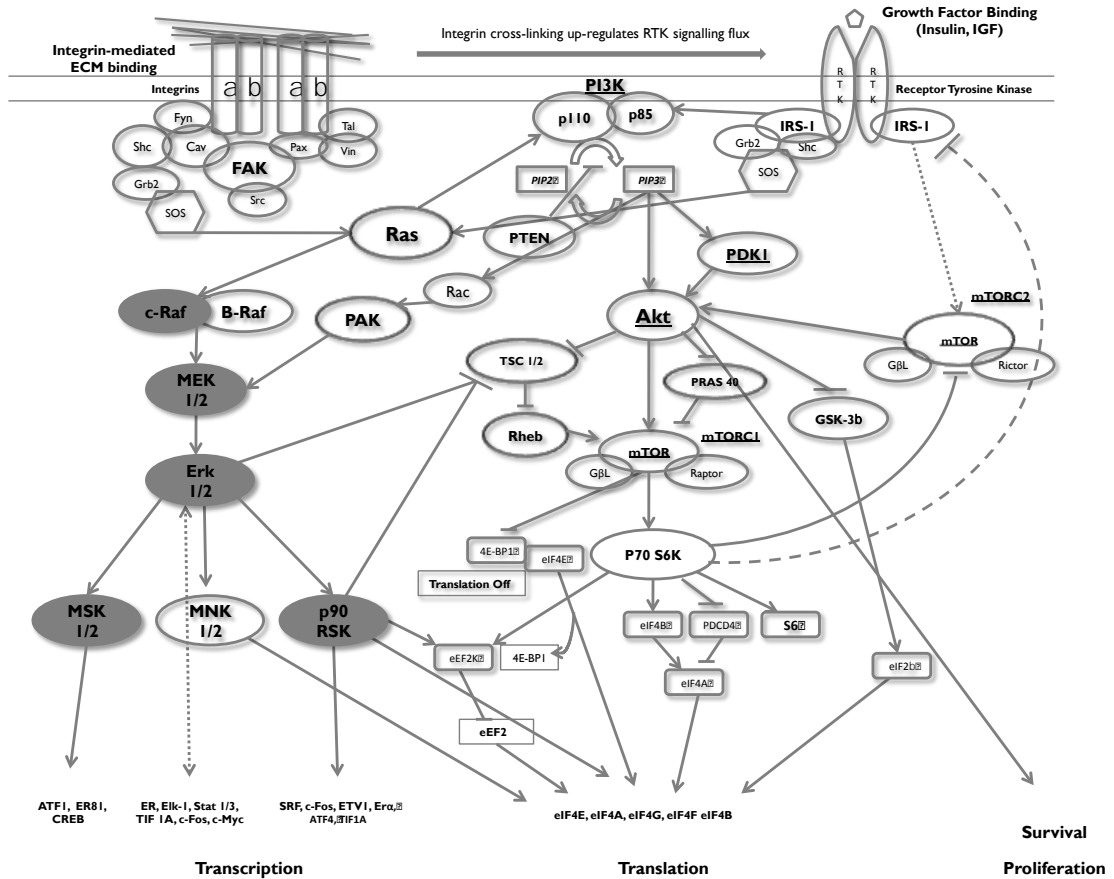
Figure 4.21: Schematic overview of the PI3K/Akt and Ras/MAPK signalling networks. The key signalling intermediates in the Ras/MAPK pathway that have been analysed by western blotting are shown in grey. Signalling network diagram generated from sources including: cellsignal.com, (Anjum and Blenis, 2008; Schwartz and Assoian, 2001; Vivanco and Sawyers, 2002).

4.3.5 Analysis of the activation status of key translational control intermediates

Downstream of mTOR lies the mitogen activated Ser/Thr protein kinase p70 S6K that is involved in cell growth and G1 cell cycle progression (Pullen and Thomas, 1997). Two key phosphorylation sites, Thr229 in the catalytic domain and Thr389 in the linker domain are critical for full kinase functions. Thr389 acts as a docking site for PDK1, allowing PDK1 to phosphorylate Thr229 which leads to kinase activation. As well as PDK1, mTOR is also a potent Thr389 kinase.

Figure 4.22 shows the total p70 S6K content and phospho-p70 S6K (Thr389) levels of the CCL-61 and CHO-SA cells growing in adherent and suspension conditions. In terms of activation at the phosphorylation site (Thr389), CCL-61 cells when growing in adherent conditions display significantly higher levels of phosphorylation than when growing in suspension conditions (CCL-61 (Sus) + Insulin, $p<0.05$, CCL-61 (Sus) – Insulin, $p<0.01$). There is a slight increase in phosphorylation level in suspension conditions with insulin compared to without insulin conditions. By comparison, CHO-SA cells when growing in suspension conditions with insulin display significantly higher levels of phosphorylation at Thr389 compared to CHO-SA growing in adherent conditions(CHO-SA (Sus) + Insulin, $p<0.01$). When insulin is removed from the culture media in suspension conditions CHO-SA cells show a slight decrease in phosphorylation at Thr389 compared to adherent cultured CHO-SA. This pattern of activation of p70 S6K is very similar to that of its upstream activators PDK1 and mTOR.

One of the targets of activation of p70 S6K is the S6 ribosomal protein (rpS6), which is a component of the 40S ribosomal subunit. The S6 ribosomal protein becomes phosphorylated at several key serine sites in response to mitogen stimulation (Hutchinson et al., 2011). p70 S6K has been shown to phosphorylate rpS6 at numerous serine sites including Ser240 and Ser244, while in an mTOR-independent mechanism, p90 RSK exclusively phosphorylates rpS6 at the serine sites Ser235 and Ser236. This RSK activation of rpS6 acts as a link between the

A)
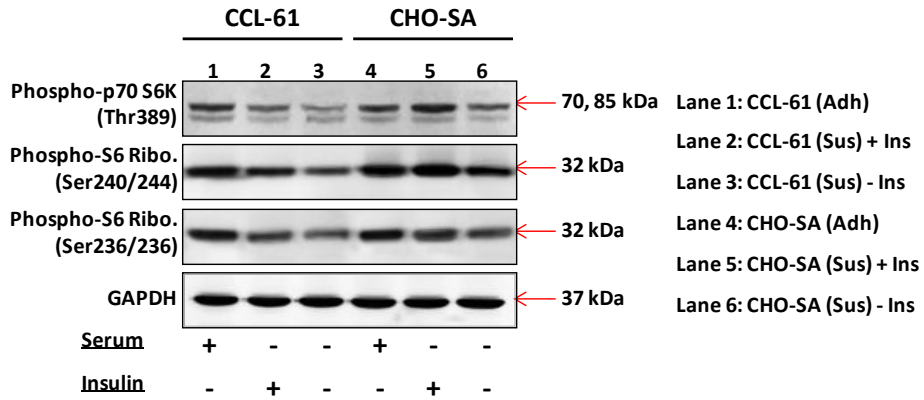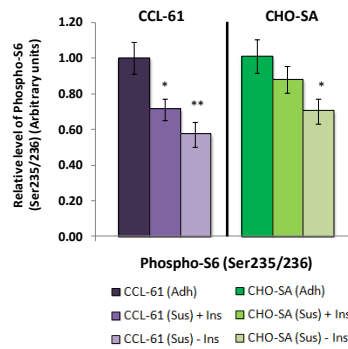
B)   **C)**   **D)**
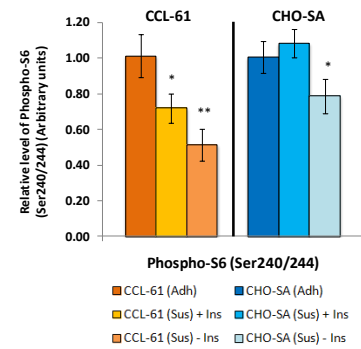
Figure 4.22: Western blotting analysis of the phospho-p70 S6K (Thr389) levels, phospho-S6 ribosomal protein (Ser235/236) levels and the phospho-S6 ribosomal protein (Ser240/244) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-p70 S6K (Thr389) levels, C) Phospho-S6 (Ser235/236) levels and D) Phospho-S6 (Ser240/244) were calculated relative to the level of activation of the CCL-61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

Ras/MAPK pathway and the cells translational machinery, that is indepdendent of mTOR (Roux et al., 2007). Figure 4.22 shows the relative activation levels of rpS6 at the key phosphorylation sites Ser240/244 and Ser235/236. At phospho-site Ser235/236, CCL-61 cells have a significantly higher level of phosphorylation when growing in adherent conditions compared to when they are growing in suspension conditions (CCL-61 (Sus) + Insulin, $p < 0.05$, CCL-61 (Sus) – Insulin, $p < 0.01$). When insulin is included in the growth medium in suspension conditions, there is a slight increase in phosphorylation at Ser235/236 compared to when the cells are growing in suspension conditions without insulin in the growth medium. In terms of CHO-SA cells, when cultured in suspension conditions with insulin there is only a slight decrease in phosphorylation at this site compared to CHO-SA cells growing adherently. When insulin is removed from the suspension culture media as in CHO-SA (Sus) – Insulin, there is a significant decrease in activation at Ser235/236 compared to adherent conditions ($p < 0.05$).

The pattern of activation observed at phosphorylation site Ser240/244 is similar to that of Ser235/236 for the CCL-61 cells cultured in adherent and suspension conditions. CCL-61 cells growing in suspension conditions with and without insulin have significantly lower levels of activation at this site compared to adherent conditions (CCL-61 (Sus) + Insulin, $p < 0.05$, CCL-61 (Sus) – Insulin, $p < 0.01$). By comparison, when CHO-SA cells are transferred to suspension conditions with insulin a slight but not significant increase in activation compared to adherent conditions is observed. When insulin is removed from the culture media as in CHO-SA (Sus) – Insulin, a significant decrease in activation compared to adherent conditions is observed ($p < 0.05$).

4.3.5 Analysis of the activation status of Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a tyrosine kinase that has a critical role in regulating integrin-mediated signalling activation (Zhao and Guan, 2011). As integrins do not posses any tyrosine kinase activity, they recruit FAK, which colocalises with integrins at cell contact sites with the ECM. Integrin localisation/ aggregation at the cell surface caused by ligand binding leads to the activation of FAK at the autophosphorylation site Tyr397 (Kumar, 1998; Liu et al., 2000).

As shown in figure 4.23, there is a significant change in FAK activation when both CCL-61 and CHO-SA cells are transferred from adherent to suspension conditions. Both CCL-61 and CHO-SA cells show a significant decrease in phosphorylation at the activation site Tyr397 when cultured in suspension conditions (CCL-61 (Sus) + Insulin, $p < 0.001$, CCL-61 (Sus) – Insulin, $p < 0.001$), (CHO-SA (Sus) + Insulin, $p < 0.0001$, CHO-SA (Sus) – Insulin, $p < 0.0001$). This is to be expected as lack of integrin mediated attachment, as in suspension conditions, leads to the dephosphorylation of FAK at site Tyr397.

A)
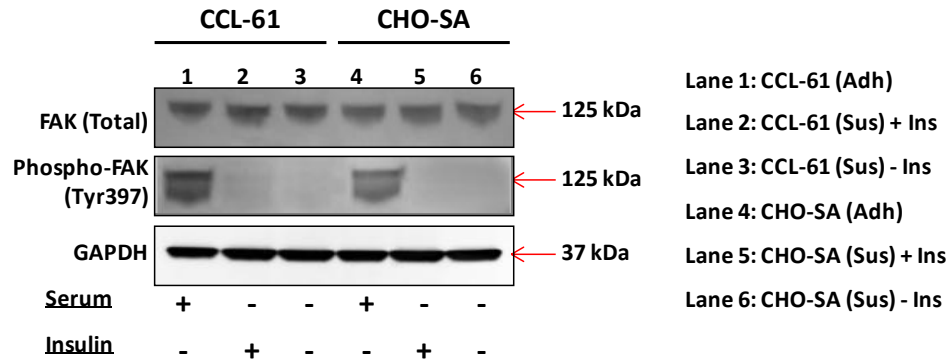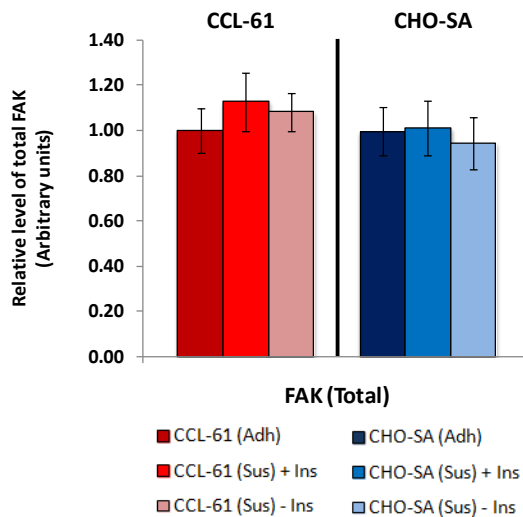
B)   **C)**

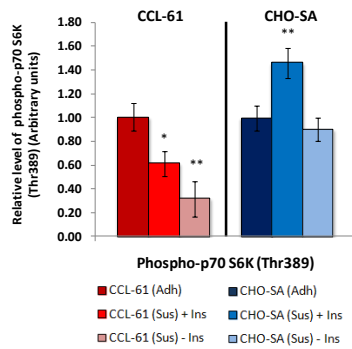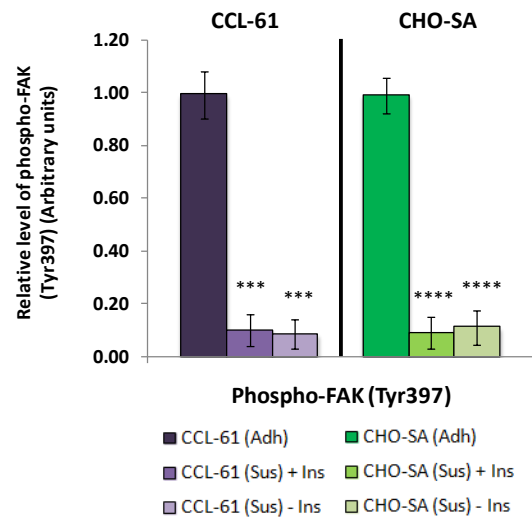**Figure 4.23: Western blotting analysis of the total FAK content and phospho-FAK (Tyr397) levels of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions.** Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Total FAK levels and C) Phospho-FAK (Tyr397) levels were calculated relative to the level of activation of the CCL61 and CHO-SA cell lines growing in adherent conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 or CHO-SA growing in adherent conditions, * is $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Data points are $\pm$ S.D. (n=3).

4.3.5 Signalling pathway analysis – Days 1 and 6 of Culture

As the previous western blot data illustrates there is a shift in the signalling pathway activation profile when cells are growing in suspension compared to adherent conditions at 72 hours (mid-exponential phase). In order to validate these results it was important to select other time points through culture to analyse the signalling profile at to make sure these results were not transient and would be different at different stages in culture. Ideally, 5 or 6 time points through culture would be selected and each signalling intermediate would be analysed at that time point. However, with only limited resources of antibody available this would be not be viable, so instead it was decided that a few key signalling intermediates would be analysed at different time points.

The first time point selected was 24 hours (day 1) post seeding into adherent and suspension conditions. This would then provide a valuable insight into the pathway activation status of the CCL-61 and CHO-SA cells early on in culture in the various growth environments, before cells have entered into the exponential growth phase. The second time point was 144 hours or day 6 or culture as by this time point in culture, cells have left the exponential growth phase and have entered stationary phase of growth or in some cases death phase.

In order to validate this shift in signalling pathway activation, the activation of some key intermediates was analysed at 24 hours in culture and 144 hours in culture. The intermediates analysed at these time points include, PI3K and AKT, from the PI3K/Akt pathway, c-Raf and Erk 1/2, from the Ras/MAPK pathway and FAK.

Figure 4.24 shows the western blotting analysis of phospho-Akt (Ser473), Phospho-Erk 1/2 (Thr202/Tyr204) (Thr185/Tyr187) and phospho-FAK (Tyr397) at days 1 and 6 of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. The westerns shown at day 1 of culture illustrate that the pattern of signalling activation seen at day 3 of culture is

A) Day 1 – Western immunoblots
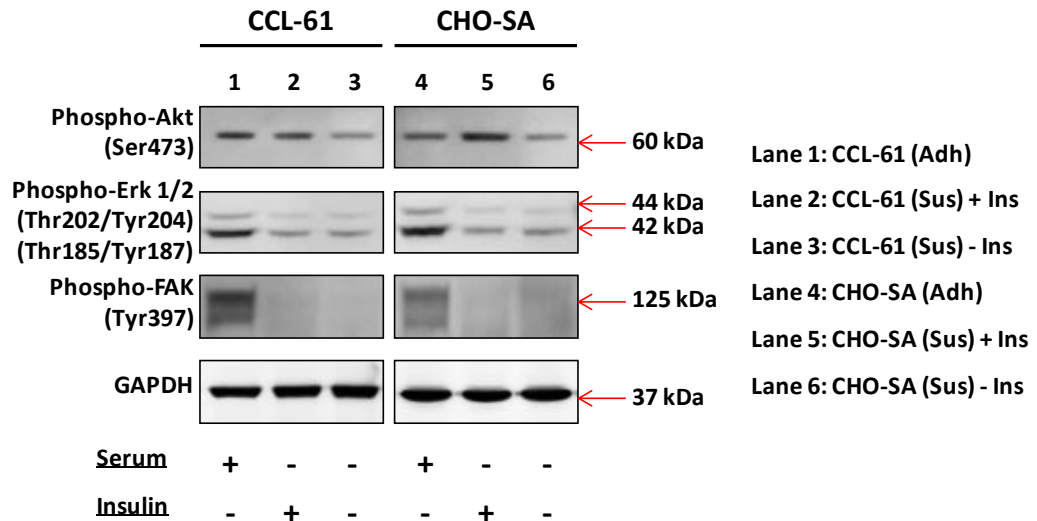
B) Day 6 – Western immunoblots
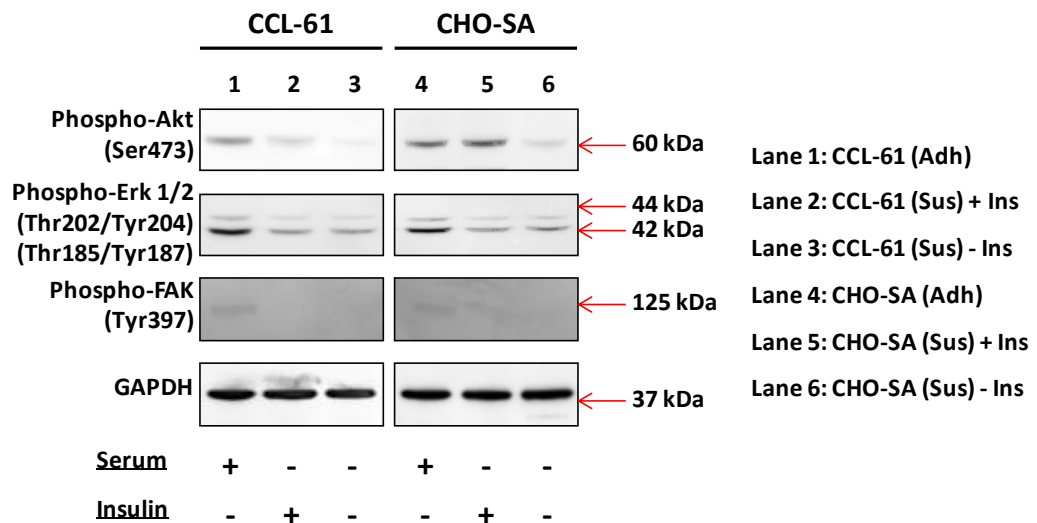
Figure 4.24: Western blotting analysis of various signalling intermediates at days 1 and 6 of the CCL-61 and CHO-SA cell lines growing in adherent and suspension conditions. Extracts from the two cell lines growing in standard adherent and suspension conditions were taken at 24 and 144 hours in culture. The western blots shown above are from one representative experiment. All western blots were performed three times. A) Western blots from cell extracts taken at day 1 of culture and B) Western blots from cell extracts taken at day 6 of culture. GAPDH was used as a loading control.

also observed at day 1 in the signalling intermediates shown. There is clearly higher levels of phosphorylation of both FAK and Erk 1/2 when CCL-61 and CHO-SA are growing in adherent culture conditions. In terms of Akt activation at site Ser473, only CHO-SA cells growing in suspension conditions with added insulin show a clear increase in activation compared to cells growing in adherent conditions, mirroring what is observed at day 3 of culture.

Figure 4.24.B shows the western blots for day 6 of culture. The westerns for phospho-FAK (Tyr397) and phospho-Erk 1/2 (Thr202/Tyr204) (Thr185/Tyr187) shows that there is still a slight increase in activation at these key phosphorylation sites, when CCL-61 and CHO-SA cells are cultured in adherent conditions. The western showing Akt activation at site Ser473 also shows a similar pattern of activation to the equivalent western blots at days 1 and 3, in that only CHO-SA cells growing in suspension with insulin show a slight increase in phosphorylation levels compared to cells in adherent conditions.
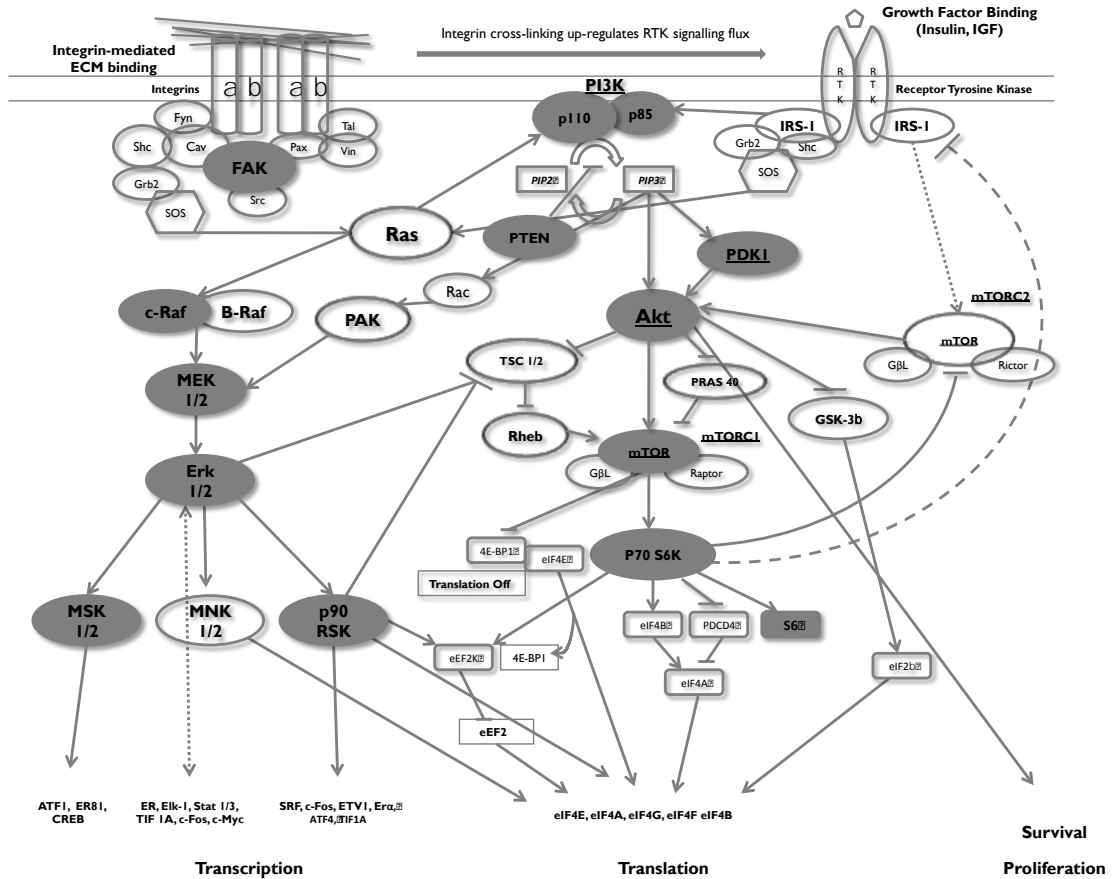
Figure 4.25: Schematic overview of the PI3K/Akt and Ras/MAPK signalling networks. The key signalling intermediates in both the PI3K/Akt and Ras/MAPK pathways that have been analysed by western blotting are shown in grey. Signalling network diagram generated from sources including: cellsignal.com, (Anjum and Blenis, 2008; Schwartz and Assoian, 2001; Vivanco and Sawyers, 2002).

4.3.6 Overview of the signalling activation of key intermediates in the PI3K/Akt pathway.

Signalling Intermediate	Key Phospho-Site(s)	Regulation		CCL-61 (Adh)	CCL-61 (Sus) + Ins	CCL-61 (Sus) - Ins		CHO-SA (Adh)	CHO-SA (Sus) +Ins	CHO-SA (Sus) - Ins
PI3K	p85 (Tyr458)	+ ve		-	*	**		-	*	*
	p55 (Tyr199)	+ ve		-	**	***		-	**	**
	p85 (Phospho-Tyr)	+ ve		-	*	*		-		
PTEN	Ser380	- ve		-		*		-		
PDK1	Ser241	+ ve		-		*		-	*	
Akt	Ser473	+ ve		-		**		-	***	
	Thr308	+ ve		-	*			-	**	
mTOR	Ser2448	+ ve		-	**	**		-	*	**
	Ser2481	+ ve		-	**	**		-	**	*

Table 4.1: Tabular overview of the activation of key intermediates in the PI3K/Akt pathway in CCL-61 and CHO-SA cells growing in adherent and suspension conditions. Signalling intermediates are listed in the first column, with the key regulatory phosphorylation site(s) of that intermediate listed in second column. The type of regulation of that particular phosphorylation site is given in column three. A traffic light system is used to denote the significant increase or decrease in activation in suspension compared to CCL-61 and CHO-SA growing in adherent conditions. The activation of each intermediate in CCL-61 and CHO-SA cells in adherent conditions is given an amber colour and (-) symbol as this is used in each case as the relative activation control. Green = significant activation increase, Amber = no significant increase or decrease, Red = significant decrease. * is p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

4.3.7 Overview of the signalling activation of key intermediates in the Ras/MAPK pathway.

Signalling Intermediate	Key Phospho-Site(s)	Regulation		CCL-61 (Adh)	CCL-61 (Sus) + Ins	CCL-61 (Sus) - Ins		CHO-SA (Adh)	CHO-SA (Sus) +Ins	CHO-SA (Sus) - Ins
c-Raf	Ser338	+ ve		-	**	***		-	**	***
	Ser259	- ve		-	***	***		-	***	**
	Ser289/296/301	+ ve		-	**	**		-		*
MEK 1/2	Ser217/221	+ ve		-	*	**		-	**	***
Erk 1/2	Erk 1 (Thr202/Tyr204)	+ ve		-	***	***		-	***	***
	Erk 2 (Thr185/Tyr187)	+ve		-	**	**		-	*	**
MSK1	(Thr581)	+ ve		-	***	****		-	***	****
p90RSK	Ser2448	+ ve		-	***	***		-	***	***

Table 4.2: Tabular overview of the activation of key intermediates in the Ras/MAPK pathway in CCL-61 and CHO-SA cells growing in adherent and suspension conditions. Signalling intermediates are listed in the first column, with the key regulatory phosphorylation site(s) of that intermediate listed in second column. The type of regulation of that particular phosphorylation site is given in column three. A traffic light system is used to denote the significant increase or decrease in activation in suspension compared to CCL-61 and CHO-SA growing in adherent conditions. The activation of each intermediate in CCL-61 and CHO-SA cells in adherent conditions is given an amber colour and (-) symbol as this is used in each case as the relative activation control. Green = significant activation increase, Amber = no significant increase or decrease, Red = significant decrease. * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

4.3.8 Overview of the signalling activation of other key intermediates.

Signalling Intermediate	Key Phospho-Site(s)	Regulation		CCL-61 (Adh)	CCL-61 (Sus) + Ins	CCL-61 (Sus) - Ins		CHO-SA (Adh)	CHO-SA (Sus) +Ins	CHO-SA (Sus) - Ins
p70 S6K	Thr389	+ ve		-	*	**		-	**	
S6 Ribosomal Protein	(Ser240/244)	+ ve		-	*	**		-		*
	(Ser235/236)	+ve		-	*	**		-		*
FAK	Tyr397	+ ve		-	***	***		-	****	****

Table 4.3: Tabular overview of the activation of other key signalling intermediates in CCL-61 and CHO-SA cells growing in adherent and suspension conditions. Signalling intermediates are listed in the first column, with the key regulatory phosphorylation site(s) of that intermediate listed in second column. The type of regulation of that particular phosphorylation site is given in column three. A traffic light system is used to denote the significant increase or decrease in activation in suspension compared to CCL-61 and CHO-SA growing in adherent conditions. The activation of each intermediate in CCL-61 and CHO-SA cells in adherent conditions is given an amber colour and (-) symbol as this is used in each case as the relative activation control. Green = significant activation increase, Amber = no significant increase or decrease, Red = significant decrease. * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

4.4 Discussion:

Following on from the cell characterisation work performed in chapter 3, the main aim of chapter 4 was to deduce how cells change their signalling pathway activation profile in relation to their growth environment. As showed in chapter 3, CCL-61 and CHO-SA cells growing in adherent conditions display very similar growth characteristics. In contrast, when transferred to suspension conditions, the non suspension-adapted CCL-61 cells with or without added insulin are not able to grow and proliferate like a "normal" suspension cell line. The suspension-adapted CHO-SA cell line, is able to grow and proliferate in suspension conditions but only in the presence of insulin in the cell culture media. When insulin is removed from the media, CHO-SA cells show a significant decrease in their maximum growth and proliferation capabilities.

The results from the western blotting experiments carried out in this chapter show that two distinct signalling mechanisms are altered when CCL-61 and CHO-SA cells are cultured in both adherent and suspension conditions.

The first signalling mechanism involves integrin-mediated adhesion to the ECM. The western data generated appears to show that when non-suspension adapted CCL-61 and suspension adapted CHO-SA cells (reversion phenotype) are cultured in adherent conditions, a higher level of signalling flux via the Ras/MAPK pathway is observed compared to when the two cell lines are cultured in suspension conditions. Both CCL-61 and CHO-SA cells growing adherently show significantly increased levels of activation of key intermediates in the Ras/MAPK pathway including, c-Raf, MEK 1/2, Erk 1/2, MSK1 and p90RSK, compared to when the two cell lines are growing in suspension conditions. This is also reflected in the activation profile for Focal Adhesion Kinase (FAK), thus indicating that Ras/MAPK pathway activation is linked to the integrin-mediated activation of FAK.

The hypothesis for this signalling mechanism is that when integrin-mediated attachment to the ECM is intact (as occurs in adherent culture condtions), FAK is activated along with the downstream Ras/MAPK signalling pathway. When integrin-mediated attachment to the ECM is abolished (as occurs in suspension culture conditions) the levels of FAK activation are significantly down-regulated and the signalling flux via the Ras/MAPK is significantly reduced.

The second signalling mechanism involves the stimulation of the Ras/MAPK and PI3K/Akt signalling pathways in suspension conditions in response to the inclusion of added growth factors, in this case insulin.

In terms of PI3K/Akt pathway activation, CCL-61 cells show significantly higher levels of activation at many of the key intermediates in the PI3K/Akt pathway when they are growing adherently, compared to when they are growing in suspension. When non-suspension adapted CCL-61 cells, are transferred to suspension conditions without insulin, they display a minimal level of activation in terms of signalling flux via the Ras/MAPK or PI3K/Akt pathways. When insulin is added to the suspension culture media, CCL-61 cells show a minimal response in terms of activation of the Ras/MAPK pathway and they show only a slight response in terms of the activation of the PI3K/Akt pathway. By comparison, the suspension-adapted CHO-SA cells when growing in suspension with added insulin, display a significant increase in activation of many of the key intermediates in the PI3K/Akt compared to CHO-SA cells growing adherently. When insulin is removed from the suspension culture media, CHO-SA cells show significantly reduced levels of activation of key intermediates in the PI3K/Akt pathway compared to when insulin is present in the culture media. This level of insulin responsiveness of the suspension cultured CHO-SA cells, is only displayed in terms of activation of the PI3K/Akt pathway, as when CHO-SA cells are cultured with or without insulin in suspension conditions, there is only a slight response to the inclusion of insulin in terms of the activation of the Ras/MAPK pathway.

The hypothesis for this signalling mechanism is that when cells are growing in suspension, integrin-mediated activation of the Ras/MAPK and PI3K/Akt pathways is significantly reduced. Therefore, cells growing in suspension are relying upon other means of stimulating pathways responsible for cell growth, proliferation and survival. In this model system, the growth factor insulin is added to the growth media in order to stimulate such pathways. From the data obtained from this western analysis, it would appear that insulin signalling is mediated to a greater degree through the PI3K/Akt pathway rather than the Ras/MAPK pathway, as shown in the activation profile of CHO-SA cells growing with insulin in suspension. It would also suggest that growth factor mediated signalling via the Ras/MAPK pathway is reduced in suspension cells, but this is not the case for the PI3K/Akt pathway.

These two hypotheses are supported by evidence in the literature from work done by many other groups. In terms of Ras/MAPK activation, numerous reports have shown that growth factor-mediated Erk signalling is up-regulated in cells adhered to ECM proteins (Galownia et al., 2007; Lee and Juliano, 2004; Roovers et al., 1999; Schwartz and Assoian, 2001). While both FAK and Shc have been implicated in the activation of the Ras/MAPK signalling cascade when integrins, which are bound to the ECM, form complexes with the adaptor proteins talin, paxillin, vinculin as well FAK and Shc (Giancotti and Ruoslahti, 1999). Other work done previously by many research groups has shown that integrin mediated cell adhesion leads to the activation of the MAPK signalling cascade. Early research focusing on integrin-MAPK signalling, postulated that integrin-mediated adhesion or integrin clustering at the cell surface can activate both the tyrosine kinase FAK as well as the MAPK cascade (Lin et al., 1997).

As reported in the review by Mendoza et al, the Ras/MAPK and PI3K/Akt pathways are activated by different sets of mitogens/agonists only some of which overlap (Mendoza et al., 2011). PMA (phorbol 12-myristate 13-acetate) has been shown to be a strong activator of the Ras/MAPK pathway. Whereas, insulin and insulin growth factor-1 (IGF1) are weaker activators of the

Ras/MAPK but strong activators of the PI3K/Akt pathway (Clerk et al., 2006; Mendoza et al., 2011; Weng et al., 2001).

In summary, the results generated from the characterisation and western blot analysis of CCL-61 and CHO-SA cells growing in adherent and suspension conditions, has provided a valuable insight into how cells alter their signalling pathway activation profiles in response to their culture environments. Both CCL-61 and CHO-SA cells growing adherently are able to activate both the Ras/MAPK and PI3K/Akt pathways to a sufficient level in order for the cells to grow, proliferate and survive in those particular culture conditions. When un-adapted CCL-61 cells are transferred to suspension conditions, they are unable to utilise added growth factors (insulin) to activate the PI3K/Akt pathway to a sufficient level for adequate levels of cell growth. In contrast, the suspension-adapted CHO-SA cells do posses the ability to utilise added insulin, to activate the PI3K/Akt pathway to a sufficient level where cells are able to grow and proliferate as expected. This difference in growth factor responsiveness between non-suspension adapted and suspension adapted cells, is perhaps one of the key underlying mechanisms which has been unwittingly selected for by taking the CCL-61 cells through rounds of suspension adaptation to generate the CHO-SA cell line.

Chapter 5

Signalling pathway analysis of CHO cell lines growing in suspension conditions

Acknowledgements

The data, figures and text in the following chapter are the work of the candidate.

Chapter 5 Aims

The main aim of this chapter is to analyse the signalling pathway activation of different CHO cell lines growing in serum-free suspension conditions. The relative activation status of both the Ras/MAPK and PI3K/Akt pathways will be mapped in three different cell lines that have been through varying rounds of adaptation to suspension cell growth.

5.1 Introduction

The ability of CHO cells to grow in serum-free suspension conditions in large scale bioreactors, is one the key principles that underpins the process of recombinant protein production from this cell line. As CHO cells typically grow as an adherent monolayer in the presence of serum, this synthetic environment should not be conducive for high levels of CHO cell growth and proliferation.

As described previously, many of the CHO cell lines that are currently used today in the biopharmaceutical industry are originally descended from the parental CHO-K1 (CCL-61) adherent cell line. In order to generate cells that were capable of proliferating and surviving in suspension conditions, long-winded empirical adaptation strategies were used where cells were gradually adapted over time to grow in such synthetic conditions (O'Callaghan and James, 2008; Sinacore et al., 2000).

In this study, the CCL-61 cell line was adapted over-time to generate the suspension cell line, CHO-SA which grows in suspension conditions in cell culture media that is supplemented with insulin. As discussed previously, insulin is most frequently added growth factor that is added to serum-free suspension CHO cell cultures in order to stimulate cell growth and proliferation and maintain high levels of cell viability (Morris and Schmid, 2000). The CHO-SA cell line was then taken through further rounds of adaptation to generate the Mab producing cell lines Cell Line (CL1) and Cell Line (CL2), as shown in figure 5.1.
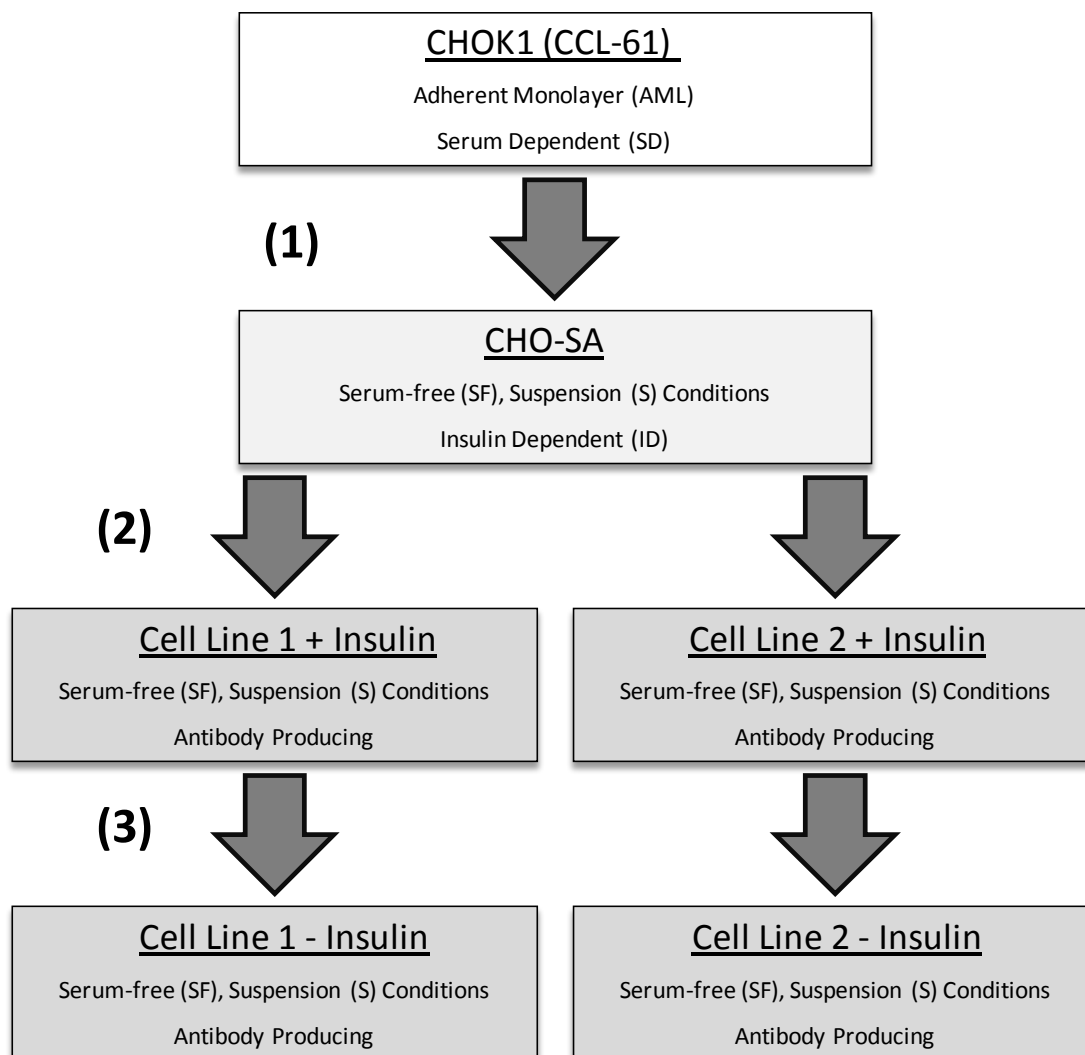
Figure 5.1: Cell line history of suspension cell lines received from Pfizer. The suspension cell lines received from Pfizer and used in this chapter are all descended from the parental adherent CHO-K1 (CCL-61) cell line. (1) As described previously, CCL-61 cells were adapted using a 3 step adaptation strategy to generate the suspension cell line, CHO-SA. (2) CHO-SA cells were split two ways and were transfected to become stable Mab producers, generating Cell Line 1 and Cell Line 2. The cells were transferred to new media, proprietary suspension media 2. (3) Cell Line 1 and Cell Line 2 cells were adapted to grow in PSM2 without added insulin.

The CL1 and CL2 cell lines were adapted over time to grow at higher viable densities and were then transfected to become stable Mab producing cell lines.

In order to analyse the changes that have occurred in signalling pathway activation over the course of these adaptations, cell lines that have undergone various levels of adaptation were compared in their ability to grow in serum-free suspension conditions with and without insulin. The cell lines selected were the non-suspension adapted CCL-61 cells, the suspension adapted CHO-SA cell line, the Mab producing cell lines CL1 and CL2 and the non-related cell line CHO-S. CHO-S was used as an example of a typical CHO suspension cell line with which cell growth characteristics of the other cell lines could be compared against.

The first stage of analysis involved looking at the cell growth and viability characteristics of the various cell lines growing in suspension conditions with and without insulin. The second stage of analysis involved mapping the signalling pathway activation status of three of the cell lines via western blotting analysis.

5.2 Methodology

5.2.1 Suspension characterisation

To analyse the growth characteristics of the nine cell lines in suspension conditions, all cell lines were harvested at day 3 of culture (growing in their standard conditions). All cell lines were washed in PBS and then seeded into standard suspension conditions and batch cultured for 12 days. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser.

5.2.2 Harvesting suspension cells

For the western immunoblotting analysis all nine cell lines that were cultured in suspension conditions were harvested at three separate time points, exactly 72 hours in culture (day 3), 144 hours in culture (day 6) and 216 hours in culture (day 9). As described in detail in materials and methods section 2.2, approximately 5×10^6 cells were harvested from each flask at each time point and cells were lysed and proteins extracted using RIPA buffer.

5.2.3 Calculating protein content from cell extracts

As described in materials and methods section 2.2.4 the BCA total protein assay was used to calculate the total protein from every protein extraction performed. The results from the BCA assay were then used to calculate the amount of each extract to load into each well of the SDS-PAGE gel to give a loaded concentration of 25 µg of protein per well.

5.2.4 SDS-PAGE and western immunoblotting

All the western blots shown in this chapter were performed using the method given in materials and methods section 2.3. All western immunoblots that are shown in this chapter are representative of three repeated blots for each protein/phospho-protein of interest. As shown in section 4.3.1, all western immunoblots performed included a biotinylated protein ladder to confirm that the bands observed are approximately at the correct molecular weight for the target protein.

Cell Line	Growth Conditions	Other
CHO-K1 (CCL-61) + Insulin	PSM 1+ 10 mg/ L Insulin	Non-producer
CHO-K1 (CCL-61) - Insulin	PSM1 - 10 mg/ L Insulin	Non-producer
CHO-SA + Insulin	PSM1 + 10 mg/ L Insulin	Non-producer
CHO-SA - Insulin	PSM 1- 10 mg/ L Insulin	Non-producer
Cell Line 1 + Insulin	PSM2 + 10 mg/ L Insulin	Antibody producing, DHFR expression system
Cell Line 1 - Insulin	PSM2 – 10 mg/ L Insulin	Antibody producing, DHFR expression system
Cell Line 2 + Insulin	PSM2 + 10 mg/ L Insulin	Antibody producing, DHFR expression system, Clumpy cell line
Cell Line 2 - Insulin	PSM2 – 10 mg/ L Insulin	Antibody producing, DHFR expression system, Clumpy cell line
CHO-S	CD-CHO	8mM L-Glutamine and 1% H/T supplementation

Table 5.1: Outline of suspension cell line(s) culture conditions. CCL-61 and CHO-SA cells were cultured in suspension conditions in PSM1 with and without insulin added. Cell Line 1 and Cell Line 2 were cultured in PSM2 with and without added insulin, both cell lines are Mab producers. Cell Line 2 is a noticeably "clumpy" cell line with cells aggregating together in suspension culture. CHO-S was used as a standard suspension cell line with which to compare the other cell lines against.

5.3 Results

5.3.1 Growth characterisation results of CHO cell lines growing in suspension culture conditions

Before analysing the signalling pathway activation of the nine cell lines in suspension culture, it was imperative to carry out growth characterisation studies. Figures 5.2 to 5.5 show the results of these growth characterisation studies.

The through culture viability and viable cell density of the nine cell lines growing in suspension is shown in figure 5.2. As shown in figure 5.2.A, there is substantial variability between the cell lines in terms of their viability through culture. Most of the cell lines maintain a viability of >90% up until 168 hours in culture, after which a drop in viability to less than 90% is observed. Two noticeable exceptions to this are the CCL-61 – Ins cell line and the CHO-SA + Ins cell line. The non-suspension adapted CCL-61 cell line growing without insulin displays a substantial drop in viability from 48 hours onwards. While the suspension adapted CHO-SA cell line cultured with insulin drops below 90% viability after 120 hours in culture.

Figure 5.2.B shows the viable cell density of the nine cell lines throughout suspension culture. Again there is substantial variability between the growth capabilities of the different cell lines. The non-suspension adapted, CCL-61 + Ins and CCL-61 - Ins cells, by comparison to the other cell lines, reach very low maximum viable cell densities (VCD) of less than 1×10^6 cell/ mL^{-1}. The suspension adapted, CHO-SA cell line growing with insulin reaches a significantly higher maximum VCD of 5.35×10^6 cell/ mL^{-1}, however when insulin is removed from the culture, a substantial drop in the maximum VCD reached is observed. The Mab producing cell lines, Cell Line 1 and Cell Line 2, that were adapted from the CHO-SA cell line, all reach maximum VCDs that are above 9×10^6 cell/ mL^{-1}. In each case for Cell Lines 1 and 2, the cells growing with insulin reach slightly higher cell densities than the cell lines growing without insulin. The CHO-S cell line reached the highest maximum VCD of 15.79×10^6 cell/ mL^{-1}.

A)

B)
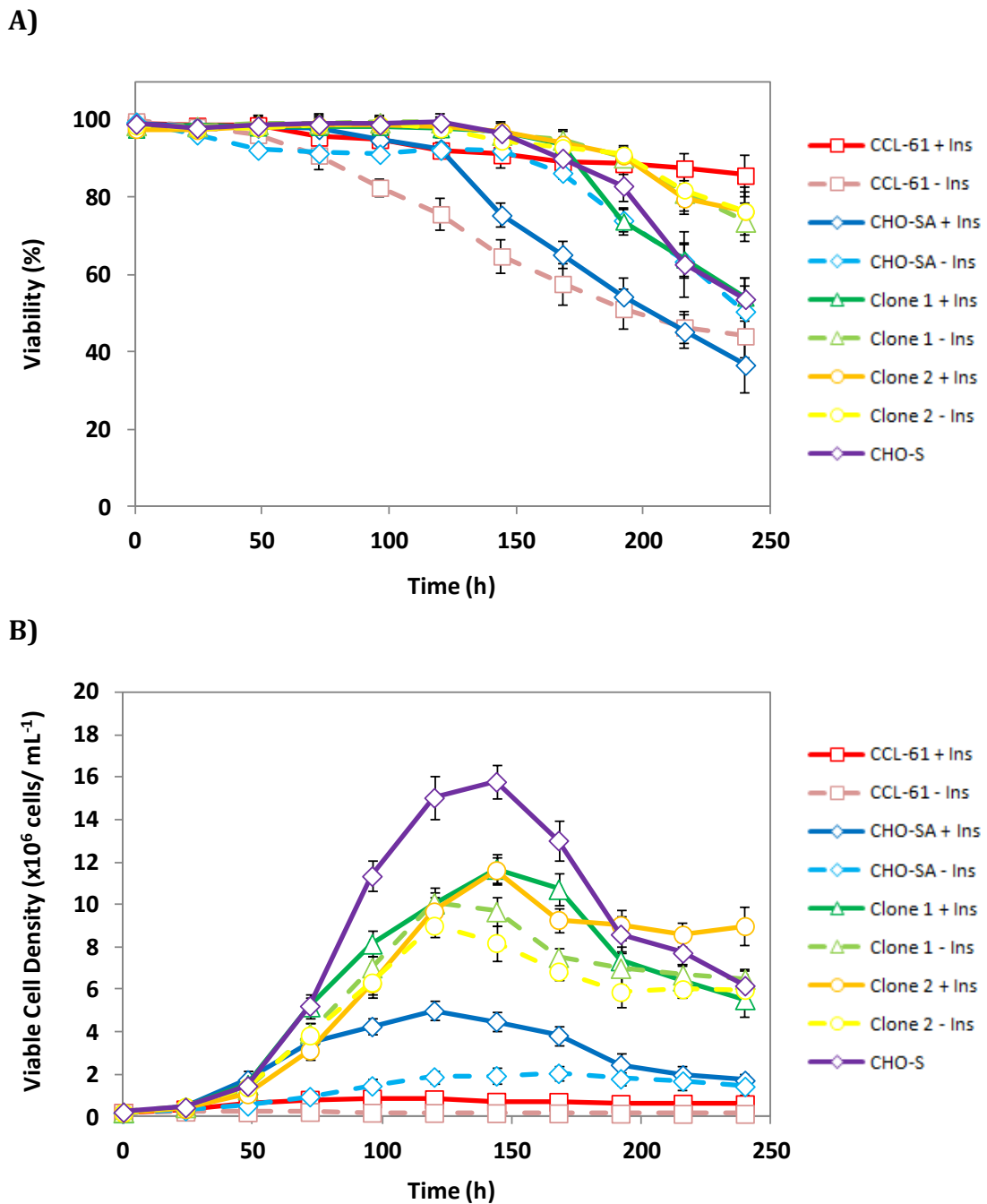
Figure 5.2: Characterisation results of the viability and viable cell density of the nine cell lines growing in suspension culture conditions. All cell lines were batch cultured in 500 mL Erlenmeyer flasks for 240 hours in standard suspension growth conditions. A) The viability of all 9 cell lines through culture. B) The viable cell density of all nine cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

Figure 5.3.A, shows the cell specific growth rate (μ) of the various cell lines throughout culture. The CCL-61 + Insulin, CCL-61 – Insulin and CHO-SA – Insulin cells, display a different trend to the other cell lines in that they display no significant increase in μ in the first few days in culture and after that they display a slow gradual decline in growth rate. The CHO-SA + Insulin cell line shows a large increase in μ between 24 and 48 hours in culture after which a substantial and sharp decline in growth rate is observed. All the other cell lines follow a general trend, in that an increase in μ is observed up until 72 hours in culture, after which a decline in growth rate is observed.

Figure 5.3.B shows the IVCC of the various cell lines throughout culture. From these results it is clear to see that there is substantial variation between the cell lines. From these IVCC results, the various cell lines could be classed into three groups, "Low Growers", "Medium Growers" and "High Growers". The CCL-61 + and – Insulin cell lines, as well as the CHO-SA – Insulin cell lines could be classed as low growers as they have a final IVCC that is substantially lower than all the other cell lines. The CHO-SA + Insulin cell line could be classed as a medium grower as it reaches a final IVCC that is approximately half way between the low growers and high growers. By comparison the Cell Line 1 and 2 cell lines as well as the CHO-S cell line reach significantly higher IVCCs and therefore could be classed as high growers.

Figure 5.4.A shows the cell volume of the various cell lines throughout suspension culture. Most of the cells display a general trend in that an initial increase in cell volume is observed at the start culture, before a decline in volume is observed. During the last few days in culture the cell volume of the cell lines levels off. By comparison the CCL-61 and CHO-SA cells growing without insulin, show an initial decrease in volume at the start of culture followed by a long period where the cell volume remains fairly constant. Figure 5.4.B shows the protein content off the cell lines through culture. In a similar manner to the cell volume, most of the cell lines show an initial increase in protein content at the start of culture before a decline phase is observed. Again the CCL-61 cell line

A)

B)

Figure 5.3: Characterisation results of the cell specific growth rate and IVCC of the nine cell lines growing in suspension culture conditions. All cell lines were batch cultured in 500 mL Erlenmeyer flasks for 240 hours in standard suspension growth conditions. A) The cell specific growth rate (μ) of all 9 cell lines through culture. B) The IVCC of all nine cell lines through culture. The cell specific growth rate and IVCC were calculated using the viable cell density. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

A)

B)

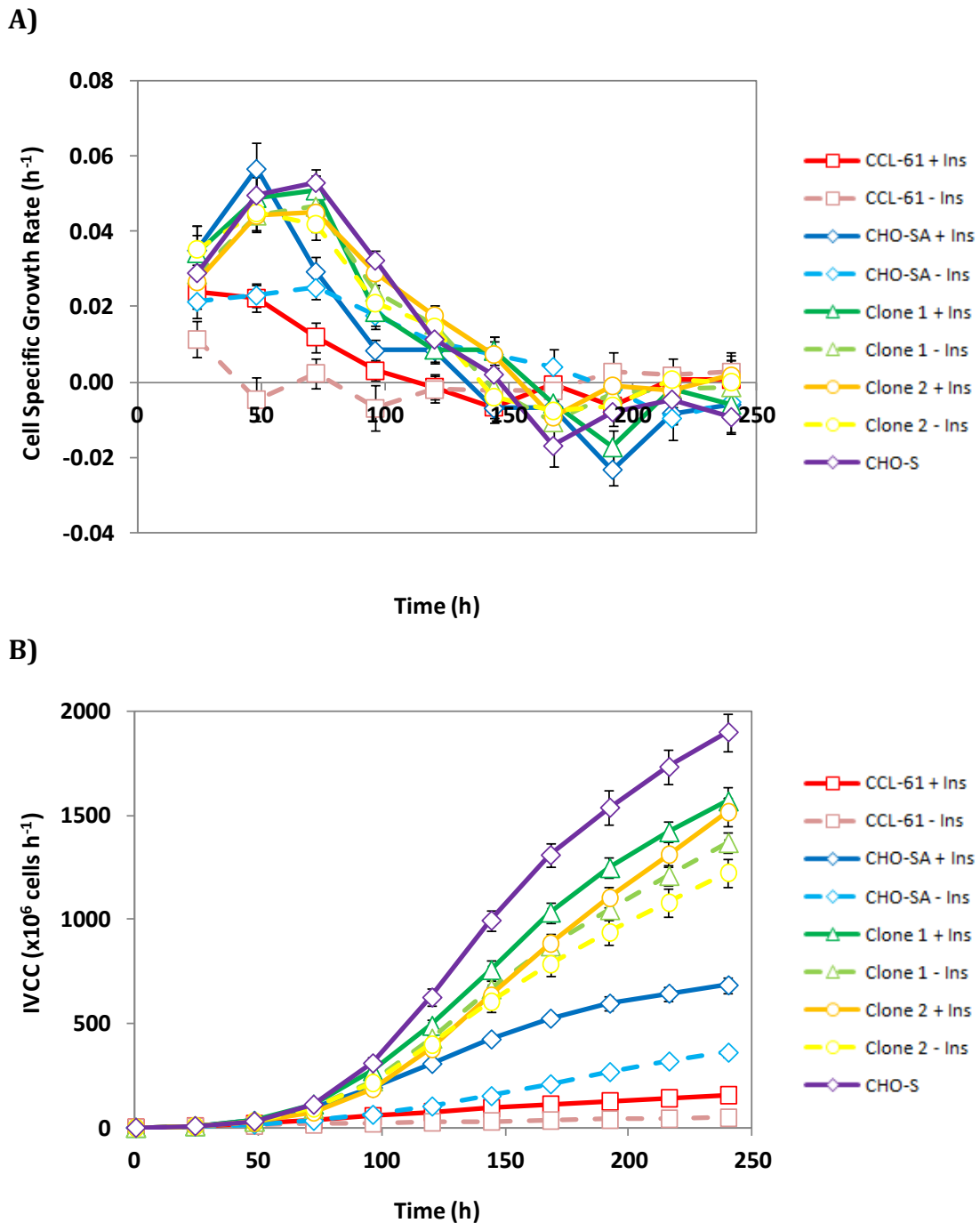**Figure 5.4: Characterisation results of the cell volume and protein content of the nine cell lines growing in suspension culture conditions.** All cell lines were batch cultured in 500 mL Erlenmeyer flasks for 240 hours in standard suspension growth conditions. A) The cell volume of all 9 cell lines through culture. B) The cellular protein content of all nine cell lines through culture. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.
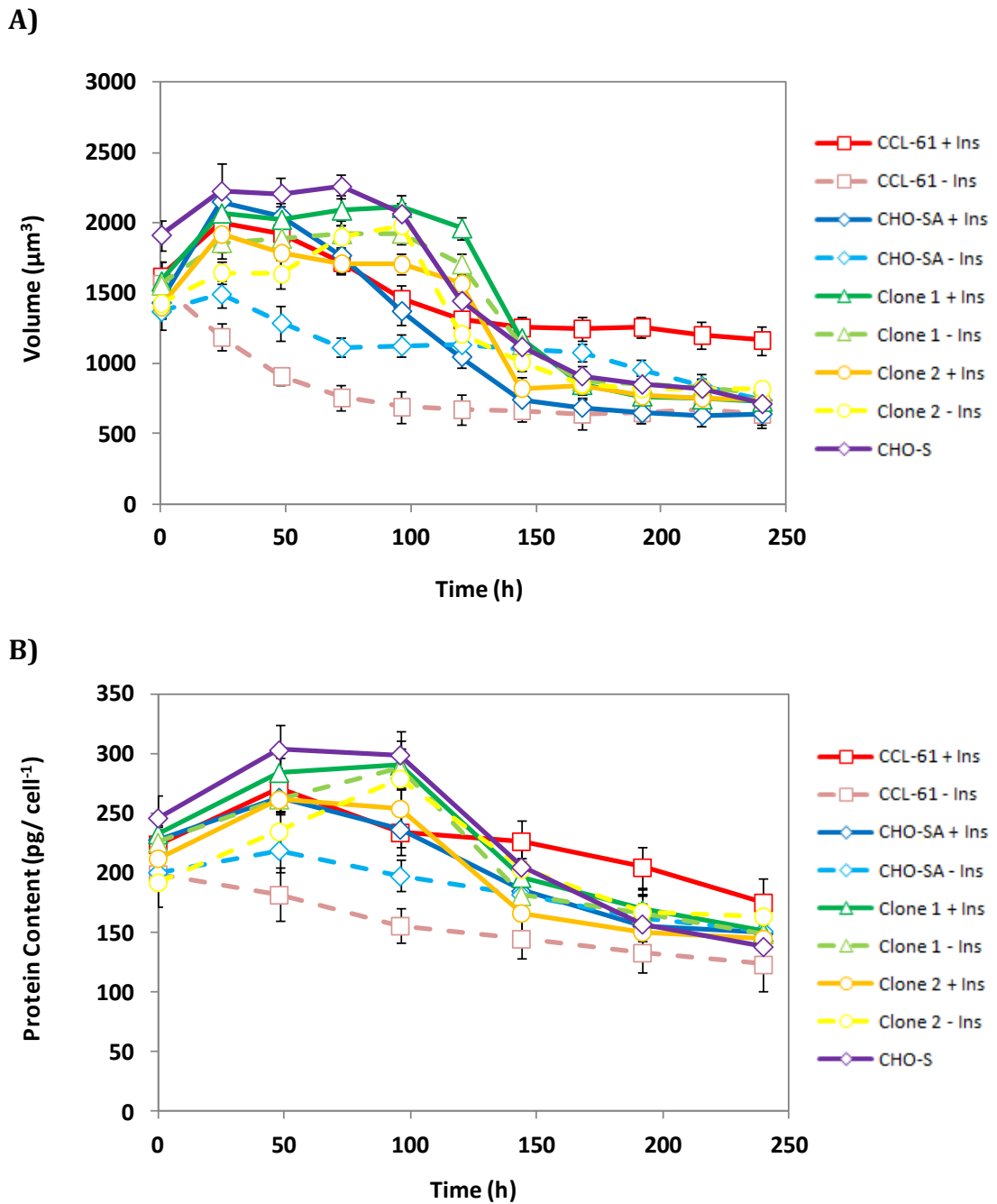
growing without insulin does not follow this trend in that it's cellular protein content declines from the start of culture.

Cell Line	Max. VCD (x10^6 cells/ mL^{-1}) (±S.D.)	Max. μ (±S.D.)	Average μ (h^{-1}) Day 0-6	IVCC (x10^6 cells h^{-1}) (±S.D.)
(CCL-61) + Insulin	0.89 (0.09)	0.025 (0.005)	0.0088 (0.012)	157.12 (9.62)
(CCL-61) - Insulin	0.24 (0.04)	0.012 (0.003)	0.0003 (0.006)	48.20 (4.27)
CHO-SA + Insulin	5.35 (0.30)	0.057 (0.007)	0.0217 (0.022)	685.20 (28.89)
CHO-SA - Insulin	1.85 (0.19)	0.025(0.004)	0.0155 (0.007)	209.32 (18.46)
Cell Line 1 + Insulin	11.68 (0.62)	0.052 (0.004)	0.0283 (0.019)	1569.93 (57.10)
Cell Line 1 - Insulin	10.10 (0.54)	0.046 (0.005)	0.0262 (0.018)	1369.60 (50.91)
Cell Line 2 + Insulin	11.62 (0.55)	0.045 (0.004)	0.0283 (0.014)	1517.92 (56.94)
Cell Line 2 - Insulin	9.10 (0.48)	0.045 (0.003)	0.0256 (0.018)	1226.24 (55.70)
CHO-S	15.79 (0.75)	0.053 (0.004)	0.0295 (0.020)	1902.08 (79.21)

Table 5.2: Table showing the maximum viable cell density, maximum μ, average μ (days 0-6) and IVCC of the nine cell lines growing in suspension conditions. All cell lines were batch cultured in 500 mL Erlenmeyer flasks for 240 hours in standard suspension growth conditions. Samples for Vi-Cell analysis were taken every 24 hours. From the viable cell density readings obtained the cell specific growth rate (μ) and IVCC were calculated. The data presented are the average values of triplicate samples analysed in triplicate. Standard deviation is given in the brackets.

5.3.2 Analysis of the activation of the Ras/MAPK and PI3K/Akt signalling pathways in suspension cultured CHO cell lines

From the initial characterisation results conducted, three of the CHO cell lines were selected in order to conduct further analysis in regards to their signalling pathway activation status. The three cell lines selected were: the non-suspension adapted CCL-61 cell lines growing with insulin, as this cell line has not been adapted to grow in suspension culture conditions and displays low levels of growth in suspension culture conditions. The next cell line selected was the CHO-SA cell line growing with insulin, as this cell line has been adapted to grow in suspension conditions but it does not reach as high a viable cell density as the other suspension cell lines. The last cell line selected was Cell Line growing with insulin, as this cell line reaches a significantly higher viable cell density than the other two cell lines. Cell Line 1 (CL1) is also one of Pfizers Mab producing platform cell lines, therefore the analysis will be comparing a non-suspension adapted cell line (CCL-61), a suspension adapted non-producing cell line (CHO-SA) and a high growing Mab producing cell line (Cell Line 1). All the cell lines were cultured with insulin in order to analyse how cell lines that have been through various rounds of adaptation to suspension culture conditions respond to the inclusion of growth factors in the cell culture media.

Figure 5.6 shows the relative activation levels of PI3K in the three cell lines cultured in suspension conditions. As the western blots and densitometry data shows, at the key phosphorylation site phospho-PI3K p85 (Tyr458), there is a significant increase in activation between the CCL-61 and CHO-SA cells ($p<0.01$) and an even greater increase in activation between CCL-61 and CL1 cells ($p<0.0001$). This increase in activation of PI3K at this phosphorylation site is interesting as PI3K is a key upstream signalling intermediate in the PI3K/Akt signalling network. Downstream of PI3K, lies another key signalling intermediate in the PI3K/Akt pathway, in Akt. The activation of Akt is regulated by two key phosphorylation sites, Ser473 and Thr308. Figure 5.7 shows the relative activation of Akt of the three cell lines growing in suspension culture conditions.

A)
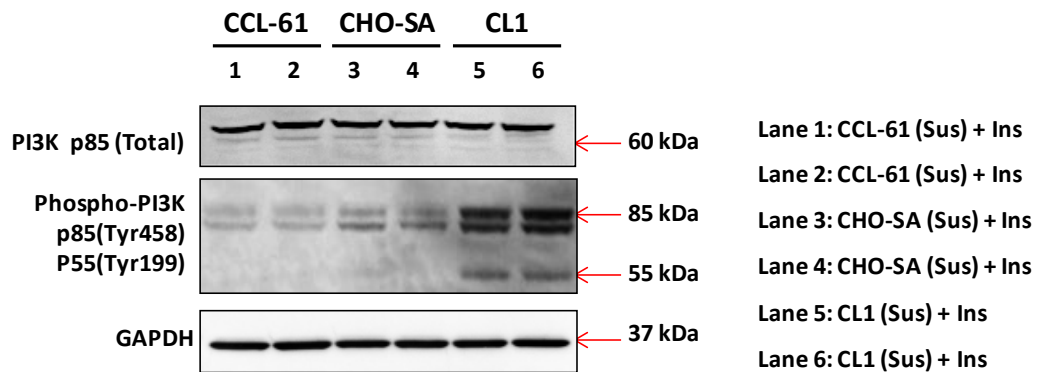
B)
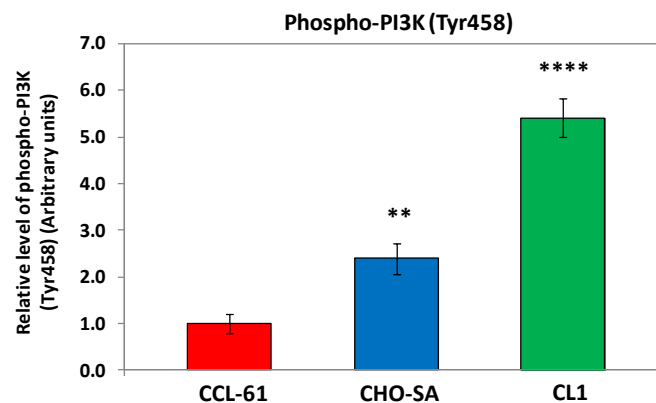
Figure 5.6: Western blotting analysis of the total PI3 Kinase p85 content and phospho-PI3K p85 (Tyr458) p55 (Tyr199) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-PI3K p85 (Tyr458) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
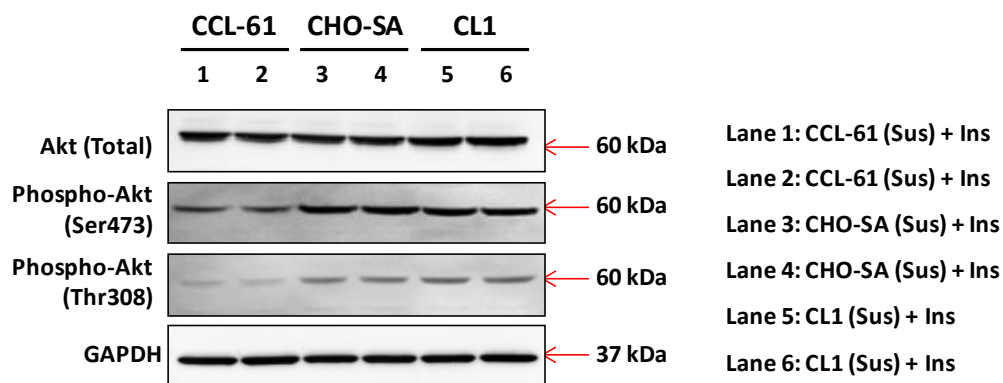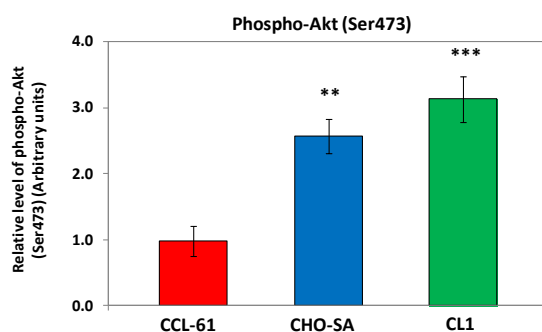
B)   **C)**
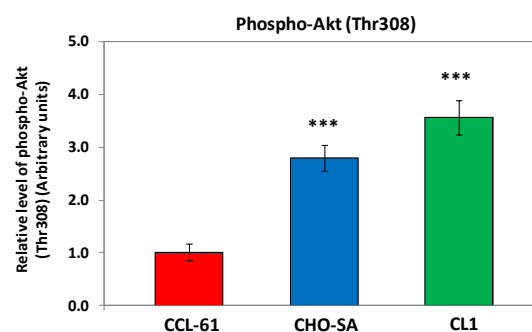
Figure 5.7: Western blotting analysis of the total Akt content and phospho-Akt (Ser473) and phospho-Akt (Thr308) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-Akt (Ser473) and C) Phospho-Akt (Thr308) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

As observed in the western blots and from the densitometry data, CCL-61 cells growing in suspension show relatively low levels of activation at both of these key Akt activation sites. Focusing on activation site Ser473, there is a significant increase in phosphorylation between the CCL-61 and CHO-SA cells ($p < 0.01$) and an even greater increase between the CCL-61 and CL1 cells ($p < 0.001$). This increase in activation is mirrored in the activation profile at site Thr308. Both CHO-SA and CL1 cells show a significant increase, compared to CCL-61, in phosphorylation at this key activation site, (CHO-SA, $p < 0.001$, CL1, $p < 0.001$). This confirms that Akt in a similar manner to PI3K, is activated to a higher degree in CHO-SA cells compared to CCL-61 and that CL1 cells show even higher levels of activation of these two key signalling intermediates.

Further downstream of PI3K and Akt, lies the signalling intermediate mTOR. mTOR is also regulated via phosphorylation at two key sites, Ser2448 and Ser2481. As observed in the upstream intermediates, PI3K and Akt , there is a significant difference in the relative activation between the three different cell lines. CCL-61 cells growing in suspension show relatively low levels of phosphorylation at these two key activation sites. In comparison, CHO-SA cells show significantly higher levels of activation at both sites when compared to CCL-61 cells (Site Ser2448, $p < 0.01$, Site Ser2481, $p < 0.05$). CL1 cells show even higher levels of relative activation when compared to the CCL-61 cells than the CHO-SA cells (Site Ser2448, $p < 0.001$, Site Ser2481, $p < 0.01$).

Figure 5.9, shows the relative activation levels of two intermediates involved in the regulation of translation, p70 S6K and the S6 ribsomal protein. The p70 S6K protein is regulated by phosphorylation at the key activation site Thr389. At this activation site, CCL-61 cells show relatively low levels of phosphorylation when compared to CHO-SA and CL1 cells growing in suspension. CHO-SA cells show a significant increase in activation at this site when compared to CCL-61 cells ($p < 0.001$). CL1 cells also show a significant increase in activation at this site compared to CCL-61 cells ($p < 0.001$). In terms of activation of the S6 ribosomal protein, a similar pattern of activation between the three cell lines is observed.

A)
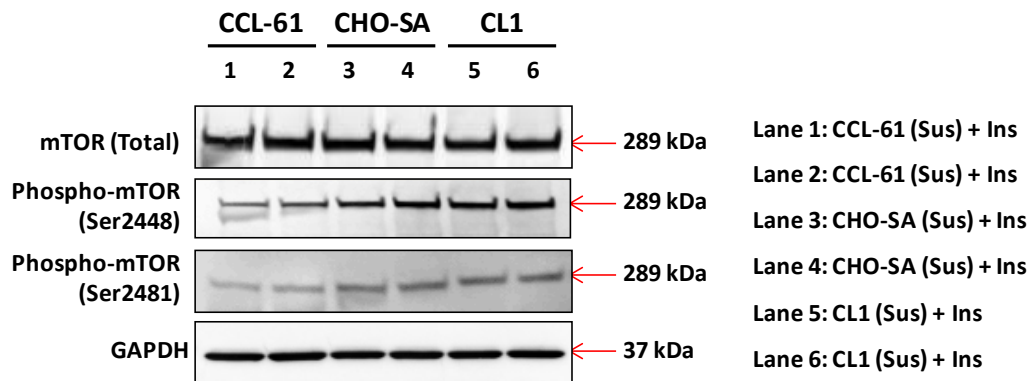
B)
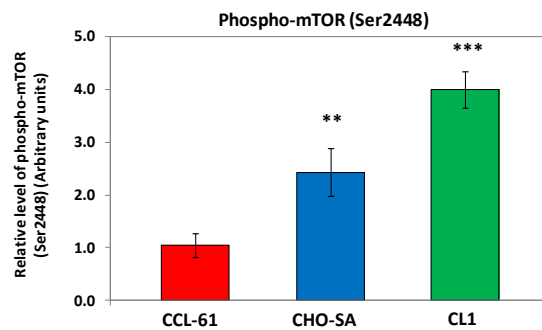
C)
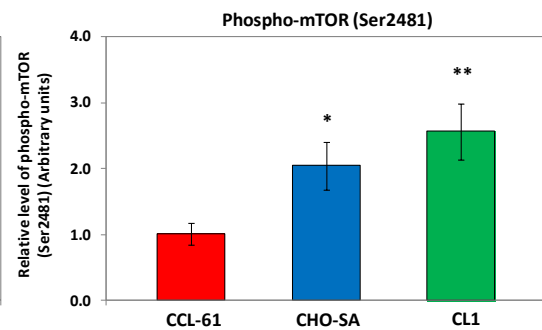
Figure 5.8: Western blotting analysis of the total mTOR content and phospho-mTOR (Ser2448) and phospho-mTOR (Ser2481) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-mTOR (Ser2448) and C) Phospho-mTOR (Ser2481) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
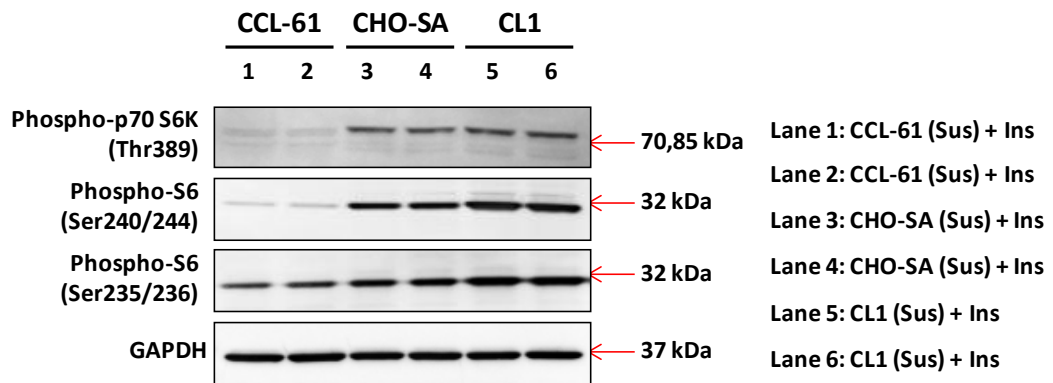
B)
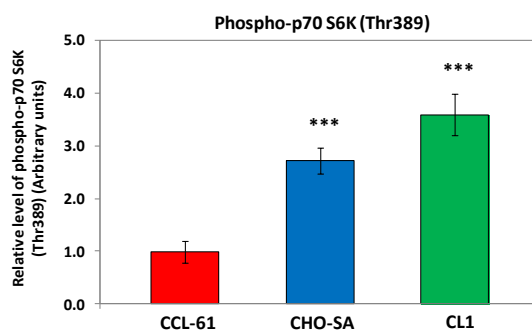
**C)**

D)
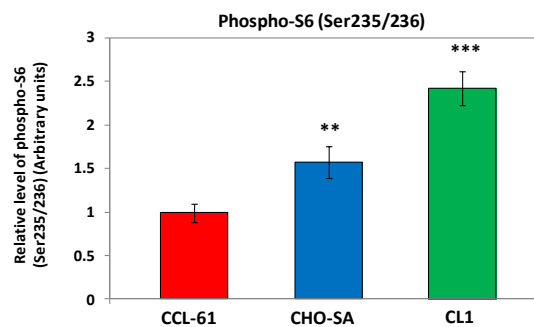
Figure 5.9: Western blotting analysis of the phospho-p70 S6K (Thr389) levels and phospho-S6 (Ser240/244) and phospho-S6 (Ser235/236) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-p70 S6K (Thr389), C) Phospho-S6 (Ser240/244) and D) Phospho-S6 (Ser235/236) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

At the key activation site Ser240/244, CHO-SA cells have a significantly higher level of phosphorylation than CCL-61 cells growing in suspension ($p<0.0001$). CL1 cells growing in suspension also show a significant increase in activation at this key phospho site, compared to CCL-61 cells ($p<0.0001$). This increase in activation is mirrored at the phosphorylation site Ser235/236, with CHO-SA cell showing again showing a significantly higher level of activation at this site compared to CCL-61 cells ($p<0.01$). An even more significant increase in activation at site Ser235/236 is observed between CCL-61 and CL1 cells growing in suspension ($p<0.001$).

Following the analysis of the activation status of key signalling intermediates in PI3K/Akt pathway and the related downstream translational control proteins, the next step was to analyse the activation status key intermediates in the Ras/MAPK pathway. Figure 5.10 shows the activation levels between the three cell lines of the key upstream signalling intermediate in the Ras/MAPK pathway, c-Raf. At the key phosphorylation activation site Ser338, both the CHO-SA ($p<0.05$) and CL1 ($p<0.01$) cells show significantly higher levels of activation when compared against the CCL-61 cells growing in suspension. This increase in phosphorylation is also observed at the inhibitory binding site Ser259. Again, both CHO-SA ($p<0.05$) and CL1 ($p<0.01$) cells show significantly higher levels of phosphorylation, compared to the CL-61 cells, at this phosphorylation site. This observation is interesting as, the level of phosphorylation at this inhibitory binding site is thought to be regulated by Akt. This is one of numerous examples where cross-talk inhibition is involved in the regulation of the Ras/MAPK and PI3K/Akt pathways. At the c-Raf hyperphosphorylation site (Ser289/296/301), the CHO-SA cells show a slight but not significant increase in activation when compared to the CCL-61 cells. By comparison a significant increase in activation is observed between the CCL-61 and CL1 cells ($p<0.05$).

Downstream of c-Raf lies another key intermediate(s) in the Ras/MAPK pathway, in Erk 1/2. The Erk 1/2 proteins are regulated by phosphorylation at two key sites, Thr202/Tyr204 of Erk1 and Thr185/Tyr187 of Erk2. Phosphorylation at

A)
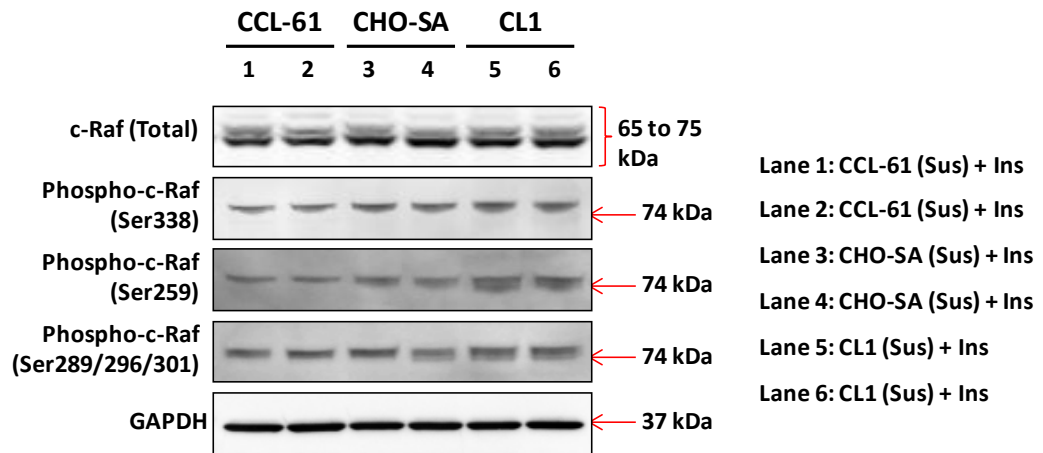
B)
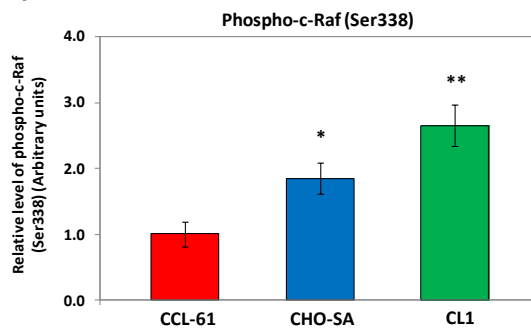
C)
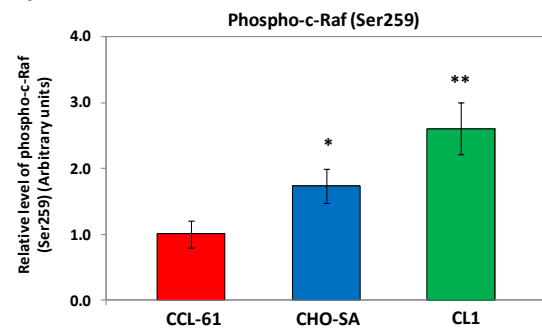
D)
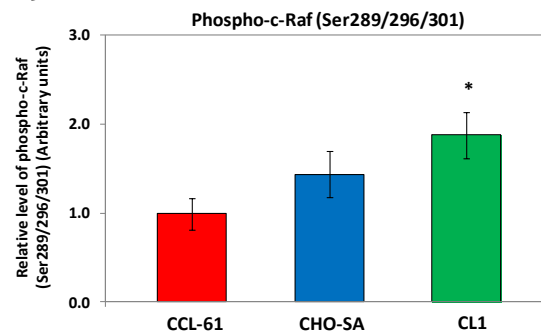
Figure 5.10: Western blotting analysis of the c-Raf (Total) content and phospho-c-Raf (Ser338), phospho-c-Raf (Ser259) and phospho-c-Raf (Ser289/296/301) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-c-Raf (Ser338), C) Phospho-c-Raf (Ser259) and D) Phospho-c-Raf (Ser289/296/301) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

A)
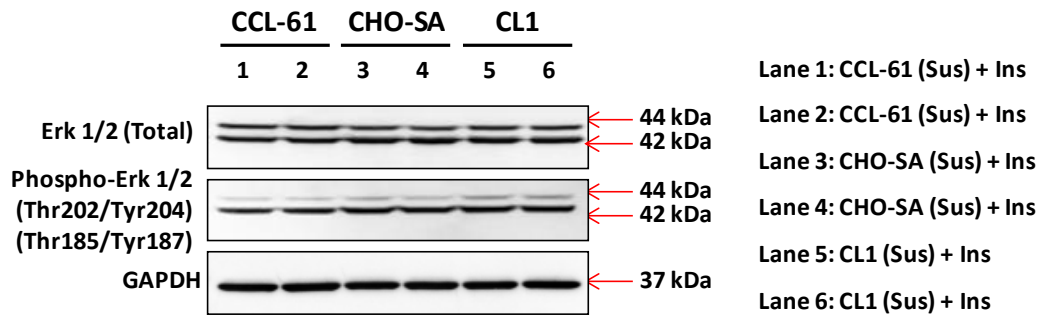
B)
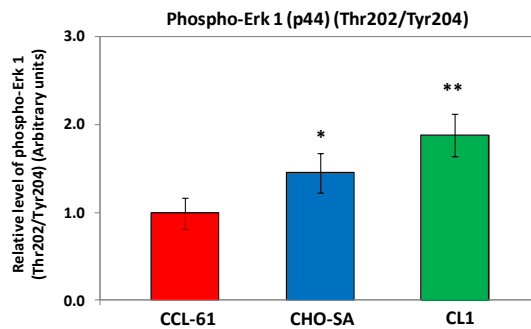
C)
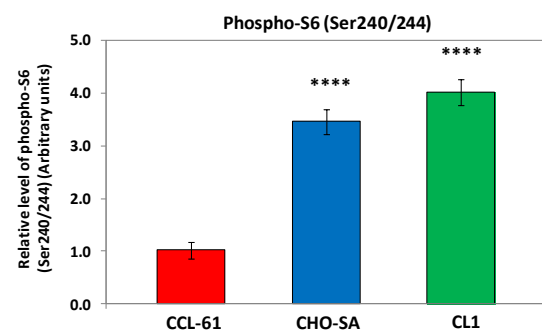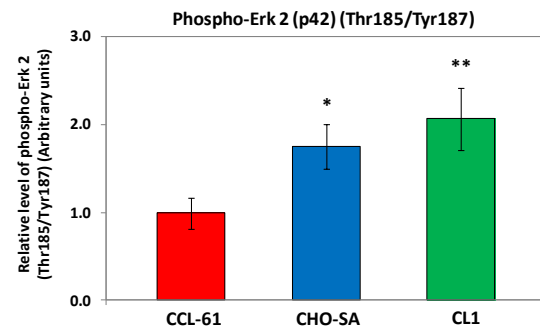

Figure 5.11: Western blotting analysis of the Erk 1/2 (Total) content and phospho-Erk1 (Thr202/Tyr204) and phospho-Erk2 (Thr185/Tyr187) levels of the CCL-61, CHO-SA and CL1 cell lines growing in suspension conditions. Extracts from the three cell lines growing in suspension conditions were taken at 72 hours in culture (mid-exponential phase). A) The western blots shown above are from one representative experiment. All western blots were performed three times. B) Phospho-Erk1 (Thr202/Tyr204) and C) Phospho-Erk2 (Thr185/Tyr187) levels were calculated relative to the level of the CCL-61 cell lines growing in suspension conditions. GAPDH was used as a loading control. * = significant difference from CCL-61 in suspension conditions, * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Data points are $\pm$ S.D. (n=3).

these two key sites is responsible for regulating the levels of downstream flux from Erk 1/2. As can be seen in figure 5.10, there is a significant increase at both of these key phosphorylation sites when comparing CHO-SA cell growing in suspension to CCL-61 cells (Site Thr202/Tyr204, ($p<0.05$), Site Thr185/Tyr187, ($p<0.05$)). In comparison, CL1 cells show even higher levels of activation at both these key phosphorylation sites when compared to CCL-61 cells (Site Thr202/Tyr204, ($p<0.01$), Site Thr185/Tyr187, ($p<0.01$)). These results indicate that there is a higher level of signalling flux occurring through the Ras/MAPK pathway in both CHO-SA and CL1 cells cultured in suspension conditions compared to CCL-61 cells.

Signalling Intermediate	Key Phospho-Site(s)	Regulation		CCL-61 (Sus)	CHO-SA (Sus)	CL1 (Sus)
PI3K	p85 (Tyr458)	+ve		-	**	****
Akt	Ser473	+ve		-	**	***
	Thr308	+ve		-	***	***
mTOR	Ser2448	+ve		-	**	***
	Ser2481	+ve		-	*	**
c-Raf	Ser338	+ve		-	*	**
	Ser259	-ve		-	*	**
	Ser289/296/301	+ve		-		*
Erk 1/2	Erk 1 (Thr202/Tyr204)	+ve		-	*	**
	Erk 2 (Thr185/Tyr187)	+ve		-	*	**
p70 S6K	Thr389	+ve		-	***	***
S6 Ribsomal Protein	Ser240/244	+ve		-	****	****
	Ser235/236	+ve		-	**	***

Table 5.2: Tabular overview of the activation of key intermediates in the PI3K/Akt, Ras/MAPK and translational control pathways in CCL-61, CHO-SA and CL1 cells growing in suspension conditions. Signalling intermediates are listed in the first column, with the key regulatory phosphorylation site(s) of that intermediate listed in second column. The type of regulation of that particular phosphorylation site is given in column three. A traffic light system is used to denote the significant increase or decrease in activation in suspension compared to CCL-61 cells in suspension conditions. The activation of each intermediate in CCL-61 cells in suspension conditions is given an amber colour and (-) symbol as this is used in each case as the relative activation control. Green = significant activation increase, Amber = no significant increase or decrease, Red = significant decrease. * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

5.4 Discussion

As discussed previously, the underlying changes in signalling pathway activation that allow CHO cells to survive in a serum-free synthetic environment, free of integrin-mediated attachment is poorly understood. One of the main aims of this chapter was to decipher the levels of activation in the Ras/MAPK and PI3K/Akt pathways of different CHO cell lines growing in suspension conditions.

The characterisation work done in section 5.3.1 showed that CHO cells that have gone through various rounds of adaptation, are able to grow, proliferate and survive in suspension conditions to significantly different levels. Cells that have not been through rounds of suspension adaptation, such as the CCL-61 cells, display very low levels of growth in suspension conditions. By comparison, the CHO-SA cells that have been through one adaptation stage, are able to grow and proliferate to a higher level than the CCL-61 cells, but only in the presence of an added growth factor. These cells could therefore be classed as semi-suspension adapted cells. This is because, cells that are descended from CHO-SA cells, in this case the CL1 and CL2 cell lines, have been through even more rounds of adaptation to give a cell phenotype that is able to reach even higher cell densities and maintain a higher viability throughout culture. Therefore these cells could be classed as fully adapted suspension cell lines.

In order to characterise the signalling pathway flux occurring in these CHO cell lines that have been through various rounds of adaptation, three cell lines were chosen to take forward for further analysis via western blotting. The cell lines selected were the CCL-61, CHO-SA and CL1 cell lines as they have all been through different levels of adaptation. All the cell lines were cultured in the presence of insulin, in order to analyse their respective abilities to respond to an added growth factor.

As shown in results section 5.3.2, the activation status of the Ras/MAPK and PI3K/Akt signalling pathways was analysed by western immunoblotting. Cells were harvested at mid-exponential phase in culture (72 hours) in a similar

manner to the western analysis conducted in chapter 4. Key intermediates in both the Ras/MAPK and PI3K/Akt pathway were then selected for analysis.

From the western immunoblotting experiments, a clear pattern of activation is observed between the three different cell lines growing in suspension culture conditions. The non-suspension adapted CCL-61 cells, show relatively minimal levels of activation of either signalling pathway, when compared to the CHO-SA and CL1 cells. This is somewhat expected, as the CCL-61 cells usually grow as an adherent monolayer that requires surface attachment to the extra-cellular matrix to survive. Also as demonstrated in chapter 4, the CCL-61 cells when transferred to suspension conditions display lower levels of responsiveness to added insulin in the culture media compared to CHO-SA cells. By comparison, the semi-suspension adapted CHO-SA cell line, which is directly descended from the CCL-61 cell line shows significantly higher levels of activation in key intermediates in both the Ras/MAPK and PI3K/Akt pathways. This increase in activation level of signalling flux via the PI3K/Akt pathway is similar to the results seen in chapter 4, where CHO-SA cells were shown to increase their activation of key intermediates in the PI3K/Akt in response to the addition of insulin to the culture media. However the increase in activation of the Ras/MAPK between the CCL-61 cells and CHO-SA cells growing in suspension is not mirrored in the results observed in chapter 4. This is probably due to the method of normalisation used in chapter 4, where the relative activation levels of each intermediate, in each cell line were, normalised again that particular cell line growing in adherent conditions. When CCL-61 cells are directly compared against CHO-SA growing in suspension conditions (as performed in this chapter), there is a significant increase in signalling flux via this pathway. Although as table 5.2 two illustrates, the increase in signalling activation between the CCL-61 and CHO-SA cells growing in suspension is more pronounced in some of the intermediates in the PI3K/Akt pathway. This again indicates that one of the main signalling mechanisms that occurs when, CCL-61 cells are adapted over-time to generate the suspension cell line CHO-SA, is that cells are able to utilise added growth factors to the culture media by stimulating the PI3K/Akt pathway.

In terms of the CL1cell line, when compared to the CCL-61 cell line there is a significant increase in key pathway intermediates in both the PI3K/Akt and Ras/MAPK pathways. Again, in a similar manner to the CHO-SA cells, there is a larger increase in activation of intermediates in the PI3K/Akt pathway (particularly Akt and PI3K) between the two cell lines than the Ras/MAPK pathway. This provides further evidence to the hypothesis that one of the key mechanisms behind cell adaptation to suspension growth is the ability to activate the PI3K/Akt pathway. One key regulator in this pathway is PI3K which is shown in these experiments to be increased in activation above 5-fold in CL1 cells, compared to CCL-61 cells. As PI3K is one of the most upstream intermediates in the PI3K/Akt pathway and is also involved in cross-talk between the two pathways mentioned, this appears to be a vital node in terms of signalling activation in suspension conditions. Further experiments using chemical inhibitors against PI3K, will demonstrate its functional role in regulating signalling flux in suspension conditions.

Chapter 6

Cell surface analysis of CHO cells in adherent and suspension conditions

Acknowledgements

The data, figures and text in the following chapter are the work of the candidate.

Chapter 6 Aims

In this chapter the relationship between the cell surface molecules that mediate cell adhesion (integrins) and growth factor binding (receptor tyrosine kinases) is investigated. How these cell surface effectors link to their respective intracellular signalling networks is also analysed.

6.1 Introduction

6.1.1 Integrins

Integrins are a family of heterodimeric transmembrane glycoprotein receptors which provide a vital link between the cell environment and the interior of the cell. One of the major functions of integrins is to mediate the process of cell adhesion to the extra-cellular matrix as well as to other cells. Integrins also play a vital role in regulating the cells morphology as they are connected to the cell cytoskeleton. Downstream they regulate several intracellular signal cascades that control many vital cellular processes such as cell growth, differentiation, proliferation and survival (Reddig and Juliano, 2005).

Integrins are heterodimers that consist of an alpha and beta subunit. Each subunit consists of a large extracellular domain that is involved in ligand binding, a single hydrophobic transmembrane domain and a short cytoplasmic domain (Hynes, 1992; Liu et al., 2000). In mammals, there are currently 18 alpha and 8 beta subunits that are known, which together can form 24 specific integrin combinations (Hynes, 1992; Regent et al., 2010). These different subunit combinations mean that integrins posses the ability to interact with a wide range of substrates. The integrin family includes receptors for collagen (β1 and α1, α2, α10 and α11), laminin (β1 and α3, α6 and α7) and the RGD motif (α5β1, α8β1 and αV + β3, β5, β6, β8). RGD receptors recognise the RGD tri-peptide motif (Arg-Gly-Asp) that is located in many ECM components such as fibronectin and vitronectin (Danen and Sonnenberg, 2003; Regent et al., 2010). An overview of the integrin family and their specific ligands is shown in table 6.1.

Integrin		Ligand	
Alpha Subunit	**Beta Subunit**	**ECM**	**Cell-Cell**
α1	β1	Collagen, Laminin	
α2	β1	Collagen, Laminin	(E-cadherin)
α3	β1	Collagen, Laminin	
α4	β1	Fibronectin, Osteopontin	VC AM-1
	β7	Fibronectin, Osteopontin	VC AM-1
α5	β1	Fibronectin, Fibrin, Osteopontin	
α6	β1	Laminin	
	β4	Laminin	
α7	β1	Laminin	
α8	β1	Fibronectin, Osteopontin, Vitronectin	
α9	β1	Osteopontin, Tenascin	VCAM-1
α10	β1	Collagen, Laminin	
α11	β1	Collagen	
αV	β1	Fibronectin, Osteopontin	
	β3	Fibronectin, Vitronectin, Laminin, Thrombospondin, Tenascin	PECAM-1
	β5	Fibronectin, Osteopontin, Vitronectin	
	β6	Fibronectin	
αIIb	β3	Fibronectin, Thrombospondin	

Table 6.1: An overview of the integrin subunit combinations and their various ligands. Table adapted from (Humphries et al., 2006; Hynes, 1992; Stupack and Cheresh, 2002).

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As shown in table 6.1, ligand specificity is dictated by the combination of the relative alpha and beta subunits. Specific integrin-ligand interactions in vivo are determined by the relative integrin expression and activation levels as well the abundance of the particular ligand (van der Flier and Sonnenberg, 2001).

6.1.2 Integrin activation and signalling

In order for integrins to bind to their specific ligands, they are required to be in an activated state. This activation can be mediated by many intracellular mechanisms, therefore the term given to this type of integrin activation is "inside-out signaling/activation" (Ginsberg et al., 1992). Following ligand binding integrins mediate numerous cellular processes including cell spreading and migration, actin cytoskeleton restructuring and the activation of downstream signaling pathways that regulate cellular processes such as cell growth, proliferation and survival. This type of activation is given the term "outside-in signaling"(Ginsberg et al., 1992; Hynes, 1992).

As described previously, one of the key processes that integrins regulate is the prevention of "detachment induced apoptosis" called anoikis (Grossman, 2002). This integrin-mediated cell survival has shown to be regulated by activation of FAK in a mechanism that involves the key intermediates in the PI3K/Akt pathway (Frisch and Ruoslahti, 1997). While Reddig and Juliano postulated that overcoming this "anoikis barrier" is a key step in cells being able to survive in anchorage independent conditions (Reddig and Juliano, 2005). Work conducted by many groups has shown that in adherent cells a specific class of integrins, the alpha5 beta1 sub-class, regulate cell survival by up-regulating Bcl-2 expression in a mechanism involving that also involves Akt and FAK (Lee and Ruoslahti, 2005; Zhang et al., 1995).

In terms of their ability to trigger downstream signalling cascades, one of the key structural domains of integrins, is the cytoplasmic domain. As well as their integral in role in terms of forming interactions with the actin cytosekeleton, the cytoplasmic domains, of the β integrins also regulate outside-in and inside-out

signalling by providing binding sites for various integrin cytoplasmic domain binding proteins (Liu et al., 2000; Wiesner et al., 2005). Two key proteins that bind to integrin β tails are the kinases, focal adhesion kinase (FAK) and integrin linked kinase (ILK). Focal adhesion kinase has several tyrosine phosphorylation sites which regulate FAK kinase activity and mediate its interaction with various SH2-domain containing proteins (Zhao and Guan, 2011). One key family of proteins that FAK interacts with is the Src family of kinases. Activated FAK is able to form a complex with these Src proteins and consequently numerous downstream signalling pathways including the Ras/MAPK and PI3K/Akt pathways(Liu et al., 2000).

In terms of deciphering the functional role of integrins in adherent and suspension conditions, three main experiments were conducted. Firstly, the expression levels of three key integrins (alpha 1, alpha 5 and beta 1) were measured on CCL-61 cells growing in adherent conditions as well as the CHO-SA and CL1 cells growing in suspension conditions. Next, confocal microscopy was used to analyse the conformation and distribution of integrins on the cell surface of CHO cells growing in adherent and suspension conditions. Finally, the cross-talk between integrin-mediated signaling pathways and the effect of growth factor mediated binding to specific cell surface receptors is investigated.

6.2 Methodology

6.2.1 Flow cytometry analysis

In order to analyse the expression levels of various integrins on the surface of both the adherent CCL-61 and suspension adapted CHO-SA cell lines, a number of different antibodies were tested.

For this particular experiment the following antibodies were found to work in CHO cells:

- Anti-Integrin alpha V antibody, mouse monoclonal (Clone 272-17E6), Abcam
- Anti-Integrin beta 1 antibody, mouse monoclonal (Clone 3B6), Abcam
- Anti-Integrin alpha 4 antibody, mouse monoclonal (Clone R1-2), Miltenyi Biotec
- Anti-Integrin alpha 1 antibody, mouse monoclonal (Clone Ha31/8), BD Biosciences
- Anti-Integrin alpha 5 antibody, mouse monoclonal (Clone 5H10-27), BD Biosciences

All the antibodies listed above are unconjugated and therefore require the use of a secondary antibody. For each antibody a relevant secondary antibody was used, with all the secondary antibodies used being linked to an Alexa 488 fluorochrome. Flow cytometry experiments were conducted as outlined in the materials and methods.

Median fluorescence intensity (MFI) was calculated by dividing the fluorescence intensity for positively stained cells by the fluorescence intensity for the secondary antibody used in each experiment.

6.2.2 Confocal microscopy analysis

6.2.2.1 Cell staining for integrin analysis

The following antibodies were used for confocal microscopy analysis on integrin distribution and conformation:

Integrin beta 1 antibody – Clone 9EG7 (BD Biosciences)

Integrin alpha 1 antibody – Clone Ha31/8 (BD Biosciences)

Integrin alpha 5 antibody - Clone R1-2 (Miltenyi Biotec)

Confocal analysis was performed as described in materials and methods and figure legends.

6.2.2.2 Cell staining with actin

Actin or more specifically F-actin, staining was performed using Alexa Fluor® 546 phalloidin (Molecular Probes, Paisley, UK). The freeze dried phalloidin was reconstituted in methanol giving a stock solution of 200 units/mL and was then stored at -20°C in the dark. Confocal analysis was performed as described in materials and methods and figure legends.

6.2.3 Cell adhesion assay

The cell adhesion assay was performed using a Cultrex CultreCoat Fibronectin Cell Adhesion Assay kit (Trevigen). The assay was performed according to the manufacturer's instructions. Briefly, CCL-61 and CHO-SA cells were labelled with 2 µM Calcein AM for one hour and then washed with PBS. Cells were suspended in the provided adhesion buffer and diluted to the optimal seeding density. Cells were then seeded onto the fibronectin coated plates at the appropriate density and the plates were incubated for 1 hour at 37°C, 5% CO_2. The plate was then removed from the incubator and the fluorescence read at 485 nm excitation/520 nm emission. Non-attached cells were removed the plate and the plate was washed gently three times with wash buffer. 100 µl of adhesion buffer was added per well and then the fluorescence was read again at 485 nm excitation and 520 nm emission.

6.3 Results

6.3.1 Validation of flow cytometry results

A) Unstained Cell Sample   **B) Secondary Antibody Staining**
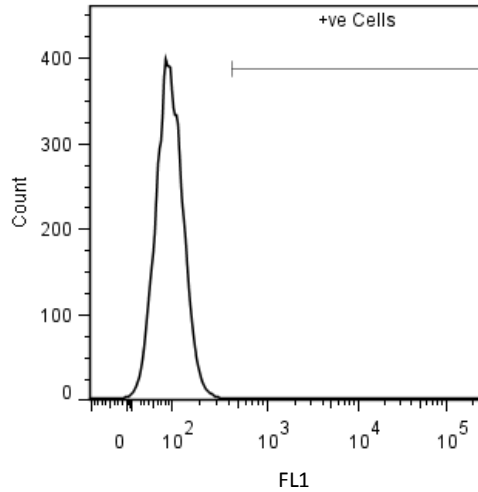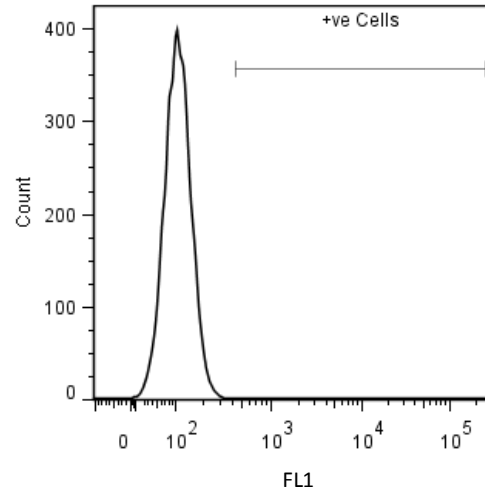
C) Integrin Beta 1 Staining   **D) Overlay of three samples for Beta 1**
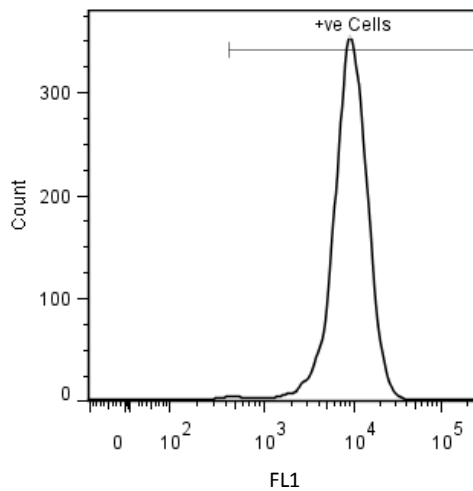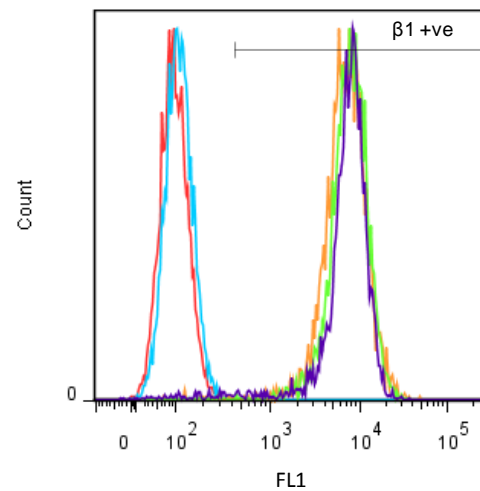
Figure 6.1: Validation of staining method for integrin analysis. The three example histograms are an example of a set of results obtained for staining of fixed CCL-61 cells for beta 1 integrin. The histograms above represent: A) unstained CHO-SA cells, B) staining with the relevant secondary antibody only, C) complete staining with the relevant primary and secondary antibodies. D) overlay of triplicate samples for beta 1 integrin. As the histograms above show, there is only a small shift in median fluorescence intensity of CCL-61 cells stained with secondary antibody only compared to the unstained CCL-61 cells. By comparison, there is a significant shift in the population of CCL-61 cells stained specifically for integrin beta 1.

6.3.2 Expression levels of integrins alpha 1, alpha 5 and beta 1 on CCL-61, CHO-SA and CL1 cells

In order to assess the levels of integrin(s) alpha 1, alpha5 and beta 1 expression on CCL-61, CHO-SA and CL1 cells growing in their standard conditions, flow cytometry staining analysis was used. Figure 6.2 shows a set of example histograms for the staining of the CCL-61, CHO-SA and CL1 cells for integrin beta 1. The histograms in this figure show that there is only a small shift in median fluorescence intensity of each cell line stained with secondary antibody only compared to the unstained cells. By comparison, there is a significant shift in the population of cells stained specifically for integrin beta 1. For all three cell lines a single peak for positively stained cells is observed. Although there is not a drastic change in peak form between the different cell lines, there is a shift in the median fluorescence intensity (MFI) observed as indicated by the calculated MFI in the table shown. It is also apparent that there is not a large shift between the different cell lines, in terms of the autofluorescence peak of unstained cells.

Figure 6.3 shows a set of example histograms for the staining of CCL-61, CHO-SA and CL1 cells for integrin alpha 1. In a similar manner to the staining results observed for integrin beta 1, there is only a slight shift in median fluorescence intensity for each line stained only with the relevant secondary antibody compared to the unstained cells. Again there is a significant shift in the population cells that are positively stained for integrin alpha 1. For all three cell lines there is a single peak for cells that are positively stained for integrin alpha 1. By comparison to the results seen in figure 6.4, there is a more pronounced change in peak form between the different cell lines, as well as an observable shift in the MFI as shown in the table in figure 6.5.D.

In figure 6.4, a set of example histograms are shown for the staining of CCL-61, CHO-SA and CL1 cells for integrin alpha 5. As observed with the staining results seen for the previous two integrins, there is a minimal shift in median fluorescence intensity when each cell line is stained with the secondary antibody

Integrin Beta 1 Analysis:

A) CCL-61 cells

B) CHO-SA cells

C) CL1 cells

D)
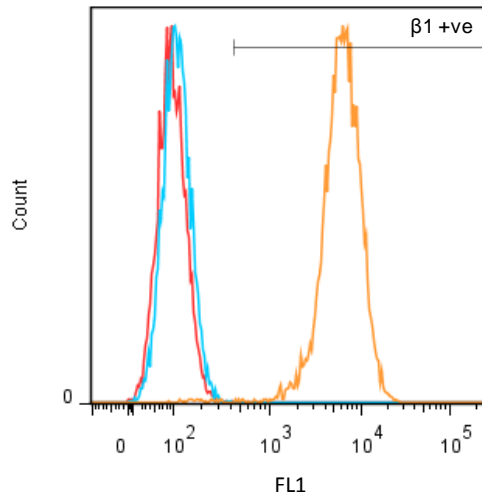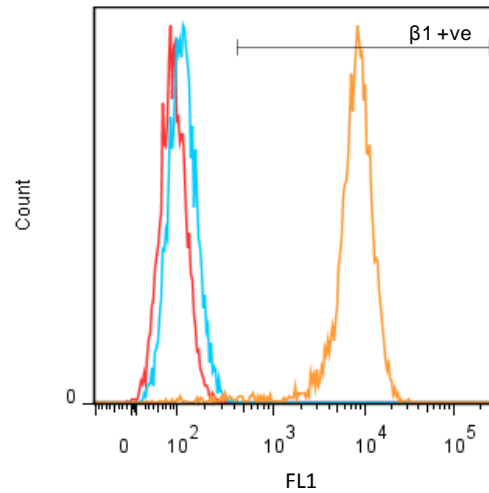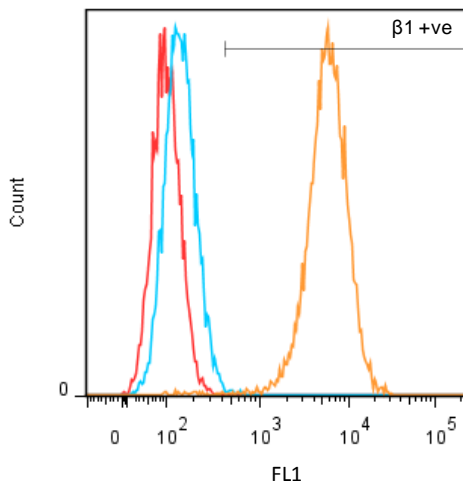
Cell Line	Median Fluorescence Intensity
CCL-61	14.28 ± 1.39
CHO-SA	16.61 ± 2.34
CL1	12.81 ± 1.41

Figure 6.2: The fluorescence intensity for integrin β1 staining of CCL-61, CHO-SA and CL1 cells. The three histograms above are an example of a set of results obtained for staining of A) CCL-61 cells B) CHO-SA and C) CL1 cells for integrin β1. Unstained cells are shown in red, secondary antibody only stained cells are shown in blue, cells stained for specifically for integrin β1 are shown in orange. The histograms above show a single sample, which are representative of triplicate samples. D) The average and standard deviation of the median fluorescence intensity for each population of cells.

Integrin Alpha 1 Analysis:

A) CCL-61 cells   **B) CHO-SA cells**
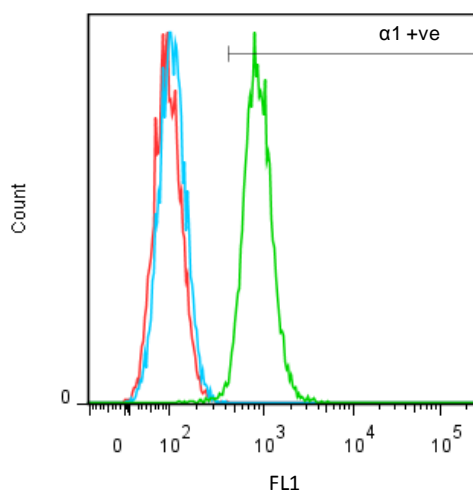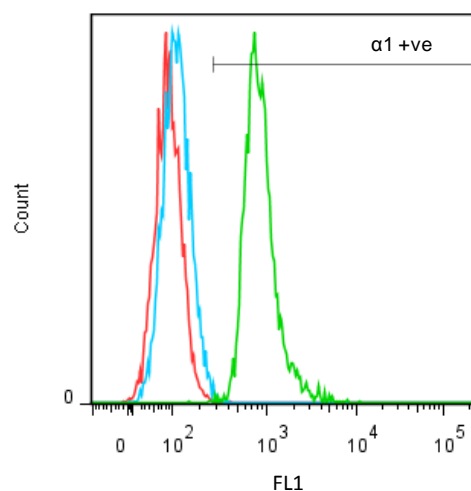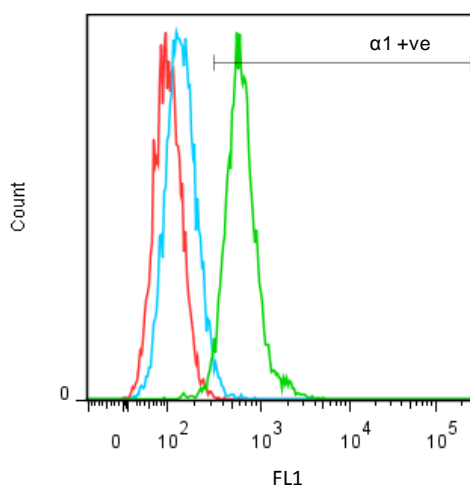
C) CL1 cells   **D)**

Cell Line	Median Fluorescence Intensity
CCL-61	1.92 ± 0.39
CHO-SA	1.81 ± 0.15
CL1	1.35 ± 0.18

Figure 6.3: The fluorescence intensity for integrin α1 staining of CCL-61, CHO-SA and CL1 cells. The three histograms above are an example of a set of results obtained for staining of A) CCL-61 cells B) CHO-SA and C) CL1 cells for integrin α1. Unstained cells are shown in red, secondary antibody only stained cells are shown in blue, cells stained for specifically for integrin α1 are shown in green. The histograms above show a single sample, which are representative of triplicate samples. D) The average and standard deviation of the median fluorescence intensity for each population of cells.

Integrin Alpha 5 Analysis:

A) CCL-61 cells   **B) CHO-SA cells**
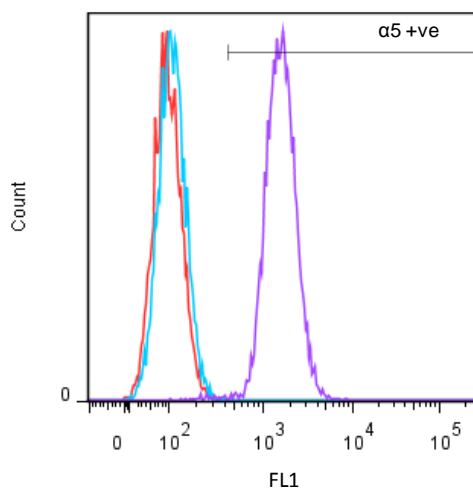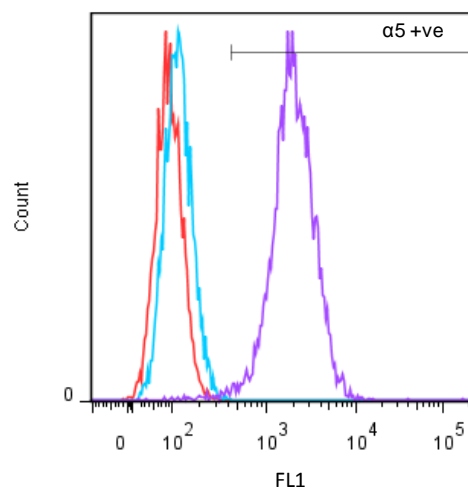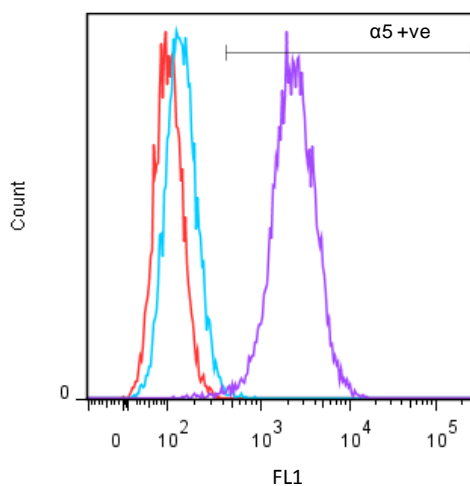
C) CL1 cells   **D)**

Cell Line	Median Fluorescence Intensity
CCL-61	3.44 $\pm$ 0.49
CHO-SA	4.32 $\pm$ 0.75
CL1	4.48 $\pm$ 0.68

Figure 6.4: The fluorescence intensity for integrin α5 staining of CCL-61, CHO-SA and CL1 cells. The three histograms above are an example of a set of results obtained for staining of A) CCL-61 cells B) CHO-SA and C) CL1 cells for integrin α5. Unstained cells are shown in red, secondary antibody only stained cells are shown in blue, cells stained for specifically for integrin α5 are shown in green. The histograms above show a single sample, which are representative of triplicate samples. D) The average and standard deviation of the median fluorescence intensity for each population of cells.

only compared to the MFI for unstained cells. There is also a noticeable shift in the population of cells that are positively stained for integrin alpha 5. Again, for all three different cell lines there is a single peak observed for positively stained cells. As expected, there is a slight change in peak form between the different cell lines, as well as an observable shift in the MFI observed.

The data from figures 6.2 to 6.4 is summarised in figure 6.5. Figures 6.7 A-C show the relative expression for each integrin on the three model cell lines, CCL-61, CHO-SA and CL1. While figure 6.5.D shows the results of the western blot experiments conducted to also analyse the expression of integrins alpha 1, alpha 5 and beta 1 on the three model cell lines. As shown in figure 6.5.A, integrin beta1 is expressed on CCL-61 cells growing adherently and also on CHO-SA and CL1 cells that are growing in suspension. The level of expression does not significantly change between the three cell lines (One-way ANOVA, Tukey's Multiple Comparison Test, p=0.097). Although, there is a slight increase in expression of integrin beta 1 on CHO-SA cells growing in suspension compared to CCL-61 and CL1 cells. From these results it is clear to see that integrin beta 1 expression does not significantly change when cells are adapted to suspension growth conditions. Figure 6.5.B shows the levels of integrin alpha 1 expression on the three model cell lines. As the figure shows integrin alpha 1 is expressed on all three cell lines growing in their standard culture conditions. The level of integrin alpha 1 expression does not significantly alter between the cell lines (One-way ANOVA, Tukey's Multiple Comparison Test, p=0.079). Despite this, it is clear to see CL1 cells growing in suspension have slightly lower levels of integrin alpha 1 compared to CCL-61 and CHO-SA cells. As shown in figure 6.5.C all three cell lines growing in their standard culture conditions do express integrin alpha 5. Again, there is not a statistically significant change in the expression level of integrin alpha 5 between the three cell lines (One-way ANOVA, Tukey's Multiple Comparison Test, p=0.188). However from the analysis conducted, there does appear to be a slight increase in the levels of integrin alpha 5 in CHO-SA and CL1 cells that are cultured in suspension conditions. As well as the flow cytometry analysis of integrin expression, western blotting was also conducted in order to

A) Integrin β1 Expression
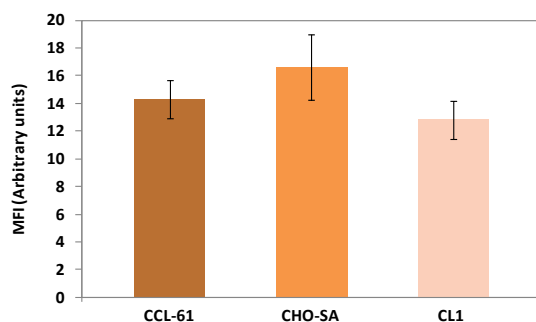
B) Integrin α1 Expression
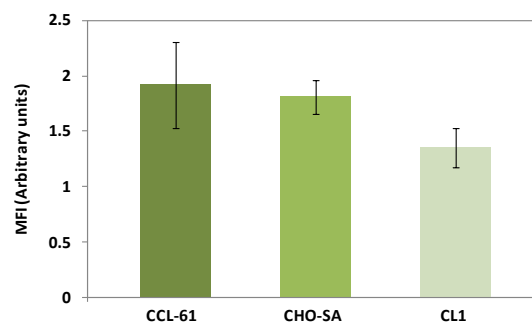
C) Integrin α5 Expression
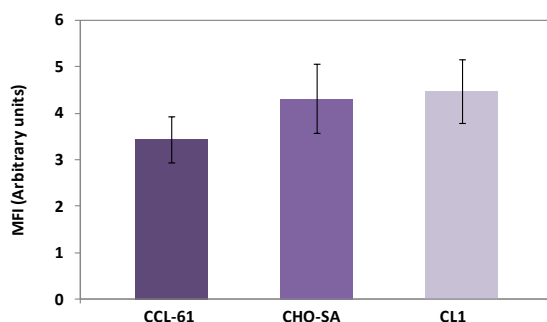
D) Western blot analysis
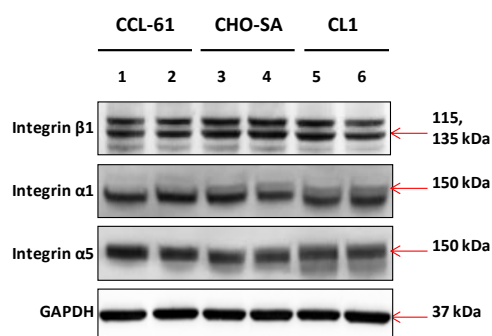
Figure 6.5: The expression levels of integrins α5, α1, and β1 on CCL-61, CHO-SA and CL1 cells cultured in the standard growth conditions. CCL-61 cells were cultured in standard adherent conditions, while CHO-SA and CL1 cells were cultured in standard suspension conditions. Staining for the three integrins was conducted as described in section 6.2. Triplicate samples were run for each cell line for each integrin. From the triplicate samples the average MFI was calculated. MFI was calculated as described previously. A) Flow cytometry results of staining for integrin β1. B) Flow cytometry results of staining for integrin α1. C) Flow cytomtery results of staining for integrin α5. D) Western blotting results for all three integrins in the three model cell lines. The western blots shown above are from one representative experiment. All western blots were performed three times.

confirm the results already obtained. Figure 6.5.D shows the results of the western blot analysis of the integrin alpha 1, alpha 5 and beta 1 content of the three model cell lines. The expression of all three integrins on all three cell lines is confirmed by the western blots shown. In terms of the expression levels between the cell lines, the western blotting analysis conducted shows that there is no significant up or down regulation of these three integrins between these three cell lines grown in their standard culture conditions.

The results of the flow cytometry and western blotting analysis of integrin expression shows that the expression of integrins alpha 1, alpha 5 and beta 1 is not significantly decreased on cells that are growing in suspension conditions. Despite the fact that cells growing in suspension do not require integrins to mediate attachment to the extracellular matrix, they still appear to express these integrins to a similar level as cells cultured in adherent conditions. Therefore further questions emerge as to the functional role of these expressed integrins on the surface of suspension CHO cells, as well as whether the integrins present are functionally active or not.

6.3.3 Confocal microscopy analysis of the changes in cell morphology and cell surface properties of adherent and suspension CHO cells

Following on from the flow cytometry experiments that showed that integrin expression is not significantly decreased on suspension cultured cells, further experiments were conducted to analyse the conformation on integrins on both adherent and suspension cells. In order to analyse the conformation of cell surface integrins on both adherent and suspension cells, cells were single stained for either integrin beta 1, alpha 1 or alpha 5 and then analysed by confocal microscopy as described in section 6.2. The confocal microscopy images shown in the chapter are of the CCL-61 cells growing in adherent conditions and the CHO-SA and CL1 cells growing in suspension conditions.

As well as looking at the conformation of integrins on cells in adherent and suspension conditions, cells were also stained for their actin content and conformation.

Figure 6.6 shows the results of a control experiment that was performed to check the background staining levels with the relevant secondary antibodies that were used in these experiments. As can be observed in set of images (A), when CHO-SA cells were stained with the relevant secondary antibodies only, an extremely weak background signal was observed, making these antibodies ideal for use in these confocal microscopy experiments.

Figure 6.7 shows the images from the conformational analysis of integrins beta 1, alpha 1 and alpha 5 on CCL-61 cells growing in standard adherent conditions. The images shown are representative examples from the confocal analysis conducted. As the images in figure 6.7 show the CCL-61 cells when growing in standard adherent conditions, show a typical "spread-out" morphology. The images in this figure show that the three integrins analysed are evenly distributed over the surface of the CCL-61 cells. Small clusters of integrins are observed on the cell surface, but there is no particular area of localisation observed for all three integrin images.

Integrin β1   **A)**   **B)**

Integrin α1   **A)**   **B)**

Integrin α5   **A)**   **B)**
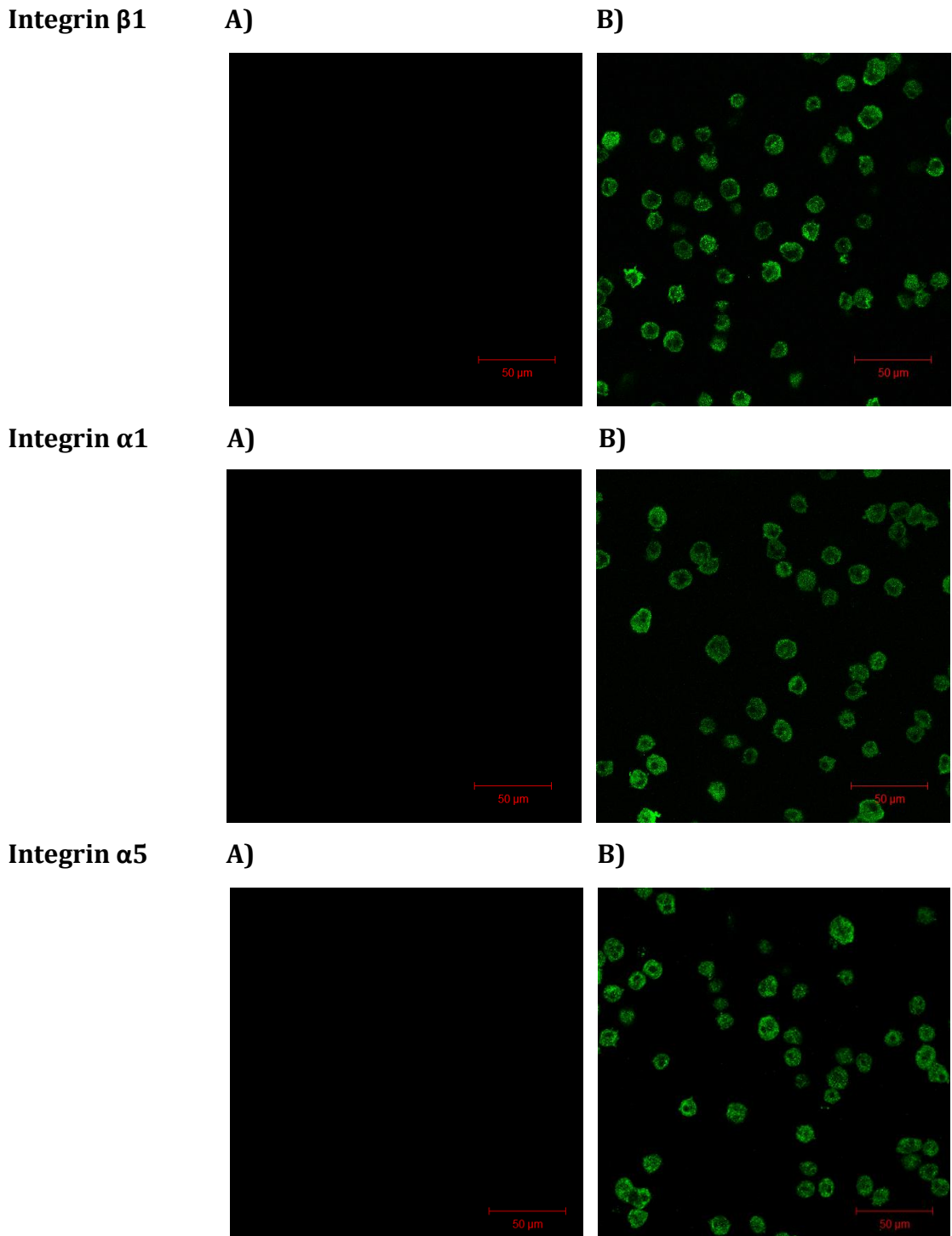
Figure 6.6: Validation of staining method for integrins α5, α1 and β1 on CHO-SA cells cultured in standard suspension conditions. CHO-SA cells were seeded into standard suspension conditions and were A) stained with secondary antibody only B) specific primary antibody and secondary antibody (complete staining). Cells were analysed by confocal microscopy using an inverted Zeiss LSM510 Meta Confocal Microscope. Samples were analysed with a Plan-Neofluar 20x/1.3 Oil DIC objective and the pinhole channel was set to 79 µm.
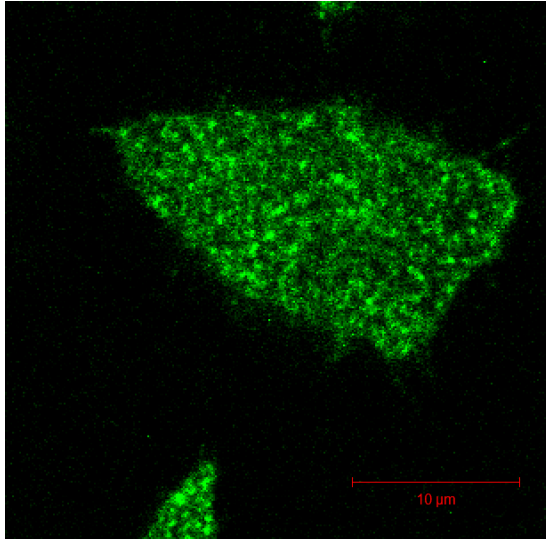
A) Integrin β1
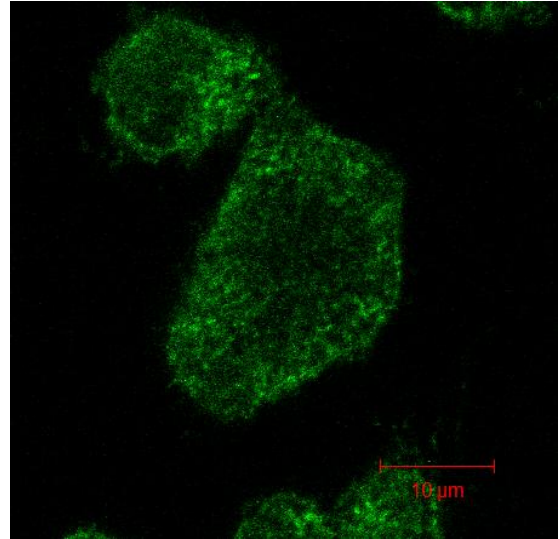
B) Integrin α1
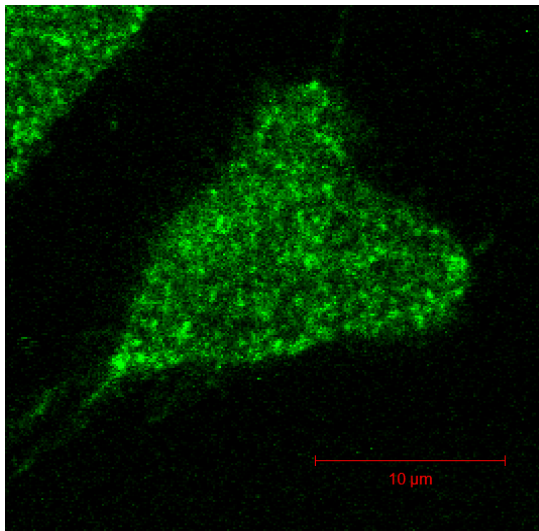
C) Integrin α5

Figure 6.7: Confocal microscopy analysis of the conformation of integrins α5, α1 and β1 on CCL-61 cells cultured in standard adherent conditions. CCL-61 cells were seeded into standard adherent conditions and were stained with the relevant specific primary antibody and secondary antibody (complete staining). Cells were analysed by confocal microscopy using an inverted Zeiss LSM510 Meta Confocal Microscope. Samples were analysed with a Plan-Neofluar 40x/1.3 Oil DIC objective and the pinhole channel was set to 56 μm.

Figure 6.8 shows the conformation of integrins beta 1, alpha 1 and alpha 5 on A) CHO-SA and B) CL1 cells growing in standard suspension conditions. As can be observed in the images shown, there is clearly clusters of integrins on the cell surface for all three integrins. As can be seen for integrin beta 1 in both CHO-SA and CL1 cells cultured in suspension conditions, the integrins are clustered into almost a sphere like shape, reflecting the morphology of a cell growing as a spherical individual cell in suspension. For integrin alpha 1, clusters of integrins are also observed but the spherical clustering pattern is less pronounced. This is also the case for the alpha 5 integrin in CHO-SA cells, although in the image shown there is a larger area where some of these clusters are grouped together. In terms of alpha 5 integrin distribution on CL1 cells, there is a more pronounced spherical shaped clustering of integrins, although not as pronounced as for integrin beta1.

Figure 6.9 shows the results of the actin staining experiments for both CCL-61 and CHO-SA cells growing in both adherent and suspension conditions. As the images show, there is a distinct change in the actin cytoskeleton morphology when cells are transferred from adherent to suspension conditions. CCL-61 and CHO-SA cells growing in standard adherent conditions, display fibres of actin that spread across the surface of the cell as well as a distinct pattern of actin fibre formation at the very edges of the cells. The morphology of the actin cytoskeleton reflects the expected shape that is usually observed when cells are growing in adherent conditions. By contrast, when both CCL-61 and CHO-SA cells are transferred to suspension culture conditions, no actin filaments that traverse across the middle of the cell are observed, instead the actin cytoskeleton forms into a distinct sphere shape, that mimics what the shape of standard suspension cell is expected to be. This change is the actin cytoskeleton also mirrors the change in the distribution of the beta 1 integrin in suspension cultured cells, thus suggesting that in suspension, the morphology of a cell is dictated by the interaction of the actin cytoskeleton with certain cell surface integrins.

A) Integrin β1

A)   **B)**

B) Integrin α1

A)   **B)**

C) Integrin α5

A)   **B)**
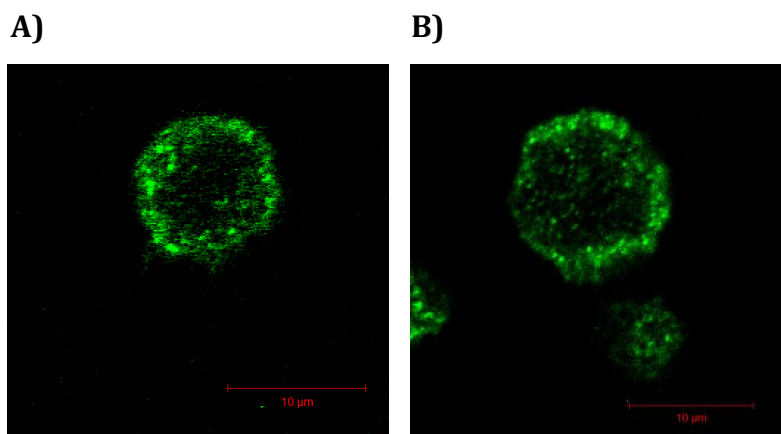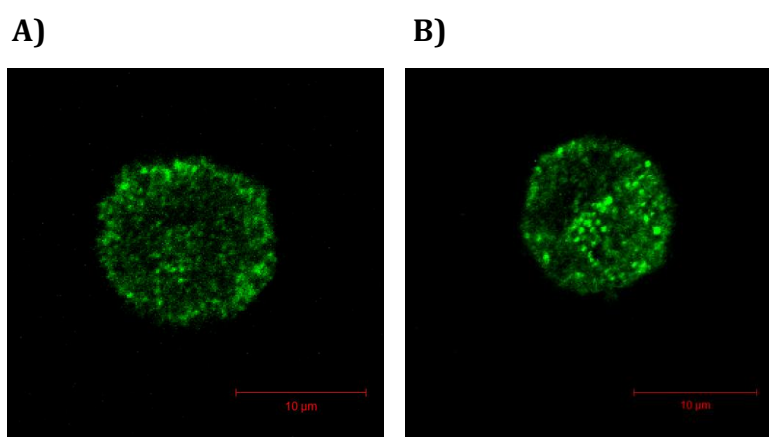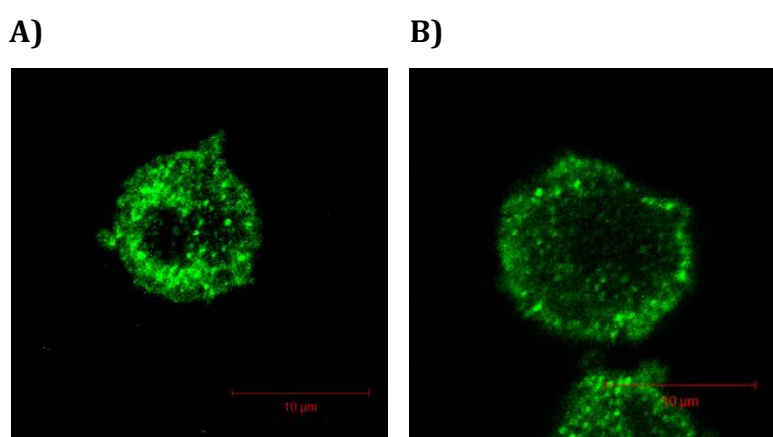
Figure 6.8: Confocal microscopy analysis of the conformation of integrins α5, α1 and β1 on CHO-SA and CL1 cells cultured in standard suspension conditions. A)CHO-SA and B)CL1 cells were seeded into standard suspension conditions and were stained with the relevant specific primary antibody and secondary antibody (complete staining). Cells were analysed by confocal microscopy using an inverted Zeiss LSM510 Meta Confocal Microscope. Samples were analysed with a Plan-Neofluar 40x/1.3 Oil DIC objective and the pinhole channel was set to 56 µm.
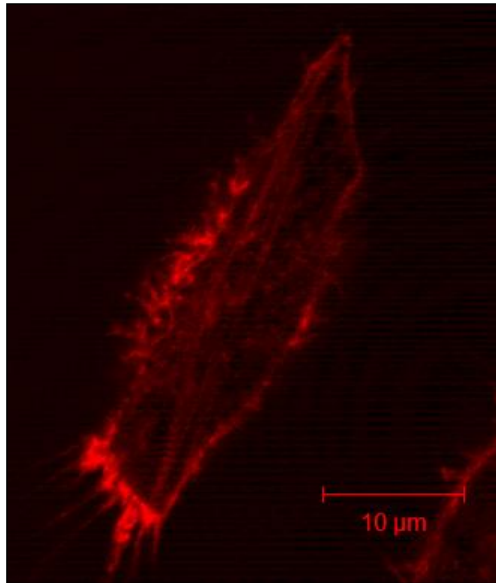
A) CCL-61

Adherent   **Suspension**
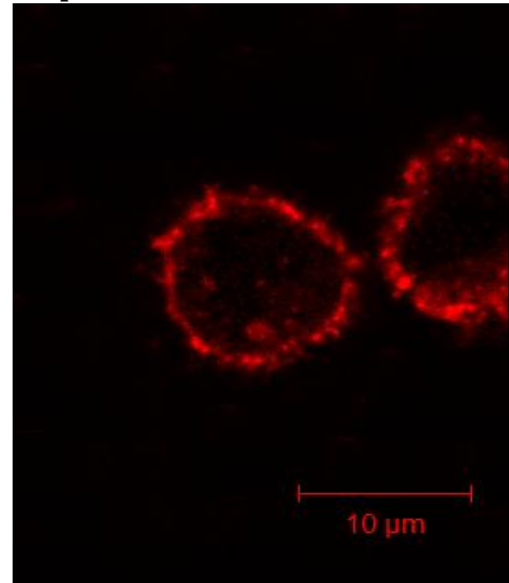
B) CHO-SA
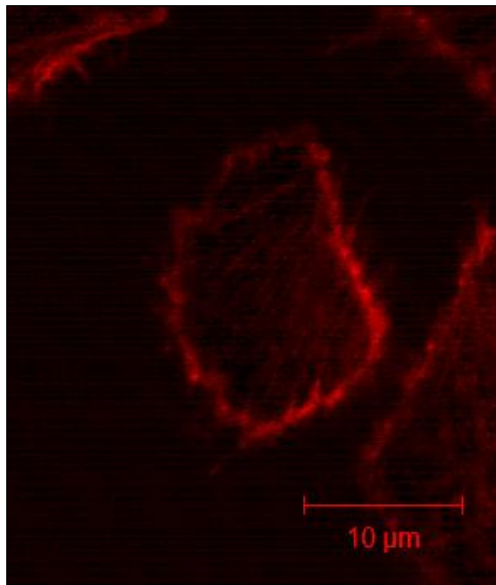
Adherent   **Suspension**
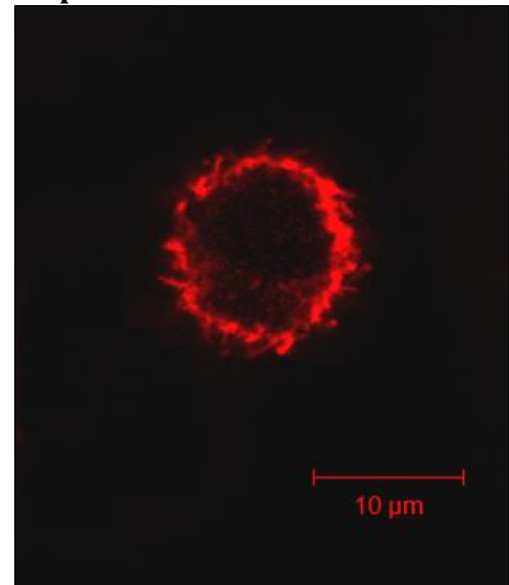
Figure 6.9: Actin staining of CCL-61 and CHO-SA cells cultured in both standard adherent and suspension conditions. CCL-61 and CHO-SA were seeded into standard adherent conditions (left images) and standard suspension conditions (right images). Cells were stained with Alexa Fluor® 546 phalloidin and were analysed by confocal microscopy using an inverted Zeiss LSM510 Meta Confocal Microscope. Samples were analysed with a Plan-Neofluar 40x/1.3 Oil DIC objective and the pinhole channel was set to 79 µm.

6.3.4 The interaction of integrins with growth factor receptors

Two of the main mechanisms that are being investigated in this study are the ability of cells to grow with and without integrin-mediated attachment as well the ability to respond to growth factor stimulation. It is well established that integrin attachment to the extra-cellular matrix (ECM) potentiates growth factor signalling, while some research groups have shown that growth factor stimulation increases cell adhesion to the (ECM). Therefore it is important to understand how these two processes, that interact with one another, affect downstream activation of linked signalling pathways. Work by many different groups has shown that CHO cells adhere to the ECM protein fibronectin by the $\alpha5\beta1$ integrin receptor (Brown and Juliano, 1985; Guilherme et al., 1998). While the paper by Guilhereme et al, showed how insulin treatment of CHO-T cells (which overexpress human insulin receptors (hIr)) promotes CHO cell adhesion to a fibronectin matrix (Guilherme et al., 1998). Interestingly, the paper by Fujita et al, showed that IGF binding to the alpha6 beta4 sub-class of integrins potentiated IGF mediated signalling in anchorage-independent cells (Fujita et al., 2012). This indicates that the relationship between integrins and specific growth factor receptors is not necessarily reliant on cell adhesion.

In order to analyse the cross-talk between growth factor receptors and key integrins on the cell surface, a cell adhesion assay was conducted, where the adhesion capability of cells that were treated with and without insulin and various inhibitors was measured. Both CCL-61 and CHO-SA were treated with and without insulin and were transferred into wells coated with fibronectin. The main integrins responsible for the binding of cells to fibronectin are the alpha 5 and beta 1 integrin subclasses, which links to the previous work conducted. Cells were then allowed to adhere to the fibronectin for 1 hour before the percentage cells attached was calculated.

Figure 6.10 shows that a significant increase in the percentage cells attached to the fibronectin matrix occurs when both CCL-61 and CHO-SA cells are pre-

A)
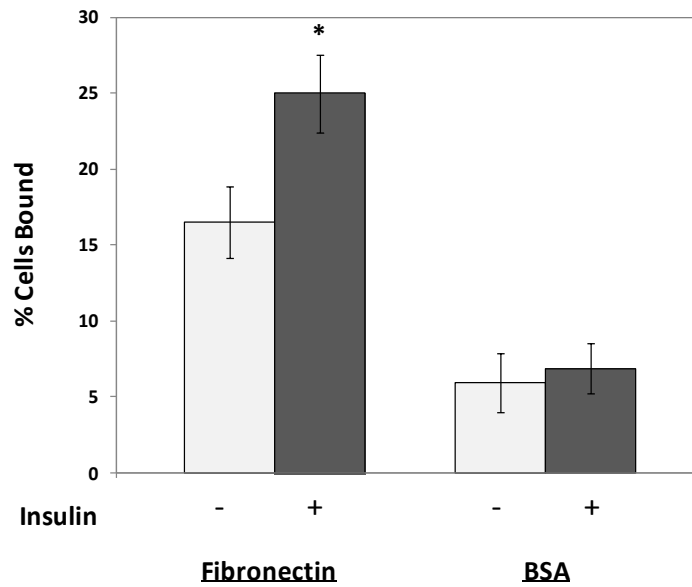
B)
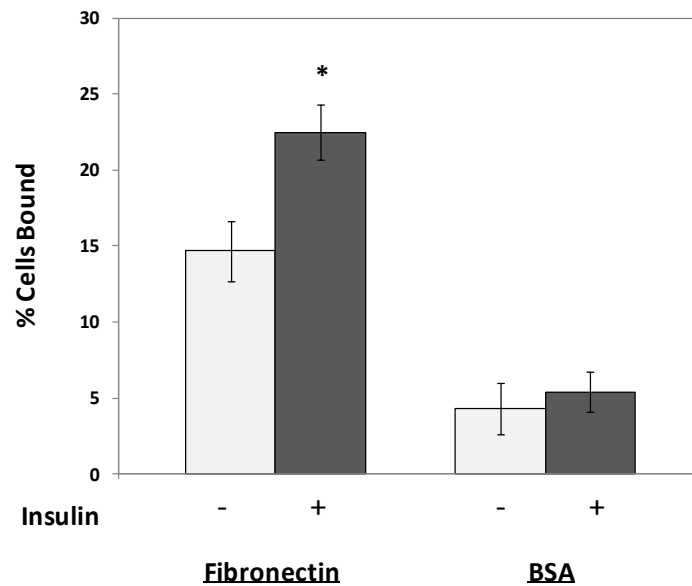
Figure 6.10: The effect of insulin on integrin-mediated binding of CCL-61 and CHO-SA cells onto fibronectin. CCL-61 and CHO-SA cells pre-labelled with Calcein-AM were treated with or without 100 nM insulin for 15 minutes and then re-plated onto various substrata-coated wells and allowed to adhere for 60 minutes.   A) % of CCL-61 cells bound to fibronectin and BSA (non-specific binding control) after treatment with (+) or without (-) 100 nM insulin. B) % of CHO-SA cells bound to fibronectin and BSA after treatment with or without 100 nM insulin. The data presented are the average values of three independent experiments ± S.D. * indicates a significant difference between insulin treated and un-treated samples, where * is $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

treated with 100 nM insulin ($p<0.05$). While figure 6.11 shows how the treatment of both cell lines with the PI3K inhibitor LY294002 substantially inhibits insulin-stimulated, fibronectin-mediated attachment. This puts forward the hypothesis that insulin stimulated integrin-mediated attachment is regulated through a PI3K-dependent mechanism. Finally, in figure 6.12, the effect of treating both the CCL-61 and CHO-SA cells with FAK inhibitor 14 on their cell attachment capabilities is shown. The figure shows that by treating CCL-61 and CHO-SA cells with FAK inhibitor 14, significantly less cells are able to attach to the fibronectin matrix. This indicates that FAK is a vital intermediate in regulating integrin-mediated attachment signalling.

A)
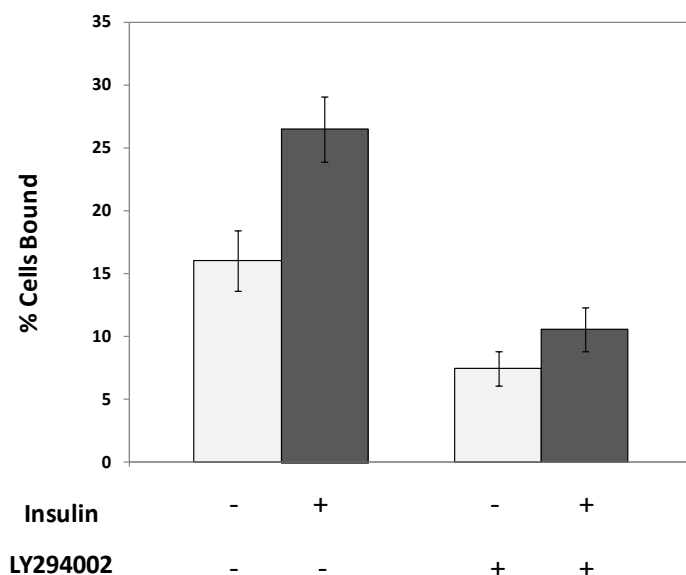
B)
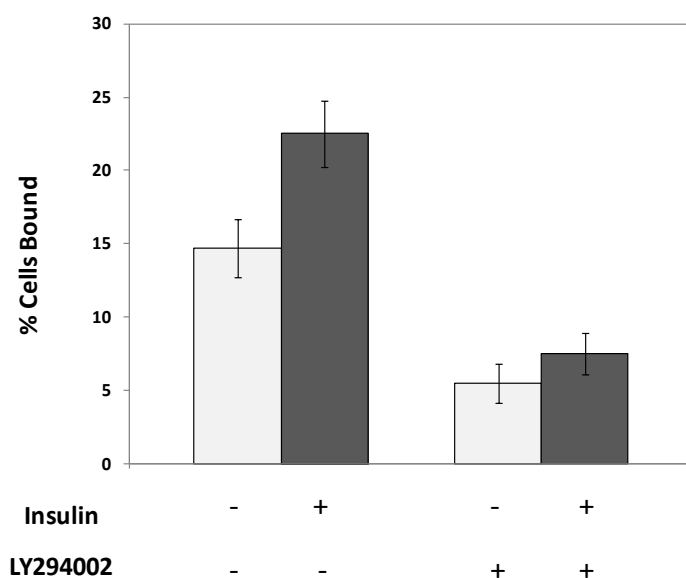
Figure 6.11: LY294002 inhibits the effect of insulin on integrin-mediated binding of CCL-61 and CHO-SA cells onto fibronectin. CCL-61 and CHO-SA cells pre-labelled with Calcein-AM were treated with or without 50 µM LY294002 for 15 minutes and then were treated with or without 100 nM insulin for 15 minutes and then re-plated onto various substrata-coated wells and allowed to adhere for 60 minutes. A) % of CCL-61 cells bound to fibronectin after treatment with (+) or without (-) 50 µM LY294002. B) % of CHO-SA cells bound to fibronectin treatment with (+) or without (-) 50 µM LY294002. The data presented are the average values of three independent experiments $\pm$ S.D.

A)
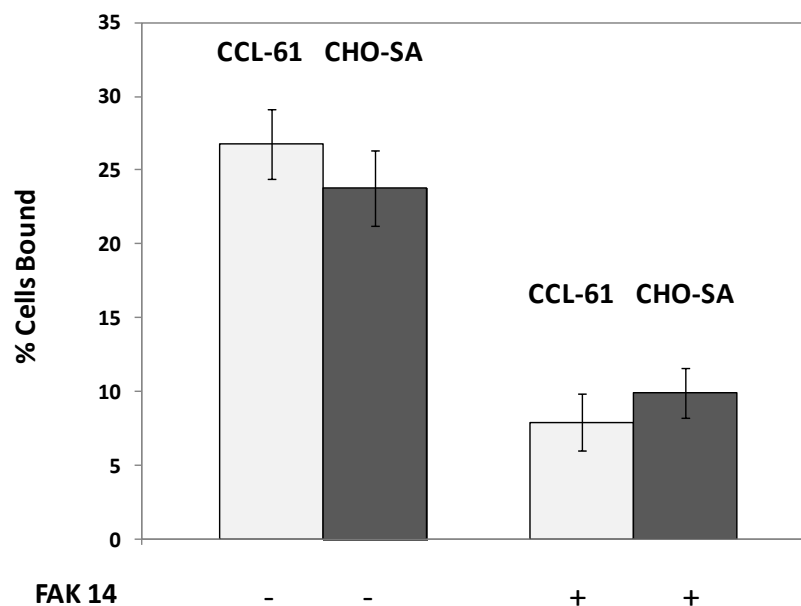
Figure 6.12: FAK inhibitor 14 inhibits the ability of CCL-61 and CHO-SA cells to adhere to fibronectin. CCL-61 cells pre-labelled with Calcein-AM were treated with or without 50 μM of FAK inhibitor 14 for 15 minutes and were then re-plated onto various substrata-coated wells and allowed to adhere for 60 minutes. The figure above shows the % of CCL-61 and CHO-SA cells bound to fibronectin after treatment with (+) or without (-) 50 μM of FAK inhibitor 14. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

6.4 Discussion

As shown in earlier chapters 4 and 5, there are significant changes in signalling pathway activation that occur, when cells are transferred from an adherent growth environment to a suspension growth environment. Therefore it is important to see if these changes in intracellular processes are reflected at the cell surface.

One of the key mechanisms involved in cells maintaining an adherent phenotype is the attachment of the cell to the extracellular matrix via integrins. As suspension cells do not require this integrin-mediated attachment, one might hypothesise that suspension cells do not need integrins and therefore their expression would be down-regulated in suspension cells. Flow cytometry experiments carried out showed that the levels of integrins alpha 1, alpha 5 and beta 1 are not significantly down-regulated when comparing the adherent cultured cell line, CCL-61, to the suspension cell lines CHO-SA and CL1. While there is some variation in the expression of these three integrins, the results show that all three integrins are indeed expressed on suspension adapted cells. This data was backed up western blotting analysis looking at the total integrin content of integrins alpha 1, alpha 5 and beta 1 in the three model cell lines.

Following on from the flow cytometry analysis, confocal microscopy analysis was conducted to analyse the conformation and distribution of integrins alpha 1, alpha 5 and beta 1 on adherent cultured cells and suspension cultured cells. Cells cultured in adherent conditions displayed a uniform distribution of three of the integrins across the cell surface. While cells that were cultured in suspension conditions showed a different integrin conformation. In suspension cells, integrin beta 1 was shown to be clustered and arranged in a sphere shape that mimics the shape of the cell. This arrangement is somewhat apparent for integrins alpha1 and alpha 5 but is more pronounced in terms of integrin beta1. Further confocal microscopy analysis of the morphology of the actin cytoskeleton showed that when cells are cultured in adherent conditions they display actin filaments that spread across the surface of the cell as well as a

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distinct pattern of actin fibres at the edge of the cell that reflect the adherent cell morphology. However, when cells are transferred to suspension conditions a substantial change in the actin cytoskeleton occurs, where actin forms a distinctive sphere-like shell that is similar to the sphere like conformation of integrin beta 1. As described in the papers by Regent et al and Wiesner et al, the actin cytoskeleton is major target of integrin-mediated attachment signalling (Regent et al., 2010; Wiesner et al., 2005). Therefore a possible mechanism is put forward where cells that are transferred to suspension conditions alter their cell morphology in a mechanism that involves the rearrangement of both the actin cytoskeleton and certain key cell surface integrins, in this case integrin beta 1.

Further cell adhesion assay experiments were performed in order to decipher the relationship between cell surface integrins and cell surface receptor tyrosine kinases. The specific relationship investigated focused on the effect of insulin stimulation on cell adhesion to the ECM glycoprotein fibronectin via the alpha 5 and beta 1 integrins. Insulin was found to significantly stimulate cell adhesion of both CCL-61 and CHO-SA cells to fibronectin. Treatment of the cells with the PI3K inhibitor, LY294002 showed that this mechanism of insulin stimulated cell adhesion is mediated via PI3K. A final experiment treating both the CCL-61 and CHO-SA cells with the focal adhesion inhibitor FAK-14 showed that integrin-mediated attachment and the related signalling pathway activation is mediated in a FAK-dependent manner.

Chapter 7

Dissection of signalling pathways using chemical inhibitors

Acknowledgements

The data, figures and text in the following chapter are the work of the candidate.

Chapter 7 Aims

In this chapter the two main signalling networks that are being investigated in this study, the Ras/MAPK and PI3K/Akt pathways, are dissected by using various chemical inhibitors, against specific nodes in each pathway, when cells are growing in both adherent and suspension culture conditions.

7.1 Introduction

CHO cells growing in a synthetic environment respond to various signal inputs that control many key cellular functions, such as cell growth, proliferation, migration and survival. These signal inputs are transmitted throughout the cell, via complex signalling networks that work in conjunction with one another, to cause changes in gene expression, protein function and cell morphology to enable a particular cellular response (Logue and Morrison, 2012).

As mentioned previously, the Ras/MAPK and PI3K/Akt signalling networks are two of the most important pathways that regulate such processes in mammalian cells and are therefore also commonly activated during oncogenesis (Mendoza et al., 2011). Therefore these two pathways are ideal targets for therapeutic inhibition in cancer cells. Extensive use of particular chemical inhibitors, against specific nodes in these two pathways, in various cancer cell models, has provided a wealth of information regarding how these pathways are regulated.

In CHO cells, many different groups have used various chemical inhibitors to decipher the activation and regulation of different signalling networks. Targeted inhibition of particular signalling intermediates has also been used as a method of altering cell growth characteristics and increasing recombinant protein production in CHO cells.

Section 7.1.1 lists the specific inhibitors used in this study to dissect the Ras/MAPK and PI3K/Akt pathways and study how they are regulated in CHO cells growing in adherent and suspension conditions.

7.1.1 Inhibitors of the PI3K/Akt pathway

7.1.1.1 LY294002 – PI3K inhibitor

LY294002 has been shown when used *in vivo* to be a highly selective inhibitor of PI3 Kinase. It specifically inhibits the PI3K subunits p110α (IC50 – 0.5 μM), p110δ (IC50 -0.57 μM) and p110β (IC50 – 0.97 μM). LY294002, when used at a concentration of ∼ 50 μM, has been shown to inhibit PI3 Kinase activity and also have no inhibitory effect on other lipid/ protein kinases including PI4 kinase, PKC and MAP kinases (Vlahos et al., 1994). By inhibiting PI3K, LY294002 inactivates Akt, and thereby arrests cell proliferation and induces apoptosis.

In some colon cancer cell lines, LY294002 has been shown to inhibit cell growth via reducing the expression of phospho-Akt (Ser473) (Semba et al., 2002). In melanoma cells, LY294002 treatment causes G1 phase cell cycle arrest and consequently cell proliferation inhibition. This combined effect of LY294002 on cell cycle progression and PI3K/Akt signalling has provided an insight into the possible links between the two in cancer cell growth (Hu et al., 2000). In myeloma cells it has been observed that treatment with LY294002 lead to a decrease in the active form of Akt and an increase in lactate consumption (Mulukutla et al., 2012).

In CHO cells, LY294002 has been extensively used to study the role of PI3K in cell growth and survival. Work done by Lee and Ruoslahti, showed that attachment of CHO cells to fibronectin, via the α5β1 integrins, up-regulates the expression of the anti-apoptotic protein Bcl-2 via a PI3K/Akt-dependent mechanism. Treatment of these cells with LY294002 led to a decrease in Akt phosphorylation and subsequently a down-regulation in Bcl-2 levels (Lee and Ruoslahti, 2005). Other work by Xu and others, showed that CHO cells transfected with the homeobox transcription factor Prox1, showed increased levels of proliferation through activation of the PI3K/Akt signalling pathway. Treatment of cells with LY294002 led to decreased levels of Akt phosphorylation and PI3K expression as well as a reduction in cell proliferation rate(Xu, 2010).

7.1.1.2 MK-2206 – Akt inhibitor

MK-2206 is a highly selective inhibitor of Akt that has been used primarily in preclinical studies for cancer therapies. In these preclinical studies MK-2206 has been shown to have a high level of efficacy in terms of Akt inhibition as well as the ability to promote to cancer cell death when used as a single chemotherapeutic agent as well as in conjunction with other agents (Cheng et al., 2012; Pant et al., 2012).

As shown in the paper by Pant et al, Ishikawa cells that were treated with MK-2206 showed decreased levels of phospho-Akt (Ser473) and phospho-Akt (Thr308) as well as the Akt downstream protein phospho-PRAS40 (Thr246). Further experimental work conducted showed that levels of phospho-Erk 1/2 (Thr202/Tyr204) and phospho-SAPK/JNK (Thr13/Tyr185) remain unchanged after treatment with MK-2206 thus demonstrating the Akt specificity of MK-2206 (Pant et al., 2012).

As MK-2206 is primarily used as a chemotherapeutic agent, the effects of MK-2206 on Akt inhibition has not been extensively used as a research tool to study CHO cells. However there are some examples where MKK-2206 has been utilised in studies involving CHO cells. Work done by Ulu et al, studying the transactivation of the epidermal growth factor receptor (EGFR) by the alpha 1-adrenoceptor in CHO cells, showed that this transactivation is mediated by PI3K in an independent manner that does not require the activation of Akt or Erk 1/2. The authors demonstrated this by using specific inhibitors against Akt and Erk 1/2, such as MK-2206, which did not influence the levels of transactivation of EGFR by alpha 1A-AR (Ulu et al., 2013).

7.1.1.3 Rapamycin – mTOR inhibitor

Rapamycin, a specific mTORC1 inhibitor, is a lipophilic macrocyclic lactone that has antifungal and immunosuppressant activities (Dumont et al., 1990). It forms a complex with the immunophilin FK-506-binding protein 12 (FKBP12) which

then inactivates mTORC1 (Lee and Lee, 2012). This leads to the downstream dephosphorylation and inactivation of p70 S6 Kinase as well as the dephosphorylation of 4E-BP1, thus enhancing its binding to and inhibition of eIF4E (Jefferies et al., 1997).

In terms of studying the signalling dynamics of the PI3K/Akt/mTOR signalling pathway and related signalling networks, Wan et al, showed that mTOR/S6K1 inhibition by rapamcyin results in a negative feedback signalling loop that leads to the activation of Akt signalling (Wan et al., 2007). In hybridoma cells, rapamycin has been shown to reduce cell death and enhance monoclonal antibody production (Balcarcel and Stephanopoulos, 2001). Work done by the group Chaturvedi et al, has shown that in various cell types rapamycin inhibition of mTOR triggers the activation of cell survival signalling pathways by activating two key pro-survival MAP kinases Erk 1/2 and p90RSK (Chaturvedi et al., 2009).

In CHO cells, rapamycin has been widely used to study the dynamics of mTOR mediated signalling as well as the effect of mTOR activation on CHO cell growth and productivity. Work done by numerous groups has shown that the activity of the mTOR complex 1 has been shown to correlate with the relative productivity and growth of Mab producing CHO cell lines.

Lee and Lee showed that treating two CHO cell lines, a DG44 host cell line and an antibody producing recombinant CHO cell line (rCHO), growing in serum-free suspension conditions with rapamycin, lead to a delay in a drop in viability and the induction of apoptosis as well as an increase in maximum antibody titre (Lee and Lee, 2012). In hybridoma cells rapamycin treatment has been shown to reduce cell death and also enhance mAb production (Balcarcel and Stephanopoulos, 2001).

7.1.2 Inhibitors of the MAPK pathway

7.1.2.1 U0126 – MEK 1/2 inhibitor

U0126 is a specific inhibitor against the MAPK pathway signalling intermediates MEK 1 and MEK 2. U0126 has been used as a tool to demonstrate the role of MEK activation in cell survival signalling (Ballif and Blenis, 2001). As well as U0126, another MEK inhibitor, PD98059, has been widely used to show MEK dependency in cell survival. U0126 and PD98059 both bind to MEK 1 in a mutually exclusive manner which suggests that they act at the same site on MEK 1 (Favata et al., 1998). However, U0126 has the ability to inhibit both MEK 1 and MEK 2 compared to PD98059 which inhibits MEK 1 more effectively than MEK 2.

The use of both U0126 and PD98059, to elucidate the mechanisms of MEK signalling and MEK dependent cell survival, is discussed in detail in the paper by Ballif and Blenis (Ballif and Blenis, 2001).

Dougherty et al, showed that treatment of NIH3T3 cells with U0126 disrupted the downstream feedback inhibition of Raf-1 by ERK. Following stimulation with PDGF, U0126 treatment prevented the hyperphosphorylation and consequent inactivation of Raf-1, thus indicating that signalling downstream of MEK 1/2 is required for this feedback inhibition Raf-1 (Dougherty et al., 2005). Roux et al, utilised the inhibitory effect of U0126 to show how ERK signalling contributes to the regulation of the ribosomal S6 protein via phosphorylation at site Ser 235/236 in an mTOR-independent but MEK 1/2 dependent manner. HEK293 cells that were treated with U0126 showed significantly lower levels of phospho-S6 (Ser235/236) but levels of phospho-S6 (Ser240/244) were not affected (Roux et al., 2007).
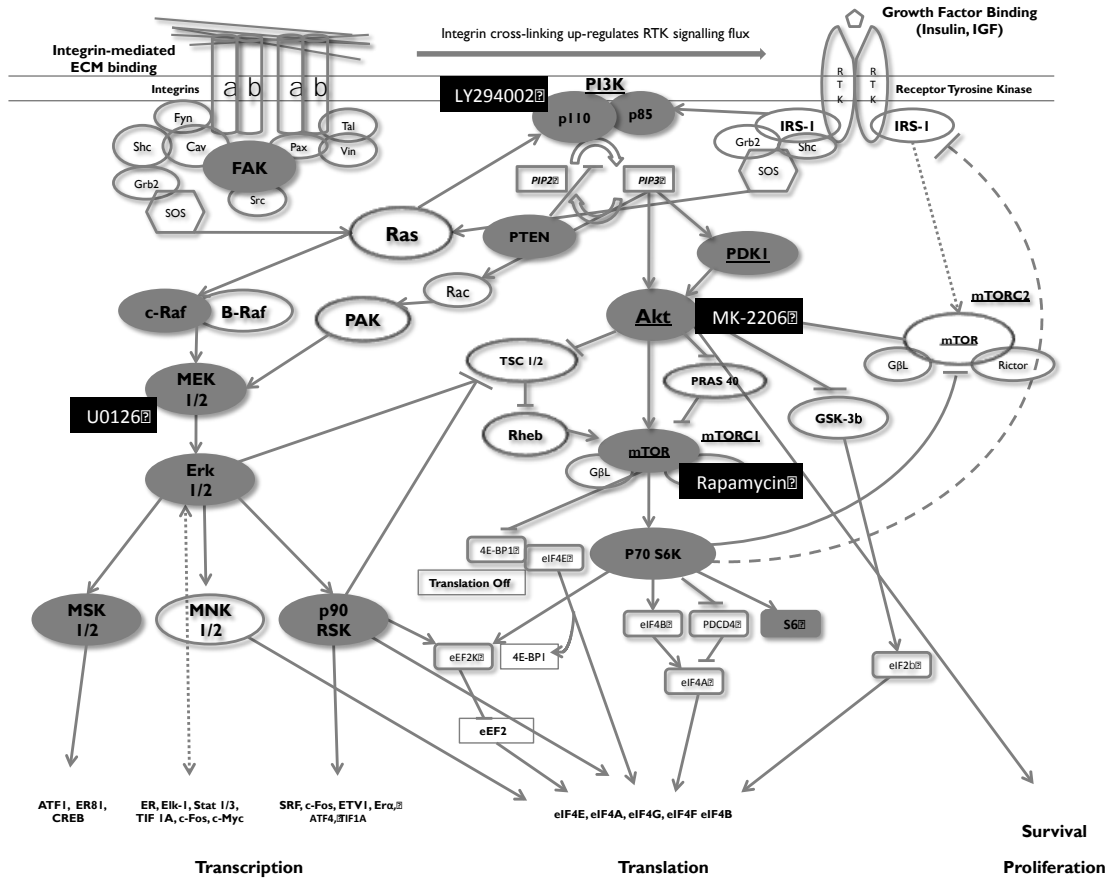
Figure 7.1: Schematic overview of the PI3K/Akt and Ras/MAPK signalling networks and the chemical inhibitors used to dissect these networks. The key signalling intermediates in both the PI3K/Akt and Ras/MAPK pathways that have been analysed by western blotting are shown in grey. The specific chemical inhibitors used in this chapter (LY294002, MK-2206, Rapamycin and U0126) are shown in the black boxes. Signalling network diagram generated from sources including: cellsignal.com, (Anjum and Blenis, 2008; Schwartz and Assoian, 2001; Vivanco and Sawyers, 2002).

7.2 Materials and Methods

7.2.1 Dissection of signalling pathways in adherent conditions

In order to analyse the growth characteristics of the CCL-61 cell line treated with various chemical inhibitors in adherent conditions, the CCL-61 cells were harvested at day 3 of culture (growing in their standard conditions). CCL-61 cells were washed in PBS and then seeded into standard adherent conditions and batch cultured for 8 days. After 24 hours in culture the relevant inhibitor at the appropriate concentration was added to the CCL-61 cells. For the control or untreated CCL-61 cells, the inhibitor vehicle at the appropriate concentration was added after 24 hours in culture. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser.

7.2.2 Dissection of signalling pathways in suspension conditions

To analyse the growth characteristics of the CHO-SA cell line treated with various chemical inhibitors in suspension conditions, the CHO-SA cells were harvested at day 3 of culture (growing in their standard conditions). CHO-SA cells were washed in PBS and then seeded into standard suspension conditions and batch cultured for 8 days. After 24 hours in culture the relevant inhibitor at the appropriate concentration was added to the CHO-SA cells. For the control or untreated CHO-SA cells, the inhibitor vehicle at the appropriate concentration was added after 24 hours in culture. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser.

7.2.3 Dissection of signalling pathways of the Mab producer Cell Line 1 growing in suspension conditions

The growth and productivity characteristics of the Mab producing cell line, Cell Line 1 (CL1), growing in suspension conditions, were analysed in the presence of various chemical inhibitors over 10 days in culture. CL1 cells were harvested at day 3 of culture (growing in their standard conditions), were washed in PBS and then seeded into their standard suspension conditions and batch cultured for 12 days. Post 24 hours, the relevant chemical inhibitor at the appropriate concentration was added to the CL1 cells. For the control or untreated CL1 cells,

the inhibitor vehicle at the appropriate concentration was added after 24 hours. Viability and viable cell density measurements were taken every 24 hours using a Vi-Cell Cell Viability Analyser. Mab supernatant samples were also taken every 24 hours and 5×10^6 cells were harvested every two days for western immunoblot analysis.

7.3 Results

7.3.1 Dissection of signalling pathway activation of cells growing in adherent and suspension conditions

In order to further consolidate the work previously done in chapters 3,4,5 on the differences in signalling pathway activation of cells growing in adherent and suspension conditions, chemical inhibitors were used to dissect these signalling networks and the subsequent effect on cell growth and viability was analysed.

For these experiments, the model system for cells growing in adherent conditions were the CCL-61 cells growing in standard adherent conditions. For suspension conditions, the CHO-SA cells growing in standard suspension conditions with insulin were used.

The effect of the mTOR complex 1 inhibitor, rapamycin, on cells growing in adherent and suspension conditions is shown in figure 7.2. Figure 7.2.A shows the effect of rapamycin on CCL-61 cells growing in adherent conditions. Compared to the control conditions, the 100 nM rapamycin treatment of CCL-61 cells leads to a delay in the drop in viability, with cells maintaining a viability of >95% until the end of culture at 192 hours. In terms of viable cell density, CCL-61 cells treated with rapamycin reach a maximum viable cell density of 0.32×10^6 cells/ cm^2 at 120 hours in culture compared to the control conditions where CCL-61 cells reach a maximum VCD of 0.41×10^6 cells/ cm^2 at 96 hours in culture.

In suspension culture conditions, CHO-SA cells treated with rapamycin also display a delay in the drop in cell viability compared to the control conditions. Cells treated with rapamycin have a final viability of >75% at the end of culture, while the control cells show a decrease in viability from >90% at 120 hours to a final viability of 54% at the end of culture (196 hours). Focusing on viable cell density, control CHO-SA cells reach a maximum VCD of 5.0×10^6 cells/ mL^{-1} at 120 hours in culture, compared to rapamcyin treated CHO-SA cells which reach a maximum VCD of 4.15×10^6 cells/ mL^{-1} at 120 hours in culture.

A) CCL-61 cells in standard adherent conditions
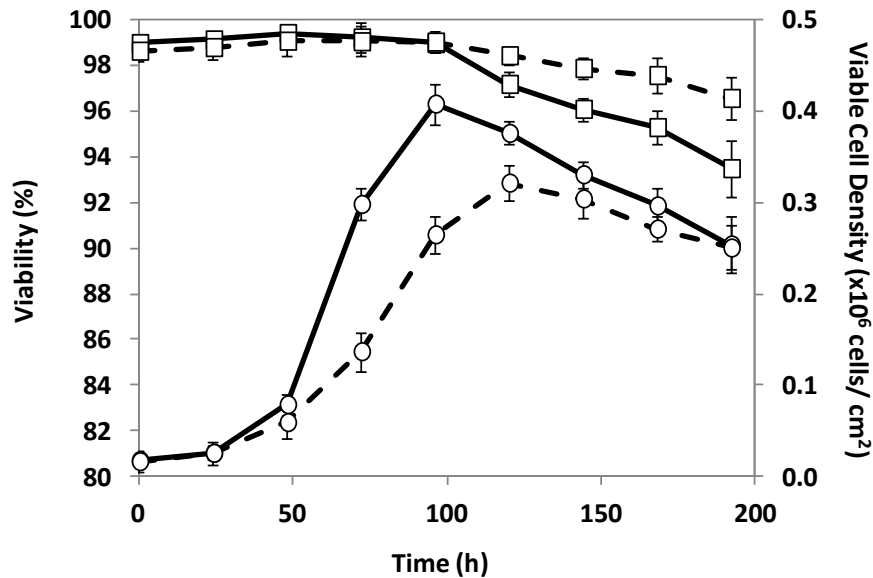
B) CHO-SA cells in standard suspension conditions
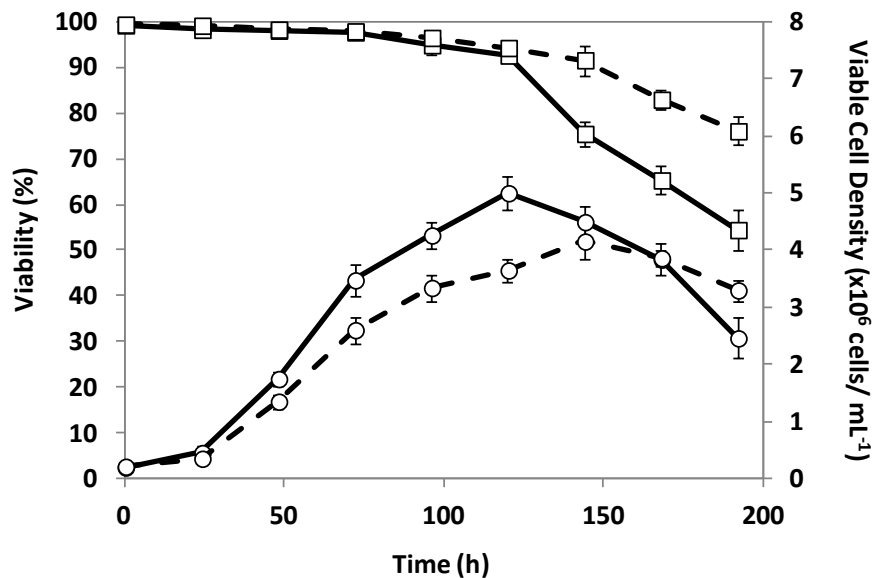
Figure 7.2: Treatment of CCL-61 and CHO-SA cells with 100 nM Rapamycin.
CCL-61 and CHO-SA cells were cultured in standard adherent and suspension conditions, respectively, for 192 hours. For each condition, cells were also treated with 100 nM rapamycin which was added after 24 hours in culture. A) The viability and viable cell density of CCL-61 cells, untreated and treated with rapamycin, growing in adherent conditions. B) The viability and viable cell density of CHO-SA cells cells, untreated and treated with rapamycin, growing in suspension conditions. Viability is represented by square markers and viable cell density by circle markers. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

These results appear to show that rapamycin has similar inhibitory effects on cells growing in adherent and suspension conditions. In both conditions, rapamycin delays the drop in cell viability but also leads to a reduction in maximum viable cell density achieved.

Figure 7.3 shows the results of when CCL-61 cells and CHO-SA cells, growing in adherent and suspension conditions respectively, are treated with 10 µM of the MEK 1/2 inhibitor U0126. Compared to control conditions, CCL-61 cells treated with U0126 show a significant drop in viability from >90% viability at 72 hours in culture to <25% viability at the end of culture. U0126 treated cells also reach a much lower maximum viable cell density of 0.15 x 10^6 cells/ cm^2 at 72 hours in culture compared to the control conditions (0.41 x 10^6 cells/ cm^2 at 96 hours in culture).

In suspension culture conditions CHO-SA cells, treated with U0126, maintained a viability of >95% up to 96 hours in culture after which a sharp decrease in viability is observed over the next 96 hours in culture. At the end of culture CHO-SA cells treated with U0126 had a final viability of 42%. This decrease in viability is observed 24 hours earlier than decrease observed in the control CHO-SA cells, which maintain a viability of >90% up to 120 hours in culture. In terms of viable cell density CHO-SA cells treated with U0126, show a considerable reduction in the maximum viable cell density reached compared to control CHO-SA cells. Control CHO-SA cells follow a relatively normal suspension growth profile, reaching a maximum viable cell density of 5.0 x 10^6 cells/ mL^{-1} at 120 hours in culture. By comparison, CHO-SA cells treated with U0126 have a much shorter exponential phase of growth, reaching a maximum VCD of 2.59 x 10^6 cells/ mL^{-1} at 96 hours in culture. Post 96 hours in culture cells appear to enter death phase and the viable cell density decrease until the end of culture.

Figure 7.4 shows the results of the treatment of CCL-61 and CHO-SA cells with the Akt inhibitor MK-2206 (1 µM). Figure 7.4.A shows the effect of treating CCL-61 cells growing in adherent conditions with the Akt inhibitor MK-2206. In a similar manner to the control CCL-61 cells, CCL-61 cells treated with MK-2206,

A) CCL-61 cells in standard adherent conditions
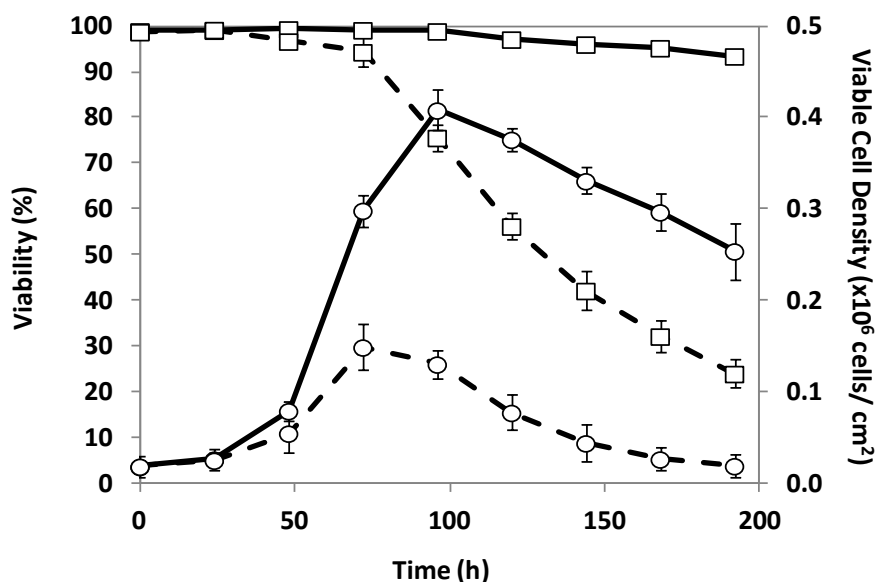
B) CHO-SA cells in standard suspension conditions
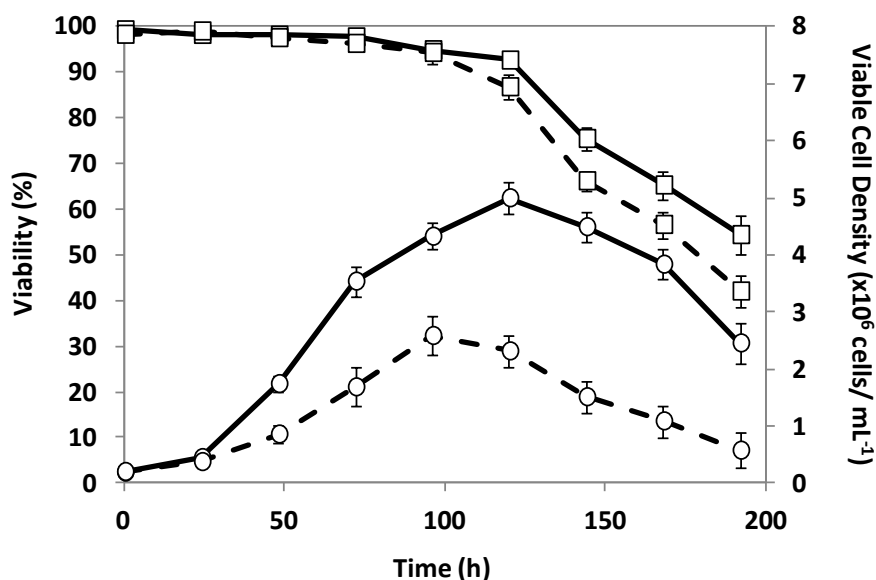
Figure 7.3: Treatment of CCL-61 and CHO-SA cells with 10 μM U0126.
CCL-61 and CHO-SA cells were cultured in standard adherent and suspension conditions, respectively, for 192 hours. For each condition, cells were also treated with 10 μM U0126 which was added after 24 hours in culture. A) The viability and viable cell density of CCL-61 cells, untreated and treated with U0126, growing in adherent conditions. B) The viability and viable cell density of CHO-SA cells cells, untreated and treated with U0126, growing in suspension conditions. Viability is represented by square markers and viable cell density by circle markers. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

A) CCL-61 cells in standard adherent conditions

B) CHO-SA cells in standard suspension conditions

Figure 7.4: Treatment of CCL-61 and CHO-SA cells with 1 µM MK-2206.
CCL-61 and CHO-SA cells were cultured in standard adherent and suspension conditions, respectively, for 192 hours. For each condition, cells were also treated with 1 µM MK-2206 which was added after 24 hours in culture. A) The viability and viable cell density of CCL-61 cells, untreated and treated with MK-2206, growing in adherent conditions. B) The viability and viable cell density of CHO-SA cells cells, untreated and treated with MK-2206, growing in suspension conditions. Viability is represented by square markers and viable cell density by circle markers. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

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maintain a viability of above 90% throughout culture. In terms of viable cell concentration, MK-2206 treated CCL-61 cells display a longer exponential growth phase but despite this reach a lower maximum VCD of 0.30 x 10^6 cells/ cm^2 at 144 hours compared to control CCL-61 cells which reach a maximum VCD of 0.41 x 10^6 cells/ cm^2 at 96 hours in culture.

In suspension conditions, when CHO-SA cells are treated with MK-2206, a noticeable delay in the drop of viability is observed compared to the CHO-SA control cells. MK-2206 cells treated CHO-SA cells maintain a viability of >95% up to 144 hours in culture. Between 144 hours and the end of culture a drop in viability from 96% to 82% is observed. In contrast, control CHO-SA cells display a significant drop in viability from 120 hours onwards until the end of culture. In terms of viable cell density, MK-2206 treated CHO-SA cells have a much lower maximum VCD of 2.81 x 10^6 cells/ mL^{-1} observed at 144 hours in culture, compared to the control CHO-SA cells which have a maximum VCD of 5.00 x 10^6 cells/ mL^{-1} observed at 120 hours in culture.

Figure 7.5 shows the effects of treating CCL-61 and CHO-SA cells with the PI3K inhibitor LY294002. In adherent conditions, CCL-61 cells treated with 50 μM LY294002 show a significant drop in viability post 96 hours in culture. At 96 hours in culture CCL-61 cells have a viability of 95%, which drops over the next 96 hours in culture to 46%. By contrast, CCL-61 control cells maintain a viability of >90% throughout the duration of culture. In terms of viable cell density, CCL-61 cells treated with LY294002 show a considerable drop in the maximum VCD reached of 0.16 x 10^6 cells/ cm^2 at 96 hours compared to control CCL-61 cells which reach a maximum VCD of 0.41 x 10^6 cells/ cm^2 at 96 hours in culture.

In suspension conditions, CHO-SA cells treated with LY294002 display a much earlier decrease in viability compared to control CHO-SA cells. Post 72 hours a significant drop in viability from 93% to 24% at the end of culture is observed. In control CHO-SA cells a viability of greater than 90% is observed up until 120 hours in culture. In terms of viable cell density, treated CHO-SA cells reach a considerably lower maximum VCD of 1.38 x 10^6 cells/ mL^{-1} observed at 72 hours
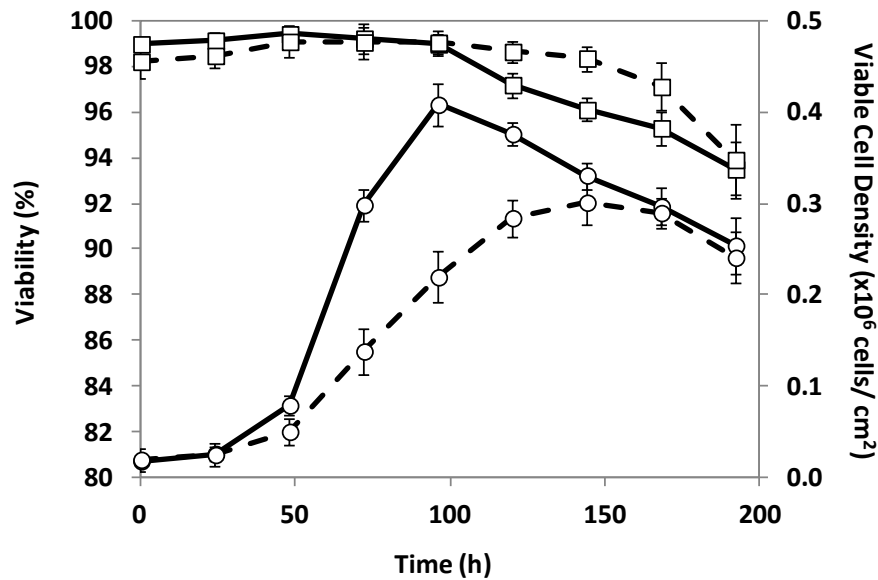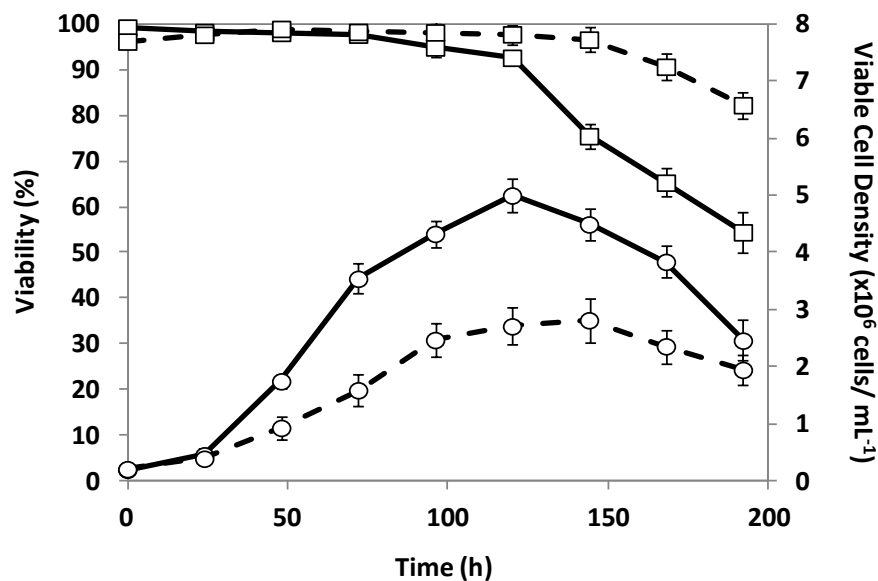
A) CCL-61 cells in standard adherent conditions
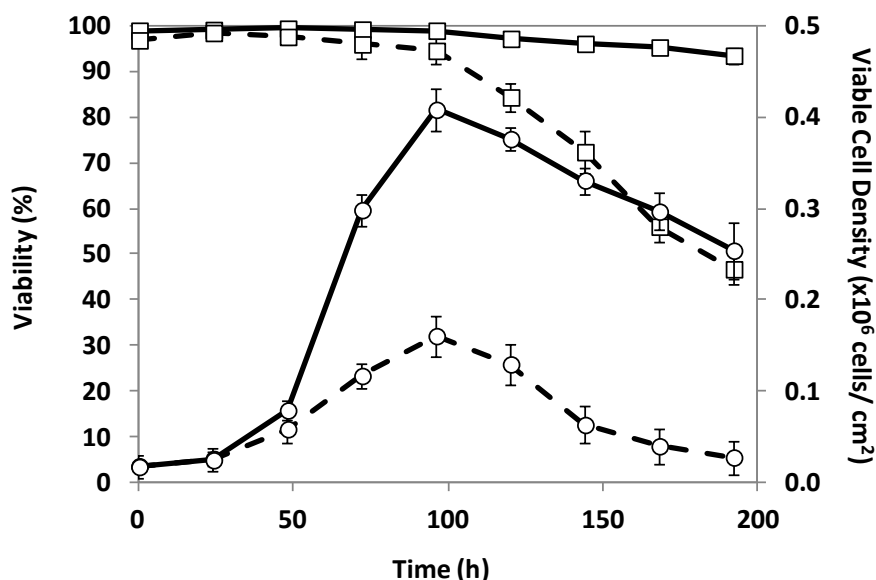
B) CHO-SA cells in standard suspension conditions
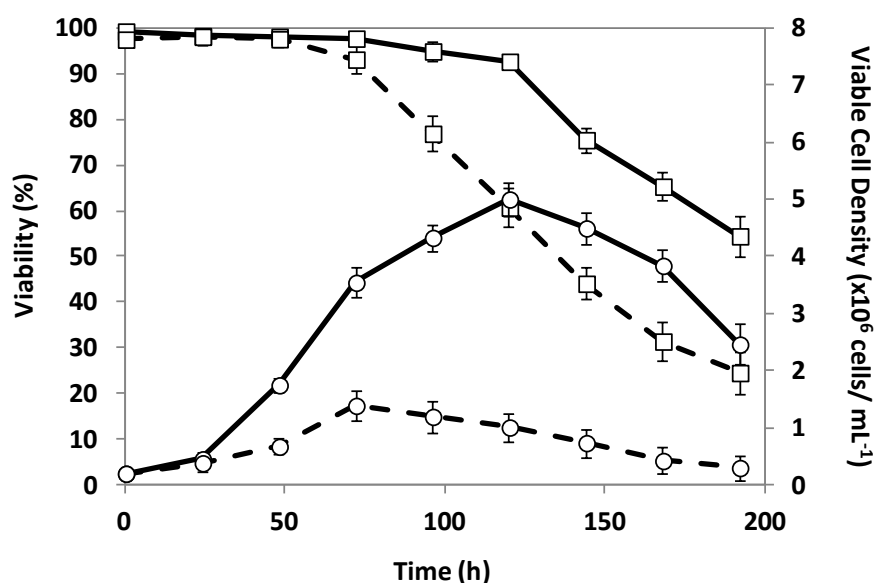
Figure 7.5: Treatment of CCL-61 and CHO-SA cells with 50 μM LY294002.
CCL-61 and CHO-SA cells were cultured in standard adherent and suspension conditions, respectively, for 192 hours. For each condition, cells were also treated with 50 μM LY294002 which was added after 24 hours in culture. A) The viability and viable cell density of CCL-61 cells, untreated and treated with LY294002, growing in adherent conditions. B) The viability and viable cell density of CHO-SA cells cells, untreated and treated with LY294002, growing in suspension conditions. Viability is represented by square markers and viable cell density by circle markers. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

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in culture, compared to the control CHO-SA cells which have a maximum VCD of 5.00×10^6 cells/ mL^{-1} observed at 120 hours in culture. CHO-SA cells treated with LY294002 have an extremely short exponential growth phase from 48 to 72 hours in culture. Post 72 hours in culture, treated cells have a long protracted death phase which lasts up until the end of culture.

7.3.2 Dissection of the signalling pathway activation of the Mab producing Cell Line 1 growing in standard suspension conditions

Further experimental work was performed focusing on the dissection of the signalling networks that are activated in Cell Line 1 (CL1) cells growing in standard suspension conditions. The CL1 cell line is a Mab producing cell line that is descended from the CHO-SA suspension cell line, as explained in section 5.1. As well as analysing the growth characteristics of CL1 cells treated with various inhibitors, their Mab productivity characteristics were also recorded. In addition western blotting analysis, from cell extracts taken every two days, were performed, to analyse how inhibitor treatment affects the signalling pathway activation profile of CL1 cells growing in suspension.

Figure 7.6 shows the results of treating the CL1 cell line with 100 nM rapamycin. In a similar manner to CCL-61 and CHO-SA cells, CL1 cells treated with rapamycin show a delay in the decrease in cell viability compared to the control CL1 cells. Rapamycin treatment also has a minimal effect on viable cell density, with CL1 cells reaching a maximum VCD of 11.26×10^6 cells/ mL^{-1} at 168 hours compared to control CL1 cells which reach a maximum VCD of 11.68×10^6 cells/ mL^{-1} at 144 hours in culture. In terms of Mab productivity, rapamycin treated CL1 cells have a final Mab titre of 923 μg mL^{-1} which is actually slightly higher than that of the control cells (894 μg mL^{-1}).

Western blotting analysis of untreated CL1 and rapamycin treated CL1 cells shows how rapamycin treatment of CL1 leads to a delay in terms of the phosphorylation mediated activation of the mTOR downstream intermediates

A)

B)

C)

D)

E)

F)
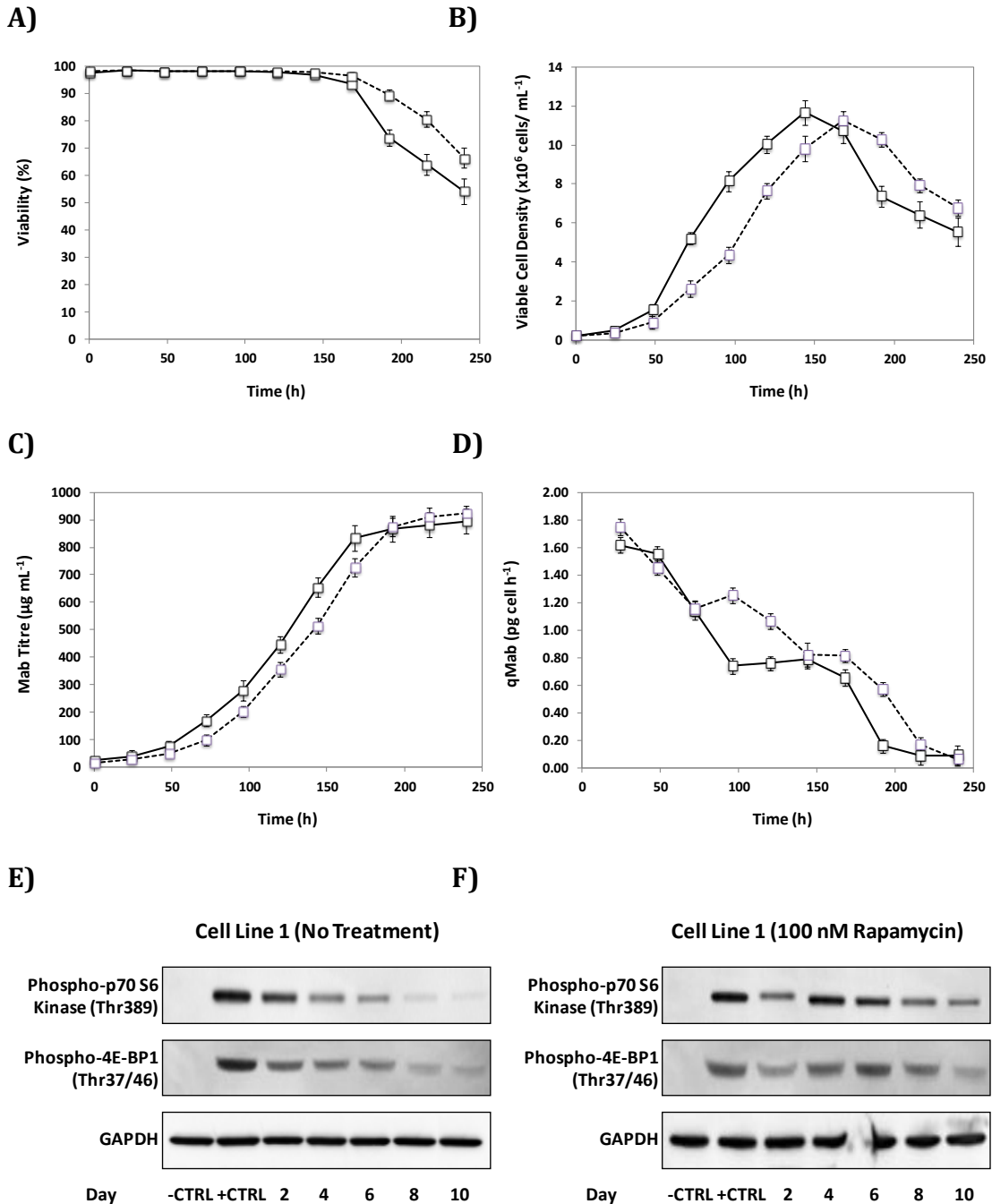
Figure 7.6: Treatment of Mab producing Cell Line 1 with 100 nM Rapamycin. Cell Line 1 was cultured in normal suspension conditions for 240 hours. Cells were also treated with 100 nM Rapamycin which was added after 24 hours in culture. A) The viability of CL1 cells, untreated and treated with Rapamycin. B) The viable cell density of CL1 cells, untreated and treated with Rapamycin. C) The cumulative Mab titre of CL1 cells, untreated and treated with Rapamycin. D) qMab of CL1 cells, untreated and treated with Rapamycin. E) Key western blots of untreated CL1 cells. F) Key western blots of Rapamycin treated CL1 cells. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D.

p70 S6 Kinase and 4E-BP1. These western blots show that rapamycin treatment does affect the signalling activation of intermediates downstream of mTOR.

Figure 7.7 shows the effect of treatment of the Mab producing cell line CL1 with the MEK 1/2 inhibitor U0126. CL1 treatment with 10 μm U0126 leads to a significant drop in viability from 120 hours onwards in culture, which is slightly earlier than the decrease in viability observed in control CL1 cells. CL1 treated cells also have a significantly reduced maximum VCD of 5.34 x 10^6 cells/ mL^{-1} at 120 hours compared to control CL1 cells which reach a maximum VCD of 11.68 x 10^6 cells/ mL^{-1} at 144 hours in culture. A substantial drop in final Mab titre is also observed in CL1 treated cells, of 430 μg mL^{-1}, compared to the final titre of the control cells of 894 μg mL^{-1}. Western blotting analysis conducted, shows that treatment of CL1 cells with U0126 leads to a high level of activation inhibition of the MEK 1/2 downstream signalling intermediate Erk 1/2.

Treatment of the Mab producing cell line CL1 with the Akt inhibitor MK-2206 is shown in figure 7.8. When CL1 cells are treated with 1 μM MKK-2206 a slight delay in the drop in viability is observed compared to untreated CL1 cells. In terms of viable cell density, treated CL1 cells have a maximum VCD of 5.81 x 10^6 cells/ mL^{-1} at 168 hours compared to control CL1 cells which reach a maximum VCD of 11.68 x 10^6 cells/ mL^{-1} at 144 hours in culture. CL1 treated cells appear to have a longer and more protracted lag phase compared to untreated CL1 cells. In terms of Mab productivity CL1 cells treated with U0126 have a final Mab titre of 476 μg mL^{-1}, which is considerably lower when compared to the final titre of the control cells of 894 μg mL^{-1}. In terms of western blotting analysis, the western blots shown illustrate how treatment of CL1 cells with MK-2206, leads to a delay in the activation of Akt in terms of phosphorylation at the key regulatory site Ser473.

The treatment of CL1 cells with the PI3K inhibitor LY294002 is shown in figure 7.9. CL1 treatment with LY294002 leads to a considerable drop in viability from

Figure 7.7: Treatment of Mab producing Cell Line 1 with 10 μM U0126. Cell Line 1 was cultured in normal suspension conditions for 240 hours. Cells were also treated with 10 μM U0126 which was added after 24 hours in culture. A) The viability of CL1 cells, untreated and treated with U0126. B) The viable cell density of CL1 cells, untreated and treated with U0126. C) The cumulative Mab titre of CL1 cells, untreated and treated with U0126. D) qMab of CL1 cells, untreated and treated with U0126. E) Key western blots of untreated CL1 cells. F) Key western blots of U0126 treated CL1 cells. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

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A)

B)

C)

D)

E)

Cell Line 1 (No Treatment)

F)

Cell Line 1 (1 μM MK-2206)
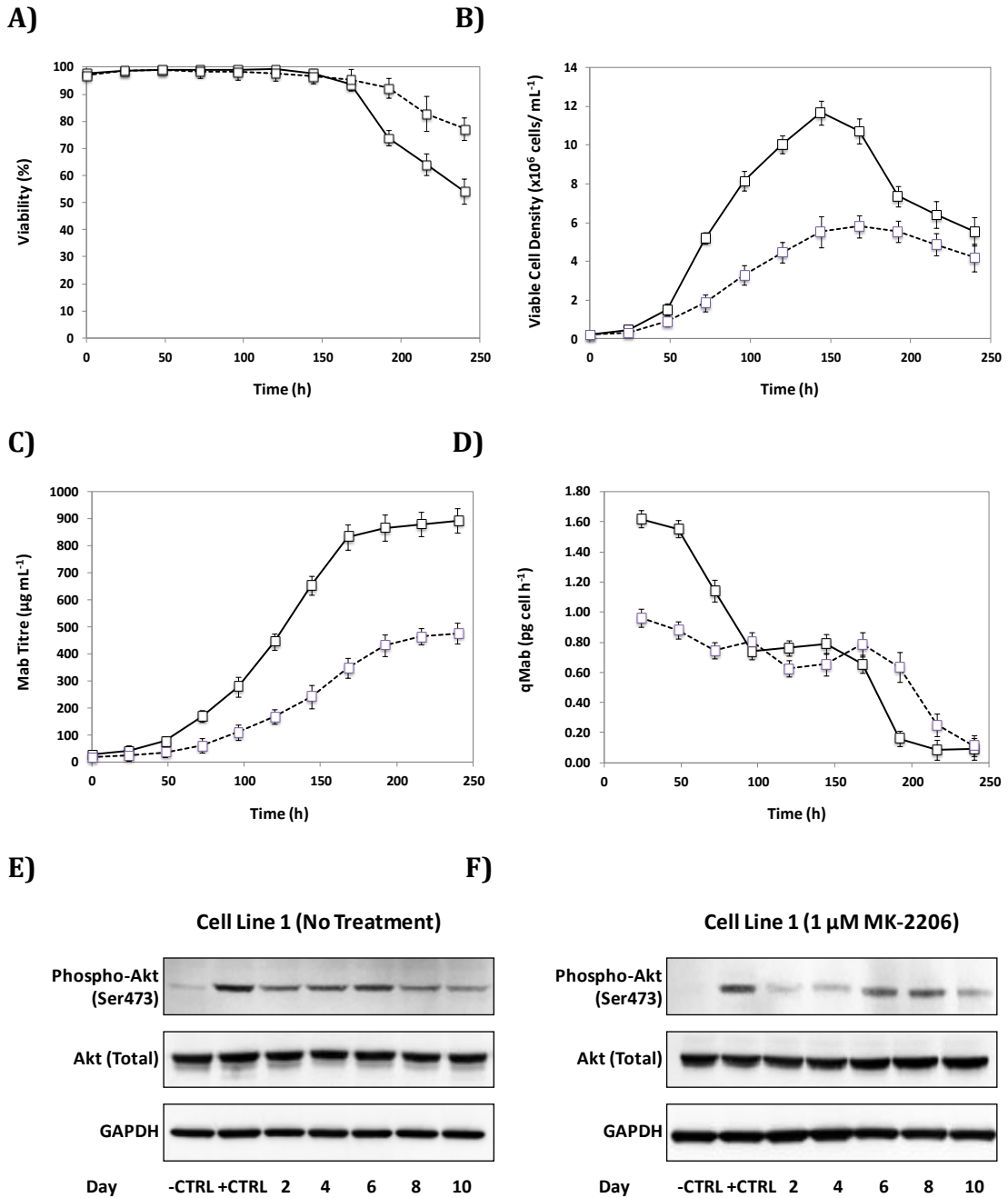
Figure 7.8: Treatment of Mab producing Cell Line 1 with 1 μM MK-2206. Cell Line 1 was cultured in normal suspension conditions for 240 hours. Cells were also treated with 1 μM MK-2206 which was added after 24 hours in culture. A) The viability of CL1 cells, untreated and treated with MK-2206. B) The viable cell density of CL1 cells, untreated and treated with MK-2206. C) The cumulative Mab titre of CL1 cells, untreated and treated with MK-2206. D) qMab of CL1 cells, untreated and treated with MK-2206. E) Key western blots of untreated CL1 cells. F) Key western blots of MK-2206 treated CL1 cells. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

188

A)

B)

C)

D)

E)

Cell Line 1 (No Treatment)
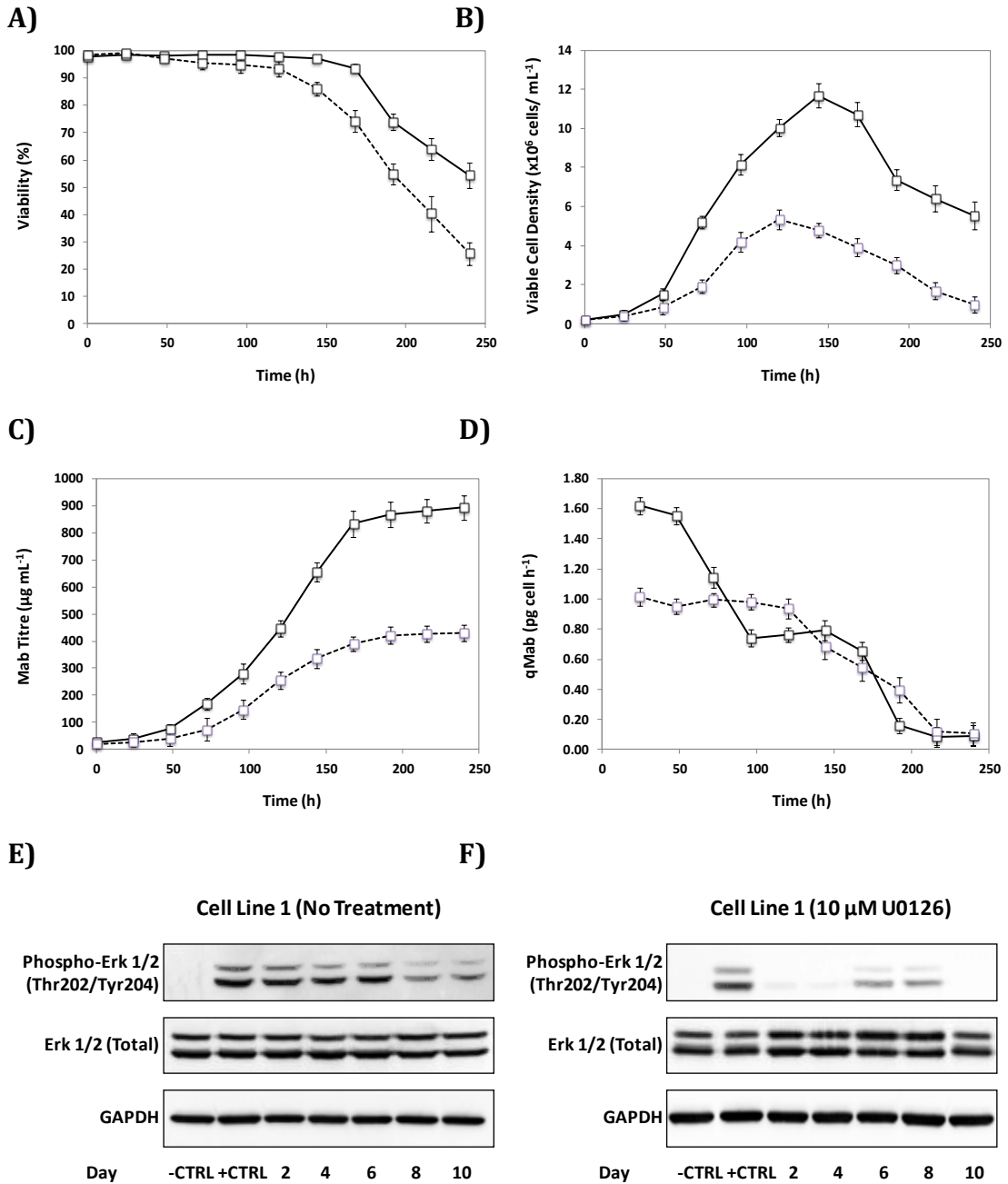

F)

Cell Line 1 (50 µM LY294002)
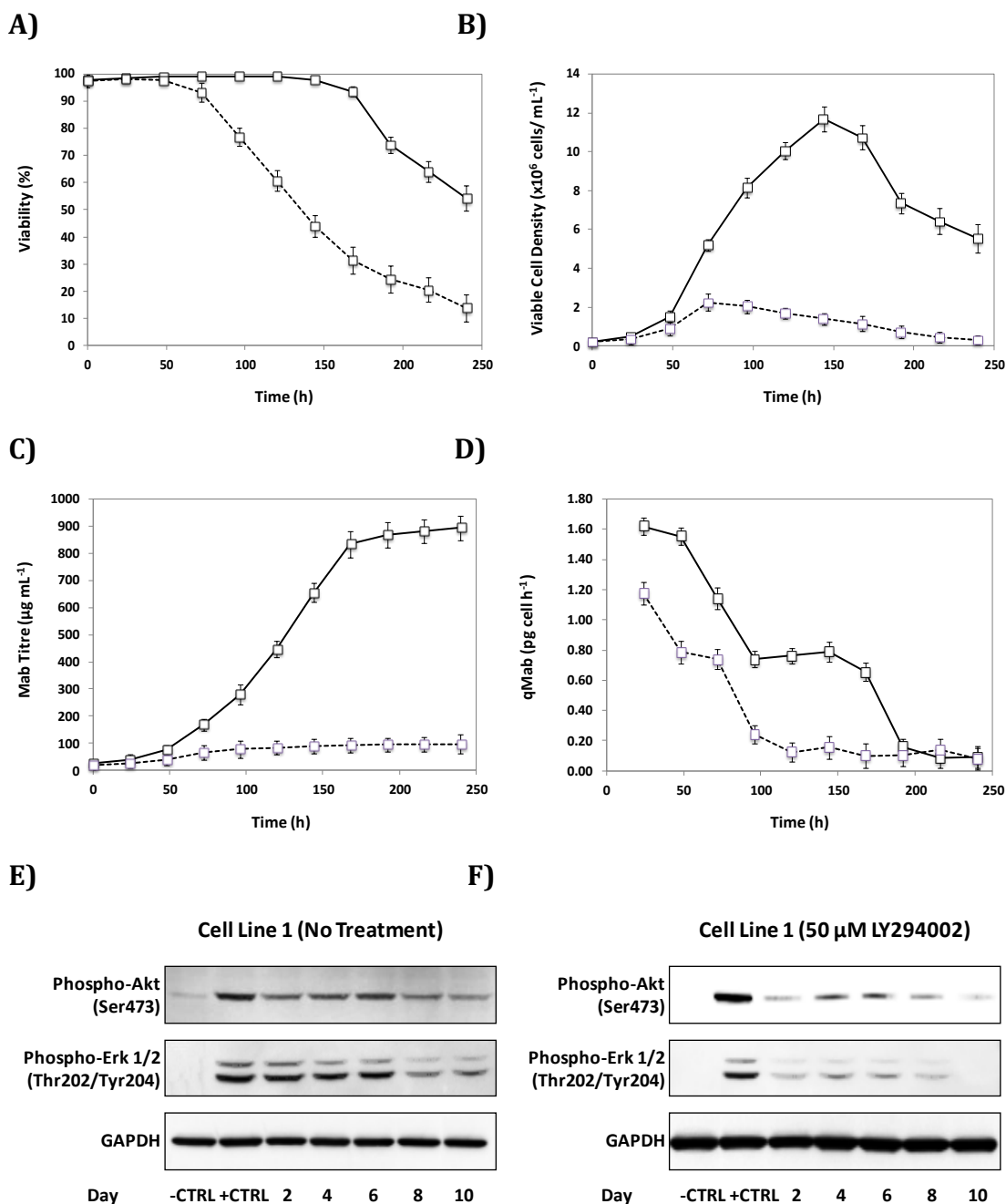
Figure 7.9: Treatment of Mab producing Cell Line 1 with 50 µM LY294002. Cell Line 1 was cultured in normal suspension conditions for 240 hours. Cells were also treated with 50 µM LY294002 which was added after 24 hours in culture. A) The viability of CL1 cells, untreated and treated with LY294002. B) The viable cell density of CL1 cells, untreated and treated with LY294002. C) The cumulative Mab titre of CL1 cells, untreated and treated with LY294002. D) qMab of CL1 cells, untreated and treated with LY294002. E) Key western blots of untreated CL1 cells. F) Key western blots of LY294002 treated CL1 cells. Solid lines represent untreated (control) conditions, dashed lines represent inhibitor treated conditions. The data presented are the average values of triplicate samples analysed in triplicate ± S.D.

72 hours onwards in culture. This is reflected in the viable cell density profile of treated CL1 cells which reach a maximum VCD of 2.25 x 10^6 cells/ mL^{-1} at 72 hours compared to control CL1 cells which reach a maximum VCD of 11.68 x 10^6 cells/ mL^{-1} at 144 hours in culture. Post 72 hours in culture treated CL1 cells go through a long protracted death phase which lasts up until the end of culture. In terms of Mab productivity, LY294002 treated CHO cells have a significantly lower final Mab titre of 97 μg mL^{-1}, when compared to the final titre of the control cells of 894 μg mL^{-1}. Western blotting analysis of CL1 cells treated with LY294002, shows how the activation of two downstream intermediates of PI3K, Akt and Erk 1/2, are affected by this mechanism of inhibition. LY294002 treatment of CL1 cells causes a high level of inhibition in terms of phosphorylation at phospho-Akt (Ser473), which is expected as Akt lies downstream of PI3K. Further western blotting analysis also showed that inhibiting PI3K with LY294002 also leads to the de-activation of Erk 1/2, providing evidence that PI3K is also a regulator of the MAPK signalling cascade.

Finally, figure 7.10 shows a summary of the IVVC of the CCL-61, CHO-SA and CL1 cells when cultured in their standard growth environments and treated with various chemical inhibitors. Figure 7.10.A shows that when compared to the control CCL-61 cells growing in adherent conditions, treatment with all four inhibitors had a significant effect on the final IVCC (One-way ANOVA, Dunnett's Multiple Comparison Test, p<0.0001). In adherent conditions the two most effective inhibitors in terms of cell growth inhibition were U0126 and LY294002. Figure 7.10.B shows the IVCC of CHO-SA cells cultured in standard suspension conditions and their relative response to the inhibitors used. In suspension conditions, only rapamycin treated cells did not differ significantly from the control cells in terms of IVCC (One-way ANOVA, Dunnett's Multiple Comparison Test, p<0.0001). The relative response, in terms of IVCC, of the CL1 cells growing in suspension conditions, to the four inhibitors tested, is shown in figure 7.10.C. In a similar manner to the CHO-SA cells, only rapamycin treated CL1 cells did not differ significantly from the control CL1 cells in terms of IVCC (One-way ANOVA, Dunnett's Multiple Comparison Test, p<0.0001). In both the suspension cultured

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A)
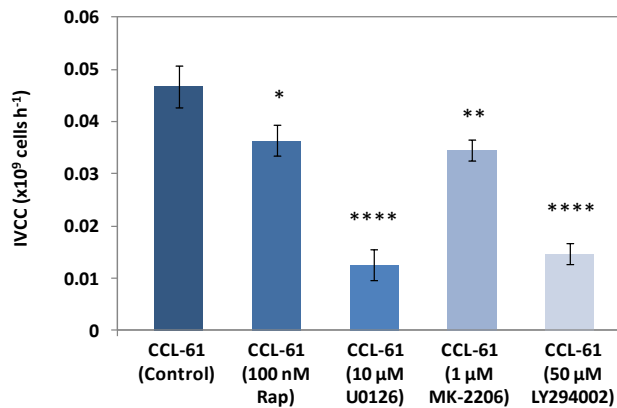
B)
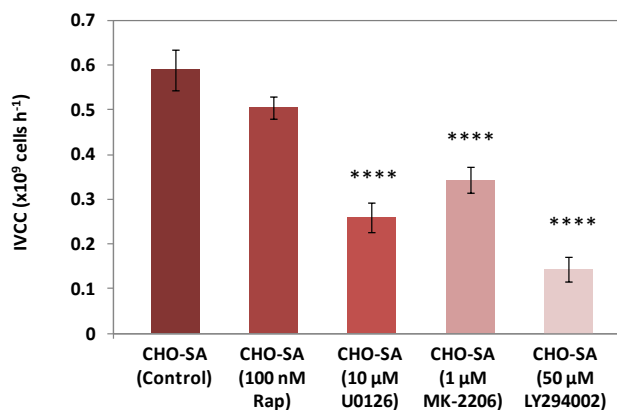
C)
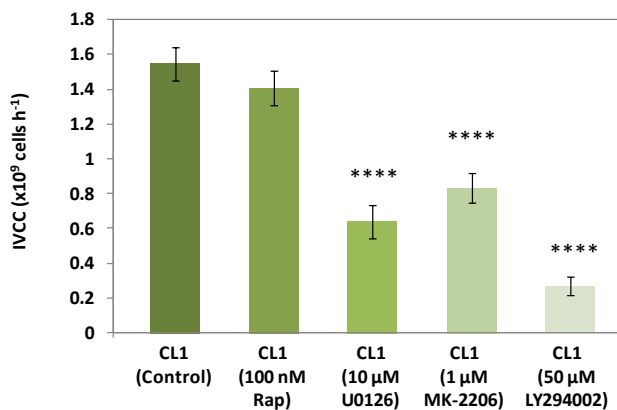
Figure 7.10: IVCC of CCL-61, CHO-SA and CL1 cells treated with various chemical inhibitors. A) The IVCC of CCL-61 cells cultured in standard adherent conditions and treated with various chemical inhibitors. B) The IVCC of CHO-SA cells cultured in standard suspension conditions and treated with various chemical inhibitors. C) The IVCC of CL1 cells cultured in standard suspension conditions and treated with various chemical inhibitors. The data presented are the average values of triplicate samples analysed in triplicate $\pm$ S.D. * = significant difference from control, * is $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

CHO-SA and CL1 cells, the most effective inhibitor in terms of growth inhibition the most effective inhibitor was LY294002.

7.4 Discussion

In terms of gaining a deeper level of understanding of the signalling pathway activation profile, of CHO cells growing in different synthetic environments, chemical inhibitors have proved to be an invaluable tool. The four inhibitors used this study (LY294002 (PI3K inhibitor), U0126 (MEK 1/2 inhibitor), MK-2206 (Akt inhibitor), Rapamycin (mTORC1 inhibitor) were selected as they inhibit key intermediates at various upstream/downstream levels of both the Ras/MAPK and PI3K/Akt pathways.

The data presented in this chapter shows that LY294002 is potent inhibitor of PI3K signalling in CHO cells growing in both adherent and suspension conditions. As shown previously in chapter 4, one hypothesis put forward was that CHO cells adapted to grow in suspension conditions, are able to utilise growth factors included in the cell culture media by activating the PI3K/Akt pathway. This would confer PI3K as a key regulator in suspension growth conditions. This is confirmed by the data presented in this study, as the growth and proliferation of both CHO-SA and CL1 cells growing in suspension conditions is significantly inhibited by LY294002. What is also apparent is that PI3K is also a key signalling intermediate when CCL-61 cells are growing in adherent conditions. As again shown in chapter 4, cells growing adherently utilise both the Ras/MAPK and PI3K/Akt pathways. As LY294002 significantly reduces the IVCC of CCL-61 cells growing in adherent conditions, this would indicate that PI3K is linked to the regulation of the Ras/MAPK pathway as well as the PI3K/Akt pathway. Such signalling cross-talk has been previously discussed in publications such as the paper by Mendoza et al (Mendoza et al., 2011). Various upstream intermediates in the Ras/MAPK pathway, including Ras itself, have been shown to directly activate PI3K (Kodaki et al., 1994; Rodriguez-Viciana and Downward, 2001).

The relationship between PI3K and its downstream signalling targets including cross-talk with the MAPK pathway has also been described in several reports. While numerous groups have shown that activation of the MAPK pathway by growth factors, including insulin, is sensitive to the PI3K inhibitors Wortmannin and LY294002 (Cross et al., 1994; Versteeg et al., 2000).

Experiments conducted with the MEK 1/2 inhibitor, U0126, provide further evidence that both the Ras/MAPK and PI3K/Akt pathways play vital roles in adherent and suspension growth. In both adherent and suspension culture environments, treatment of CCL-61, CHO-SA and CL1 cells with U0126 leads to a significant inhibition of cell growth, indicating that even low level Ras/MAPK activation is vital for cell growth and survival. As discussed in chapter 4, suspension cells may utilise the PI3K/Akt pathway to a greater degree, in terms of their response to insulin, but the data presented in this chapter also shows that suspension cells do utilise the MAPK pathway to some degree. This is to be expected as previous work conducted has shown that the MAPK pathway plays a key role in cell survival (Ballif and Blenis, 2001). As well, the MAPK signalling network plays a key role in the co-regulation of the mTORC1 signalling complex (Costanzo-Garvey et al., 2009).

After looking at the activation of the upstream intermediate PI3K, the next intermediate in the PI3K/Akt to focus upon was Akt. As discussed earlier in this chapter, the Akt inhititor MK-2206, has not been widely used in CHO cells therefore considerable experimental work was conducted testing different concentrations and dose-response times prior to these experiments. Treatment with MK-2206 of CCL-61 cells growing adherently and CHO-SA and CL1 growing in suspension, leads to a significant decrease in the IVCC of all three cell lines in their respective growth environments. This would again provide evidence that Akt activation is required for cells growing in both adherent and suspension conditions. It is widely accepted that Akt is a key regulator of numerous cellular processes such as cell survival, proliferation and metabolism. In adherent cells, integrin-mediated cell survival has been shown to be regulated by a mechanism that involves the activation of Akt (Lee and Ruoslahti, 2005; Matter and

Ruoslahti, 2001). While cells in suspension have been shown to utilise the PI3K/Akt pathway as a method of circumventing anoikis (Khwaja et al., 1997).

Downstream of Akt, lies the key regulator of protein synthesis, mTOR. In this study the mTORC1 inhibitor rapamycin was used to inhibit mTOR activation in cells growing in both adherent and suspension conditions. The results shown in this chapter appear to show that rapamycin only has a significant effect on CCL-61 cells cultured in adherent conditions. In CHO-SA cells there is a minimal affect on cell growth by treatment with rapamycin. In the Mab producing CL1 suspension cells, treatment with rapamycin has a minimal on cell growth, prolongs cell viability and actually slightly increases Mab production when compared to the control untreated cells. These results are similar to what has been observed by other groups such as Lee and Lee, and Balcarel and Stephanopoulos, where recombinant protein production was increased by treating cells with rapamycin (Balcarcel and Stephanopoulos, 2001; Lee and Lee, 2012). From these results a number of conclusions can be drawn. The first is that rapamycin is perhaps not an ideal inhibitor for long term studies (days rather than hours) focusing on the inhibition of mTOR (Huo et al., 2011). The second conclusion is that a number of different signalling intermediates including p90RSK, Akt and TSC1/2 are responsible for the upstream regulation of mTOR (Nave et al., 1999; Roux and Blenis, 2004; Roux et al., 2007). Finally, with mTOR being a key regulator involved in the regulation of cell growth, protein translation, amino acid transport, autophagy and many other processes, it is an ideal target when looking to engineer cells to improve protein production capacity. This has already been shown in the study by Dreesen and Fussenegger, where overexpression of mTOR, increased cell proliferation rate and antibody production (Dreesen and Fussenegger, 2010).

Chapter 8

Discussion and Future Work

The overall aim of this study was to gain a greater understanding of the changes that occur in terms of signalling pathway activation when CHO cells are adapted to grow in serum-free suspension conditions. The ability of CHO cells to reach high cell densities in suspension conditions, in chemically-defined media free of serum, is a key principle that underpins the process of recombinant protein production from mammalian cells growing in culture. The adaptation procedures that have been developed in order to generate such suspension CHO cell lines that reach high cell densities in suspension culture are extremely well-defined processes. However, the level of understanding behind the biological changes that occur, to allow cells to survive in such an environment, is minimal.

8.1 Conclusions from work conducted

The starting point for this study was to look back at the cell line history of the suspension cell lines received from Pfizer. In order to generate their platform Mab producing cell lines, Pfizer have taken the parental CCL-61 cell line, which grows as an adherent monolayer in the presence of serum, and taken it through numerous rounds of adaptation. The end result is a cell line is able to reach a high cell density, maintain a high viability and produce a large quantity of the recombinant protein product.

In order to generate their production cell lines, the first adaptation step that Pfizer carried out, was to take the CCL-61 cells and gradually adapt them to serum-free suspension culture conditions, thus generating the CHO-SA cell line. In order to understand the changes that have occurred to the cells that have been through this adaptation process, growth characterisation studies were

conducted, looking at the ability of the CCL-61 and CHO-SA cell lines to grow in both adherent and suspension conditions. From these studies it was ascertained that CHO-SA cells are able to revert back to an adherent growth phenotype in a similar manner to the CCL-61 cells. For the suspension growth characterisation studies both cell lines were cultured in proprietary suspension media with and without added insulin. Only the CHO-SA cell line growing in the presence of added insulin was able to reach sufficiently high cell densities to be classed as a cell line capable of growing in suspension culture conditions. From this it was concluded that cells that CCL-61 cells are not-adapted to grow in suspension, as they display a low level growth response to added insulin in the culture media. Whereas, the suspension-adapted cell line, CHO-SA, displays a higher level growth response to added insulin and can therefore grow and proliferate in suspension conditions.

The next stage of analysis involved mapping the changes that occur, in terms of signalling pathway activation, when cells are transferred from adherent conditions to suspension conditions with and without added insulin. Two key signalling pathways were identified that are closely linked to the regulation of cell attachment mediated signalling as well as growth factor signalling. Both the Ras/MAPK and PI3K/Akt pathways were analysed in detail in order to decipher the changes that occur from adherent mediated signalling to suspension – growth factor mediated signalling. Extensive western blotting analysis of key intermediates in the Ras/MAPK and PI3K/Akt pathways when the CCL-61 and CHO-SA cell lines are growing in both adherent and suspension conditions, revealed two distinct cell signalling mechanisms are regulating cell growth. In adherent conditions, when integrin mediated-attachment signalling is occurring, both the Ras/MAPK and PI3K/Akt pathways are activated to a certain level. When all the cell lines are transferred to suspension growth conditions, there is significantly less signalling activation occurring through the Ras/MAPK pathway, thus indicating that the Ras/MAPK pathway is activated in response to cell adherence to the extra-cellular matrix. The second mechanism of signalling that was uncovered, was the regulation of the PI3K/Akt pathway in response to insulin in suspension conditions. All the cell lines cultured in suspension

conditions showed relatively low levels of increased activation of the Ras/MAPK pathway in response to the addition of insulin. By contrast, the CHO-SA cell line showed a significant increase in PI3K/Akt activation when cultured in the presence of insulin in suspension. These results indicate that the CHO-SA cell line that has been adapted to suspension growth, is able to grow in suspension conditions by utilising the insulin-mediated activation of the PI3K/Akt pathway.

After mapping the changes that occur in terms of signalling pathway activation from adherent to suspension growth conditions, the next stage was to map the changes that occur when suspension cells are further adapted, over time, to grow at higher densities in suspension. Cell Line 1 was a cell line, received from Pfizer, that was adapted from the CHO-SA cell lines to grow in high density suspension conditions and that had been transfected to become a stable recombinant Mab producing cell line. In order to gain an insight into the pathway activation profile of cells growing in suspension, three different cell lines were selected that reflected the various stages of adaptation that had been performed to generate Cell Line 1. The pathway activation status of the Ras/MAPK and PI3K/Akt pathways was measured when CCL-61 (non-suspension adapted), CHO-SA (semi-suspension adapted) and CL1 (fully suspension adapted) cell lines were cultured in suspension conditions with added insulin. The results revealed that CCL-61 cells show relatively low levels of activation of both the Ras/MAPK and PI3K/Akt pathways when compared to CHO-SA and CL1 cells cultured in suspension. When compared to the CCL-61 cell line, the CHO-SA cell line shows an increase in activation of the Ras/MAPK pathway but an even greater increase in the activation levels of intermediates in the PI3K/Akt pathway is also observed. This is mirrored in the activation profile of the CL1 cells which displays an even greater increase in activation of the PI3K/Akt pathway. CL1 cells also display a large increase in activation of the key upstream signalling intermediate PI3K, implicating PI3K activation as a key feature in the ability of cells to reach high cells densities in suspension culture.

After deciphering the activation status of the Ras/MAPK and PI3K/Akt pathways in different growth environments it was important to analyse how these

pathways are regulated by changes at the cell surface interface. As postulated in many studies, integrins play a vital role in anchoring adherent cells to the extracellular matrix and are therefore implicated in the regulation of anoikis. As suspension cells do not require such integrin mediated attachment in order to grow, proliferate and survive, it was hypothesised that integrin expression levels would be down-regulated on suspension adapted CHO cells. Flow cytometry and western blotting analysis of the expression levels of the integrins alpha1, alpha5 and beta1 shows that all three integrins are expressed on CCL-61 cells, cultured in standard adherent conditions, and CHO-SA and CL1 cells that are growing in suspension conditions. There was slight but not significant variation in the expression of all three integrins on the three cell lines analysed, thus disproving the hypothesis that integrin expression is down-regulated on suspension adapted cells.

To investigate the role of integrins on suspension adapted CHO cells, confocal microscopy analysis was used to look at the conformation and distribution of the alpha1, alpha5 and beta1 integrins on the cell surface of adherent and suspension cells. The confocal microscopy images revealed that a change in integrin distribution is observed when cells are adapted to suspension growth environments. Cells that are adapted to suspension growth, have integrins that are clustered together on the cell surface and that are fairly evenly distributed over the surface of the cell, almost forming a sphere shape. This is particularly the case for integrin beta 1. This sphere like arrangement of integrins on suspension cells is mirrored by changes observed in the actin cytoskeleton of cells growing in suspension conditions compared to adherent conditions. When cells are transferred from adherent to suspension conditions, the actin cytoskeleton changes to form a sub-cortical actin ball that acts almost like a shell, encapsulating the inside of the cell. This change in integrin and actin conformation in suspension cells, perhaps indicates a shift in the role of integrins between adherent and suspension adapted CHO cells.

Adhesion assay experiments were then performed in order to investigate the how growth factor receptors and integrins interact on the cell surface. Previous

research has indicated that activation of specific growth factor receptors on the cell surface potentiates integrin mediated attachment to various proteins that form the extra-cellular matrix such as fibronectin and vitronectin. Experiments conducted in this study showed that treatment, of both non-suspension adapted CCL-61 cells and semi-suspension adapted CHO-SA cells, with insulin significantly increased the integrin-mediated binding capabilities of both cell lines to the ECM protein fibronectin. Further treatment of both cell lines with the PI3K inhibitor, LY294002, significantly inhibited this insulin stimulated and integrin-mediated adhesion to fibronectin, indicating that PI3K is a key signalling intermediate in regulating both adherent-mediated signalling and growth factor mediated signalling. Treatment with the focal adhesion kinase inhibitor FAK-14, also showed that FAK is a key intermediate in terms of regulating cell adhesion to the ECM.

The final set of experiments conducted involved dissecting the activation status of both the Ras/MAPK and PI3K/Akt pathways in cells growing in both adherent and suspension conditions. This was done by using specific chemical inhibitors that inhibit selected nodes in each of pathway of interest. Treatment of both CCL-61 cells growing adherently and CHO-SA and CL1 cells growing in suspension, with various inhibitors, showed that cells cultured in both adherent and suspensions conditions require the activation of both the Ras/MAPK and PI3K/Akt in order to survive, grow and proliferate. Even in suspension culture conditions, where previous work done has demonstrated that signalling flux occurs to a greater level through the PI3K/Akt pathway, cells treated with the MEK 1/2 inhibitor U0126 show significantly reduced levels of growth and a reduced viability earlier in culture. Further use of the PI3K inhibitor, LY294002, showed that PI3K is vital signalling intermediate for cell growth and survival in both adherent and suspension culture environments.

8.2 Future work/directions for research

The main aim of this study was to analyse the underlying changes that occur in terms of signalling pathway activation, when CHO cells are transferred to different synthetic growth environments. The work conducted so far has provided an insight into some of the changes that occur in both the Ras/MAPK and PI3K/Akt pathways in response to changes in the cell growth environment. However, in order to generate such changes that allow cells to survive and proliferate at high densities in suspension conditions, as well as to produce high levels of correctly folded recombinant protein, long-lasting empirically driven adaptation processes are still utilised. Therefore, one of the main goals of such research, has to be to gain the ability to engineer cells that can circumvent such long-winded processes.
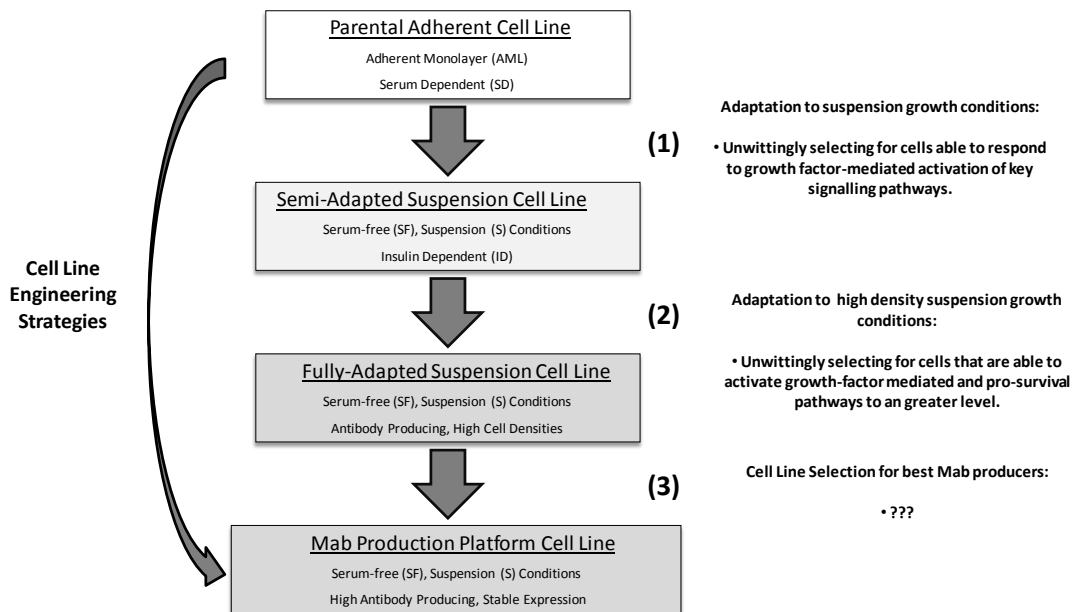
Figure 8.1: Overview of a cell line adaptation strategy. An overview of a cell line adaptation strategy used to generate a platform Mab producing CHO cell line. Targeted cell line engineering strategies provide a possible solution in avoiding such time and labour consuming processes.

8.2.1 Cell engineering strategies

Cell engineering strategies that have been employed so far range from one gene-based targeted engineering (where the improvement of one cellular characteristic is focused upon) to multi-gene-based strategies and also the improvement of many cellular characteristics by directed genetic engineering (Dreesen and Fussenegger, 2010; Fussenegger et al., 1998). There are numerous examples of such strategies being used to improve cell growth, survival and protein production capabilities.

Work done by Mastrangelo et al, focused upon improving mammalian cell survival in bioreactor conditions, by the overexpression of the anti-apoptotic proteins bcl-2 and bcl-x(L) (Mastrangelo et al., 2000). Dreesen et al, looked at improving various cell traits including, cell line viability, proliferation and protein production, by ectopically expressing the downstream PI3K/Akt signalling intermediate mTOR (Dreesen and Fussenegger, 2010). While in the paper by Matter and Ruoslahti, the authors looked at the overexpression of a number signalling intermediates in both the Ras/MAPK and PI3K pathways that lead to increased bcl-2 expression and therefore increased cell survival in suspension conditions(Matter and Ruoslahti, 2001).

8.2.2 Insulin signalling

A large portion of the research conducted in this study focused upon the activation of the Ras/MAPK and PI3K/Akt pathways in response to the growth factor insulin. Whilst the understanding of the insulin mediated activation of each pathway has been investigated, the actual upstream events that dictate this activation have only been touched upon. Some work (that was not included in this thesis) was conducted looking at the activation of the insulin receptor and IRS-1 proteins in response to insulin in different culture environments; however this was not of sufficient depth or quality to be relied upon. Work done by Heller-Harrison et al , showed that the IRS-1 protein is a key intermediate in relaying the effects of insulin binding at the cell surface to downstream PI3K

activation(Heller-Harrison et al., 1995). While work done by Guilherme et al, showed that tyrosine phosphorylation of the insulin receptor and IRS-1 protein is lower in cells that are cultured unattached to the ECM (Guilherme et al., 1998). This is a possible explanation of why non-suspension adapted CCL-61 cell growing in suspension are less responsive to insulin. Further work investigating the effect of integrin mediated potentiating of growth factor signalling as described in work by Fujita et al and Guilherme et al, could also be conducted(Fujita et al., 2012; Guilherme et al., 1998).

8.2.3 Pathway analysis in fed-batch and bioreactor cultured cells

The experiments done in this study involved looking at the growth of various cell lines in suspension culture conditions, that were cultured in low-volume Erlenmeyer flasks. All the experiments conducted were in batch culture conditions. These conditions are not truly reflective of what usually occurs in the biopharmaceutical industry in terms of Mab production from CHO cells. When production runs using CHO cells are performed, the cells are usually cultured in large-scale bioreactors with a fed-batch strategy. Therefore a further step in this research could be to analyse how cells alter their signalling pathway activation profile, throughout culture, in response to high cell densities, the burden of protein production and the differing feed strategies used. Work done by Prentice et al, has previously shown that cells that have evolved by being cultured in bioreactor conditions display increased growth and productivity levels (Prentice et al., 2007). This indicates that there are underlying changes at the cell signalling that have occurred to allow cells to evolve these properties.

8.2.4 Signalling pathway regulation and metabolism

The links between the Ras/MAPK and in particular the PI3K/Akt pathways and cell metabolism regulation have been become well established in the published literature, with many groups showing that alterations in these pathways can cause significant changes in the cells metabolism behaviour. Mulukutlia et al, showed that a metabolic shift to consuming lactate late in culture is regulated by

many different processes including Akt signalling(Mulukutla et al., 2012). In terms of amino acid metabolism, the synthesis and transport of key amino acids, including L-glutamine, have been shown to be key factors regulating mTOR activation(Nicklin et al., 2009). While Zoncu et al, showed that mTOR activation and translocation is closely regulated by amino acid levels(Zoncu et al., 2011). Therefore it would be interesting to look at how activation of these two pathways is altered in response to changes in the cellular metabolism and amino acid availability through culture.

8.2.5 Comparing cell lines with different Mab productivity characteristics

One final avenue of future work, would be to analyse the activation status of key intermediates in the Ras/MAPK and PI3K/Akt pathways in suspension cell lines that have different levels of Mab productivity. As shown earlier in this study, cells that have been through extensive rounds of adaptation to generate a cell line capable of growing to high densities and to produce relatively high levels of recombinant protein, display a different pathway activation profile to that of a non-suspension adapted cell line. However, very little work has been conducted looking at the possible difference(s) in the pathway activation profile between Mab producing cell lines that are relatively high, medium and low Mab producers.

Chapter 9

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