Tamoxifen resistance in breast cancer: A proteomic approach

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Abstract

Tamoxifen is an effective and well-tolerated treatment for early disease and/or pre-menopausal patients with breast cancer (BC); although many women go on to develop resistance. Currently the five-year survival rate following Tamoxifen resistance (TR) is < 20%; hence the mechanisms need to be better understood. Recent research has focussed on specific pathways, however additional mechanisms are involved and we investigated these using cell line models of BC (MCF7) and TR using a variety of proteomic approaches.

Differential expression and phosphorylation of proteins between the MCF7 and TR cell lines were detected by antibody arrays; which detected changes in Mitogen Activated Protein Kinases and Receptor Tyrosine Kinases family members, and in apoptosis related proteins. There were 21 novel proteins found to be altered in TR.

262 quantifiable proteins were found using SILAC; 29% over expressed in resistance and 25% down regulated. 5 were subsequently picked for validation by Western blot and 2 of these (IQGAP1 and cortactin) were chosen for further investigation with siRNA and functional assays.

IQGAP1 was found to play a role in TR; as decreasing expression of IQGAP1 using SiRNA decreased the proliferation of TR cells and significantly modulated the TR cells ability to invade matrigel.

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I would lastly like to thank my husband John. I thank you for putting up with/loving me during the writing process, and through everything. You have been my rock, and you rock! I love you and am very much looking forward to getting my life back with you, xxxxxxxxxxx.

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Abbreviations

#K	Lysine (C_{13} heavy label)
4 – OH	4 Hydroxy
AA	Amino acid
aCHCA	α-Cyano-4-hydroxycinnamic acid
ACN	Acetonitrile
AF2	Activating function 2
AI	Aromatase Inhibitors
AIB1	Amplified in breast cancer 1
AP1	Transcription factor activator protein 1
ARF6	ADP ribosylation factor 6
BCL XL	B-cell lymphoma-extra large
BCL2	B-cell lymphoma 2
BIG 1-98 trial	Breast International Group 1-98 trial
BSA	Bovine Serum Albumin
Cas	Src substrate p130
CID	Collision induced ionisation
csFCS	Charcoal stripped Foetal Calf Serum
CYP2D6	Cytochrome P450 2D6
CYP3A4	Cytochrome P450 3A4
DCIS	Ductal carcinoma in situ
Diablo	Direct IAP Binding Protein with Low PI
DTT	Dithiothreitol
E_2	Estrodiol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ER	Oestrogen receptor
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ErbB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ErbB4	v-erb-b2 erythroblastic leukemia viral oncogene homolog 4
ERE	Oestrogen response element
ERK1 & 2	Extracellular signal-regulated kinase 1 and 2
ESI	Electrospray Ionisation
ESI	Electrospray ionisation
FA	Formic acid
FAK	Focal adhesion kinase

FCS	Foetal Calf Serum
FOXP3	Forkhead box P3
GTP	Guanidine tri-phosphate
HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
HSP90	Heat shock protein 90
IAP	Inhibitor of apoptosis
ICPL	Isotope-coded protein label
IGF1	Insulin-like growth factor 1
ITRAQ	Isobaric tags for relative and absolute quantitation
JNK	Jun-amino-terminal kinase
K	Lysine
LCIS	Lobular carcinoma in situ
LDS	Lithium dodecyl sulfate
LHRH	Leutenising
LN_2	Liquid Nitrogen
MALDI	Matrix assisted laser desorption ionisation
MAPK	Mitogen Activated Protein Kinase
MDR1	Multidrug resistance 1, also known as P-gylycoprotein
MeOH	Methanol
MES	2-(*N*-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
mTOR	Mammalian Target of Rapamycin
NF Kappa B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Health and Clinical Excellence
NPC	Naso-pharangeal Cancer
PAX2	Paired box gene 2
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PELP1	Proline glutamic acid and leucine-rich protein 1
PI3K	Phosphoinositide 3-kinase
PKCd	Protein kinase C delta
PRMT1	Protein arginine methyltransferase 1
PTM	Post translational modification
PUMA	p53-upregulated modulator of apoptosis
PVDF	Polyvinylidene Fluoride
R	Arginine
RF	Radio frequency
rt	Room temperature
ROS	Reactive oxygen species

RSK	90 kDa ribosomal S6 kinase 1
RTK	Receptor Tyrosine Kinase
SELDI	Surface enhanced laser desorption ionization
SERDs	Selective Estrogen Receptor Downregulator
SERMs	Selective Estrogen Receptor Modulators
SILAC	Stable isotope labeling with amino acids in culture
siRNA	Silencing RNA
SMAC	Second Mitochondria-derived Activator of Caspases
SPA	Sinapinic Acid
Src	Tyrosine-protein kinase Src
SRM/MRM	Selected reaction monitoring/ multiple reaction monitoring
STAT3	Signal transducer and activator of transcription 3
TBS-T	Tris buffered Saline – Tween20
TEAM trial	Tamoxifen Exemestane Adjuvant Multinational trial
TFA	Tri-fluoroacetic acid
TGFb	Transforming growth factor beta
TMA	Tissue micro array
TNF	Tumor necrosis factor
VEGFR2	Vascular endothelial growth factor receptor 2
WB	Western blot

1 Introduction

1.1 Proteomics

"Proteomics includes not only the identification and quantification of proteins, but also the determination of their localization, modifications, interactions, activities, and, ultimately, their function." (Fields, 2001)

Proteomics can be defined as the study of proteins within a system. A system can encompass a whole cell, a particular sub-cellular fraction or components of the cell for example the nucleus, protein secretions from cell lines (secretomes), human body fluids such as nipple aspirate, serum, plasma, urine and cerebral spinal fluid. A whole cell lysate sample contains thousands of proteins whereas a more defined experiment (for example, immunoprecipitation) would contain fewer.

Proteomics is still in its infancy compared to genomics. The human genome was sequenced in 2001 (Lander et al., 2001 and Venter et al., 2001) whereas an understanding of the entire proteome is expected to be years away, this is in part due to the dynamic nature of proteins. Analysis of proteins is also hampered by the fact that there is no way in which a protein can be amplified as there is in molecular biology (polymerase chain reaction, PCR for example). In proteomics, the instrumentation has to become more sensitive to analyse the less abundant proteins. In recent literature the idea of global assessment of proteins in a sample, with a view to elucidating fundamental mechanisms of disease has become tangible; even reasonable. An organism's genome is reasonably

stable unlike an organism's proteome in which the proteins are in a constant state of flux. The flux of a proteome can include processes such as post translational modifications (PTMs), localisation, proteolysis, increased and decreased expression, interactions (which can vary with differing conditions within the cell), and signalling cascades can be triggered, all of which can be captured in a "snap-shot" look at the cell proteome using proteomics. Messenger RNA expression does not always correlate with protein expression (Celis et al., 2000) and investigation of the genome only tells part of the story. There are approximately 21,000 human protein encoding genes in the human genome, but the total human proteome may well reach approximately 1,000,000 proteins (Jensen, 2004) and approximately 3000 of these represent druggable targets.

One aspect of proteomics is the identification of biomarkers. Biomarkers are biological markers often used in diagnostics for the detection and diagnosis of early stage disease (such as cancer), the prognosis or stage and the potential of therapy response. Biomarkers have been found using various proteomic technologies including Matrix Assisted Laser Desorption Ionisation (MALDI) and Surface Enhanced Laser Desorption Ionisation (SELDI) (detailed below - 1.1.4)

Untargeted platforms for proteomics, for instance global shotgun approaches, can be used to compare superficial or complex proteomes at the first stages of experimentation. This provides a way in which to identify novel proteins that exhibit differences in abundance and are generally considered to be the mainstay of proteomic based experiments. However, a more targeted proteomic approach is becoming more popular; tissue microarrays, multiplexed Western blots, peptide microarray and antibody arrays. The latter two being considered for a larger scale analysis of samples, and are

among the more versatile tools as long as there is an antibody available that can be immobilised. Antibody arrays were used in this study and are detailed below in section 1.1.1.

1.1.1 Antibody based proteomics

Tissue microarrays (TMA) and multiplexed Western blots are considered more targeted approaches to antibody based proteomics whilst antibody and protein micro-arrays are suitable for high throughput analysis. Antibody arrays enable the detection of multiple proteins in parallel and have proven useful in many instances, including basic biological and applied research. Over the past few years the range of commercially available antibodies that have a highly selective binding capacity to the native protein or the post translational modifications of proteins, has become extensive. There are now many commercially available (and bespoke platform) antibody array technologies based upon the high affinity antibodies which are being used extensively in studies to quantify antigens (Kolch et al., 2010). In many ways the antibody array has become complementary to mass spectrometry based technology, potentially acting as a control if the same protein is identified. Antibody arrays have been used to identify possible biomarkers in cancer for instance gastric (Mohri et al., 2009) and oesophageal squamous cell carcinoma (Shao et al., 2009). They have been used to profile cytokines in samples (Ghoniem et al., 2011) and glycopeptide profiling in colorectal cancer serum (Pedersen et al., 2011). Profiling of receptor tyrosine kinase (RTK) signalling and protein-protein interactions have also been investigated using this technology (Engels et al., 2011 and Patel et al., 2011). The arrays can be used to look at a multitude of different samples;

tissue, cell culture lysates, serum, plasma and conditioned media. When used on samples such as conditioned media and secreted proteins, there is minimal preparation work involved as the proteins are already soluble. This application could be utilised in the profiling of cytokines, signalling growth factors, and potential biomarkers.

When used in a clinical setting the antibody arrays can provide a potentially valuable tool for the prognosis, diagnosis, and determination of therapy/drug response. Combinations of markers (multi-markers) (Mion et al., 2007) have been proven to produce less false positives in a clinical setting. If a number of markers in combination were used on an array, it could provide a test that could be carried out using relatively low volumes of sample and allowing the parallel detection of proteins in one sample. Away from the clinical setting, antibody arrays have great potential in basic and applied biological research. They allow the co-ordinated changes in pathways known to be important in several diseases including cancer, such as mitogen activating protein kinase (MAPK), to be investigated fully in several experimental models simultaneously. As the whole proteome is very complex it is unlikely that the development of an array that targets a significant portion of the total proteome will come to fruition for many years. The largest array commercially available is from Sigma –Aldrich which assays 725 proteins. Antibody arrays are evolving rapidly into being extremely useful tools in both basic and applied research.

1.1.2 Mass spectrometry based proteomics

Mass spectrometers are essentially analytical tools that can determine the elemental composition, or chemical structure, of a compound or a sample. They are, in

the most basic sense comprised of 3 parts; an ion source, a mass analyser, and a form of mass detection unit (see Figure 1-1 below).

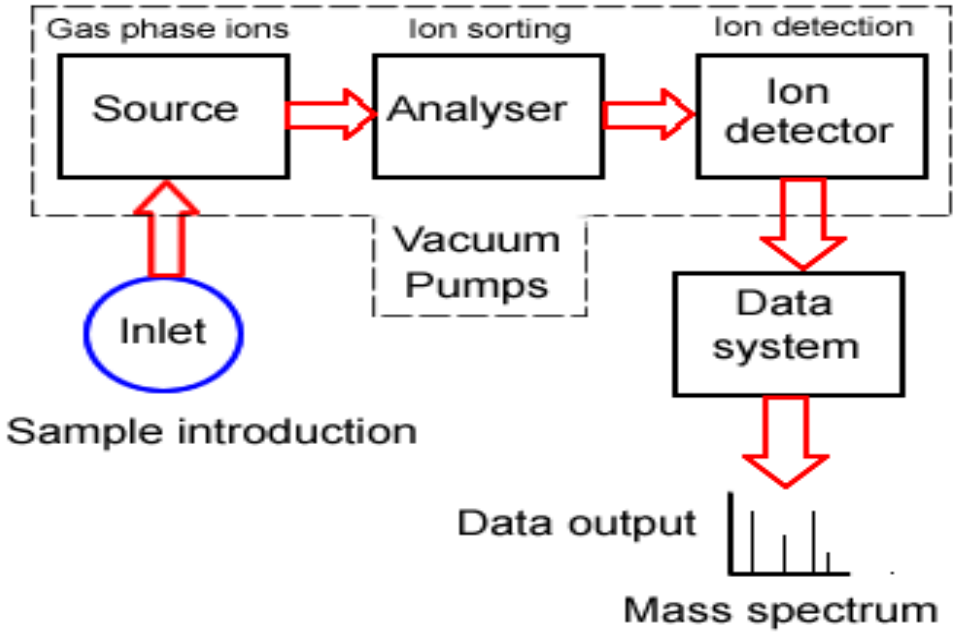


Figure 1-1 A basic figure of a mass spectrometer work flow. The sample is introduced at the inlet, followed by the ion source where the ions are made into gas phase ions, this is followed by the mass analyser where the ions are sorted by size and then the ions are detected using a mass detection unit. The data gathered from this is then analysed in the mass spectrometer software and results produced in the form of a mass spectrum. Adapted from www.ionsource.com (February 2010)

The ion source is used to create a charged analyte, the analyser is used to record the ratio of mass to charge (m/z) of the ion and the detector measures the relative abundance of the ion. Normally, protein samples are enzymatically digested (often using porcine trypsin) producing much smaller, structurally less complicated peptides. When proteins are digested with trypsin to form peptides they are cut at the C terminal side of lysine (K) or arginine (R) amino acid residues, allowing prediction of the cleavage sites. When proteins are trypsinised to form peptides it increases the number of separate entities in the

sample, therefore it is often necessary to separate the peptides so that relatively few are introduced into the mass spectrometer simultaneously. Reverse phase – high performance liquid chromatography (RP HPLC). It is often used to separate the peptides and this less complicated mixture of peptides is then introduced into the mass spectrometer for analysis.

Electro spray Ionisation (ESI) (see Figure 1-2) is one of the most common mass spectrometry methods for biological sample analysis. The analytes leave the HPLC and are sprayed into the mass spectrometer via a needle. The analyte passes out of the needle and is ejected in a particular shape, known as a Taylor cone (Taylor, 1964) (see Figure 1-2). This shape occurs because of the large potential difference (1-5Kv) and high temperature (approximately 200°C) between the needle and the mass spectrometer inlet. The desolvation of the analyte is aided by these conditions.

There are several ways in which the instrumentation can be optimised to deal with peptides including using a low flow rate (0.1-1µl/min) which allows the samples to be concentrated more effectively and also aids with the desolvation process. The high potential difference between the needle and the inlet helps to draw the positively charged peptide ions (after desolvation) into the ion trap.

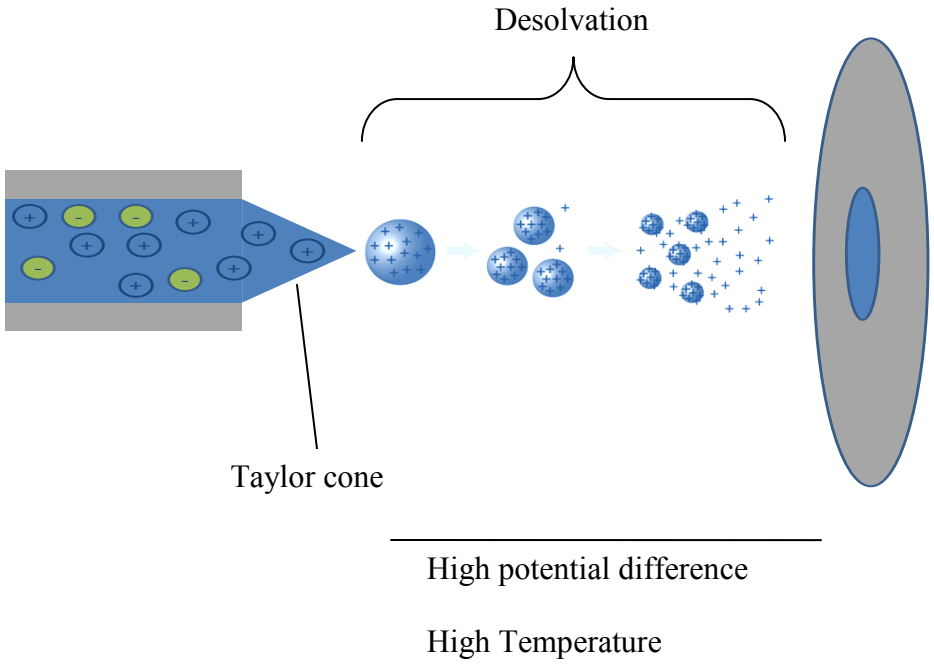


Figure 1-2 Electro Spray Ionisation (ESI) The analytes are dissolved in a volatile solvent, usually a mixture of acetonitrile, water and a source of protons (diluted in the mobile phase). The analytes are sprayed in to the mass spectrometer via a needle producing a cone shape (known as Taylor cone). Desolvation of the analyte takes place over a high potential difference and temperature and the positively charged analyte then enters the mass spectrometer.

In this study an ion trap mass spectrometer was used; inside an ion trap there is a high vacuum (created using vacuum pumps), the purpose of this is to eliminate the prospect of the peptide ions colliding with molecules from the atmosphere. The path that the ions take into the ion trap decreases in pressure, ultimately finishing at 10 e-3 Torr. When in the trap the ions are manipulated; firstly they are trapped, then isolated followed by excitation and finally ejected from the trap. Trapping is where all the ions are allowed to enter the ion trap, there is a direct relationship between the mass and the charge of an ion and the position it is in relative to the radio-frequency (RF) voltage (RF voltage is used to control the ions within the trap). This relationship allows all the ions within a

defined range of mass to be trapped. The isolation step can be used to isolate ions of a particular size (within a mass tolerance) which then can be used in the excitation step.

Collision induced disassociation (CID) is a process used to fragment the ions (of a particular mass) for MS/MS and was used in this study. In CID helium is used as a neutral gas to collide with the peptides with sufficient energy to allow the intra-peptide bonds to break – frequently the middle amide bond between the adjacent amino acids. The charge can either be retained on the N-terminal of the fragment the ion which is then classified as a B ion, or the charge is retained on the C-terminus of the fragment then the ion will be classified a Y ion (see Figure 1-3). The detected mass of the fragments is what is used to determine the mass "fingerprint" of the ion and eventually lead to the sequence being resolved. The peptides do not fragment sequentially it is a random process. The final step in the ion trap is ejection; ions are scanned out of the trap in increasing m/z order where the mass of the ion is recorded.

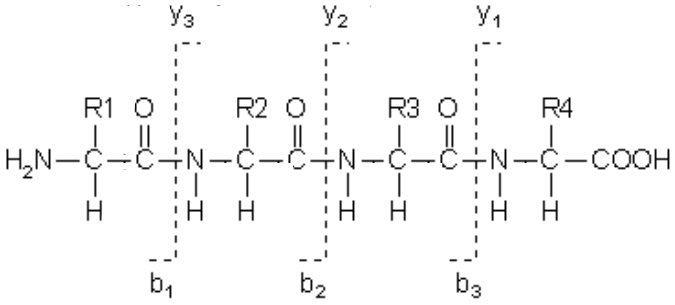


Figure 1-3 Fragmentation showing B and Y ions of a peptide.

The analysis of peptides by mass spectrometry is done in two stages; in the first stage, MS analysis, the m/z ratio of the peptide ion is recorded collectively with the ions relative abundance (the intensity of the ions signal). The second stage of peptide analysis

is determination of the mass fingerprint (MS/MS analysis) of a peptide can be then used to determine the sequence of the tryptic peptides using computer programs such as Sequest. The m/z ratio and the fragmentation pattern enable the peptide sequence to be predicted using suitable search algorithms. This sequence identification is achieved when the mass of the first ion is compared with the next 500 peptides that are closest in mass to the trypic fragment; these 500 predicted peptides are sourced from searchable database such as Human_FASTA ipi. From the list of predicted matches the software makes a comparison with the predicted and the actual peptide fragment and the closest match is likely to be the sequence of the actual peptide. Multiple peptides from the same protein increase the confidence in the identification.

Predicted peptides are given a score, the more accurate the match the higher the score. This is done using Bioworks browser software and Sequest, and the score given to the peptide is called Xcorr. Xcorr is an indicator of accuracy and is a raw cross-correlation score, derived from several relevant parameters.

1.1.3 Labelling strategies – Quantitation in Mass spectrometry

Use of a labelled approach helps to lessen the effect of ion suppression, the origin and mechanisms of which are not fully understood. It describes a change in the intensity which is often observed as a loss in response. The term ion suppression was introduced originally by Buhrman et al., 1996 and it is usually apparent in the early stages of the ionization process in LC–MS, usually when a component is eluted from the HPLC column which influences the ionisation of a co eluted analyte. Ion suppression can occur when elution of more than one analyte occurs in the same retention window as the

analyte of interest. As the peptides in SILAC (heavy and light) are chemically indistinct they elute in the same elution window and are subject to the same level of ion suppression.

There are now many labelling strategies for mass spectrometry (MS) based quantitation. MS is an ideal technology for the identifying peptides and /or proteins. It can be used in conjunction with a labelling technology so that the relative protein abundance in the sample can also be assessed. This is frequently carried out through stable isotope labelling (which can be broadly classified into a tag based technology or a biological incorporation technology); stable (non-radioactive) heavier isotopes of carbon (^{13}C), hydrogen (^{2}H), oxygen (^{18}O) or nitrogen (^{14}N) are incorporated into one experimental sample and the corresponding light isotopes in another. Early strategies included isotope-coded affinity tags (ICAT) labelling cystine residues (Gygi et al., 1999) and $H_2{}^{18}O$ labelling (Heller et al., 2003) (trypsin catalysed) and the now preferred techniques of stable isotope labelling in amino acids in cell culture (SILAC) (Ong et al., 2002) which was used in this study, isobaric tags for relative and absolute quantitation (iTRAQ) (Cong et al., 2006) and isotope coded protein label (ICPL) labelling (Kellerman, 2008) are generally used. There is also label free quantitation which is increasingly being used (Wright et al., 2011), unfortunately the performance of the mass spectrometers and software used during this project could not be used in conjunction with iTRAQ or label free quantitation such as spectral counts (Friso et al., 2011) and Selected Reaction Monitoring (SRM) (Lange et al., 2008). ICPL is a more recent advance in labelling strategy and was not available for this study.

The addition of a heavy stable isotope to one sample and a light stable isotope to the other allows for the simultaneous measurement of the same molecule and both the heavy and light isotopes, all subjected to the same environment and subsequently the relative intensities are deemed quantative (Pieniaszek et al., 1999).

SILAC was first described in 2002 (Ong et al., 2002) who showed the incorporation of specific amino acids into all cellular proteins *in vivo.* In this they used deuterated leucine residues as the label, but more recently papers cite labelled arginine and lysine as the preferred residues for the label. The ability to measure the same molecule but distinguish between two samples; heavy and light labelled which co-elute so the MS analysis takes place simultaneously, this makes the ratio of peptides reliable. This is important as it forms the basis of the MS based quantitative analysis of peptides and proteins as the ratio of intensities of the peptide peaks in a mass spectrum provides us with a relative ratio of abundance between the two species.

As the cells are grown in the heavy and light media they begin the incorporation of the particular isotopic variants into newly synthesised proteins and after a minimum of six population doublings achieve 95% incorporation (Ong et al., 2002). The distinct mass difference (6Da in ^{13}C lysine) and the common retention time of the co-eluting peptides is the key to identifying from which sample the peptide originates. The difference between heavy and light isotopes does not affect the cells (morphological, biochemical, or proliferative differences are not observed) as the isotopes are chemically indistinct.

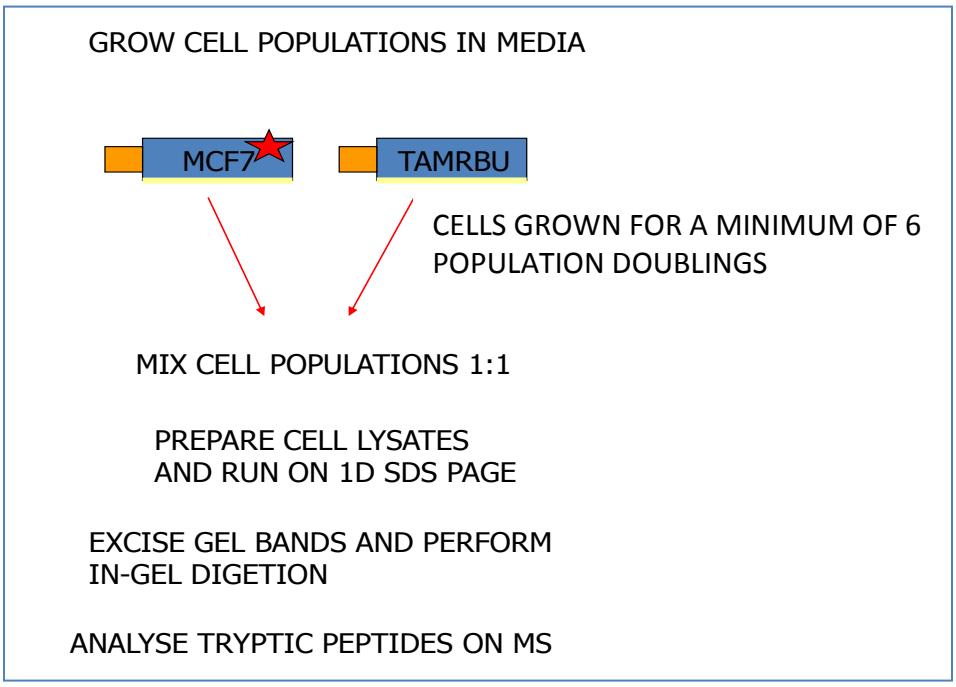


Figure 1-4 SILAC overview work flow: two populations of cells (MCF7 and TAMRBU shown here) MCF7 here are grown in Heavy isotope labelled media (designated with a red star) and TAMRBU are grown in the C12 light isotope labelled media. These are grown for a minimum of 6 population doublings to ensure high level incorporation of the isotope. The cells are then counted and mixed, then lysed. A protein assay is carried out and the protein run on a gel, in-gel digested and the tryptic peptides analysed on the mass spectrometer.

A distinct advantage of SILAC is that the incorporation of the isotope can approach 100% and the samples from two populations can be mixed at a very early stage then processed by further fractionation as one sample eliminating the potential for experimental variance between samples which may otherwise be seen if processing was done separately. In the literature, SILAC has been used to investigate a great number of processes; including MAPK pathway analysis (Blagoev et al., 2006), protein interactions (Thomas et al., 2011), cisplatin resistance in HeLa cells (Chavez et al., 2011), secretome

analysis of gliobastomas (Formolo et al., 2011), and profiling Myc associated proteins (Agrawal et al., 2010).

1.1.4 Matrix Assisted Laser Desorption Ionisation (MALDI) and Surface Enhanced Laser Desorbtion/Ionisation (SELDI)

MALDI is a laser based soft ionisation technique that has been proven to be one of the most popular methods of MS for protein analysis and can be used for the identification of xenobiotics and also in MS imaging. MALDI and SELDI based technology allows for rapid production of protein profiles from complex mixtures such as; cell lysates, conditioned media, serum, plasma and tissue. They have been used with some success to detect biomarkers and SELDI based proteomics is used to detect peptides and small proteins (less than 20 kDa).

SELDI is a modified form of MALDI based on a chip which was developed by Ciphergen. The sample is spotted onto the chip which has a modified surface with different chemical functionality, allowing some proteins to bind while others are washed away. The sample that is retained on the chip surface has appropriate matrix applied to it and measured directly in the MALDI MS. The chromatographic surface operates as a separation step and the sample is decreased in complexity. The chip surfaces available include: CM10 (weak-positive ion-exchange), H50 (hydrophobic surface – this is similar to reverse phase separation), IMAC30 (metal-binding surface- this can be loaded with different metal salts such as copper sulphate), and Q10 (strong anion exchange). Surfaces can also be used in conjunction with antibodies (for immuno-MS).

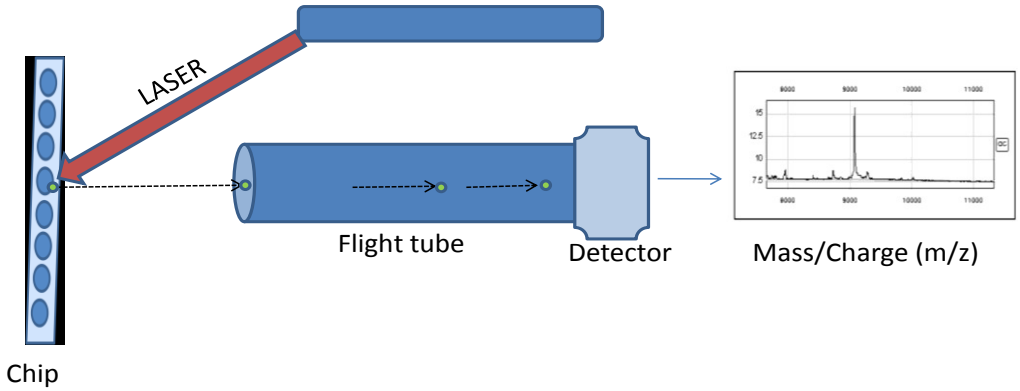


Figure 1-5 A basic diagram of the SELDI/MALDI system. Samples are spotted on to a SELDI chip with its retentate surface and processed with matrix. A laser ionises the peptides from the sample/matrix crystals on the spot. These ions are accelerated down an electric potential and down a flight tube to the detector. The m/z can be determined by the time taken to travel the flight tube and the kinetic energy of the ions in the electric field. Relative peak intensities from the MALDI can be used as a semi quantitive measurement of the protein presence in the sample.

A chemical matrix is required for MALDI investigation of samples. The sample is acidified using a weak organic acid such as tri fluoroacetic acid (TFA) and is either laid under, in or on the matrix which is always in a molar excess (compared to the sample). The matrix helps to produce intact gas-phase ions from biomolecules such as proteins and oligonucleotides or large inorganic compounds and synthetic compounds; it does this through absorbing the laser light energy which is then transferred causing the target substrate to vaporise. It is vital to use the correct one; there are a number of matrices for protein/ peptide analysis such as alpha cyano-4-hydroxycinnamic acid (αCHCA). Matrix properties must include vacuum stability, ability to co-crystallise with analytes, be stable in solvents such as acetonitrile (ACN), able to cause co-desorption of the analyte when laser stimulated, and be able to absorb the laser wavelength.

1.2 Cancer

Cancer is the result of many complex changes occurring in a "normal" cell, progressing through to malignant and potentially metastatic. The 6 hallmarks of cancer as outlined in Hanahan and Weinburg's review in 2000 are shown in Figure 1-6 below.

Cells commonly become cancerous when they acquire irreparable DNA damage, changing the sequence of genes which code for important regulatory proteins. After replication the mutations are passed down to the next generation of cells which can lead to deregulated growth, tumour progression and invasion through the basement membrane.

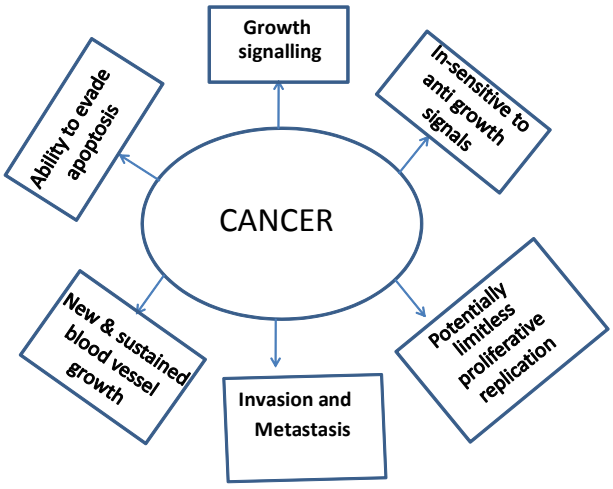


Figure 1-6 The 6 hallmarks of cancer (adapted from Hanahan & Weinburg, 2000)

Accumulations of successive DNA mutations arise over the course of a person's life time (somatic gene changes) which is why age is often a risk factor in cancer. Also, lifestyle factors such as diet, alcohol, stress and tobacco have all been implicated in either

causing or increasing the risk of cancer. The environmental exposure to chemical carcinogens and the ultra-violet rays of the sun have also been shown to result in DNA damage and result in cancer. There are examples of gene mutations that are hereditary for instance BRCA1 and BRCA2 which are tumour suppressor genes involved in DNA repair of double stranded breaks. Mutations in these genes can cause instability of the human genome. Women that have heterozygous germ-line mutations in BRCA1 or BRCA2 have a substantially increased risk of highly penetrative breast cancer and ovarian cancer. Patients that are positive for this mutation can chose to have pre-emptive mastectomies or preventative Tamoxifen treatment (Reding et al., 2010).

1.3 Breast Cancer

Breast cancer is now the most common cancer in the United Kingdom with approximately 125 women diagnosed each day. In 2008 around 12,000 women and 70 men died from breast cancer. (CRUK – "cancerstats -key facts." Updated Nov 2010). The incidence of breast cancer increases with age (around 80% of diagnosed cases are in women over the age of 50) and there are other risk factors; such as obesity, high socio-economic group, alcohol use, expression of BRCA1 or BRCA2, ethnicity, early menarche, and childbirth late in life.

There are many types of breast cancer; ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) are early, non-invasive types. There is also invasive lobular and invasive ductal carcinoma, or more rare types such as inflammatory breast cancer, medullary breast cancer, mucinous breast cancer, metaplastic breast cancer and papillary breast cancer. The stage and grade of a tumour, as well as the presence of oestrogen

receptor, progesterone receptor and ErbB2 are used to decide the treatment options for the patient. The patient can be classed as “triple negative” where they do not express any of these 3 receptors.

A unique feature of oestrogen receptor positive breast cancer is the dependence on the endogenous steroid oestrogen. This is a feature that can be, and is, frequently used in the prevention of development of tumours and in the treatment of them. Manipulation of the oestrogen receptor can be carried out using therapeutic agents such as selective oestrogen receptor modulators (SERMs) (section 1.5.1), selective oestrogen receptor down regulators (SERDs) (section 1.5.2), luteinizing hormone-releasing hormone (LHRH) inhibitors (section 1.5.3), aromatase inhibitors (AI) (section1.5.4) or using physical ablation of the ovaries.

When this project started in 2007, the majority of oestrogen receptor positive breast cancer patients in the United Kingdom received Tamoxifen either pre or post surgery, however, the use of AI’s has significantly increased in post menopausal women. The TEAM (Coombes and Kilburn, 2011) and BIG 1-98 (Kelly and Buzdar, 2010) trials have since reported that there is still value in patients being treated with Tamoxifen (2.5years) prior to a switch to AI therapy for post menopausal women. For women with breast cancer Tamoxifen is still a standard component of therapy.

1.4 Oestrogen and oestrogen receptors

Oestrogen is an important regulator in the development and progression of breast cancer and also in the development of normal breast. More than a century ago, Scottish surgeon George Beatson performed an oophrectomy and noted this procedure induced regression

of breast cancer which was later proven to be due to a reduction in systemic oestrogen levels (Stockwell, 1983). Oestrogen mainly originates in the ovaries in pre-menopausal women, whereas in post menopausal women the main source is in the aromatisation of androgens in adipose tissue. This accounts for the difference in therapeutic regimen between the two groups of women.

Oestrogen functions by activating two nuclear steroid receptors: ERα and ERβ. Both receptors bind oestrogen and initiate gene transcription through ERE (oestrogen response elements) in oestrogen target tissues but have distinct functions and tissue distribution (Sommer and Fuqua, 2001). In most breast carcinomas (~70%) ERα is highly expressed which results in the increased rate of proliferation without differentiation or apoptosis. ERα is exclusively epithelial (Saunders et al 2002 and Speirs et al 2004) whereas the distribution of ERβ in breast cancer is exclusively nuclear but expressed in multiple cell types (stromal fibroblast, endothelial and immuno-infiltrating cells) its expression has also been shown to correlate with an increase in aggression of the tumour (Younes and Homna, 2011). ERβ expression has been reported to range from 26% to 94%, the lowest of which was in a Japanese study, this implicates that the expression could be related to ethnicity. When Caucasian cohorts of patients were compared, the range was less extensive at 74%-94% (Speirs, 2004). The expression of ERβ in the breast has been well described although its usual function, clinical value in carcinogenesis, and its relevance to the pathological diagnosis of breast cancer, is yet to be determined (Speirs, 2004).

Oestrogen receptors that are not bound to ligand are inactive and usually sequestered in multi-meric protein complexes organised around the molecular chaperone heat shock protein 90 (HSP90). ER signalling pathways can be classed into 4 main mechanisms of action: Classical, ERE independent, ligand independent and non-genomic Figure 1-7).

Classically the cytoplasmic ER translocates into the nucleus upon ligand binding. In the cytoplasm, the receptor dimerises, transcription factors and co-activation proteins are recruited, and the target genes are then activated through an oestrogen response element (ERE). Oestrogen activates genes that are involved in survival and cell proliferation amongst other actions (Elliston et al., 1990; Frasor et al., 2003 and Wilson et al., 2006). Oestrogen bound ER interaction with Fos and Jun, which dimerise and become part of the activator protein-1 (AP-1) complex modulating gene expression. This ligand bound ER gene modulation can also occur with GC box bound specificity protein -1 (SP-1) this is ERE independent modulation. ER can act independently of oestrogen by being phosphorylated on multiple residues (see Figure 1-8) within the receptor after growth factor activation. The phosphorylation of ER leads to the dimerisation of the receptor, DNA binding and ultimately activation of transcription (Murphy et al., 2011; Le Goff et al., 1994 and Chen et al., 1999).

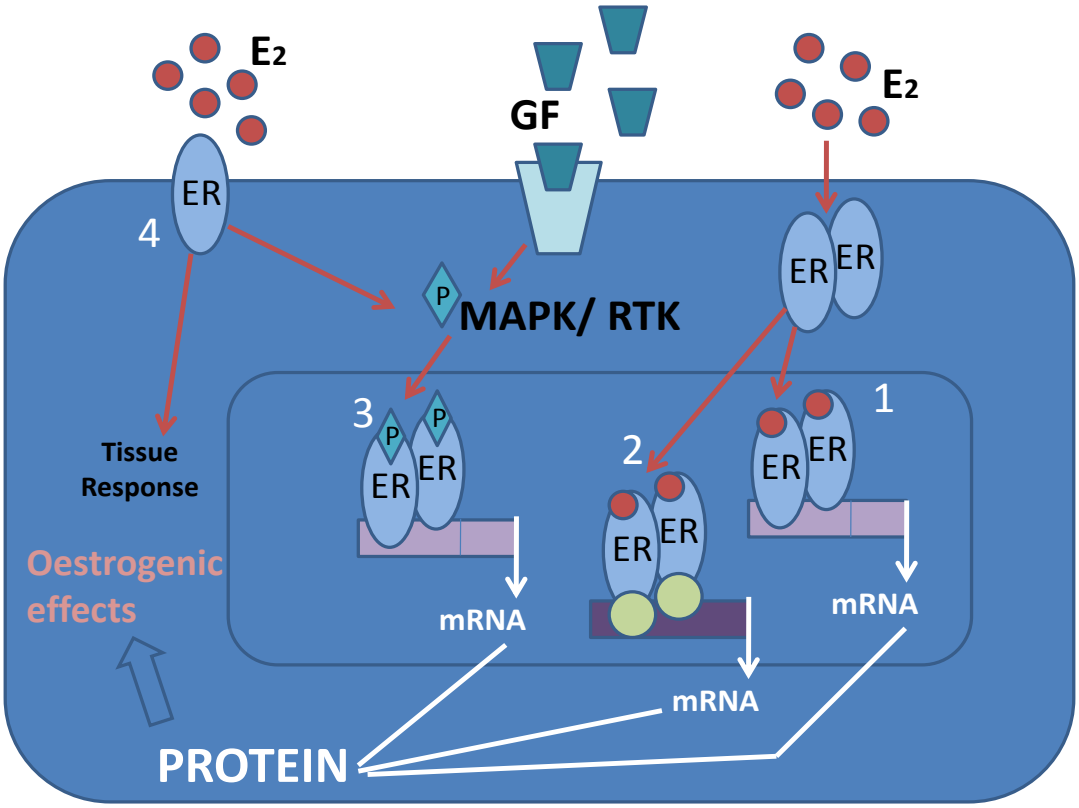


Figure 1-7. A simplified view of the 4 mechanisms by which oestrogen can exert its action upon a cell (roles and names of co-factors have been omitted). 1, Classical pathway: This is a genomic, ligand dependent mechanism; where estradiol (E_2) bound dimers bind to the EREs (in lilac) in target promoter genes – this leads to an up regulation or down regulation of gene transcription and resulting in the oestrogenic effects being seen in the cell. 2, ERE independent pathway: This is also a genomic, ligand dependent mechanism where oestrogen bound ER dimers bind to alternative gene response elements such as AP-1 through Fos and Jun association and thus lead to gene transcription. 3, Ligand independent mechanism: This is a genomic ligand independent mechanism where the phosphorylation and subsequent activation of the ER, following growth factors and/or signalling molecules stimulation leads to target gene transcription. 4, the non-genomic ligand dependent mechanism; this is where the estradiol binds to a membrane associated receptor activating intracellular signalling generating rapid oestrogenic effects in the cell. Figure adapted from Hall et al., 2001

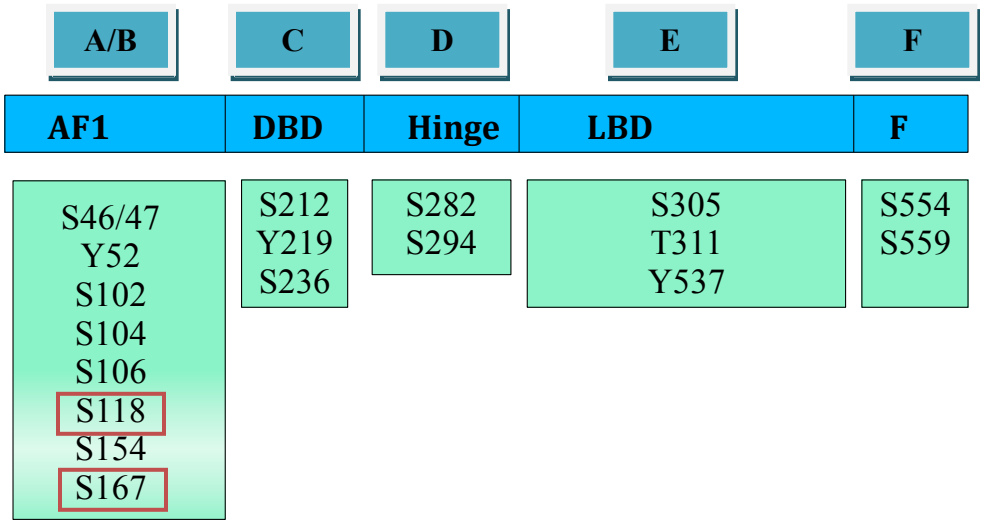


Figure 1-8 Shows a basic example of ERα and its regions A/B - Activating factor 1 domain (AF1), C -DNA binding domain (DBD), D – Hinge domain, E- Ligand binding domain, and F domain. Multiple phosphorylated sites can be seen in the light blue boxes below the ERα domain where they have been identified by a variety of approaches. S118 and S167 (highlighted with a red box) have been associated with Tamoxifen resistance. Figure adapted from Murphy et al., 2011.

Oestrogen can also activate membrane bound ER and cause a rapid oestrogen signalling response through non-genomic activation (Levin et al., 2005 and Song and Santen, 2006). The non-genomic simulation of the oestrogen receptor is a rapid response to the ligand binding and its response in the cell is independent of the gene transcription. Oestrogen receptors act through a complex interplay of signalling cascades; such as insulin-like growth factor 1 receptor (IGF-1 R) (Song et al., 2010), EGFR (Fox et al., 2008), G-proteins, Src, and PI3K (Wu et al., 2011).

1.5 Therapeutic agents

Development of new therapeutics for breast cancer has resulted in new agents directed against certain molecular targets such as c-met (Eder et al., 2009), HSP90 (Wang et al., 2011), or angiogenesis related targets such as VEGFR2 (De Luca and Normanno, 2010), signalling pathways such as mTOR (O'Regan and Hawk, 2011) all of which could prove to aid in treatment of the heterogeneous disease and a more unique patient treatment regimen. Presently, the majority of these new therapeutic agents are not clinically available and most are at preliminary laboratory stage of investigation. The therapeutic agents in clinical practice are detailed below and can be seen in Figure 1-9.

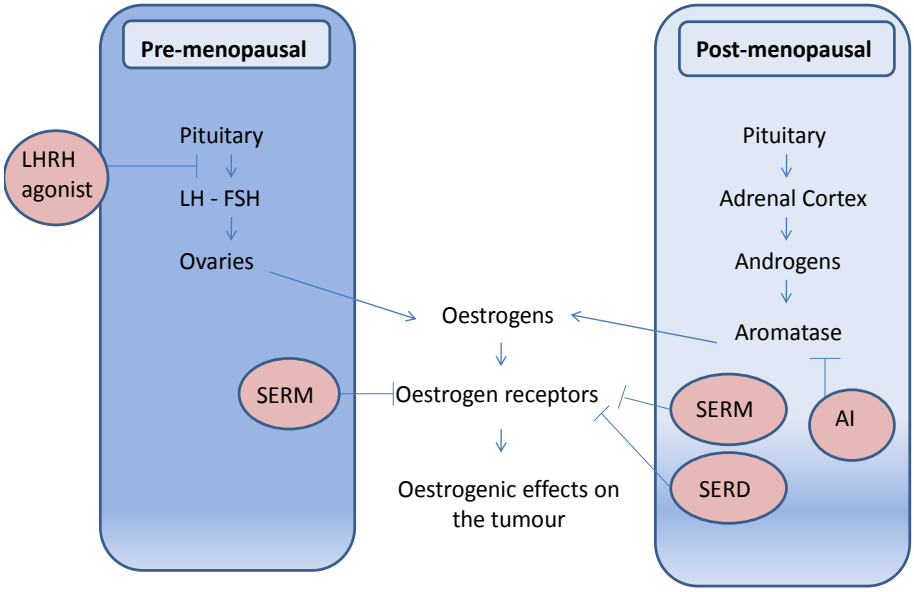


Figure 1-9 Endocrine therapeutic agents can be used to treat ER positive breast cancer. The treatment regimen usually differs between pre and post menopausal women. In pre-menopausal women the LHRH agonists work by blocking the production of oestrogen by the ovaries. SERMs such as Tamoxifen can also be used to partially block the oestrogen receptors. In post menopausal women AIs stop the enzyme aromatase from working and this prevents the conversion of androgens to oestrogens. SERMs can also be used in post-menopausal women and also SERDs can be used to prevent ER mediated cell replication by potentially binding and degrading the oestrogen receptor.

1.5.1 Tamoxifen & Selective oEstrogen Receptor Modulators

Tamoxifen (Nolvadex) is a selective oestrogen receptor modulator (SERM) that is classed as a partial antagonist due to having oestrogenic effects in some tissues such as the endometrium and bone, and anti-oestrogenic effects in tissues such as breast and the mammary epithelium. In breast cancer, oestrogen has been shown to promote development of the disease (as described above in section 1.4), so the interference in its action can be used to help slow/stop the progression of breast cancer. Tamoxifen is a hormonal treatment licensed as a first line adjuvant therapy in the treatment of early oestrogen-receptor positive breast cancer in the UK (NICE guidelines). In the UK, 5 years of Tamoxifen therapy has become standard adjuvant hormonal treatment for postmenopausal women with early, oestrogen-receptor-positive breast cancer. Over the decades that Tamoxifen has been available it has become the most widely used endocrine therapy for ER+ women. It has been proven that the use of Tamoxifen significantly improves the survival of women with early stage breast cancer (Clark, 2006). Tamoxifen also provides protection against bone fractures in postmenopausal women, due to its oestrogenic effects in bone (Section 1.4) and it lowers serum cholesterol levels. The side effects associated with long term use of Tamoxifen have been associated with vaginal bleeding, endometrial thickening, and increased risk of endometrial cancer and thrombo-embolic events.

Patients may initially respond to the Tamoxifen, but unfortunately, almost all patients eventually acquire resistance over the course of the therapeutic regimen and the disease progresses. Several mechanisms of Tamoxifen failure have been suggested, some of which will be discussed further in section 1.7.

1.5.2 Selective Estrogen Receptor Down-regulators

Selective oestrogen receptor down regulators (SERDs) such as fulvestrant, bind the oestrogen receptor and abrogate oestrogen signalling in cells. Fulvestrant has been shown to have no agonistic activity (unlike SERMs such as Tamoxifen) and therefore it does not preserve bone density of patients, but conversely does not increase the risk of blood clots or increase the risk of endometrial cancer. Fulvestrant may provide a beneficial effect in the treatment of oestrogen receptor positive patients that are Tamoxifen resistant, though it is mainly used for the treatment of postmenopausal women. Use in premenopausal women has also been suggested as beneficial by in an adjuvant setting (Young et al., 2008).

1.5.3 Luteinising Hormone Releasing Hormone agonists

Luteinising Hormone Releasing Hormone (LHRH) agonists (such as goserelin) are another strategy in adjuvant endocrine therapy for pre menopausal women. They work by temporarily suppressing the synthesis of ovarian oestrogen; this is done by desensitising the hypothalamus/ pituitary/ ovarian axis (Huirne and Lambalk, 2001). This is used rather than the permanent effects of oopherectomy or radiotherapy that were traditionally used. LHRH agonists have proved to be a reliable and reversible agent to the ablation of ovarian oestrogen synthesis. The role of LHRH agonists in the treatment of breast cancer patients remains unclear as it was deemed that not enough evidence was available to compare LHRH agonists directly to (Goel et al., 2009). However, it has been indicated in the literature that the combination of treatments, LHRH agonist and Tamoxifen, is

superior to the treatment of LHRH agonist treatment alone in women who are premenopausal with advanced breast cancer (Klijin et al., 2001).

1.5.4 Aromatase Inhibitors

Aromatase inhibitors (AIs) are a class of drugs that work by stopping the biosynthesis of oestrogen from the circulating androgens in tissues such as the breast and adipose tissue. This biosynthesis is carried out using the enzyme aromatase and the drug works by inhibiting this enzyme. AIs such as anastrozole, letrozole and exemestane have been shown in the literature and in trial data to be a superior treatment to Tamoxifen in the treatment of advanced breast cancer in post menopausal women (Nabholtz and Gligorov, 2006; Mouridsen, 2007 and Coombes and Kilburn, 2011). Other trials have shown very similar efficiency for sequential regimens with AI and Tamoxifen and a combined regimen might reduce side effects of the treatments (Van de Velde, 2011). AI's are not an effective treatment for pre-menopausal women with an intact hypothalamus/ pituitary/ ovarian axis and can potentially only be safely used in this group of patients if the axis is suppressed (either by LHRH agonists, surgery, or radiotherapy). Tamoxifen in combination with chemotherapy and Tamoxifen in combination with LHRH agonists remain the only proven endocrine intervention in the treatment of women with pre-menopausal breast cancer. There are now known to be cases of AI resistance (Ma et al., 2011) and the literature supports the hypothesis that the acquisition of resistance is predominantly mediated by cellular signalling events that lead to the constitutive activation of ERα and growth factor miscommunication (Santen et al., 2005; Riggins et al., 2005 and Masri et al., 2008).

1.6 Mechanisms of Tamoxifen action

Tamoxifen is a non-steroidal triphenylethylene derivative and works by binding to the oestrogen receptor. It works strongly as an anti-oestrogen within mammary epithelial cells, but does also act as an oestrogenic in some tissues such as bone. It was originally screened as a contraceptive agent that was effective in rats but not humans. In the early 1970's Tamoxifen was shown to be useful for clinical palliative care for advanced breast cancer and approved for the treatment of post menopausal patients with metastatic breast cancer in 1973 (UK) and 1977 (USA).

The mechanism by which Tamoxifen works in the treatment of breast cancer is complex. Its principal mechanism of action is stopping the proliferation of the cells that is initiated by oestrogen stimulation (cytostatic); this action is mediated by the drug competing with oestrogen and subsequently binding to the oestrogen receptor in mammary epithelia to form a nuclear complex that decreases DNA binding and oestrogenic effects (see Figure 1-10). Tamoxifen and its metabolites such as 4-hydroxy Tamoxifen (4-OH Tamoxifen) (see Figure 1-11) bind to oestrogen receptors with a similar affinity to that of oestrogen, and have been shown (Dowsett et al., 2005) to saturate the ER present in post-menopausal women (99.9% saturation).

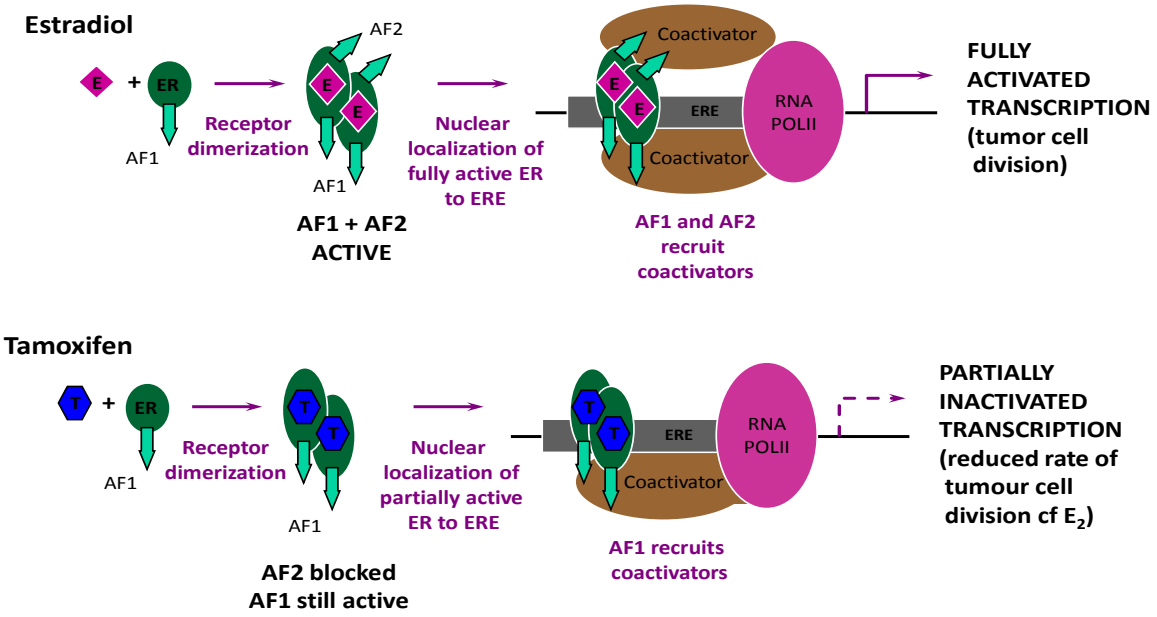


Figure 1-10. The action of Tamoxifen (T) compared to the action of Estradiol (E). When stimulated with E the ER dimerises and both AF1 and AF2 are active. The receptor dimer moves to the nucleus Estrogen Response Element (ERE). AF1 and 2 recruit co-activators and E stimulated tumour growth is activated. When Tamoxifen is added the receptor dimerises, AF1 is active however, AF2 is blocked. The receptor dimer moves to the ERE however due to AF2 blockage only AF1 is able to recruit co-activators and this subsequently leads to partially inactivated transcription. Adapted from Buzdah et al., 2004.

Often genes that are down regulated upon oestrogen signalling are transcriptional repression genes and pro-apoptotic genes and conversely the genes that are up-regulated are ones that are related to pro-growth and survival (Frasor et al., 2003). Up regulation of these genes is mediated by activating function 1 (AF-1) and activating function 2 (AF-2), these are regions located on ERα (ERβ does not possess an AF-1 region, (Kong et al., 2003) which assist in the recruitment of co-regulatory proteins.

Tamoxifen competes with oestrogen; it binds to ER and the receptor dimerises, however, AF-2 is not activated (Figure 1-10) as AF-2 activation is hormone dependent.

AF-1 action is not reliant on ligand binding but is mediated by phosphorylation and therefore retains activity, thus leading to only partially inactivated transcription. Genes that only require the action of AF-1 are still transcribed, and the preference for AF-1 or AF-2 in different tissues such as the bone, uterus and breast, goes some way to explaining the selectivity of Tamoxifen efficacy in different tissues.

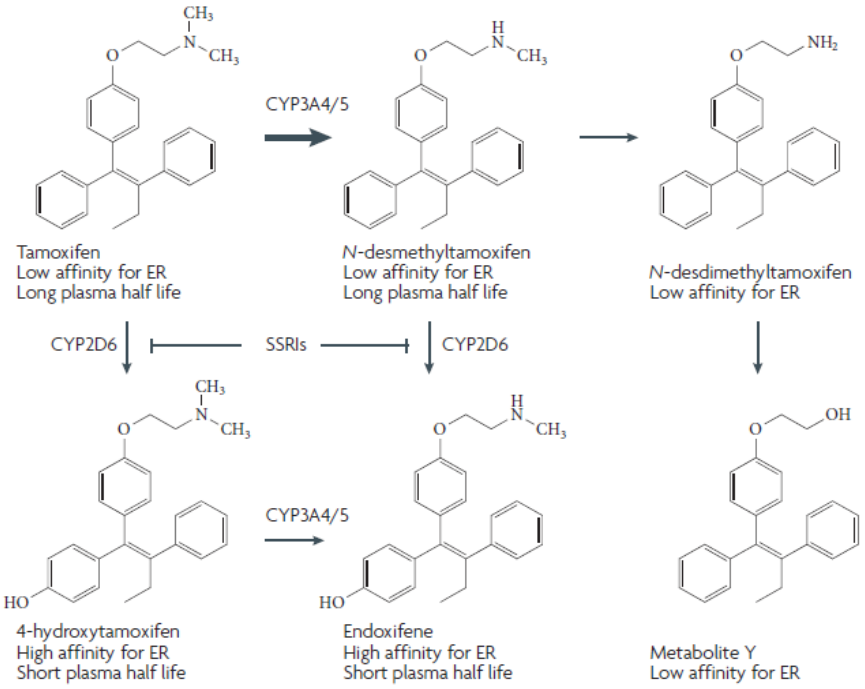


Figure 1-11 Tamoxifen and its metabolites in human treatment. Tamoxifen is given at 20mg per day. It is metabolised by the cytochrome P450 family. The main metabolites that have a high affinity for oestrogen receptors are 4 Hydroxy Tamoxifen (4OH Tamoxifen) and Endoxifene. Selective Serotonin Reuptake Inhibitors (SSRIs) have the ability to bind to the CYP2D6 preventing the hydroxylation of the Tamoxifen and N-desmethyltamoxifen preventing the formation of these important metabolites. (Jordan, 2007).

An alternative mechanism for Tamoxifen's anti-proliferative action is the production of transforming growth factor beta (TGFβ). It has been suggested that the anti-oestrogen signal transduction pathway involves sequential activation of p38 and

TGFβ pathways in order to control the growth inhibition (Buck et al., 2004). Moreover, activation of TGFβ has also been shown in ER negative cells (mesenchyme and stromal cells) which suggest this interaction does not rely wholly upon the oestrogen receptor, and could be a possible explanation for some effectiveness seen in cells negative for oestrogen receptor (Charlier et al., 1995). Some studies have also shown that circulating levels of IGF-1 can be lowered by exposure to Tamoxifen; this may abrogate the action of the potent mitogen on the breast cancer cells and aid in the suppression of the cancer growth (Ho et al., 1998). Therapeutic concentrations of Tamoxifen have also been reported to inhibit protein kinase C (PKC) and calmodulin-dependent cAMP phosphodiesterase enzymes; this could impact upon the proliferation of the cells and Tamoxifen's anti tumour activity as these proteins are known to have a role in growth regulation (O'Brian et al., 1990).

1.7 Mechanisms of acquired Tamoxifen resistance

The development of therapeutic anti-oestrogens such as Tamoxifen has proven to be effective in the treatment of hormone responsive breast cancer but has led to the discovery of a very real clinical problem: antioestrogen resistance. This type of resistance is now a common feature of the treatment and therefore limits the anti-oestrogen's therapeutic success. Some hormone responsive patients do not respond to treatment with Tamoxifen; this is called intrinsic or *de novo* resistance. Patients that are initially treated successfully with Tamoxifen frequently acquire resistance over time, often relapsing to a more metastatic state. Acquisition of resistance to Tamoxifen plausibly involves

carcinoma cells developing further elaborate changes; it is unlikely that one mechanism will fully elucidate the mechanism of resistance.

Altered ER and ER signalling

The effects of Tamoxifen are largely mediated via the oestrogen receptor, and expression of oestrogen receptor is the decisive factor which treatment is based on therefore it is natural to imagine that a loss or decrease in ER expression in the cell could confer the acquisition of Tamoxifen resistance. One cause of intrinsic resistance is decreased or absence of ER expression due to the hyper methylation of ER genes. However, a decrease in the ER level of expression only affects a minority of resistant cancers (15-20%), and does not appear to be involved in acquired resistance (Guiterrez et al., 2005). This was further confirmed when AI was given as a follow up treatment to patients who had relapsed on Tamoxifen, as most responded to AI (see section 1.5.4) treatment, or to the SERD (see section 1.5.2) Fulvestrant, which suggests that the ER continues to be functionally active in many Tamoxifen resistant breast cancers (Howell, 2002).

Early studies suggested that mutations in the oestrogen receptor could confer resistance, but, this has now been shown to be present in only 1% of ER positive breast cancers (Clarke et al., 2003; Riggins, 2007 and Herynk and Fuqua, 2004). Mutations have been generated in laboratories which can confer resistance, some of which have been detected in patients for instance, Fuqua et al., 2000 found that a single amino acid substitution was found in approximately one third of the hyperplastic breast lesions investigated, (changing K303 to L303). Further investigation showed that this mutation lead to a hypersensitive ER that was able to bind co-activators in the presence of low

oestrogen. It was found to enhance ER mediated cellular growth by modulating the cross-talk between ERα and pathways that down regulate ER signalling (Barone et al., 2010). Altered regulation such as this could assist in acquisition of resistance to hormone therapy, however, the occurrences of mutations are few and often resistance occurs without the presence of mutations. It is therefore unlikely that mutations of the oestrogen receptor fully explain the mechanism of Tamoxifen resistance.

A truncated form of ERα, ERα36, has also been discovered to correlate with diminished responsiveness to Tamoxifen in the presence of the full form of ERα (Shi et al., 2009).

The transient methylation of the ERα at arginine position 260 (R260) aids in the formation of multi-meric complexes containing PI3K, FAK, Src (tyrosine kinase) and ERα (via a protein known as protein arginine N-methyltransferase 1 – PRMT1); these complexes activate Akt which is known to be associated with endocrine therapy response (Le Romancer et al., 2008).

Altered expression of co-regulatory proteins

Co-regulatory proteins (activator and repressor) have very important roles to play in the transcription mediated by the ER. Altered patterns and expression of these proteins could contribute to Tamoxifen resistance.

Co-activators include the p160 steroid receptor family which function to stimulate the ER activity via an interaction with AF-2. The family includes AIB1, SRC-1 and TIF2 (Webb et al., 1998). The co-activator AIB1 has been shown in the literature to be over expressed in over 50% of breast cancers (Anzick et al., 1997) it has also been shown to

have a pivotal role in MCF7 cells (List et al., 2001) which are the cells used in this study. It has been shown to be a predictive factor of Tamoxifen sensitivity; high expression of AIB1 correlates with a worse patient outcome (Alkner et al., 2010). It has also been proposed that it could provide a limiting factor in non-oestrogen driven, EGF stimulated growth through EGFR and ErbB2 in Tamoxifen resistant cells in culture (Zhao et al., 2009). Other literature has provided an insight into how AIB1 and Paired Box 2 (PAX2) compete for regulation of ERBB2. Increased expression of AIB1 outcompetes PAX2 binding, which would normally repress ERBB2 transcription, and therefore directly results in increased expression of ErbB2 (Hurtado et al., 2008). Increased ErbB2 protein activation has been indicated as a possible mechanism of Tamoxifen resistance (for more detail see below – RTKs).

Another co-activator, PELP1, has been reported to have a role in resistance to Tamoxifen. It has been shown to function as a scaffold protein modulating the interaction of Src and the ER leading to the downstream activation of MAPKs, such as the ERKs, Src and the promotion of PI3K through oestrogen signalling (Gururaj et al., 2007).

Aberrant growth factor signalling

The modification and/ or altered expression and activation of several growth factors, growth factor receptors, and their downstream signalling molecules have been implicated in Tamoxifen resistance. Growth factors mediate their diverse biological actions; such as cellular proliferation, migration, metabolism and differentiation by acting upon receptors such as Receptor Tyrosine Kinases (RTK) on the cell surface. These receptors in turn activate many more signalling molecules such as the MAPK family (see below). RTKs

function in many critical roles within the cell, and their activity under normal circumstances is under tight control. When this control breaks down it results in aberrant signalling which can lead to tumourigenesis, and influence cell motility, invasion, angiogenesis, proliferation and survival. Aberrant signalling of some RTKs has also been linked with Tamoxifen resistance, including IGF-1R, EGFR and ErbB2 (Knowlden et al., 2005).

Evidence suggests that abnormalities that occur in the EGFR and ErbB2 signalling pathways could dramatically manipulate the way in which the cancer reacts to hormone action and ultimately, is thought to be critical to Tamoxifen resistant breast cancer growth in both patients and cell culture models (McClelland et al., 2001; Knowlden et al., 2003 and Hutcheson et al., 2003). Patients that express both ER and EGFR respond less well to Tamoxifen (Nicholson et al., 2002). ErbB2 over-expression is one of the better characterised mechanisms of Tamoxifen resistance.

Studies have shown that loss of transcriptional repressors alongside amplification of ERBB2 is the major factor responsible for this increase of ErbB2 expression. This loss of transcriptional repressors is mediated by Zinc finger transcription factor (GATA4) and X-linked Forkhead box P3 (FOXP3) tumour suppressor; a direct link between GATA4 and FOXP3 and Tamoxifen resistance has not yet been established (Musgrove and Sutherland, 2009).

Inhibition of ErbB2 using the inhibitor Trastuzumab (Herceptin) restored the inhibitory activity of Tamoxifen (Kurokawa et al., 2001). Herceptin alongside the EGFR inhibitor (Iressa) should theoretically provide us with useful tools in the treatment of ER positive breast tumours that are also positive for EGFR and/or ErbB2; such as in

Tamoxifen resistance. The results from a recent clinical trial showed that acquired resistance to Tamoxifen to be partially mediated through EGFR signalling which can be decreased using Iressa, (Gutteridge et al., 2010).

The Insulin-like growth factor 1 receptor (IGF-1R) is part of the RTK super family. It has been considered to be a potential oncogene and plays a role in proliferation, cellular interactions, survival, and transformation (Casa et al., 2008). In the clinical setting the over expression of IGF1-R is seen frequently; it is phosphorylated in all breast cancers, and related to a poor outcome in patients (Fagan and Yee, 2008). It has been associated with Tamoxifen resistance in a number of ways: Cross talk between the IGF1R and ERα signalling pathways (Fagan and Yee, 2008) when up-regulated IGF1R acts upon EGFR and ErbB2 (Massarweh et al., 2008 and Knowlden et al., 2005) and it is also thought that IGF1R may act in an ER independent manner where it continues to signal upon stimulation with IGF1 despite continuous suppression of ER transcription with Tamoxifen (Zhang et al., 2011).

The Mitogen Activated Protein Kinase (MAPK) pathway has been implicated in the progression of cancer (Hanahan and Weinberg, 2000) and is also a known aberrant pathway resistance to endocrine therapy (Riggins et al., 2007). There are three major sub groups in the MAPK family: ERK, JNK, and p38 MAPK. Of these the ERKs are mainly involved in stimulation of cellular growth, usually activated via growth factors. JNK and P38 MAPK are activated via many different stimuli and the response (cellular growth or death) is dependent on the stimulus (cytokines, cellular stress, growth factors). Increased ERK phosphorylation and activation have been shown to lead to a reduced survival in breast cancer patients and acquisition of Tamoxifen resistance (Riggins et al., 2007).

Phosphorylation of Ser118 of the ERα has been shown to be induced by members of the MAPK (Joel et al., 1998), however, Weitsman et al., 2006 reported that the phosphorylation of Ser118 is not due to constitutive activation of MAPK pathway although the phosphorylation was reported to have a functional role in oestrogen regulated signalling. Ribosomal S6 Kinase (RSK), when activated by ERK1 and ERK2, has been shown to phosphorylate ERα at position Ser167 in the AF-1 domain (Joel et al., 1998) see Figure 1-8.

Reactive oxygen species (ROS) induce cellular stress and can induce the activation of ERK and Akt signalling (Weitsman et al., 2009). Both of these signalling kinases have been implicated in the down-regulation of ER and also in the constitutive activation of ERα and therefore may contribute to the development of resistance to Tamoxifen (Weitsman et al., 2006).

PI3K and Akt and their signalling pathways provide another viable mechanism of acquired Tamoxifen resistance. Akt is a signalling molecule and a downstream target of PI3K (Datta et al., 1999). Increased activation of Akt1 has been described as a potential contributing factor in the aggressive phenotype that is often seen in Tamoxifen resistant ER positive breast cancers and cell culture models (Jordan et al., 2004). Activation of Akt can promote cellular proliferation, and avoidance of cell death. Akt has been reported to phosphorylate Serine167 on the AF-1 (Figure 1-8) domain of the ERα, consequentially instigating the ligand- independent activation of the receptor (Campbell et al., 2001) and subsequently has the potential to be a contributing factor in development of acquired Tamoxifen resistance. Increased expression and activation of p38 MAPK has been

observed in Tamoxifen resistant tumours, it has been described to be involved in TGFβ signalling (See section 1.6) and is also part of a paracrine signalling loop with the angiogenesis related protein Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) (Linderholm et al., 2011).

The Src family of non receptor tyrosine kinases and their downstream targets have often been associated with breast cancer. They have been shown to be frequently over-expressed (in particular Src) in breast cancer (Biscardi et al., 2000) and Tamoxifen resistance (Morgan et al., 2009). One substrate of Src, Cas, is a focal adhesion adaptor molecule which has been shown to have a role in Tamoxifen resistance when the protein was induced *in vitro.* An elevated activity of Src promotes motility and invasion in Tamoxifen resistant cells (Hiscox et al., 2006 and Hiscox et al., 2007). It has also been reported that the localisation of activated Src may be important, elevated levels located in the cytoplasm of breast tumours was significantly associated with a decreased patient survival whereas a nuclear expression of activated Src was associated with a more favourable response to endocrine therapy (Morgan et al., 2009).

Pharmacological mechanisms

A frequent mechanism of general drug resistance is altered efflux or influx of the drug, causing altered (often decreased) concentrations of the drug in the cell. This is a process that is often controlled by membrane pumps such as p-glycoprotein (MDR1) (Saeki et al., 2005). It has been shown in the literature that patients with acquired Tamoxifen resistance show a lower concentration of Tamoxifen in serum (Johnston et al., 1993). In this study there were no significant differences seen in between the *de novo* resistant

group of patients and the acquired resistant group. When the drug concentration was measured in the tumour the concentration was lower which may imply that there is a connection between low concentrations of the drug in serum and tumour and acquired resistance (Johnston et al., 1993).

Tamoxifen is a pro-drug that is metabolised into many metabolites (see Figure 1-11). It is therefore possible that altered metabolism of the drug could explain, at least partially, acquired resistance to Tamoxifen. It has been postulated that a large increase in oestrogenic metabolites of Tamoxifen could compete with the anti-oestrogenic metabolites for activation of the oestrogen receptor (Clarke et al., 2001). Yet it would require a very large shift to alter the levels of Tamoxifen in the tumour and the levels of metabolites in patient serum has been monitored over several years and indicate that the metabolites remain largely constant over the treatment regimen.

Often involved in *de novo* resistance, genetic polymorphisms of cytochrome P450 enzymes CYP2D6 and CYP3A4 are used in the bio-activation of Tamoxifen to form its major metabolites (see Figure 1-11) could also play a role in acquired resistance. In plasma, endoxifen has been observed to have a large variability between patients. CYP2D6 is of particular interest as patients that have decreased CYP2D6 or genetic variants, have lower endoxifen concentrations (Stearns et al., 2003). Increased endoxifen levels have been reported to circumnavigate resistance associated with increased ErbB2 expression (Goetz, 2009). Investigation of variants of CYP2D6 could lead to a more personalised drug regimen by predicting outcomes of Tamoxifen therapy, although this proposal remains controversial (Kuderer & Peppercorn, 2009).

Cell cycle regulators

Tamoxifen is a cytostatic; the presence of Tamoxifen in culture leads to G_1 phase specific arrest consequently leading to decreased proliferation and growth (Dowsett et al., 2006). Key cell cycle regulators such as Cyclin D's, Cyclin E's and Myc are often over expressed in cancer and are frequently observed with a decrease of p21 or p27 expression which act as inhibitors of cyclin dependent kinases (CDKs) concomitantly. These observations have been associated with Tamoxifen resistance. Abukhdeir et al. showed in 2008 that breast epithelial cell lines that had the p21 gene deleted displayed a growth proliferative response when treated with Tamoxifen. The cell cycle inhibitor p27 has been reported to mediate the G1 arrest when Tamoxifen is used, however, it has been shown that activation of the MAPK pathway and decreases the expression of the p27 protein which was suggested to be a contributing factor in Tamoxifen resistance (Donovan et al., 2001). Src has also been shown to regulate p27 stability through phosphorylation (pY74 and pY88) leading to decreased p27 expression (Chu et al., 2007).

Growth factors have been shown to induce cell cyclin D1 expression and it has also been shown to interact with several transcription factors such as ERα and signal transducer and activator of transcription 3 (STAT3) (Ishii et al., 2008 and Coqueret et al., 2002). The over expression of cyclin D1 is frequent and is seen in approximately 50% of breast cancers (Zwijsen et al., 1997).

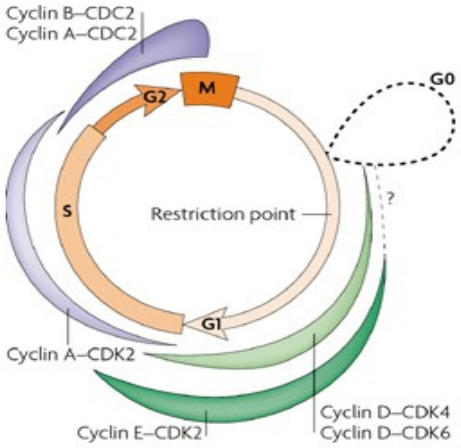


Figure 1-12 This figure shows the phases of a cell cycle. G1 phase. G0 phase (shown as an out-of-cycle state that cells might enter at a point in G1) S phase, G2 phase and M phase are also shown. Cyclin–cyclin-dependent kinase (CDK) complexes are shown, their position demonstrating where they are active. Tamoxifen is known to act through G1 phase specific arrest. From: Coller, 2007.

Myc is an activator of RNA polymerase II transcription (Hynes and Stoelzle, 2009) influencing a wide range of cell functions such as cell cycle, protein synthesis and apoptosis. Myc is involved in normal mammary development but is also frequently deregulated in mammary tumours. This deregulation is caused by multiple mechanisms including altered signalling pathways, genetic amplification, and altered transcriptional activity. ERα, among others, binds the Myc promoter, and stimulate transcription. ErbB2 activation via ERα has been shown to stimulate ERK and PI3K/Akt pathways, increasing RNA and protein Myc expression (Hynes and Stoelzle, 2009). Musgrove et al., 2008 showed that the over expression of Myc was associated with a Tamoxifen resistance phenotype and was accompanied by a repression of p21 (tumour suppressor) allowing the cyclin E1-CDK2 (Figure 1-12) complexes to facilitate the proteolysis of p27 (decrease in

p27 expression has been associated with Tamoxifen resistance, detailed above). Tamoxifen resistant cells consistently express high levels of cyclin E1 and CDK2 (Louie et al., 2010). The over expression of Myc and cyclin D1 have been reported to be associated with Tamoxifen resistance *in vitro* which is now becoming more apparent in a clinical setting (Caldon et al., 2006 and Butt et al., 2005). There is also evidence that cyclin E1 over expression and inactivation of the retinoblastoma (RB) tumour suppressor facilitates a decreased clinical response to the treatment, probably via decreased expression of p27 (Chu et al., 2008).

Cellular survival and Apoptosis

One way in which cells potentially acquire resistance to Tamoxifen is avoiding drug-induced cell death (apoptosis). Initiation of apoptosis through the mitochondrial pathway is shown in Figure 1-13. Studies have shown that cross-talk between the apoptotic effectors of Tamoxifen, the tumour necrosis factor (TNF) pathway and the increased survival signalling pathways such as Akt/PI3K and NF-KappaB have been associated with the evasion of Tamoxifen induced cell death (deGraffenreid et al., 2004).

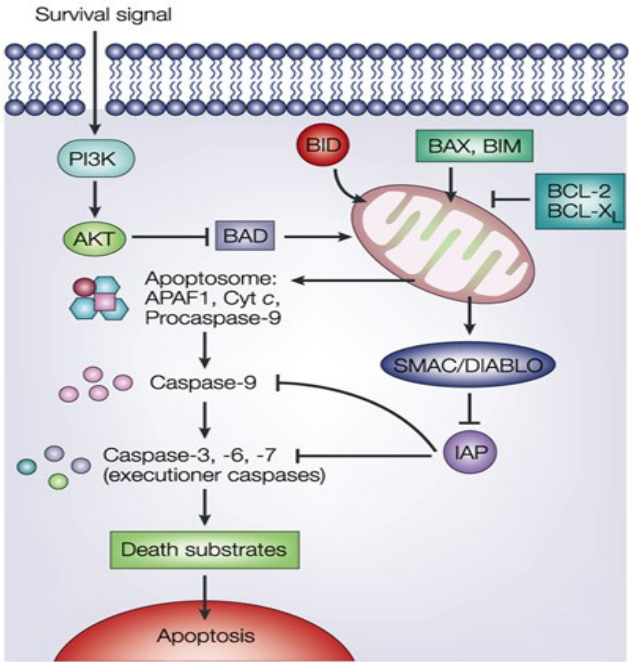


Figure 1-13 This figure shows the initiation of apoptosis through the mitochondrial pathway. Pro-apoptotic BCL2 family proteins, for example; BAX, BID, BAD and BIM are important mediators of these signals. Apoptosis through mitochondria can be inhibited on different levels by anti-apoptotic proteins, including the anti-apoptotic BCL2 family members BCL2 and BCL-X_L and inhibitors of apoptosis proteins (IAPs), which are regulated by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI). Another way is through survival signals, such as growth factors and cytokines that activate the phosphatidylinositol 3-kinase (PI3K) pathway. PI3K activates AKT, which phosphorylates and inactivates the pro-apoptotic BCL2-family member BAD. (Igney and Krammer, 2002)

It has been shown that inhibition of NF-KappaB expression can restore sensitivity to Tamoxifen in resistant cells and can also further sensitise the MCF7 (Tamoxifen sensitive) cells to 4-OH Tamoxifen. These data suggest that this effect is not dependent on cell cycle or autophagy (Nehra et al., 2010). It is thought that the mechanism, through which NF-KappaB inhibition restores the cell's sensitivity to the drug is through a reduction in BCL2 expression and subsequent induction of the capsize 8 caspase 8, initiating Tamoxifen driven apoptosis (Nehra et al., 2010).

There is mounting evidence in the literature that an increased expression of anti-apoptotic molecules (such as the BCL2 and BCL-XL) and a decrease in pro-apoptotic molecules (for example Caspase 9 and BAK) are observed in Tamoxifen resistant cell models and patients (Riggins et al., 2005). The attenuation of response to Tamoxifen could be due to the acquisition of pro-survival pathway activations (such as PI3K/Akt via ERα RTK activation).

The BCL-2 homology-3 (BH3)-only, pro-apoptotic regulator PUMA (p53 up regulated modulator of apoptosis) has been identified recently as an oestrogen target gene (Roberts et al., 2011). In response to oestrogen stimulation PUMA is highly down-regulated in many breast cancer cell lines. Also, patients with low PUMA mRNA expression correlated with a poor patient prognosis in ERα positive, Tamoxifen treated patients indicating that PUMA may contribute to the mediation of apoptotic response to Tamoxifen. There is therefore an increasingly convincing body of evidence justifying investigation of new therapeutic approaches that target apoptotic pathways to modulate response to endocrine therapy.

1.7.1 Aim of this project

Establish and characterise a robust cell model of Tamoxifen resistance and ascertain the phenotype of the cells. This was done through chemical selection, exposing the MCF7 (ERα positive) cells to increasing concentrations of Tamoxifen (4 OH Tamoxifen) over a period of time. The phenotype of these cells was compared to an established Tamoxifen resistant cell line (TAMR), developed in Cardiff, using proteins previously shown to be altered in resistance such as ERK1/2 and PKCδ. These marker proteins were used throughout the project to ensure that there was no phenotypic drift over time. After a robust cell model of Tamoxifen resistance was established, the aim was the identification of alterations in protein expression and/ or phosphorylation between parental MCF7cells and the models of Tamoxifen resistance. Any altered expression and phosphorylation was assessed through thorough proteomic investigation; utilising technologies such as antibody arrays and mass spectrometry in conjunction with the best available labelling strategy for the instruments and software available in our laboratory.

Following the identification of altered proteins; further investigation and determination of their biological significance in Tamoxifen resistance was investigated. This was done using functional assays to measure characteristics such as proliferation used alongside siRNA used to determine if decreasing the expression of the protein of interest effects the characteristics of the cells. Novel mechanisms of Tamoxifen resistance that have not hitherto been investigated may be identified, increasing our understanding of the cell biology and ultimately possibly allowing the development of novel therapeutic approaches to decrease the occurrence of Tamoxifen resistance.

2 Materials and Methods

All chemicals were from Sigma-Aldrich unless otherwise stated.

2.1 Cell culture:

All cell culture work was carried out in a class II biological hood under sterile working conditions. All general tissue culture plastic used was from Corning and sterile for single use. Cell lines were grown in incubators set to controlled conditions, 5% CO_2 humidified atmosphere at 37˚C. Cells were counted on a glass haemocytometer.

2.1.1 Cells

MCF7 are a mammary cell line originally acquired from a metastatic pleural effusion. TAMRBU are cells which were derived from MCF7 cells using pharmacological selection (section 2.1.2), resulting in a resistant phenotype. The MCF7 and TAMR were a kind gift from the Tenovus Center for Cancer Research at Cardiff University. TAMRBU were developed for the purpose of this project (Chapter 3). All cell lines express oestrogen receptor and are adherent. All cells were routinely tested for the presence of Mycoplasma.

2.1.2 Establishment of the Tamoxifen resistant (TAMRBU) cell line.

The Tamoxifen resistant cell line (TAMRBU) was established by the MCF7 parental cell line (sensitive to Tamoxifen) being continually exposed to 4-OH Tamoxifen (10^{-7}M) diluted in ethanol and added freshly into experimental media (Table 2-1) for the routine maintenance of these cells for a period of 8 months. During this time the cells were

passaged when necessary. For the initial 3 months of the TAM treatment regimen the cells exhibited a substantial growth inhibition and some cell death. Subsequently, the cells became more proliferative until similar growth rates to the parental MCF7 cells were achieved.

2.1.3 Charcoal stripping the FCS

Charcoal stripped FCS (csFCS) was prepared as follows: A charcoal/dextran solution was prepared using activated charcoal (11.1%) and dextran C (0.06%) This mixture was then mixed vigorously for 1 hour. FCS was adjusted to pH 4.2 using 4M HCl and allowed to equilibrate for 30 mins at 4˚C. 5ml of charcoal solution was added to every 100ml of FCS and incubated over night (16 hours) with gentle agitation at 4˚C. The charcoal was removed from the FCS using centrifugation at 12,000g for 45 mins followed by filtration using Whatman number 4 papers to remove more traces of the charcoal. The FCS was readjusted to pH7.2 using 5M NaOH and filtered again using a 0.2µM membrane syringe filter in a class-2 culture hood to remove any micro-organisms and fine charcoal impurities. This was stored at -80˚C until required.

2.1.4 Sterile cell culture solutions

Phosphate buffered saline (PBS); 137mM sodium chloride, 2.7mM potassium chloride, 4.3mM disodium hydrogen phosphate and 1.47mM potassium dihydrogen phosphate. pH 7.4. Stored at room temperature (rt).

Trypsin EDTA purchased as a 1x solution from Invitrogen.

2.1.5 Routine seeding from stock

Cells were removed from the gas phase of the liquid nitrogen (LN_2), warmed quickly at 37˚C, added to a T25 flask with pre-warmed appropriate growth medium (Table 2-1). The cells were then left overnight (16hours) to attach, the media was removed, the cells washed in warmed PBS (2.1.4) and fresh media added.

2.1.6 Cell Passage

Cells were passaged approximately once a week upon reaching 90% confluency.
The culture medium was aspirated the cells washed with warmed PBS. Trypsin / EDTA (3ml) (2.1.4 above) was added to the flask, rocked gently and placed in the 37˚C incubator for 5 mins until the cells were rounded and detached. 8 mls normal media (Table 2-1) was added to the cells to inactivate the trypsin and centrifuged at 1350g for 5mins at rt. The supernatant was aspirated and the cell pellet re-suspended in 10 ml of culture medium. 2.5 ml of the re-suspended cell mixture was then added to a T75 flask with 7.5 ml of culture medium added and placed in an incubator. The media was changed every four days, until required or until 90 % confluent.

	Media		FCS (vol/vol)	Penicillin (10IU/ml) Streptomycin (10μg/ml)	Glutamine (4mM)	4-OH Tamoxifen (10^{-07}M)		DMSO (vol/vol)
Normal – MCF7	RPMI 1640 (phenol red)		Yes - 5%	Yes	Yes	No		No
Normal – TAMR & TAMRBU	RPMI 1640 (no phenol red)		Yes-5% Charcoal Stripped	Yes	Yes	Yes		No
Experimental	RPMI 1640 (no phenol red)		Yes-5% Charcoal Stripped	Yes	Yes	No		No
SILAC	RPMI 1640 (no phenol red)		Yes-5% Dialysed Charcoal Stripped	Yes	Yes	MCF7	TR	No
	MCF7 "Heavy" (C^{13}Lys) 40mg/L (C^{12}Arg) 200mg/L	TR "Light" (C^{12}Lys) 40mg/L (C^{12}Arg) 200mg/L				No	Yes *	No
Freezing	RPMI -1640 (phenol red)		Yes 50%	No	Yes	No		Yes 10%

Table 2-1 The media used for culturing the cell lines for the purposes of normal culture, experimental, SILAC and cryopreservation. All media used was RPMI 1640 from Invitrogen with the exception of the SILAC media where it was purchased from Geneflow. All FCS was from PAA, with the exception of the SILAC media where dialysed serum was purchased from Invitrogen and then charcoal stripped (2.1.3). To avoid the unwanted mild oestrogenic properties of the phenol red, phenol-red free media was used in experiments. It was also necessary to use steroid-depleted charcoal stripped foetal calf serum (csFCS) in experiments. The serum is dialysed for SILAC as it removes all unwanted amino acids that would otherwise "contaminate" the heavy and light preparations.
*Tamoxifen was removed from the media 7 days prior to the experiment.

2.1.7 Stable Isotope Labelling of proteins with Amino acids in Cell culture (SILAC)

MCF7 cells and TAMRBU cells were washed thoroughly in PBS and then MCF7 cells grown in "heavy" media and TAMRBU in "light" media (Table 2-1). Cells were cultured routinely in the "heavy" or "light" media for at least 6 population doublings to ensure near 100% incorporation of the [^{13}C] or [^{12}C] isotope of the amino acid (Isotopic equilibrium see 5.1). Both MCF7 and TAMRBU cells grown in SILAC media showed an initial slowing of growth.

2.1.8 Cryopreservation.

Cells were trypsinised as in section 2.1.6; the pellet was re-suspended gently in freezing media (Table 2-1) so that the cells are at a density of 10^6 cells per ml. The re-suspended cells were then aliquotted into 1ml cryotubes (Nunc) and placed in "Mr. Freeze" (Nunc) at -80˚C overnight and then the cryotubes were then transferred to the LN_2 for storage at -140˚C. The "Mr. Freeze" and the DMSO help to reduce the formation of ice crystals in the cells and therefore reduce cell death during the cryopreservation process.

2.2 Protein Chemistry

2.2.1 Lysis of cells

The cells were lysed using NP-40 lysis buffer (detailed below) with gentle agitation for 30 minutes at 4˚C, samples were sonicated (sonicating bath for 10 minutes for mass spectrometry experiments, a series of 5 second pulses using the sonicating probe for

Western blots) followed by another 10 mins at 4˚C with gentle agitation. Samples were centrifuged at 10, 000 x g for 30 minutes and the supernatant collected.

Nonidet-P40 (NP-40) Lysis buffer: 20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol (v/v),1% nonidet P-40 (Igepal)(v/v) and 2 mM EDTA. All chemicals used were from Sigma –Aldrich. Protein inhibitor cocktail tablets (Roche) and PhosSTOP phosphatase inhibitor tablets (Roche) were added on the day of lysis.

2.2.2 Determination of protein concentration; BCA assay

25 µL of each of the BSA standard (0-1000µg/ml) and 1:10 dilutions of unknown samples were pipetted, in triplicate, into a 96 well flat bottomed plate. The BCA working reagent was prepared at 50 parts BCA reagent A to 1 part reagent B (Pierce). 200 µL working reagent was added to each well and the plate mixed using the microplate mixer for 1 minute. The plate was then incubated at 37˚C for 30 minutes and cooled to room temperature before reading at 562nm.

2.2.3 Sodium Dodecyl Sulfate -polyacrylamide gel electrophoresis (SDS-PAGE)

Selections of pre-cast gels (Invitrogen) were used for SDS-PAGE. The use of these was especially important for mass spectrometry experiments as they were free from contaminating proteins. Gels used included 10, 12, or 1 well 4-12% Bis-Tris gels, 10 well 3-8% Tris acetate gels, and 4-20% Tris Glycine gels. Typically, 4-12% Bis-Tris gels were used as they provided good separation of proteins, separating as low as 3KDa using MES running buffer.

The buffers used are as follows:

3-(N-morpholino) propanesulfonic acid (MOPS) Buffer: MOPS 50mM, Tris Base 50mM, 0.1% SDS, 1mM EDTA, pH 7.7.

2-(*N*-morpholino) ethanesulfonic acid (MES) Buffer : MES 50mM, Tris Base 50mM, 0.1% SDS, EDTA 1mM, pH 7.3.

Tris-Acetate buffer (Invitrogen): Tris Base 50mM, Tricine 50mM, 0.1% (v/v) SDS, pH 8.2

Sample loading buffer: Lithium Dodecyl Sulphate (LDS): 10% (v/v) Glycerol, Tris Base 141mM, Tris HCl 106mM, 2% LDS, EDTA 0.51mM, Coomassie Blue G250 0.22mM, Phenol Red 0.175mM, pH 8.5. Reducing conditions were implemented on the day of use by adding 50mM dithiothreitol (DTT) to disrupt the disulphide bonds in the sample.

2.2.4 Western blotting

Samples were prepared in sample buffer (2.2.3) and separated using SDS PAGE as described previously (2.2.3). Western blotting was performed on either semi-dry or wet platforms, using apparatus from Geneflow and Invitrogen respectively. For semi dry method: PVDF membrane (Milipore) was used, proteins were transferred for 90 minutes with 100 mA constant current and a maximum setting of 25 Volts or 60 minutes with 30 Volt constant, and 170 mA to 110 mA using the wet method. The PVDF membrane washed briefly in TBS-T then incubated, with gentle agitation, with an appropriate blocking solution for 60 minutes (powdered non fat skimmed milk (Marvel) 5% w/v in TBS-T or, for phospho- antibodies 5% BSA in TBS-T). The membrane was then incubated with the appropriate primary antibody in either milk or BSA (dependant on the

manufacturer's directions), overnight (16 hours) at 4˚C. This was followed by 3x 5 minute washes and 1x 15 minute wash in TBS-T and then incubation in the appropriate secondary antibody conjugated to horse-radish peroxidase (HRP) for 60 minutes, followed by 3x 5 minute washes and 1x 15 minute wash in TBS-T. All excess liquid was drained from the membrane and a 1:1 mixture of ECL Western blotting detection reagent (GE Healthcare) (125µL/ cm^2 PVDF) was added for 1 min. Excess reagent was removed and autoradiography film (GE Healthcare) was used to develop the blot for an appropriate time. The working dilutions and sources of the antibodies can be found in Table 2-5.

Buffers and solutions used for Western blots:

Tris Buffered Saline (TBS): 50 mM Tris HCl at pH 7.4 and 150 mM NaCl. TBS-T was used with the addition of Tween-20 detergent at a final concentration of 0.1%.

Transfer buffer: NuPAGE transfer buffer: Bicine 25mM, Bis-tris (free base) 25mM, EDTA 1.0mM, Chlorobutanol 0.05mM, pH 7.2. Methanol at 10%, this was removed for the larger receptor proteins such as EGFR. When transferring two membranes using the wet method, the methanol content was increased to 20%.

2.3 Proteomics (mass spectrometry)

2.3.1 Preparation of samples for analysis by reverse- phase (RP) High performance Liquid Chromatography (HPLC) and Electrospray ionisation mass spectrometry (ESI MS)

Cell lysates were run on SDS-PAGE gel and then stained using colloidal coomassie. Colloidal coomassie stain: 0.08% Coomassie Brilliant Blue G250 (CBB 250), 1.6% orthophosphoric Acid, 8% ammonium sulphate, and methanol added on the day of staining to a final concentration of 20%. The gel was placed in a plastic sandwich box with the stain and was agitated for a minimum of 6 hours to overnight (16 hours) at rt. The gel was then de-stained in 1% acetic acid (analytical grade - Fisher Scientific) until non- stained areas of the gel developed a clear appearance.

After de-staining the SDS PAGE gel the sample lane was cut into gel pieces, (40 bands/pieces of a 1 well gel for SILAC experiment) this was done on a clean glass plate with a sterile scalpel blade. To minimise contamination from keratins/dust the excision and processing took place in a positive pressure hood.

Gel pieces were washed twice with 500µl of 50% acetonitrile/50mM ammonium bicarbonate for 20-45mins at 37°C with agitation. The gel pieces were then dried down using a vacuum drier and 250µl of 50mM DTT (made up in 10% acetonitrile/50mM ammonium bicarbonate) added and incubated at 56°C for 1 hour, after which the supernatant removed. 100mM Iodoacetamide (250µl) (made up in 10% acetonitrile/50mM ammonium bicarbonate) was added and then incubated at rt in the

dark for 30 minutes after which the supernatant was removed. The gel piece was then washed 2x in 500µl of 10% acetonitrile in 50mM ammonium bicarbonate for 15-30 mins at room temperature with agitation and the bands thoroughly dried using the vacuum dryer. Promega trypsin (sequencing grade, modified, porcine) was prepared by adding 200µl of the provided trypsin resuspension buffer (dilute acetic acid used to lower the pH) to 20µg of trypsin to give a 200µg/ml stock solution. Immediately prior to use this was diluted 1 in 8 with 10% acetonitrile/50mM ammonium bicarbonate. This raises the pH and activates the trypsin, and is subsequently used in rehydrating the dried gel slices with 50µl for 1 hour (rt). 20µl of 10% acetonitrile/50mM ammonium bicarbonate was added to ensure that there is sufficient liquid to completely rehydrate the gel piece these were incubated at 37°C overnight. Supernatants were collected and 30µl 3% Formic acid in water was added and incubated at 37°C for 1hour. Supernatants were collected again and added to the previous supernatant. Another 30µl 3% Formic acid was added and incubated at 37°C for 30 mins and supernatants were again collected. Supernatants were put in snap cap tubes and run straight away or stored at -80ºC.

2.3.2 Separation of peptides by reverse phase HPLC and direct detection using electro spray ionisation mass spectrometry

The gradient of the solvents used in the reverse phase HPLC was over 2 hours and can be seen in Figure 2-1 below, details of software and equipment associated with the use of ESI MS can be found in Table 2-2. Peptides were rapidly retained on a C18 trap column and unbound material was washed through to waste. Next the valve switching the flow was directed to the C18 analytical column and the ion trap and the peptides are eluted

from the column using the gradient of solvents see Buffer A and B in Table 2-2 and detailed in Figure 2-1 (flow rate of 1µL a minute). This is detailed in Section 1.1.2.

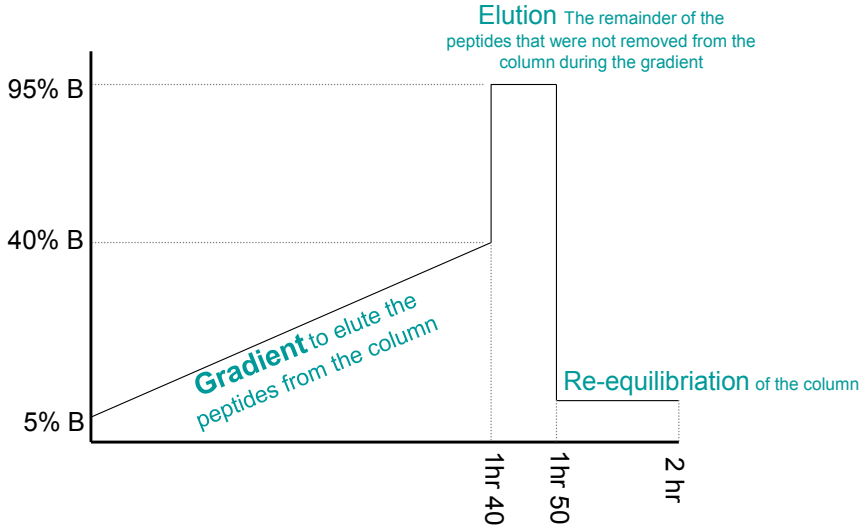


Figure 2-1. Reverse phase HPLC gradient used in the separation of peptides.
Buffer A = 100% water, 0.1% Formic acid. Buffer B =5% water, 95% ACN, 0.1% formic acid.

2.3.3 Database searching

The RAW MS/MS data was searched against an indexed non-redundant human fasta database (NCBI) to establish identification of peptides and proteins using TurboSEQUEST search within the Thermo Electron Company software, BioWorksBrowser 3.1 SR1©. The data was searched with an Xcorr value of 1.5 for singly charged peptides, 2.0 for doubly charged and 2.5 for triply charged peptides. Xcorr (Cross correlation) is a means of measuring the similarity between the RAW peptide data and the peptide contained within the database so that an increasing Xcorr value indicates that the identification is more accurate. Data that did not reach these criteria were removed from the resulting analysis.

Equipment / information	Supplier
Autosampler	Surveyor (Thermo Finnigan)
HPLC	Surveyor MS pump (Thermo Finigan)
Ion Trap Mass spectrometer	LCQ DECA XP Plus (Thermo Finnegan)
Software	Xcalibur ™ 1.3 SR1 © Thermo
Sample vials	Electron corporation 11mm PP vial crimp/snap 250mandrel (national scientific company)
Peek Tubing	50µm diameter tubing (Altech)
C18 trap column	Dionex
C18 analytical column	150 x 0.18mm 5µm BioBasic C18 KAPPA
Buffer A	100% water, 0.1% Formic acid
Buffer B	5% water, 95% ACN, 0.1% formic acid
Flow rate at detection	1µL per minute.
Water	HPLC grade (ChromoNorm)
Acetonitrile (ACN)	HPLC grade (LGC promochem)
Formic Acid	100% stock

Table 2-2 Table of equipment and general information useful to the process of proteomics using a mass spectrometer.

2.3.4 SILAC labelling efficiency

A flask of "heavy" labelled cells (6 population doublings) was lysed in NP-40 lysis buffer (section 2.2.1), run on a 1D SDS PAGE and stained (sections 2.2.3 and 2.3.1). The colloidal coomassie gel can be seen in Figure 5-1. From this gel, 3 bands were excised (outlined in red) and digested using the method outline in section 2.3.1 The identified

peptides from the excised bands were manually scanned for the presence of K# (# = heavy) residues. Not all peptides have a K residue present in a trypsin digestion as trypsin can cut peptides at both K or R residues (see section 1.1.2). All peptides with an absence of K were excluded from the calculation. All peptides with a K residue were counted and a percentage of labelled to non-labelled was calculated.

2.3.5 Manual Quantitation of isotopically labelled peptide pairs in SILAC

Cells grown in SILAC media (heavy or light Lysine) outlined in Table 2-1 were prepared as in section 2.1.7 and 2.2.1 and analysed using the mass spectrometer. Using the BioWorks software and the TurboSEQUEST search allowed for the heavy and light modification to be taken into account and identifications of peptide sequences from their proteins of origin. This allowed the heavy and light version of the same protein to be identified and thus quantified using the XPRESS function. The XPRESS function calculates the relative abundance of SILAC pairs using the calculated ion current (XIC) for each peptide. This was then confirmed using Xcalibur 3.1 for each pair manually and averaged if there was more than one labelled peptide pair identified in the protein.

2.4 Antibody based proteomics

2.4.1 Proteome Profiler; phospho MAPK

The phospho- MAPK array (R & D systems) was used to determine the relative levels of phosphorylation of the three main families of Mitogen Activated Protein Kinases; the (ERKs) Extra-cellular signal Related Kinases, (JNKs) c-Jun N-terminal Kinases, and the P38 family. It uses capture and control human antibodies that have been pre-spotted on to

a nitrocellulose membrane in duplicate. Cell lysates from TAMRBU, TAMR and MCF7 were prepared as described below (section 2.4.2). The array was blocked for one hour with the provided buffer and the samples added and left overnight at 4˚C with gentle rocking. The array was then washed 3x for 10 mins (provided wash buffer) and the detection antibody cocktail added which was incubated with the array for 2 hours. The detection cocktail was removed and the array washed as previously described, streptavadin horse radish peroxidase was added at 1:2000 (diluted with array buffer) and incubated for 30 mins with gentle rocking. After briefly washing the array ECL detection agent was added and the arrays wrapped in plastic wrap and exposed to autoradiography film. All densitometry was read using a BioRad GS800 reader and all analysis was completed using MiniTab 15 using t-tests or one way unstacked ANOVA. The box plots show the median value and the range.

2.4.2 Cell lysate preparation for MAPK, RTK and apoptosis arrays.

Cells were grown to 80% confluence in a T75 flask and rinsed with PBS and lysed with the lysis buffer provided although cell lysates for RTK array were prepared using the NP-40 lysis buffer (section 2.2.1) .The cells were solubilised at a concentration of $1x10^6$ cells per ml of lysis buffer by agitating gently for 30minutes, microcentrifuged at 14 000 x g for 5 mins and the supernatant transferred into a clean microcentrifuge tube. The protein concentration was determined using a BCA assay (section 2.2.2). 200µg of protein was used and a 5:1 ratio with the provided array buffer 2/3 solution. Thawed lysates were always kept on ice.

2.4.3 Proteome Profiler; RTK (Receptor Tyrosine Kinase)

The RTK proteome profiler was used to determine the relative protein phosphorylation levels of 42 receptor tyrosine kinases. This array uses capture and control antibodies developed using cell lines, or where this was not available, recombinant protein. They are spotted in duplicate onto nitrocellulose membranes. The array was assayed as in section 2.4.1.

2.4.4 Proteome Profiler; Apoptosis

The apoptosis array was used to detect the relative levels of expression of 35 apoptosis related proteins using antibodies that selected using cellular extracts expressing the protein target. They are spotted in duplicate onto nitrocellulose membranes. The array was assayed as in section 2.4.1.

2.5 Functional Assays

Once Western blots had confirmed the identity of proteins found using MS (see Western blotting section 2.2.4 and antibodies used in the study Table 2-5), functional assays were used to determine the functionality of any interesting proteins that were differentially expressed or modulated between the resistant cell lines and the parental. The main assays used for this purpose were: the cell growth assay using AlamarBlue (Invitogen) and an adherence assay, in combination with temporal knock downs of the protein of interest using siRNA, detailed below.

2.5.1 Adherence assay

The adhesion assay was used to investigate the difference in adherence between the parental and resistant lines and if siRNA transient knockdowns of the proteins of interest affected the adherence. A 96 well plate was coated with 0.1% gelatin or left with no attachment factor and left for 1 hour at 37˚C. The wells were washed in 0.1% BSA in RPMI1640 2x, then blocked with 0.5% BSA in RPMI1640 for 45minutes at 37˚C and washed with PBS. MCF7 and TAMRBU cells were detached from the T75 flask using enzyme free cell dissociation buffer (Invitrogen) and counted. 10,000 cells in 50µL were added per well then incubated for 45minutes. After the incubation period the plate shaker was used to shake at 1500rpm for 10 seconds. Wells were washed with PBS 3 times and then fixed with 4% formaldehyde (Pierce) for 15 minutes and subsequently washed with 0.1% BSA in RPMI. Crystal violet (5mg/ml in 2% Ethanol) was added for 10 minutes, aspirated and washed with dH_2O. The plate was inverted to dry completely. Once dry 2% SDS solution was added to each well and incubated on the plate shaker at room temperature for 30 minutes. The concentration of crystal violet released from the adherent cells was assayed on the BioRad 680 microplate reader at 550nM.

2.5.2 AlamarBlue™ Assay for cell growth assay.

The amount of fluorescence produced in this assay is reflective of the number of living cells present, as this corresponds to the cells metabolic activity. Damaged and non-viable cells have a lower metabolic activity, and thus, generate a proportionally lower signal than healthy cells. The assay incorporates a specially selected oxidation-reduction (REDOX) indicator (Figure 2-2), that both fluoresces and undergoes colorimetric change

in response to cellular metabolic reduction. Fluorescence was monitored at 560 nm excitation wavelength and 590 nm emission wavelengths.

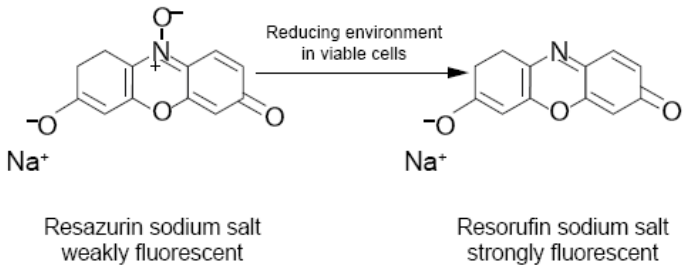


Figure 2-2 The REDOX reaction of the resazurin sodium salt, the indicator in AlamarBlue™. From: Invitrogen product manual.

AlamarBlue™ reagent (Invitrogen) was added to cells that had been plated at least 6 hours, at a concentration of 10% of the sample volume and incubated for 6 hours at 37ºC. The resulting fluorescence was read on a plate reader or fluorescence spectrophotometer.

2.5.3 Invasion assay

For the invasion assay BD Matrigel Matrix (phenol red free) was used as a representative basement membrane in which the cells could invade. The Matrigel matrix was thawed, diluted 1:2 with cooled culture media and mixed to ensure homogeneity. Cooled pipette tips were used to apply a thin layer (10µL) of matrigel to the (pre cooled) 12µm Millipore filter insert and warmed at 37°C for 1 hour. Cells were detached using cell dissociation buffer (Invitrogen) and counted. 400µl of cells ($1x10^5$ cells/ml) were seeded on top of the filters and immersed into the feeder wells of a 24 well plate containing 600µL of full culture media (Table 2-1) and incubated for 24hours at 37°C which allows for the invaded cells to attach to the underside of the filter immersed in the feeder tray. Cells that

had not invaded (on top of the filters) were removed using a sterile cotton bud. Alamar blue was added to the media in the filter tray to a final concentration of 10% and the plate further incubated at 37°C for 6 hours. 200µL of conditioned culture medium was transferred into a flat bottomed 96 well plate and the fluorescence monitored at 560 nm excitation wavelength and 590 nm emission wavelengths. The % alamar blue reduction of invaded cells was normalised to the % of alamar blue reduction of an equal number of cells in the feeder tray with no filter in normal media.

2.5.4 siRNA protocol

siPORT NeoFX (Ambion) was used as the siRNA transfection agent for siRNA assays. It uses a lipid-based formulation to efficiently transfect adherent cells as they are subcultured. 1 hr or less before transfection, MCF7 and TAMRBU cells were trypsinised and re-suspended in normal growth medium (Table 2-1) at a suitable concentration as outlined below in Table 2-3 and set aside at 37°C in the incubator while the transfection complexes were prepared as below in Table 2-3 different assays were carried out on different plate sizes (e.g. proliferation 96well and invasion 24well).

The reagents were all brought up to room temperature. The diluted siPORT *NeoFX* agent into OPTI-MEM I (Invitrogen) medium and the Diluted RNA in OPTI-MEM I medium were then mixed and incubated at rt for 10 mins to allow transfection complexes to form. This mixture was added to the well of a culture plate and the cells were gently added on top. The complexes were mixed gently by tilting the plate and then incubated for at least 48 hours. To ensure the knock down was achieved, Western blots were performed and densitometry data was gathered (section 2.2.4.)

Table 2-3 Number of cells used in siRNA transfections in differing well plate formats.

	96-well	24-well	12-well	6-well
Cell overlay volume	80 µL	450 µL	900 µL	2.3 mL
Total number of cells	6×10^3	4×10^4	8×10^4	2×10^5
Final transfection vol.	100 µL	500 µL	1 mL	2.5 mL

Table 2-4 A, B & C. Directions for preparation of the transfection complexes and cell volume needed.

A Diluted siPORT *NeoFX* agent into OPTI-MEM I medium

	96-well	24-well	12-well	6-well
siPORT™ *NeoFX™* agent	0.5 µL	1 µL	3 µL	5 µL
OPTI-MEM® to:	10 µL	25 µL	50 µL	100 µL

B Diluted siRNA in OPTI-MEM I medium as follows

	96-well	24-well	12-well	6-well
OPTI-MEM® to:	10 µL	25 µL	50 µL	100 µL
Add the following amounts of different types of small RNA:				
1 µM *Silencer®* Select siRNA	0.5 µL	2.5 µL	5 µL	12.5 µL

C Cell volume needed and final transaction volume required.

	96-well	24-well	12-well	6-well
Cell overlay volume	80 µL	450 µL	900 µL	2.3 mL
Total number of cells	6×10^3	4×10^4	8×10^4	2×10^5
Final transfection vol.	100 µL	500 µL	1 mL	2.5 mL

Table 2-5 List of Antibodies used in the study.

Antibody / protein	Company	WB dilution
Primary EGFR	Santa Cruz	1:1000
Primary ERα	Santa Cruz	1:500
Primary ERK1+2	Abcam	1:2000
Primary p-ERK1+2 (Thr202/Tyr204)	Cell Signalling Technology	1:1000
Primary PKCδ	BD Bioscience	1:2000
Primary p-PKCδ (Thr 505)	Cell Signalling Technology	1:1000
Primary Akt	Cell Signalling Technology	1:1000
Primary p-Akt (Ser 473)	Cell Signalling Technology	1:1000
Primary IQGAP1	Upstate/Milipore	1:1000
Primary Cortactin	Upstate/Milipore	1:1000
Primary Talin-1	Cell Signalling Technology	1:5000
Primary Cathepsin D	Santa Cruz	1:5000
Primary Ribophorin II	Santa Cruz	1:2500
Primary β Actin	Abcam	1:50,000
Primary GAPDH	Ambion	1:2000

3 Development of the Tamoxifen resistant cell line TAMRBU

3.1 Introduction

Tissue samples are often difficult and/or painful to obtain, and breast tissue is a fatty tissue which can complicate sample preparation for proteomics. An alternative is cell culture. Cell lines are an important tool in scientific research, and tissue culture is a routine laboratory technique. In 2000, historians of science Meyer Friedman and Gerald W. Friedland named it one of the 10 greatest discoveries in medicine *"It made possible the study of living organisms at the cellular and even the molecular level and the development of modern vaccines ... and abetted the search for the causes of cancer (and AIDS). Indeed, because of tissue culture, more has been learned about the basic mechanisms of disease in the past 50 years than in the previous 5,000"* (Freidman and Freidland, 2000). Though using cells in culture is known to have disadvantages such as the cells are not *in situ* and are therefore are not growing in the 3D architecture of the body or are they subject to paracrine stimulation; it does have the advantage of simplifying the sample with the presence of only one cell type, as opposed to the stromal cells, endothelial cells and "normal" non- cancerous epithelial cells that would be present in a small biopsy. Obtaining a biopsy of tissue is also normally an invasive procedure and therefore the use of cell culture as a tool to study disease models has proven to be useful.

MCF7 cells are one of the most widely used cancer models in the world, retaining functional oestrogen receptors that are able to process oestrodiol effectively. They were used as the parental cell line to establish both the TAMR and the TAMRBU cell lines. MCF7 are an adherent, epithelial mammary cell line originally derived from a female breast cancer patient with metastatic disease via pleural effusion. Initially, MCF7 cells respond to the growth inhibitory effects of Tamoxifen, however the MCF7 cell line, with constant exposure to Tamoxifen over a period of time can provide a sub-population that can circumvent the effects of Tamoxifen and go on to develop the resistant phenotype allowing for the development of an experimental Tamoxifen resistance model. The use of this cell culture model to investigate the issue is well established.

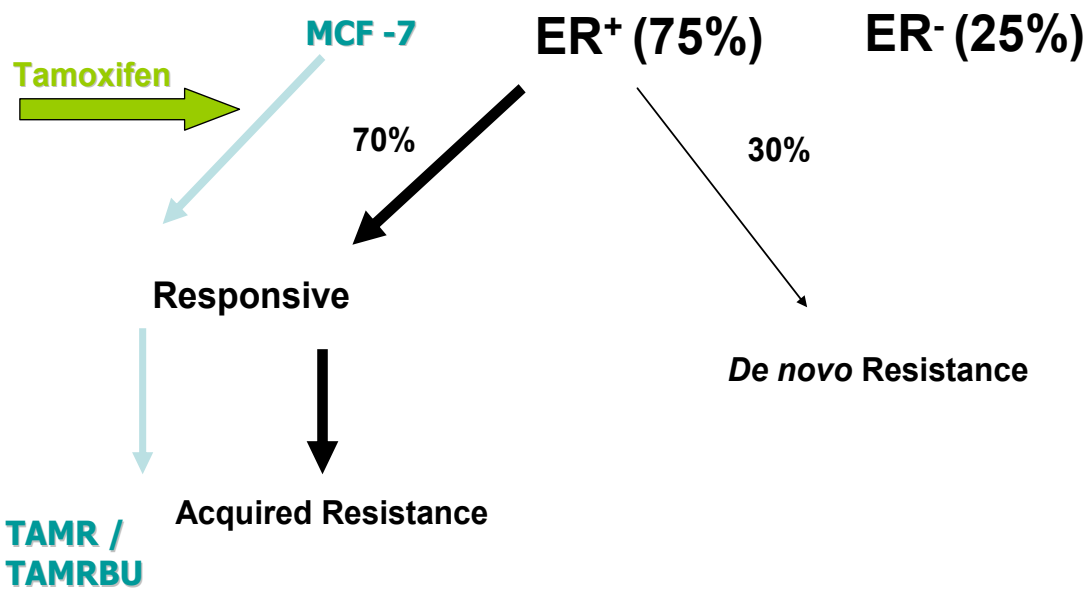


Figure 3-1 Model of Tamoxifen resistance

Above is a simplified model showing the development of resistance. Of all breast carcinoma cases, the majority (approximately 75%) are oestrogen receptor positive. Of the oestrogen receptor positive patients that take Tamoxifen, approximately 30% do not respond to treatment (*de novo* resistant). Approximately 70% of patients that take Tamoxifen initially respond to the treatment, the majority of which will have acquired Tamoxifen resistance during the course of their 5 year treatment.

3.2 Results

To develop a meaningful Tamoxifen resistant cell line it is necessary to mirror the clinical path to resistance, see Figure 3-1. The morphological changes of the MCF7 cell line can be seen as it goes through the chemical selection process and develops into the resistant phenotype sub-line TAMRBU (Figure 3-2). Using Tamoxifen continuously in culture selects for the resistant phenotype as the cells adapt to the drug's presence. Initially the time taken for the cells to become confluent was increased dramatically from 5 days to 4 weeks (with regular changes of media) as the majority of cells were phenotypically more rounded and many had died/detached from the flask surface. The time taken to reach confluence was used as a rough measure of cell growth/survival in the presence of Tamoxifen. With continuous exposure, the time taken for the cells to reach confluence in the flask decreased, after a period of 20 weeks the cells were proliferating at a similar rate to the parental MCF7 cells but in the presence of Tamoxifen, and the cells were deemed resistant as shown in the graph in Figure 3-3.

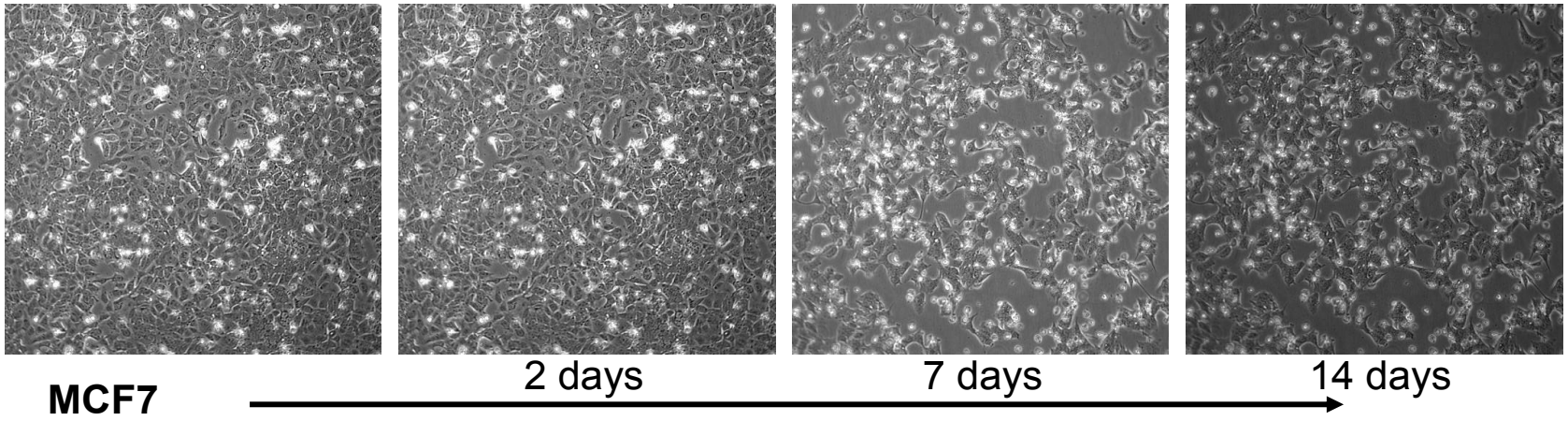
3.2.1 Determination of the relative protein concentration of oestrogen receptor in the MCF7, TAMR and TAMRBU cell lines.

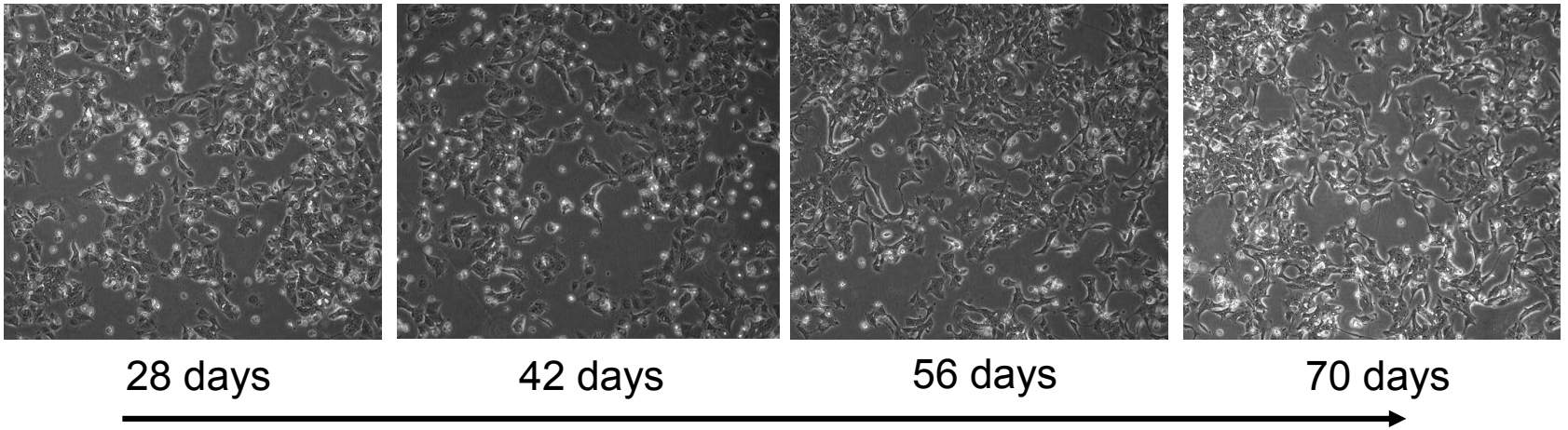
In order for a breast cancer patient to be treated with Tamoxifen they are screened for the presence of oestrogen receptor α as it is the target receptor. It has been previously shown that the majority of patients that go on to develop acquired Tamoxifen resistance have cells that are still expressing functional oestrogen receptors (Henryk and Fuqua, 2007). Therefore, it was important to determine that the models of resistance used in this study are oestrogen receptor positive. To verify that we could detect this in the TAMR and

TAMRBU cell lines, the oestrogen receptor positive MCF7 cells were compared to the TAMR and TAMRBU cell lines using Western blot analysis (Figure 3-4). The blots were additionally probed for β Actin as a control for overall proteins expression level loading accuracy. The Rf values were determined for this and some other characterisation proteins to confirm protein size.

Rf = distance protein migration\solvent front

It was seen that the resistant cell lines both expressed ERα; the protein was shown to be expressed but to a lesser extent than in the parental MCF7 line.


MCF7
2 days
7 days
14 days
Plus 10^{-7}M 4 OH Tamoxifen


28 days
42 days
56 days
70 days
Plus 10^{-7}M 4OH Tamoxifen

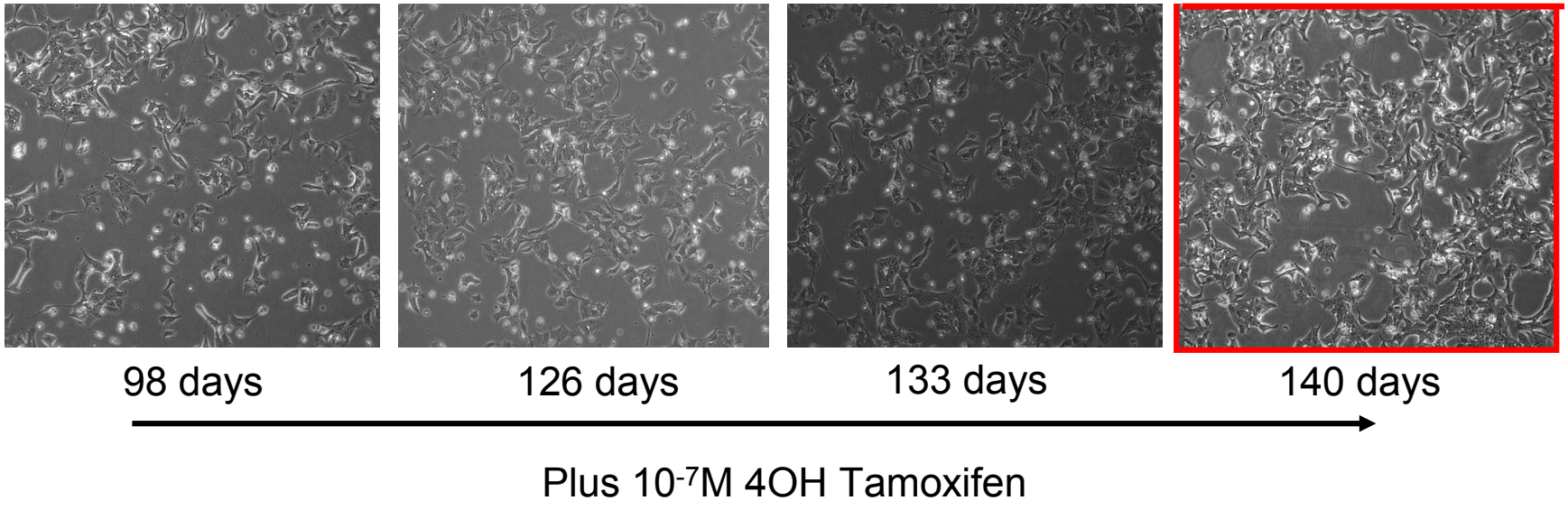
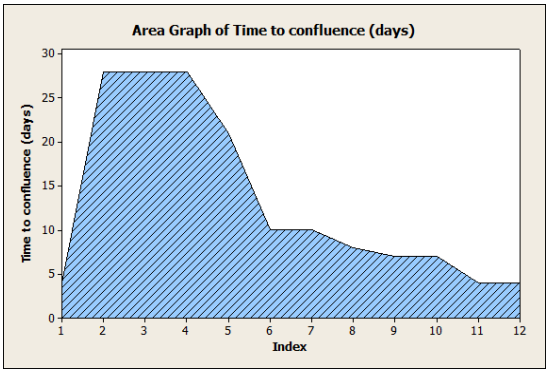


Figure 3-2 Investigating morphological changes occurring while developing the Tamoxifen resistant cell line TAMRBU. Images were taken the microscope on bright field mode (x10). The MCF7 cells were grown in media containing 5% csFCS with Tamoxifen present as a chemical selection process in T25 flasks and images were taken periodically of the development of resistance from the MCF7 cells to the TAMRBU cells. Over a period of 10 weeks the cells began to grow well in the presence of Tamoxifen. This is depicted in graphical form in Figure 3-3.



MCF7 plus TAM (days)	Time to confluence (days)
0	4
2	28
7	28
14	28
28	21
42	10
56	10
70	8
98	7
126	7
133	4
140	4

Figure 3-3 Time line of the development of the resistant cells TAMRBU.

3.2.2 Determination of the relative protein concentration of "characterisation" proteins.

3.2.2.1 Characterisation proteins

These proteins were selected as they had previously been shown to be differentially expressed between MCF7 and TAMR cells. They were used in this project to conform the establishment of the TAMRBU cell model but also as a characterisation panel to monitor the quality of the cell culture model in subsequent experiments throughout this study thus ensuring that the incidence of cell drift was avoided.

Legend for Figures 3-4 to 3-11.Western blot analysis was carried out on whole cell extracts taken from MCF7, TAMR and TAMRBU cell lines. These were grown to 80% confluence in media containing 5% csFCS. The SDS PAGE gels were 4-12% Bis – Tris gradient gels, and blotted with primary antibodies (Table 2-5.) All blots were probed for β actin as a loading control and all densitometry was carried out on the Bio-Rad G800 densitometry reader. The resulting blots were normalised to β actin and illustrated as a percentage of the loading control concentration. All experiments were carried out independently five times (with the exception of p-Akt at n=3, Figure 3-11) which were carried out and show standard error of the mean.
**Phospho PKCδ (Thr505), Figure 3-9, the densitometry was measured on the upper band only. The lower band was presumed to be a degradation product as known to be liable to break down.

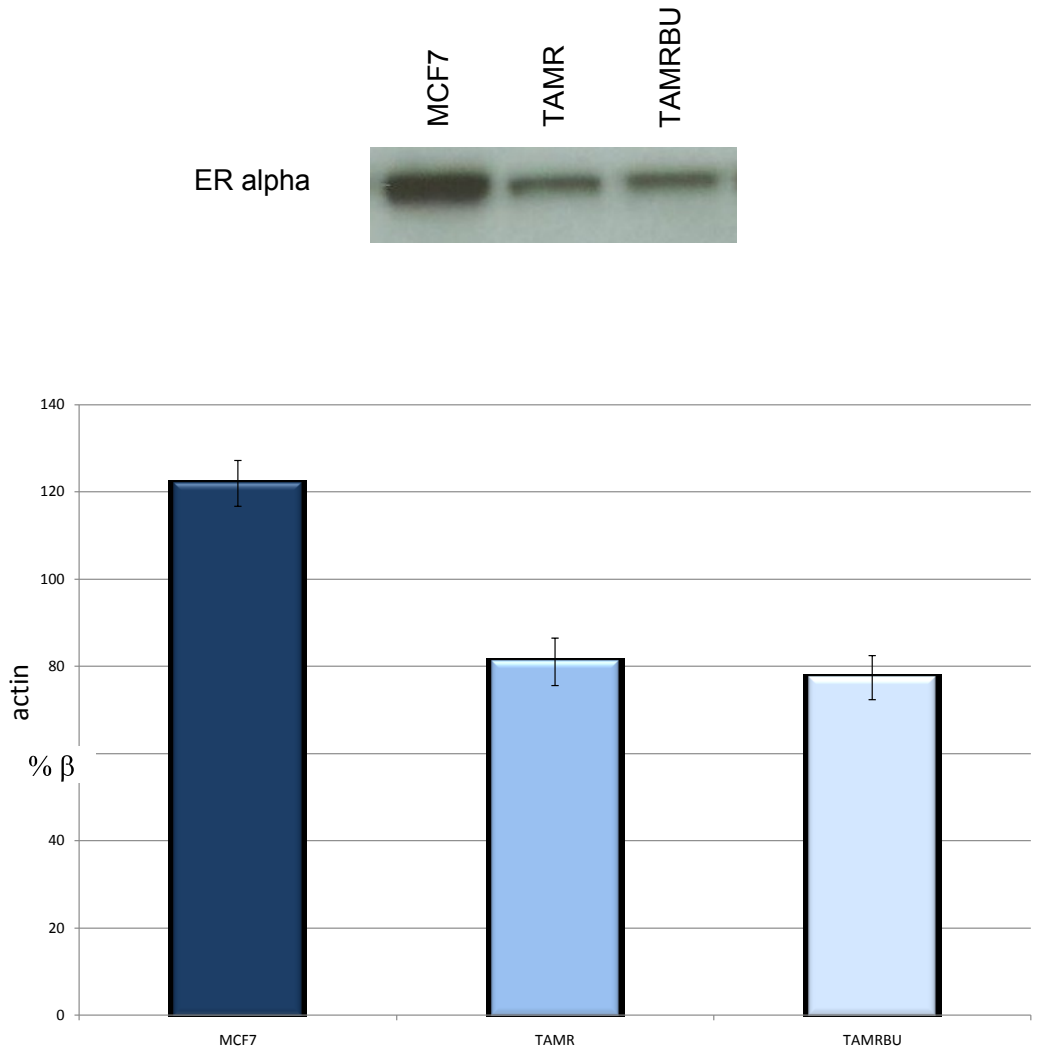


Figure 3-4 Relative ERα protein expression between the MCF7 parental cell line and the TAMR and TAMRBU, Tamoxifen resistant cell lines.

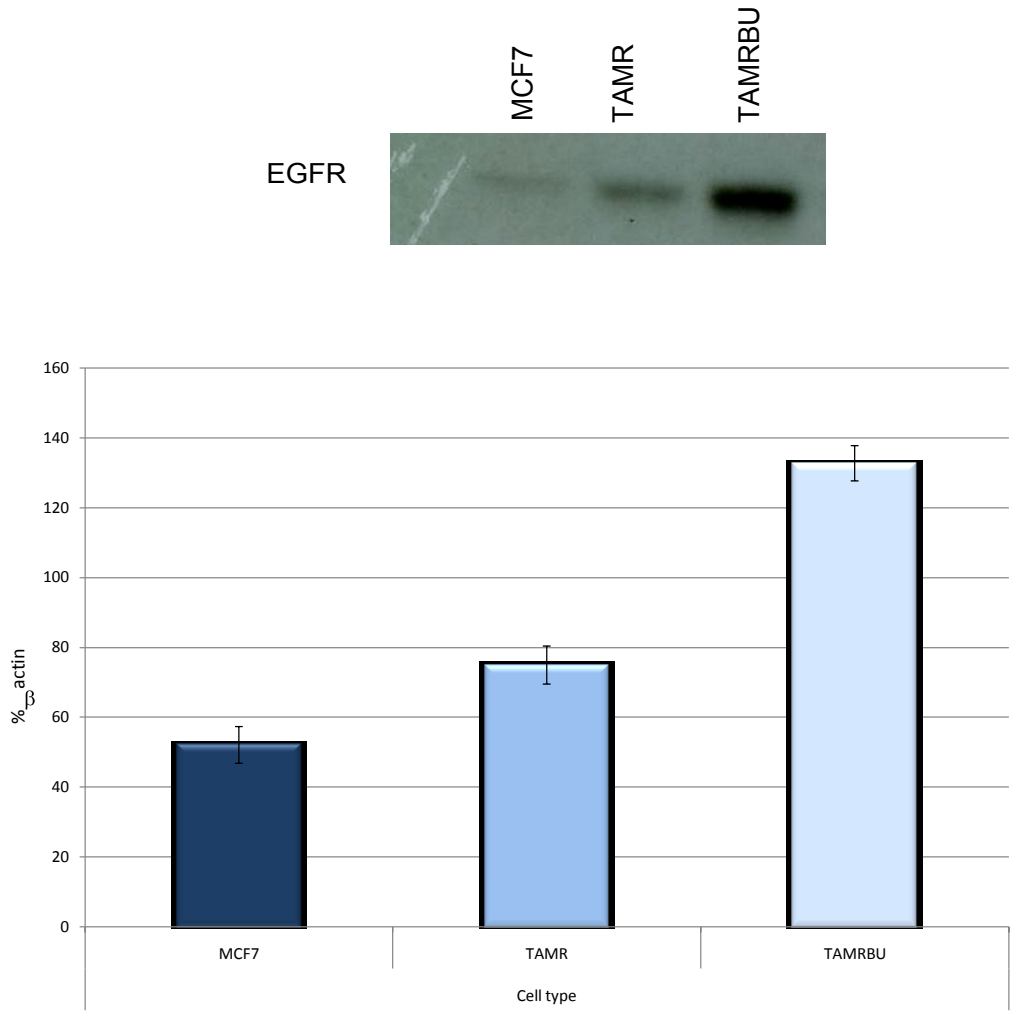


Figure 3-5 Relative EGFR protein expression between the MCF7 parental cell line and the TAMR and TAMRBU, Tamoxifen resistant cell lines.

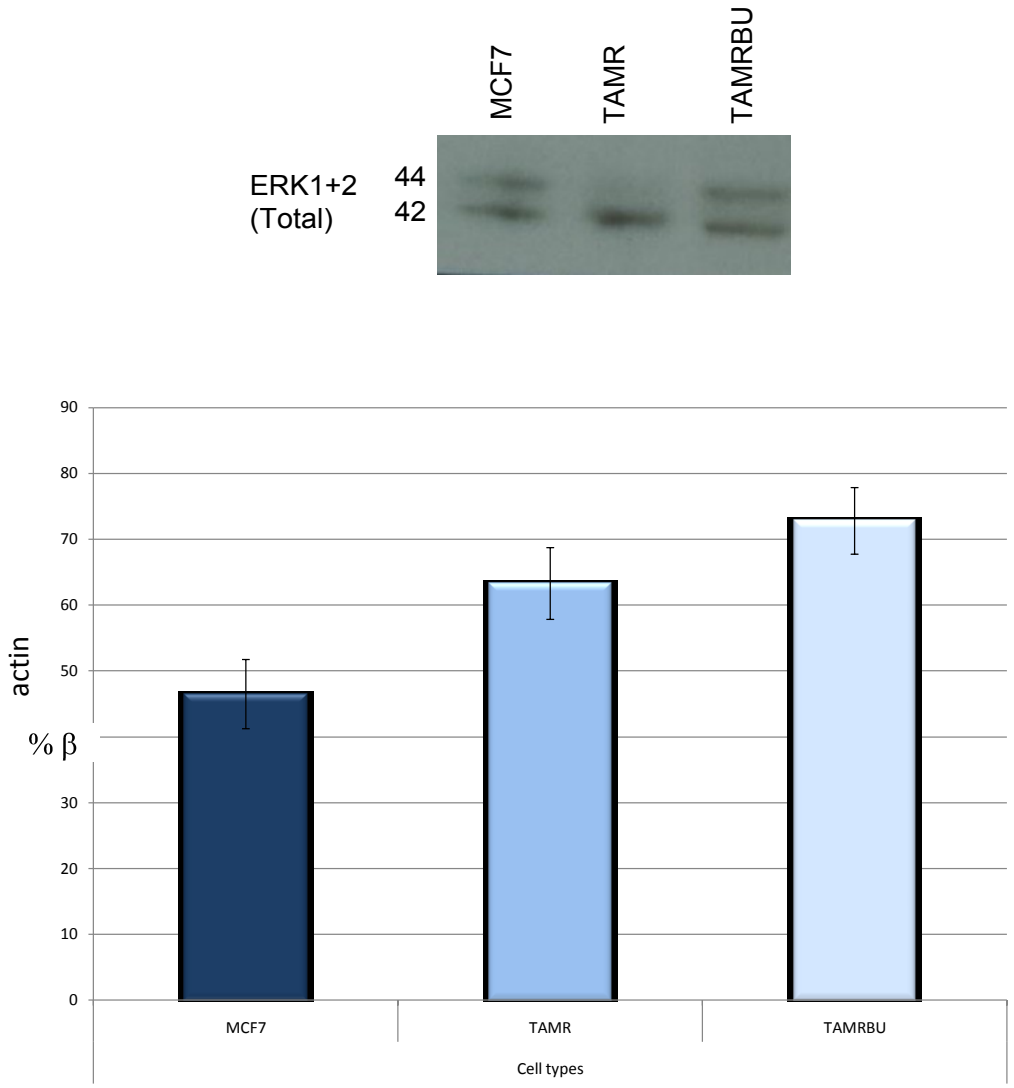


Figure 3-6 Relative ERK1+2 protein expression between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines.

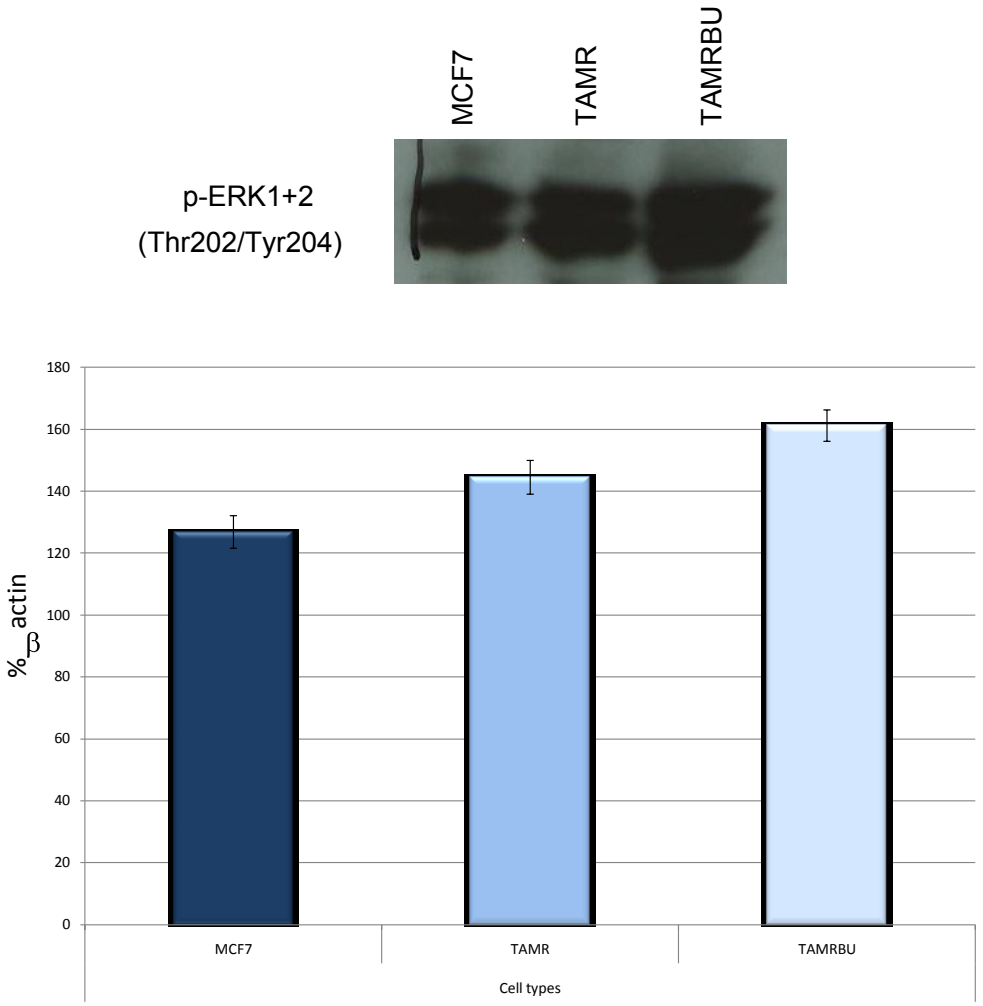


Figure 3-7 Relative ERK1+2 protein phosphorylation between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines.

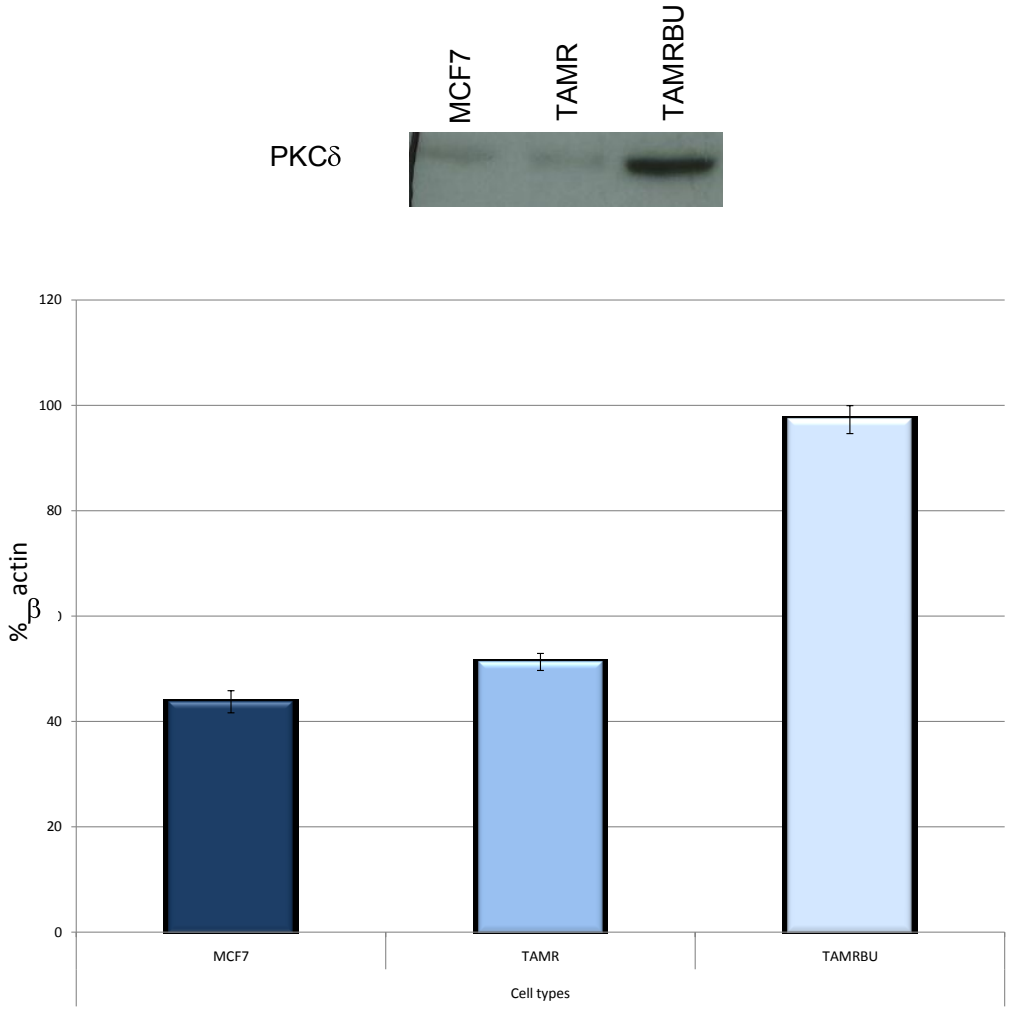


Figure 3-8 Relative PKCδ protein expression between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines.

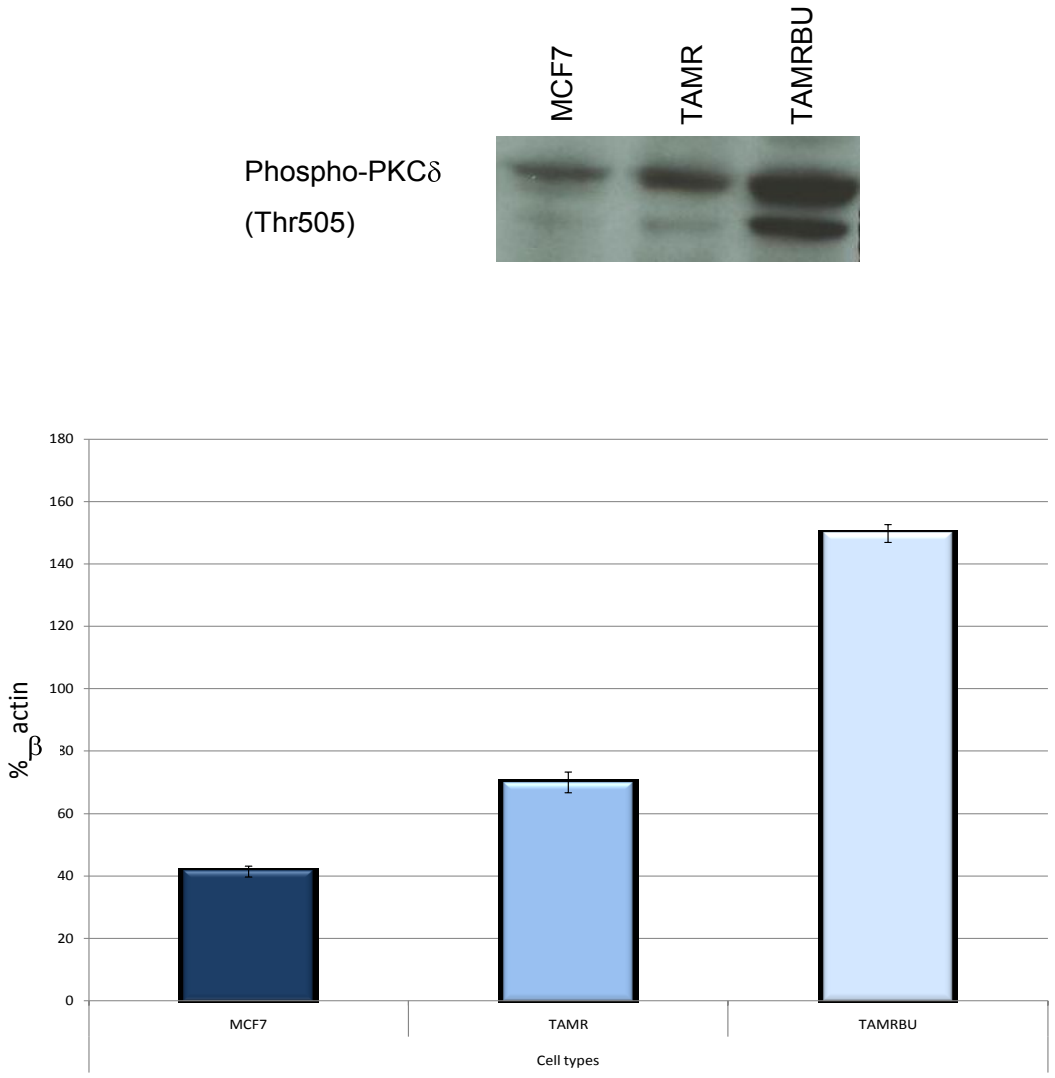


Figure 3-9 Relative p-PKCδ protein phosphorylation between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines.

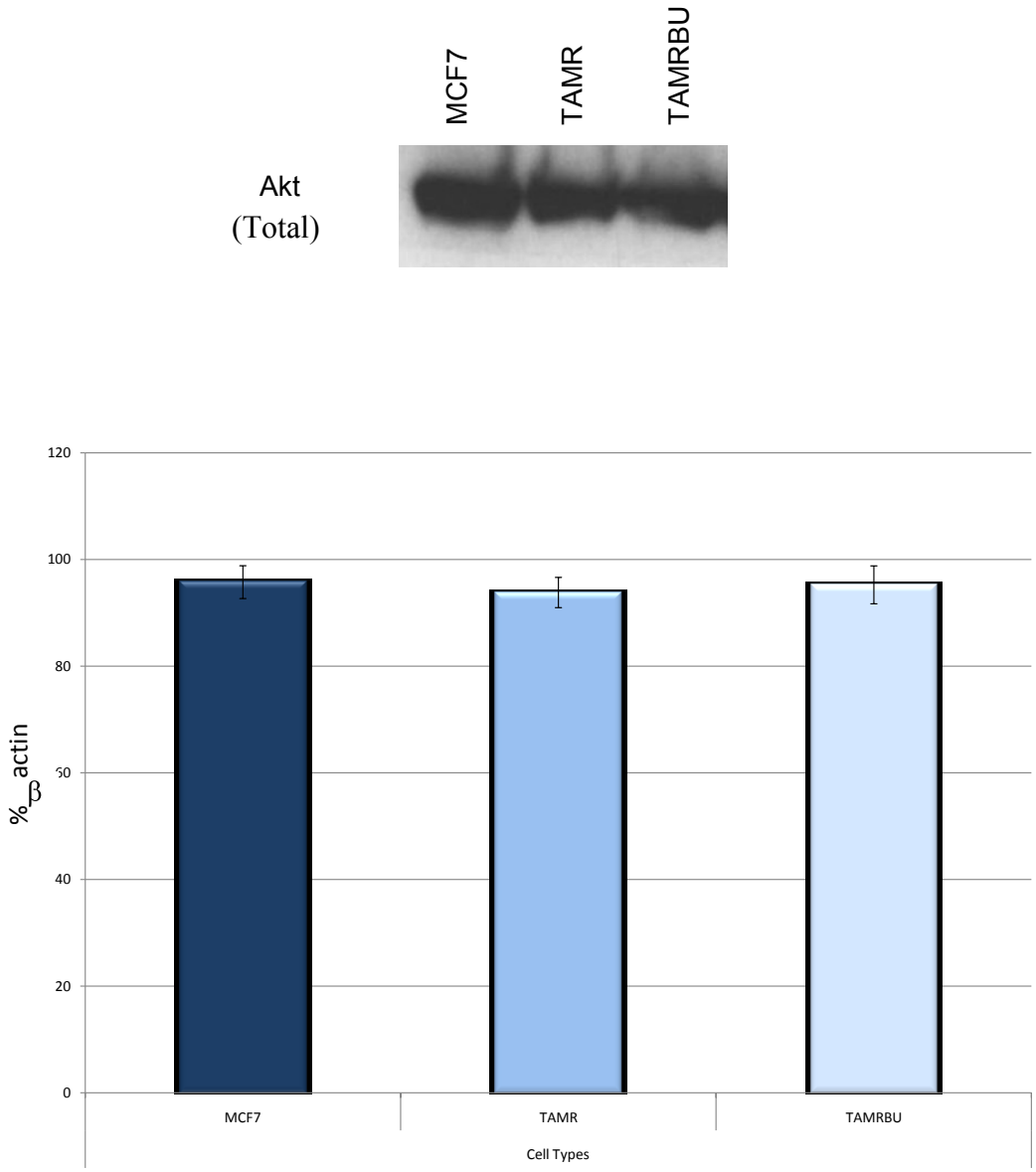


Figure 3-10 Relative Akt protein expression between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines.

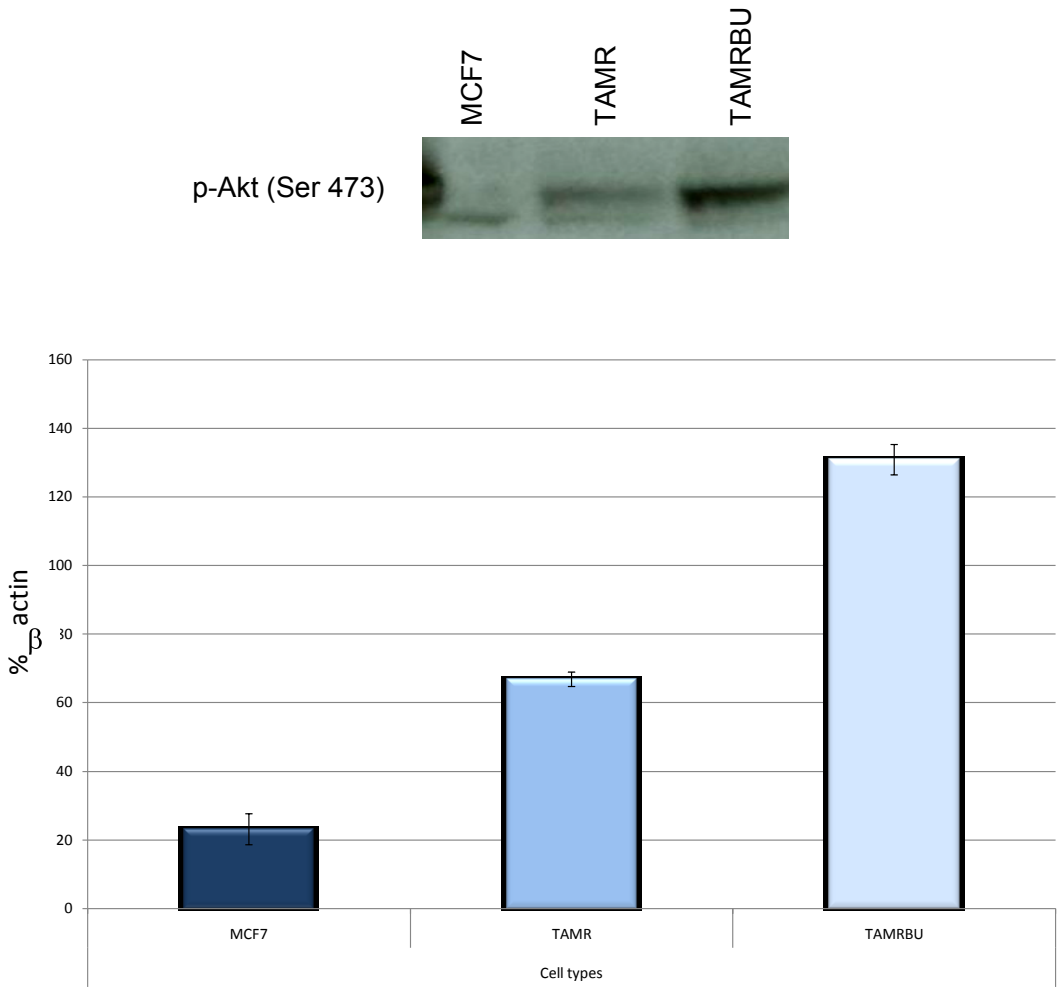


Figure 3-11 Relative Akt (Ser473) protein phosphorylation between the MCF7 parental cell line and the TAMR and TAMRBU Tamoxifen resistant cell lines (N=3).

3.2.3 The effect of long term removal of Tamoxifen from the culture growth media.

The changes seen in the cells when they have acquired Tamoxifen resistance should be the effect of acquiring resistance rather than the short term effect of Tamoxifen presence in the culture media. To verify this, TAMRBU cells were cultured in the presence of Tamoxifen or the absence of Tamoxifen for 1, 2, 3, 4, 5 and 6 weeks. The cellular proteomes from the TAMRBU cells, along with MCF7 control cells, were extracted and the lysate was run on a SDS PAGE gel and then Western blotted, investigating the stability of the EGF receptor expression. (Figure 3-12). Western blot analysis was performed and was additionally probed for β Actin as a control for overall proteins expression level loading accuracy.

The level of EGFR expression in the resistant cell line was consistently up compared with the MCF7 parental cell line and was stable in the presence of Tamoxifen and when Tamoxifen was withdrawn on a long term basis. This was not tested on other characterisation proteins, but does infer that the proteins expressed from the TAMRBU cells are stably expressed and are not affected by the presence or absence of Tamoxifen.

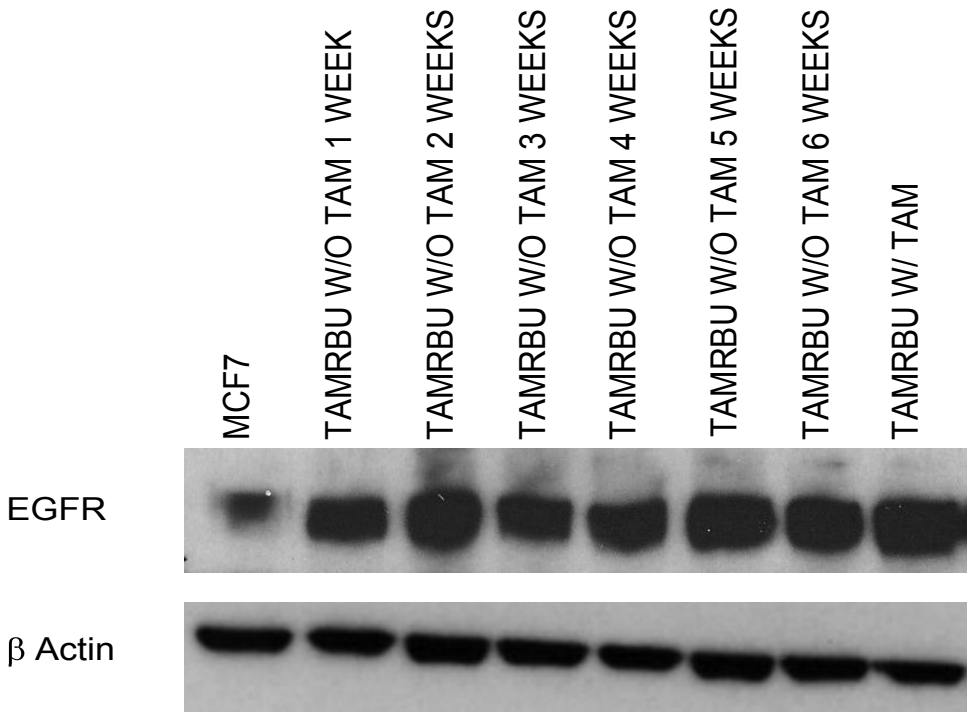


Figure 3-12 Effect of Tamoxifen and its absence from the growth media on stability of the relative expression of EGFR over a period of 6 weeks.
Western blot analysis was carried out on whole cell extracts taken from MCF7 and TAMRBU breast cancer cells. The cells were grown to 80% confluence in media containing 5% csFCS with Tamoxifen present (W/) and Tamoxifen absent (W/O) over varying period of time, cells were passaged at 80% confluence when necessary. The 1D SDS PAGE gels were 4-12% Bis-Tris gradient gels, and the blotted membranes were probed with primary antibodies for EGFR and β Actin.

3.2.4 Investigation into the growth patterns of the cell culture models

MCF7 should not thrive in an environment where Tamoxifen is present. To investigate cell growth; AlamarBlue™ (Invitrogen) was used. The amount of fluorescence produced in the assay is reflective of the number of living cells present as it corresponds to the cells metabolic activity. Damaged and non-viable cells have a lower metabolic activity, and thus, generate a proportionally lower signal than healthy cells. AlamarBlue was used to determine the effect of different treatments on MCF7 and TAMRBU cells. The effect of Tamoxifen, oestrodiol, and Tamoxifen and oestrodiol in combination, as shown in Figure 3-13; ethanol was the vehicle, so the effect of ethanol was also investigated. The results indicate that the TAMRBU and MCF7 cells were stimulated by the addition of oestrodiol to the media: thus inferring the oestrogen receptor is functional in both cell models. The TAMRBU cell line appears to proliferate more in the presence of Tamoxifen in the media, but the MCF7 cells growth is significantly subdued to an average of 50% of the growth of MCF7 cells with no treatment. When used in combination Tamoxifen and oestrodiol seem to effect the MCF7 cells as they do not grow as effectively as the cells that have had no treatment but consistently grow more than MCF7 that have been solely subjected to Tamoxifen. The effect of the vehicle was minimal on both cell lines. The proliferation rate of the Tamoxifen resistant TAMRBU cells and the MCF7 sensitive cells independent of the treatments did not differ significantly between the cell lines.

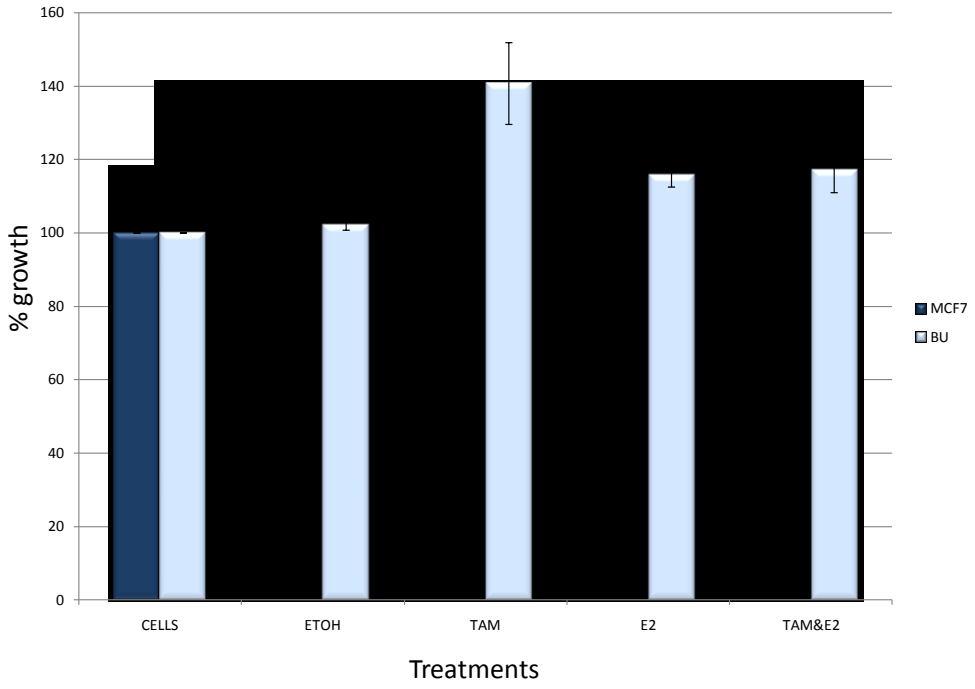


Figure 3-13 Alamar blue assay used for the measurement of proliferation.

Alamar Blue proliferation assay was carried out on MCF7 and TAMRBU (BU) cells. Prior to the experiment the cells were grown to 80% confluence in media containing 5% csFCS with Tamoxifen absent for a period of 3 days. Optimisation of plating density had been carried out and subsequently 10,000 cells per 0.32cm^2 were chosen as the optimum number of cells to receive an adequate reading on the plate reader after a period 72 hours. Treatments: ETOH vehicle, TAM= Tamoxifen 10^{-7}M, E_2= Oestrodiol 10^{-9}M, and TAM&E_2= Both Tamoxifen 10^{-7}M and Oestrodiol 10^{-9}M.
Results above are derived from 5 independent experiments normalised to cells with no treatment ± SEM.

3.3 Discussion

The development of the Tamoxifen resistant cells "in house" enabled us to follow the process that the Tenovus Centre for Cancer Research in Cardiff had used to develop their resistant cells – this was seen as good practice and carried the added benefit of producing a robust cell line, which behaved similarly to the TAMR cell line but with a lower passage number. The characterisation profiles of the two cell lines were very similar or followed the same trend. Some proteins were more highly expressed in the TAMRBU cells and this was putatively explained by the lower passage number of the cells. The TAMRBU cells after a period of 20 weeks proliferated at a similar rate to TAMRs and MCF7 cell lines, and grew well in the presence and in the absence of Tamoxifen. The results indicate subsequent to the removal of the Tamoxifen from the media, that the cells were not reliant on the presence of the drug to grow and the Western analysis determined that the absence of Tamoxifen over a period of 6 weeks did not attenuate the increase in expression of the EGF receptor, inferring the presence/absence of the drug was not altering the receptor expression in a short term manner but rather, this was a stable change in the cells generated by acquired resistance to Tamoxifen.

The characterisation of the two Tamoxifen resistant cell lines shows that they are consistent with the previously published characteristics (ability to proliferate in the presence of Tamoxifen, and high level of expression of specific proteins such as EGFR) and therefore a robust and reproducible model to be used in the subsequent experiments discussed in the following chapters - 4, 5, and 6.

4 Antibody based proteomic investigation

4.1 Introduction

Several commercially available antibody arrays were used to investigate the potential proteomic differences in expression / phosphorylation status of many proteins between MCF7 and Tamoxifen resistant cells. Antibody arrays enable multiplex high-throughput protein expression profiling. They do not provide complete global assessment of the proteins in the sample as mass spectrometry does, but provide the ability to analyse over 30 proteins at once (depending upon the array), many focussed on a particular group of proteins such as Mitogen Activated Protein Kinase family. The arrays used in this study were chosen specifically for their signalling pathway focus MAPK, or the protein family focus such as Receptor Tyrosine Kinase (RTKs) and apoptosis. All of these arrays contained at least one protein that had been previously described as altered in Tamoxifen resistance.

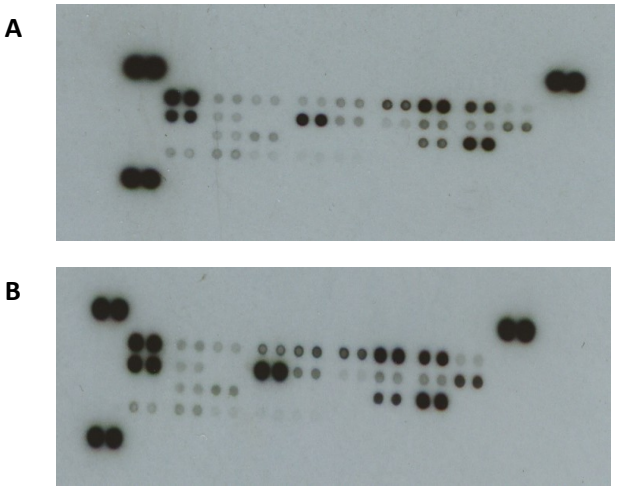


Figure 4-1 Example of Proteome profilers. An example of the differences seen between Tamoxifen sensitive (A) and Tamoxifen resistant cells (B) using a proteome profiler array (R and D systems).

Many different samples can be measured on antibody arrays including tissue lysates, cell lysates, conditioned media, and in some cases serum and plasma. Cell lysates from the MCF7, TAMR and TAMRBU were used on the selected antibody arrays. All densitometry analysis was read using a BioRad GS800 reader and all analysis was completed using MiniTab 15 using t-tests or one way unstacked ANOVA. The box plots show the median value and the range. Detailed methodology of the antibody arrays is located within the material and methods chapter (section 2.4).

4.2 Human phospho- MAPK proteome profiler

This array analysed the phosphorylation status of all three of the major MAPK families as well as additional intercellular protein kinases such as Akt, p70 S6 Kinase, and GSK-3. The full details of the proteins used on the array can be seen in Table 4-1 and an example of the array can be seen in Figure 4-1. The array included a positive and negative control as detailed in Table 4-1 and the average reading of the negative controls was subtracted from the densitometry values. The MAPK proteome profiler was assayed on three independent occasions on three separate cell populations. Significance of results and fold change seen in the MAPK profiler is summarised in Table 4-2.

4.2.1 Controls

The three positive control spots were analysed on each array for each of the three replicates. These were then analysed using a one way ANOVA (unstacked) giving a P value of 0.880. These data enable confidence in the arrays as they do not differ significantly between experiments.

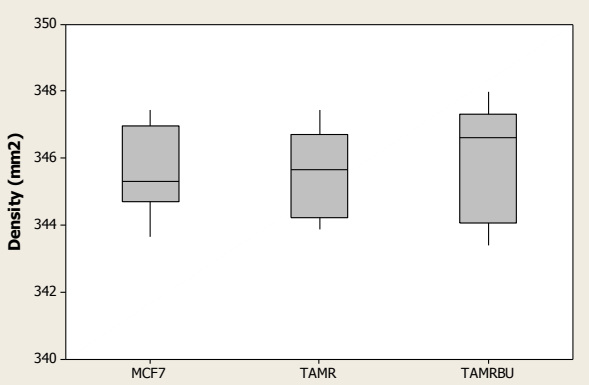


Figure 4-2 Box plot showing the positive control spots; no significant difference can be seen between the cell lines and between the three replicates.

Table 4-1 MAPK proteome profiler antibody array.

Target Protein/Control	Phosphorylation Site Detected
Positive control	/
Negative control	/
ERK 1	T202/Y204
ERK 2	T185/Y187
JNK 1	T183/Y185
JNK 2	T183/Y185
JNK 3	T221/Y223
JNK pan	T183/Y185, T221/Y223
P38 α	T180/Y182
P38 β	T180/Y182
P38 δ	T180/Y182
P38 γ	T183/Y185
RSK1	S380
RSK2	S386
MSK2	S360
Akt1	S473
Akt2	S474
Akt3	S472
Akt pan	S473, S474, S472
GSK3α/β	S21/S9
GSK3β	S9
HSP27	S78/S82
P70S6K	T421/S424

A table showing the antibodies spotted onto the MAPK proteome profiler antibody array; including the targeted sites of phosphorylation, and alternative nomenclature. Table adapted from the R and D systems product sheet.

4.2.2 Densitometry analysis of the MAPK proteome profiler: ERK 1 and ERK 2.

As we have already shown ERK (extracellular signal-regulated kinase) 1 and 2 to have increased phosphorylation in the resistant cell lines using Western blotting (Figure 3-6). These proteins acted as an internal control for this array experiment. ERK1 and ERK2 phosphorylation on the antibody array was obviously increased in resistance and a one way un-stacked ANOVA analysis of both gave a p value of less than 0.001 between the MCF7 and resistant lines (Figure 4-3). A paired t-test showed both TAMR and TAMRBU to have significantly increased phosphorylation of ERK1 when compared to MCF7 cells, but not significantly different to each other, (P=0.457). The increase in phosphorylation was 55.1% in TAMR and a 61.6% in TAMRBU from the MCF7 parental line (Table 4-1). ERK2 was also increased in the resistant lines significantly in t-test (TAMR, P=0.007 and TAMRBU, P=0.008). They do slightly differ from each other as the TAMRBU showed marginally lower phosphorylation than the TAMR, this however was not significant (P=0.171). The phosphorylation is increased by 76.5% in TAMR and 54.6% in TAMRBU from the MCF7 parental line (Table 4-2).

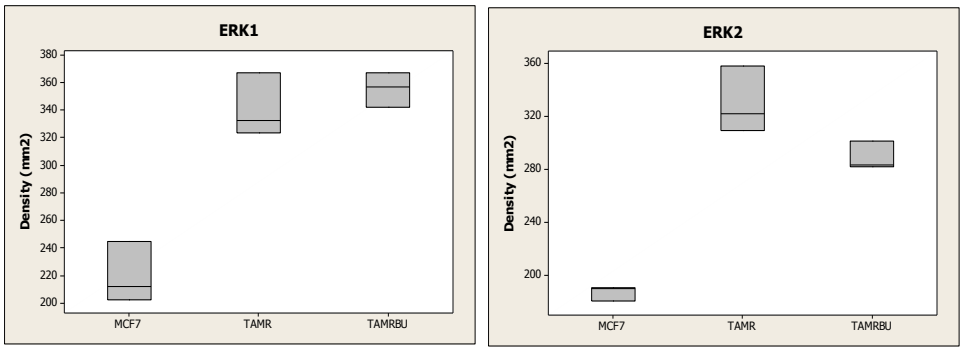


Figure 4-3 Box plots of the phosphorylation of ERK1 and ERK2. A comparison of MCF7, TAMR and TAMRBU. It shows that both the resistant cell lines have increased phosphorylation of both proteins. ERK1, P=<0.001. ERK2, P=<0.001. N=3.

4.2.3 Densitometry analysis of the MAPK proteome profiler: JNK family.

The density of the pixels on the proteome profiler was inconsistent for the JNK family of proteins, so although there was a general trend of increased phosphorylation for the JNK family members assayed only JNK1 showed a statistically significant increase (Figure 4-4). JNK1 showed an increase of 11.9% (TAMR) and 6% (TAMRBU) when compared with the phosphorylation of JNK1 in MCF7 cells. This was a statistically significant using ANOVA (P=0.003), and t-test comparison between the MCF7 and TAMR gave a P value of 0.001, whilst between MCF7 and TAMRBU gave 0.076. The resistant lines did not significantly differ from each other (P=0.111).

JNK2 showed a 5.1% (TAMR) and 5.8% (TAMRBU) increase in phosphorylation, however, the difference between MCF7 and TAMR values gave a P value of 0.371 whereas MCF7 and TAMRBU gave a P value of 0.022. TAMR and TAMRBU did not differ significantly (P=0.911). JNK3 showed increased phosphorylation in both resistant lines (TAMR; 14.3%, TAMRBU; 15.6%) in comparison to MCF7. However, the ANOVA and individual t-tests did not indicate any significance as the MCF7 results was so variable. Total (or pan) JNK phosphorylation showed a minimal increase in phosphorylation (TAMR; 2.1%, TAMRBU; 4.5%) the values did not reach significance (Table 4-2).

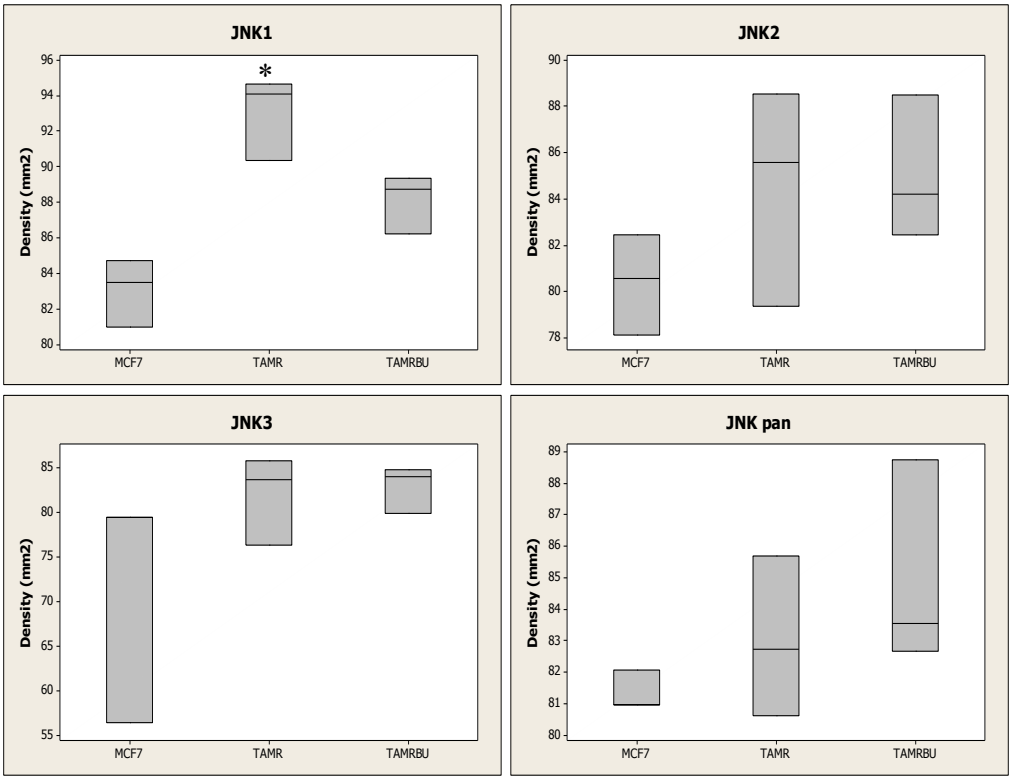


Figure 4-4 Box plots of the JNK family of MAPK. JNK1 is shows a statistically significant difference in phosphorylation between the three cell populations with an ANOVA P value of 0.003, however the JNK2, JNK3 and total JNK (JNK pan) shows no overall significant difference. * indicates significance $P<0.05$. N=3

4.2.4 Densitometry analysis of the MAPK proteome profiler: p38 family.

These data (Table 4-1 & Figure 4-5) show the p38 family had increased phosphorylation in both resistant cell lines when ANOVA is performed: p38α has a P value of 0.001, p38β the P value is 0.026, whilst p38δ and p38γ have P values of less than 0.001. The increases in phosphorylation seen between the MCF7 and resistant lines are very apparent with p38α increased by 25.4% (TAMR) and 39.0% (TAMRBU), p38β increased by 24.9% (TAMR) and 26.4% (TAMRBU), p38δ increased by 24.1% (TAMR) and 36.8% (TAMRBU) and p38γ increased by 38.6% (TAMR) and 40.7% (TAMRBU). The

differences seen between the MCF7 and either resistant line were all significant and there was no statistically significant difference between TAMR and TAMRBU, see Table 4-2.

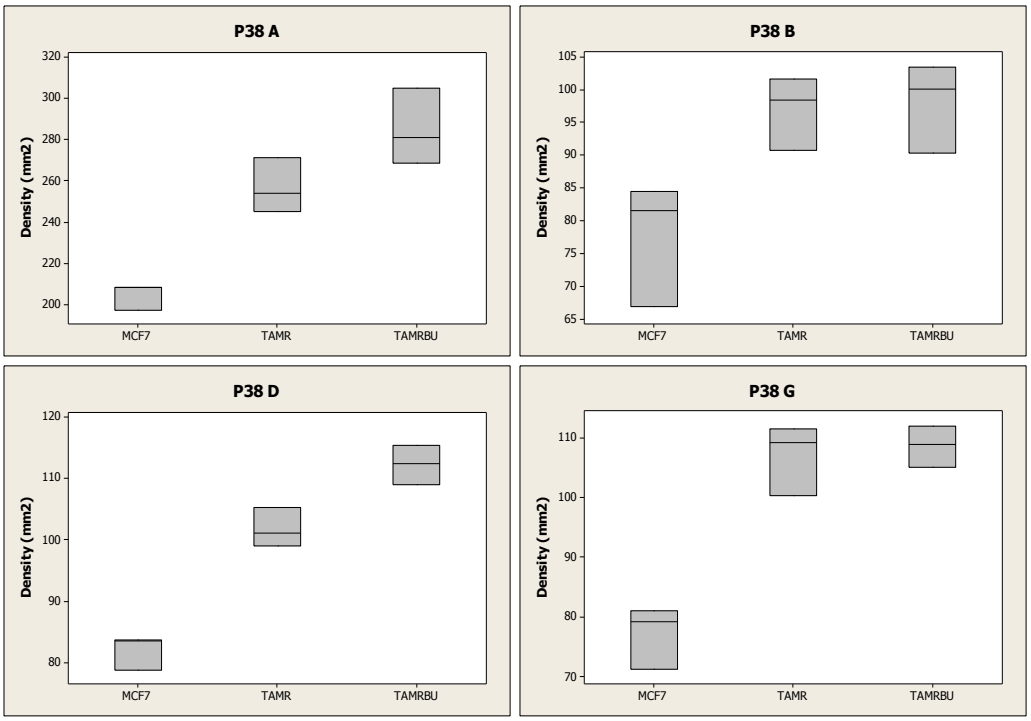


Figure 4-5 These Box plots for the p38 family of MAPK show the three cell lines and an increase in phosphorylation in both resistant cells. p38α has a P value of 0.001, for p38β the P value is 0.026, whilst p38δ and p38γ have P values of less than 0.001. N=3.

4.2.5 Densitometry analysis of the MAPK proteome profiler: RSK1 and RSK2.

RSK1 demonstrated an increased phosphorylation of TAMR and TAMRBU compared to MCF7(115.1% & 23.2% respectively).These increases are seen to be significant by t-test with P=values at 0.040 (TAMR) and 0.017 (TAMRBU). The resistant lines were not significantly different from each other with a P value of 0.289. The ANOVA gave a P value of 0.017. The RSK2 isoform showed no significance with ANOVA (0.229). The

median expression of RSK2 remained similar across the three cell lines. None of the values reached significance (see Table 4-2).

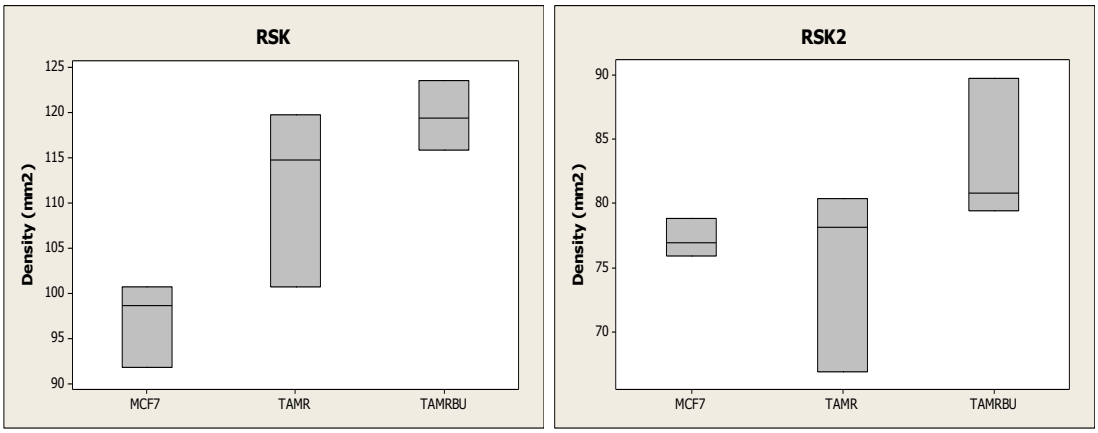


Figure 4-6 Box plots of the RSK1 and RSK2 protein phosphorylation. The ANOVA result for each plot is RSK1 P= 0.017 and RSK2 P=0.229. N=3

4.2.6 Densitometry analysis of the MAPK proteome profiler: Akt 1, 2, 3 and total.

Akt1, 2, 3 and total illustrated a general trend of increased phosphorylation in resistance with Akt1 showing the largest percentage increase compared with MCF7 cells with a 43.9% (TAMR) and 49.9% (TAMRBU) increase. T-tests indicated that the increase was significant with P values of 0.026 and 0.044 respectively and there was no significant difference between the two resistant lines (0.728). Akt2 showed a smaller increase in phosphorylation (10.4%; TAMR and 17.8%; TAMRBU) and neither of these increases reach significance on t-test analysis (Table 4-2). Akt3 demonstrated a similar increase in phosphorylation (11.4% TAMR; 13.5% TAMRBU) but TAMRBU was significantly different to MCF7 by t-test analysis (0.008). Akt pan (total) showed a marked increase in phosphorylation, with TAMR increased by 50.8% and TAMRBU 56.4%. The ANOVA

suggests a high degree of variance (P= less than 0.001) and the t-tests performed between MCF7 and TAMR (P=0.001) and MCF7 and TAMRBU (P=0.014) showed that these increases were significant. The t-test between the resistant lines indicated that there was no difference between them, with respect to Akt phosphorylation (P=0.559).

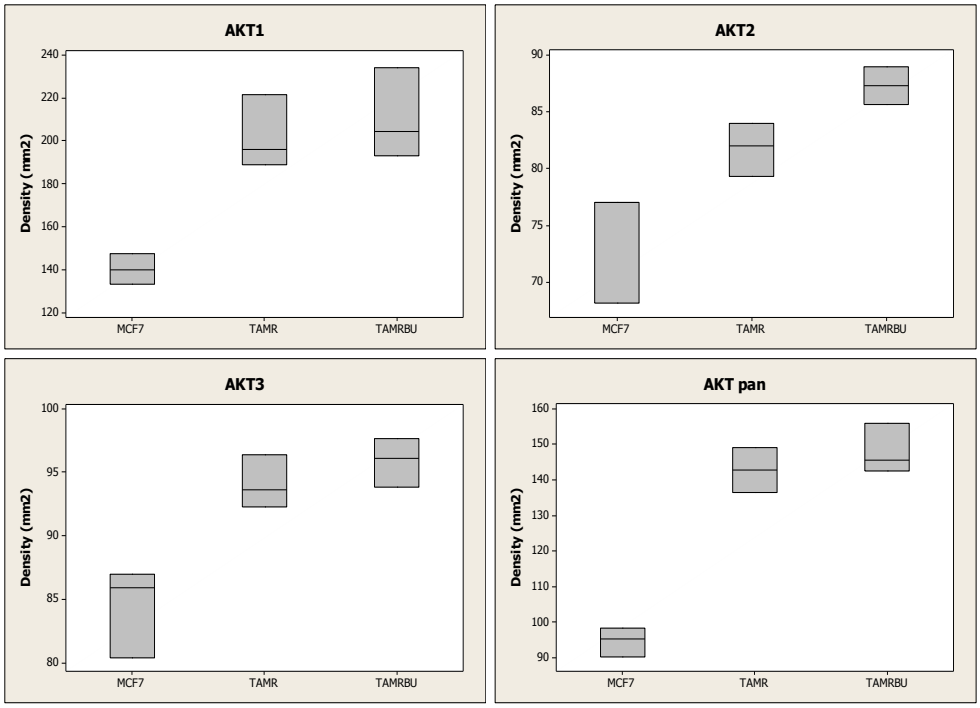


Figure 4-7 These Box plots for Akt 1, 2, 3 and total (pan) show the three cell lines MCF7, TAMR and TAMRBU and an increase in phosphorylation in both resistant cells compared to the parental. Akt1has a P value of 0.003, Akt2 0.009, Akt3 0.004 and Akt pan less than 0.001. N=3.

4.2.7 Densitometry analysis of MAPK proteome profiler: GSK3 α/β and GSK β

The protein phosphorylation of GSK3 α/β and GSK3 β in Tamoxifen resistant cells showed a small decrease when compared to the MCF7 cells. GSK3 α/β densitometry displayed a 3.3% decrease (TAMR) and a 7.9% decrease (TAMRBU). ANOVA analysis indicated that there was a significant variance within the data (P=0.037) but individual t-

tests did not produce significant values. GSK3 β showed a 9.8% decrease (TAMR) and a 7.4% decrease (TAMRBU). ANOVA did not suggest that there was a significant variance in these data (P=0.402) the individual t-tests did not give significant results. See Table 4-2 for values.

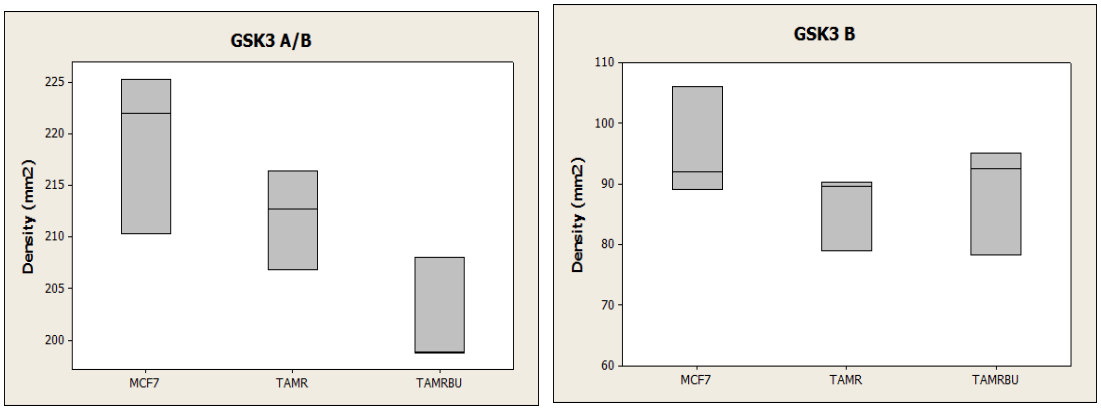


Figure 4-8 Box plots of GSK3 α/β and GSK3 β phosphorylation between MCF7, TAMR and TAMRBU cells. ANOVA analysis indicates that GSK3 α/β is significant (P=0.037) and GSK3 β is not (P=0.402). N=3.

4.2.8 Densitometry analysis of the MAPK proteome profiler: P70S6K and HSP27.

The proteome profiler array data showed increased phosphorylation of P70S6K in both of the resistant cell lines (TAMR; 14.7%, TAMRBU; 39.2%) compared to MCF7. The ANOVA P value for P70S6K of less than 0.001 indicated that there was a very significant amount of variance between the groups. The individual t-test between MCF7 and TAMR gave a P value of 0.055 and between MCF7 and TAMRBU the P value was 0.006. The t-test between the resistant cell lines implied that the difference clearly seen in Figure 4-9 between TAMR and TAMRBU was significant (P=0.032).

HSP27, as shown in Figure 4-9, had an increased level of phosphorylation in the Tamoxifen resistant cells (TAMR; 4.8%, TAMRBU; 24.5%). This increase was not statistically significant when comparing TAMR and MCF7 cells, however, it was very significant in the MCF7 and TAMRBU t-test analysis (P=0.006). There was also a difference seen between the resistant lines (P=0.032). The statistically significant differences seen between resistant lines for both HSP27 and P70S6K was partly due to the variance seen in the TAMR cell line across the three replicates, but could also imply a degree of clonal variance between the resistant cell lines or perhaps result from the lower passage number of the TAMRBU cells, discussed further in section 4.5.1.

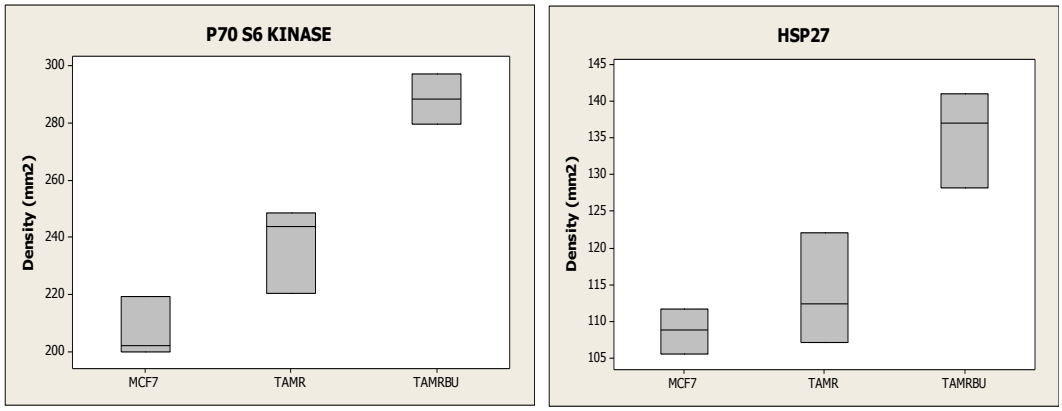


Figure 4-9 The Box plots above show the phosphorylation of P70 S6 Kinase and Heat shock protein 27. The proteins both show an increase in the phosphorylation in the resistant cell lines. The ANOVA P value for P70S6K was less than 0.001 and for HSP27, 0.004. N=3.

Table 4-2 A summary of the MAPK antibody array results.

Protein	Significance M:T	Significance M:B	Significance T:B	ANOVA M:T:B	Normalised Fold change %			Proteins of interest
					M	T	B	
ERK1	0.000	0.013	0.457	0.000	100	155.1	161.6	*
ERK2	0.007	0.008	0.171	0.000	100	176.5	154.6	*
JNK1	0.001	0.076	0.111	0.003	100	111.9	106.0	
JNK2	0.371	0.022	0.911	0.273	100	105.1	105.8	
JNK3	0.173	0.211	0.597	0.267	100	114.3	115.6	
JNK pan	0.447	0.230	0.092	0.260	100	102.1	104.5	
p38α	0.011	0.017	0.053	0.001	100	125.4	139.0	*
p38β	0.132	0.006	0.885	0.026	100	124.9	126.4	
p38δ	0.004	0.007	0.093	0.000	100	124.1	136.8	*
p38γ	0.002	0.024	0.777	0.000	100	138.6	140.7	*
RSK1	0.040	0.017	0.289	0.017	100	115.1	123.2	*
RSK2	0.715	0.126	0.380	0.229	100	97.3	107.9	
MSK2	0.052	0.150	0.443	0.056	100	120.3	127.0	
Akt1	0.026	0.044	0.728	0.003	100	143.9	149.9	*
Akt 2	0.053	0.074	0.113	0.009	100	110.4	117.8	
Akt 3	0.093	0.008	0.503	0.004	100	111.4	113.5	
Akt pan	0.001	0.014	0.559	0.000	100	150.8	156.4	*
GSK3α/β	0.392	0.066	0.222	0.037	100	96.7	92.1	
GSK3 β	0.402	0.564	0.277	0.402	100	90.2	92.6	
HSP27	0.332	0.006	0.032	0.004	100	104.8	124.5	
P70S6K	0.055	0.001	0.032	0.000	100	114.7	139.2	

M=MCF7, T=TAMR, B=TAMRBU. Significance indicates P value obtained using a student paired t-test analysis between the cell lines. ANOVA is the P value obtained from one-way unstacked ANOVA analysis of variance between all three cell lines. Fold change in phosphorylation is normalised to the MCF7 levels and displayed as a percentage. Proteins of interest are proteins that have reached significance in both resistant cell lines. Significance is reached when $p \leq 0.05$.

4.3 Human phospho- Receptor Tyrosine Kinase (RTK) proteome profiler

This array was used to detect changes in pan tyrosine phosphorylation of 42 receptor tyrosine kinases between the MCF7, TAMR and TAMRBU cellular lysates. Full details of the proteins and controls used on this array are included in Table 4-3. The RTK proteome profiler was assayed on three independent occasions on three separate cell populations. Significance of results and fold change seen in the RTK profiler are summarised in

Table 4-4.

4.3.1 Controls

The array included a positive and negative control as detailed in Table 4-3 and the average readings from the negative controls were subtracted from the densitometry values. The densitometry values for the three (duplicated) positive phospho-tyrosine antibody spots were averaged for each of the array replicates and ANOVA analysis suggested no statistical variance (P=0.555). The t-tests carried out between the cell lines did not reach significance, see Table 4-4 for values.

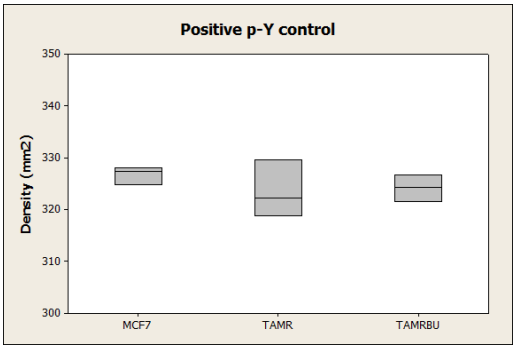


Figure 4-10 Box plot showing the densitometry values for the positive phospho tyrosine control spots. ANOVA analysis gives a P value of 0.555. N=3.

Table 4-3 A table showing the proteins, and their families spotted onto the Human Phospho-Receptor Tyrosine Kinase Proteome profiler array, alongside controls.

Proteins spotted on to the RTK Proteome profiler antibody array.	
+ Control	ROR2
EGFR	TIE1
ErbB2	TIE2
ErbB3	TrkA
ErbB4	TrkB
FGFR1	TrkC
FGFR2a	VEGFR1
FGFR3	VEGFR2
FGFR4	VEGFR3
Insulin R	MuSK
IGF-1R	EphA1
Axl	EphA2
Dtk	EphA3
Mer	EphA4
HGFR (met)	EphA5
MSPR	EphA6
PDGFRa	EphA7
PDGFRb	EphB1
SCFR	EphB2
Flt-3	EphB4
M-CSF-R	EphB6
c-RET	
ROR1	

4.3.2 Densitometry analysis of EGF receptor family members.

EGFR has long been reported to be elevated in TAMR cells. We therefore used EGFR as a "characterisation protein" in chapter 3 and again show the expression of this protein to be increased by array analysis. The phosphorylation of this receptor was increased by 80.9% (TAMR) and 88.2% (TAMRBU) using this RTK array. ANOVA analysis suggested a high degree of statistically significant variance (P= less than 0.001). The t-test values for EGFR, as listed in Table 4-4, showed a high degree of statistical significance between the MCF7 cell line and both TAMR and TAMRBU, but there was no significance between the two resistant lines (P=0.643).

ErbB2 also had a high degree of statistical significance using ANOVA (P= 0.001). The phosphorylation of ErbB2 was increased by 159.4% (TAMR) and 164.4% (TAMRBU) compared to MCF7. These were statistically significant changes as the t-test showed, TAMR; P=0.001, and TAMRBU; P=0.002. There were no differences between the resistant cell lines TAMR and TAMRBU, P=0.617.

ErbB3 (ANOVA, P=0.189) and ErbB4 (ANOVA, P=0.125) showed an increase in phosphorylation in the Tamoxifen resistant cell lines (see Table 4-4) but due to the variability seen in ErbB3 and ErbB4 TAMR samples, the increase was not significant. When a paired t-test was performed between the MCF7 and the TAMRBU cells, the outcome was significant with ErbB3 having a P value of 0.042 and ErbB4 (0.013). When a paired t-test was performed between the two resistant cell lines they showed no significant difference (ErbB3; P= 0.710, ErbB4; P=0.892).

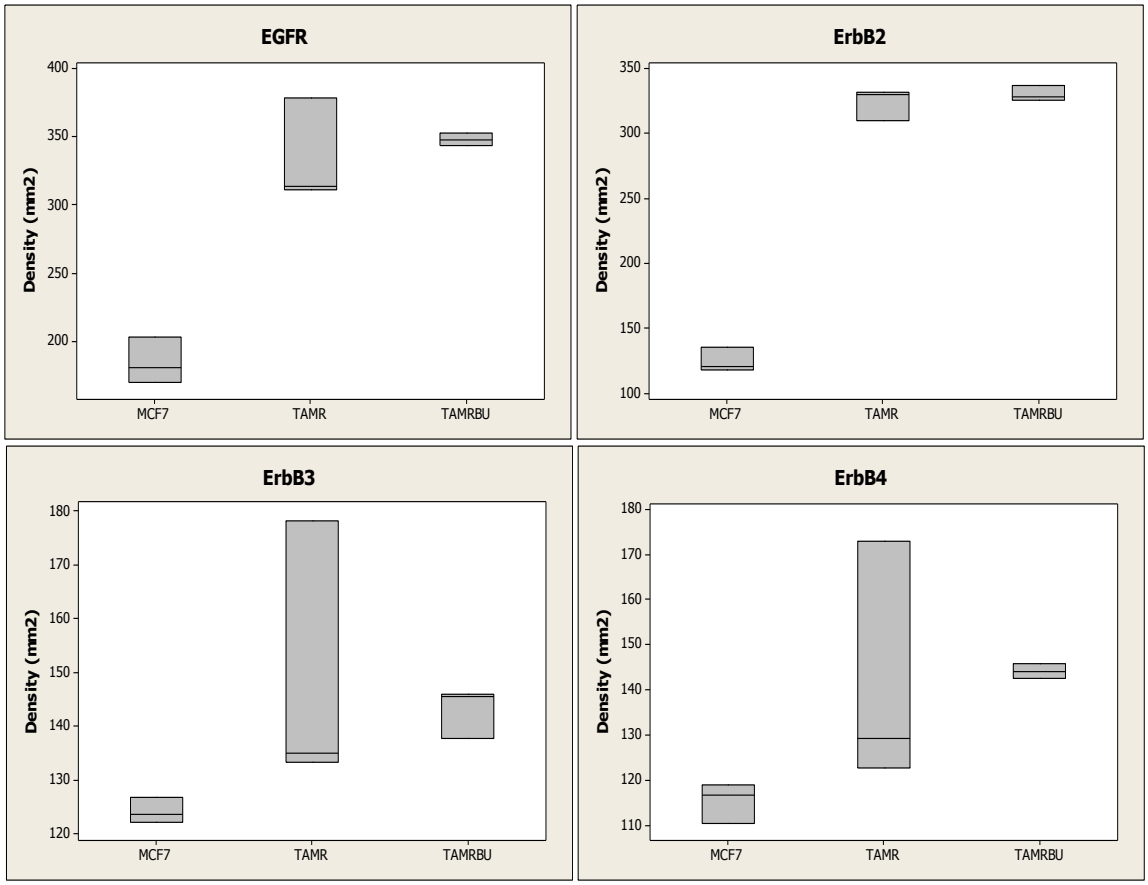


Figure 4-11 The phospho-RTK data for EGFR (p= less than 0.001), ErbB2 (p= 0.001), ErbB3 (p=0.189) and ErbB4 (p=0.125). All of these proteins show an increase in phosphorylation in the Tamoxifen resistant cell lines but due to the variability seen in ErbB3 & ErbB4 TAMR samples the increase was not significant.

4.3.3 Densitometry analysis of Eph Receptors.

The densitometry analysis for the EphB receptors 1, 2, 4 and 6 is shown in Figure 4-12. EphB2, 4 and 6 showed marginal increases in phosphorylation; however, as these changes were small and the data variable, there was a lack of statistical significance by ANOVA. Nevertheless, a t-test showed that the difference between the phosphorylation of MCF7 and TAMRBU for EphB4 (4.8% increase) was significant (P=0.026). Comparing the two resistant lines, no significantly difference in EphB2 and EphB4

phosphorylation was seen, but for EphB6 a significant result was seen (P=0.034). Moreover, EphB1 showed a more marked increase in phosphorylation (TAMR; 48.0%, TAMRBU, 31.1%), which illustrated statistical significance with a P value of 0.047 (ANOVA). Individual t-tests showed TAMR phosphorylation to be significantly higher than in MCF7 cells (P=0.045) and TAMRBU nearly reached significance (P=0.051).

The EphA receptor analysis is shown below in Figure 4-13. EphA1 showed a statistically significant increase in phosphorylation when compared to the MCF7 parental line (89.6%; TAMR, P=0.014 and 108%; TAMRBU, P=0.013). The full statistical data can be seen in Table 4-4. Similarly, EphA2 exhibited an increase in phosphorylation in both resistant lines although the magnitude of change was smaller (TAMR; 18.3%, TAMRBU; 20.1%). An unstacked ANOVA analysis suggested that there was a degree of variance in the samples (P=0.049) which was confirmed in t-test analysis, TAMR P= 0.016 and TAMRBU, P=0.085, both compared to MCF7 cells.

In marked contrast EphA3 showed little increase in phosphorylation and was not significant in any analysis. Both EphA4 and EphA5 showed increases in phosphorylation in the resistant cells (EphA4 - 40.3%;TAMR and 33.7% TAMRBU) but when a t-test was performed it did not prove to be statistically different, due to intra assay variation. Interestingly, EphA6 and EphA7 showed a decrease in phosphorylation in resistance (EphA6 -2.4%; TAMR and 10.6%; TAMRBU). However, this did not reach significance in EphA6 (P=0.172 for MCF7 compared to TAMR and 0.088 for MCF7 compared to TAMRBU). Whereas it did not for EphA7, comparing MCF7 and TAMRBU cells produced a P value of 0.050 (difference= -14.8%).

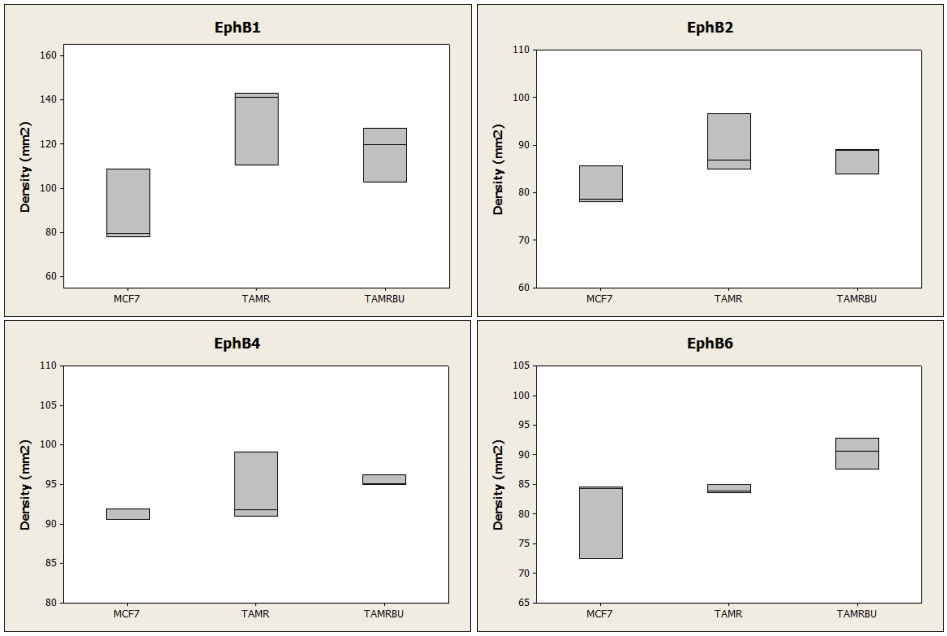


Figure 4-12 Densitometry analysis of the EphB receptors, these box plots show a marginal increase in most of the EphB receptors with EphB1 showing the most increase in phosphorylation, which is statistically significant with a P value of 0.047. However the others do not show significance by ANOVA; EphB2 is 0.134, EphB4 0.193 and EphB6 0.770. N=3.

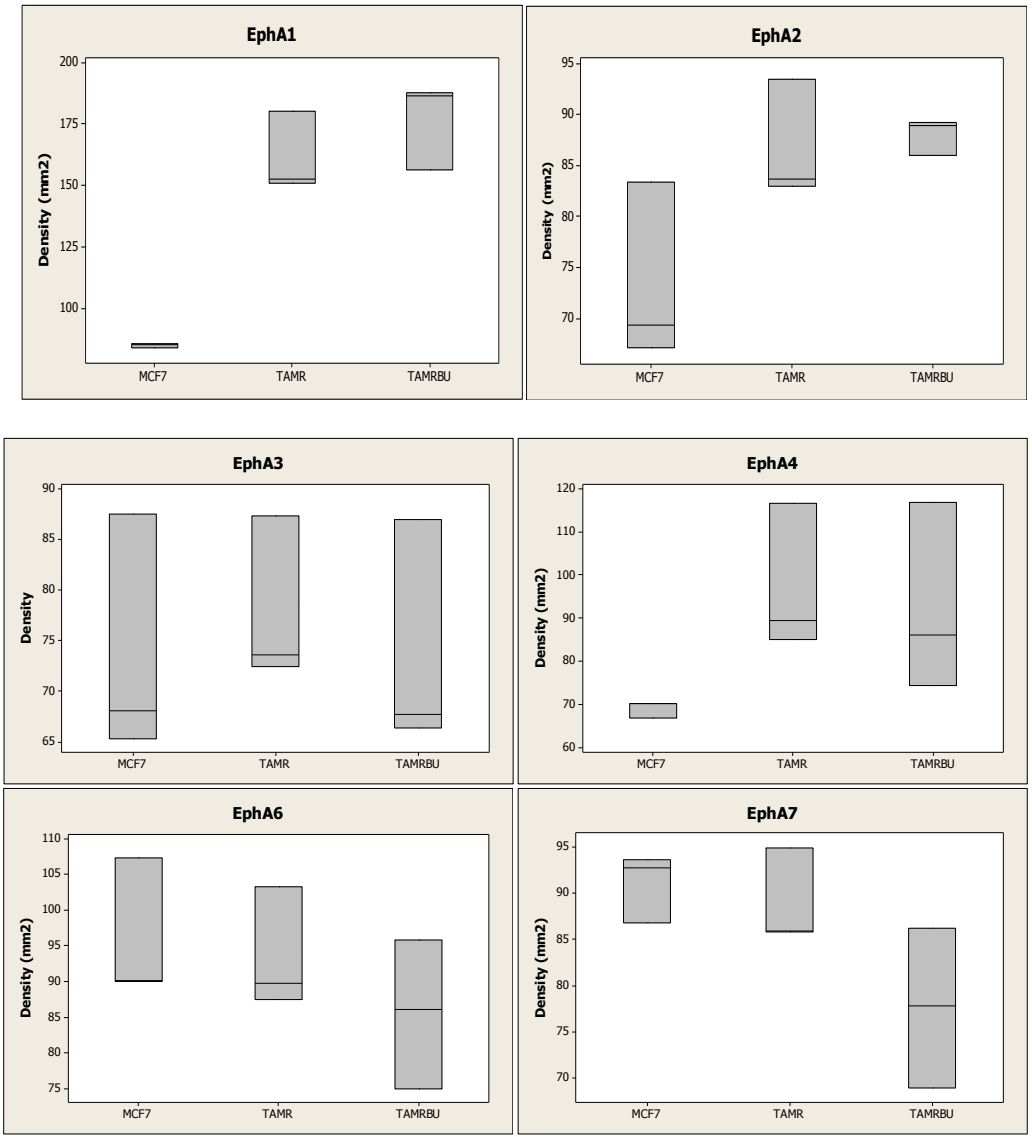


Figure 4-13 Box plots showing densitometry analysis of EphA receptors. Only EphA1 and EphA2 show any significantly significant differences between resistance and MCF7 with P values of less than 0.001 and 0.049 respectively. N=3.

4.3.4 Densitometry analysis of Vascular Endothelial Growth Factor Receptors 1, 2, and 3.

The VEGF receptors 1, 2 and 3 all showed a trend of increased phosphorylation in the resistant cell lines (VEGFR1; 9.5% and 8.4%, VEGFR2; 59.6% and 77.4%, VEGFR3; 35.8% and 24.6% - for TAMR and TAMRBU compared to MCF7 in each case). Of these increases in phosphorylation, VEGFR2 was statistically significant by t-test (TAMR, 0.020; TAMRBU 0.010) as was VEGFR3, with P values of 0.028 (TAMR) and 0.048 (TAMRBU). The resistant lines were not statistically different from each other (VEFGR2; 0.348 and VEGFR3; 0.127).

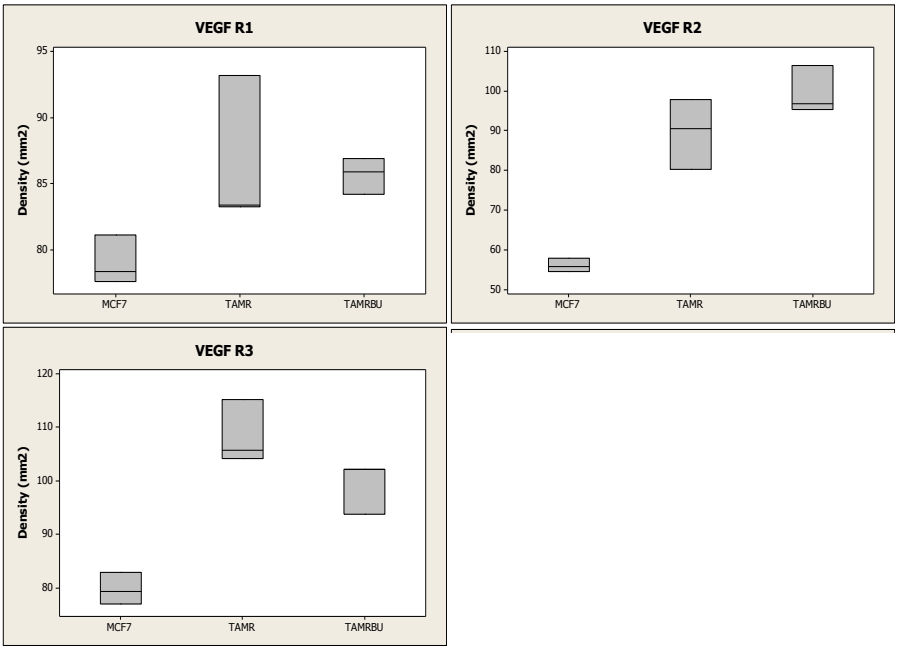


Figure 4-14 The VEGF receptor family of RTKs. Box plots showing densitometry analysis of VEGF receptors family (1, 2 and 3) producing P values of 0.077, less than 0.001 and 0.001 respectively using ANOVA. N=3.

4.3.5 Densitometry analysis of Fibroblast Growth Factor receptors (FGFRs) 1, 2α, 3 and 4.

FGFR1 and FGFR4 showed no significant difference between any of the cell lines (see Table 4-4). FGFR3 exhibited a statistically significant increase in phosphorylation between the MCF7 and TAMRBU lines with a 35.4% increase (P=0.042). FGFR2a presented a statistical difference using ANOVA analysis of variance (P= less than 0.001) which was also conveyed when analysed using t-tests; MCF7 and TAMR (less than 0.001), MCF7 and TAMRBU (0.002) and TAMR with TAMRBU (0.008).

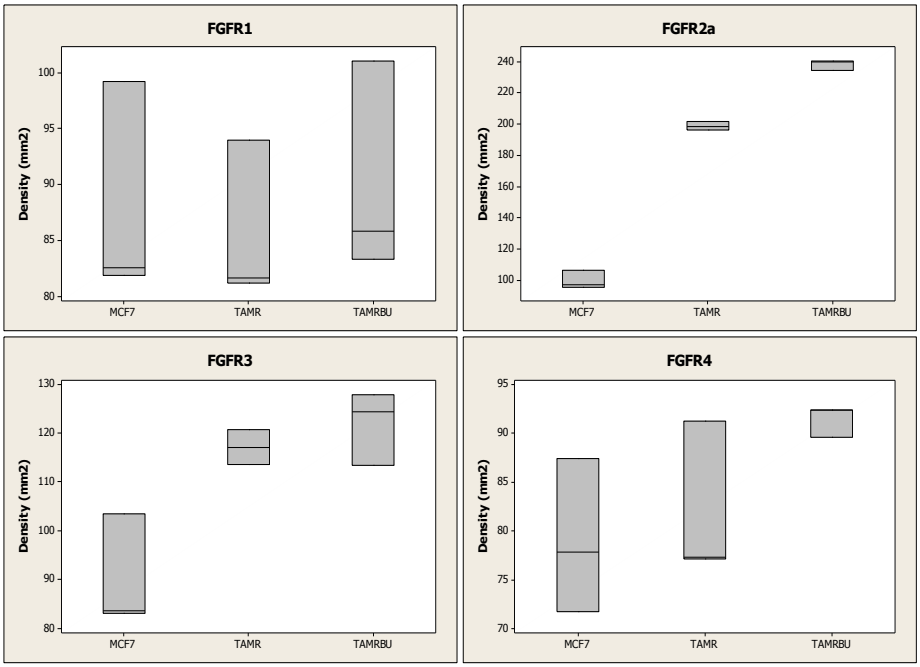


Figure 4-15 FGF receptor family densitometry data. These box plots show the FGF receptor family (1, 2α, 3 and 4), P values from ANOVA are as follows; 0.835, less than 0.001, 0.007, and 0.130, respectively. (N=3).

4.3.6 Densitometry analysis of Platelet Derived Growth Factor receptors (alpha and beta).

The densitometry values in the MCF7 sample for PDGFRα showed considerable variance, and the phosphorylation was relatively similar between cell lines with no statistical variation, see Table 4-4. The PDGFRβ however, showed a much more marked change in phosphorylation between MCF7 and resistant lines (TAMR; 17.4% and TAMRBU; 36.9%) though only the difference between MCF7 and TAMR reaches significance (P=0.017), due to the TAMRBU assay variance.

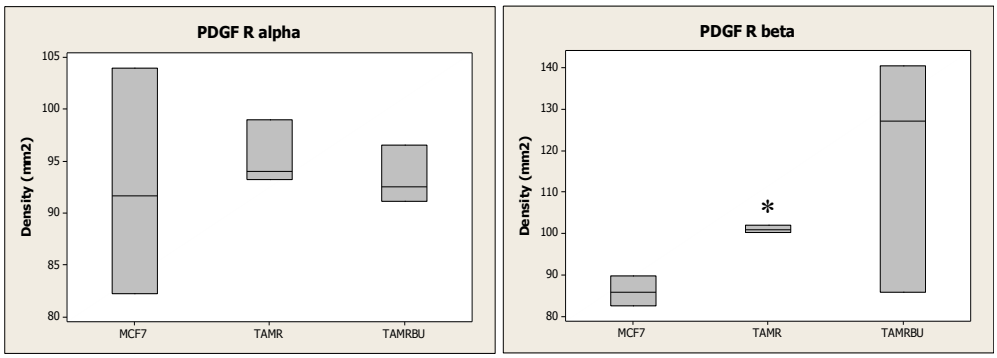


Figure 4-16 Densitometry analysis of the PDGFR alpha and beta. The ANOVA analysis of variance does not show significance (P=0.875 and P=0.142 respectively) However the increase in phosphorylation between TAMR and MCF7 is significant (P=0.012) and denoted by *. N=3.

4.3.7 The densitometry analysis of TIE1 and TIE2 receptors.

The TIE1 receptor showed an increase in phosphorylation, which was significant in TAMR cells, (see Table 4-4 for values). TIE2 receptor showed a marked increase of tyrosine phosphorylation (49.9% and 87.0%) in both the TAMR and TAMRBU cell lines.

These increases were statistically significant by t-test giving P values of 0.032 and less than 0.001.

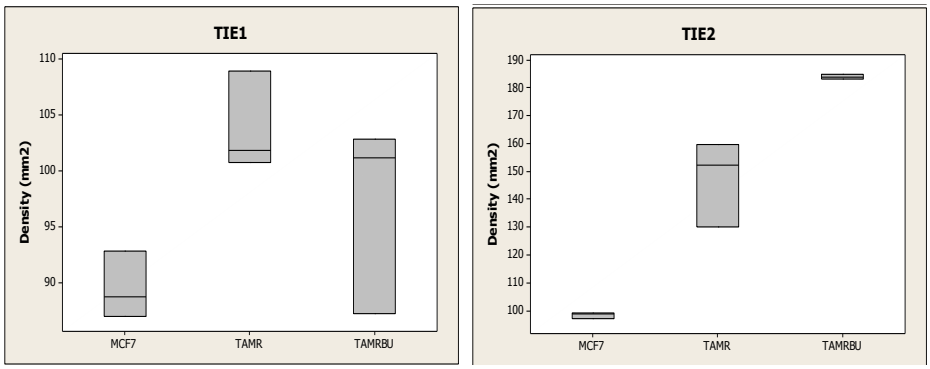


Figure 4-17 The TIE receptor phosphorylation data. Box plots showing the difference in phosphorylation of the TIE receptor family. ANOVA values indicate a degree of significance P=0.064 and P ≤0.001 respectively. N=3.

4.3.8 Densitometry analysis of ROR1 and ROR2 receptors.

ROR receptor 1 showed statistically increased phosphorylation in the TAMR and TAMRBU cell lines compared to the MCF7 parental cell lines with the TAMR cells showing the greatest increase (23.4%), (Seen in Figure 4-18 and Table 4-4). In contrast, ROR2 receptor only reached significance when TAMR and MCF7 were compared by t-test analysis (P=0.008).

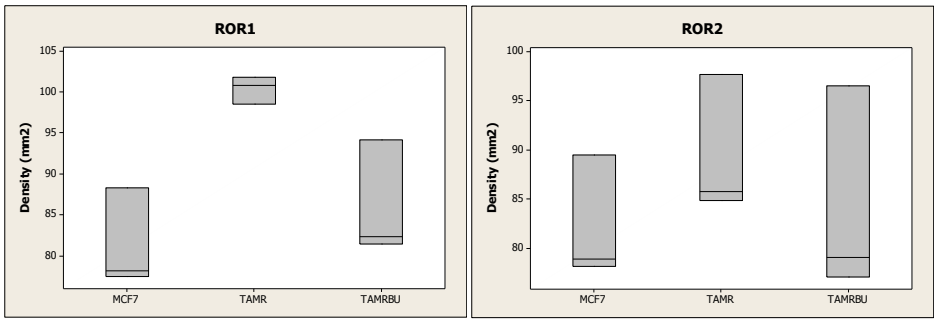


Figure 4-18. Densitometry data for ROR1, ROR2; the TAMRs show a greater increase in phosphorylation than TAMRBU. ANOVA analysis provides P values of 0.013 for ROR1 and 0.574 for ROR2. N=3.

4.3.9 The densitometry analysis of TrkA, B and C receptors.

TrkA receptor phosphorylation exhibited no statistical difference between any of the cell lines. Interestingly, TrkB showed a statistical difference with a 9.3% decrease in phosphorylation between TAMRBU and MCF7, with a P value of 0.030. This is in contrast to TrkC which showed a 12.4% increase in phosphorylation of the TAMRBU cells when compared to MCF7, which is also statistical when analysed by t-test P=0.035.

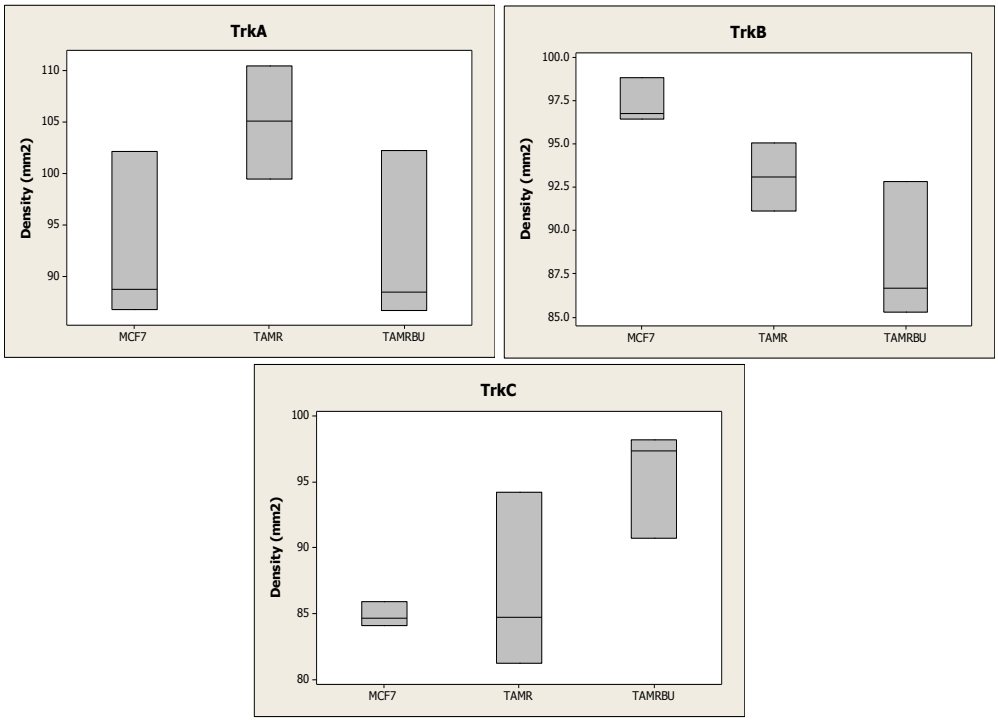


Figure 4-19 Densitometry analysis of TrkA, B and C. The box plots show the varying degree of phosphorylation between the parental and resistant lines giving ANOVA analysis P values of 0.145, 0.017 and 0.062. N=3.

4.3.10 Densitometry analysis of M-CSF receptor (c-fms) phosphorylation

The phosphorylation of this proto-oncogene related protein was increased by 12.7% and 14.2% in TAMR and TAMRBU respectively, compared with phosphorylation of the receptor in MCF7 cells. These increases were statistically significant (P=0.014, TAMR and P=0.003 TAMRBU). There was no statistical difference between resistant lines (P=0.573).

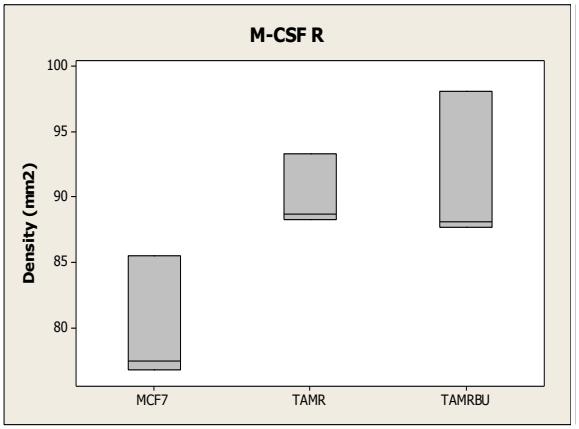


Figure 4-20 Densitometry analysis of M-CSF receptor tyrosine phosphorylation between MCF7 and Tamoxifen resistant cells. ANOVA analysis performed gave a P value of 0.046. N=3.

4.3.11 Densitometry analysis of Hepatocyte Growth Factor Receptor

HGF receptor phosphorylation was increased by 28.3% in TAMR and 29.9% in TAMRBU. Both of the increases seen were statistically significant (0.008 and 0.017 respective P values by t-test).

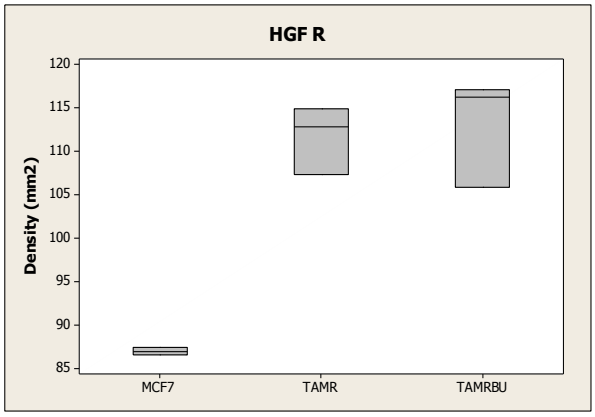


Figure 4-21. The densitometry analysis of HGFR and the differences in tyrosine phosphorylation seen between the MCF7 and resistant lines. ANOVA analysis ($P \leq 0.001$). N=3.

4.3.12 Densitometry analysis of TAM family of receptors: Tyro3 (Dtk), AxI and Mer

AxI receptor phosphorylation did not change significantly between cell lines. However Dtk and Mer showed marked increases in phosphorylation in both TAMR and TAMRBU. Dtk showed 91.1% increase in the TAMR and a 99.8% increase in the TAMRBU cells. These data were significant statistically; t-test showed P values of 0.026 and 0.003 respectively. The increase in Mer was not as large at 62.5% TAMR and 56.2% TAMRBU but still a marked increase. These were also statistically significant using t-test analysis with P values of 0.026 and 0.002 for TAMR and TAMRBU. The resistant lines did not significantly differ from each other.

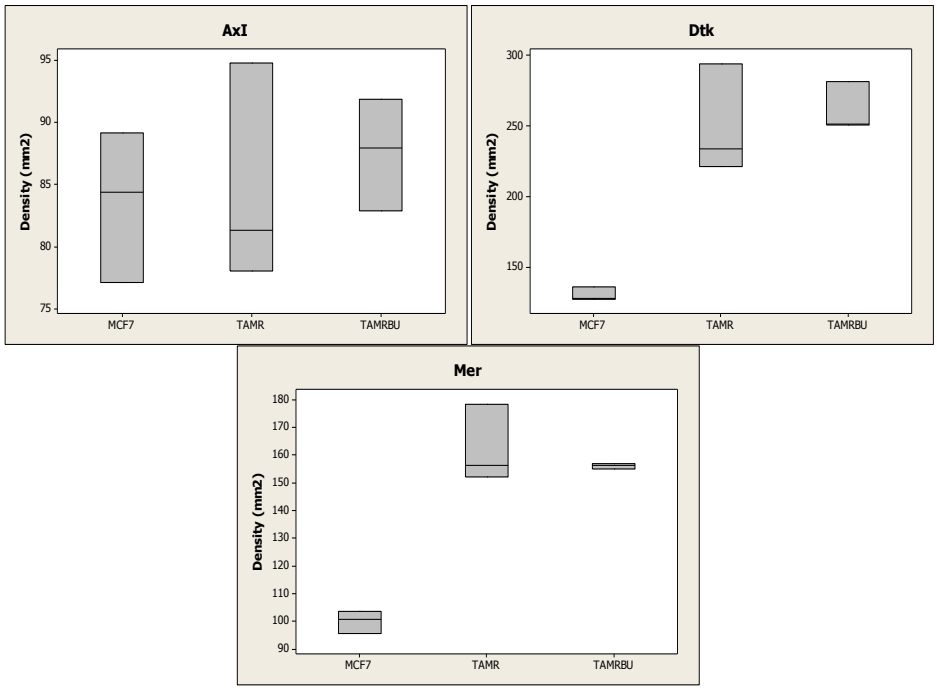


Figure 4-22 Densitometry analysis of the TAM family of receptors, showing a marked increase in phosphorylation in the resistant lines for Dtk and Mer receptors giving ANOVA P values of 0.001 and less than 0.001 respectively. N=3.

Table 4-4 A summary table of results from the RTK array.

Protein	Significance M:T	Significance M:B	Significance T:B	ANOVA M:T:B	Normalised Fold change %		
					M	T	B
+ Control	0.521	0.112	0.893	0.555	/	/	/
EGFR *	0.028	0.002	0.634	0.000	100	180.9	188.2
ErbB2 *	0.001	0.002	0.617	0.000	100	259.4	264.4
ErbB3	0.256	0.042	0.710	0.189	100	119.9	115.3
ErbB4	0.221	0.013	0.892	0.125	100	122.8	125.0
FGFR1	0.271	0.056	0.105	0.835	100	97.4	102.5
FGFR2α *	0.000	0.002	0.008	0.000	100	199.3	238.8
FGFR3	0.062	0.042	0.533	0.007	100	130.7	135.4
FGFR4	0.259	0.084	0.156	0.130	100	103.7	115.7
Insulin R	0.078	0.232	0.648	0.655	100	106.9	110.0
IGF-1R	0.399	0.009	0.425	0.126	100	109.5	117.4
Axl	0.689	0.049	0.438	0.765	100	101.4	104.8
Dtk *	0.026	0.003	0.461	0.001	100	191.1	199.8
Mer *	0.026	0.002	0.513	0.000	100	162.5	156.2
HGFR *	0.008	0.017	0.793	0.000	100	128.3	129.9
MSPR	0.076	0.010	0.222	0.030	100	188.0	136.7
PDGFRα	0.609	0.922	0.576	0.875	100	103.0	100.8
PDGFRβ	0.017	0.160	0.408	0.142	100	117.4	136.9
SCFR	0.189	0.111	0.403	0.070	100	112.7	108.8
Flt-3	0.146	0.063	0.981	0.077	100	113.6	113.8
M-CSF-R *	0.014	0.003	0.573	0.046	100	112.7	114.2
c-RET *	0.011	0.143	0.636	0.036	100	123.1	119.8
ROR1 *	0.030	0.030	0.071	0.013	100	123.4	105.7
ROR2	0.008	0.509	0.142	0.574	100	108.8	102.4
TIE1	0.004	0.178	0.213	0.064	100	116.0	108.5
TIE2 *	0.032	0.000	0.052	0.000	100	149.9	187.0
TrkA	0.175	0.942	0.151	0.145	100	113.5	99.9
TrkB	0.098	0.030	0.180	0.017	100	95.6	90.7
TrkC	0.648	0.035	0.173	0.062	100	102.1	112.4
VEGFR1	0.205	0.041	0.762	0.077	100	109.5	108.4
VEGFR2 *	0.020	0.010	0.348	0.000	100	159.6	177.4
VEGFR3 *	0.028	0.048	0.127	0.001	100	135.8	124.6
MuSK	0.050	0.163	1.000	0.050	100	143.5	143.5
EphA1 *	0.014	0.013	0.507	0.000	100	189.6	208.2
EphA2	0.016	0.085	0.702	0.049	100	118.3	120.1
EphA3	0.229	0.914	0.178	0.865	100	105.7	100.1
EphA4	0.126	0.231	0.476	0.154	100	140.3	133.7
EphA5	0.280	0.664	0.154	0.286	100	122.3	110.3
EphA6	0.172	0.088	0.179	0.451	100	97.6	89.4
EphA7	0.467	0.050	0.060	0.078	100	97.6	85.2
EphB1 *	0.045	0.051	0.061	0.047	100	148.0	131.1
EphB2	0.231	0.250	0.589	0.134	100	110.7	108.1

EphB4	0.410	0.026	0.638	0.193	100	103.2	104.8
EphB6	0.421	0.067	0.034	0.077	100	104.6	112.2

M=MCF7, T=TAMR, B=TAMRBU. Significance indicates P value obtained using a student paired t-test analysis between the cell lines. ANOVA is the P value obtained from one-way unstacked ANOVA analysis of variance between all three cell lines. Fold change in phosphorylation is normalised to the MCF7 levels and displayed as a percentage. * denotes significant proteins of interest, significance reached when $p \leq 0.05$.

4.4 Human Apoptosis proteome profiler array

The human apoptosis proteome profiler analysed the expression of thirty five apoptosis related proteins, full details of which can be seen in Table 4-5, so that they could be compared between the cell lines. The apoptosis proteome profiler was assayed on 2 independent occasions on TAMRBU and MCF7 cell populations, the TAMR and TAMRBU had followed similar trends on the previous antibody arrays and so due to financial constrainsts it was deemed reasonable that only the TAMRBU and MCF7 cells be analysed using the apoptosis array. The statistically significant results are displayed below graphically, all other results are summarised in Table 4-6.

4.4.1 Controls

The array included positive and negative controls as detailed in the table below.

The average reading of the negative controls was subtracted from all densitometry values. As the experiment had only been carried out at two independent occasions the conclusions drawn cannot be relied on as truly significant. The statistics were carried out using the individual spots as independent values rather than averaging them as was done in the MAPK and RTK profilers, although not strictly correct this gave an indication of

which protein expression differs in resistance. “Significance” of results and fold change can be seen in the apoptosis array summary Table 4-6

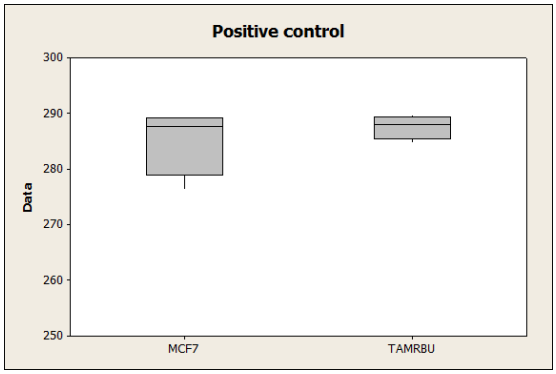


Figure 4-23 the box plot showing the positive control values between experiments. ANOVA shows a P value of 0.485 and t-test 0.560.

Table 4-5 A table of the proteins that are targeted on the apoptosis array proteome profiler

Protein Targets of the Apoptosis Proteome Profiler	
HSP70	HO-1/HMOX1/HSP32
HSP27	HSP60
HO-2/HMOX2	Bcl-2
HTRA2/Omi	Bcl-x
Bad	Bax
Livin	Pro-Caspase-3
PON2	Cleaved Caspase-3
p21/CIP1/CDNK1A	Catalase
p27/Kip1	cIAP-1
Phospho-p53 (S15)	cIAP-2
Phospho-p53 (S46)	Claspin
Phospho-p53 (S392)	Clusterin
Phospho-Rad17 (S635)	Cytochrome c
SMAC/Diablo	TRAIL R1/DR4
Survivin	TRAIL R2/DR5
TNF RI/TNFRSF1A	FADD
XIAP	Fas/TNFRSF6
HIF-1α	
Negative Control	Positive control

4.4.2 Densitometry analysis of some members of the Bcl family

The Bcl family has many members including Bcl-2 (B cell lymphoma -2), BAX (Bcl-2 like protein 4), BAD (Bcl-2 antagonist of cell death) and Bcl-X (Bcl-2 like protein 1) which were on the apoptosis proteome profiler. The Bcl-2 protein had decreased expression in the resistant cell lines (11.8%) this was a statistically significant decrease with P values from ANOVA and t-test giving 0.010 and 0.032 respectively. The BAD protein also showed a significantly decreased expression when compared to the MCF7 cells (20.1%) with P values of 0.014 and 0.041 from ANOVA and t-test analysis. BAX also showed a statistically significant decrease (21.7%). In keeping with the increased growth of the resistant cell lines ANOVA gave a P value of 0.004 and t-test gave 0.016. Bcl-X, however, showed a statistically significant increase (18.9%), P values were 0.004 (ANOVA) and 0.008 (t-test).

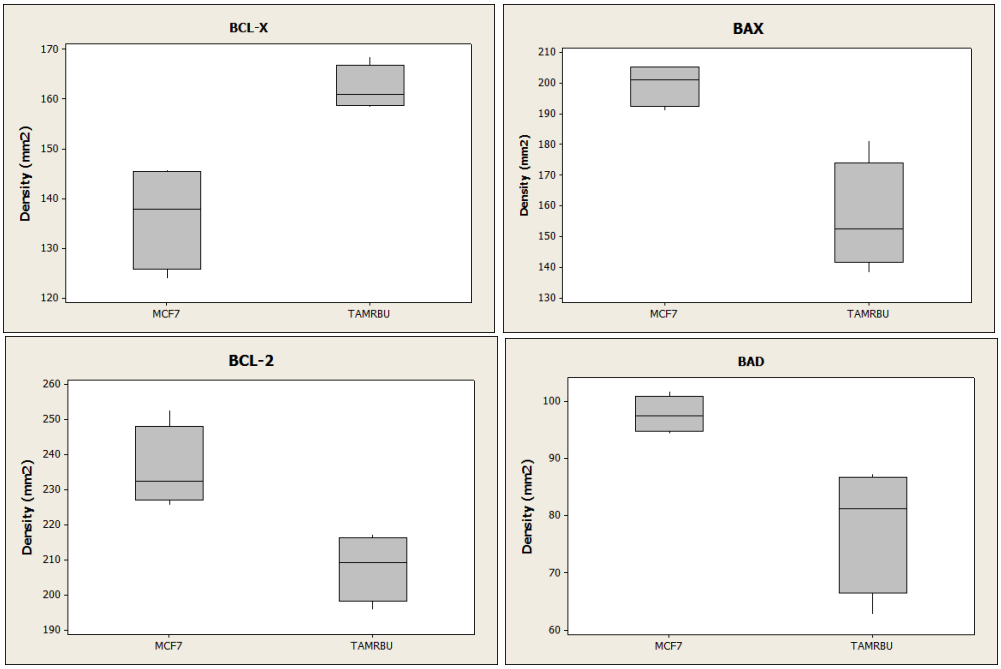


Figure 4-24 Box plots showing the general decrease in expression of the Bcl family of proteins in Tamoxifen resistant cell line, TAMRBU. The Bcl-X protein however, shows an increase. The differences are all statistically significant using ANOVA.

4.4.3 Densitometry analysis of catalase

The protein catalase shows a marginal, yet statistically significant, increase in Tamoxifen resistant cell lines (3.1%). The P value from ANOVA is 0.022 and from t-test 0.011. This is a small increase, which though interesting, may be artefactual due to the lack of replicate experiments n=2.

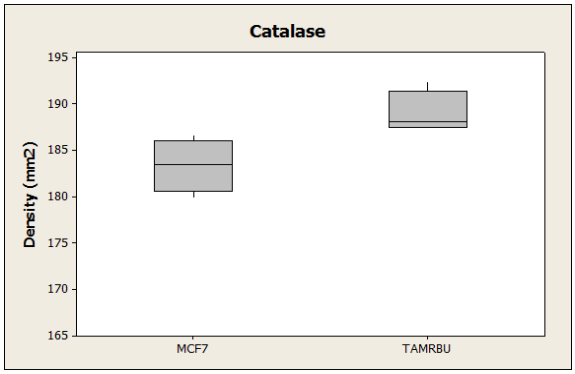


Figure 4-25 Box plot showing the statistically significant increased expression of catalase (P=0.011, t-test; P=0.022, ANOVA)

4.4.4 Densitometry analysis of the inhibitors of apoptosis proteins

c-IAP1 (cellular-Inhibitor of Apoptosis Protein 1), c-IAP2 (cellular-Inhibitor of Apoptosis Protein 2) and X-IAP (X linked -Inhibitor of Apoptosis Protein) all showed a statistically significant increases in the Tamoxifen resistant cell line TAMRBU when compared with MCF7 cells. The c-IAP1 shows the most marked increase at 48.3% (P less than 0.001 ANOVA; P=0.001 t-test), X-IAP was increased by 20.2% (P less than 0.001 ANOVA, P=0.007 t-test) and c-IAP2 by 17.4% (P=0.008 ANOVA, P=0.008 t-test).

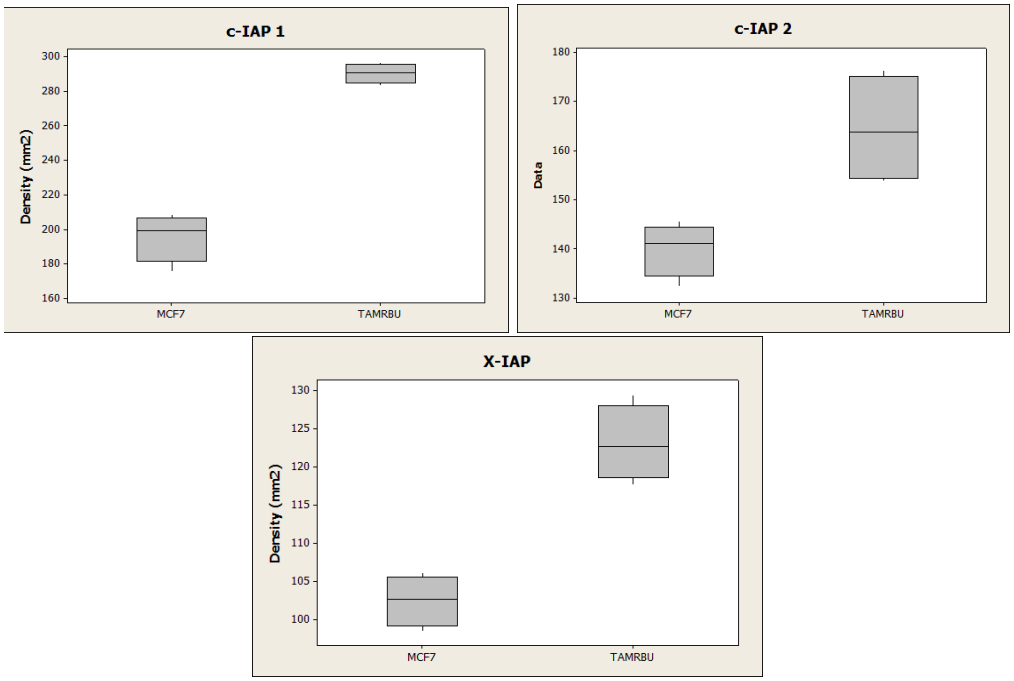


Figure 4-26 Densitometry analysis box plots, showing a statistically significant increase in IAP expression in resistance (Table 4-6).

4.4.5 Densitometry analysis of HIF1 α

HIF1α (Hypoxia inducible factor 1 alpha) shows a statistically significant increase in Tamoxifen resistant cells (18.5%) when compared with the sensitive parental cells MCF7. ANOVA analysis shows high variance (P value = less than 0.001) and t-test shows P=0.003. This result is interesting as the cells were not under hypoxic conditions, the flasks of cells (before harvesting lysates) were grown in the same incubator and the cells were at a similar cell density.

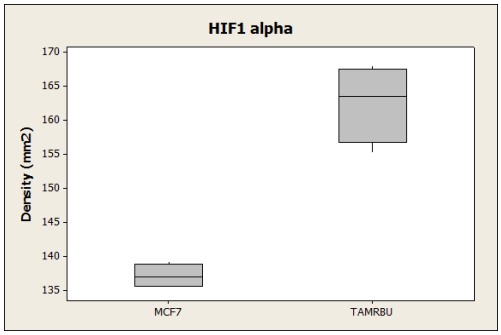


Figure 4-27 Densitometry analysis of HIF1α showing a statistically significant increase expression in resistance.

4.4.6 Densitometry analysis of Heat shock protein 70 (HSP70)

Heat Shock Protein 70 was one of three Heat Shock Proteins (HSP) that were spotted on the apoptosis array; however, HSP70 was the only one that showed a significant change in expression, (P=0.003 ANOVA, P=0.011t-test) with an increase of 16.5% in TAMRBU compared to the MCF7 cells.

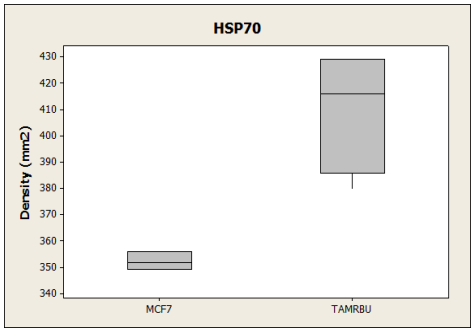


Figure 4-28 The densitometry analysis of the Heat shock protein 70 expression showing high levels in both cell lines but significantly increased in TAMRBU.

4.4.7 Densitometry analysis of Paroxanase 2 expression

PON2 (Paroxanase 2) showed a statistically significant increase of 15.9% in resistant cells (TAMRBU) when compared with the MCF7. ANOVA (P=0.006) and t-test (P=0.001).

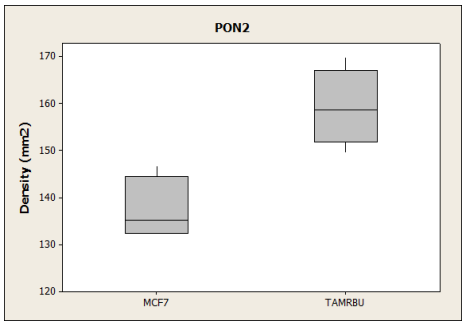


Figure 4-29. Densitometry analysis of the PON2 antioxidant protein showing a significant increase in expression in resistance.

4.4.8 Densitometry analysis of RAD17 phosphorylated at Serine 635

The cell cycle checkpoint protein RAD17 illustrated an increase in phosphorylation (34.5%) in resistance when compared with the MCF7 cells. This was significant with both ANOVA (0.032) and t-test (0.009).

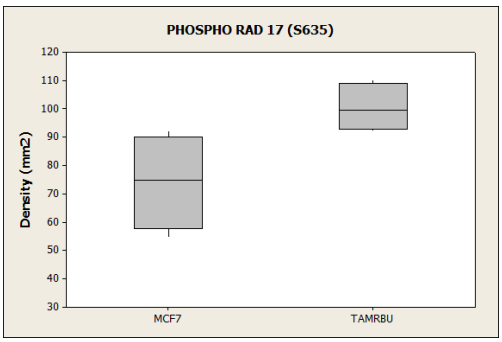


Figure 4-30 Densitometry analysis box plots showing a statistically significant increase in phospho RAD17 in acquired resistance.

4.4.9 Densitometry analysis of SMAC/DIABLO protein

SMAC/DIABLO protein showed a decrease in expression in resistant cells in comparison with MCF7 cells (31.9%). This was a marked decrease that was statistically significant ANOVA showing P value of less than 0.001 and t-test 0.001. The protein is involved with the BCL2 family of proteins and also the inhibitors of apoptosis. This result is discussed in more detail below (Discussion; section 4.5).

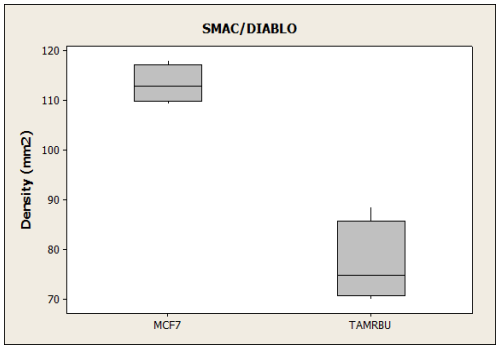


Figure 4-31 Box plots showing the densitometry analysis of SMAC DIABLO protein. The resistant line is significantly decreased when compared with the MCF7 parental line.

4.4.10 Densitometry analysis of Survivin protein expression

Survivin, exhibited an increase of 20.5% which was statistically significant with both ANOVA (P=0.004) and t-test (P=0.046).

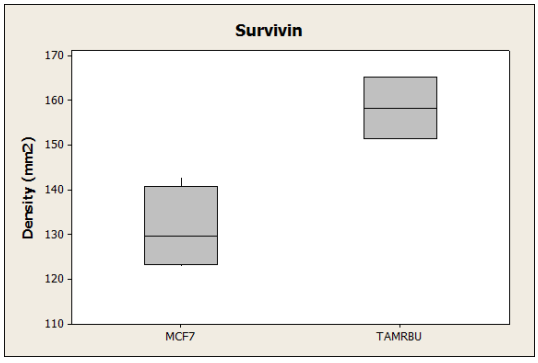


Figure 4-32 the box plot showing the increased expression of survivin expression in resistance, this is statistically significant in both ANOVA and in t-test.

Table 4-6 A summary table of results from the apoptosis proteome profiler array

Protein	Significance	ANOVA	Normalised Fold change %	
	M:B	M:B	M	B
Positive control	0.560	0.485	/	/
HIF-1α *	0.003	0.000	100	118.5
HO1/HMOX1/HSP32	0.395	0.640	100	102.8
HSP60	0.030	0.478	100	104.8
Bcl-2	0.032	0.010	100	88.2
Bcl-x *	0.008	0.004	100	118.9
Bax *	0.016	0.004	100	78.3
Pro-Caspase-3	0.151	0.189	100	96.1
Cleaved Caspase-3	0.103	0.458	100	98.1
Catalase	0.011	0.022	100	103.1
cIAP-1 *	0.001	0.000	100	148.3
cIAP-2 *	0.008	0.008	100	117.4
XIAP *	0.007	0.000	100	120.2
Claspin	0.655	0.573	100	102.4
Clusterin	0.154	0.042	100	102.4
Cytochrome c	0.844	0.817	100	100.9
TRAIL R1/DR4	0.096	0.147	100	91.9
TRAIL R2/DR5	0.091	0.135	100	93.7
FADD	0.140	0.081	100	93.4
Fas/TNFRSF6	0.590	0.605	100	98.5
HSP70 *	0.011	0.003	100	116.5
HSP27	0.180	0.069	100	111.4
HO-2/HMOX2	0.289	0.777	100	103.2
HTRA2/Omi	0.353	0.500	100	97.4
Bad *	0.041	0.014	100	79.9
Livin	0.416	0.383	100	105.3
PON2 *	0.001	0.006	100	115.9
p21/CIP1/CDNK1A	0.557	0.604	100	102.2
p27/Kip1	0.795	0.712	100	101.7
Phospho-p53 (S15)	0.245	0.604	100	98.3
Phospho-p53 (S46)	0.421	0.344	100	105.1
Phospho-p53 (S392)	0.202	0.309	100	104.9
Phospho-Rad17 (S635) *	0.009	0.032	100	135.4
SMAC/Diablo *	0.001	0.000	100	68.1
Survivin *	0.046	0.004	100	120.5
TNF RI/TNFRSF1A	0.690	0.622	100	100.8

M=MCF7, B=TAMRBU. Significance indicates P value obtained using a student paired t-test analysis between the cell lines. ANOVA is the P value obtained from one-way unstacked ANOVA analysis of variance between both cell lines. Fold change in phosphorylation is normalised to the MCF7 levels and displayed as a percentage. Proteins of interest are highlighted *. Significance reached when $p \leq 0.05$.

4.5 Discussion

Several mechanisms, proteins and pathways have been implicated in the development of Tamoxifen resistance including EGFR, ERK1/2, and FGFR. The antibody arrays used in this chapter were specifically chosen to include some of the proteins previously shown (by us and others) to have an involvement in acquired resistance to Tamoxifen therapy. This is useful as they were used as positive controls for the array experiments. Utilisation of Antibody arrays in this project was a useful tool as they offered an opportunity to screen a whole pathway simultaneously whilst providing a targeted approach to proteomic evaluation of the mechanisms of acquiring resistance.

4.5.1 MAPK

MAPKs are involved in important cellular pathways mediating cell proliferation, and cell survival pathways. Expression and activation of these pathways have been shown to play an important role in the development and progression of cancer (Keshet and Seger., 2010) and other findings show that it also has a role to play in Tamoxifen resistance (Hutcheson et al., 2003). Recent data in the literature suggests that the proteins in the complex MAPK signalling pathways can modulate oestrogen receptor activity (Yamnik and Holz, 2010). Using this MAPK focussed array has enabled us to see that several members of the MAPK family have modulated activity in the Tamoxifen resistant cell lines, see Table 4-2.

We previously showed increased phosphorylation of ERK1 and ERK2 in the TAMR and TAMRBU cell lines by Western blotting (Figure 3-7) and the proteome MAPK profiler mirrors this increase, this gives confidence in the array data as the ERKs act as an internal validation of the data.

The p38 Mitogen-activated Protein Kinases (MAPKs) are a family of four related Serine /Threonine kinases which can be activated by pro-inflammatory cytokines as well as environmental stresses. All four of the p38 family members, alpha, beta, gamma, and delta, are phosphorylated at specific threonine and tyrosine positions. Once activated, p38 phosphorylates a number of targets, inducing nuclear transcription factors. The whole family shows statistically significant increases (TAMR; 25.4%, 24.9%, 24.1% and 38.6%; TAMRBU; 39%, 26.4%, 36.8% and 40.7% for α, β, δ, γ respectively). Although these data show only small increases this still may affect the balance of signalling in the cell. Total p38 MAPK has also been shown in the literature to have a role in resistance (within an autocrine loop with VEGF/VEGFR2 and p38 (Aesoy et al., 2008)) and Linderholm et al., 2011 have suggested the use of p38 as a potential biomarker of intrinsic Tamoxifen resistance. This study reported this finding for total p38 however the data in this Thesis suggest that there are some differences seen between the isoforms.

Akt 1, 2 and 3 are a family of serine/threonine protein kinases that are thought to have a role in multiple cellular processes; cell proliferation, inhibition of apoptosis, transcription and cell migration as well as tumourigenesis. They are often activated downstream of growth-factor receptors and PI3 Kinase. Akt has previously shown to have a role to play in acquired Tamoxifen resistance (Pancholi et al., 2008). The activation of Akt 1 by phosphorylation at Ser473 was shown by Western blotting in Figure 3-11. The MCF7 and TAMR displayed predominately Akt1, but also increased Akt 2 and 3 phosphorylation. It is worth noting that an increased expression of Akt3 has been shown in the literature to be associated with a more aggressive clinical phenotype (Nakatani et al., 1999). Interestingly, this isotype was shown to have an increased

phosphorylation in resistance, which reached statistical significance in the TAMR cells moreover, a cell line that had been stably transfected with constitutively active Akt was protected from apoptosis induced by Tamoxifen (Shin and Arteaga, 2006). Our Tamoxifen resistant cells show an increase in Akt activity and also showed anti apoptotic mediators (see section 4.5.3).

RSK (90 kDa ribosomal S6 kinase) is an effector of both Ras/MEK/MAPK and PI3K/PDK1 pathways. There are four isoforms but only RSK1 and RSK2 are spotted onto this array and only RSK1 significantly increased. RSK can be activated by ERK in response to growth factors, polypeptide hormones, chemokines and other stimulus. It has been shown to be over expressed in breast cancer (Yamnick and Holtz, 2010) but not specifically cited to have a role in Tamoxifen resistance. ERK response is up regulated in our Tamoxifen resistant cell lines (Figure 3-7) this is known to activate RSK so the increase in RSK1 phosphorylation seen on this array fits with this being a functionally important pathway in Tamoxifen resistance.

MAPK antibody array showed major differences in ERK1 and 2, p38's, and Akt as would be predicted but interestingly this array also identified other significantly altered proteins which are novel findings (see Table 4-2).

4.5.2 RTK

RTKs aid cells to interpret the diverse range of signals and stimulus that cells come in to contact with, ensuring cells respond to the external stimulus appropriately. The activation of these receptors in the cell is tightly controlled and allows the cell to respond to its environment effectively. Cancer evades the normal mechanisms that control the cell and the regulation of the response to external stimulus causing

uncontrolled proliferation. The aberrant signalling of RTKs and their role in human cancers has been well known for many years. The constitutive activation of many proteins in this family of receptors has been shown to be important in tumour proliferation and the progression of cancer.

The receptors themselves are divided into sub families, according to their structural characteristics but all have a single trans-membrane domain separating the intra cellular tyrosine kinase region from the extra-cellular portion. Adaptor molecules allow for the activation of RTKs to be linked to downstream signalling pathways such as MAPK pathway (which is crucial for RTK induced cellular proliferation).

The EGF receptor family members (which include EGFR, ErbB2, ErbB3 and ErbB4) are tyrosine kinase receptors involved in the regulation of cell growth and differentiation. Differential expressions of the EGFR family members have been shown in breast carcinomas to influence different clinical behaviours, (Knowlden et al., 2003). It is thought that acquired resistance to anti-oestrogens such as Tamoxifen is facilitated by increased EGFR expression which enables stimulation of proliferation and phosphorylation of the ER on specific serine residues (Gee et al., 2005). ErbB2 also showed a statistically significant increase in phosphorylation; this has been described before in Tamoxifen resistance (Gee et al, 2005). Patients whose tumours have increased amplification of ErbB2 often go on to develop resistance to therapy (Knowlden et al., 2003) and often also have different responses to other therapeutic agents such as Taxanes (Mokbel and Elkak, 2001). In these patients Herceptin is an important treatment. Herceptin is a humanised IgG monoclonal antibody specific to the growth factor ErbB2

(aka HER2) and is used in ErbB2 amplified cancers such as some breast and also some upper gastrointestinal cancers.

Eph Receptors represent the largest group of RTKs; their expression controls many cellular processes through binding of the ligand Ephrin. In a cancer environment the EphR's have been shown to play a role in aberrant cell – cell communication. They are thought to influence cancer progression, though the mechanisms through which they do this are poorly understood due to the complexity of EphR signalling (Pasquale, 2010). Both increased and decreased EphR expression has been linked with cancer progression, leading to promotion of tumourigenesis, as well as inhibition of it (Truitt et al., 2010). Eph RTKs regulate attachment to extra cellular matrix in several cell types, including tumour cell lines, but have been shown to promote rounding and detachment from the matrix in some breast tumour cell lines, (Truitt et al., 2010). This expression and conflicting evidence that EphR's are involved in many aspects of cancer development and progression seems to suggest that their bidirectional signals may contribute to this. They have been shown to affect growth, migration and invasion in cultured cancer cells and *in vivo* affecting tumour growth, metastasis, and angiogenesis. For these reasons, the Eph receptors could be promising new therapeutic targets in cancer. It has been suggested in the literature that the over expression of EphA2 infers a more aggressive breast cancer phenotype, and decreases breast epithelial cells sensitivity to Tamoxifen (Brantley-Sieders et al., 2008 and Lu et al., 2003) More recently, Gokmen-Polar et al., 2011 have shown that dual targeting of EphA2 and ER restores Tamoxifen sensitivity in ER/EphA2 positive breast cancer. Interestingly, EphA2 was shown to have increased phosphorylation in our resistant cells, this reached significance in TAMR ($p=0.016$), for

EphA1 however, the tyrosine phosphorylation was increased and again reached significance. This has not previously been shown in the literature, however, EphA1 expression was shown to be increased in gastric cancer correlating with increased stage and invasion and a poor patient survival (Wang et al., 2010).

There are five FGFRs (1, 2, 3, 4, and 5) that mediate FGF activity, each contains three Ig domains with acidic amino residues between the first and second of these residues. All of the receptors, bar FGFR5, have a cytoplasmic split tyrosine kinase domain. Alternative splicing leads to multiple forms of FGFR1, 2 and 3. The only receptor that showed a significantly altered phosphorylation was FGFR2a; this has not been shown before. However, Meijer et al., 2008 has shown that altered expression of FGF17 causes Tamoxifen resistance in vitro and that FGFR4 is involved in the acquisition of Tamoxifen resistance. Interestingly, FGFR4 showed an insignificant increase in expression in our experiments; although this may have been partly due to the low levels of expression detected and the variation in the MCF7 cells expression. Further investigation of this result (by Western blot) would be warranted. A recent paper has also suggested that an over expression/ amplification of FGFR1 has a causal role in the progression of treatment resistance in breast cancer (Turner et al., 2010) no evidence of a change of phosphorylation was seen in this study, expression of FGFR1 was not investigated. The FGF signalling pathway could be a valuable target in the treatment of breast cancer patients resistant to endocrine treatment, but further investigation into which receptors are increased is needed.

The VEGFR family belong to the class III subfamily of receptor tyrosine kinases All three receptors in the family contain seven immunoglobulin-like repeats in their

extracellular domain and kinase insert domains in their intracellular region. They are perhaps best known for their role in regulating VEGF family-mediated vasculogenesis, angiogenesis, and lymphangiogenesis. VEGFR2 is thought to be the primary inducer of VEGF-mediated blood vessel growth, while VEGFR3 plays a significant role in VEGF-C and VEGF-D-mediated lymphangiogenisis. These are the two receptors that have statistically significant increases in tyrosine phosphorylation in our cell model. This is a potentially interesting result as the cells are epithelial in nature and the VEGFR family are endothelial receptors, although their expression has previously been described in MCF7 cells (Aesoy et al., 2008). The up-regulation of VEGFR2 phosphorylation (Figure 4-14) and p38 phosphorylation (Figure 4-5) in this study has also been reported in the literature in acquired Tamoxifen resistance (Aesoy et al., 2008) who reported that MCF7 cells with acquired Tamoxifen resistance have increased VEGF secretions and subsequently increased signalling through VEGFR2. It was elucidated that this increased signalling was acting through p38, the expression and phosphorylation of which was found to be increased in resistance, as we also report in this Thesis. Aesoy et al., 2008 showed that inhibition of p38 in resistant cells led to a decreased proliferation of both resistant and sensitive cells and a connection (signalling loop) was established between VEGFR2 and p38 using knock downs of VEGFR2. The p38 pathway is involved with growth, and VEGFR2 pathway is involved in angiogenesis and anti-apoptosis, modulation of these pathways may provide a way to overcome Tamoxifen resistance. Our data would fit well with such a model.

Tie-2 is a receptor for the angiopoietins (ANG 1 and 2) and is involved in vascular stabilization and remodelling in endothelial cells both in physiological and

pathological conditions. The phosphorylation of Tie2 was found to be statistically increased in both resistant cell lines (Figure 4-17). Tie 2/Ang signalling has been found to regulate the survival and apoptosis of endothelial cells, control vascular permeability, and regulates capillary sprouting. In cancer, Tie2 was originally found to be over expressed in tumour blood vessels; however, it has been recently shown in the literature that Tie2 is also expressed outside the vascular partition in several types of cancer, including breast tumours (Martin et al., 2008). Our results show a statistically significant increase in Tie2 phosphorylation in both resistant cell lines and add weight to these findings and suggest that the role of Tie2 in tumour cells needs further exploration.

HGF receptor (HGFR), a product of the proto-oncogene c-met, is a heterodimeric transmembrane glycoprotein that has tyrosine kinase activity and is activated by HGF. In this chapter it was shown to have increased phosphorylation in the Tamoxifen resistant cell lines (Figure 4-21). The receptor has been shown in the literature to have increased expression when MCF7 and T47D breast cancer cells are exposed to chronic doses of Fulvestrant, leading to the activation of HGF secreting fibroblasts and activation of a number of signalling proteins including Src, ERK1 and 2 and Akt, all of which contribute to a more aggressive phenotype in breast cancer cells (Hiscox et al., 2006). Here we show that Tamoxifen resistance also leads to statistically significant increases in HGFR activation. Modulation of cell motility, cellular adhesion, resistance to apoptosis and anchorage independent growth have all been associated with HGFR in cancer. Clinically, over expression of HGFR may confer a tendency for breast tumours to metastasise, as it allows the surrounding stromal cells (i.e. fibroblasts) to contribute additional stimulating signals. This would fit with the known increases seen in TAMR cells.

The Macrophage stimulating factor receptor (M-CSFR), shown to have increased phosphorylation in resistant cells (Figure 4-20), is the product of the c-fms proto-oncogene. Abnormal expression has previously been shown in the breast, ovary and endometrium (Maher et al., 1998) whilst the activation of the receptor by the ligand has been reported to regulate invasion and anchorage independent growth in breast cancer cells. The increased phosphorylation of M-CSFR seen in this study would fit with these findings. M-CSFR has been investigated as a prognostic marker in ipsilateral breast cancer reoccurrence in clinical samples but showed no correlation between receptor expression and metastasis and was thought to be a poor prognostic marker (Maher et al., 1998).

Axl, Dtk, and Mer share a common ligand; GAS6 (Growth arrest specific 6). GAS6 has highest affinity for Axl, followed by Dtk, then Mer. Axl, Dtk and Mer are a sub family of receptor tyrosine kinases whose extracellular domain contains motifs similar to those found in many cell adhesion molecules, they have been implicated in tumourigenesis, cell survival, proliferation and adhesion. GAS6 has been shown to be regulated by oestrogen (Mo et al., 2007) and an ERE has been identified in the GAS6 promoter. Axl was only shown to have significantly increased phosphorylation in TAMRBU cells and this was a marginal increase of 4.8%. The other two receptors showed greater phosphorylation and this was significant in both resistant cell lines (see Table 4-4). Less is known about these receptors and their role in cancer but they have been found to be over expressed in a number of human cancers (Linger et al., 2008). The clinical ramifications of the increases have not yet been elucidated so this represents a novel and interesting finding which requires further investigation as one could suggest

that an increase in the phosphorylation of these receptors of an oestrogen regulated ligand could represent a mechanism for evading oestrogen/Tamoxifen regulated growth.

4.5.3 Apoptosis

Apoptosis is the process of programmed cellular death, and it plays a critical role in normal processes whilst inappropriate apoptosis tends to lead to diseases, including cancer. It comprises of complex cascades that lead to cellular changes such as membrane blebbing, DNA degradation, chromatin condensation and ultimately the formation of apoptotic bodies that are cleared by phagocytosis (Elmore, 2007).

Apoptosis and proliferation play an important role in normal breast development; the balance of these two mechanisms is therefore crucial in the determination of overall growth and regression of cancer cells and also in the cells response to therapeutics such as chemotherapy, radiotherapy and hormonal therapy such as Tamoxifen, as the action of these therapeutics, at least in part, is based on induction of apoptosis.

The Bcl-2 family of proteins consist of both promoters and inhibitors of apoptosis. The pro-apoptotic Bad (Bcl-2-antagonist of cell death) is a pro-apoptotic protein and its expression was significantly decreased in TAMRBU, this was also the case of the pro-apoptotic protein Bax (See section 4.4.2). These data would fit with the increased proliferation rate seen in the resistant cell model. Bax has been shown to be crucial for inducing the permeability of the outer mitochondrial membrane and therefore the release of the promoters of apoptosis such as SMAC/Diablo. Again, the decrease in expression of the proteins suggests that the TAMRBU cells are evading the apoptotic cascade in order to survive. However, the results for the pro-survival proteins Bcl-x and Bcl-2 was conflicting. There was an increase in Bcl-x protein but not of Bcl-2 (section 4.4.2). The

decrease in Bcl-2 also conflicts with reports in the literature (Planas-Silva et al., 2007) where it is implied that an increase is associated with a more aggressive phenotype and therefore an increase in our Tamoxifen resistance cell model would have predicted. This would be an interesting result to repeat and validate by Western blot.

Heat shock proteins 27 and 70 have previously been associated with clinical resistance to Tamoxifen (Ciocca et al., 1998). HSP27 is an oestrogen-regulated protein and HSP70 is part of the chaperone machinery that works in the assembly and function of steroid receptors. In addition, HSPs have been shown in the literature to assist in protecting cells against stress, and their expression has been linked to drug resistance (Calderwood et al., 2006). The HSP60 showed a small increase in expression in this study (see Table 4-7) and has also been shown to be up regulated in many cancer types including breast (Desmetz et al., 2008). The role of HSP60 in cancer is still poorly understood, and the relationship between HSP60 and Tamoxifen resistance is novel.

As already mentioned, mitochondrial pro-apoptotic protein SMAC/Diablo expression in TAMRBU was statistically decreased (Figure 4-31). This is interesting as it is a likely promoter of apoptosis, reported to inhibit IAP activity (Fandy et al., 2008). During the process of apoptosis, Smac/DIABLO gets released into the cytosol of the cell and becomes bound to an inhibitor of apoptosis XIAP which acts as an antagonist to XIAP's interaction with caspase 9. This action promotes caspase 9 activity, followed by caspase 3 and apoptosis commences. It has been shown in the literature (Mizutani et al., 2005) that patients with metastatic disease Renal Cell Carcinoma have lower Smac/DIABLO levels than those with localised disease. It has also been shown that Smac/Diablo when artificially over expressed in cancer cells is able to enhance the

sensitivity of the cells to various therapeutic agents including Tamoxifen (Fandy et al., 2008).

The cellular IAPs (Inhibitor of Apoptosis) proteins were increased in the TAMRBU cell line (Figure 4-30). IAPs inhibit the activity of mature caspases. It has been speculated that cellular caspase activity may be restored by proteins such as SMAC/Diablo (Wilkinson et al., 2004) which links with the decrease in SMAC/Diablo expression seen in TAMRBU cells in this Thesis (see above). XIAP (X-linked inhibitor of apoptosis) is also a member of the IAP family. XIAP inhibits the activity of caspase 3, 7, and 9 (Suzuki et al., 2001). However, no change in caspase expression was seen in these cells.

Increased Survivin has been detected in most types of cancer but is rarely expressed in the "normal" equivalent tissue, so this could be a potential promising biomarker of cancer. It has been associated with markers of poor prognosis in breast cancer patients in the literature (Ryan et al., 2006) which would appear to support the increase seen in Tamoxifen resistance here (Figure 4-32). It has also been associated with a resistance to Tamoxifen induced apoptosis in MCF7 cells (Moriai et al., 2009).

HIF1α was shown to be up regulated in TAMRBU cells on the apoptosis array (Figure 4-27). It is understood from the literature that HIF1α activates the transcription of many genes in prostate cells under hypoxic conditions; these include glucose transporters and vascular endothelial growth factor (Mori et al., 2010). Whilst these cells were certainly not hypoxic, the expression of this protein is the prime mechanism for induction of tumour angiogenesis (Quintero et al., 2004), and the up regulation is again in keeping

with the fact that the resistant cell lines are rapidly proliferating and therefore potentially would lead to the growth of large tumours which need more vascularisation.

PON2 has recently been reported to contribute to tumourigenesis and evasion of apoptosis (Witte et al., 2011). PON2 was found to be over expressed in a number of human cancers including prostate, endometrium and liver; furthermore, its over expression was shown to be associated with resistance to various therapeutic agents such as imatinib, doxorubicine, staurosporine in leukaemic cell line models (Witte et al., 2011). It was also suggested that high expression of PON2 lowered ROS and limited the formation of caspase activation; however, caspase 3 activation was not altered in TAMRBU cells (Table 4-6). Witte et al., 2011 reported that a decrease in JNK activation (Thr183 and Tyr185) was seen in cells that over expressed PON2, however, JNK activation was not significantly altered in TAMRBU cells (Figure 4-4).

Antibody arrays have shown value in the discovery of proteins associated with Tamoxifen resistance as many of the proteins found to have altered expression or phosphorylation in Tamoxifen resistant cell lines are novel findings as shown in the summary Table 4-7.

Table 4-7. A table of all the proteins that were statistically different in the both Tamoxifen resistant cell lines (where applicable), whether the modulation has been described in resistance previously and the reference of the paper if it has. Phosphorylation is designated by (P)

Protein Name	Increased/ decreased in TR	Previously found in TR?	Reference
EGFR (P)	Increased	YES	Knowlden et al 2003
ErbB2 (P)	Increased	YES	Knowlden et al 2003
FGFR2a (P)	Increased	NO	
Dtk (P)	Increased	NO	
Mer (P)	Increased	NO	
HGFR (P)	Increased	YES	Hiscox et al 2006
M-CSF-R (P)	Increased	NO	
ROR1 (P)	Increased	NO	
Tie2 (P)	Increased	NO	
VEGFR2 (P)	Increased	YES	Svensson et al 2005
VEGFR3 (P)	Increased	NO	
EphA1 (P)	Increased	NO	
EphB1 (P)	Increased	NO	
ERK1 (P)	Increased	YES	Gee et al 2001
ERK2 (P)	Increased	YES	Gee et al 2001
P38 (P)	Increased	YES	Aesoy et al 2008
RSK1 (P)	Increased	NO	
Akt (P)	Increased	YES	Clark et al 2002
HIF1α	Increased	YES	Martinez-Outschoom et al 2010
Bcl-2	Decreased	NO	
Bcl-X	Increased	NO	
Bad	Decreased	YES	Cannings et al 2007
Bax	Decreased	NO	
Catalase	Increased	NO	
cIAP1	Increased	NO	
cIAP2	Increased	NO	
XIAP	Increased	NO	
HSP70	Increased	NO	
PON2	Increased	NO	
p-RAD17 (S635) (P)	Increased	NO	
SMAC/Diablo	Decreased	NO	
Survivin	Increased	YES	Moriai et al 2009

5 Mass spectrometry based proteomic investigation

Quantitative proteomics has traditionally been based on 2D SDS PAGE but there are other emerging, relatively new technologies that can overcome some of the disadvantages of the 2DE (such as poor membrane protein analysis). These include labelling technologies and strategies such as ITRAQ, O_{18} labelling, ICAT and SILAC all of which were introduced in section 1.1.3

SILAC is the approach that was chosen for this project. It is a form of metabolic labelling where one cell population is grown in a media containing a heavy labelled lysine and one in normal lysine (this is outlined in more depth in the methods section, 2.1.7, and in the main introduction 1.1.3).

The ability to use mass spectrometers to investigate cellular proteomes allows us to assess many proteins and their expression in one spatial time-point. This is a valuable tool in the field of Tamoxifen resistance providing the potential to discover novel changes.

5.1 SILAC labelling and global proteomics

5.1.1 Labelling efficiency.

The SILAC experiment did not commence until the extent of labelling had been established (see section 2.3.4). This was done as a simple calculation. The % of K inclusive peptides with labelled K residues was 98%. This was a high incorporation of label and further cell population doublings were deemed unnecessary.

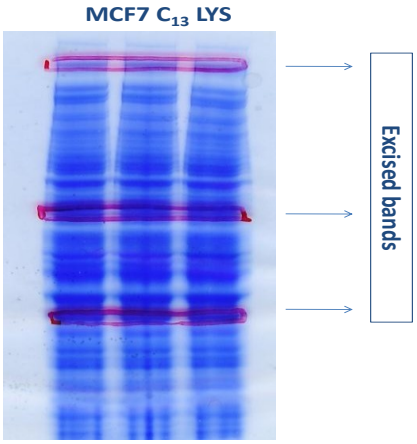


Figure 5-1. 4-12% Bis-Tris gel to obtain the SILAC labelling efficiency.

5.1.2 Shotgun global SILAC experiment

Briefly, the cells were trypsinised (section 2.1.6) and counted. $5x10^6$ cells of each "heavy" and "light" respectively were counted and mixed into one tube which was then lysed using NP-40 lysis buffer (2.2.1) and a BCA assay completed to determine protein concentration. 200µg of protein lysate was loaded into a 4-12% Bis-Tris 1 well gel (Figure 5-2), run and stained with colloidal Coomassie. A paper stencil was used as a guide enabling 40 even, manageable slices to be cut manually from the gel; this enabled the proteins to be separated by their size before they were typically digested, with the largest proteins in slice 1 and smallest in slice 40.

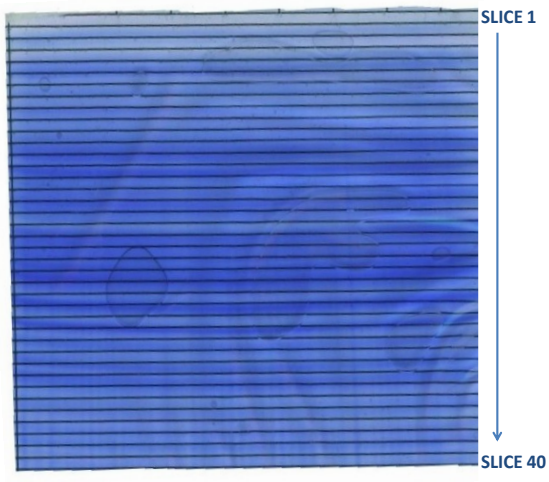


Figure 5-2. 4-12% Bis-Tris one well gel used to separate the whole cell lysates from the mixed heavy and light labelled cells. This was then cut using this template to create 40 gel bands to perform in-gel digestion on and extract peptides from.

In total, 262 quantifiable proteins were identified using FASTA database (ipi.HUMAN.v3.51.fasta). Each protein included in the study had 2 or more peptides found, these peptides were only included if they reached an XCorr value of 1.5 for singly charged peptides, 2.0 for doubly charged and 2.5 for triply charged peptides. Data that did not reach these criteria were removed from the resulting analysis. Raw data is available in Appendix.

The data from each slice was analysed and the Xpress ratio (ratio Heavy: Light) data was manually checked; due to the frequently seen irregularity in the software's peak picking. The peaks that the software picked were often incorrect, an example of which is shown in Figure 5-3b, and subsequently each Xpress ratio was checked manually.

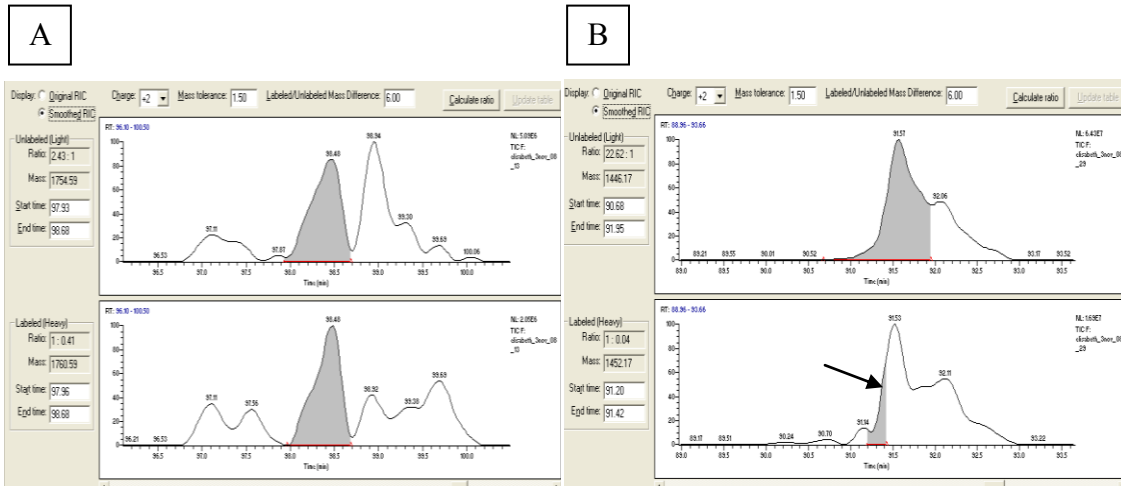


Figure 5-3 Examples of good (A) and poor (B) peak picking. The shaded grey area under the peak gives the value on which the Xpress ratio is based.

Of the 262 quantifiable proteins found in the global SILAC proteomic experiment, the difference between over expressed proteins and under expressed proteins are fairly evenly spread, with 25% down regulated and 29% up-regulated in resistance. A large proportion (46%) presented as neutral (no difference between heavy and light peptides). This can be seen in a pie chart below (Figure 5-4) and also in the formation of the waterfall plot (Figure 5-8) the average of the LogBase2 is 0.334 – showing a slight skew as more proteins are up-regulated.

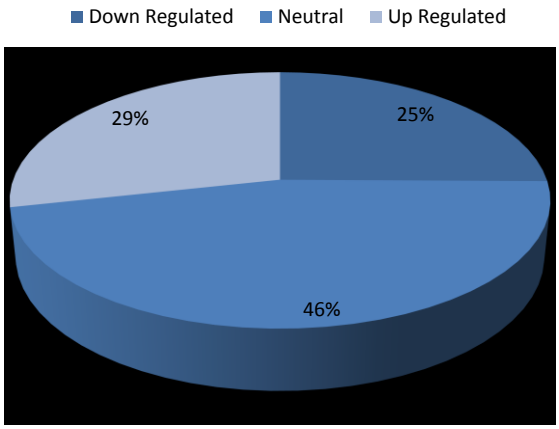


Figure 5-4. 46% of the labelled proteins found were neutrally expressed between heavy MCF7 and light TAMRBU. There were marginally more up-regulated in resistance.

The ontological data of the proteins found in this experiment was gathered from UniProt knowledge database. The cytoplasm is the largest (by volume) portion of the cell and therefore it is unsurprising that the largest proportion of the proteins found were cytoplasmic with 67% (**Error! Not a valid bookmark self-reference.** A). The ontology also showed that 19% of the proteins found were nuclear proteins. Unfortunately there were few membrane proteins and no receptors found. This would have been interesting to collate with the data from the RTK antibody array (Section 4.3). The functionality of the proteins found was also investigated and the Uniprot knowledge database was again used. This could not classify a function for 48% of the proteins and they were therefore labelled "unknown". Enzymes accounted for 28% of the proteins found. This could be due to the higher metabolic rate/proliferation that is seen in the resistant cell lines (alamar blue assay – section 3.2.4). There were also kinases, phosphatases and transcription regulators found by the experiment. It has been estimated that 20% of the human genome encodes

for signalling proteins such as kinases, phosphatases and transcription factors (Taylor et al, 2004). The resistant cells are known to have aberrant signalling pathways and therefore it is good that the experiment identified some proteins in these groups.

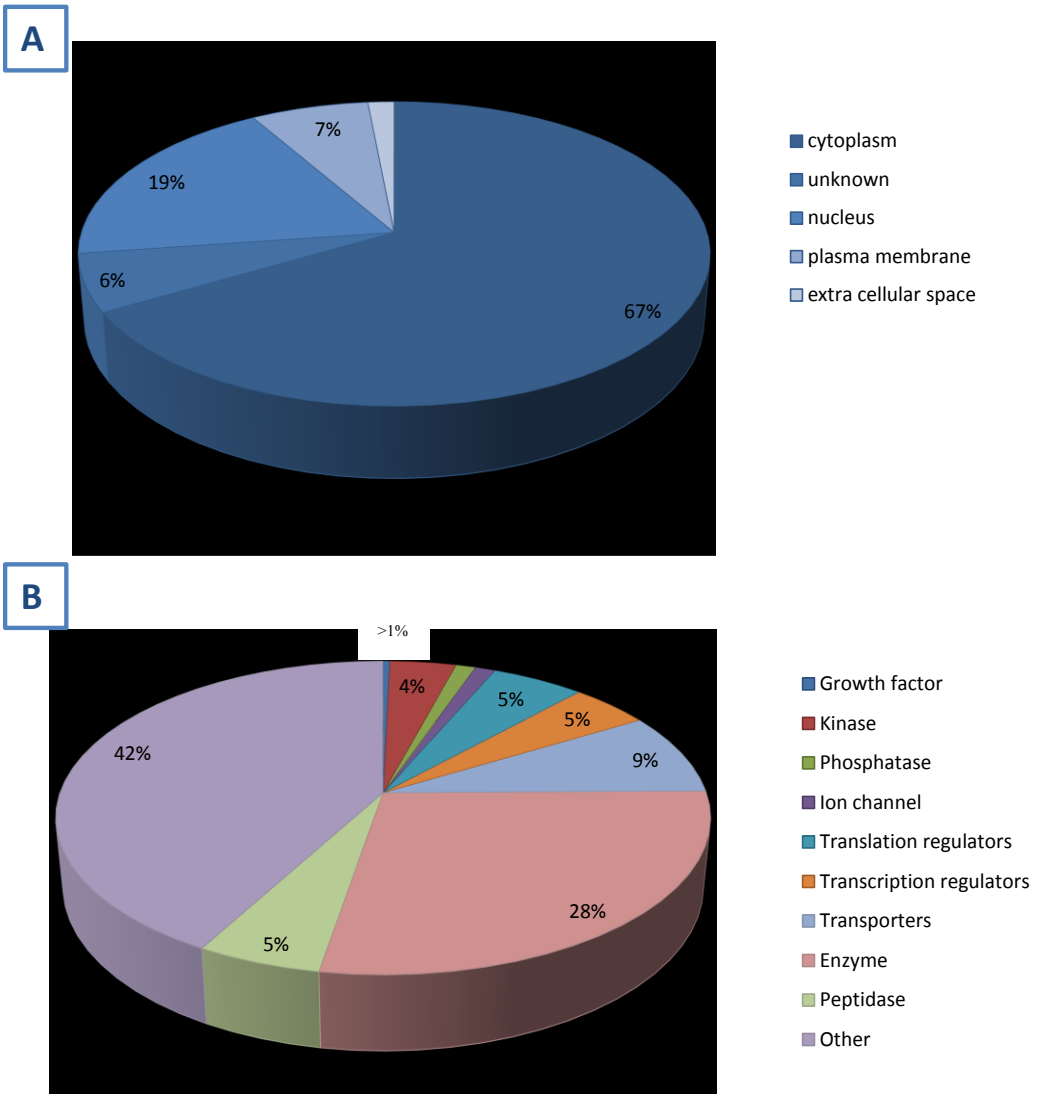


Figure 5-5. Ontological information pertaining to the quantifiable protein identifications obtained using the global SILAC approach. A, shows the largest proportion of proteins identified were cytoplasmic. Part B shows of the total identified 42% did not specify role and were given the sub-group of "other". Ontological information gathered from UniProt knowledge database.

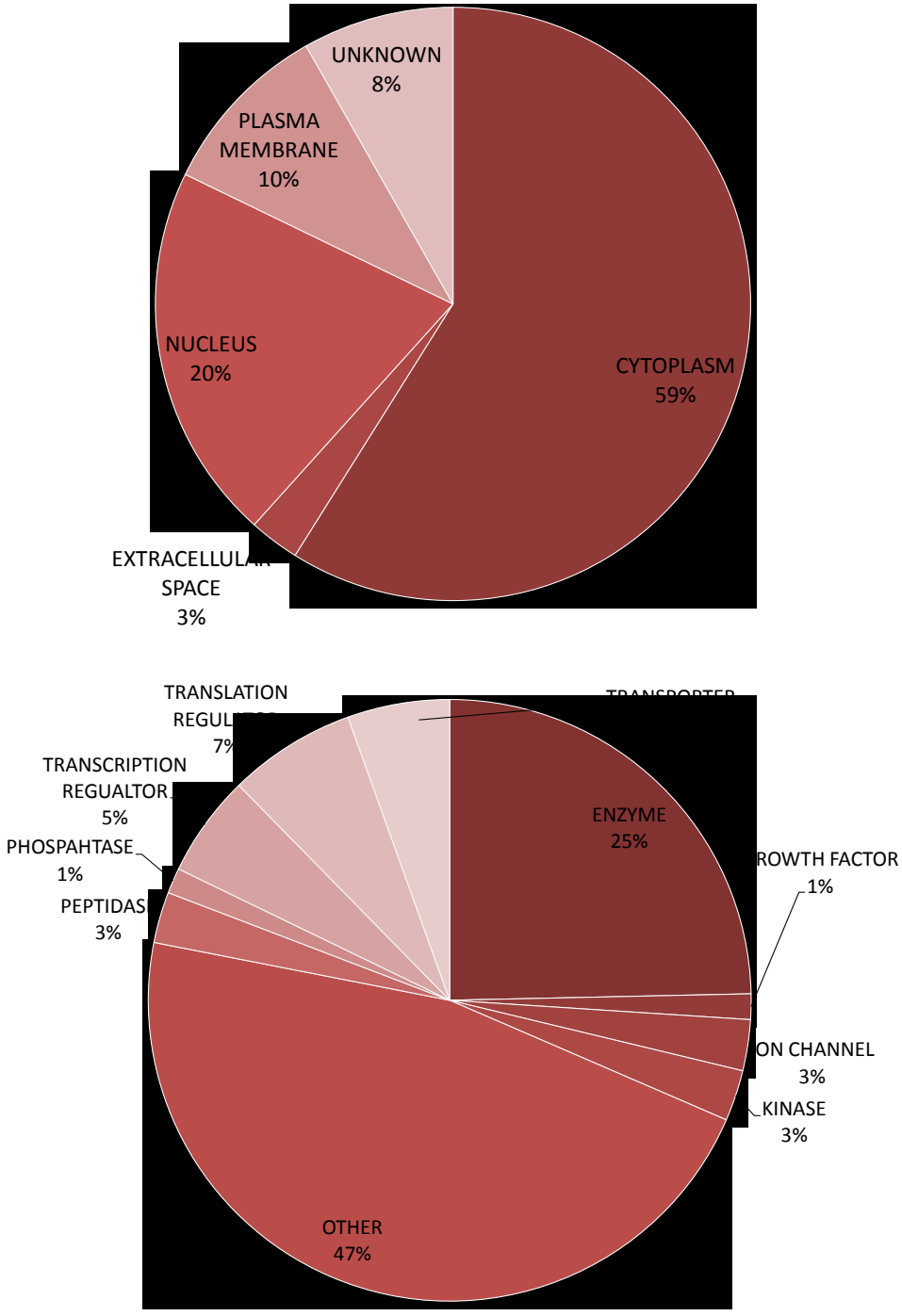


Figure 5-6 Ontological Information of the proteins that are over expressed in the SILAC experiment. Of the proteins showing increased expression in resistance 59% were cytoplasmic, 20% nuclear, and 10% plasma membrane proteins. The fewest proteins were found to be associated with the extracellular space and 8% were of undesignated localisation. Nearly half of the proteins were of unknown specific function and 25% were enzymes.

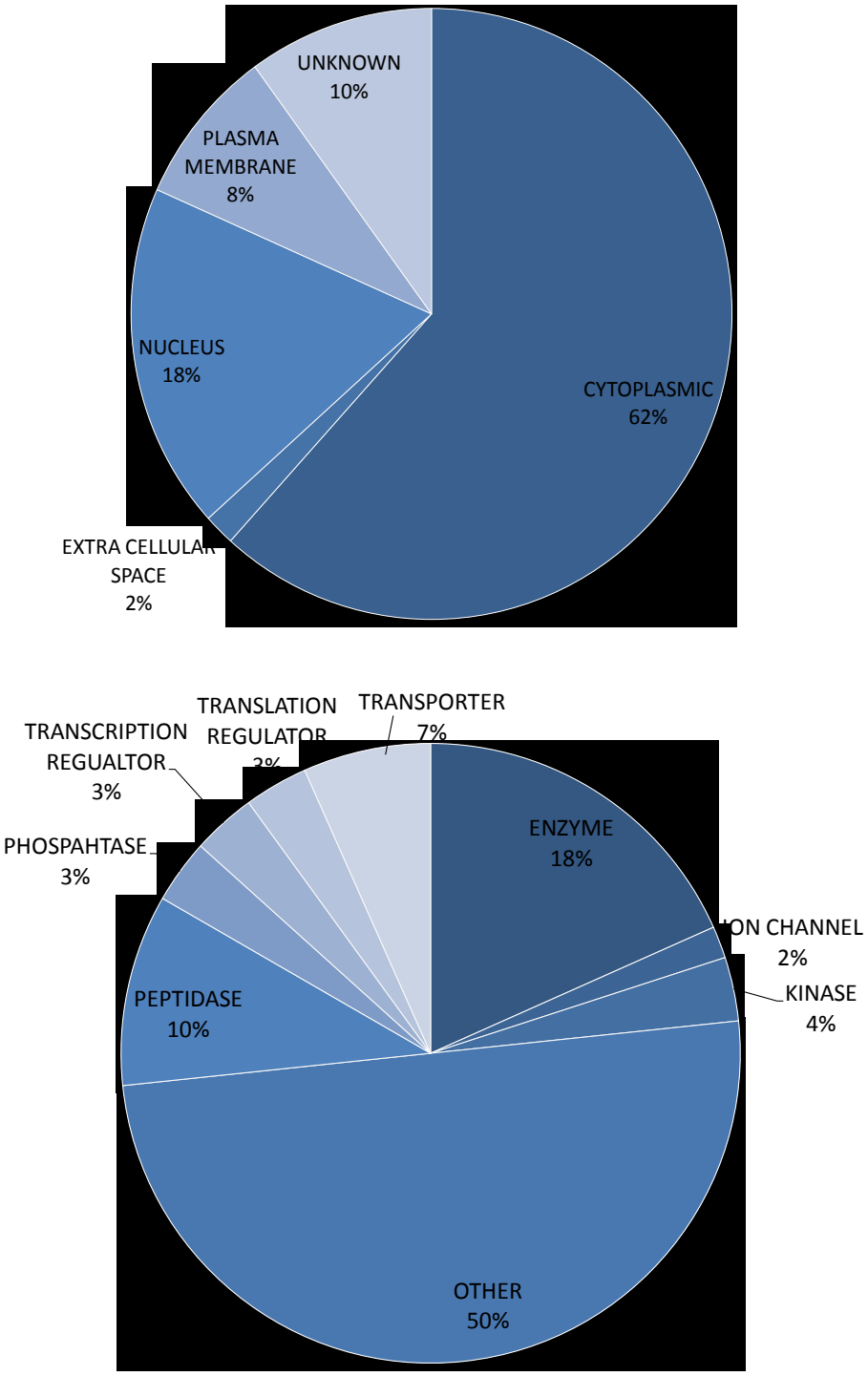


Figure 5-7 Ontological information of the proteins that showed down regulation in TAMRBU using SILAC. The majority of the proteins with lower expression in TAMRBU were cytoplasmic (62%) and 50% of the down regulated proteins were classified as having no specific function, 18% were enzymes and 10% were peptidases.

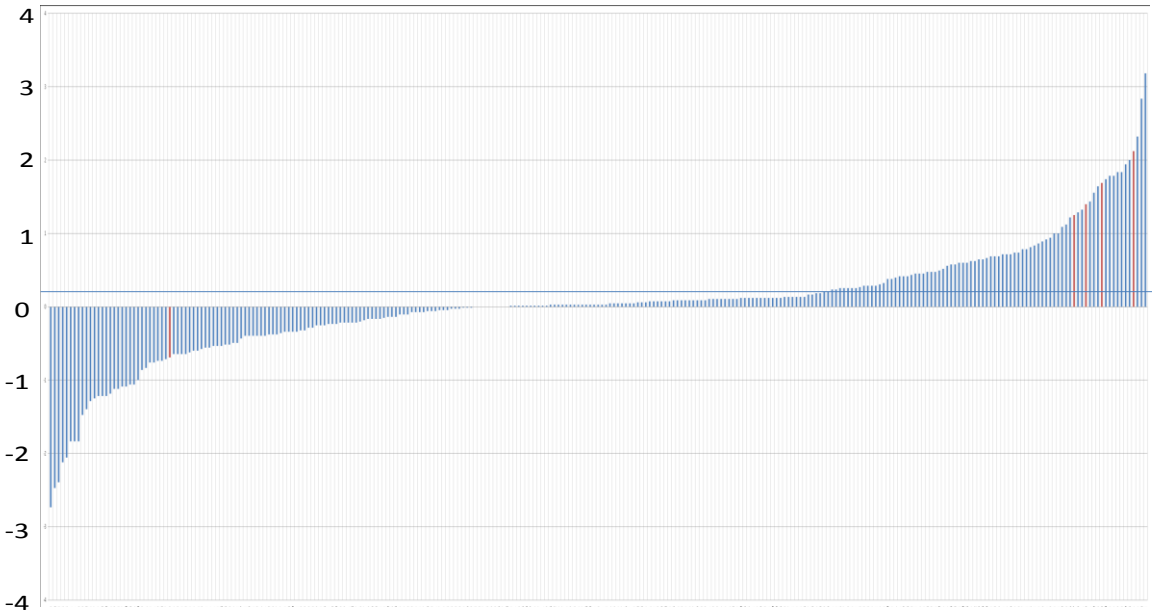


Figure 5-8 A waterfall plot of the 262 quantifiable proteins that were identified, each blue bar represents a protein, the 5 red bars are the proteins that have been further validated by Western blot analysis. The blue line that crosses the bars represents the average (0.334). More proteins were over expressed in resistance. The Y axis represents LogBase2 of the ratio between heavy and light.

Table 5-1. A table describing 15 example proteins that showed increased expression in the Tamoxifen resistant cell model TAMRBU found using SILAC labelling MS approach.

Protein name (plus abbreviation)	Gene	Accession	Fold Change	Log_2
BCL2-Associated athanogene (BAG-1)	BAG1	Q99933	7.14	2.84
Calpain-1	CAPN1	P07384	3.44	1.79
Chloride intracellular channel 1 (CLIC-1)	CLIC1	O00299	3.85	1.94
Cortactin	CTTN	Q14247	2.38	1.25
Epithelial cell adhesion molecule (EpCAM)	EPCAM	P16422	9.09	3.18
Hepatoma-derived growth factor (HDGF)	HDGF	P51858	2.17	1.12
High-mobility group box 1 (HM Box-1)	HMGB1	P09429	3.57	1.84
IQ motif containing GTPase activating protein 1 (IQGAP1)	IQGAP1	P46940	2.66	1.40
Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1 (P-Rex)	PREX1	Q8TCU6	3.57	1.84
Protein phosphatase 1, gamma isozyme (PP1γ)	PPP1CC	P36873	1.63	0.86
Prohibitin	PHB	P35232	2.00	1.00
Proliferation-associated 2G4, (PA2G4)	PA2G4	Q9UQ80	3.33	1.74
Ribophorin - 2	RPN2	P04844	4.35	2.12
Staphylococcal nuclease and Tudor domain containing 1 (SND-1)	SND1	Q7KZF4	3.44	1.79
Talin-1	TLN1	Q9Y490	3.22	1.69

Table 5-2 A table that represents 15 example proteins that were down regulated in the TAMRBU resistance cell culture model compared to MCF7 are shown, as identified using SILAC and MS approach.

Protein (Plus abbreviation)	Gene	Accession	Fold Change	Log $_2$
α Actinin 4	ACTN4	O43707	-1.6	-0.43
AFG3-Like protein 2	AFG3L2	Q9Y4W6	-4.16	-2.05
Programmed cell death 6 interacting protein ALIX	PDCD6IP	Q8WUM4	-1.5	-0.25
Calponin-2	CNN2	Q99439	-2.32	-1.22
Cathepsin D	CTSD	P07339	-1.6	-0.69
Centaurin δ–3	ARAP3	D3DQE3	-1.56	-0.62
Cytochrome C1	CYC1	P08574	-1.5	-0.64
Ezrin-radixin-moesin binding phosphoprotein-50 (EBP-50)	SLC9A3R1	O14745	-5.55	-2.47
Protein FAM49B (L1)	FAM49B	Q9NUQ9	-6.66	-2.74
Lamin B1	LMNB1	P20700	-2.32	-1.21
nuclear transcription factor, X-box binding-like 1	NFXL1	Q6ZNB6	-4.3	-2.40
Nuclear migration protein NUDC	NUDC	Q9Y266	-3.57	-1.83
SET	SET	Q01105	-2.08	-1.05
Vigilin	HDLBP	Q00341	-2.27	-1.18

5.2 Western Blots of five candidate proteins found using MS SILAC

Of the 262 proteins found using SILAC (see CD appendix) 5 were chosen for further validation using Western blotting. Validation of MS analysis is routinely used in the literature and it was seen as good practice before further, more functional work was carried out.

The 5 proteins that were chosen for further study were selected for several reasons; IQGAP1 had been shown in the literature to be important in EGFR signalling and also ERK1 and 2 signalling, these are known to be key factors in Tamoxifen resistance acquisition. It also was quantified using over 10 peptide pairs giving more confidence in the data. Cortactin and Talin were shown in the literature to play a role in actin cytoskeleton reorganisation and attachment, and have been shown to interact with many proteins that are implicated in acquired Tamoxifen resistance in the literature. Ribophorin- 2 was chosen for further investigation as there was little known data for function of this protein with the exception of an increase of expression shown in docetaxel resistance and the quantitation from SILAC suggested that this protein also shown an increased expression in resistance (4.35 increase in TAMRBU). It was also seen as good practice to validate a protein that was decreased in resistance, for this reason cathepsin D was chosen.

5.2.1 Ras GTPase activating like protein

Ras GTPase activating like protein (IQGAP1) is a protein that has many roles in the normal function of a cell, as discussed in detail in section 5.3.1. Here we found via SILAC that TAMRBU showed an increase in IQGAP1 of 166.8% (p = less than 0.001) compared to the expression seen in the MCF7 cells. This trend is also seen to a lesser extent (81.7%, p ≤0.001) in the Western blot densitometry analysis as can be seen in Figure 5-9.

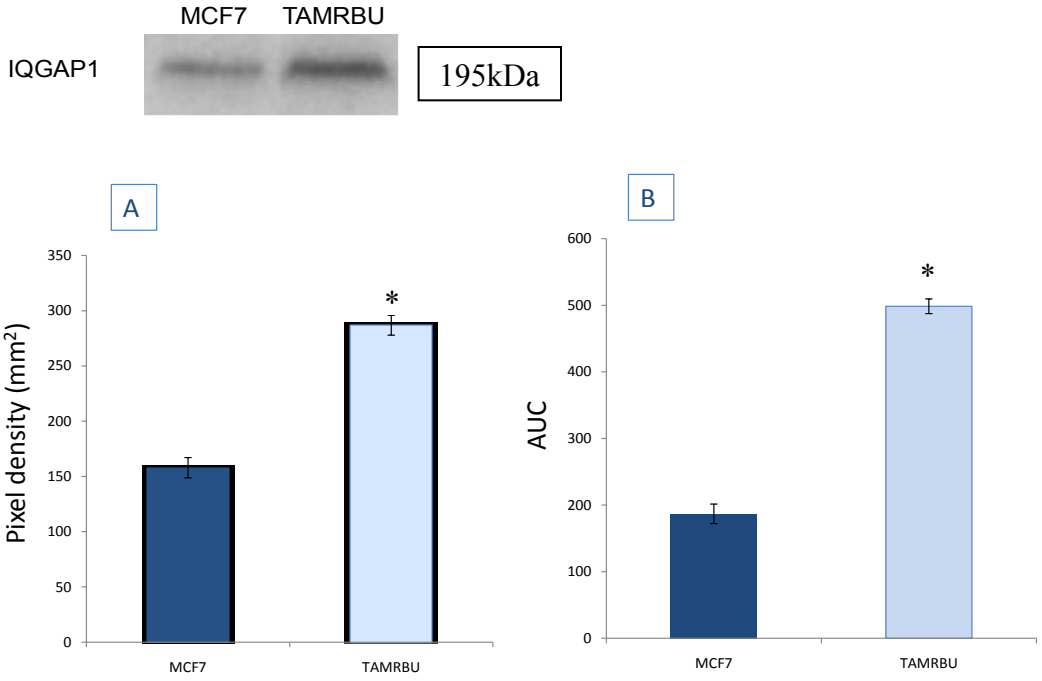


Figure 5-9 Composite showing A, the densitometry analysis of the Western blot (shown above chart) showing an 87.1% increase in TAMRBU pixel density (p<0.001 by students T test) when compared to MCF7. B, shows the comparison of the area under the curve (AUC) value for the quantitative peptides analysed in SILAC, this shows a 113.1% increase in the TAMRBU when compared to MCF7 with p value <0.001.

5.2.2 Src substrate Cortactin

Over expression of cortactin has been associated with an increase in motility and has been frequently reported in many types of cancer which is discussed in more detail in section 5.3.1. Using SILAC, cortactin was shown to be increased by 138.4% in resistance, compared with MCF7 cells. There is no statistical analysis of this data as there were only 2 quantifiable peptide pairs found. In the Western blot analysis there was an increase seen in resistance, however it is to a lesser extent (33% increase in resistance) this was statistically significant (p=0.01) by student t-test.

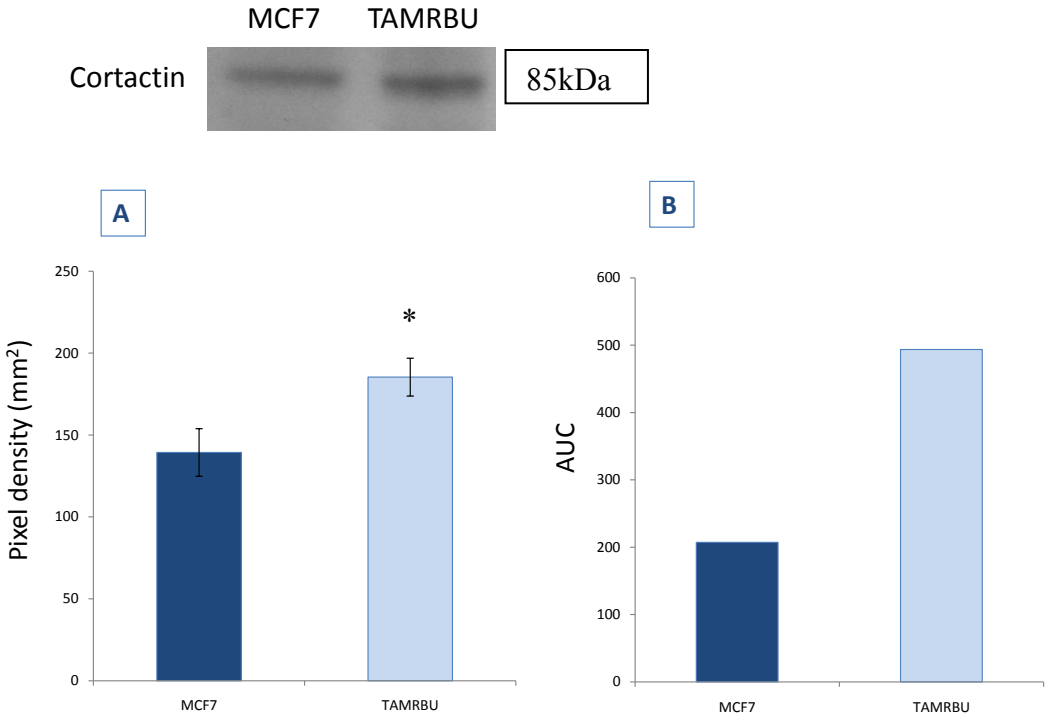


Figure 5-10 Composite showing A, the densitometry analysis of the Western blot (shown above chart) showing a 33% increase in TAMRBU pixel density (p=0.010 by students T test) when compared to MCF7. B, shows the comparison of the area under the curve (AUC) value for the quantitative peptides analysed in SILAC, this shows a 138.4% increase in the TAMRBU when compared to MCF7, there are no statistics available on the SILAC data as only two peptide pairs were found. * denotes statistical significance.

5.2.3 Talin-1

Talin-1 is a protein involved in motility and potentially involved in Akt signalling which is discussed further in section 5.3.1. The SILAC data showed a 3.22 fold change increase in TAMRBU cells. This was calculated from one quantifiable pair of peptides for the protein so no statistics could be used. When Western blots were used to validate the result, TAMRBU showed a 90.6% increase in expression when compared to the expression seen in MCF7 ($p=0.002$). Both SILAC and Western blots showed an increase of expression of Talin-1 in resistance.

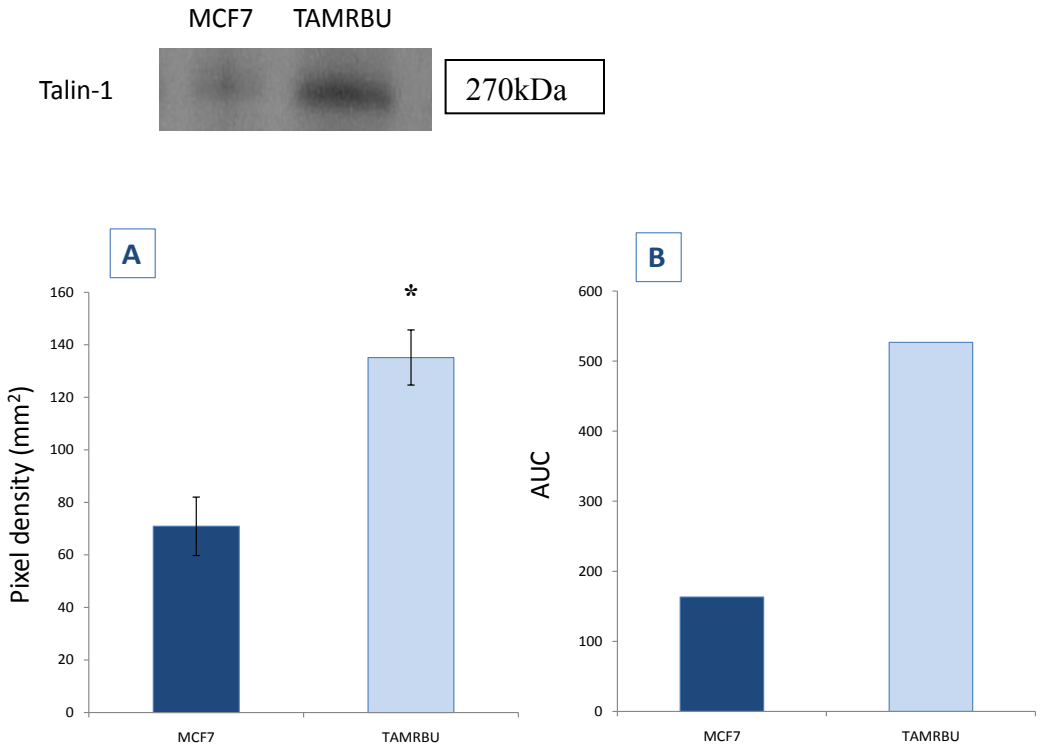


Figure 5-11 Composite showing A, the densitometry analysis of the Western blot (shown above chart) showing a 90.6% increase in TAMRBU pixel density ($p=0.002$ by students T test) when compared to MCF7. B, shows the comparison of the area under the curve (AUC) value for the quantitative peptides analysed in SILAC, this shows a 222.6% increase in the TAMRBU when compared to MCF7, there are no statistics available on the SILAC data as only one peptide pair was found. * denotes significance.

5.2.4 Ribophorin 2

The validation of Ribophorin 2 using Western blot analysis did not agree with the result from SILAC and therefore the result seen in SILAC that ribophorin was increased by 155.6% was not validated. Indeed, the Western blot analysis showed a statistically significant decrease in expression (48.6%, p=0.019).

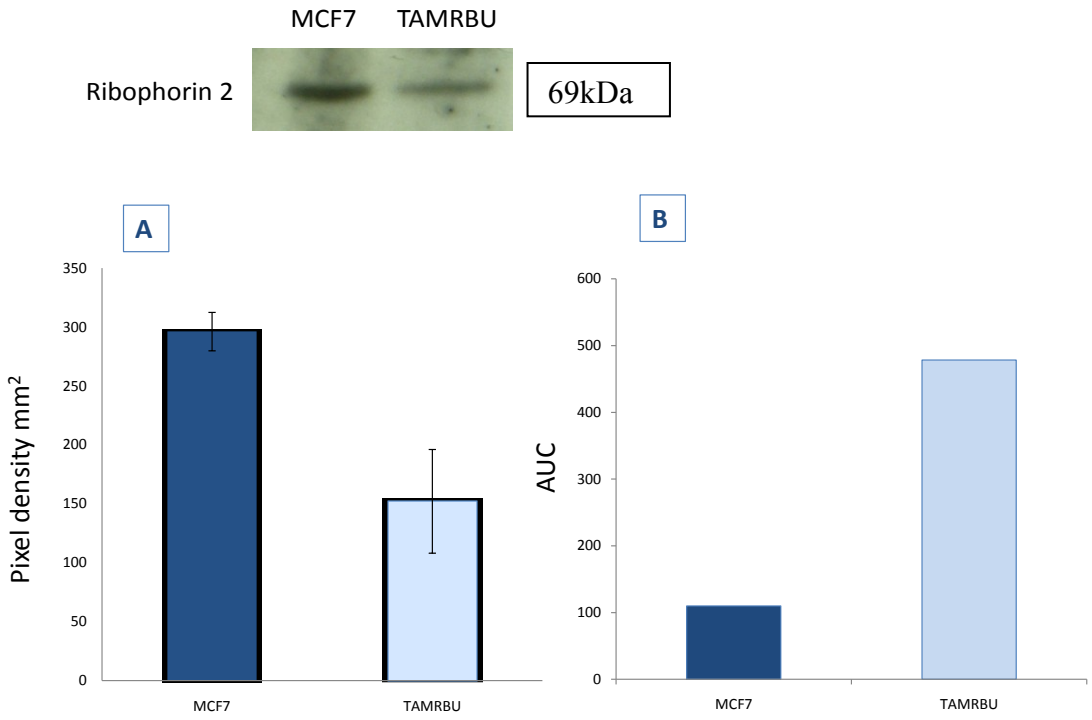


Figure 5-12 Composite showing A, the densitometry analysis of the Western blot (shown above chart) showing a 48.6% decrease in TAMRBU pixel density (p=0.019 by students T test) when compared to MCF7. B, shows the comparison of the area under the curve (AUC) value for the quantitativ eptides analysed in SILAC, this shows a 155.6% increase in the TAMRBU when compared to MCF7, there are no statistics available on the SILAC data as only two peptide pairs were found.

5.2.5 Cathepsin D

Cathepsin D and its potential role in Tamoxifen resistance is discussed in detail in section 5.3.2. Validation of the SILAC is shown in Figure 5-13; the TAMRBU showed a 37.6% decrease in Cathepsin D when compared to MCF7 cells. There was only one pair of quantifiable peptide pairs found for this protein and therefore statistics could not be performed. When Western blot densitometry analysis was performed it showed a 61.6% decrease in resistance (p=0.002). Thus both the SILAC and the Western blots show a decrease in expression.

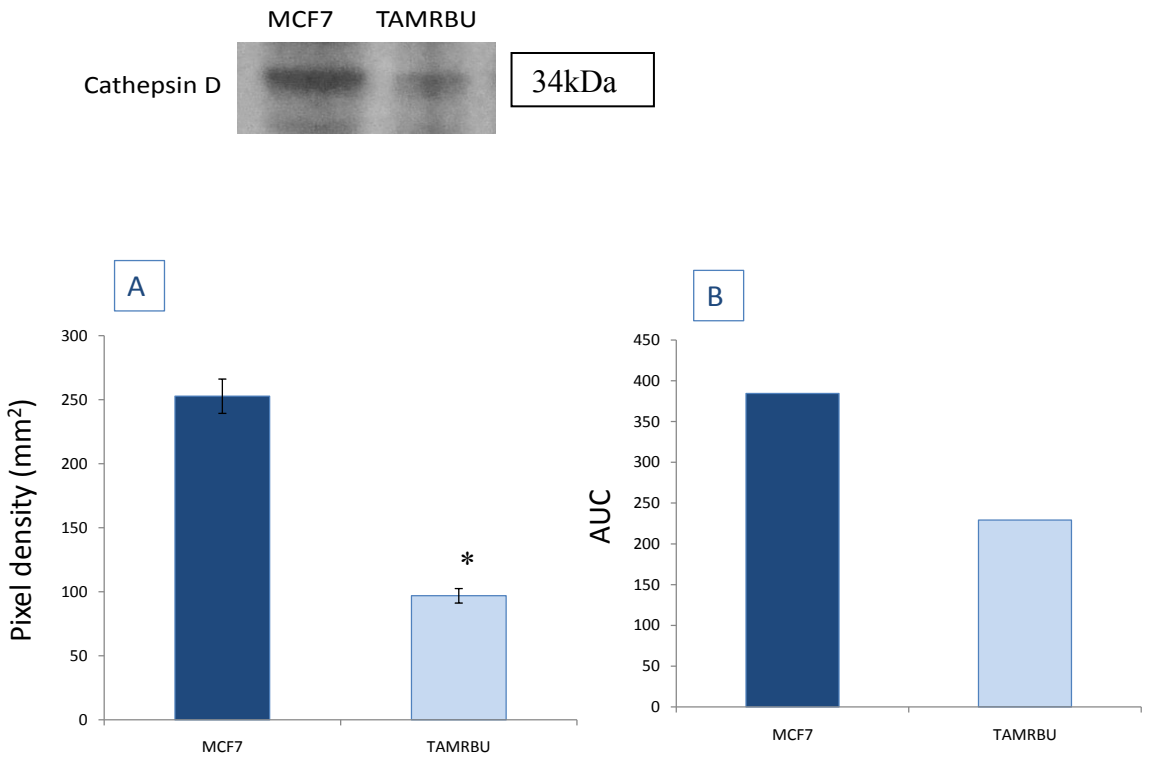


Figure 5-13 Composite showing A, the densitometry analysis of the Western blot (shown above chart) showing a 61.6% decrease in TAMRBU pixel density (p=0.002 by students T test) when compared to MCF7. B, shows the comparison of the area under the curve (AUC) value for the quantitative peptides analysed in SILAC, this shows a 37.6% decrease in the TAMRBU. There is no statistical data for B as there was only one pair of quantifiable proteins. * denotes statistical significance.

5.3 Discussion

The SILAC labelling approach that was used in section 5.1 is a technique used to investigate different protein expression profiles using relative quantitation. SILAC as a labelling strategy is a tool for the quantitative analysis of global differential protein expression, and when used in combination with differential MS analysis; provides us with a powerful tool to identify and quantify complex protein samples. The majority of proteins found were cytoplasmic. There were no receptors found, we know that many receptors have altered expressed and modulation in acquired Tamoxifen resistance, this can however be explained by the ability of the mass spectrometers that were used in the analysis. The way in which a mass spectrometer analyses proteins is outlined in section 1.1.2. Briefly, after fragmentation of the first ion it analyses the next most abundant and then the next, etcetera. The low abundant proteins may not be analysed as the time is taken up on the more abundant proteins in the sample which are analysed first. Newer instruments would be more sensitive as they are able to cycle more rapidly to reach less abundant peptides. The lysis buffer used on the SILAC labelled cells was identical to the lysis buffer used in preparation of lysates for Western blots; and large receptors such as EGFR were identified from the lysates using Western blot (Figure 3-5) indicating that the lysis protocol was not the issue. The use of a multi dimensional HPLC run may have produced a less complex sample for the mass spectrometer to analyse. However, as the SILAC experiment provided us with a large number of leads to follow without this extra step, it was deemed unnecessary for this study. It may provide more leads to follow in the future (Section 7.2).

There was no cross over between the proteins found in the arrays and proteins found via SILAC, potentially due to the arrays being focussed on low abundance proteins such as receptors which the mass spectrometer used in this study could not detect. Ideally, some proteins would have been detected by both methods, thus acting as internal standards and providing further confirmation of hits. As there was no cross-over, Western blots were used as further validation of the SILAC results (see section 5.2). The 5 proteins that had a Western blot carried out on them for validation purposes are highlighted in red, Figure 5-8.

The importance of performing validation of the SILAC results is paramount to confirm the data and is reported widely in the literature. The SILAC method provides an opportunity to identify novel changes that occur between two cell populations. There are limitations to the experiment in this study; it was only carried out on one occasion and only heavy lysine was used to label the peptides. More recent studies use dual labelling with arginine and lysine giving a better coverage of the proteins identified. However, arginine has been shown to convert to proline, potentially affecting the quantification if labelling with arginine only, as the basis of the heavy isotope incorporation relies upon cellular metabolism, there are methods available to decrease/prevent this conversion (Lossner et al., 2011).

A selection of proteins that showed differential expression between the MCF7 and the TAMRBU cells were chosen for further investigation in the literature, all proteins were chosen as they exhibited clear MS spectra and had quantitation scores that were the result of multiple peptide pairs (increased confidence), the quantitation scores were either above or below 1.5 fold change difference MCF7 to TAMRBU.

5.3.1 Over expressed proteins

BCL-2 athanogene-1: Fold change = 7.14

BCL-2 athanogene-1 (BAG1) is a pro-survival, multifunctional protein that can associate with steroid hormone receptors, such as ERα, and BCL-2 protein (described above in section 4.4.2) (Townsend et al., 2005). To this end, BAG1 is able to regulate oestrogen dependent transcription and also exhibits an anti-apoptotic action. It has the ability to directly interact with the chaperone protein HSP70 (which expression was shown in 4.4.6 to be increased in TAMRBU cells). HSP70 is rapidly induced by the cell during times of cellular stress which may support the many functions that BAG1 participates in (Linman et al., 2005). BAG1 has been shown to be frequently over-expressed in breast cancer (Anderson et al, 2010) in which it plays an important but contentious function. High expression of BAG1 has been implicated in poor outcome for patients in some studies (Tang, 2002) but others have failed to confirm this observation. The implication of a high level of BAG1 in acquired Tamoxifen resistance could be very important. However, some studies have contradicted our data; for example, Millar et al., 2009 showed that improved patient responsiveness to Tamoxifen and a better patient outcome was associated with a high expression of BAG-1, this seems counter-intuitive as the protein is a known pro-survival anti-apoptotic protein. Furthermore, Tang et al., 1999 showed that BAG-1 is over expressed in clinical samples of invasive breast carcinomas and the over-expression was associated with a shorter disease-free and decreased overall survival outcome of patients.

Calpain-1: Fold change = 3.44

Calpain-1 belongs to a conserved family of cysteine proteinases first described in 1964. The activity of this family has been implicated in several fundamental cellular processes such as signalling, migration, apoptosis and cell survival (Huang and Wang, 2001). The expression of calpain-1 is altered during tumourigenesis and proteolysis of substrates such as FAK and Talin (Talin was also found to be over expressed using SILAC), as well as proto-oncogenes such as Myc; showing that it may have a role in pathogenesis. It has also been suggested in the literature that Src and EGFR stimulated cell motility is dependent of the activation of the calpain family (Carrager and Frame, 2002). The calpain family have been implicated in the protein tyrosine phosphatase 1B and src mediated induction of invadapodia (Cortesio et al., 2008). It has also been revealed to be involved in cortactin (also over expressed in TAMRBU) mediated actin reorganisation and laemellipodia and pseudopodia formation at the leading edge of the cell (Perrin, 2006).

There has been recent evidence that the increased expression of Calpain-1 may influence the response to some therapies used in the treatment of cancer (Storr et al, 2011). For example, it has been associated with a poor patient response to Trastuzumab therapy in ErbB2 positive breast cancer patients. Moreover, it has been shown that the inhibition of calpain-1 modulates signalling in several tumour types in particular ErbB2 positive breast cancer (Kulkarni et al., 2010) and also prostate cancer where it facilitates signalling independent of androgen stimulation (Pelley et al 2006). Tamoxifen resistant cells show aberrant cell signalling and over express ErbB2 and here we have shown that calpain-1is also over expressed in the Tamoxifen resistant cells. Calpain activity has been revealed to be both pro-survival and pro-apoptotic, it is known to cleave caspases 10 and

7, and also Bcl-2 family members such as BAX. Evidence in the literature suggests that its survival activity is mediated through the p53 tumour suppressor and NF κ B. Calpain is able to cleave p53 subsequently preventing p53 mediated apoptosis (Pariat et al., 1997) and also promote survival through the activation of NF κ B. Calpain mediates the cleavage of the NF κ B inhibitor, IκBα, in response to tumour necrosis factor (TNF) (Han et al., 1999) and also through the activation of ErbB2 signalling via PI3K and Akt in breast cancer cells (Pianetti et al., 2001).The proteolysis of the inhibitor IκBα can be suppressed by the over expression of the endogenous, ubiquitously expressed inhibitor of calpain, calpastin (Chen et al., 2000). The roles that calpain-1 has in cancer can be conflicting, and its influence exerted may depend on its cellular location.

CLIC1: Fold change = 3.85

The chloride channel family are a varied group of proteins that are thought to be involved in many cellular processes such as maintenance of intracellular pH, the regulation of cell volume and the stabilisation of the cellular membrane potential (Averaimo et al., 2010). It is reported that the protein localises primarily to the cell nucleus and displays both nuclear and plasma membrane chloride ion channel activity. CLIC1 is a member of the p64 family (along with parchorin). They are widely expressed channels with a reported ability to auto insert and self assemble as intracellular anion channels with a potential function in apoptosis and secretion of signalling proteins (Ashley, 2003). This is a controversial report as the receptors exist as a soluble protein and a membranous form and the way in which they transform from one to the other is yet to be elucidated. A differential expression of CLIC1 has not previously been described in breast cancer or

Tamoxifen resistance. However, it has been shown to be up regulated in gastric carcinoma (Chen et al., 2007), colorectal cancer (Petrova et al., 2008), gall bladder cancer (Wang et al., 2009) and nasopharyngeal carcinoma (Chang et al., 2009). Interestingly, CLIC1 has been associated with increased expression of multidrug resistance protein (MDR) in gastric cancer patients being treated with chemotherapeutic agents (Chen et al., 2007). In endothelial cells the decreased expression of CLIC1 inhibited directed migration in wound healing assays (Tung and Kitajewski, 2010).

Cortactin: Fold change = 2.38

Cortactin is a src kinase substrate that is frequently over expressed in many types of cancer including; laryngeal cancer (Gibcus et al., 2008), colorectal cancer (Cai et al., 2010), head and neck cancer (Timpson et al., 2007) and breast cancer (Bowden et al., 2006). There are many reports that this increase in cortactin expression consistently correlates with a poor patient outcome by increasing the aggressive nature of the tumour. The way in which it does this is potentially by promoting the invasive nature of the cells, most likely through the formation of invadapodia and remodelling of the actin cytoskeleton but also it has been suggested that the promotion of metastasis is driven through the degradation of the extra cellular matrix by the cortactin expressing cancer cells (Clark et al., 2007).

Over expression of cortactin has also been implicated in sustaining EGFR signalling by preventing the ligand induced breakdown of the receptor in cancer (van Rossum et al., 2005) and an increase in cortactin expression is associated with resistance to gefitinib (EGFR antagonist) in head and neck carcinoma (Timpson et al., 2007).

Notably, the cortactin expression is up in the Tamoxifen resistant cells in this Thesis, as is the expression and activation of EGFR (Figure 3-5 and Figure 4-11).

The gene that encodes cortactin (EMS1) is frequently seen over expressed in breast cancers (Hui et al., 1998) and in cell lines (Campbell, 1996). EMS1 is on Chromosome 11q and this amplification is also seen in many cancers, with the region known to code for many oncogenes (including Cyclin D1). The increased expression of Cyclin D1 and its role in resistance has been reported in the literature (Kilker and Planas-Silva, 2006) and is often co-localised together with cortactin (Lundgren et al., 2008) and may even function synergistically.

One of the main functions of cortactin within the cell is in actin organisation. Arp2/3 is a molecule that is able to nucleate branched actin filament networks providing the cell with structural support as well as enabling vesicle trafficking and the persistence of protrusions such as invadapodia (Goley et al., 2006). The localisation of cortactin to such cellular protrusions raises the prospect of cortactin expression playing a role in motility in cancer. This has been investigated in a number of ways including transwell migration and wound closure assays, in conjunction with siRNA knock down of cortactin showing that cortactin over expression is closely linking to an increase in cell motility in cancer (Bryce et al., 2005 and Rothschild et al., 2006). This may be relevant as Tamoxifen resistant cells have a more metastatic phenotype (Hiscox et al., 2004) and will be investigated further in the next chapter.

Cortactin is also known to be post translationally modified by numerous protein kinases (src, ERK, met, syk and pak). When the N-terminal region of cortactin is phosphorylated by pak at position Ser113, it decreases its actin binding potential

(Martinez-Quiles et al., 2004). This raises the issue that phosphorylation of cortactin may be important to the functional role of the protein as well as its degree of expression. Further investigation of this, either through Western blotting or phosphoproteomics may provide more clues as to its function in Tamoxifen resistance.

Epithelial Cell Adhesion Molecule: Fold change 9.09

Epithelial Cell Adhesion Molecule (EpCAM) is an epithelial cellular adhesion membrane glycoprotein and is highly expressed in numerous carcinomas (Van de Gun, 2010) and frequently over expressed in human invasive breast cancer (Gostner, 2011) in which it has been reported to be a marker of poor prognosis (Gastl et al., 2000). It has been explored as a marker of cancer initiating cells (cancer stem cells) (Lugli et al., 2010) and has a controversial biological role, having been associated with increasing and also preventing metastasis and also acting as a tumour suppressor (Shiah et al., 2009 and Spizzo et al., 2011). The role of EpCAM in cancer needs further investigation to fully elucidate its role in the process of carcinogenesis although it is likely that expression, signalling of the protein and impact are linked to the stage, type, and possibly even the microenvironment. The expression levels of the EpCAM antigen shown by immunohistochemistry by Spizzo et al., 2002 correlated with a lower patient survival and this was decreased further when the patients also over expressed ErbB2 (as our cells do). There have been a number of trials of a monoclonal antibody (mAb) that targets EpCAM, one of which has recently been approved by the European Commission for the treatment of EpCAM positive patients (Schmidt et al., 2010).

A decrease of EpCAM has previously been associated with induction of epithelial mesenchymal transition (EMT) in non small cell lung and head and neck cancer cells (Frederick et al., 2007) however, this does not fit with our results as EMT like phenotype is displayed in Tamoxifen resistance cells (TAMR) (Hiscox et al, 2006) and also exhibited in TAMRBU cells but EpCAM is up regulated. In renal and thyroid cancer show up regulation of EpCAM to be associated with a better patient survival however in many other cancers (bladder, gall bladder, pancreas, ovarian, breast) increased expression of EpCAM is associated with a decrease in patient survival and in breast cancer it was also associated with large, less differentiated tumours, the presence of nodal metastasis as well as overall worse survival (Spizzo et al., 2004). Cimino et al., 2010 reported that there also was an increase in EpCAM in the metastasis site when compared to the matched primary site.

Hepatoma derived growth factor (HDGF): Fold change = 2.17

HDGF is a nuclear protein that has been shown to be mitogenic in a wide variety of cells (Yu et al., 2011). Its increased expression has been associated with poor prognosis in Nasopharyngeal carcinoma (NPC) (Wang et al., 2011) and also correlating with tumour progression, and poor patient disease free and overall survival in oesophageal cancer in Japan (Yamamoto et al 2007). Lee et al., 2010 showed that HDGF regulates the BAD mediated apoptotic pathway and the induction of VEGF secretion in gastric cancer cells. When the HDGF was silenced using shRNA pro-apoptotic BAD was induced and there was an inactivation of ERK, this in turn de-phosphorylated BAD at Ser112 and Ser136 and this induced apoptosis. Down regulation of HDGF led to a decrease in cell

proliferation and a less invasive phenotype in gastric cancer cells. These results would be in keeping of our observation of increased HDGF, increase in BAD (Figure 4-24) and increased ERK activity in TAMRBU cells (Figure 3-7). Recently, Guo et al, 2011 investigated the role of HDGF in breast and prostate cancer. They showed siRNA knock down of HDGF expression in MCF7 cells significantly decreased cell proliferation. This leads to the prospect that HDGF has a role in cell growth, invasion and avoidance of apoptosis all of which are known to be characteristic of resistance.

<u>High mobility group box 1:</u> Fold change = 3.57

High mobility group box 1 (HMGB1) also known as amphoterin is an architectural transcription factor, as it has the ability to bend DNA to regulate gene activity (Boonyaratanakornkit et al., 1998). It was shown to have increased expression in Tamoxifen resistant TAMRBU cells via SILAC in this Thesis. HMGB1 may be involved in EMT (Lynch et al., 2010) as HMGB1 expression induces morphological changes consistent with the EMT phenotype (decreased E cadherin and increased cell migration). This was seen in renal proximal tubular epithelial cells and may be induced, at least partly, by receptor for advanced glycation end products (RAGE) through the induction of TGFβ secretion. HMGB1 has also been implicated in the process of autophagy, a tightly controlled process that occurs ubiquitously and contributes to the routine turnover of proteins in the cytoplasm. It can be induced by changes in environmental conditions such as drug treatment and nutrient depletion. Paradoxically, the process has also been implicated in cancer serving to protect the cell and also contribute to cell damage (Dagenhardt, 2006 and Eisenberg-Lerner and Kimchi, 2009). Damage associated

molecular pattern (DAMP) molecules can initiate immune responses to damage, and also mediate autophagy contributing to drug resistance in cancer (Lui et al., 2011). HMGB1 is released in tumour cells after a dose of treatment cytotoxicity is induced. The release of HMGB1 activates autophagy through the PI3K-MEK-ERK pathway, in leukaemia and siRNA's directed at these signalling pathway reverses the drug resistance (Tang et al., 2010 and Yang et al., 2011). HMGB1 is a prototypical DAMP and subsequently acts on the DAMP receptors (i.e. RAGE) the sustained release of HMGB1 indicates that autophagy will also be sustained. (Lui et al., 2011). Interestingly, HMGB1 expression is regulated by transcription factors such as p53 and c-myc. It has DNA chaperone functionality allowing it to participate in fundamental processes such as DNA replication, transcription and repair. This is of particular interest as it is thought that HMGB1 enhances the transcription factors involved in the progression of cancer such as p53 and p73 and also modulates the nuclear hormone receptors such as ERα (Ohmori et al., 2011).

Increased expression of HMBG1 has previously been shown to have a role in which cells develop drug resistance/exhibit phenotype of drug resistance; increased invasion and metastasis (mediated through HMGB1, Matrix metallo proteinases (MMPs) and RAGE), sustained angiogenesis (activated through HMGB1 and VEGF secretion), insensitivity to antigrowth signals (increased HMGB1 increases expression of Cyclin D1), self sufficiency growth signalling (increased HMGB1 and Akt, MAPK and NFκB) and evasion of apoptosis (increased HMGB1, working through BCL-2 and c-IAPs) (Tang et al, 2010).

IQ motif containing GTPase activating protein: Fold change = 2.66

IQ motif containing GTPase activating protein **(**IQGAP1) is a ubiquitously expressed scaffold protein that contains multiple protein interaction domains enabling it to interact with many molecules and proteins – such as Rac1, cdc42 and ErbB2. Interestingly IQGAP1 has been reported to play a role in the progression of cancer, in particular cell motility and invasion (Mataraza et al., 2003 and Mataraza et al., 2007).

IQGAP1 more recently has been implicated in Trastuzumab resistance (White et al., 2011) who report that IQGAP1 is able to bind directly to the ErbB2 protein and is involved in signalling pathways associated with resistance. When over expressed IQGAP1 is knocked down, ErbB2 expression and phosphorylation is also decreased, causing a decrease in the proliferation that is often associated with breast cancer with over expressed ErbB2 receptors. When IQGAP1 expression was knocked down, p27 (a protein which binds to and prevents the activation of cyclins and subsequently controls the cell cycle progression at G1) is up regulated. White et al., 2011 showed that blocking this up-regulation reduces the growth inhibition caused by the knock down of IQGAP1, implying that IQGAP1 has a role in cell cycle progression in cancer. They also report that IQGAP1 was over expressed in trastuzumab-resistant breast cells, and the inhibition of IQGAP1 function restores functionality of Trastuzumab in ErbB2 over expressing cells. In this project we have shown that IQGAP1 is over expressed in TAMRBU cells, as is ErbB2 activity (Figure 4-11). In the next chapter we will investigate the effects of knocking down IQGAP1 with siRNA.

Phosphatidylinositol-3, 4, 5-trisphosphate-dependent Rac exchange factor 1: Fold change = 3.57

Phosphatidylinositol-3, 4, 5-trisphosphate-dependent Rac exchange factor 1 (P-Rex1) is an essential protein implicated to have a role in fundamental cellular functions such as growth and motility. It has also been revealed to play a role in tumourigenesis in breast cancer through ErbB receptors (Sosa et al., 2010). It is an essential mediator of the small GTPase Rac1 and its mediators and effector proteins are established as mediators of mitogenic and motile signalling by RTKs in breast cancer (Sosa et al., 2010). Moreover, the over expression of P-Rex1 seen in our TAMRBU cells is also mirrored in the majority of human breast cancers and breast cancer derived cell lines (Montero et al., 2011). Qin et al., 2009 identify an increased expression of P-Rex1 in prostate cancer which promotes metastasis. There has been development of therapeutic inhibitors (Sosa et al., 2010 and Vigil et al., 2010) which could work well for patients with acquired Tamoxifen resistance, as a pharmacological inhibitor of Rac1 has been shown to reverse Tamoxifen resistance in resistant cell lines (Felekkis et al., 2005).

Protein phosphatase 1 gamma: Fold change = 1.63

Protein phosphatase 1 gamma (PP1 γ) is a ubiquitous serine/threonine phosphatase that is involved in regulating many cellular processes including protein synthesis and glycogen metabolism; it also has an essential role in cell division. PP1γ has been shown in the literature to be over expressed in invasive DCIS when compared to mammary dysplasia and fibroadenoma, the same research group also implicated PP1γ in the increased proliferation in breast cancer cell lines (Sogawa et al., 1997). As we have shown

PP1γ was increased in TAMRBU cells using SILAC this change could be associated with an increased metabolic activity required for increased proliferation.

It has also been show to be responsible for the de-phosphorylation of Histone H3 after DNA damage (Shimada et al., 2010) and it has been shown to play a protective role in vascular smooth muscle cells against oxidative stress induced apoptosis. This apoptotic process begins with an increase in ROS (mitochondrial), leads to caspase 3/7 activation and DNA fragmentation, this decreased mitochondrial membrane potential in the cells and PP1γ expression and activity is decreased. When the PP1γ expression was decreased using siRNA it led to an increase in apoptosis, conversely, the over expression of PP1γ protected against apoptosis by attenuating the caspase 3/7 activation (Tchivilev et al., 2008).

Prohibitin: Fold change = 2.00

Prohibitin is a multifunctional protein and is implicated to have roles in proliferation, transcription and apoptosis (Theiss and Sitaraman, 2011). Prohibitins are mainly located in the mitochondria but can also be found in the nucleus (McClung et al., 1995). Prohibitin has been shown to interact with PI3K/Akt and transforming growth factor-β (TGF-β) as well as Ras/MAPK/ERK (Rajalingham and Rudel, 2005 and Mishra et al., 2010). The proteins that prohibitin interacts with have all been implicated to have a role in Tamoxifen resistance. He et al., 2011 showed that prohibitin is regulated by oestrogen in murine uterine cells. When prohibitin expression was down regulated using shRNA in HELA cells there was a significant decrease in the rate of cell division which led to the degradation of a fusion protein (OPA1) which is a Dynamin-related GTPase required for

mitochondrial fusion and regulation of apoptosis. The morphology of the mitochondria did not alter; however, degradation of OPA1 reduced the ability of some cells to adhere to ECM and showed a decreased capability of anchorage independent growth (Sievers et al., 2010).

Proliferation associated protein 2G4: Fold change = 3.33

Proliferation associated protein 2G4 also known as ErbB3 binding protein 1 (EBP1) was increased in TAMRBU cells. The gene PA2G4 encodes for the EBP1 protein which is often associated with growth regulation of cells but also may be involved in ribosomal assembly and RNA processing. The increased expression of this protein in Tamoxifen resistance is surprising as it has been previously shown to have decreased expression in Tamoxifen resistance (Lu et al, 2011) and in hormone therapy refractory prostate cancer (Zhang et al., 2008). In effect, the result seen in our SILAC experiment is opposite to that seen in the literature.

The literature describes EBP1 as a tumour suppressor, and also suggests that there is an important interaction with EBP1 and ErbB2 and 3 which contributes to the signalling that regulates growth. As has been discussed in section 4.5.2, Erb signalling in breast cancer plays a critical role in its progression. It has been reported that EBP1 inhibits EGF stimulated breast cancer growth, contributing to Tamoxifen sensitivity by decreasing the expression of ErbB2 (Lu et al, 2011). It was also shown that expression of EBP1 accelerated the rate of ErbB2 degradation and that this process could be reversed by inhibiting proteosome action (Lu et al, 2011) Furthermore, knock down of EBP1 led to Tamoxifen resistance which could be abrogated by inhibition of ErbB2 activity.

In TAMRBU, the SILAC experiment showed that EBP1 was over expressed but in TAMRBU ErbB2 signalling was also over expressed (Figure 4-11). EBP1s effect on Tamoxifen sensitivity is mediated by its ability to modulate ErbB2. Perhaps in TAMRBU cells this modulation is avoided/ altered. However, this was not one of the 5 proteins selected for validation by Western blotting and did not feature on the antibody arrays used in this Thesis. As illustrated by the results from Ribophorin 2 in this project (Figure 5-12) it is possible to get peaks from peptides which do not agree with results from Western blotting. The variation may be due to the mass spectrometer detecting peptides in a different region of the protein to that that is targeted by the antibody.

Ribophorin 2: Fold change = 4.35

Ribophorin 2 is a member of a ribosome receptor family of proteins that are associated with binding of ribosomes to the rough endoplasmic reticulum and as such is directly linked to the translation of proteins. There is not a large amount of literature regarding Ribophorin 2 in cancer. However, one report implicates Ribophorin 2 in docetaxel resistant breast cancer. The siRNA treatment of Ribophorin 2 decreased protein levels and caused a decrease in glycosylation of P-glycoprotein, conferring sensitivity to the MCF7 –ADR (Docetaxel resistant cells) in the presence of the drug (Honma et al., 2008). Interestingly in our study Ribophorin 2 was found to be over expressed in the TAMRBU cells via SILAC but in Western blot validation it was down regulated. As the two techniques are identifying different regions of the protein this could be accounted for by detection of degradation products or simply illustrates the need for cross validation of the SILAC results.

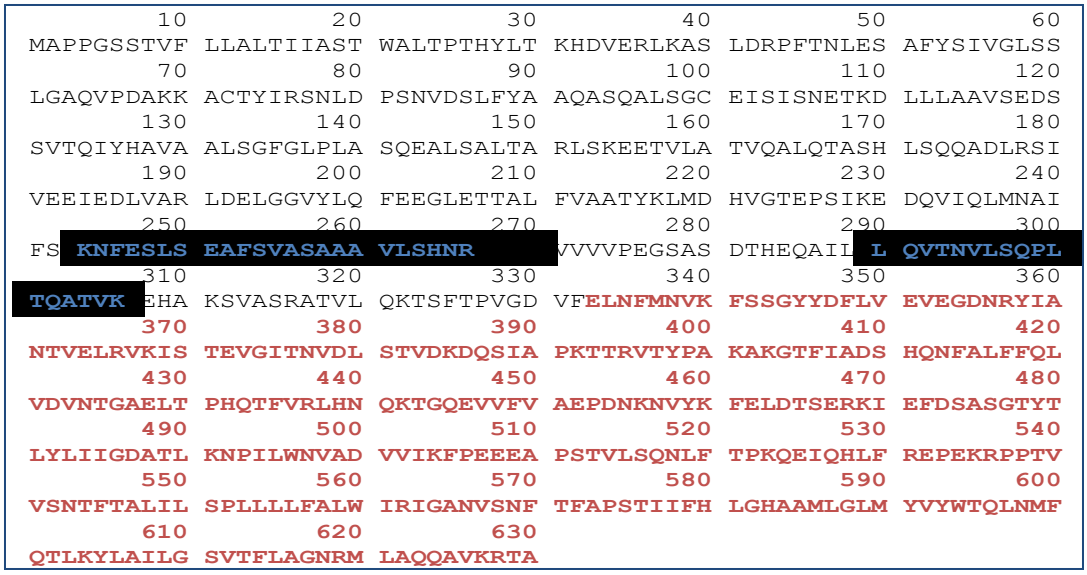


Figure 5-14 Peptide sequence of Ribophorin 2. The region of the protein highlighted in red is targeted by the antibody. Peptides in blue were found via SILAC.

<u>Staphylococcal nuclease domain-containing protein 1:</u> Fold change = 3.44

Ho et al., 2009 investigated novel breast cancer metastasis associated proteins found through the use of an iTRAQ labelled ESI MS approach on a panel of cells which was then validated using tissue microarrays; SND1 protein was more highly expressed in metastatic breast cancers. Higher expression of SND1 mRNA has also been reported in colon cancer (Tsuchiya et al., 2007) correlating with increasing grade and aggressiveness of the cancer. Its higher expression in TAMRBU cells would therefore be in keeping with the literature and this more aggressive phenotype.

<u>Talin-1</u>: Fold change = 3.22

Talin-1 is a focal adhesion complex protein that regulates interactions between intergrins and the extracellular matrix (ECM). This interaction has been implicated in many

functions such as regulation of cell signalling and cell adhesion regulated by cytoskeleton dynamic organisation. Some intergrins have been implicated in tumour invasion and also in resistance to cisplatin therapy in oral carcinomas as were intergrin effectors proteins such as p130cas, src and talin (Sansing et al., 2011). Knock down of the effector proteins in this study showed that they were required for invasion through Matrigel. Furthermore, decreasing talin expression increased cisplatin resistance, inhibited spread on collagen and laminin, and reduced cell proliferation (Sansing et al., 2011). Talin-1 over expression has also been reported in prostate cancer where it enhanced migration and invasion, the ability to avoid anoikis (attachment mediated programmed cell death) and increased cell adhesion (Sakamoto et al., 2010).

Talin-1 has also been reported to interact with Akt signalling through phosphorylation by focal adhesion complex proteins such as focal adhesion kinase (FAK) and src. FAK has been shown as over expressed and have increased activation in many cancers, correlating with invasion and metastasis (Gabarra-Niecko, 2003) and has been implicated in Tamoxifen resistance (Hiscox et al., 2011).

5.3.2 Proteins down regulated in resistance

α Actinin 4: Fold change = - 1.6

α Actinin 4 is a protein that is ubiquitously expressed and maintains cellular morphology and structure by cross linking actin filaments. It is also able to interact with nuclear receptors which is mediated by the LXXLL (nuclear receptor binding motif) functional motif, which allows it to interact with ERα, and the over expression of α Actinin 4 potentiates ERα mediated transcription thought to be in part through an association with

HDAC7 promoting the proliferation of MCF7 cells (Khurana et al., 2011). Knock down of α Actinin 4 has been shown to down regulate ERα target gene expression which may infer a role in Tamoxifen resistance, as in TAMRBU this protein was shown to be decreased (Khurana et al., 2011). This down regulation of ERα target gene expression may have an effect on the cells ability to bypass the oestrogen proliferative stimulus by using alternative signalling pathways to increasingly proliferate and grow. However, location could be a key factor in the regulation of this protein as Honda et al., 1998 showed that α Actinin 4 when localised in the cytoplasm is associated with an infiltrative histological phenotype correlating to significant poor prognosis of breast cancer patients (Honda et al., 1998). This paper suggests a role for cytoplasmic α Actinin 4 in the regulation and organisation of the actin cytoskeleton and cell motility.

ATPase family gene 3, yeast like protein 2: Fold change = - 4.16

The gene encoding ATPase family gene 3, yeast like protein 2 (AFG3L2) was first identified by Banfi et al., 1999 and was thought to be a novel paraplegin-related gene and was reported to have a similar expression pattern to paraplegin. The protein is also known as paraplegin like protein. It is a multi-pass membrane metalloprotease and it contains one AAA (ATPase associated with diverse cellular activities) domain. There is minimal literature on this protein but it is thought to be involved in signal transduction and potentially chaperone like activities and has been shown to form a complex with paraplegin in the mitochondria, potentially regulating the quality of protein by degrading the misfolded proteins and regulating ribosome assembly. This appears to be the first report to associate AFG3L2 protein with cancer. However, it has been previously shown

to be involved in ataxia; a misssense mutation in the proteolytic domain accounts for approximately 1.5% of European ataxias (Cagnoli et al., 2010).

ALG-2-interacting protein X: Fold change = - 1.5

ALG-2-interacting protein X (ALIX) or Programmed Cell Death 6 Interacting Protein (PCD6IP) is a protein that has been implicated to have many roles in the cell. It has been shown to regulate intergrin mediated cell adhesions and extra cellular matrix assembly (Pan et al., 2008). In Hela cells increased expression of ALIX promoted flattening and alignment of cells, and a decrease in expression produced the opposite effect (Cabezas et al., 2005). A decrease in ALIX expression is seen in the TAMRBU cells and this may be related to the more disorganised growth patterns seen in the Tamoxifen resistant cells (Figure 3-2). This protein is hypothesised to act as a switch between cell death, and cell proliferation (Krebs and Klemenz, 2000) found that phosphorylation of ALIX allowed binding of ALG-2 (a calcium ion binding protein involved in apoptosis) and subsequent recruitment of SETA (SH3 domain-containing adapter molecule) formed a complex which promoted cell survival by avoiding the inhibition of PI3K and activating Akt. It has been inferred that ALIX cooperates with ALG-2 to promote apoptosis, and that an increase in ALIX expression increases cell death (Blum et al., 2004 and Hemming et al., 2004) possibly by an ALIX regulated caspase activation and apoptosis (Mahul-Mellier et al., 2006). The decreased expression of ALIX seen in TAMRBU cells may be part of the process by which the resistant cells avoid apoptosis in resistance. As phosphorylation and binding of ALG-2 also stops an apoptotic signal providing a way of avoiding cell death

further investigation of the phosphorylation status of this protein could add extra functional information and would be interesting to investigate further in the future.

Another role for ALIX is in the negative regulation of growth factor receptor endocytosis. Endocytosis is the first step to long term down regulation of activated EGFR and precedes the delivery of the receptor to the lysosomes via the multi vesicular body (MVB). The C terminus of the receptor undergoes ubiquitination, which triggers the endocytic process. As EGFR expression and activation has a role in Tamoxifen resistance ALIXs involvement in the turnover of the EGFR could be very important. Schmitt et al., 2004 showed that over expression of ALIX produced a drop in EGFR internalisation and siRNA transient knock down of ALIX enhanced the endocytosis of EGFR. This may seem counter intuitive as it implies that the decrease in ALIX seen in TAMRBU cells would increase of endocytosis of EGFR; however, this could infer that there is a greater turnover of the receptor.

Calponin-2: Fold change = -2.32

Calponin -2 has been shown to have a role in the regulation of cell proliferation, migration, and vascular construction in cancer cells (Wu and Jin, 2008). Some studies have implicated this protein in the growth and prognosis of cancer for example Dang et al., 2006 showed that an increase in CNN2 (the gene encoding the calponin-2 protein) was associated with skin cancer. However, this is the opposite of what we saw in our SILAC experiment where there was a decrease in calponin-2 expression.

This protein is thought to play a role in cell adhesion and actin remodelling (it can bind actin, calmodulin, troponin and tropomysin). Calponin-2 shows a broader tissue

expression than that of calponin 1 and 3. This protein has been shown to bind ERK1 and 2 through a calponin homology (CH) domain. ERK1 and 2 have increased expression and activation in Tamoxifen resistance (Figure 3-6) and the binding of calponin-2 to the MAPKs suggest that it may have a role in ERK signalling (Rozenblum et al., 2008).

Cathepsin D: Fold change = -1.6

Cathepsin D is a ubiquitously expressed lysosomal proteinase implicated in proteolytic degradation, cell invasion and apoptosis (Steinfeld et al., 2006). Evidence in the literature seems to be contradictory, predicting good clinical outcome or associated with poor prognosis. Increased expression of Cathepsin D has been associated with an increased invasive and metastatic phenotype in breast cancer (Ohri et al., 2007 and Berchem et al., 2002). Its role in Tamoxifen resistance has also been investigated (Long and Van den Berg, 1996) showing a decrease in expression and secretion of cathepsin D was associated with acquisition of Tamoxifen resistance and/or oestrogen independent growth (in both the pro-cathepsin form and the mature form) This is in agreement with what is reported in this Thesis as the TAMRBU showed a decrease in expression of cathepsin D. An increase in expression has also been shown clinically in breast cancer to correlate with node negative, progesterone receptor positive tumours which show a favourable response to Tamoxifen (Ferno et al., 1994) which correlates with the decrease in cathepsin D in Tamoxifen resistant cells seen in the SILAC experiment although a causative role was not established for deregulated Cathepsin D in this Thesis.

Centaurin δ–3: Fold change = -1.56

This protein is also called ARAP3 and is a multi-modular signalling protein that is a substrate of src family kinases. It was recently shown (Gambardella et al., 2011) that with a transient but complete knockdown of ARAP3 in murine neutrophils the cells are activated and exhibit increased β2 Intergrin affinity and avidity, these cells also showed a hyper response to adhesion response stimulus which was also demonstrated under flow conditions. If the same was seen in epithelial Tamoxifen resistant cells, this could implicate the decrease in ARAP3 in the metastatic potential of the cells. A decreased expression of ARAP3 has also been recently reported in gastric carcinoma (Yagi et al., 2011) which implicates its involvement in cancer. Many phospho-tyrosine activated proteins and receptors and src substrates (such as cortactin - see section 5.3.1) are up-regulated in cancer often more so in later/more aggressive stages of the disease (Yeatman et al., 2004) the opposite is true for ARAP3 in gastric cancer, it shows medium/high expression in normal gastric mucosa and the expression is decreased in poorly differentiated carcinomas which is similar to the result seen in here in Tamoxifen resistance which has a more aggressive phenotype.

Expression of ARAP3 inhibits cell-extracellular matrix attachment and invasion in vitro (Yagi et al., 2011). ARAP3 is also thought to suppress PI3K effector proteins (Figure 5-15) which link ARAP3s expression to the regulation of actin cytoskeleton, the formation of actin stress fibres, cell spreading and the formation of lamellapodia (Krugmann et al., 2001 and Krugmann et al., 2006). PI3K mediates a wide range of

activities in the cell including cell proliferation; motility and survival (see Figure 5-15) its expression has also been associated with angiogenesis (Raajimakers et al., 2007).

ARAP3 also undergoes tyrosine phosphorylation in response to EGF and PDGF stimulus; this is most likely to involve src-kinases (Frame, 2002) and could have implications for Tamoxifen resistance however, the phosphorylation of ARAP3 was not investigated in this Thesis, though this may provide interesting information if looked at in the future.

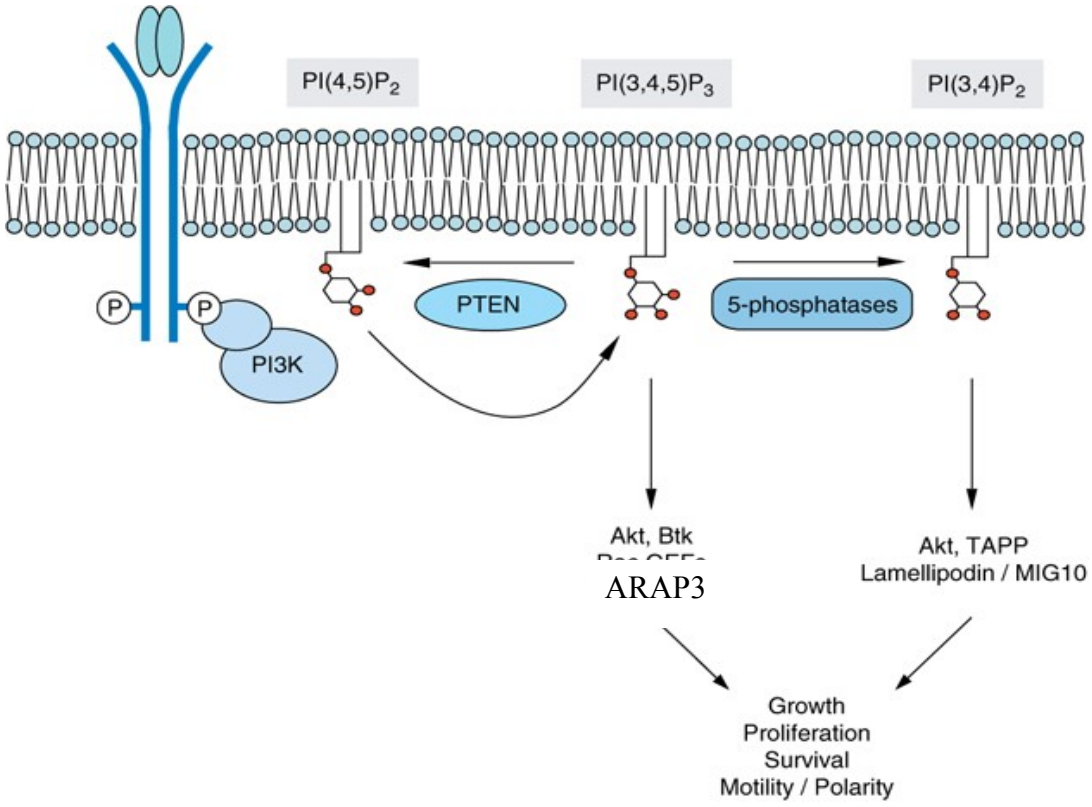


Figure 5-15 Shows a model of the phosphoinositide 3-kinase (PI3K) dependent signalling. The synthesis of PI(3,4,5)P3 from PI(4,5)P2 through the phosphorylation of the inositol ring at the 3rd position by PI3K. PTEN catalyses the conversion of PI(3,4,5)P3 back to PI(4,5)P2. Other proteins such as SHIP (member of the 5-phosphatases family) convert PI(3,4,5)P3 to PI(3,4)P2 which both share downstream

signalling effects increasing growth, proliferation, survival and motility. ARAP3 is an effector molecule of PI(3,4,5)P3. Adapted from Leslie et al., (2008).

<u>Cytochrome c1</u>: **Fold change = - 1.5**

Cytochrome c1 is a molecule found in the mitochondrial respiratory chain (RC) (see Figure 5-16). The RC can be affected when cancer cells become hypoxic and switch from oxidative phosphorylation to glycolysis which is often seen in both early and later stages of tumour growth and is the result of the cancer needing to acquire more energy in order to grow (Gatenby and Gilles, 2004).

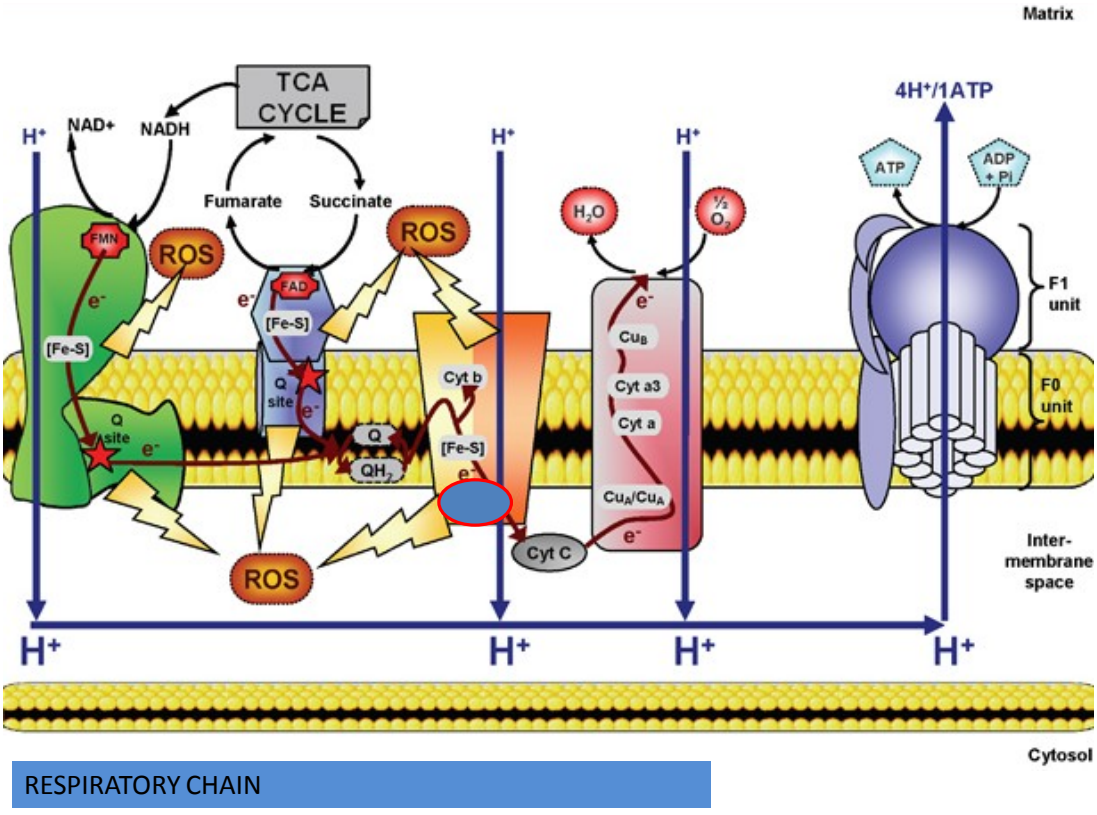


Figure 5-16 Figure shows the mitochondrial respiratory chain (RC) and the complexes provide energy through ATP production. Cytochrome c1 is circled in red and is has a lower expression in TAMRBU cells. Adapted from Lamarie and Grimm (2011).

This shift is mediated by an up regulation of hypoxia inducible factor 1 α (HIF1α) which the work presented in this Thesis has shown to be statistically increased in Tamoxifen resistant cells (see section 4.4.5). There may also be a role for cytochrome c1 in activating apoptosis as its downstream binding partner is apoptotic protease activating factor (APAF-1). APAF-1 is known to interact with caspases and Bcl like 1 protein (see Figure 1-13). When cytochrome c1 is bound to APAF-1 it forms an apotosome which is a catalytic multi-protein platform that activates caspase-9 most likely through autocatalysis (Martin et al, 2005). There is less cytochrome c1 seen in the Tamoxifen resistant cells this may be related to the way in which the cells avoid apoptosis.

Ezrin-radixin-moesin binding phosphoprotein: Fold change = 5.55

Ezrin-radixin-moesin binding phosphoprotein (EBP-50) showed one of the largest decreases in expression seen in the Tamoxifen resistant cells. It is also known as Na_2/H_2 exchanger regulatory factor 1 (NHERF1). It is a scaffold protein that recruits membrane receptors and transporters and cytoplasmic signalling proteins forming functional complexes and interestingly has been shown to interact with many proteins involved in cancer progression including phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Takahashi et al., 2006), EGFR (Lazar et al., 2004) and β – catenin (Kreimann et al., 2007). Research suggests that EBP50 may have a tumour suppression role in breast cancer (Song et al., 2007), and in cell lines its up regulation correlated with a lower, less invasive tumour grade (Schindelmann et al., 2002). Over expression of EBP50 has been

shown to increase apoptosis, inhibit cellular growth and reduce the level of ERK1 and 2 activation in MDA—MB- 231cells (an aggressive ER negative cell line) (Belizzi et al., 2010). The role of EBP50 as a regulator of cell growth is however controversial as it has also been shown that an increase in EBP50 expression correlated with poor patient prognosis and high tumour staging (Belizzi et al., 2010) yet Pan et al., 2006, transiently knocked down the expression of EBP50 in MCF7 cells and found that it enhanced cell growth. A large decrease in EBP50 has been shown here in TAMRBU compared with the parental MCF7, for the first time. It could be postulated that there is a role for EBP50 in Tamoxifen resistance potentially acting through the ERK/ MAPK pathway and the avoidance of apoptosis, further investigation would have to be done to corroborate this.

FAM49B: Fold change = - 6.66

There is little information available on this protein and its role in cancer. The human protein atlas (Colwill et al., 2011 through Sigma-Aldrich UK website) shows weak immunohistochemistry (IHC) staining in normal glandular breast tissue whilst there was moderate staining in breast cancer tissue. Its expression was also looked at in MCF7 cells, where weak staining was established. There is no literature suggesting any role for this protein in breast cancer or Tamoxifen resistance which makes this an interesting protein to have identified, especially since it was one of the more decreased expressions seen in the TAMRBU cells. FAM49B is known to be able to interact with inhibitor of Kappa light polypeptide gene enhancer in B cells kinase epsilon (IKBKE) which is an oncogene amplified in 30% of breast cancers (Hutti et al., 2009).

Lamin B1: Fold change = - 2.32

The lamin family of proteins are thought to provide a platform for the binding of proteins and chromatin (Dechat et al., 2009). They have been implicated in various roles within the cell in particular roles such as DNA repair and genome organisation and stabilisation. Lamin B1 specifically is a protein kinase C (PKC) binding protein (Tabellini et al., 2002) which makes this an interesting finding as if it could be involved in PKCδ signalling (increased activation of which is seen in Tamoxifen resistance, see Figure 3-9). Lamin B1 is essential for cell survival and is expressed in most cells and expression of lamins is usually found to be decreased in carcinomas for example Moss et al., 1999 showed that there was a decrease in nuclear lamin in neoplasm's of the gastro-intestinal tract, but has been shown to be increased in some such as prostate cancer (Coradeghini et al., 2006).

Lamin B1 has been implicated in numerous cellular processes including apoptosis, most likely mediated through the Bcl-2 pathway as Bcl-2 prevents apoptosis in part by stopping lamin B1 degradation (Mandal et al., 1996). An avoidance of apoptosis seems to have a role to play in Tamoxifen resistance (see section 4.4) and this protein found in SILAC, is an interesting finding and would require further investigation.

Nuclear migration protein nudC: Fold change = - 4.3

Nuclear migration protein nudC (NudC) has chaperone activity with no catalytic activity; there is some evidence in the literature that it may have a poorly defined role to play in cellular division and proliferation. Zhou et al., 2003 showed that NudC was essential for

cytokinesis, which is the last stage of mitosis where cells pull apart. The Human Protein Reference Database (HPRD--http://www.hprd.org Kesheva Prashad et al., 2009) has established that NudC is a cell cycle control protein, which is involved in communication and signalling within the cell. It has also been suggested that NudC has a role in the microtubule movement of the nucleus by association with dynein (a microtubule motor protein) potentially interacting with components of the dynein/dynactin complex. Microtubules play a central role in the coordination of a multitude of cell functions and can be targeted for therapy. NudC is seen in prostate cancer cells and it's over expression led to a block on cell division (Lin et al., 2004). A decrease of NudC expression is observed in the TAMRBU cells and this could potentially correlate to this finding as the population of resistant cells have increased cell proliferation (see section 3.2.4).

SET: Fold change = - 2.08

SET is an onco-protein, also known as PHAPII, which has a role in multiple cellular functions including metastasis, migration, apoptosis, and nucleosome formation. The majority of the literature available indicates that increased expression of SET protein is associated with cancer progression and more proliferative cells, for instance in ovarian cancer (Ouellet et al., 2006). The SET protein affects multiple cellular processes, in particular inhibiting the tumour suppressor protein phosphatase 2A (PP2A) (Switzer et al., 2011). However, the protein is decreased in the TAMRBU cells. This result would correlate with the observation by Fukukwa et al., 2005 that SET negatively regulates the MEK/ERK pathway. They showed that siRNA depletion of SET resulted in enhanced cellular proliferation via the enhanced activation of ERK. When SET expression was

decreased using siRNA the cell cycle was altered. The cells in G_1 phase of the cell cycle (where the cells increase in size) was decreased but there was an increase in S phase (where DNA replication takes place) indicating that SET also negatively regulates G_1/S transition (see Figure 1-12 for basic cell cycle).

Vigilin: Fold change = - 2.27

In human cells vigilin functions as part of a complex of proteins involved in RNA binding and translation factors and has been implicated in translocation of the complex to the cytosol from the nucleus (Kruse et al., 2003). It has also been suggested that vigilin is induced by oestrogen (Dodson and Shapiro, 1997) where it binds to the 3' untranslated region (3'UTR) of oestrogen stabilised vitellogenin mRNA, where vigilin may have a role in the hormonal control of mRNA metabolism. It was recently found that a decreased expression of vigilin conferred an increase in the expression of c-fms (macrophage colony-stimulating factor receptor) at the protein and mRNA level (Woo et al., 2011). This is relevant as the Tamoxifen resistant cells have a decreased expression of vigilin observed here and the increased phosphorylation of macrophage colony-stimulating factor receptor (see section 4.3.10).

Woo et al., 2011 also propose that vigilin and ELAV-like protein 1 (HuR) compete to bind to the same 69 nucleotide sequence in the 3'UTR of the c-fms mRNA. This dynamic relationship between the ratio of vigilin and HuR affects the ability of these proteins to effectively associate with the mRNA, which in turn regulates the expression of c-fms which is then phosphorylated. The study also showed a correlation between HuR and vigilin expression when motility and invasion in breast cancer cell lines were

investigated; the protein vigilin is shown to suppress cell motility and invasion this may link to a tumour suppressive role of vigilin and one could hypothesise that the decreased level of vigilin seen in the TAMRBU cells be linked concurrently to the increased activity seen in the M-CSF receptor (Figure 4-20).

The importance of validating results from SILAC is widely reported in the literature, our validation blots are shown in Figure 5-9, Figure 5-10, Figure 5-11, Figure 5-12and Figure 5-13. The need to validate can be seen in this thesis as the SILAC data shows that the expression of ribophorin 2 is increased, when this was validated used Western blots to be decreased (Figure 5-12). This could be due to the antibody corresponding to another epitope of the peptide to what was found in the SILAC sample as shown in Figure 5-14.

SILAC provides a straight forward method, depending on cellular metabolism to incorporate the isotopes into the proteins. It can be used to generate large amounts of data which can subsequently lead to a more hypothesis driven investigation.

6 Functional assessment of IQGAP1 and Cortactin

Functional assays are very useful, in combination with siRNA knockdowns, to analyse the role of proteins found to be differentially expressed by proteomic methods. The siRNA used in this study were silencer select validated siRNA from Ambion. They have been verified by Ambion experimentally to reduce the target gene expression by at least 80% 48hours after transfection. Where negative control siRNA was used it was silencer select negative control #1 siRNA from Ambion or where this was not available the NeoFX transfection agent was used as a control. In this study they have been used to determine the functionality of IQGAP1 and Cortactin, two proteins identified by SILAC in the mechanisms of Tamoxifen resistance. These proteins were chosen for further investigation as they both have potentially interesting roles in progression of cancer but neither has previously been reported in the literature as having a role in Tamoxifen resistance. The proteins had been successfully validated by Western blot in section 5.2. Therefore assays to investigate their role in adhesion, proliferation, and invasion were utilised in this chapter to assess if a role in resistance to Tamoxifen is apparent.

The role of adhesion in cancer is a contentious one; up regulation and down regulation both being associated with an increase in cancer progression. In order to progress there must be changes in the adhesive properties, as the cells must have the ability to detach from the primary tumour site in order to metastasise to a secondary site. However, the cell must then be able to attach to the site of the secondary cancer. A change in the adhesive properties has been established in Tamoxifen resistance (Hiscox et al., 2007). Increased invasion is generally accepted as a mark of more aggressive/

metastatic phenotypes as cells have to be able to invade through the basement membrane in order to metastasise. Increases in proliferation are seen in more aggressive cancers. These phenotypic behaviours are all described as "hallmarks of cancer" (Hanahan and Weinberg, 2000).

6.1 Functionality of IQGAP1 in Tamoxifen resistance

6.1.1 siRNA & Western blot

siRNA was used to transiently knock down the expression of IQGAP1 using the method described in section 2.5.4. Figure 6-1 shows the knock down of IQGAP1 in both MCF7 and TAMRBU cells. The effect of Tamoxifen presence was also shown as it further decreases the expression of IQGAP1. Beta actin was used as a loading control.

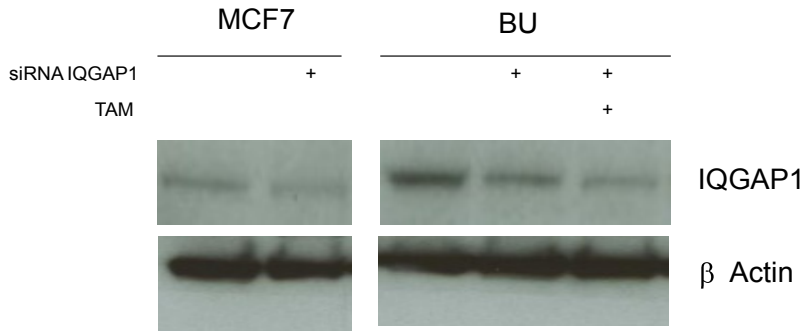


Figure 6-1 Composite of the Western blot performed to determine extent of decreased expression when the siRNA IQGAP1 had been used (48 hours).
Western blot analysis was carried out on whole cell extracts taken from siRNA treated MCF7 and TAMRBU breast cancer cells. The cells were grown to 80% confluence in media with Tamoxifen present or Tamoxifen absent. The 1D SDS PAGE gels were 4-12% Bis-Tris gradient gels, and the blotted membranes were probed with primary antibodies for IQGAP1 and β Actin.

6.1.2 AlamarBlue proliferation assay

The AlamarBlue proliferation assay was performed in conjunction with siRNA to assess the functionality of IQGAP1 in acquired Tamoxifen resistance. The siRNA protocol was carried out as shown in section 2.5.4 and used with the AlamarBlue assay that was used to assess the cells in section 3.2.4. In Figure 6-2 we show that the vehicle had no effect on the cell growth. However, when the Tamoxifen treatment was applied to the MCF7 siRNA negative and siRNA IQGAP1 treated cells, both showed a decrease in cell growth when compared to cells receiving no treatment.

When the TAMRBU siRNA negative cells were treated with Tamoxifen there continued to be a significant increase in cell growth (as shown in and not statistically significantly different from the observations in section 3.2.4). However, in the presence of siRNA IQGAP, Tamoxifen caused the cells growth to be significantly decreased (-38% compared to siRNA negative) p=0.002. Treatment with siRNA to IQGAP1 also significantly decreased (-18%, p=0.008) oestrodiol stimulated TAMRBU cell proliferation. When Tamoxifen and oestrogen treatments were added simultaneously to the MCF7 cells treated either with negative control siRNA or IQGAP1 siRNA there was no difference in cell proliferation. However, a statistically significant difference (-24%, p=0.012) was seen when TAMRBU cells grown in the presence of siRNA negative control or siRNA to IQGAP1, were treated with oestrodiol plus Tamoxifen. Again the siRNA to IQGAP1shows a significant decrease in the oestrodiol plus Tamoxifen stimulated growth.

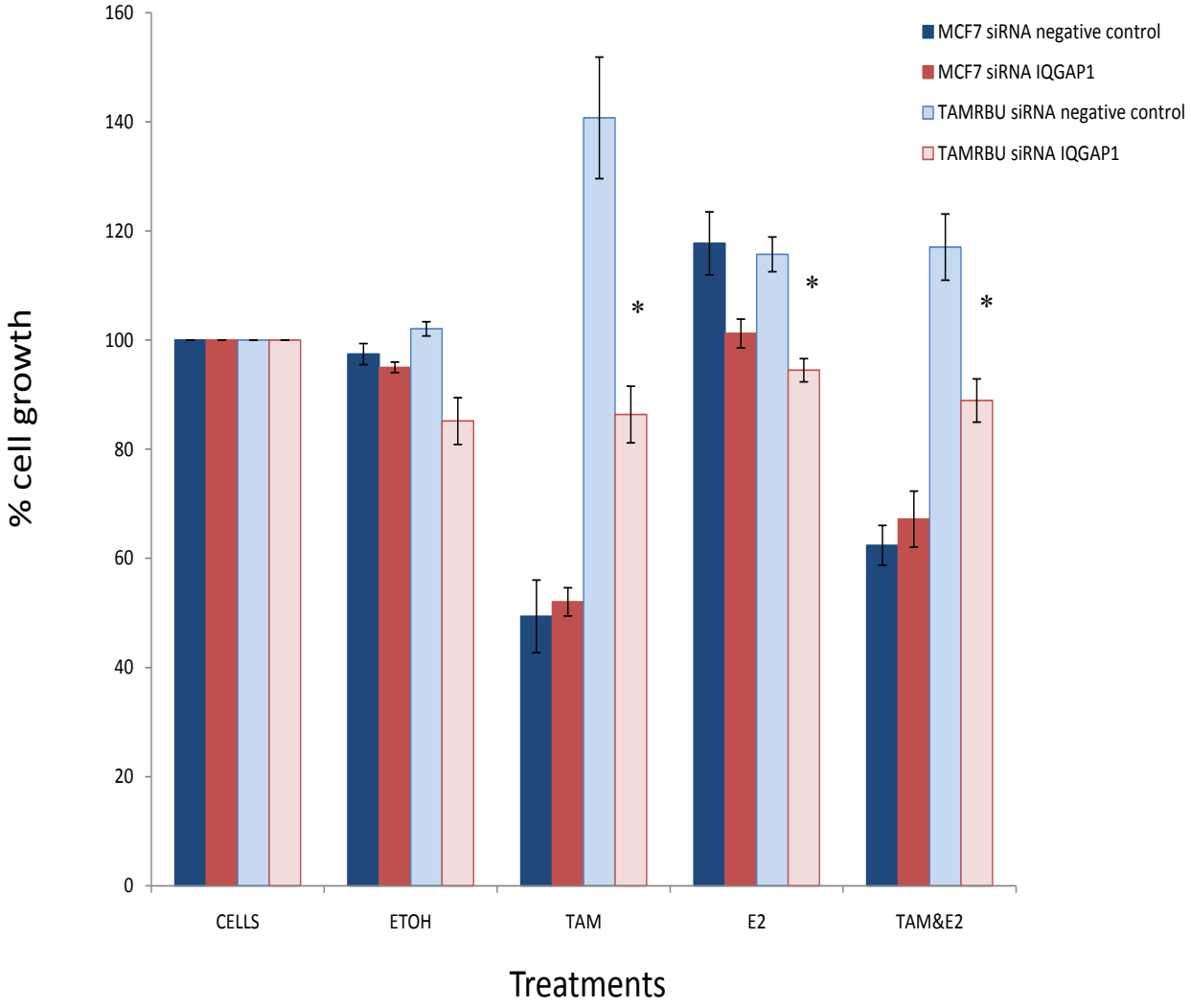


Figure 6-2 Alamar blue proliferation assay showing the response of the cells to different treatments in the presence or absence of siRNA IQGAP1.
The * denotes statistical significance ($p \leq 0.05$).
Treatments: ETOH vehicle, TAM= Tamoxifen 10^{-7}M, E2= Oestrodiol 10^{-9}M, and TAM&E2= Both Tamoxifen 10^{-7} M and Oestrodiol 10^{-9} M.
Results derived from 5 independent experiments normalised to cells with no treatment ± SEM.

In summary, there was no significant difference seen between the siRNA negative and the siRNA to IQGAP1 in MCF7 treated cells when treated with vehicle, Tamoxifen, oestrogen, or a combination of Tamoxifen and oestrogen. However, statistically significant differences were seen between TAMRBU cells treated with siRNA negative control or to siRNA IQGAP1, when the cells were subsequently treated with Tamoxifen, oestrogen or the combination of Tamoxifen and oestrogen treatments.

6.1.3 Adherence assay

An adherence assay was used to determine the effect of a siRNA to IQGAP1 on the cells' ability to attach to a surface coated with attachment factor (0.1% Gelatin) after 45mins (see section 2.5.1 for methodology). Figure 6-3 shows that there was no significant difference between the adherences in TAMRBU and MCF7 cells treated with vehicle. Interestingly , siRNA knockdown of IQGAP1 in MCF7 cells reduced their ability to adhere to the surface. However, TAMRBU cells were still able to adhere to the surface to the same extent as in control cells, despite treatment with siRNA to IQGAP1, perhaps due to increases in other adherence mechanisms or as a result of the higher "normal" expression of IQGAP1 in TAMRBU.

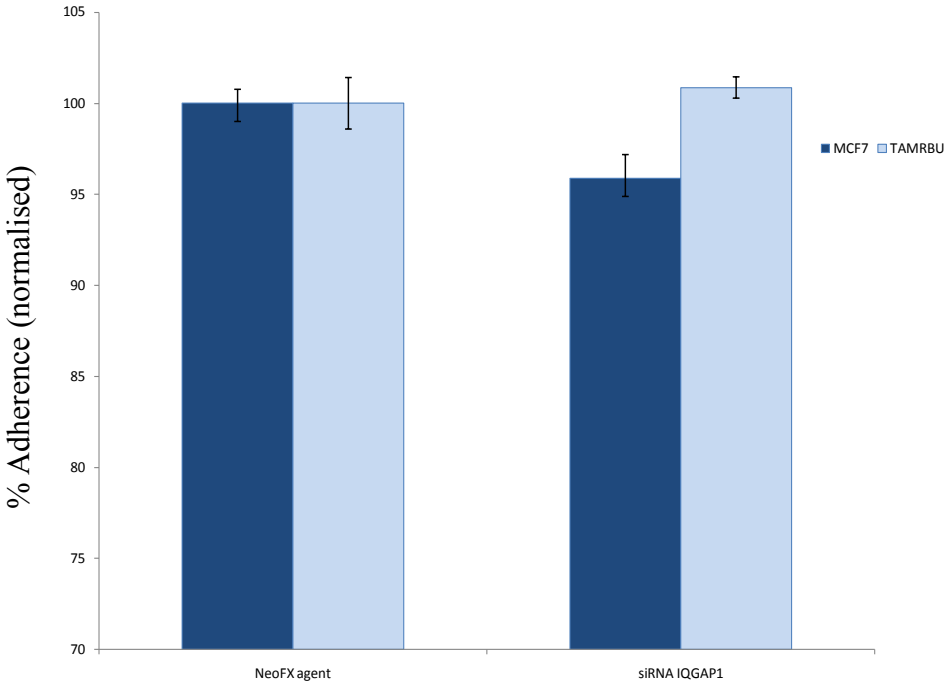


Figure 6-3 Adherence assay results showing no difference in adhesion when IQGAP1 is knocked down in TAMRBU but a small (not statistically significant difference) in MCF7cells. Experiment carried out on 4 independent occasions.

6.1.4 Invasion assay

A Matrigel invasion assay was used to determine the effect of siRNA to IQGAP1 on the cells ability to invade an extracellular matrix (see section 2.5.3) the method is adapted from Al-Nasiry et al, 2007. The assay indicated that the TAMRBU cells showed a more invasive phenotype (p=0.017) (Figure 6-4) which is an observation that has previously been reported in the literature (Hutcheson et al, 2007). Here, we have shown that knocking down IQGAP1 using siRNA lessened the invasive potential of the TAMRBU cells. The TAMRBU cells ability to invade the Matrigel was significantly decreased by 52.1% when compared to the negative control (p=0.004). The % invasion was worked out using % AlamarBlue reduction of invaded cells ÷ % AlamarBlue reduction of cells

without filters x 100. 100% would be the equivalent of all the cells within the inserts migrating through into the feeder wells. This allows for any differences between in proliferation seen between the cell lines.

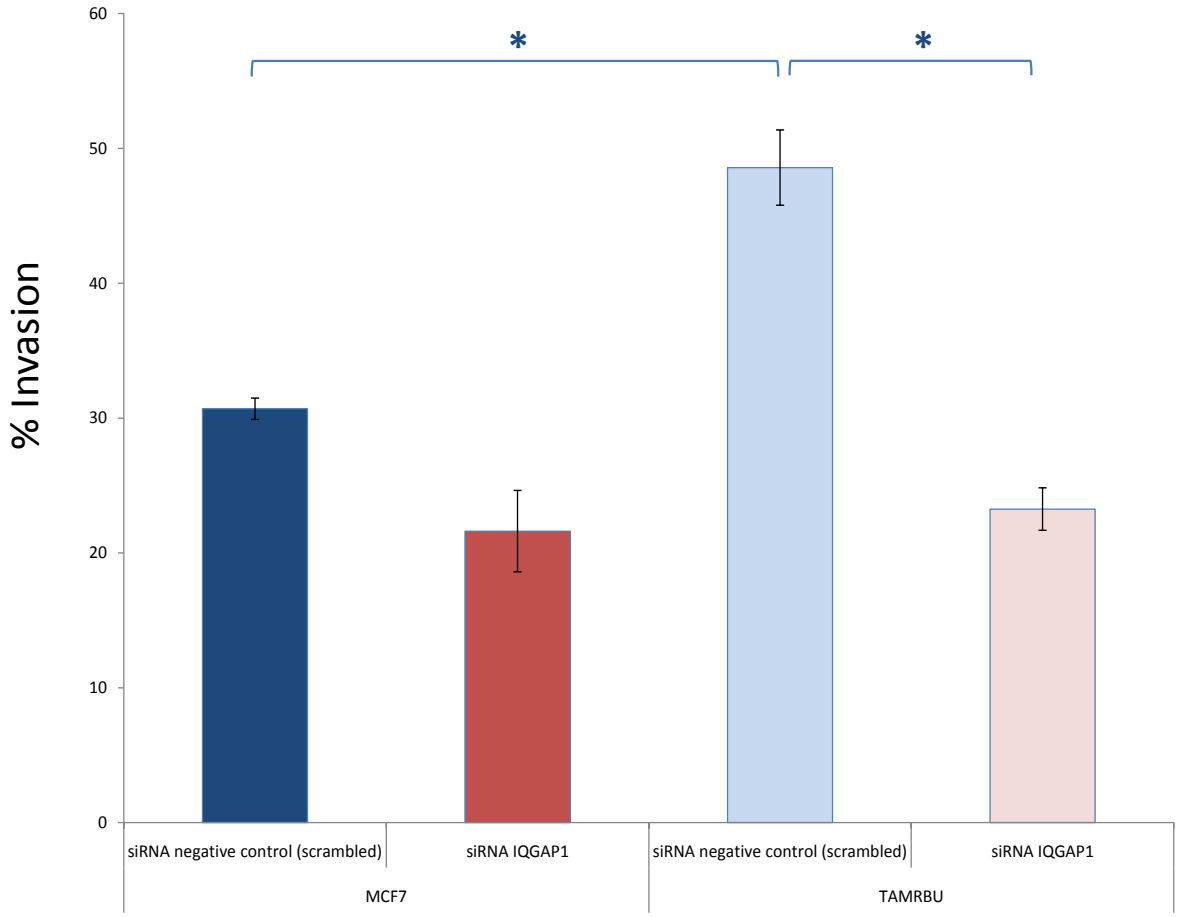


Figure 6-4 Alamar blue Invasion assay showing the ability of the cells to invade when treated with siRNA to IQGAP1. Results above are derived from 3 independent experiments normalised to cells with no treatment ± SEM. The * denotes statistical significance $p \leq 0.05$.

6.2 Functionality of Cortactin in Tamoxifen resistance

6.2.1 siRNA & Western blot

siRNA was used to transiently knock down the expression of cortactin using the method described in section 2.5.4. The figure below (Figure 6-5) shows the knock down of cortactin in both MCF7 and TAMRBU cells. Beta actin was used as a loading control. The siRNA to cortactin decreased its expression in both MCF7 and TAMRBU cells, although to a lesser extent if Tamoxifen was present in the media.

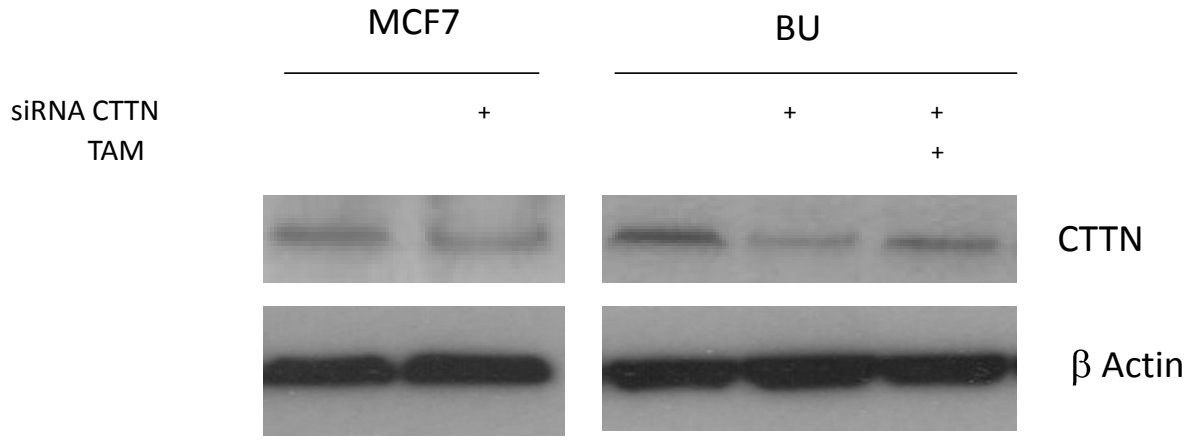


Figure 6-5 Western blot showing the decreased cortactin (CTTN) expression when the siRNA to CTTN had been used (48 hours).
Western blot analysis was carried out on whole cell extracts taken from siRNA treated MCF7 and TAMRBU breast cancer cells. The cells were grown to 80% confluence in media with Tamoxifen present and Tamoxifen absent. The 1D SDS PAGE gels were 4-12% Bis-Tris gradient gels, and the blotted membranes were probed with primary antibodies for CTTN and β Actin.

6.2.2 AlamarBlue proliferation assay.

The proliferation assay was the same as the one performed to examine the cells after they had been developed in Chapter 3 (Section 3.2.4). The results of this assay, when used in conjunction with the siRNA knockdown of cortactin, did not show any distinct differences in response to treatments between the siRNA negative treated cells (Figure 6-6) and the non-siRNA treated cells shown in Figure 3-13.

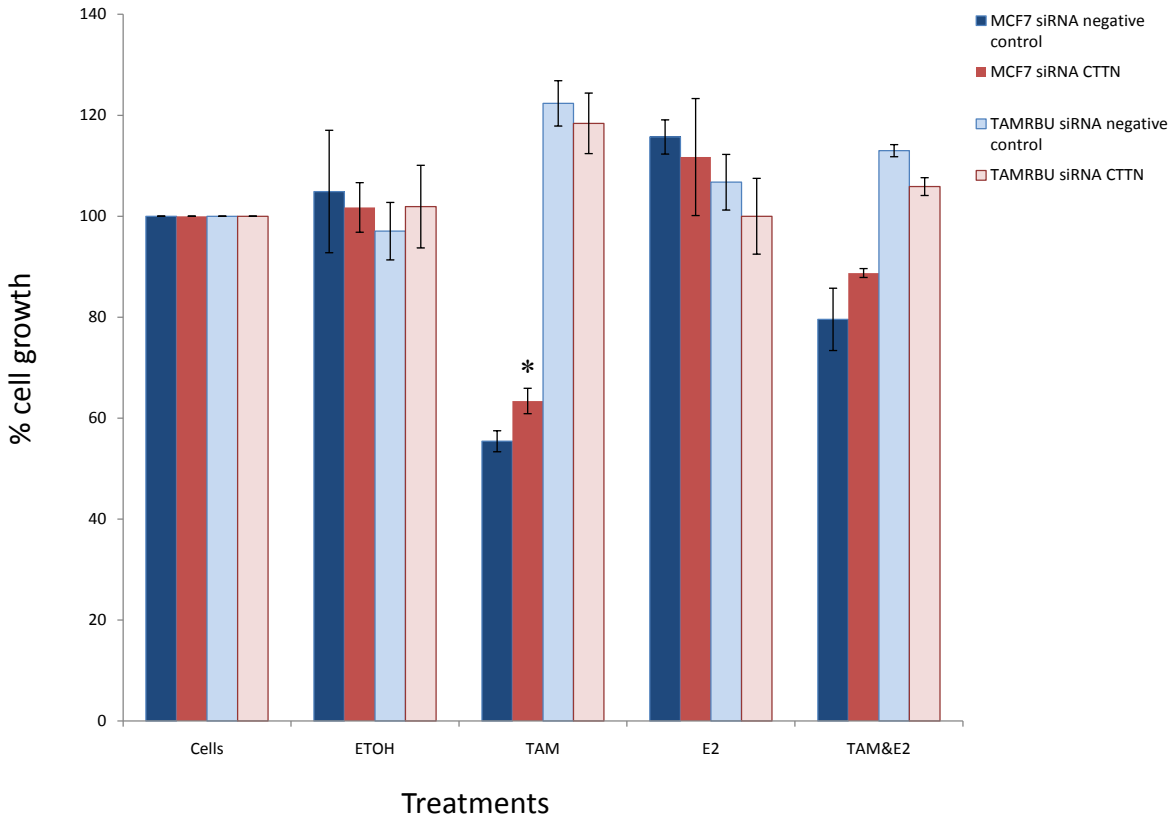


Figure 6-6 Alamar blue proliferation assay showing the response of the cells to different treatments when siRNA cortactin was used. Alamar Blue proliferation assay was carried out on MCF7 and TAMRBU cells treated with siRNA (negative control and CTTN). Treatments: ETOH vehicle, TAM= Tamoxifen 10^{-7}M, E2= Oestrodiol 10^{-9}M, and TAM&E2= Both Tamoxifen 10^{-7} M and Oestrodiol 10^{-9} M.
Results above are derived from 5 independent experiments normalised to cells with no treatment ± SEM. The * denotes statistical significance $p \leq 0.05$.

Interestingly, and in marked contrast to our results with siRNA IQGAP1 knockdown (Figure 6-2) the only statistically significant difference seen between a negative control and a cortactin knockdown siRNA was seen in MCF7 cells treated with Tamoxifen. Here we show a small (7.9%) statistically significant (p=0.03) increase in growth where the cortactin has been knocked down

6.2.3 Adherence assay

The adherence assay was used to determine the effect of siRNA knockdown of cortactin on the cells ability to attach to a surface coated with attachment factor (0.1% Gelatin) after 45mins (see section 2.5.1 for methodology). There was no significant difference seen between the MCF7 cells treated with vehicle and MCF7 with siRNA to cortactin (Figure 6-8). There were also no significant differences seen between TAMRBU cells in vehicle those treated with siRNA to cortactin.

Interestingly, when the siRNA to CTTN and IQGAP1were used simultaneously a much more marked decrease in adherence was observed in the TAMRBU cells than was seen when either cortactin or IQGAP1 were knocked down alone (Figure 6-8), perhaps suggesting that these molecules can compensate for the loss of the other one, but the cells cannot adapt to this if both are lost.

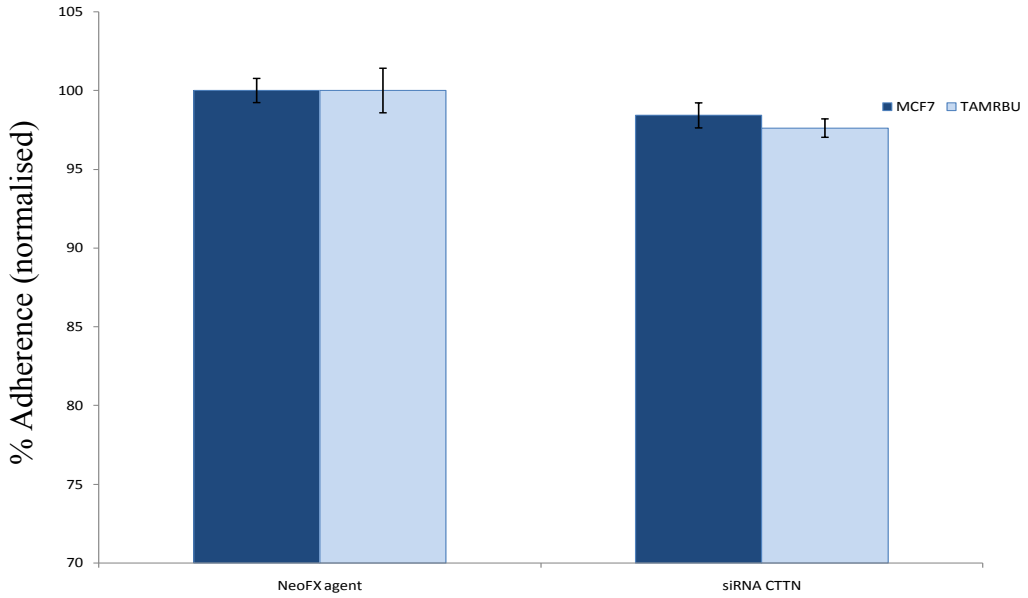


Figure 6-7 Adherence assay results showing no statistical significant difference in adhesion when cortactin is knocked down in TAMRBU or MCF7 cells. Experiment carried out on 4 independent occasions.

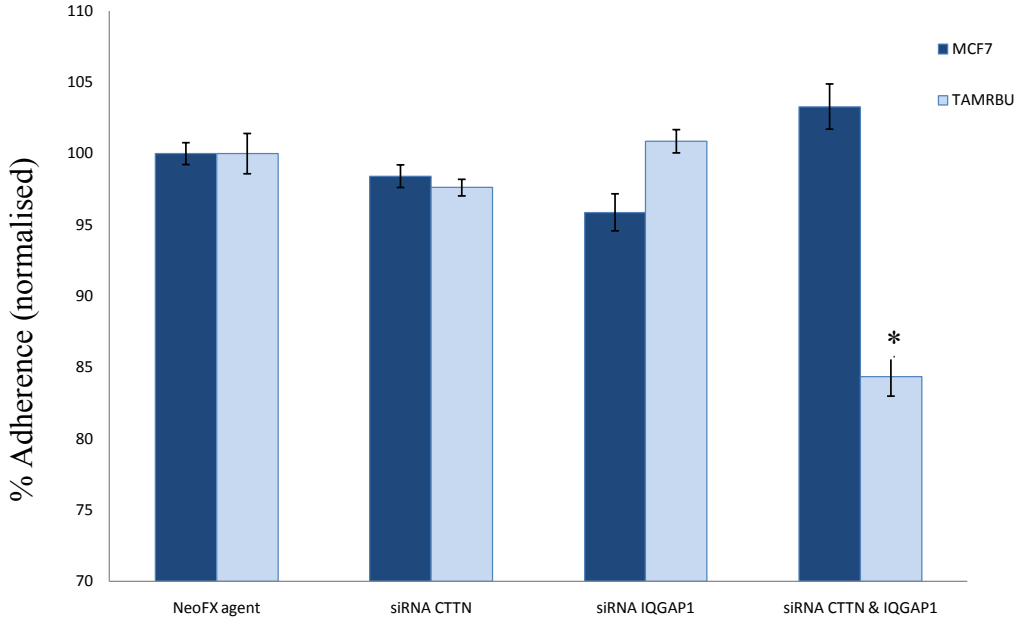


Figure 6-8 Adherence assay normalised to cells treated with neoFX transfection agent. The independent action of siRNA to cortactin or IQGAP1 appears to have no statistical affect upon the adherence of MCF7 or TAMRBU to 0.1% gelatin. However, a statistically significant (* $p \leq 0.05$) reduction in adherence is seen with a combination of siRNA IQGAP1 and cortactin ($p=0.012$). Experiment N=4.

6.3 Discussion

IQGAP1 and cortactin were investigated further in this section after being successfully validated by Western blot analysis (Figure 5-9) and (Figure 5-10). These proteins were specifically chosen as they have been associated with breast cancer progression; increases in motility, proliferation and invasion. These cancer characteristics are well known in the acquisition and progression of cancer to a more aggressive phenotype and drug resistance (Hanahan and Weinberg, 2000). In this study proliferation, invasion and crude adherence, in combination with siRNA knockdowns were investigated to further assess the functionality of IQGAP1 and cortactin.

Figure 6-2 shows that the transient knock down of IQGAP1 by siRNA treatment attenuated the increased cell proliferation seen in cells when treated with Tamoxifen, oestrogen or a combination of Tamoxifen and oestrogen suggesting a role of IQGAP1 in increased growth of these cells. Increased expression of IQGAP1 has been associated with breast cancer progression (Jadeski et al., 2008) and Trasuzumab resistance (White et al., 2011) but this is the first time it has been reported for Tamoxifen resistance. IQGAP1 is a protein that has many roles in the normal function of a cell, including acting as a scaffold protein (Sacks, 2006). Its role is executed through interactions with a range of specific target proteins through conserved domains (White et al., 2009) as discussed in section 5.3.1. The IQGAP1 protein has been shown to bind proteins such as calmodulin, cadherin, actin and β cateninin and also has been shown to regulate the function of many of the proteins that it binds to (Kuroda et al., 1998). The diversity of the functions IQGAP1 is associated with is revealed by the large number of proteins that it binds to.

IQGAP1 has been documented to be a scaffold protein for proteins in the MAPK pathway; members of which are known to be involved in acquired Tamoxifen resistance (see section 4.5.1). IQGAP1 can bind directly to B-Raf, MEK1/2, ERK1/2 (Ren et al., 2007, Roy et al., 2004 and Roy et al., 2005) and has also been shown to modulate MAPK activity when activated with EGF, CD44 and also N-cadherin (Roy et al., 2004, Bourguignon et al., 2005, and Schrick et al., 2007). Ren et al., 2007 showed that decreasing IQGAP1 expression attenuated the ability of the cells to activate MAPK following stimulation with VEGF, EGF, or PDGF. The mechanism by which IQGAP1 connects MAPK and these growth factors has not been fully elucidated. Recently, McNulty et al., 2011 have demonstrated that EGF activation of EGFR catalyses the phosphorylation IQGAP1 on Ser^{1443}, this phosphorylation enhances the EGF stimulated EGFR tyrosine phosphorylation potentially through a positive feedback mechanism able to modulate EGFR activation. This could have implications on the increased expression of IQGAP1 and its subsequent knock down having a role in cell growth, as activated EGFR triggers downstream signalling induction of the MAPK cascade. EGFR signalling is known to be involved in Tamoxifen resistance (Knowlden et al., 2003), the signal cascade is multifaceted and perhaps relies upon proteins such as IQGAP1, a scaffold protein, to form protein signalling complexes. The TAMRBU cells are known to express more EGFR (Figure 3-5), have increased activation of the MAPK pathway (section 4.5.1) which would fit with the increased IQGAP1 expression (Figure 5-9). This indicates that IQGAP1 may have a role in Tamoxifen resistance through modulation of EGFR/oestrogen signalling cross talk. The increase in EGFR expression, activation and downstream functions have long been associated with increased in proliferation in cancer

and Tamoxifen resistance, however, a direct involvement of IQGAP1 would have to be further investigated, perhaps by using the MAPK and RTK profilers used in section 4 following siRNA knockdown of IQGAP1. Also, Wang et al., 2008 reported that phosphorylation of IQGAP1 appears to have an important role in its function; further proteomic investigation using instrumentation capable of phosphoproteomics to help elucidate its function in EGFR signalling/proliferation within Tamoxifen resistance. IQGAP1 may have a role in oestrogen signalling crosstalk between ERα and EGFR as in Figure 6-2 there was a decrease in cell growth when siRNA IQGAP1 treated TAMRBU cells were stimulated with oestrogen, compared to siRNA negative cells (Figure 6-2). Cross talk has already been reported in Tamoxifen resistance (Britton et al., 2006) however, IQGAP1s involvement in this has not been previously described.

The adherence assay investigated the ability of the cells to adhere to their surroundings. It showed that there was very little difference seen in the cells attachment to the 0.1% gelatin coated wells, with cells treated with neoFX transfection agent as the negative control in this experiment. There was no statistical difference in adhesion seen between the MCF7 and TAMRBU cells in the negative control. It has been previously demonstrated that proteins such as Src, known modulators of cell-matrix adhesion, are elevated in Tamoxifen resistance (Hiscox et al., 2007) who showed that the Tamoxifen resistant cells had the ability to adhere more rapidly, and showed a higher affinity to bind to matrix which was decreased with inhibitors of Src. In that report, a range of matrix substrates were used which may explain the differences seen here, as 0.1% gelatin provides a non specific binding platform. There was also no significant difference in adherence seen between the MCF7 and the TAMRBU when the expression of IQGAP1

was decreased with siRNA (Figure 6-3) inferring that there is no role for IQGAP1 in the attachment of the cells to the 0.1% gelatin.

Our results (Figure 6-4), clearly show that the ability of TAMRBU cells to invade Matrigel (mimicking the cells' ability to invade the basement membrane) is severely diminished ($p=0.004$) when IQGAP1 expression is decreased to a level which is comparable to the capability of the MCF7 cell line. Over expression of IQGAP1 has been associated with invasive potential in many cancers including ovarian (Dong et al., 2008), colorectal (Hayashi et al., 2010), gliomas (Hu et al., 2009), thyroid (Lui et al., 2009) and breast (Jadeski et al., 2008). Jadeski et al., 2008 reported that when MCF7 cells that over expressed IQGAP1 formed invasive tumours when injected in to immune compromised mice, whereas, when IQGAP1 was stably knocked down there was still tumour formation yet the tumours were smaller and less invasive. This indicates that IQGAP1 has a role in the proliferation and the invasive potential of breast cancer in line with the results reported in this thesis. IQGAP1's role in invasion has been linked with HGF stimulation (Hu et al., 2008) in glioma cells. They also reported that siRNA knockdown of IQGAP1 significantly inhibits the ADP-ribosylation factor 6 (ARF6) (a member of the Ras super family of small GTPases), induced Rac1 activation and cell migration. Decreasing ARF6 protein expression through siRNA led to decreased recruitment of IQGAP1 and attenuated protrusion and production of invasive structures.

The implication that IQGAP1 having a role in Tamoxifen resistance is interesting as it has been implicated in the progression of a number of cancers and in epithelial mesenchymal transition in NPC (Lui et al., 2009); Tamoxifen resistant cells appear to

have acquired a phenotype that show increased progression and an EMT like phenotype (Hiscox et al., 2006).

Increased expression and altered phosphorylation of cortactin has been associated with cancer progression and modulation of cell invasion (Cai et al., 2010). Cortactin is a protein known to bind to F-actin, complexing with cortical actin involved in lamelopodia formation and membrane ruffling (Kelley et al., 2010). It is also known to be a substrate of src suggesting a role in cell motility (Wu and Parsons, 1993) and is also discussed in section 5.3.1. Proliferation of MCF7 or TAMRBU cells was not statistically altered in cells that had cortactin transiently knocked down with siRNA (Figure 6-6), however, when the cells were treated with Tamoxifen the MCF7 cells with decreased expression of cortactin showed a slight (7.9%) statistically significant increase in cell growth. No previous reports in the literature have shown this induction of proliferation; in fact, there have been no reports that cortactin has been associated with breast cancer proliferation. Van Rossum et al., 2005 showed that in HBL-100 breast cancer cell lines there was a decrease in invasion and migration with siRNA cortactin treated cells. They also reported that the decreased expression had no role in proliferation which would fit with the findings in this Thesis. In head and neck cancer it has been reported that over expression has an oncogenic role attenuating the ligand induced down regulation of EGFR in turn leading to sustained EGFR signalling through the MAPK family specifically ERK (Timpson et al., 2007). This finding indicates that there may be an oncogenic proliferative role for cortactin in breast cancer, as increased EGFR and ERK signalling seen in Tamoxifen resistance (section 4.2.2 and 4.3.2) however, decreasing the protein expression of cortactin using siRNA did not alter the proliferation of the TAMRBU cells

in this study. This may imply cortactin's role is not significant or more likely, the decrease in cortactin expression achieved in the experiments was insufficient to produce a significant decrease in proliferation. This opens up the whole debate about how much expression is required to have an effect in the cell.

The adherence assay showed that there was very little difference in the cells attachment to the 0.1% gelatin coated wells. NeoFX transfection agent was used as the negative control in this experiment. There were no differences seen between MCF7 cells and TAMRBU negative control treated cells. There was also no difference observed when expression of cortactin was decreased with siRNA. As discussed above, it has been previously demonstrated that Tamoxifen resistant cells show altered adherence properties (Hiscox et al., 2007). The lack of change shown in our experiments may be due to the 0.1% gelatin providing non specific binding platforms for the cells. Interestingly however, there was a small but significant loss in adhesion when IQGAP1 and cortactin were knocked down simultaneously ($p=0.012$). This reduced adhesion was only seen in the TAMRBU cells, this could be as a result of the TAMRBU cells over expressing both of these proteins. Knocking the proteins down may have additional effects on the resistant phenotype as it is possible that the cells have become more reliant on the proteins (or the pathways that the proteins are implicated in). It would be interesting to repeat these knock downs and perform more specific adhesion assay methods for some of the interesting proteins found to be differentially expressed in section 5.1, such as Talin-1, cortactin and IQGAP1which have been shown in the literature to have roles in cell adhesion, actin re-modelling, cell motility and invasion.

7 Discussion & Future work

7.1 General discussion

Modern biological research and the rise of "omic" technology have provided useful and powerful tools for biological research. The generation of phenomenal amounts of data can confuse the actual reason for the research although it could be argued that biology is far too complex for hypothesis driven research. A combination of both "omics" and hypothesis driven approaches could deliver a more complete understanding of the biology, with "omics" providing leads which can then be used for important hypothesis driven experiments providing functional data. The combination of proteomics and functional biology was used in this research and has provided many interesting leads, of which 2 we have investigated further to investigate potential functional roles for these proteins in resistance (section 6). Several more proteins have been identified and could be investigated further after Western blot validation (Appendix (CD)).

In the antibody array section of this work a total of 32 proteins, statistically different in expression or phosphorylation between the MCF7 and the resistant lines were identified. Of these proteins, 11 have been previously described in Tamoxifen resistance (see Table 4-7) and 21 proteins that have not been previously reported. This shows that using antibody array technology for signalling pathway and protein family analysis has led to a greater knowledge into the changes that occur in the Tamoxifen resistance cellular proteome.

Using mass spectrometry analysis, a total of 262 proteins were found (each with at least one quantifiable peptide pair) 5 proteins were further investigated by Western blotting

and in 4 cases this confirmed the SILAC observations. In one case the result was not confirmed, perhaps indicating detection error in the MS analysis, or more likely due to differences in the specific peptides found within the protein detection using the two methods. For IQGAP1 and cortactin validation and further investigation into their function and role within Tamoxifen resistance proved interesting and more of the proteins found to be differentially expressed by SILAC could prove to be important in the acquisition of Tamoxifen resistance.

Of the 262 proteins, 59% were over-expressed in resistance and 62% were decreased. The majority of them were cytoplasmic (67%). There is a slight bias of up regulated proteins but this is expected as biologically it is expected that the cell would turn on pathways and increase protein expression to overcome the presence of the drug. There were no receptors found in the MS SILAC data. The ontological profile of the proteins remained similar between the up and down regulated proteins however there were some differences; more transporters and peptidases were decreased in resistance.

Bioactive peptides on the cell surface can be associated with a cancer phenotype (Nanus et al., 2003). Degradation of peptides is the job of the peptidases, which could be seen as a step in controlling growth and differentiation of normal cells by degrading the peptide before it induces tumour initiation and progression. In Tamoxifen resistance there was a decrease of peptidases seen; one could imagine that the loss or decreased expression of peptidases could result in a normal cells ability to inactivate a bioactive peptide leading to the stimulation of migration, invasion, cell growth or promotion of cell survival (Nanus et al., 2003). Conversely, 3% of peptidases increased in resistance. This could conceivably contribute to a gain in function through the increased expression of

certain peptidases normally expressed at low levels, potentially converting a pro-peptide to a bio-active form to produce greater catalytic activity enabling the cell to become more proliferative (Nanus et al., 2003).

Enzymes also showed a difference between proteins identified to be up and down regulated in resistance 25% were up, 18% down. Many classes of enzymes have been associated with increased metabolism and are often seen to be increased in more proliferative cancerous cells (Sabbisetti et al., 2009). They are thought to do this to fuel their increased growth. The increased expression of enzymes seen in resistance could conceivably be the cells needing more energy as they proliferate more rapidly. The resistant cells are also shown in this Thesis to be more invasive, specific enzymes such as MMPs are required to degrade the basement membrane in order to invade. However, there are also decreases in enzymes seen in the SILAC data which could be another class of enzyme, for example the cytochrome P450 2DS and sulfotransferases, which are used to convert Tamoxifen (pro-drug) to the active metabolite (Figure 1-11). These may well be decreased in Tamoxifen resistance as Wegman et al., 2005 showed that increasing these enzymes therapeutically could benefit the patients taking Tamoxifen.

When the data gathered with SILAC and the data from the antibody arrays are studied collectively; a clearer picture starts to emerge. The global SILAC approach did not find any proteins that had been found with the antibody arrays (however, it did find proteins that had been reported in the literature to have a role in Tamoxifen resistance such as Cathepsin D), so the two approaches have an additive effect. To analyse these sets of data collectively KEGG pathway analysis was used (Kanehisa, 2002). The results of this analysis can be seen in Figure 7-1, Figure 7-2 below.

Figure 7-1 The actin cytoskeleton is regulated by many proteins and complex networks as is displayed in the below figures. Below are the proteins identified in the pathways by the KEGG ontology pathway analysis program (http://www.genome.jp/kegg/pathway.html) (Red indicates an increase in expression, Blue a decrease)

Figure 7-1 Regulation of actin cytoskeleton - Homo sapiens (human) (10 proteins)

ACTN1; actinin, alpha 1
ACTN4; actinin, alpha 4
EGFR; epidermal growth factor receptor
EZR; ezrin
FGFR2; fibroblast growth factor receptor 2
IQGAP1; IQ motif containing GTPase activating protein 1
MAPK1; mitogen-activated protein kinase 1
MAPK3; mitogen-activated protein kinase 3
MYH14; myosin, heavy chain 14, non-muscle
PPP1CC; protein phosphatase 1, catalytic subunit, gamma isozyme

Figure 7-2 Cancer is known to be regulated by many pathways. The pathways and proteins known to be associated with cancer are displayed below. Red indicates an increase in expression, Blue a decrease. Below are the proteins identified in the pathways by the KEGG ontology pathway analysis program (http://www.genome.jp/kegg/pathway.html)

Figure 7-2 Pathways in cancer - Homo sapiens (human) (18 proteins)

AKT1; v-akt murine thymoma viral oncogene homolog 1
BAD; BCL2-associated agonist of cell death
BAX; BCL2-associated X protein
BCL2; B-cell CLL/lymphoma 2
BCL2L1; BCL2-like 1
BIRC2; baculoviral IAP repeat containing 2
BIRC3; baculoviral IAP repeat containing 3
BIRC5; baculoviral IAP repeat containing 5
CSF1R; colony stimulating factor 1 receptor
CTNNA1; catenin (cadherin-associated protein), alpha 1, 102kDa
EGFR; epidermal growth factor receptor
ERBB2; v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2
FGFR2; fibroblast growth factor receptor 2
HIF1A; hypoxia inducible factor 1, alpha subunit
HSP90AA1; heat shock protein 90kDa alpha (cytosolic), class A member 1
MAPK1; mitogen-activated protein kinase 1
MAPK3; mitogen-activated protein kinase 3
XIAP; X-linked inhibitor of apoptosis

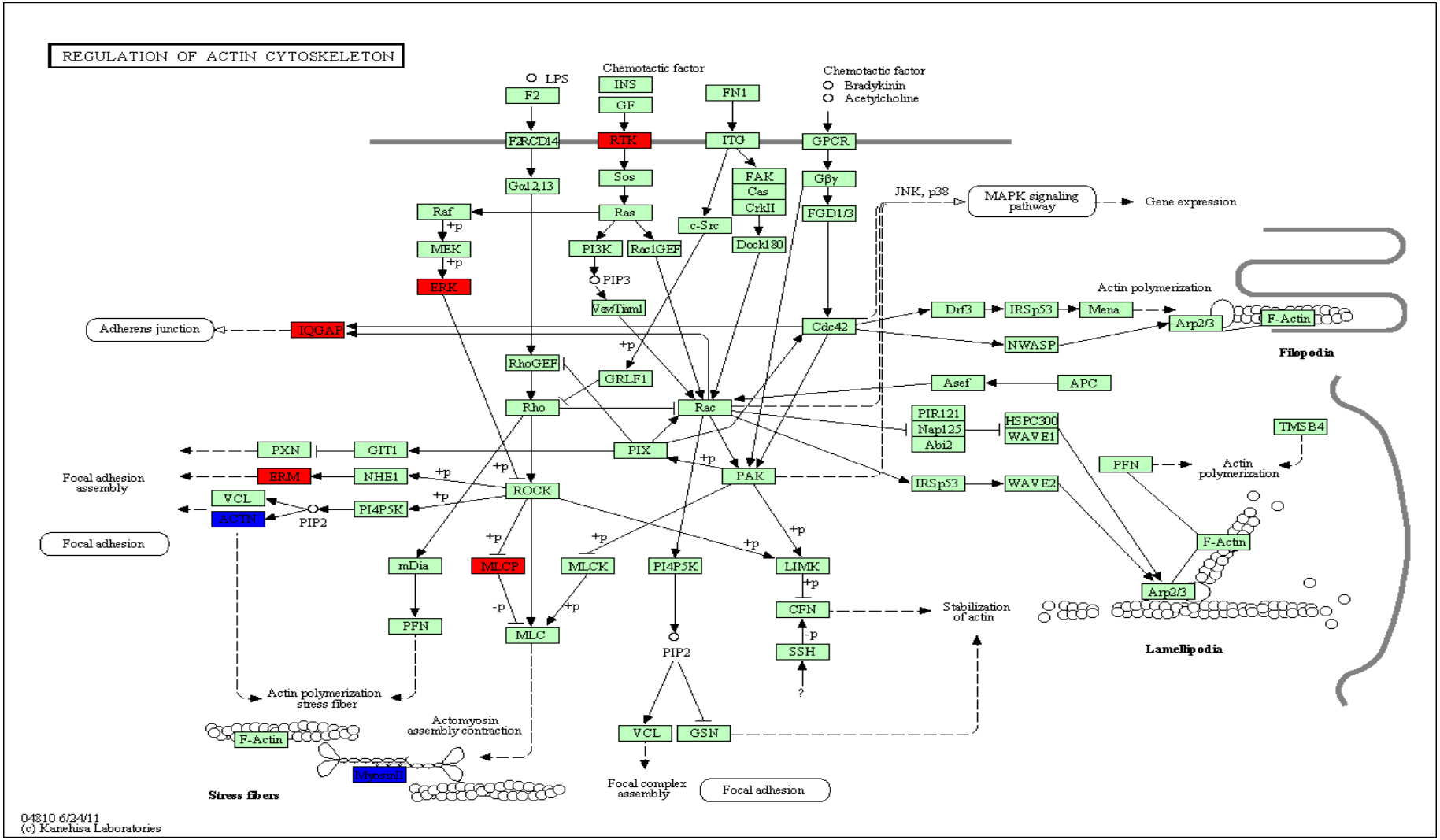

REGULATION OF ACTIN CYTOSKELETON
LPS
F2
FZRCD14
Chemotactic factor
INS
GF
RTK
Sos
Ras
PI3K
Rac1GEF
PIP3
Vav/Tiam1
Chemotactic factor
Bradykinin
Acetylcholine
FN1
ITG
FAK
Cas
CrkII
c-Src
Dock180
GPCR
Gβγ
FGD1/3
Gα12,13
Raf
MEK
ERK
JNK, p38
MAPK signaling pathway
Gene expression
Actin polymerization
Drf3
IRSp53
Mena
Arp2/3
F-Actin
Filopodia
NWASP
Adherens junction
IQGAP
Cdc42
RhoGEF
GRLF1
Asef
APC
Rho
Rac
PIR121
Nap125
Abi2
HSPC300
WAVE1
TMSB4
PXN
GIT1
PIX
PAK
PFN
Actin polymerization
Focal adhesion assembly
ERM
NHE1
ROCK
IRSp53
WAVE2
VCL
ACTN
PIP2
PI4P5K
Focal adhesion
mDia
MLCP
MLCK
PI4P5K
LIMK
F-Actin
Arp2/3
PFN
MLC
CFN
SSH
Stabilization of actin
Lamellipodia
Actin polymerization stress fiber
Actomyosin assembly contraction
F-Actin
Myosin II
Stress fibers
PIP2
VCL
GSN
Focal complex assembly
Focal adhesion
04810 6/24/11
(c) Kanehisa Laboratories

Figure 7-1

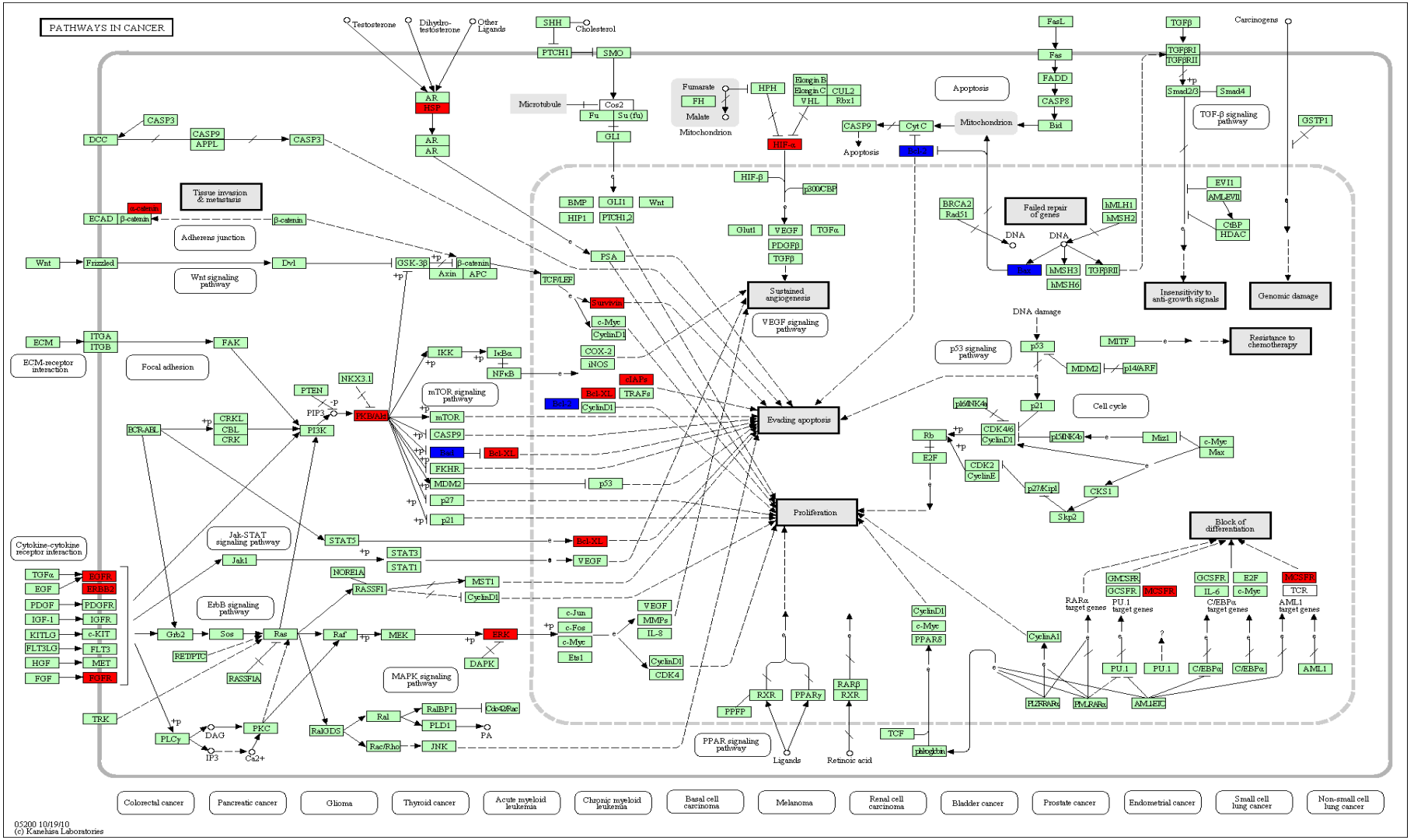

PATHWAYS IN CANCER
Testosterone
Dihydro-testosterone
Other Ligands
AR
HSP
AR
AR
SHH
Cholesterol
PTCH1
SMO
Microtubule
Cos2
Fu
Su (fu)
GLI
Fumarate
FH
Malate
Mitochondrion
HPH
Elongin B
Elongin C
VHL
CUL2
Rbx1
HIF-α
HIF-β
p300/CBP
Glut1
VEGF
TGFα
PDGFβ
TGFβ
Sustained angiogenesis
VEGF signaling pathway
Apoptosis
CASP9
Cyt C
Apoptosis
Bcl-2
Mitochondrion
FasL
Fas
FADD
CASP8
Bid
TGFβ
TGFβRI
TGFβRII
Smad2/3
Smad4
TGF-β signaling pathway
GSTP1
Carcinogens
CASP3
DCC
CASP9
APPL
CASP3
Tissue invasion & metastasis
α-catenin
ECAD
β-catenin
β-catenin
Adherens junction
Wnt
Frizzled
Dvl
GSK-3β
β-catenin
Axin
APC
Wnt signaling pathway
TCF/LEF
Survivin
c-Myc
CyclinD1
COX-2
iNOS
BMP
GLI1
Wnt
HIP1
PTCH1,2
PSA
BRCA2
Rad51
Failed repair of genes
DNA
DNA
Fas
hMLH1
hMSH2
hMSH3
hMSH6
TGFβRII
EVI1
AML-EVI1
CtBP
HDAC
Insensitivity to anti-growth signals
Genomic damage
DNA damage
p53 signaling pathway
p53
MDM2
p14/ARF
MITF
Resistance to chemotherapy
ECM
ITGA
ITGB
ECM-receptor interaction
FAK
Focal adhesion
IKK
IκBα
NFκB
cIAPs
Bcl-XL
Bcl-2
CyclinD1
TRAFs
mTOR signaling pathway
mTOR
CASP9
Bad
Bcl-XL
FKHR
MDM2
p53
p27
p21
NKX3.1
PTEN
PIP3
PKB/Akt
BCR-ABL
CRKL
CBL
CRK
PI3K
Evading apoptosis
p16/INK4a
p21
Cell cycle
Rb
E2F
CDK4/6
CyclinD1
p15/INK4b
Miz1
c-Myc
Max
CDK2
CyclinE
p27/Kipl
CKS1
Skp2
Proliferation
Block of differentiation
Cytokine-cytokine receptor interaction
TGFα
EGF
PDGF
IGF-1
KITLG
FLT3LG
HGF
FGF
EGFR
ERBB2
PDGFR
IGFR
c-KIT
FLT3
MET
FGFR
Jak-STAT signaling pathway
Jak1
STAT5
STAT3
STAT1
Bcl-XL
VEGF
ErbB signaling pathway
Grb2
Sos
Ras
Raf
MEK
ERK
RETFPTC
RASSF1A
NORE1A
RASSF1
MST1
CyclinD1
DAPK
MAPK signaling pathway
c-Jun
c-Fos
c-Myc
Ets1
VEGF
MMPs
IL-8
CyclinD1
CDK4
TRK
PLCγ
DAG
IP3
Ca2+
PKC
RalGDS
Ral
RalBP1
PLD1
PA
Cdc42/Rac
Rac/Rho
JNK
PPFP
RXR
PPARγ
RARβ
RXR
PPAR signaling pathway
Ligands
Retinoic acid
TCF
pklogklbn
CyclinD1
c-Myc
PPARδ
CyclinA1
GMCSFR
GCSFR
MCSFR
PU.1 target genes
PU.1
PU.1
GCSFR
IL-6
E2F
c-Myc
C/EBPα target genes
C/EBPα
C/EBPα
MCSFR
TCR
AML1 target genes
AML1
RARα target genes
PLZF/RARα
PML/RARα
AML1/ETO
Colorectal cancer
Pancreatic cancer
Glioma
Thyroid cancer
Acute myeloid leukemia
Chronic myeloid leukemia
Basal cell carcinoma
Melanoma
Renal cell carcinoma
Bladder cancer
Prostate cancer
Endometrial cancer
Small cell lung cancer
Non-small cell lung cancer
05200 10/19/10
(c) Kanehisa Laboratories

Figure 7-2

Proteins found in this study have been implicated as key players in pathways in cancer (Figure 7-2) and also some have been shown to be over-expressed in Tamoxifen resistance such as EGFR, survivin and HIFα and these can be seen in this Figure 7-2 coloured in red whilst those proteins that have lower expression in resistance can be seen coloured in blue, such as Bad. This could imply that the acquisition of Tamoxifen resistance may alter some of the most important pathways in cancer. This is already known in some respects as the ErbB2 and MAPK signalling pathway increases are well described for Tamoxifen resistance. Similarly for the evasion of apoptosis with the increase in survivin (which has been discussed in the literature Vanderlaag et al., 2010) however, the change in IAP expression has not been previously described.

It is unlikely that there is one simple mechanism that underlies Tamoxifen resistance such as the loss of ERα signalling, or increased EGFR signalling. Many mechanisms have previously been implicated in Tamoxifen resistance (detailed in section 1.6) in this study we have found evidence for many more. There were ten proteins with changes seen in Tamoxifen resistance associated with actin remodelling in cells, as can be seen in Figure 7-1. Actin and associated proteins such as RhoA-C, Rac1, Cdc42 (all in the Rho family of GTP-binding proteins), also IQGAP1 and cortactin are important in functions such as regulation of polymerisation of actin in the production of lamellapodia, stress fibres, membrane ruffles and invadapodia usually in response to extracellular signals, or proliferation or apoptosis. It has been reported in the literature that silencing of Rho Guandine dissociation inhibitors (Rho GDI) affect the metastatic phenotype and the responsiveness of breast cancer cells to Tamoxifen as investigated with xenograft models of resistance (Barone et al., 2011) Whilst these changes are observed in our TAMRBU

cells, whether they have a causal role in Tamoxifen resistance, or merely a consequence or requirement of the resistant phenotype has not been investigated in this Thesis.

There have been several studies that show an association between Tamoxifen resistance and the pathways and signalling networks that regulate the cytoskeleton. Tamoxifen therapy has also been implicated in the aberrant localisation of actin and fibronectin and actin remodelling in endometrial cancer cells (Albright et al., 1997). Over expression of Ras-GDP exchange factor AND34/BCAR3, known to associate with focal adhesion proteins and reported to modulate the actin cytoskeleton, gives the cells the ability to grow in the presence of Tamoxifen (Cai et al., 2003).

The remodelling of actin and the motile and invasive (Figure 6-4) phenotype seen in Tamoxifen resistance are important steps in the development of a metastatic phenotype. The two proteins identified in this study and chosen for more in-depth investigation, IQGAP1 and Cortactin, are both known to have roles in development and progression of a more metastatic, invasive phenotype and are known modulators of the actin cytoskeleton. In this study we showed that IQGAP1over expression has a potential role in Tamoxifen resistance. How this over expression may fit with this role is discussed further below.

IQGAP1 is a ubiquitously expressed 189KDa scaffold protein (White et al., 2011), it has many interaction domains including; calmodulin binding (IQ domains), polyproline binding (WW domain), calponin domain and Ras GAP related region (White et al., 2009). There are many proteins that can bind through these domains; including Rac1/cdc42, actin, calmodulin, MAPK pathway components such as ERK1 and 2, adenomatous polyposis coli, VEGFR2, EGFR, β catenin, E-Cadherin (Johnston et al,.

2009). Through the binding and association with these proteins IQGAP1 is functional in many cell activities as was described in section 5.3.1. Through its involvement in these processes and its association with these proteins there is increasing evidence that IQGAP1 plays a major role in tumourigenesis (Johnson et al., 2009 and White et al., 2009) many of the proteins that IQGAP1 binds to have roles in tumour progression or/and the initiation of cancer. Over expression of IQGAP1 has been described in several cancers as discussed above in section 5.3.1 and this together with the functions of its binding proteins infer that IQGAP1 has an oncogenic role in cancer. It has been shown in the literature that modulation of IQGAP1 expression in breast cancer cell lines (MCF7 and MDA-MB 231) significantly alters their tumorigenicity and siRNA knockdown decreases this effect (Jadeski et al., 2008). It has recently been shown that IQGAP1 has a role in Trasuzumab (Herceptin) resistance (White et al., 2011) where it was shown that IQGAP1 binds directly to ErbB2 eliciting several effects in the cell including the stabilisation of ErbB2 expression (demonstrated in SkBR3 breast cancer cells) however, the mechanism by which this occurs remains elusive. White et al., 2011 also suggest that IQGAP1 is necessary for the phosphorylation of ErbB2; we have shown that ErbB2 phosphorylation is statistically up regulated in TAMR and TAMRBU cells (section 4.3.2) and this may contribute to the activation of the PI3K/Akt and MAPK signalling seen in Tamoxifen resistance (see section 4.2) as some studies have indicated these signalling pathways are inhibited when the phosphorylation of ErbB2 is reduced (Shattuck et al., 2008 and Nahta et al., 2004). It has also been reported in the literature that IQGAP1 has a role in these pathways (PI3K/Akt and MAPK) (Roy et al., 2004, Roy et al., 2005 and Ren et al., 2007). Yamaoka-Toja et al., 2004, showed that the knockdown of IQGAP1 impairs

Akt activation by VEGF and White et al., 2011 show that knock down of IQGAP1 increases p27 (a CDK inhibitor that causes cell cycle arrest and apoptosis through the reduction of CDK2 activity) and subsequently reduces Akt phosphorylation and its ability to induce cell proliferation.

IQGAP1 has also been associated with invasion in cancer which may result from the increased motile potential, the actin remodelling involved in invasive structures and also targeting of matrix metallo proteinase 1 (MMP1) to the invadapodia. The latter is imperative for the invading tumour cells to degrade the ECM to undergo metastasis as Bao et al., 2010 expression of MMP1 can also be ErbB2 mediated show in gastric cancer. IQGAP1 has been reported to bind to Sec3 and 8 (components of the exocyst complex) in order to target the MMP1 to the invadapodia (Sakurai-Yageta et al., 2008) this was regulated by Cdc42 and RhoA GTPase. This may be carried out through IQGAP1s function in the actin cytoskeleton modelling allowing the exocyst vesicles to be delivered to the invadapodia or through stimulation of actin to form the protrusions and exocytic complexes.

The ability of IQGAP1 to interact with CD44, a hyaluron receptor, could also be important in the invasive potential of the cells. CD44 has been associated with many cancers and is regularly reported to be related to metastasis. It has also been shown to be elevated in the TAMR cell line (Baruah et al., 2009) where it was found that the over expression of CD44 sensitises cells to EGF and Hyaluron (HA). IQGAP1 over expression shown here and the CD44 over expression by Baruah et al., 2009 may act synergistically increasing the malignant phenotype of the Tamoxifen resistant cells.

The modulation of IQGAP1 expression (over expression and silencing) results in changes in the cytoskeleton associated with exhibition of both lamellopodia and membrane ruffles (Figure 7-3). At these structures, IQGAP1 acts as a scaffold protein to join actin and microtubule related proteins to proteins such as APC, β catenin and cadherin (Johnson et al., 2009).

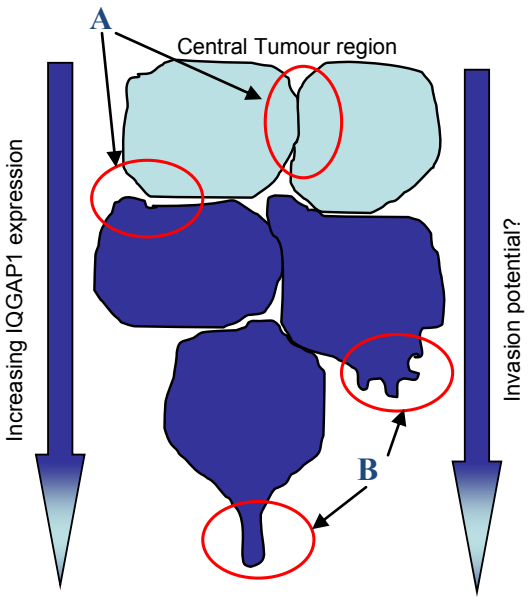


Figure 7-3 The potential role of IQGAP1 in malignant cell invasion. A basic figure showing that increased expression of IQGAP1 in cancer cells is localised in the invasive structures (B); membrane ruffles and invadapodia. Low expression of IQGAP1 is associated with low invasion and cell-cell contact (A).

Here, in this study (sections 5.3.1 and 5.2.1) we showed that IQGAP1 is over expressed in our Tamoxifen resistant cell line TAMRBU by means of SILAC and validated this by Western blot. We also showed that it has a functional role in Tamoxifen resistance effecting cellular proliferation, adherence and invasion, by using functional assays in conjunction with transient knock down of the protein expression (Figure 6-1, Figure 6-2, Figure 6-3, and Figure 6-4). This would seem to fit in with the literature,

discussed above. The expression of IQGAP1 protein has not previously been associated with Tamoxifen resistance in breast cancer.

7.2 Future work

The discovery of a role for IQGAP1 in acquired Tamoxifen resistance could just be a starting block for further work into exactly how this protein acts in order to have an effect on the phenotype of the resistant cells and through the binding of which proteins. There are several ways in which this could be studied; siRNA knockdowns of IQGAP1 in the MCF7, TAMR and TAMRBU cells could be investigated using the MAPK and the RTK antibody arrays. This would enable us to see whether the over expression of IQGAP1 in resistant cells has an effect on the MAPK and RTK family of proteins as the literature suggests that it can work with EGFR and ERK. This approach would also allow us to see whether any of the other family members are involved. It would be interesting to see whether EGFR and ErbB2 are involved, as they are the proteins that IQGAP1 has been associated with in the development of Traszumab resistance (White et al., 2011).

Performing an immunoprecipitation of IQGAP1 in combination with SDS PAGE and MS could indicate the binding proteins of the IQGAP1 in the Tamoxifen resistant cells. This could be performed in a series of increasingly stringent washes (by varying the amount of NaCl) allowing us to see the proteins that are more bound than others.

Stable suppression of IQGAP1 in MCF7 cells using a lentivirus would enable us to investigate whether the cells could still develop Tamoxifen resistance in culture using the chemical selection method which was used to develop the TAMRBU cell lines. In TAMRBU the decreased expression of IQGAP1 could be used to investigate whether the

resistant cells can continue to grow with Tamoxifen present in the media long term and if that is the case, whether they would develop a mechanism to overcome this decrease. Also, knocking out the IQGAP1 with lentivirus in MCF7 and TAMRBU would give us the ability to use assays which take a longer period of time to perform such as colony forming assays to investigate non-anchorage dependent growth – often used as a characteristic to show increased tumourgenicity. In order to determine whether IQGAP1 over expression is relevant in failure of Tamoxifen therapy, the expression of IQGAP1 in clinical samples from patients who have responded to Tamoxifen or failed to respond to therapy after two years should be analysed.

In this Thesis there was no discernible role seen for cortactin in the proliferation or adhesion of Tamoxifen resistant cells, the literature has suggested there may be a role for cortactin in invasion and motility; however, it seems that the function of cortactin may be reliant upon its phosphorylation status rather than its increased expression. Cortactin has been reported to be a master switch activating the maturation of invadapodia in breast cancer cells which is reliant upon the phosphorylation of cortactin (Mader et al., 2011). It is not known which kinases are responsible for this phosphorylation in the invadapodia, although an EGF induced phosphorylation (mediated by Src and Arg) has been shown to activate ECM degradation actin polymerisation in invadapodia and invasion (Mader et al., 2011). Oser et al., 2010 also reported that the phosphorylation of cortactin at particular residues was imperative for the maturation of the invadapodia but not associated with the EGFR- Src – Arg – Cortactin pathway described above. Further studies into the role of expression, and particularly phosphorylation, of cortactin in the

increased invasive potential seen in the Tamoxifen resistant TAMRBU cells would be interesting.

It would be appealing to further develop the adhesion assay into a more robust assay using various matrices such as fibronectin and collagen, to investigate whether IQGAP1 and cortactin and the suppression of their expression does have an effect on these cells. As a change in adhesion has been reported in Tamoxifen resistant cells, this is an important phenotypic change to analyse the functional role of the proteins found in this study.

In total there were 262 quantifiable proteins identified in this study (see section 5.1). Of these 29% were over expressed in resistance and 25% were down regulated. This gives lots of further target proteins which, after validation by Western blotting, could be investigated further using functional assays in combination with siRNA technology. These proteins are detailed in the Appendix (CD) in the back of this Thesis. The potential of discovering biomarkers using SELDI or MALDI of secretomes is another potential area for further investigation.

The opportunity to investigate some of the proteins found in this study in clinical samples, perhaps through tissue microarrays, would be the ultimate aim for any future work. Validating changes seen in the Tamoxifen resistant model in a clinical setting would be interesting – especially for the IQGAP1 where further work in Section 6 has shown it to have a role in Tamoxifen resistance/the progression of cancer. It would give us the opportunity to validate not only the proteins found in the study with patient samples but also have the potential to show that the changes seen in the cell culture model correlate in a clinical setting.

In conclusion, using proteomic technologies we have discovered several novel proteins that may be involved in the acquisition of Tamoxifen resistance. With further work this could help unfold the complicated network of proteins and signalling pathways that seem to play a role in Tamoxifen resistance and cancer progression. Figure 7-4 below is representative of the hallmarks of cancer (Hanahan and Weinburg, 2011) it has been adapted to show that in this study of Tamoxifen resistance there seems to be more emphasis on the hallmarks outlined in red; increased growth signalling, the ability to evade apoptosis/cell death, activation of invasion and metastasis and potentially limitless proliferation when TAMRBU are compared to the cancer cell line MCF7.

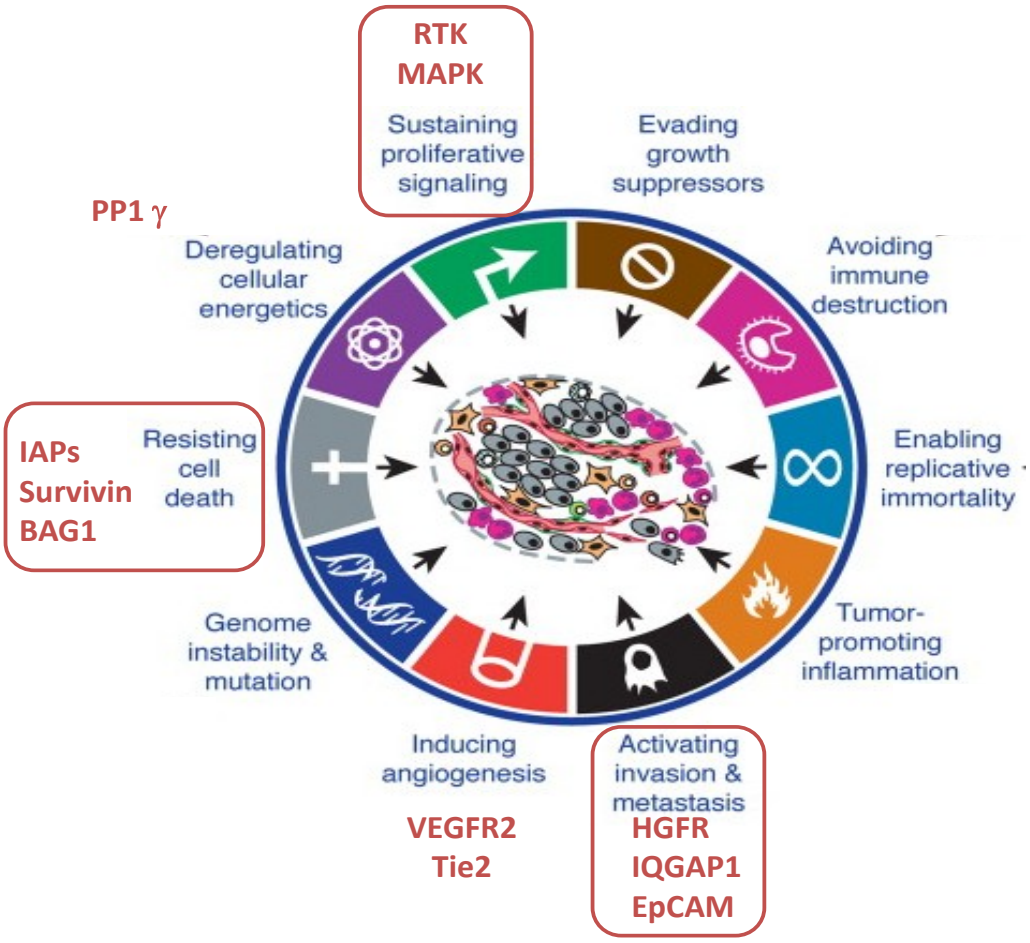


Figure 7-4 An adapted figure of the Hallmarks of cancer, this body of work suggests that Tamoxifen resistant phenotype relies upon increasing hallmarks shown here outlined in red. Adapted from Hanahan and Weinberg, 2011.

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Appendices: From CD

A. Quantifiable peptides found up regulated in TAMRBU
B. Quantifiable peptides found down regulated in TAMRBU
C. Data from SILAC experiment (with ratios)

QUANTIFIABLE PEPTIDES FOUND		MH+	Charge	XC	Score Delta Cn	Accession Sp	RSp
UPregulated in TAMRBU							
TALIN-1	-.TLAESALQLLYTAK.-	1522.77	2	3.78	0.51	1188.4	1
	-.TLAESALQLLYTAK#.-	1528.77	2	2.65	0.33	750.7	1
BCL2-ASSOCIATED ATHANOGENE							
	-.MKILEEIDTLILPENFK.-	2053.44	3	2.61	0.12	473.2	1
	-.MKILEEIDTLILPENFK#.-	2059.44	3	2.66	0.09	543.3	1
RAS GTPASE-ACTIVATING-LIKE PROTEIN IQGAP1							
	-.ATFYGEQVDYYK.-	1484.59	2	2.58	0.36	1123.0	1
	-.ATFYGEQVDYYK#.-	1490.59	2	3.03	0.45	1051.4	1
	-.TLQALQIPAAK.-	1154.39	2	3.06	0.45	1492.3	1
	-.TLQALQIPAAK#.-	1160.39	2	3.15	0.39	1363.8	1
	-.LPYDVTPEQALAHEEVK.-	1940.14	3	4.53	0.49	1318.5	1
	-.LPYDVTPEQALAHEEVK#.-	1946.14	3	4.25	0.41	1715.9	1
	-.LPYDVTPEQALAHEEVK.-	1940.14	2	4.34	0.55	802.4	1
	-.LPYDVTPEQALAHEEVK#.-	1946.14	2	2.65	0.46	376.6	1
	-.VDQIQEIVTGNPTVIK.-	1755.01	2	3.51	0.44	1454.3	1
	-.VDQIQEIVTGNPTVIK#.-	1761.01	2	2.13	0.16	829.6	1
	-.SPDVGLYGVIPEC*GETYHSDLAEAK.-	2708.91	3	5.20	0.52	986.3	1
	-.SPDVGLYGVIPEC*GETYHSDLAEAK#.-	2714.91	3	4.21	0.37	554.0	1
	-.LGLAPQIQDLYGK.-	1416.65	2	3.44	0.48	659.8	1
	-.LGLAPQIQDLYGK#.-	1422.65	2	3.01	0.36	434.5	1
	-.RLAAVALINAAIQK#.-	1458.77	2	2.60	0.20	542.5	1
	-.RLAAVALINAAIQK.-	1452.77	2	2.32	0.23	609.8	2
	-.EKLEAYQHLFYLLQTNPTYLAK.-	2685.07	3	5.20	0.39	1300.1	1
	-.EK#LEAYQHLFYLLQTNPTYLAK#.-	2697.07	3	3.85	0.31	836.2	2
	-.ILAIGLINEALDEGDAQK.-	1884.12	2	6.93	0.56	1627.6	1
	-.ILAIGLINEALDEGDAQK#.-	1890.12	2	6.63	0.58	2717.2	1
	-.FALGIFAINEAVESGDVGK#.-	1944.17	2	3.72	0.53	1251.5	1
	-.FALGIFAINEAVESGDVGK.-	1938.17	2	4.74	0.56	2082.1	1
ISOFORM 2 OF PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE-DEPENDENT RAC EXCHANGER 1 PROTEIN.							
PREX	-.IAC*YQEFAAQLK#.-	1448.64	2	2.63	0.31	694.8	1
	-.IAC*YQEFAAQLK.-	1442.64	2	3.03	0.36	1474.5	1

STAPHYLOCOCCAL NUCLEASE DOMAIN-CONTAINING PROTEIN 1							
SND1	-.KVNVTVDYIRPASPATETVPAFSER.-	2749.07	3	4.06	0.49	592.4	1
	- .K#VNVTVDYIRPASPATETVPAFSER. -	2755.07	3	2.28	0.41	407.2	1
CALPAIN-1 CATALYTIC SUBUNIT					130.23		
27.05 - 27.55	-.YLGQDYEQLR.-	1285.39	2	3.74	0.43	766.7	1
56.53 - 56.79	-.LPPGEYVVVPSTFEPNK.-	1874.13	2	2.97	0.40	694.5	1
56.73 - 57.05	-.LEIC*NLTPDALK#.-	1393.60	2	2.61	0.35	478.6	2
57.15 - 57.42	-.RPTELLSNPQFIVDGATR.-	2015.26	3	3.03	0.36	860.0	1
57.32 - 57.65	-.RPTELLSNPQFIVDGATR.-	2015.26	2	3.51	0.41	559.2	1
57.89 - 58.36	-.NYLSIFR.-	913.06	1	1.78	0.25	75.7	4
61.59 - 61.95	-.K#APSDLYQIILK#.-	1401.67	2	3.56	0.48	1372.2	1
61.61 - 62.08	-.KAPSDLYQIILK.-	1389.67	2	4.54	0.41	1208.6	1
71.73 - 71.95	-.NYPATFWVNPQFK.-	1612.81	2	3.62	0.51	936.7	1
72.67 - 72.92	-.APSDLYQIILK.-	1261.49	2	3.21	0.41	1655.0	1
78.01 - 78.26	-.LPPGEYVVVPSTFEPNKEGDFVLR.-	2691.03	3	3.89	0.51	644.5	1
78.17 - 78.45	- .LPPGEYVVVPSTFEPNK#EGDFVLR.-	2697.03	3	4.21	0.43	556.3	1
108.18 - 108.43	-.LGLVEFNILWNR.-	1474.73	2	3.37	0.42	1415.5	1
SRC SUBSTRATE CORTACTIN					50.19		
945 - 958	-.NASTFEDVTQVSSAYQK.-	1875.97	2	3.83	0.51	1266.5	1
963 - 978	-.NASTFEDVTQVSSAYQK#.-	1881.97	2	3.36	0.48	1322.1	1
1197 - 1214	-.LPSSPVYEDAASFK.-	1511.66	2	3.27	0.50	708.3	1
1200 - 1215	-.LPSSPVYEDAASFK#.-	1517.66	2	2.15	0.37	1198.9	1
3047 - 3082	-.YGLFPANYVELR.-	1442.64	2	3.08	0.43	558.7	1
RIBOPHORIN II					80.26		
2296 - 2312	-.LQVTNVLSQPLTQATVK#.-	1847.14	2	3.81	0.37	1726.3	1
2298 - 2315	-.LQVTNVLSQPLTQATVK.-	1841.14	2	5.27	0.60	1194.0	1
3531 - 3542	-.KNFESLSEAFSVASAAAVLSHNR.-	2436.67	3	5.13	0.48	2017.4	1
3539 - 3545	-.K#NFESLSEAFSVASAAAVLSHNR.-	2442.67	3	3.53	0.36	1261.0	1
Proliferation-associated protein 2G4					40.19		
1420 - 1431	-.ITSGPFEPDLYK.-	1367.53	2	2.61	0.37	710.5	1
2278 - 2315	-.SLVEASSSGVSVLSLC*EK.-	1853.06	2	3.32	0.45	528.6	1
2908 - 2928	-.HELLQPFNVLYEK.-	1630.87	2	3.81	0.29	1073.8	1
2913 - 2925	-.HELLQPFNVLYEK#.-	1636.87	2	2.37	0.05	611.8	1
HEPATOMA-DERIVED GROWTH FACTOR		**IPI00020956**			30.23		
2692 - 2728	-.GFSEGLWEIENNPTVK.-	1820.98	2	4.03	0.36	668.6	1
2720 - 2735	-.GFSEGLWEIENNPTVK#.-	1826.98	2	2.04	0.13	406.8	1
3645 - 3656	-.YQVFFFGTHETAFLGPK.-	1990.25	3	2.76	0.48	274.5	1
PROHIBITIN-2	**IPI00027252**				70.23		
302	-.IVQAEGEAEAAK.-	1216.32	2	2.15	0.24	1404.6	1
642 - 652	-.LGLDYEER.-	995.07	2	2.28	0.40	880.6	1
1332 - 1364	-.FNASQLITQR.-	1178.32	2	3.05	0.33	720.0	1
1373 - 1379	-.AQVSLLIR.-	900.10	2	2.23	0.23	643.3	4
2129 - 2159	-.LLLGAGAVAYGVR.-	1260.51	2	4.01	0.42	1385.9	1
2933 - 2951	-.IGGVQQDTILAEGLHFR.-	1855.09	2	4.60	0.49	1454.7	1
4863 - 4898	-.IPWFQYPIIYDIR.-	1725.03	2	4.36	0.49	1665.4	1

TUMOR-ASSOCIATED CALCIUM SIGNAL TRANSDUCER 1	**IPI00296215**				30.24			3 (3 0 0 0 0)
	-.AKPEGALQNNDGLYDPDC*DESGLFK.-	2754.89	3	4.73	0.42	674.3	1	28/96
3766 - 3777	-.TQNDVDIADVAYYFEK.-	1892.01	2	4.63	0.57	1639.3	1	22/30
3782 - 3832	-.TQNDVDIADVAYYFEK#.-	1898.01	2	3.38	0.42	1104.1	1	20/30
PROHIBITIN	**IPI00017334**				40.22			4 (4 0 0 0 0)
1091 - 1101	-.IFTSIGEDYDER.-	1445.51	2	2.16	0.28	749.2	1	17/22
1468 - 1492	-.FDAGELITQR.-	1150.27	2	4.20	0.50	1485.0	1	16/18
1698 - 1714	-.ILFRPVASQLPR.-	1397.69	3	2.94	0.36	366.4	1	20/44
2123 - 2164	-.K#LEAAEDIAYQLSR.-	1607.79	2	4.32	0.49	2091.2	1	20/26
	-. KLEAAEDIAYQLSR.-	1613.79	2	4.39	0.48	2091.2	1	20/26
CHLORIDE INTRACELLULAR CHANNEL PROTEIN 1	**IPI00010896**				**60.27**			**6 (6 0 0 0 0)**
2483 - 2498	-.VLDNYLTSPLPEEVDETSAEDEGVSQR.-	2994.13	2	4.39	0.50	480.5	1	21/52
2753 - 2754	-.EEFASTC*PDDEEIELAYEQVAK.-	2574.68	2	2.05	0.35	304.9	1	15/42
2895 - 2954	-.LAALNPESNTAGLDIFAK.-	1846.07	2	4.44	0.61	921.6	1	22/34
2955 - 2978	-.LAALNPESNTAGLDIFAK#.-	1852.07	2	4.50	0.48	999.0	1	23/34
2960 - 2972	-.LAALNPESNTAGLDIFAK.-	1846.07	2	5.30	0.58	1025.3	1	22/34
3240 - 3251	-.FLDGNELTLADC*NLLPK.-	1934.17	2	3.42	0.19	689.5	1	14/32
HIGH MOBILITY GROUP PROTEIN B1					*20.28*			*2 (2 0 0 0 0)*
2999 - 3008	*-.RPPSAFFLFC*SEYRPK.-*	*2003.29*	*3*	*5.64*	*0.50*	*1724.3*	*1*	*29/60*
3011 - 3027	*-.RPPSAFFLFC*SEYRPK#.-*	*2009.29*	*3*	*5.09*	*0.41*	*1478.2*	*1*	*27/60*

QUANTIFIABLE PEPTIDES FOUND
Down regulated in TAMRBU

ISOFORM 1 OF NF-X1-TYPE ZINC FINGER PROTEIN NFXL1.					28.27		3 (2 1 0 0 0)
35.04 - 35.21	-.FLDPNTLER.-	1105.23	2	3.27	0.26	656.7	14/16
69.28 - 69.89	-.LAAKLSATLEQAAATAR.-	1686.94	2	4.34	0.50	1374.3	19/32
82.83 - 83.30	-.QVVSAVTTLVEAAER.-	1573.77	2	5.37	0.50	1787.7	22/28
VIGILIN.					90.32		9 (9 0 0 0 0)
1589 - 1619	-.IREESNTKIDLPAENSNSETIIITGK.-	2874.15	3	2.89	0.30	201.1	20/100
1589 - 1619	-.IDLPAENSNSETIIIITGK.-	1916.12	2	4.26	0.43	624.4	19/34
1599 - 1620	-.IREESNTKIDLPAENSNSETIIITGK#.-	2880.15	3	2.86	0.24	234.0	20/100
1599 - 1620	-.IDLPAENSNSETIIIITGK#.-	1922.12	2	3.59	0.34	580.7	19/34
1668 - 1678	-.GNSLQEILER.-	1159.28	2	3.14	0.32	1262.0	15/18
2121 - 2140	-.ANSFTVSSVAAPSWLHR.-	1831.02	2	3.21	0.55	678.3	16/32
2740 - 2763	-.ASVITQVFHVPLEER.-	1725.97	2	2.79	0.36	551.4	13/28
2747 - 2756	-.ASVITQVFHVPLEER.-	1725.97	3	3.31	0.53	535.0	23/56
2795 - 2808	-.DKFPEVIINFPDPAQK.-	1859.12	2	2.77	0.39	660.5	16/30
ALPHA-ACTININ-4.					198.29	20 (19 1 0 0 0)	
615 - 630	-.ASFNHFDKDHGGALGPEEFK.-	2204.34	3	3.75	0.30	2494.5	31/76
1139 - 1163	-.LSGSNPYTTVTPQIINSK.-	1921.14	2	3.40	0.17	770.1	19/34
1186 - 1204	-.LSGSNPYTTVTPQIINSK#.-	1927.14	2	3.45	0.39	988.8	19/34
1304 - 1320	-.LVSIGAEEIVDGNAK.-	1515.69	2	4.60	0.54	1307.7	19/28
1312 - 1334	-.LVSIGAEEIVDGNAK#.-	1521.69	2	3.15	0.42	969.2	17/28
1342 - 1352	-.VLAGDKNFITAEELR.-	1676.90	3	3.33	0.22	654.1	22/56
1360 - 1392	-.VLAGDKNFITAEELR.-	1676.90	2	2.75	0.39	417.9	16/28
1536 - 1557	-.KAGTQIENIDEDFRDGLK.-	2050.22	3	5.49	0.54	2026.3	33/68
1775 - 1800	-.KDDPVTNLNNAFEVAEK.-	1905.06	2	5.66	0.60	1809.1	26/32
1901 - 1910	-.QLEAIDQLHLEYAK.-	1671.88	2	3.40	0.38	879.7	16/26
1950 - 1958	-.NVNVQNFHISWK.-	1486.66	2	3.68	0.48	1758.5	18/22
1965 - 1976	-.DGLAFNALIHR.-	1227.40	2	3.27	0.38	979.7	15/20
1980 - 1985	-.DGLAFNALIHR.-	1227.40	3	2.53	0.38	447.6	20/40
1983 - 1996	-.QFASQANVVGPWIQTK.-	1775.00	2	4.92	0.52	771.2	20/30
1984 - 2025	-.ETTDTDTADQVIASFK.-	1742.82	2	5.71	0.58	1757.4	21/30
2004 - 2014	-.ETTDTDTADQVIASFK#.-	1748.82	2	3.57	0.50	1405.2	19/30
2092 - 2104	-.AC*LISLGYDVENDR.-	1625.76	2	2.99	0.28	1082.9	19/26
2103 - 2121	-.IC*DQWDALGSLTHSR.-	1759.90	3	3.89	0.51	1470.3	32/56
2112 - 2124	-.IC*DQWDALGSLTHSR.-	1759.90	2	2.93	0.31	1119.0	17/28
2247 - 2294	-.DDPVTNLNNAFEVAEK.-	1776.88	2	4.54	0.57	1593.8	24/30

PDCD6IP PROTEIN					40.21		4 (4 0 0 0 0)
47.15 - 47.58	-.HYQFASGAFLHIK.-	1519.73	3	3.48	0.39	2052.3	28/48
68.14 - 68.65	-.SVIEQGGIQTVDQLIK.-	1728.97	2	4.30	0.52	1829.5	20/30
68.20 - 68.44	-.SVIEQGGIQTVDQLIK#.-	1734.97	2	2.74	0.34	1572.7	21/30
85.48 - 85.72	-.FYNELTEILVR.-	1397.60	2	3.18	0.45	1091.2	17/20
AFG3-LIKE PROTEIN 2					78.39		8 (7 1 0 0 0)
608 - 620	-.QGDMVLEKPYSEATAR.-	1796.00	2	2.02	0.19	397.5	15/30
1438 - 1449	-.GLGYAQYLPK.-	1110.29	2	2.47	0.33	1184.6	15/18
1832 - 1844	-.VGQISFDLPR.-	1132.29	2	3.11	0.30	777.0	15/18
2301 - 2310	-.VALLLLEK.-	899.15	2	2.17	0.12	806.6	13/14
2302 - 2304	-.VALLLLEK.-	899.15	1	1.72	0.17	416.1	11/14
2442 - 2450	-.NLETLQQELGIEGENR.-	1843.97	2	4.39	0.49	1364.7	19/30
2609	-.TVAYHEAGHAVAGWYLEHADPLLK.-	2649.94	3	2.55	0.22	430.0	27/92
4056	-.NAPC*ILFIDEIDAVGR.-	1804.03	2	2.48	0.44	556.7	16/30
PDCD6IP PROTEIN					70.31		7 (7 0 0 0 0)
1209 - 1220	-.LALASLGYEK.-	1065.25	2	2.02	0.16	822.4	14/18
1671 - 1684	-.HYQFASGAFLHIK.-	1519.73	3	4.59	0.45	2307.1	31/48
1674 - 1698	-.HYQFASGAFLHIK#.-	1525.73	3	2.91	0.40	1299.0	23/48
2157 - 2170	-.LANQAADYFGDAFK.-	1531.65	2	2.11	0.09	980.2	17/26
2633 - 2669	-.SVIEQGGIQTVDQLIK#.-	1734.97	2	4.80	0.52	1634.9	21/30
2636 - 2666	-.SVIEQGGIQTVDQLIK.-	1728.97	2	6.18	0.47	2649.2	25/30
3408 - 3426	-.FYNELTEILVR.-	1397.60	2	3.56	0.33	771.7	13/20
CENTAURIN-DELTA-3						20.38	
2224 - 2258	-.PDWAAVNLGVVIC*K#QCAGQHR.-	2329.66	3	2.69	0.08	231.0	2 (2 0 0 0 0)
2331 - 2368	-.PDWAAVNLGVVICK#QC*AGQHR.-	2329.66	3	2.52	0.13	162.9	17/80 18/80
LAMIN-B1.					40.29		4 (4 0 0 0 0)
1113 - 1133	-.SLETENSALQLQVTER.-	1818.96	2	5.76	0.40	2249.7	22/30
1323 - 1385	-.IESLSSQLSNLQK.-	1447.62	2	2.99	0.36	1114.3	17/24
1331 - 1389	-.IESLSSQLSNLQK#.-	1453.62	2	2.46	0.36	642.3	13/24
2111 - 2158	-.IQELEDLLAK.-	1172.35	2	2.35	0.12	607.7	14/18

PLASTIN-1					20.15			2 (2 0 0 0 0)
597 - 604	-.EGITAIGGTSTISSEGTQHSYSEEEK#.-	2705.78	3	3.00	0.30	289.2	1	25/100
2162 - 2178	-.AYFHLLNQIAPK.-	1415.66	2	2.14	0.37	853.6	1	14/22
Ezrin-radixin-moesin-binding phosphoprotein 50					30.28			3 (3 0 0 0 0)
515 - 537	-.AQEAPGQAEPPAAAEVQGAGNENEPR.-	2589.67	2	5.60	0.57	812.8	1	25/50
1062 - 1072	-.LLVVDPETDEQLQK.-	1627.82	2	3.30	0.45	725.0	1	18/26
1064 - 1077	-.LLVVDPETDEQLQK#.-	1633.82	2	3.51	0.42	1070.1	1	21/26
Cytochrome b-c1 complex subunit 2 mitochondrial					84.23			9 (8 0 0 1 0)
851 - 861	-.ATAAPAGAPPQPQDLEFTK.-	1911.11	2	3.81	0.44	495.8	1	22/36
867 - 888	-.ATAAPAGAPPQPQDLEFTK#.-	1917.11	2	3.27	0.46	480.8	1	19/36
1336 - 1350	-.NALANPLYC*PDYR.-	1567.72	2	2.37	0.31	431.9	1	13/24
1557 - 1571	-.AVAFQNPQTHVIENLHAAAYR.-	2351.61	3	3.32	0.46	973.5	1	26/80
1882 - 1893	-.YEDFSNLGTTHLLR.-	1666.82	3	3.31	0.24	702.5	1	27/52
1895 - 1930	-.YEDFSNLGTTHLLR.-	1666.82	2	2.08	0.37	410.2	11	15/26
1943 - 1959	-.VTSEELHYFVQNHFTSAR.-	2166.34	3	3.68	0.50	1008.4	1	27/68
3488 - 3508	-.LPNGLVIASLENYSPVSR.-	1930.19	2	2.85	0.41	708.4	1	14/34
3639 - 3674	-.LPNGLVIASLENYSPVSR.-	1930.19	2	4.50	0.51	1728.2	1	22/34
NUCLEAR MIGRATION PROTEIN NUDC					30.62			3 (3 0 0 0 0)
743 - 800	-.LKPNLGNGADLPNYR.-	1642.84	3	3.77	0.33	1167.8	1	29/56
802 - 814	-.LK#PNLGNGADLPNYR.-	1648.84	3	2.64	0.04	357.3	2	23/56
1899 - 1925	-.GQPAIIDGELYNEVK.-	1646.82	2	4.30	0.45	1471.1	1	22/28
ISOFORM 1 OF PROTEIN SET					20.22			2 (2 0 0 0 0)
2968 - 2987	-.IDFYFDENPYFENK.-	1841.95	2	4.48	0.44	2003.2	1	21/26
2969 - 2986	-.IDFYFDENPYFENK#.-	1847.95	2	3.58	0.27	1652.7	1	19/26
CATHEPSIN D					*40.24*			*4 (4 0 0 0 0)*
498 - 509	*-.VGFAEAAR.-*	*820.92*	*2*	*2.41*	*0.24*	*1009.7*	*1*	*13/14*
1750 - 1820	*-.VSTLPAITLK.-*	*1043.28*	*2*	*2.13*	*0.35*	*267.9*	*7*	*11/18*
1805 - 1814	*-.VSTLPAITLK#.-*	*1049.28*	*2*	*2.11*	*0.19*	*317.2*	*2*	*12/18*
4134 - 4161	*-.LVDQNIFSFYLSR.-*	*1602.82*	*2*	*4.82*	*0.46*	*1337.5*	*1*	*20/24*

VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 2					100.24			10 (10 0 0 0 0)
506 - 513	-.SC*SGVEFSTSGSSNTDTGK.-	1908.91	2	3.42	0.43	679.3	1	17/36
508	-.SC*SGVEFSTSGSSNTDTGK#.-	1914.91	2	3.05	0.54	575.5	1	17/36
1260 - 1264	-.LTFDTTFSPNTGKK.-	1557.73	2	2.53	0.33	590.7	1	14/26
1634 - 1646	-.LTFDTTFSPNTGK.-	1429.56	2	3.98	0.51	912.2	1	15/24
1700 - 1740	-.VNNSSLIGVGYTQTLRPGVK.-	2104.40	3	3.73	0.34	621.1	1	23/76
1709 - 1719	-.VNNSSLIGVGYTQTLRPGVK#.-	2110.40	3	2.90	0.37	681.9	1	26/76
1863 - 1883	-.TGDFQLHTNVNDGTEFGGSIYQK.-	2529.66	3	4.84	0.43	1096.2	1	30/88
1864 - 1887	-.TGDFQLHTNVNDGTEFGGSIYQK#.-	2535.66	3	3.21	0.21	894.4	1	27/88
2276 - 2301	-.LTLSALVDGK.-	1017.20	2	3.18	0.46	815.7	1	15/18
2280 - 2289	-.LTLSALVDGK#.-	1023.20	2	2.54	0.40	1229.1	1	16/18
CALPONIN-2					20.50			2 (2 0 0 0 0)
4134 - 4178	-.TWIEGLTGLSIGPDFQK.-	1863.10	2	4.68	0.64	1406.0	1	21/32
4179 - 4206	-.TWIEGLTGLSIGPDFQK#.-	1869.10	2	4.04	0.59	900.5	1	17/32
FAM49B					20.16			2 (2 0 0 0 0)
4565 - 4600	-.NVFDEAILAALEPPEPK.-	1854.09	2	3.11	0.37	378.3	1	15/32
4598	-.NVFDEAILAALEPPEPK#.-	1860.09	2	2.13	0.28	577.6	1	19/32

				RATIO	
				LIGHT	HEAVY
				TAMRBU	MCF7
TUMOR-ASSOCIATED CALCIUM SIGNAL TRANSDUCER 1	3 (3 0 0 0 0)	1 :0.11	P16422	9.09090	1
BCL2-ASSOCIATED ATHANOGENE (BAG-1)	2 (2 0 0 0 0)	1 : 0.14	Q99933	7.14285	1
Alpha-enolase	6 (6 0 0 0 0)	1 : 0.20	P06733	5	1
RIBOPHORIN II	4 (4 0 0 0 0)	1 : 0.23	P04844	4.34782	1
ISOCITRATE DEHYDROGENASE [NADP] CYTOPLASMIC	4 (4 0 0 0 0)	1 : 0.25	O75874	4	1
CHLORIDE INTRACELLULAR CHANNEL PROTEIN 1	11 (9 2 0 0 0)	1 : 0.26	O00299	3.84154	1
HIGH MOBILITY GROUP PROTEIN B1	2 (2 0 0 0 0)	1 : 0.28	P09429	3.57142	1
ISOFORM 2 OF PHOSPHATIDYLINOSITOL 3,4,5-TRISPHOSPHATE-DEPENDENT RAC EXCHANGER 1 PROTEIN.	10 (10 0 0 0 0)	1 : 0.28	Q8TCU6	3.57142	1
CALPAIN-1 CATALYTIC SUBUNIT	6 (6 0 0 0 0)	1 : 0.29	P07384	3.44827	1
STAPHYLOCOCCAL NUCLEASE DOMAIN-CONTAINING PROTEIN 1 (snd1)	4 (4 0 0 0 0)	1 : 0.29	Q7KZF4	3.44827	1
Proliferation-associated protein 2G4	4 (4 0 0 0 0)	1 : 0.30	Q9UQ80	3.33333	1
TALIN-1.	6 (6 0 0 0 0)	1 : 0.31	Q9Y490	3.22580	1
ISOFORM 1 OF CYTOSKELETON-ASSOCIATED PROTEIN 4.	6 (6 0 0 0 0)	1 : 0.32	Q8IYA6	3.125	1
ISOFORM 4 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A/B	4 (4 0 0 0 0)	1 : 0.34	Q99729	2.94117	1
ISOFORM A1-B OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN A1	2 (2 0 0 0 0)	1 : 0.37	P09651	2.70270	1
RAS GTPASE-ACTIVATING-LIKE PROTEIN IQGAP1	36 (36 0 0 0 0)	1 : 0.38	P46940	2.63157	1
Purine nucleoside phosphorylase	6 (6 0 0 0 0)	1 : 0.40	P00491	2.5	1
ISOFORM 1 OF EXPORTIN-2.	7 (7 0 0 0 0)	1 : 0.41	P55060	2.43902	1
SRC SUBSTRATE CORTACTIN	5 (5 0 0 0 0)	1 : 0.42	Q14247	2.38095	1
D-3-PHOSPHOGLYCERATE DEHYDROGENASE	7 (7 0 0 0 0)	1 : 0.43	O43175	2.32558	1
HEPATOMA-DERIVED GROWTH FACTOR	3 (3 0 0 0 0)	1 : 0.46	P51858	2.17391	1
VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 2	10 (10 0 0 0 0)	1 : 0.47	P45880	2.12766	1
CYTOCHROME B-C1 COMPLEX SUBUNIT 2, MITOCHONDRIAL	10 (10 0 0 0 0)	1 : 0.50	P22695	2	1
PROHIBITIN	7 (7 0 0 0 0)	1 : 0.50	P35232	2	1
Thioredoxin domain-containing protein 5	3 (3 0 0 0 0)	1 : 0.52	Q8NBS9	1.92307	1
ISOFORM 1 OF CATENIN ALPHA-1	3 (3 0 0 0 0)	1 : 0.53	P35221	1.88679	1
PUTATIVE ANNEXIN A2-LIKE PROTEIN	11 (11 0 0 0 0)	1 : 0.45	A6NMY6	1.85185	1
ISOFORM GAMMA-1 OF SERINE/THREONINE-PROTEIN PHOSPHATASE PP1-GAMMA CATALYTIC SUBUNIT	4 (4 0 0 0 0)	1 : 0.55	P36873	1.81818	1
PYRROLINE-5-CARBOXYLATE REDUCTASE 1 ISOFORM 2	4 (4 0 0 0 0)	1 : 0.56	A6NFM2	1.78571	1
14-3-3 PROTEIN EPSILON	8 (8 0 0 0 0)	1 : 0.57	P62258	1.75438	1
PLASTIN 3.	2 (2 0 0 0 0)	1 : 0.58	P13797	1.72413	1
HEAT SHOCK 70 KDA PROTEIN 4	8 (8 0 0 0 0)	1 : 0.58	P34932	1.72413	1
Rab GDP dissociation inhibitor beta	3 (3 0 0 0 0)	1 : 0.6	P50395	1.66666	1
THIOREDOXIN DOMAIN-CONTAINING PROTEIN 4.	5 (5 0 0 0 0)	1 : 0.60	Q9BS26	1.66666	1
HEAT SHOCK 70 KDA PROTEIN 1	16 (16 0 0 0 0)	1 : 0.61	P34931	1.63934	1

14-3-3 PROTEIN ZETA/DELTA	6 (6 0 0 0 0)	1 : 0.61	P63104	1.63934	1
INTERLEUKIN ENHANCER-BINDING FACTOR 2	5 (5 0 0 0 0)	1 : 0.61	Q12905	1.63934	1
ELONGATION FACTOR 1-BETA	4 (2 2 0 0 0)	1 : 0.62	P24534	1.61290	1
ISOFORM 2 OF PHOSPHATIDYLINOSITOL-BINDING CLATHRIN ASSEMBLY PROTEIN	2 (2 0 0 0 0)	1 : 0.62	Q13492	1.61290	1
ISOFORM 1 OF EXTENDED-SYNAPTOTAGMIN-1.	11 (11 0 0 0 0)	1 : 0.62	Q9BSJ8	1.61290	1
Elongation factor 1-alpha 2	15 (15 0 0 0 0)	1 : 0.63	Q05639	1.58730	1
HSP HEAT SHOCK PROTEIN 90KDA ALPHA (CYTOSOLIC), CLASS A MEMBER 1 ISOFORM 1	12 (12 0 0 0 0)	1:0.63	P07900	1.5625	1
ISOFORM 1 OF PLECTIN-1	69 (68 1 0 0 0)	1 : 0.64	Q15149	1.5625	1
GLUCOSE-6-PHOSPHATE ISOMERASE	9 (9 0 0 0 0)	1 : 0.65	P06744	1.53846	1
ISOFORM LONG OF SPECTRIN BETA CHAIN, BRAIN 1	8 (8 0 0 0 0)	1 : 0.65	Q01082	1.53846	1
GUANINE NUCLEOTIDE-BINDING PROTEIN SUBUNIT BETA 2-LIKE 1	6 (6 0 0 0 0)	1 : 0.66	P63244	1.51515	1
ISOFORM 1 OF CLATHRIN HEAVY CHAIN 1.	18 (17 1 0 0 0)	1 : 0.66	Q00610	1.51515	1
TRANSLATIONAL ACTIVATOR GCN1	8 (8 0 0 0 0)	1 : 0.66	Q92616	1.51515	1
ISOFORM 2 OF WD REPEAT-CONTAINING PROTEIN 1	2 (2 0 0 0 0)	1 : 0.67	O43379	1.49253	1
GLUTAREDOXIN-3	2 (2 0 0 0 0)	1 : 0.67	O76003	1.49253	1
ISOFORM 1 OF EXPORTIN-5	5 (5 0 0 0 0)	1 : 0.68	Q9HAV4	1.47058	1
ALPHA-ACTININ-1	13 (13 0 0 0 0)	1 : 0.70	P12814	1.42857	1
ISOFORM 1 OF FILAMIN-B	28 (26 2 0 0 0)	1 : 0.71	O75369	1.40845	1
EARLY ENDOSOME ANTIGEN 1.	5 (5 0 0 0 0)	1 : 0.72	Q15075	1.38888	1
ISOFORM 1 OF IMPORTIN-4	6 (6 0 0 0 0)	1 : 0.72	Q8TEX9	1.38888	1
cDNA FLJ13940 fis, clone Y79AA1000833, moderately similar to TUBULIN ALPHA-1 CHAIN	3 (3 0 0 0 0)	1 : 0.72	Q9H853	1.38888	1
EUKARYOTIC INITIATION FACTOR 4A-I	5 (5 0 0 0 0)	1 : 0.73	P60842	1.36986	1
Dynactin subunit 2	3 (3 0 0 0 0)	1 : 0.73	Q13561	1.36986	1
HSPA5 PROTEIN	3 (3 0 0 0 0)	1 : 0.73	Q2KHP4	1.36986	1
ISOFORM M1 OF PYRUVATE KINASE ISOZYMES M1/M2	20 (20 0 0 0 0)	1 : 0.74	P14618	1.35135	1
EZRIN.	3 (3 0 0 0 0)	1 : 0.75	P15311	1.33333	1
Rab GDP dissociation inhibitor alpha	2 (2 0 0 0 0)	1 : 0.75	P31150	1.33333	1
IMPORTIN SUBUNIT BETA-1	3 (3 0 0 0 0)	1 : 0.75	Q14974	1.33333	1
Calcium-binding mitochondrial carrier protein SCaMC-1	2 (2 0 0 0 0)	1 : 0.76	Q6NUK1	1.31578	1
ISOFORM 1 OF SQUAMOUS CELL CARCINOMA ANTIGEN RECOGNIZED BY T-CELLS 3	2 (2 0 0 0 0)	1 : 0.77	Q15020	1.29870	1
PUTATIVE NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX SUBUNIT ALPHA-LIKE PROTEIN	3 (2 0 0 1 0)	1 : 0.78	Q9BZK3	1.29870	1
Gamma-enolase	5 (2 3 0 0 0)	1 : 0.80	P09104	1.25	1
ACYL-COA DEHYDROGENASE FAMILY MEMBER 9, MITOCHONDRIAL	2 (2 0 0 0 0)	1 : 0.81	Q9H845	1.23456	1
STRESS-INDUCED-PHOSPHOPROTEIN 1	3 (2 1 0 0 0)	1 : 0.82	P31948	1.21951	1
PUROMYCIN-SENSITIVE AMINOPEPTIDASE	10 (10 0 0 0 0)	1 : 0.82	P55786	1.21951	1
Protein disulfide-isomerase A6	8 (8 0 0 0 0)	1 : 0.82	Q6ZSH5	1.21951	1

ISOFORM 1 OF KINECTIN	7 (7 0 0 0 0)	1 : 0.82	Q86UP2	1.21951	1
PHOSPHOENOLPYRUVATE CARBOXYKINASE [GTP], MITOCHONDRIAL	4 (4 0 0 0 0)	1 : 0.83	P35558	1.20481	1
ATP synthase subunit beta, mitochondrial	5 (5 0 0 0 0)	1 : 0.84	P06576	1.19047	1
PROTEIN DISULFIDE-ISOMERASE	13 (13 0 0 0 0)	1 : 0.84	P07237	1.19047	1
60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL	14 (14 0 0 0 0)	1 : 0.84	P10809	1.19047	1
UBIQUITIN-LIKE MODIFIER-ACTIVATING ENZYME 1	5 (5 0 0 0 0)	1 : 0.84	P22314	1.19047	1
Tubulin beta-8 chain	2 (2 0 0 0 0)	1 : 0.84	Q3ZCM	1.19047	1
Elongation factor 1-gamma	5 (5 0 0 0 0)	1 : 0.85	P26641	1.17647	1
Hsc70-interacting protein	3 (3 0 0 0 0)	1 : 0.85	P50502	1.17647	1
ELONGATION FACTOR 2	9 (9 0 0 0 0)	1 : 0.87	P13639	1.14942	1
ISOFORM 2 OF AP-2 COMPLEX SUBUNIT BETA-1	2 (2 0 0 0 0)	1 : 0.87	P63010	1.14942	1
ENDOPLASMIN	7 (7 0 0 0 0)	1 : 0.88	P14625	1.13636	1
Hsp90 co-chaperone Cdc37	2 (2 0 0 0 0)	1 : 0.88	Q16543	1.13636	1
ISOFORM 1 OF DNA-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT	27 (27 0 0 0 0)	1 : 0.89	P78527	1.12359	1
CYTOPLASMIC DYNEIN 1 HEAVY CHAIN 1	58 (57 1 0 0 0)	1 : 0.89	Q14204	1.12359	1
SIMILAR TO HEAT SHOCK PROTEIN HSP 90-BETA	2 (2 0 0 0 0)	1:0.91	P08238	1.09890	1
CALRETICULIN	3 (3 0 0 0 0)	1 : 0.91	P27797	1.09890	1
ELONGATION FACTOR 1-DELTA	6 (5 1 0 0 0)	1 : 0.91	P29692	1.09890	1
ISOFORM R-TYPE OF PYRUVATE KINASE ISOZYMES R/L	2 (2 0 0 0 0)	1 : 0.91	P30613	1.09890	1
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN H	2 (2 0 0 0 0)	1 : 0.91	P31943	1.09890	1
NASCENT POLYPEPTIDE-ASSOCIATED COMPLEX SUBUNIT ALPHA	5 (4 1 0 0 0)	1 : 0.91	Q13765	1.09890	1
EXPORTIN-1.	5 (5 0 0 0 0)	1 : 0.92	O14980	1.08695	1
ISOFORM 1 OF U5 SMALL NUCLEAR RIBONUCLEOPROTEIN 200 KDA HELICASE	10 (10 0 0 0 0)	1 : 0.92	O75643	1.08695	1
ARGININOSUCCINATE SYNTHASE	5 (5 0 0 0 0)	1 : 0.92	P00966	1.08695	1
ADP/ATP TRANSLOCASE 2	9 (9 0 0 0 0)	1 : 0.92	P05141	1.08695	1
BIFUNCTIONAL AMINOACYL-TRNA SYNTHETASE	12 (12 0 0 0 0)	1 : 0.92	P07814	1.08695	1
ISOFORM 1 OF POLYADENYLATE-BINDING PROTEIN 1.	12 (12 0 0 0 0)	1 : 0.92	P11940	1.08695	1
ADP/ATP TRANSLOCASE 3	3 (3 0 0 0 0)	1 : 0.22	P12236	1.08695	1
TRANSITIONAL ENDOPLASMIC RETICULUM ATPASE	8 (8 0 0 0 0)	1 : 0.92	P55072	1.08695	1
GLUCOSAMINE--FRUCTOSE-6-PHOSPHATE AMINOTRANSFERASE [ISOMERIZING] 2	2 (2 0 0 0 0)	1 : 0.92	Q06210	1.08695	1
UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 7	4 (4 0 0 0 0)	1 : 0.92	Q93009	1.08695	1
LEUCYL-TRNA SYNTHETASE, CYTOPLASMIC	6 (6 0 0 0 0)	1 : 0.92	Q9P2J5	1.08695	1
CDNA FLJ54957, HIGHLY SIMILAR TO TRANSKETOLASE	4 (4 0 0 0 0)	1 : 0.93	B4DE31	1.07526	1
ISOFORM 1 OF MYOSIN-9	43 (43 0 0 0 0)	1 : 0.93	P35579	1.07526	1
EUKARYOTIC INITIATION FACTOR 4A-III	3 (3 0 0 0 0)	1 : 0.98	P38919	1.07526	1
MALATE DEHYDROGENASE, CYTOPLASMIC.	6 (6 0 0 0 0)	1 : 0.93	P40925	1.07526	1
ISOFORM 1 OF CLATHRIN HEAVY CHAIN 2	10 (10 0 0 0 0)	1 : 0.93	P53675	1.07526	1
COMPLEMENT COMPONENT 1 Q SUBCOMPONENT-BINDING PROTEIN, MITOCHONDRIAL	3 (3 0 0 0 0)	1 : 0.93	Q07021	1.07526	1

MULTISYNTHETASE COMPLEX AUXILIARY COMPONENT P38	3 (3 0 0 0 0)	1 : 0.93	Q13155	1.07526	1
ISOFORM 1 OF DIPEPTIDYL-PEPTIDASE 3	12 (12 0 0 0 0)	1 : 0.93	Q9NY33	1.07526	1
ATP SYNTHASE SUBUNIT ALPHA, MITOCHONDRIAL	9 (8 1 0 0 0)	1 : 0.94	O43175	1.06383	1
EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT A	5 (4 0 1 0 0)	1 : 0.94	O75821	1.06383	1
PHOSPHOGLYCERATE KINASE 1	10 (10 0 0 0 0)	1 : 0.94	P00558	1.06383	1
TROPOMYOSIN 3	4 (4 0 0 0 0)	1 : 0.94	P06753	1.06383	1
L-LACTATE DEHYDROGENASE B CHAIN	5 (5 0 0 0 0)	1 : 0.94	P07195	1.06383	1
DNA TOPOISOMERASE 1.	2 (2 0 0 0 0)	1 : 0.94	P11387	1.06383	1
ISOFORM 1 OF MYOSIN-10	9 (7 1 0 1 0)	1 : 0.94	P35580	1.06383	1
TUBULIN ALPHA-4A CHAIN	16 (16 0 0 0 0)	1 : 0.94	P68366	1.06383	1
ISOFORM 2 OF SIGNAL RECOGNITION PARTICLE 68 KDA PROTEIN.	4 (4 0 0 0 0)	1 : 0.94	Q9UHB9	1.06383	1
ISOFORM 2 OF N-ACETYLSEROTONIN O-METHYLTRANSFERASE-LIKE PROTEIN.	2 (2 0 0 0 0)	1 : 0.95	O95671	1.05263	1
ISOFORM 1 OF HEAT SHOCK COGNATE 71 KDA PROTEIN.	13 (13 0 0 0 0)	1 : 0.95	P11142	1.05263	1
ATP SYNTHASE SUBUNIT BETA, MITOCHONDRIAL	10 (10 0 0 0 0)	1 : 0.95	P24539	1.05263	1
SWI/SNF-RELATED MATRIX-ASSOCIATED ACTIN-DEPENDENT REGULATOR OF CHROMATIN A4 ISOFORM D. SMARCA4	5 (5 0 0 0 0)	1 : 0.95	Q12824	1.05263	1
2,4-DIENOYL-COA REDUCTASE, MITOCHONDRIAL	7 (7 0 0 0 0)	1 : 0.95	Q16698	1.05263	1
LEUCINE-RICH REPEAT-CONTAINING PROTEIN 59	11 (11 0 0 0 0)	1 : 0.95	Q96AG4	1.05263	1
ISOFORM 2 OF U5 SMALL NUCLEAR RIBONUCLEOPROTEIN 200 KDA HELICASE	3 (3 0 0 0 0)	1 : 0.96	O75643	1.04166	1
EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT C	4 (4 0 0 0 0)	1 : 0.96	Q99613	1.04166	1
ISOFORM 3 OF OBG-LIKE ATPASE 1	3 (3 0 0 0 0)	1 : 0.96	Q9NTK5	1.04166	1
ATP-DEPENDENT RNA HELICASE DDX3X	6 (6 0 0 0 0)	0.97 : 1	O00571	1.03092	1
C-1-TETRAHYDROFOLATE SYNTHASE, CYTOPLASMIC	7 (7 0 0 0 0)	1 : 0.97	P11586	1.03092	1
ATP-DEPENDENT DNA HELICASE 2 SUBUNIT 1	5 (5 0 0 0 0)	1 : 0.97	P46063	1.03092	1

FATTY ACID SYNTHASE	62 (62 0 0 0 0)	1 : 0.97	P49327	1.03092	1
Tubulin beta-2A chain	2 (2 0 0 0 0)	1 : 0.97	Q13885	1.03092	1
SIMILAR TO BETA-ACTIN	2 (2 0 0 0 0)	1 : 0.97	Q9BYX7	1.03092	1
PCTP-LIKE PROTEIN	3 (3 0 0 0 0)	1 : 0.97	Q9Y365	1.03092	1
ISOFORM 1 OF HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN Q	6 (6 0 0 0 0)	1 : 0.98	O60506	1.02040	1
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN C-LIKE 1	5 (5 0 0 0 0)	1 : 0.98	O60812	1.02040	1
KERATIN, TYPE II CYTOSKELETAL 1	3 (2 1 0 0 0)	1 : 0.98	P04264	1.02040	1
PROTEIN DISULFIDE-ISOMERASE A4	7 (7 0 0 0 0)	1 : 0.98	P13667	1.02040	1
ELECTRON TRANSFER FLAVOPROTEIN SUBUNIT ALPHA, MITOCHONDRIAL	8 (8 0 0 0 0)	1 : 0.98	P13804	1.02040	1
PROTEASOME SUBUNIT ALPHA TYPE-4	4 (4 0 0 0 0)	1 : 0.98	P25789	1.02040	1
ISOFORM LONG OF DELTA-1-PYRROLINE-5-CARBOXYLATE SYNTHETASE	9 (8 1 0 0 0)	1:0.98	P54886	1.02040	1
ISOFORM 1 OF EUKARYOTIC TRANSLATION INITIATION FACTOR 3 SUBUNIT B	2 (2 0 0 0 0)	1 : 0.98	P55884	1.02040	1
Epiplakin	42 (40 2 0 0 0)	1 : 0.98	P58107	1.02040	1
PUTATIVE HEAT SHOCK PROTEIN HSP 90-ALPHA A2	4 (4 0 0 0 0)	1:0.98	Q14568	1.02040	1
116 KDA U5 SMALL NUCLEAR RIBONUCLEOPROTEIN COMPONENT	9 (9 0 0 0 0)	1 : 0.98	Q15029	1.02040	1
BETA-ACTIN-LIKE PROTEIN 2	4 (2 2 0 0 0)	1 : 0.98	Q562R1	1.02040	1
ISOFORM 1 OF CULLIN-ASSOCIATED NEDD8-DISSOCIATED PROTEIN 1	18 (18 0 0 0 0)	1 : 0.97	Q86VP6	1.02040	1
ISOFORM 2 OF SWI/SNF COMPLEX SUBUNIT SMARCC2	4 (4 0 0 0 0)	1 : 0.98	Q8TAQ2	1.02040	1
MYOSIN-IA.	3 (3 0 0 0 0)	1 : 0.98	Q9UBC5	1.02040	1
ISOFORM 2 OF FILAMIN-A	60 (60 0 0 0 0)	1 : 0.99	P21333	1.01010	1
PROTEIN DISULFIDE-ISOMERASE A3	12 (12 0 0 0 0)	1 : 0.99	P30101	1.01010	1
PHOSPHOENOLPYRUVATE CARBOXYKINASE, CYTOSOLIC [GTP].	2 (2 0 0 0 0)	1 : 0.99	P35558	1.01010	1
ISOFORM 1 OF TRANSCRIPTION INTERMEDIARY FACTOR 1-BETA	3 (3 0 0 0 0)	1 : 0.99	Q13263	1.01010	1
TUBULIN BETA-3 CHAIN.	3 (3 0 0 0 0)	1 : 0.99	Q13509	1.01010	1
ISOFORM 2 OF NEUTRAL ALPHA-GLUCOSIDASE AB	10 (10 0 0 0 0)	1 : 0.99	Q14697	1.01010	1
ISOFORM 4 OF ABHYDROLASE DOMAIN-CONTAINING PROTEIN 11	5 (5 0 0 0 0)	1 : 0.99	Q8NFV4	1.01010	1
ISOCHORISMATASE DOMAIN-CONTAINING PROTEIN 1	4 (4 0 0 0 0)	1 : 0.99	Q96CN7	1.01010	1
VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN 35	10 (10 0 0 0 0)	1 : 0.99	Q96QK1	1.01010	1
PUTATIVE TUBULIN-LIKE PROTEIN ALPHA-4B	2 (2 0 0 0 0)	1 : 0.99	Q9H853	1.01010	1

ISOFORM 3 OF RIBOSOME-BINDING PROTEIN 1	11 (11 0 0 0 0)	1 : 0.99	Q9P2E9	1.01010	1
CDNA FLJ55574, HIGHLY SIMILAR TO CALNEXIN.	9 (9 0 0 0 0)	1:1.00	B4DGP8	1	1
GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	26 (26 0 0 0 0)	1 : 1.00	P04406	1	1
TUBULIN BETA CHAIN	2 (2 0 0 0 0)	1 : 1.00	P07437	1	1
6-PHOSPHOFRUCTOKINASE, LIVER TYPE (EC 2.7.1.11) (PHOSPHOFRUCTOKINASE 1) (PHOSPHOHEXOKINASE) (PHOSPHOFRUCTO-1-KINASE ISOZYME B) (PFK-B). ISOFORM 2.	10 (10 0 0 0 0)	1 : 1.00	P17858	1	1
ISOFORM 1 OF VINCULIN	4 (4 0 0 0 0)	1 : 0.93	P18206	1.075269	1.07526
ALANYL-TRNA SYNTHETASE, CYTOPLASMIC.	7 (7 0 0 0 0)	1 : 1.00	P49588	1	1
ISOFORM 1 OF HOST CELL FACTOR	4 (3 1 0 0 0)	1 : 1.00	P51610	1	1
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN F	2 (2 0 0 0 0)	1 : 1	P52597	1	1
Spliceosome RNA helicase BAT1	3 (3 0 0 0 0)	0.82 : 1	Q13838	1	1
ISOFORM 1 OF MYOSIN-IB.	6 (6 0 0 0 0)	0.99 : 1	O43795	1	1.01010
ACTIN, CYTOPLASMIC 1	5 (5 0 0 0 0)	0.99 : 1	P60709	1	1.01010
ISOFORM 1 OF L-LACTATE DEHYDROGENASE A CHAIN	6 (6 0 0 0 0)	0.98 : 1	P00338	1	1.02040
NCL PROTEIN	4 (4 0 0 0 0)	0.98 : 1	P19338	1	1.02040
KINESIN-1 HEAVY CHAIN.	6 (6 0 0 0 0)	0.98 : 1	P33176	1	1.02040
ISOFORM 1 OF NADH-CYTOCHROME B5 REDUCTASE 3	4 (4 0 0 0 0)	0.97 : 1	P00387	1	1.03092
FRUCTOSE-BISPHOSPHATE ALDOLASE A.	2 (2 0 0 0 0)	0.97: 1	P04075	1	1.03092
COATOMER SUBUNIT BETA'	5 (5 0 0 0 0)	0.97: 1	P35606	1	1.03092
ISOFORM 1 OF UBX DOMAIN-CONTAINING PROTEIN 1	2 (2 0 0 0 0)	0.96 : 1	Q04323	1	1.04166
ISOFORM 2 OF RING FINGER PROTEIN 213	4 (3 0 1 0 0)	0.96: 1	Q63HN8	1	1.04166
ISOFORM 1 OF MYOFERLIN.	2 (2 0 0 0 0)	0.96 : 1	Q9NZM1	1	1.04166
ISOFORM 1 OF GELSOLIN.	10 (10 0 0 0 0)	0.95:1	P06396	1	1.05263
ISOCITRATE DEHYDROGENASE [NADP], MITOCHONDRIAL	13 (12 1 0 0 0)	0.95 : 1	P48735	1	1.05263
DOLICHYL-DIPHOSPHOOLIGOSACCHARIDE--PROTEIN GLYCOSYLTRANSFERASE 67 KDA SUBUNIT PRECURSOR	18 (18 0 0 0 0)	0.95 : 1	P54652	1	1.05263
translation elongation factor 1 alpha 1	3 (2 1 0 0 0)	0.95 : 1	Q2F837	1	1.05263
ANNEXIN VI ISOFORM 2	13 (13 0 0 0 0)	0.93 : 1	P07355	1	1.07526
4F2 CELL-SURFACE ANTIGEN HEAVY CHAIN CD98	4 (4 0 0 0 0)	0.94:1	P08195	1	1.07526
STRESS-70 PROTEIN, MITOCHONDRIAL	11 (11 0 0 0 0)	0.93 : 1	P38646	1	1.07526
MYOSIN-IC	11 (11 0 0 0 0)	1 : 0.91	O00159	1	1.09890
ATP- CITRATE SYNTHASE	10 (10 0 0 0 0)	0.91 : 1	P53396	1	1.09890
ACONITATE HYDRATASE, MITOCHONDRIAL	11 (11 0 0 0 0)	0.91 : 1	Q99798	1	1.09890
Keratin, type I cytoskeletal 18	7 (7 0 0 0 0)	0.90 : 1	P05783	1	1.11111
MALATE DEHYDROGENASE, MITOCHONDRIAL	22 (22 0 0 0 0)	0.89 : 1	P40926	1	1.12359
ISOFORM 1 OF ACETYL-COA CARBOXYLASE 1	3 (3 0 0 0 0)	0.89 : 1	Q13085	1	1.12359
POLY(RC)-BINDING PROTEIN 1	3 (3 0 0 0 0)	0.89 : 1	Q15365	1	1.12359
ISOFORM 1 OF INORGANIC PYROPHOSPHATASE 2, MITOCHONDRIAL	5 (5 0 0 0 0)	0.89 : 1	Q9H2U2	1	1.12359

KERATIN, TYPE II CYTOSKELETAL 8	6 (6 0 0 0 0)	0.88 : 1	P05787	1	1.13636
ISOFORM 1 OF CARNITINE O-PALMITOYLTRANSFERASE I, LIVER ISOFORM	2 (2 0 0 0 0)	0.87:1	P50416	1	1.14942
40S RIBOSOMAL PROTEIN S3	7 (7 0 0 0 0)	0.86 : 1	P23396	1	1.16279
ISOFORM 1 OF PROTEASOME ACTIVATOR COMPLEX SUBUNIT 3	3 (3 0 0 0 0)	0.86 : 1	P61289	1	1.16279
40S RIBOSOMAL PROTEIN S4, X ISOFORM	7 (7 0 0 0 0)	0.86 : 1	P62701	1	1.16279
MITOCHONDRIAL 2-OXOGLUTARATE/MALATE CARRIER PROTEIN	5 (5 0 0 0 0)	0.86 : 1	Q02978	1	1.16279
DYNC1H1 PROTEIN	5 (4 1 0 0 0)	0.86 : 1	Q6P2H7	1	1.16279
CDNA FLJ56425, HIGHLY SIMILAR TO VERY-LONG-CHAIN SPECIFIC ACYL-COADEHYDROGENASE, MITOCHONDRIAL	7 (7 0 0 0 0)	0.85 : 1	B4DEA8	1	1.1764
ATP DEPENDENT DNA HELICASE 2 SUBUNIT 2	14 (13 1 0 0 0)	0.85 : 1	P63010	1	1.17647
HETEROGENEOUS NUCLEAR RIBONUCLEOPROTEIN U ISOFORM A	2 (2 0 0 0 0)	0.85 : 1	Q00839	1	1.17647
NAD(P)H DEHYDROGENASE [QUINONE] 1	5 (5 0 0 0 0)	0.84 : 1	P15559	1	1.19047
MATRIN-3.	5 (5 0 0 0 0)	0.84 : 1	P43243	1	1.19047
PDCD6IP PROTEIN	4 (4 0 0 0 0)	0.84 : 1	Q8WUM4	1	1.19047
ISOFORM 1 OF TROPOMYOSIN ALPHA-4 CHAIN	2 (2 0 0 0 0)	0.82 : 1	P67936	1	1.21951
ISOFORM 1 OF MYOSIN-14	4 (4 0 0 0 0)	0.95 : 1	Q7Z406	1	1.21951
ANNEXIN A5	13 (13 0 0 0 0)	0.80 : 1	P08758	1	1.25
LANOSTEROL SYNTHASE	2 (2 0 0 0 0)	0.80 : 1	P48449	1	1.25
60S RIBOSOMAL PROTEIN L7A	4 (4 0 0 0 0)	0.79 : 1	P62424	1	1.26582
6-PHOSPHOFRUCTOKINASE TYPE C	5 (5 0 0 0 0)	0.79:1	Q01813	1	1.26582
ISOFORM 2 OF NUCLEAR MITOTIC APPARATUS PROTEIN 1	9 (8 1 0 0 0)	0.79 : 1	Q14980	1	1.26582
ISOFORM 7 OF TITIN	11 (11 0 0 0 0)	0.79 : 1	Q8WZ42	1	1.26582
TRANSALDOLASE	4 (3 0 1 0 0)	0.78 : 1	P37837	1	1.28205
TRIPARTITE MOTIF-CONTAINING PROTEIN 25	2 (2 0 0 0 0)	0.77 : 1	O75382	1	1.29870
PROTEASOME ACTIVATOR COMPLEX SUBUNIT 1	13 (13 0 0 0 0)	0.77 : 1	P61289	1	1.29870
ZINC FINGER PROTEIN 347	2 (2 0 0 0 0)	0.77 : 1	Q96SE7	1	1.29870
HUMAN IMMUNODEFICIENCY VIRUS TYPE I ENHANCER BINDING PROTEIN 1	2 (2 0 0 0 0)	0.76 : 1	P15822	1	1.31578
40S RIBOSOMAL PROTEIN S2	7 (7 0 0 0 0)	0.76 : 1	P15880	1	1.31578
ISOFORM HEART OF ATP SYNTHASE SUBUNIT GAMMA, MITOCHONDRIAL	4 (4 0 0 0 0)	0.76 : 1	P36542	1	1.31578
TRIFUNCTIONAL ENZYME SUBUNIT ALPHA, MITOCHONDRIAL	3 (3 0 0 0 0)	0.76 : 1	P40939	1	1.31578
HEAT SHOCK PROTEIN 75 KDA, MITOCHONDRIAL	9 (9 0 0 0 0)	0.76 : 1	Q12931	1	1.31578
ISOFORM 1 OF AFLATOXIN B1 ALDEHYDE REDUCTASE MEMBER 4	2 (2 0 0 0 0)	0.76 : 1	Q8NHP1	1	1.31578
ALPHA-ACTININ-4.	20 (19 1 0 0 0)	0.74 : 1	O43707	1	1.35135
ELONGATION FACTOR 1-ALPHA 2	4 (4 0 0 0 0)	0.71 : 1	P68104	1	1.40845
ISOFORM 1 OF ACYLGLYCEROL KINASE, MITOCHONDRIAL	2 (2 0 0 0 0)	0.71 : 1	Q53H12	1	1.40845
Obsolete	3 (3 0 0 0 0)	0.70 : 1	Q05524	1	1.42857
PUTATIVE UNCHARACTERIZED PROTEIN PSME2	4 (4 0 0 0 0)	0.70 : 1	Q9UL46	1	1.42857

Elongation factor Tu, mitochondrial	3 (3 0 0 0 0)	0.69 : 1	P49411	1	1.44927
ALPHA-CENTRACTIN	2 (2 0 0 0 0)	0.69 : 1	P61163	1	1.44927
ISOFORM 1 OF ACIDIC LEUCINE-RICH NUCLEAR PHOSPHOPROTEIN 32 FAMILY MEMBER B.	4 (4 0 0 0 0)	0.69 : 1	Q92688	1	1.44927
T-COMPLEX PROTEIN 1 SUBUNIT DELTA	4 (4 0 0 0 0)	0.68 : 1	P50991	1	1.47058
T-COMPLEX PROTEIN 1 SUBUNIT BETA	6 (6 0 0 0 0)	0.68 : 1	P78371	1	1.47058
VOLTAGE-DEPENDENT ANION-SELECTIVE CHANNEL PROTEIN 1	9 (9 0 0 0 0)	0.67 : 1	P21796	1	1.49253
ISOFORM LONG OF UBIQUITIN CARBOXYL-TERMINAL HYDROLASE 5	5 (5 0 0 0 0)	0.66 : 1	P45974	1	1.51515
INORGANIC PYROPHOSPHATASE	6 (6 0 0 0 0)	0.66 : 1	Q15181	1	1.51515
CENTAURIN-DELTA-3	2 (2 0 0 0 0)	0.65 : 1	D3DQE3	1	1.53846
CYTOCHROME C1, HEME PROTEIN, MITOCHONDRIAL	5 (5 0 0 0 0)	0.61 : 1	P08574	1	1.5625
THREONYL-TRNA SYNTHETASE, CYTOPLASMIC	8 (7 1 0 0 0)	0.64 : 1	P26639	1	1.5625
T-COMPLEX PROTEIN 1 SUBUNIT EPSILON	4 (4 0 0 0 0)	0.64 : 1	P48643	1	1.5625
COATOMER SUBUNIT GAMMA.	6 (6 0 0 0 0)	0.64 : 1	Q9Y678	1	1.5625
CATHEPSIN D	4 (4 0 0 0 0)	0.62 : 1	P07339	1	1.61290
T-COMPLEX PROTEIN 1 SUBUNIT ZETA	2 (2 0 0 0 0)	0.61 : 1	P40227	1	1.63934
Beta-enolase	2 (2 0 0 0 0)	0.60: 1	P13929	1	1.66666
ALPHA-ENOLASE - deleted from swiss prot	4 (4 0 0 0 0)	0.6 : 1	Q05524	1	1.66666
ISOFORM LONG OF GLUCOSE-6-PHOSPHATE 1-DEHYDROGENASE	20 (20 0 0 0 0)	0.59 : 1	P11413	1	1.69491
TUBULIN BETA-2C CHAIN	12 (12 0 0 0 0)	0.59 : 1	P68371	1	1.69491
PLASTIN-1	2 (2 0 0 0 0)	0.56 : 1	Q14651	1	1.78571
UBIQUITIN AND RIBOSOMAL PROTEIN S27A PRECURSOR	2 (2 0 0 0 0)	0.55 : 1	P62979	1	1.81818
LARGE NEUTRAL AMINO ACIDS TRANSPORTER SMALL SUBUNIT 1	3 (3 0 0 0 0)	0.50 : 1	Q01650	1	2
ISOFORM 1 OF PROTEIN SET.	3 (3 0 0 0 0)	0.48 : 1	Q01105	1	2.08333
Bullous pemphigoid antigen 1	2 (2 0 0 0 0)	0.48 : 1	Q03001	1	2.08333
KERATIN, TYPE I CYTOSKELETAL 19	4 (4 0 0 0 0)	0.47 : 1	P08727	1	2.12766
ISOFORM 1 OF REGULATOR OF NONSENSE TRANSCRIPTS 1	7 (7 0 0 0 0)	0.47 : 1	Q92900	1	2.12766
BIFUNCTIONAL PURINE BIOSYNTHESIS PROTEIN PURH	9 (9 0 0 0 0)	0.46 : 1	P31939	1	2.17391
HEAT REPEAT-CONTAINING PROTEIN 6	3 (3 0 0 0 0)	0.46 : 1	Q6AI08	1	2.17391
VIGILIN.	9 (9 0 0 0 0)	0.44 : 1	Q00341	1	2.27272
FRUCTOSE-1,6-BISPHOSPHATASE 1	2 (2 0 0 0 0)	0.43 : 1	P09467	1	2.32558
LAMIN-B1.	4 (4 0 0 0 0)	0.47 : 1	P20700	1	2.32558
CALPONIN-2	2 (2 0 0 0 0)	0.43 : 1	Q99439	1	2.32558
ISOFORM 1 OF ENOLASE-PHOSPHATASE E1	2 (2 0 0 0 0)	0.42 : 1	Q9UHY7	1	2.38095
ANNEXIN A4	6 (6 0 0 0 0)	0.41 : 1	P09525	1	2.43902
unnamed protein product (blast)	4 (4 0 0 0 0)	0.38 : 1	Unnamed	1	2.63157
ISOFORM 3 OF RUN DOMAIN-CONTAINING PROTEIN 3A	3 (2 0 1 0 0)	0.36 : 1	Q59EK9	1	2.77777
elongation factor 1-alpha 2	2 (2 0 0 0 0)	0.28 : 1	Q8IYJ1	1	3.57142
Putative uncharacterized protein	3 (3 0 0 0 0)	0.28 : 1	Q9HBQ4	1	3.57142
NUCLEAR MIGRATION PROTEIN NUDC	3 (3 0 0 0 0)	0.28 : 1	Q9Y266	1	3.57142
AFG3-LIKE PROTEIN 2	8 (7 1 0 0 0)	0.24 : 1	Q9Y4W6	1	4.16666
ISOFORM 1 OF NF-X1-TYPE ZINC FINGER PROTEIN NFXL1.	3 (2 1 0 0 0)	0.23 : 1	Q6ZNB6	1	4.34782
60S ACIDIC RIBOSOMAL PROTEIN P0	6 (6 0 0 0 0)	0.19 : 1	P05388	1	5.26315
Ezrin-radixin-moesin-binding phosphoprotein 50	3 (3 0 0 0 0)	0.18 : 1	O14745	1	5.55555
PROTEIN FAM49B	3 (3 0 0 0 0)	0.15 : 1	Q9NUQ9	1	6.66666
				357.577	**347.156**