Identification of Lyn kinase as a therapeutic target for tamoxifen resistant breast cancer

A thesis presented for the degree of

Doctor of Philosophy at Cardiff University by

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December 2011

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Summary

Tamoxifen has made a significant contribution in decreasing breast cancer related deaths for over 30 years and until recently was the gold standard for treatment of ER positive breast cancer (Fisher *et al*, 1998). Resistance to tamoxifen is however a considerable issue with cells utilising a number of molecular mechanisms to bypass the growth inhibition caused by blocking ER activity. This move towards an anti-hormone resistant state from an anti-hormone responsive state is associated with the transition to a much more aggressive phenotype including increased proliferation and also invasiveness. Thus unfortunately, acquisition of tamoxifen resistance is not only associated with a recurrence of breast cancer, but this cancer is also much more aggressive in nature with fewer treatment options available than the initial cancer.

This study has identified Lyn kinase as increased in tamoxifen resistant breast cancer cells compared to oestrogen-responsive breast cancer cells. Subsequent removal of Lyn kinase from tamoxifen resistant breast cancer cell lines using RNAi technology led to a significant decrease in cell proliferation, increased apoptosis and also a decrease in migration and invasion. A mechanism has been suggested whereby Lyn kinase is involved in a calcium dependent zinc wave which ultimately leads to the activation of tyrosine kinases.

Metastasis to other sites in the body is ultimately responsible for fatalities due to breast cancer and so being able to block its action is key to treating breast cancer in the clinic. Therefore identifying Lyn kinase as a gene target that leads to the advancement of breast cancer to a more aggressive state provides a powerful tool for treating breast cancer in the clinic.

Acknowledgements

I would like to thank Professor Nicholson for giving me the opportunity to join the Tenovus group and Cardiff University and Tenovus for funding my work.

I would like to thank all the Tenovus staff and students past and present, for all their help over the years and for always taking the time out to answer questions and provide new perspectives.

Extra special thanks go out to my supervisor Kathy, who has always been the glass half full to my glass half empty. Her support, guidance, patience and understanding during my research has been invaluable.

Last but not least I would like to thank my family who have always supported and encouraged me. I could not have done this without you and I love you all so much.

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Chapter 1: Introduction

1.1 Cancer

Cancer is the collective name for over 100 diseases characterised by loss of normal cell management leading to uncontrollable cell division and subsequent metastasis of cells to other tissues or organs. The World Health Organisation estimates that cancer was the cause of 7.9 million deaths worldwide in 2007 (WHO, 2007), accounting for around 13% of the total deaths recorded, with the five most frequently observed cancers being lung, stomach, liver, colon and breast cancer. In the UK, cancer has an incidence rate of 1 in 3 with around 245,300 new diagnoses in 2007, and over half of these new cases represented by the four most common UK cancers- breast accounting for 31% of cases in females, lung, colorectal and prostate accounting for 25% of new cases in males (Office for national statistics, 2009). Age is by far the most significant factor when addressing cancer incidence in the UK, with 75% of new cancers occurring in people aged 60 and over (Office for national statistics, 2009). Cancer survival in the UK has increased steadily in the past 20 years, mainly due to improved awareness and cancer prevention, earlier detection of cancer and also improved available treatments due to ongoing research in the field (CRUK statistics, 2008).

1.2 Breast Cancer

1.2.1 Incidence of breast cancer

Breast cancer is the most frequent type of cancer in women in the world with a 1 in 9 lifetime risk of being diagnosed and nearly 1 million new cases each year worldwide (Brekelmans *et al*, 2003). In 2006, almost 46,000 cases of

breast cancer were diagnosed in the UK with the majority (99%) of cases occurring in women (45,508 women compared to 314 men), and thus overtaking colorectal cancer as the most commonly diagnosed cancer in the UK. In 2007, 12,000 women and 92 men died from breast cancer (CRUK statistics, 2008), however mortality rates have shown a 25% decline since 1987 largely due to improved, earlier, detection, notably the implementation of a national screening programme in 1987. This led to an increase in breast cancer incidence in women over fifty years old, with previously undetected cancers being diagnosed (figure 1.1), and also ongoing improvements in breast cancer treatments.
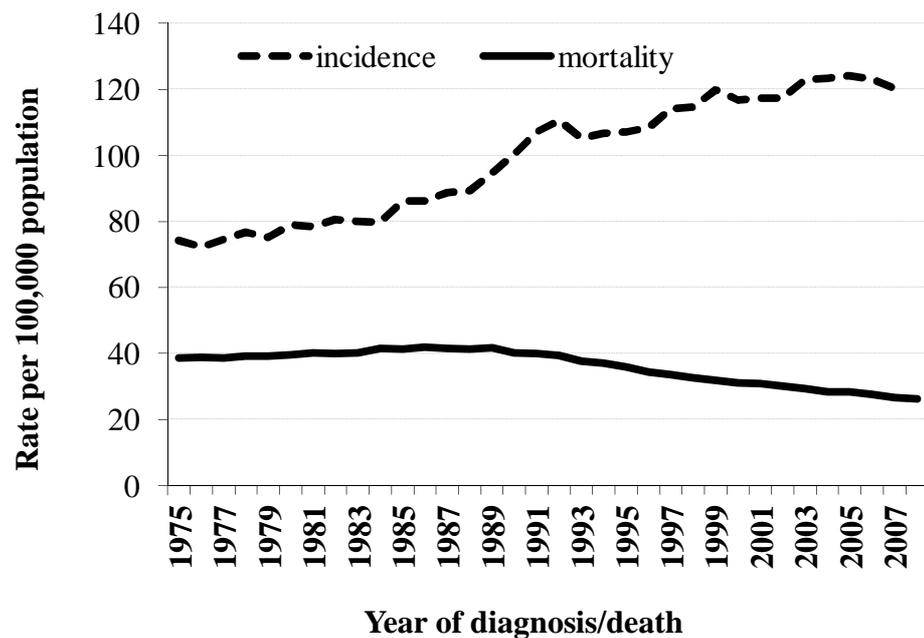
Figure 1.1: Age standardised incidence and mortality rates, breast cancer, females, GB, 1975-2008. Figure taken from CRUK statistics, 2008.

1.2.2 Risk factors associated with breast cancer

A number of risk factors are associated with development of breast cancer with the most significant (other than gender) being older age. 80% of cases occurred in women over the age of 50 years (Office for national statistics, 2005). Longer-term exposure to the steroid hormone oestrogen over a lifetime has been shown to increase the risk of breast cancer. This can be through early age at menarche (Kelsey *et al*, 1993) with each 1 year delay in onset leading to a 5% decrease in risk of developing breast cancer later in life (Key *et al*, 2001), late age at menopause, or late age at first full-term pregnancy (Layde *et al*, 1989). Exogenous exposure to oestrogen, for example the use of oral contraceptives or hormone replacement therapy, is also associated with an increased risk of developing breast cancer: Studies have also shown that the use of oral contraceptives offers protection against both ovarian and uterine cancers (Schlesselman *et al*, 1998). The use of combined oral contraceptives leads to an increased risk of around 25% of developing breast cancer (Hankinson *et al*, 1998), however the risk declines significantly following cessation of treatment and returns to baseline levels 10 years after treatment (Key *et al*, 2001). The Million Women Study conducted in 2003 and focusing on the effects of hormone replacement therapy found that 10 years use of both combined and oestrogen only replacement therapy led to a significant increase in breast cancer risk (Beral, 2003) however the benefits of hormone replacement therapy in the form of a reduction in both heart disease and osteoporosis are currently felt to outweigh the increase in breast cancer risk when prescribing. Other modifiable risk factors for development of breast cancer relate to lifestyle and include alcohol consumption (Allen *et al*, 2009),

diet, particularly consumption of saturated fat (Bingham *et al*, 2003), lack of physical activity (Monninkhof *et al*, 2007) and also high BMI (body mass index) (Reeves *et al*, 2007). It is not clear how these factors are involved in increased cancer risk however research has shown that alcohol consumption leads to an increase in sex hormones compared to those that do not consume alcohol (Rinaldi *et al*, 2006) and oestrogen levels have been shown to be reduced in postmenopausal women that exercise regularly (Chan *et al*, 2007).

The likelihood of developing breast cancer varies considerably around the world with the highest numbers seen in Western countries (North America and affluent European countries) and the lowest seen in developing countries like Africa and Asia. As countries with historically low levels of breast cancer become more 'Westernised', with changes in diet and lifestyle, the incidence of breast cancer gradually increases. Studies looking at the effect of migration from low-risk to high risk countries showed that the rates of breast cancer gradually increase in the migrant population and after two generations the levels are comparable to the native population (Key *et al*, 2001).

Whilst eighty-five percent of patients diagnosed with breast cancer have no family history suggesting no inherited factor (*Collaborative Group on Hormonal Factors in Breast Cancer*, 2001), a number of genes have been identified as increasing the likelihood of developing familial breast cancer. Five per cent of all breast cancers are caused by mutations in the breast cancer susceptibility genes BRCA1 or BRCA2 (Shih *et al*, 2002).BRCA1 mutations also increase the likelihood of developing ovarian cancer. A patient with a

mutation in the BRCA1 gene has a 50% to 80% chance of developing breast cancer in her lifetime (Casey, 1997).

1.3 Oestrogen

Oestrogen is a sex steroid that is synthesised from cholesterol and secreted primarily by the ovaries in pre-menopausal women (Nelson and Bulun, 2001). In post-menopausal women, oestrogen is produced by aromatase in peripheral tissues and by breast tumours (Nicholson and Johnston, 2005). Oestrogen occurs naturally in three forms: estrone (E_1), produced by the adrenal glands, estradiol(E_2), produced by the ovaries and estriol(E_3), which is the product of estrone and estradiol and is synthesised in the liver. These 3 naturally occurring forms of oestrogen are shown in figure 1.2. In pre-menopausal women, estradiol is the most abundant oestrogen. After menopause, estradiol levels drop and estrone becomes the main form of oestrogen present. However the most common and most biologically important form of oestrogen is 17β-estradiol (E_2) which is essential to the development and function of the female reproductive system and required for the proliferation and differentiation of healthy breast tissue (Lewis e*t al*, 2005).

1.3.1 Oestrogen receptor structure

The oestrogen receptor (ER) has two forms: ERα, which was first identified by Jensen in 1960 (Jensen and Jacobson, 1962) and ERβ, which was cloned from rat prostate and ovary tissues in 1996 (Kuiper *et al*, 1996). They are each produced by distinct genes on different chromosomes. The receptors display some homology however their distribution within tissues varies significantly.

ERα is considerably larger than ERβ with 595 amino acids compared to 530 amino acids (Fuqua *et al*, 1999). Both receptors have six domains labelled A-F (figure 1.3). The A/B domain at the N-terminus encodes a hormone independent activation function 1 (AF1). Domain C corresponds to the highly conserved DNA binding domain (DBD) and this is responsible for the binding of the receptors to specific EREs that are located upstream of oestrogen regulated genes. Region D is the hinge region between the DBD and the LBD (ligand binding domain) and Region E encodes activation function 2 (AF2)

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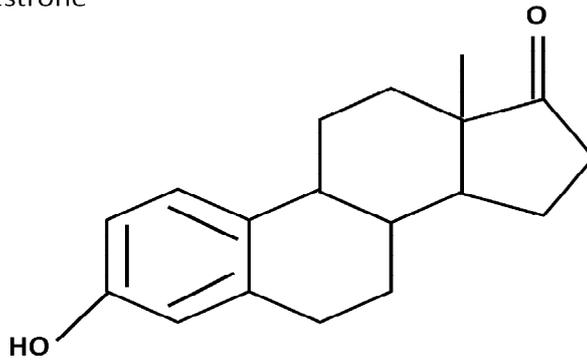
Estriol
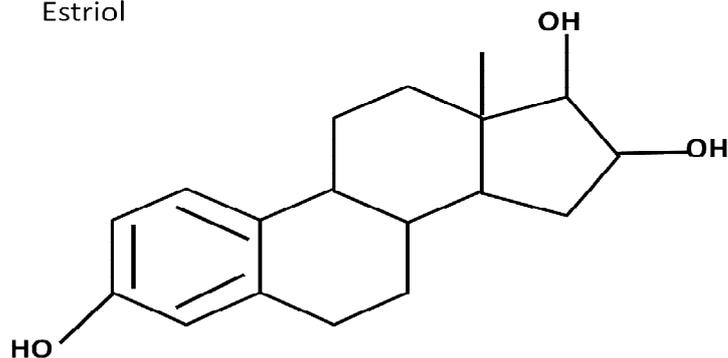
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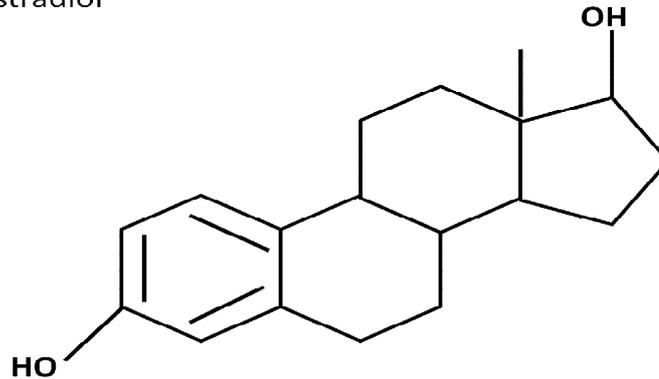
Figure 1.2: The structure of the three forms of oestrogen

and contains the LBD. The LBD consists of 12α-helices and 5 of these form the hydrophobic pocket responsible for E_2 or selective oestrogen receptor modulator (SERM) binding. The main difference between ERα and ERβ is in the AF1. In ERα, the AF1 domain is very active; however in ERβ there is negligible activity (Kuiper *et al*, 1997). This difference is thought to be the reason for the variation in responses to ligands seen between the two oestrogen receptors (Paech *et al*, 1997). The ER is distributed throughout the body, with ERα mainly expressed in breast, uterine and vaginal tissues, while ERβ is mainly expressed in the CNS, cardiovascular system and immune system.

A

B
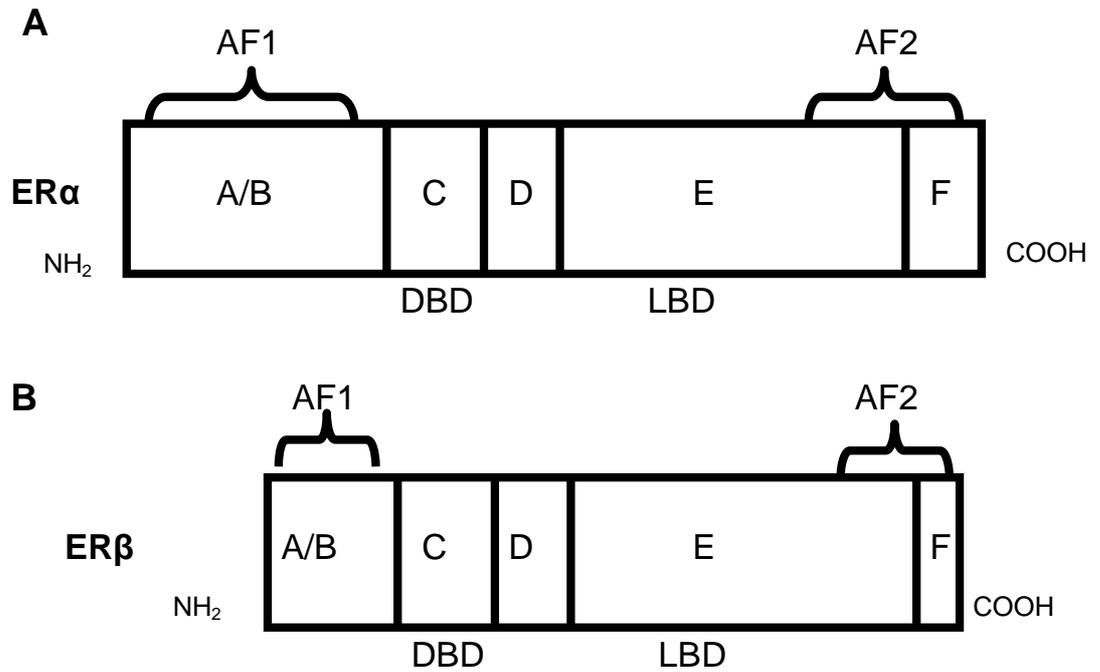
Figure1.3: Structure of ERα (A) and ERβ (B).

1.3.2 Oestrogen receptor activation

1.3.2.1 Classical oestrogen receptor signalling

Prior to activation, the oestrogen receptor exists as an inactive molecule in the nuclei that is bound and inhibited by a complex consisting of heat shock proteins HSP90 and 70, and cyclophilin-40 and p23 (Pratt and Toft, 1997). In the classical mode of action for oestrogen, E_2 diffuses into the cell, translocates to the nucleus, binds to the ER and activates it. This binding induces receptor homo- or hetero-dimerisation and phosphorylation as well as a conformational change in the ER. The heat shock proteins dissociate from the complex and the dimers then bind to Oestrogen Response Elements (EREs) in the promoter region upstream of the oestrogen responsive genes (Osborne and Schiff, 2005). Once bound to the DNA, the ER can recruit and

bind co activators that promote ER dependent gene expression or co repressors that repress ER dependent gene expression and this receptor complex can then regulate gene transcription by AF1 and AF2, acting synergistically to mediate positive regulation of gene expression (through gene activation/inactivation). Frasor *et al* have shown that after treatment with E_2 in MCF-7 breast cancer cells, many genes involved with transcription repression, growth suppression and apoptosis are down regulated, whereas genes that induce cell proliferation are up-regulated (Frasor *et al*, 2004).

1.3.2.2 Non-classical oestrogen receptor activation

Non-classical ER signalling occurs indirectly where ligand-bound ER forms protein-protein interactions with other transcription factors, for example c-fos and c-jun. This promotes the expression of genes containing the AP-1 response element in their promoter region. This demonstrates how the ER can act as a co-activator for other transcription factors.

1.3.2.3 Non-nuclear oestrogen receptor signalling

In breast cancer cells, the ER has also been shown to be present in the cytoplasm or on the plasma membrane (Losel *et al*, 2003), explaining the term non-nuclear oestrogen receptor signalling. The response to oestrogen occurs within a matter of minutes following exposure (Gee *et al*, 2005) and so cannot be due to either the classical or non-classical genomic mode of oestrogen receptor signalling. In this type of signalling, the ER at the plasma membrane

activates growth factor signalling cascades such as the epidermal growth factor (EGFR) (Fildaro *et al*, 2000) that initiates a rapid response to hormone signalling (Levin, 2003; Gee *et al*, 2005). This in turn leads to the activation of mitogen activated protein kinase (MAPK) and serine/threonine protein kinase (Akt) signalling pathways.

1.4 Treatment of Breast Cancers that are ER negative

Patients with cancers that are negative for the ER have fewer treatment options as far as endocrine therapy is concerned than those with ER positive tumours. Blocking the action of the ER will have no effect on those tumours that are ER negative. However ER negative patients derive greater benefit from chemotherapy than those with ER positive tumours. In some cases, a patient may be ER/PR negative, but be HER-2 positive, in which case Herceptin may be a treatment option. Herceptin (trastuzumab) is a monoclonal antibody (Carter *et al*, 1992) against HER-2 that has been licensed for the treatment of breast cancer since 1998 (Bange *et al*, 2001).

1.5 Endocrine Therapy in ER positive patients

Since the role of oestrogen in breast cancer is well established, with approximately 70% of breast tumours positive for ER expression (Muller-Tidow 2004), blocking the action of oestrogen has long been used in the treatment of breast cancer. The earliest example of this was ovarian ablation.

The benefit of ovarian ablation in treating breast cancer was first appreciated by the Scottish physician George Beatson in 1896 where the removal of the ovaries in pre-menopausal women led to tumour regression in some premenopausal patients (Beatson, 1896). In post-menopausal women the adrenal glands were removed to prevent the production of androgens. This was first achieved by Charles Huggins, a prostate cancer specialist in 1952.

More recently, drugs have been used which either inhibit oestrogen binding (anti-oestrogen), prevent oestrogen synthesis by inhibiting aromatase (aromatase inhibitors (AIs)) or down-regulate ER protein levels (pure anti-oestrogens). The use of endocrine drugs may be combined with surgery and/or chemotherapy or radiotherapy. The types of endocrine drugs available will be discussed in the following sections.

1.6 Anti-oestrogen drugs.

The first anti-oestrogen compounds were discovered in the 1950s. They were manufactured as fertility drugs, but the positive response of some breast cancer patients to treatment was noted (Kistner and Smith, 1960). An example of this was clomiphene (Clomid) which is still widely used as an infertility treatment. Anti-oestrogen drugs work either by competitively binding the ER or by causing the degradation of the ER in the cell. The two main classes of anti-oestrogen drugs currently used are SERMs (Selective oestrogen receptor modulators) and SERDs (Selective oestrogen receptor down-regulators).

1.6.1 Selective oestrogen receptor modulators

SERMs show tissue selectivity in that they act as agonists in some tissues for example bone (Vehmanen *et al*, 2007), but antagonists in other tissues e.g. breast (Shang *et al*, 2002). Tamoxifen has been used as an effective treatment for ER positive breast cancer for over 30 years and is therefore the most well known, having first been approved by the Food and Drug Administration in the UK in 1977 for treatment of advanced breast cancers. Until recently tamoxifen was the gold standard for treatment of ER positive breast cancer (Fisher *et al*, 1998) and has made a significant contribution to the decrease in breast cancer related deaths with 5 years of therapy with tamoxifen preventing the recurrence of second primary tumours by 50% (Gee *et al*, 2005).
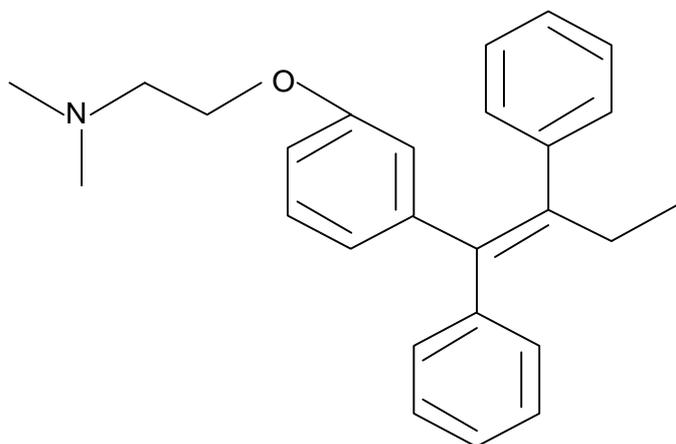
Figure1.4: Structure of Tamoxifen

1.6.1. Action of tamoxifen

When ligands bind to the ER they are enveloped in a hydrophobic pocket that is closed by helix 12 in the ligand binding domain (Region E). The positioning of helix 12 is crucial for the recruitment of co-activators to the AF2 site and the following initiation of RNA polymerase activity (Lewis *et al* ,2005). X-ray structural work has demonstrated that when different ER ligands such as estradiol and tamoxifen (and other SERMs) interact with the ligand binding domain, different conformations of the ER are induced (Shiau *et al*, 1998). It is proposed that as a result of these different ligand-induced conformations, the ER is able to recruit different co-regulating proteins and thus various outcomes of gene expression are possible (Frasor *et al,* 2004). E_2 seems to recruit transcriptional co-activators to the receptor, whereas SERMs seem to recruit co-repressors (Lavinsky *et al*, 1998). It is thought that this is the mechanism by which SERMs block the action of oestrogen. It is worth noting that tamoxifen does not inhibit the ER's AF1 region, therefore it is only a pure antagonist of genes where AF2 is required for transcription (Santen *et al*, 2003).

1.7 Resistance to Tamoxifen

Resistance to tamoxifen is of significant clinical importance. Resistance can be *de novo* or acquired. The most common mechanism of *de novo* resistance is the absence of ER/PR expression. However 25% of ER positive breast cancer does not respond to endocrine therapy from the beginning (Honig, 1996). Acquired resistance occurs where ER positive breast cancer initially

responds to endocrine treatment, but then stops responding after long term treatment (Gee *et al*, 2005). Even though millions of people gain some benefit from tamoxifen treatment, 40% of patients receiving adjuvant tamoxifen therapy develop resistance (Schiff *et al*, 2003), and unfortunately almost all metastatic patients who have benefitted from endocrine therapy will eventually develop resistance to tamoxifen. This results in a relapse of the disease with worsened outlook.

1.7.1 Mechanisms of tamoxifen resistance

Acquired resistance to tamoxifen was originally thought to be due to loss or mutation of ER signalling (Ring and Dowsett, 2004). However it has recently been demonstrated that ER levels are maintained on acquisition of tamoxifen resistance in breast tumours (Bachleitner-Hofmann *et al*, 2002) and also in breast cancer cell lines (Hutcheson *et al*, 2003). It has also been noted that ER of patients showing loss of ER expression upon recurrence of the disease (Bachleitner-Hofmann *et al*, 2002). This is supported by the clinical observation that 2 out of 3 patients that display resistance to tamoxifen still respond to pure anti-oestrogens for example fulvestrant (Robertson, 2001) or aromatase inhibitors (Buzdar and Howell, 2001). Acquired resistance to tamoxifen and subsequent disease relapse in patients is often unfortunately associated with the development of a more aggressive, invasive type of breast cancer that ultimately leads to a poorer prognosis than before (Hiscox *et al*, 2004).

It has been proposed that tumours that acquire resistance to tamoxifen use an alternative growth regulatory pathway caused by the inappropriate activation of growth factor signalling cascades. This could either be through an increased supply of growth factor ligands or through up-regulation and increased activation of growth factor receptors or their downstream signalling elements (Ring and Dowsett, 2004). Of great significance in acquired tamoxifen resistance is the observation that growth factor receptors are up-regulated, for example IGF-IR (Insulin-like growth factor receptor I) (Knowlden *et al*, 2005), HER2 (Human Epidermal growth factor Receptor 2) and EGFR, and thus their downstream kinases such as PI3K/Akt and MAP kinases (Gee *et al*, 2005; Nicholson *et al*, 2007) are also up-regulated. The activation of signalling kinases results in the phosphorylation of the AF-1 domain on the ER, activating the ER.

A model of tamoxifen resistance has been developed at the Tenovus Centre for Cancer Research by exposing the MCF-7 breast cancer cell line to long term tamoxifen treatment. Briefly, MCF-7 cells were cultured in a growth medium supplemented with 100nM 4-hydroxytamoxifen for 6 months. At first, the MCF-7 cell growth rates were significantly reduced, but after 2 months' exposure to the medium, cell growth gradually increased, indicating the development of a cell line resistant to the growth-inhibitory properties of 4-hydroxytamoxifen. The tamoxifen resistant MCF-7 cell line (TamR) was maintained for a further 4 months prior to any cell characterisation studies beginning (Knowlden *et al*, 2003).

In this model, increased expression and activity of EGFR, HER2 and IGF-IR was observed, mirroring what is seen in the clinical setting. This effect has also been observed in other *in vitro* models of acquired tamoxifen resistance.

1.7.2 Overcoming tamoxifen resistance

Since 40% of patients develop resistance to tamoxifen, a number of strategies have since been tried in order to improve outcome in ER positive breast cancer patients. These strategies include developing new therapies to target the ER more efficiently and also therapies to target growth factor signalling cascades associated with tamoxifen resistance.

1.7.2.1 Development of alternative SERMs

Since the use of tamoxifen has proved highly successful in the treatment of ER positive breast cancer, many attempts have been made to synthesise newer SERMs with better efficiency. Unfortunately, the newer generation SERMs have proved to be no more effective than tamoxifen as a first line therapy in treating ER positive breast cancer . The International Breast Cancer Study Group (IBCSG) conducted two clinical trials, IBCSG 12-93 and IBCSG 14-93 in which a comparison was made of 5 year tamoxifen treatment vs. 5 year toremifene treatment for early stage breast cancer. The study concluded that both toremifene and tamoxifen yielded similar disease free and overall survival rates following five years of treatment with similar toxicity (IBCSG, 2004). In addition, patients who have developed resistance to tamoxifen have

also proved to be resistant to alternative SERMs, for example toremifene (Stenbygaard *et al*, 1993)

1.7.2.2 Selective oestrogen receptor down regulators

SERDs act by binding competitively to the ER with a similar affinity (89%) to that of oestradiol (Osborne *et al*, 2004). The best known example of a SERD is fulvestrant, which is a steroidal analogue of 17β-oestradiol. The long alkylsulphinyl side-chain present (figure1.5), causes an abnormal conformational change of the ER protein, thus disrupting receptor dimerisation and inhibiting nuclear localisation. This complex of ER and fulvestrant is unstable and leads to the subsequent rapid degradation of ER (Osborne *et al,* 2004). Since SERDs show no agonistic effects, they are termed 'pure anti-oestrogens'. Unfortunately, fulvestrant has not demonstrated an improvement on first-line treatment such as tamoxifen or aromatase inhibitors (AIs) in the treatment of advanced breast cancers (Robertson *et al,* 2003). Whereas tamoxifen only disrupts the AF2 ligand, fulvestrant disrupts the AF1 ligand also, thus fully-blocking transcription activation resulting in no E_2-regulated gene expression. As a result, tamoxifen resistant breast cancer has been shown to respond to fulvestrant treatment in the clinic (Howell *et al*, 2000). Thus its use in the clinic following development of resistance to other anti-oestrogen therapies is beneficial (Ingle *et al,* 2006). In contrast to tamoxifen, fulvestrant does not have an agonist effect on the endometrium since it is a pure anti-oestrogen. As a result, the increased incidence of endometrial cancer

associated with tamoxifen use is not seen with fulvestrant use. (Addo *et al*, 2002)
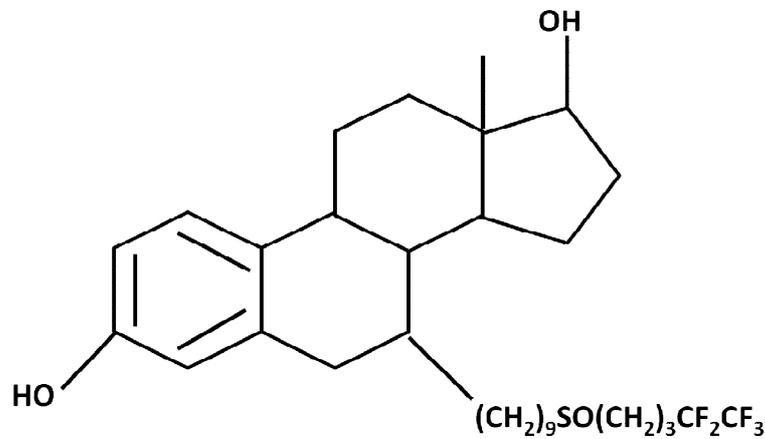
Figure1.5. Structure of fulvestrant.

1.7.2.3 Aromatase inhibitors (AIs)

The cytochrome P450 enzyme (aromatase) is an enzyme responsible for the synthesis of oestrogen. In post menopausal women, the majority of circulating oestrogen is produced in the peripheral tissues such as adipose tissue. Oestrogen is also produced in breast tumours. Aromatase inhibitors suppress aromatase both in peripheral tissues and in the tumour itself (Nicholson and Johnston, 2005). Aromatase inhibitors currently in use in the clinic include the steroidal aromatase inhibitor exemestane (Aromasin), which binds to the p450 site of the aromatase complex and the non-steroidal aromatase inhibitor anastrazole (Arimidex). The ATAC (anastrazole, tamoxifen and combination) clinical trial suggested that adjuvant anastrozole was superior to tamoxifen in terms of survival and recurrence of breast cancer in postmenopausal women with early ER-positive breast cancer (Howell *et al*, 2005), thus challenging the

current use of tamoxifen as the first-choice treatment. Anastrazole is now taking over from tamoxifen as a first-line therapy in post-menopausal women (Come *et al*, 2005), however, unfortunately, acquired resistance to aromatase inhibitors has been reported in both tamoxifen resistant breast cancer (when used as a second line therapy) and tamoxifen responsive ER-positive breast cancer patients, as well as in numerous experimental models (Nicholson and Johnston, 2005).

1.7.2.4 Targeting growth factor signalling cascades

As outlined in section 1.7.1, several growth factor signalling cascades have been associated with the development of tamoxifen resistance, both in the clinic and *in vitro* models of tamoxifen resistance notably EGFR and HER2. Using gefitinib (Iressa®), an EGFR specific tyrosine kinase inhibitor, the growth of TamR cells can be inhibited in a concentration dependent manner with 1µM gefitinib reducing cell proliferation by approximately 60% (Knowlden *et al*, 2003; Nicholson *et al*, 2004). The growth of TamR cells can also be reduced by the inactivation of the HER2 pathway following treatment with trastuzumab (Herceptin).

1.8 The tyrosine kinase family

Tyrosine kinases are a large, highly conserved, multigene family that include many growth factor receptors, cell cycle regulators and oncoproteins (Meric *et al*, 2002). They are enzymes which catalyse the phosphorylation of tyrosine residues and thus are involved in many processes, including cellular signalling

pathways and also regulate key cell functions such as proliferation, differentiation and anti-apoptotic signalling (Blume-Jensen and Hunter, 2001). 91 tyrosine kinase genes and 5 pseudogenes have been identified. These consist of 59 receptor type that are divided into 20 subfamilies (Pawson, 2002) and 32 nonreceptor type that are divided into 8 subfamilies (Robinson *et al*, 2000). At least 18 tyrosine kinases have been identified as oncogenes (Zwick et al, 2001). This oncogenic activation can involve point mutations and deletions, as well as overexpression (Blume-Jensen and Hunter, 2001).

1.8.1 Receptor tyrosine kinases

Receptor tyrosine kinases (with the exception of insulin-like growth factor receptors) exist in a monomer-dimer equilibrium (Zhang *et al*, 2010). They have an extracellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain (Arora and Scholar, 2005). The transmembrane domain acts as an anchor for the receptor in the plasma membrane, while the extracellular domain binds growth factors (Schlessinger 2000). The kinase is activated by ligand binding to the extracellular domain, which induces receptor dimerisation (Pawson, 2002). They are then able to phosphorylate the tyrosine residues outside the catalytic domain. This stabilises the dimer, and also creates docking sites for proteins which transduce signals within the cell. Members of the receptor tyrosine kinase family include the ErbB family, consisting of EGFR, ErbB2, ErbB3 and ErbB4.

1.8.2 Non-receptor (cellular) tyrosine kinases

Unlike receptor tyrosine kinases, non-receptor tyrosine kinases are located in the cytoplasm, nucleus or anchored to the plasma membrane because they do not possess a formal transmembrane domain (Abram and Courtneidge, 2000). There are eight families of non-receptor tyrosine kinases: SRC, JAK, ABL, FAK, FPS, CSK, SYK and BTK. These families have little in common structurally. SRC has some of the best characterised family members for example c-Src kinase and Lyn kinase (Abram and Courtneidge, 2000). BTK is involved in cell differentiation, particularly in β-lymphocytes (Mohamed *et al*, 2009). ABL are involved in growth inhibition (Colicelli 2010) and FAK activity is strongly associated with cell adhesion (Schlaepfer and Mitra 2003). Some members of the JAK family are involved in the cytokine receptor pathway that phosphorylates STATs (Rawlings *et al*, 2004). Thus receptor tyrosine kinases play an important role in biological processes.

1.8.3 Tyrosine kinases and human disease

In humans, tyrosine kinases have been shown to be significant in the development of many diseases including diabetes (Louvet *et al*, 2008) and almost all types of cancer (Arora and Scholar, 2005). Aberrations in tyrosine kinase signalling also exist in inflammatory diseases, by changing expression of the cytokines, which orchestrate both the duration and extent of inflammation (Page *et al*, 2009). And they have also been linked to a wide range of congenital disorders (Robinson, 2000). The ErbB family of receptor

tyrosine kinases in particular (which includes HER2 and EGFR, mentioned above) has been the subject of extensive research into the role they play in hormone dependent breast cancer (Dowsett, 2001).

1.9 Src family kinases

Src is a family of non-receptor tyrosine kinases consisting of nine members: Lyn, Hck, Lck, Blk, Src, Fyn, Yes, Fgr and Frk. They can be further grouped into two subfamilies, Lyn related (Lyn, Hck, Lck and Blk) and Src related (Src, Yes, Fyn and Fgr) (Parsons and Parsons, 2004). The Src kinase domain structure consists of 6 domains as in figure 1.6. All family members share significant homology both in sequence and structure. Each possess an N-terminal SH4 (Src homology 4) region, a unique region (50-70 amino acids) that displays high variability among the members (Boggon and Eck, 2004), a 50 amino acid Src homology 3 domain (SH3) (Koch *et al*, 1991), a 100 amino acid Src homology 2 (SH2) domain a Src homology 1 (SH1) domain or the kinase domain that is approximately 300 amino acids in length that is responsible for enzymatic activity (Ingley, 2007) and finally the C-terminal regulatory region.
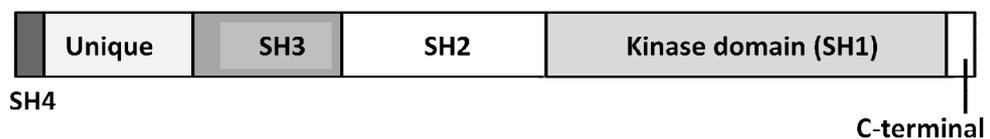
Figure 1.6: Src kinase domain structure.

The N-terminal SH4 domain is a region containing between 15 and 17 amino acid residues. It allows for permanent binding of a fatty acid called myristate. This allows the Src family kinase to bind to the plasma membrane (Tatosyan and Mizenina, 2000).

The unique region, named because of its lack of sequence homology varies in size according to family member. The high degree of variance suggests that the region has different functions in each family member (Brown and Cooper, 1996).

The SH3 domain interacts with proline-rich sequences with the sequence PXXP (where X is any amino acid) (Boggon and Eck, 2004). It controls catalytic activity, protein localisation in the cell, and mediates physical association with protein targets (Ingley, 2008).

The SH2 domain is responsible for the binding of the Src family kinase to specific amino acid sequences encoding phosphotyrosine. The preferred sequence for binding the SH2 is pYEEI, however this is not essential for SH2 binding (Tatosyan and Mizenina, 2000).

Three members of the Src family are ubiquitously expressed in humans, namely Src, Fyn and Yes, whereas the other family members are expressed differentially in different tissues. For example Lyn is present primarily in haematopoietic cells. Src has been shown to play a significant role in proliferation, migration and invasion and has been associated with numerous cancers including ovarian (Weiner *et al*, 2003), breast cancers (Verbeek *et al*, 1996), and colon cancers (Mao *et al*, 1997), specifically in tumour progression and metastasis to other tissues and organs. TamR (tamoxifen resistant MCF-7)

cells display an increased growth and a significantly increased invasive phenotype compared to their parental MCF-7 cells and additionally have significantly greater levels of Src activity (as measured by phosphorylation at Y419) (Morgan *et al*, 2006) that was not due to increases in Src protein or mRNA. Further evidence for the role of Src kinase in the aggressive phenotype of these cells was found by treatment of TamR cells with the Src inhibitor AZD0530 which led to decreased invasion across a Matrigel basement membrane complex(Morgan *et al*, 2006). This inhibition of Src was also accompanied by a reduction in EGFR signalling, suggesting that Src plays a role in the invasive behaviour seen in TamR cells (Morgan *et al*, 2006).

1.10 Zinc regulation and human disease

Zinc is an essential mineral and is involved in many cellular processes. Zinc is needed for protein, carbohydrate and lipid metabolism and is a cofactor for more than 300 enzymes (Vallee & Falchuk, 1993), playing a role in the activity or the structural stability or both of the enzyme. It plays a major role in the stabilization of a huge number of proteins, including signalling enzymes at all levels of cellar signal transduction and transcription factors (Beyersmann, 2002). Zinc is essential for multiple aspects of the function of the immune system (Rink and Gabriel, 2000) including development and function of cell-mediating innate immunity, neutrophils, and natural killer cells (Wintergerst et al, 2007; Prasad, 2009). The control of gene transcription is zinc dependent (Blanchard and Cousins, 2000) since gene transcription and replication factors contain Zn-finger motifs (Rink and Gabriel, 2000). Zinc

regulation of gene expression involves a metal-binding transcription factor (MTF) and a metal response element (MRE) in the promoter of the regulated gene (King, 2011). The MTF acquires zinc in the cytosol or nucleus and then interact with the MRE to stimulate transcription (Mocchegiani *et al*, 2010). Depending on zinc status in the cells, MTF-1 is thought to regulate numerous genes either negatively or positively (Cousins et al, 2006). As a result of this, cell proliferation does not occur in the absence of zinc, making systems displaying high levels of cell proliferation particularly vulnerable to zinc deficiency, for example skin and intestinal cells.

Given the key roles played by zinc in many processes, zinc homeostasis is very important. It is controlled by metallothioneins (MT), a group of low-molecular-weight metal-binding proteins that have high affinity for zinc (Mocchegiani et al, 2010) and two families of zinc transporters (discussed below). MT is one of the strongest cellular zinc binding proteins and is capable of binding seven zinc molecules (King, 2011), with the β domain binding 3 zinc molecules and the α domain binding 4 zinc molecules (Cousins et al, 2006). Gene expression of MT is also regulated by zinc, suggesting that MT also provides a method of maintaining zinc reserves in cells (Coyle *et al*, 2002).

1.10.1 Zinc deficiency in humans

The essential role of zinc in humans is displayed by the number of disorders that result from zinc deficiency. These include poor cell-mediated immunity

leading to increased susceptibility to infections which is one of the early manifestations of zinc deficiency. Later manifestations include dermatitis, poor wound healing, severe diarrhoea leading to malnutrition (a particular problem in developing countries) and hair loss and alopecia (Maret and Sandstead, 2006).

Plasma zinc levels decrease with age, resulting in increased susceptibility of older people to infectious diseases, autoimmunity, and cancer (Haase and Rink, 2009). Decreased zinc levels are also associated with loss of cognitive function which in turn is also associated with ageing (Chasapis *et al*, 2011). The decrease of zinc plasma levels with age has also been shown to contribute to cardiovascular disease in elderly people (Little *et al*, 2010). In addition, low zinc levels have been linked to various mood disorders such as depression. In cases of major depression treatment with zinc has been shown to have an antidepressant effect (Levenson, 2006).

Zinc is essential to β pancreatic cells for the formation of insulin crystals; zinc-insulin hexamers that are key to the storage and maturation of insulin (Kambe, 2011). Zinc deficiency in animals leads to a lack of insulin in β pancreatic cells (Rungby, 2010) and zinc metabolism is also altered in diabetes patients, with zinc supplementation proven beneficial, particularly in type 2 diabetes (Jansen, 2009). Array studies have shown that a polymorphism in the ZnT8 zinc transporter allele (discussed further below) leads to a 53% increased risk of developing diabetes (Rutter, 2010).

1.10.2 Excess zinc in humans

Conversely, excessive zinc consumption has been shown to lead to zinc toxicity. Symptoms of this in humans include nausea, vomiting, loss of appetite, abdominal cramps, diarrhoea, and headaches (Trumbo *et al*, 2001). It has also been shown to reduce immune responses and in severe cases lead to organ failure and death (Bennet et al, 1997). A major consequence of excess zinc absorption is a decrease in plasma copper levels: Metallothionein expression is up-regulated by high dietary zinc, however metallothionein binds copper with a higher affinity than zinc. The resultant complex is excreted causing low plasma copper levels. These low copper levels have been shown to result in cardiac arrhythmia, an increase in LDL cholesterol and anaemia (Plum *et al*, 2010).

Extracellular Zn metabolism is altered in Alzheimer's disease. The development of Alzheimer's disease is associated with formation of extracellular β-amyloid (Aβ) plaques in the brain and a high accumulation of zinc is found in the plaques indicating that zinc plays a role in the formation of plaques (Wang *et al*, 2010). Additionally, zinc chelating agents have been shown to inhibit plaque formation in mice adding further weight to the role of zinc in plaque formation in Alzheimer's disease (Lee *et al*, 2004).

1.10.3 Zinc in cancer

Both plasma and tumour zinc levels have been shown to be abnormal in a number of cancers thus supporting a role for zinc in cancer development (John *et al*, 2010). A decrease in plasma zinc levels is observed in cervical cancer

patients (Cunzhi *et al*, 2003), gallbladder cancer (Gupta *et al*, 2005), lung cancer (Issell *et al*, 2006), head and neck cancer (Buntzel *et al*, 2007) and breast cancer patients (Schlag *et al*, 1978). However the data available appears contradictory, with some studies finding an increase in intracellular zinc to have an adverse effect, whilst others note that decreased plasma zinc is indicative of a worsened outcome. A possible explanation for this conflict is that biomarkers such as blood and plasma tend to be poor indicators of whole body zinc status (King, 1990) as they are indicative of short term zinc exposure. A preferable method of identifying levels of zinc is to use hair or toenail samples, since these are representative of longer term zinc exposure (Navarro-Silvera and Rohan, 2007). By measuring the level of zinc in specific tissues as opposed to plasma, zinc has been shown to be increased in both breast and lung tumours compared to the corresponding normal tissue (Margalioth *et al*, 1983). Conversely, skin cancers (John *et al*, 2010) and prostate cancers (Costello and Franklin, 2006) exhibit lower levels of zinc compared to the corresponding normal tissues. In prostate cancer, it has been shown that the lower tumour zinc concentration is due to the down-regulation of the zinc transporter ZIP1 (Franklin *et al*, 2005). Dietary supplementation with high doses of zinc have however been shown to increase the risk of developing prostate cancer 2.9 fold (Leitzmann *et al*, 2003). A possible explanation for this is that in high doses, zinc has a suppressive effect on the immune system (Bennet et al, 1997).

1.10.4 Zinc in breast cancer

As previously mentioned, reported concentrations of plasma zinc in breast cancer patients have been contradictory, with some studies reporting an increase in plasma zinc concentrations and others reporting no change. However a number of studies report a decrease in plasma zinc in breast cancer patients (Schlag *et al*, 1978; Yucel *et al*, 1994). Zinc directly affects tumour cells through its regulatory role in gene expression and cell survival via tumour-induced alterations in zinc transporter expression. It also influences tumour cells indirectly by affecting the activation, function, and survival of immune cells (John *et al*, 2010). It has been shown to be increased by approximately 70% in breast tumours compared to its corresponding normal breast tissue (Margalioth *et al*, 1983). This increase is not influenced by dietary intake of zinc, with N-methyl-N-nitrosourea (MNU) induced breast cancer rat models fed either a zinc deficient or a zinc adequate diet both displaying an increase in breast tumours regardless of dietary intake (Woo and Xu, 2002), thus suggesting a role for zinc transporters in breast cancer. Another way in which zinc may directly influence tumour progression is through zinc dependent transcription factors such as SNAIL (John *et al*, 2010). SNAIL is responsible for epithelial-mesenchymal transition, a process where epithelial cells lose their polarity and are converted to a mesenchymal phenotype (Thiery, 2002). This process is important in many developmental processes. SNAIL causes loss of

cell adhesion by reducing expression of adherence genes such

as E-cadherin (Peinado et al, 2007) and its deregulation in cancer cells can lead to tumour progression with an increase in cell motility and invasiveness (John *et al*, 2010). It follows that zinc could be involved in the transition of breast cancer to a more aggressive, motile state.

1.10.5 Zinc transporters

With both zinc deficiency and also zinc toxicity clearly detrimental to human health, the balance of cellular zinc levels is clearly vital. Since zinc cannot passively diffuse across cellular membranes, two families of zinc transporters maintain zinc homeostasis: ZnT (SLC30A) and ZIP (SLC39A). It is the mutual actions of these two families that maintain cellular zinc homeostasis.

1.10.6 The ZnT family of zinc transporters

The ZnT family, also known as SLC30A (solute-linked carrier 30) consists of 10 members (ZnT1-10) and is responsible for the transport of zinc out of the cell (Palmiter and Huang, 2004). They have 6 trans-membrane domains and display histidine rich motifs located in the cytoplasmic loop between trans-membrane domains 4 and 5 (figure 1.7) that may bind metal during transport (Eide, 2006).
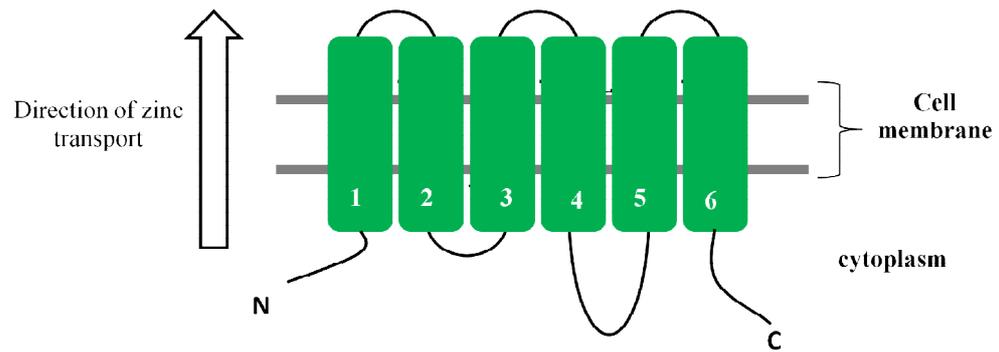
Figure 1.7: Structure of ZnT proteins

1.10.7 The ZIP family of zinc transporters

The ZIP (Zrt- and Irt-like proteins), also known as SLC39A (solute-linked carrier 39) is named after family is named after the yeast Zrt1 protein and the Arabidopsis Irt1 protein, the first identified members (Eide, 2006). It consists of 14 members (ZIP1-14) and is responsible for the transport of zinc into the cell (Eide, 2004). The ZIP family have 8 transmembrane domains, including a histidine rich region located in the cytoplasmic loop between trans-membrane domain 3 and 4 (figure 1.8).
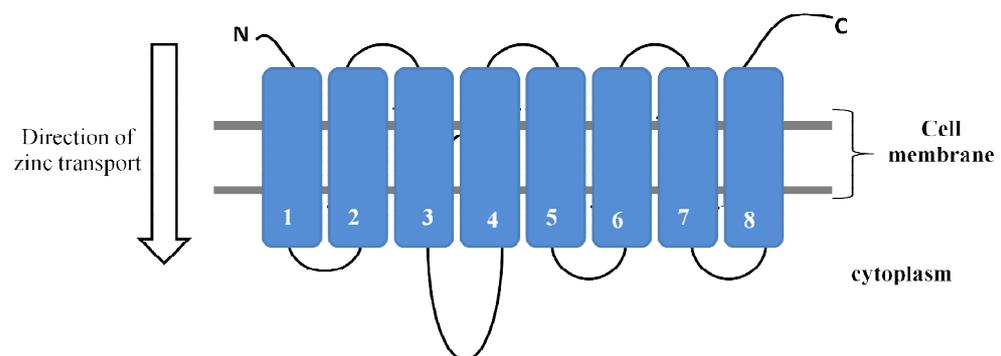
Figure 1.8: Structure of ZIP proteins

1.10.8 Zinc transporters and cancer

A number of zinc transporters have been linked to the development and progression of mammalian cancers.

ZIP4 has been shown to be over-expressed in 94% of pancreatic adenocarcinoma samples compared to the normal tissues adjacent to it. In addition, affymetrix analysis revealed that ZIP4 mRNA expression is significantly higher in human pancreatic cancer cells than normal pancreatic cells (Logsdon *et al*, 2003). Over-expression of ZIP4 increased intracellular zinc levels 2-fold *in vitro*, and significantly increased tumour volume by 13-fold in a nude mice model with subcutaneous xenograft compared with the control cells. Cell proliferation was also increased. (Li *et al*, 2004). These data indicate that aberrant ZIP4 up-regulation may contribute to the pancreatic cancer pathogenesis and progression and provides a possible target for future pancreatic cancer therapies.

ZIP1, ZIP2 and ZIP3 have all been demonstrated to be down-regulated in prostate cancer, displaying an important role in the unique ability of prostate cells to accumulate high cellular zinc levels (Franklin and Costello 2007). The three transporters have been shown to be down-regulated in malignant prostate cells (Desouki et al, 2007). This is consistent with the lowering of tumour zinc concentration associated with prostate cancer (Costello and Franklin 2006). These data indicate a role for ZIP1, ZIP2 and ZIP3 as tumour suppressor genes in prostate cancer. ZIP6, also known a LIV-1 has also been shown to be involved with prostate cancer by driving epithelial-to-mesenchymal transition (EMT). When over-expressed in ARCaP cells

(androgen-refractory prostate cancer cells), a model of prostate cancer bone metastasis, ZIP6 promoted EMT irreversibly (Lue et al, 2011).

In addition to prostate cancer, ZIP 6 has also been associated with breast cancer. It has been shown to be predominantly expressed in oestrogen receptor positive breast cancer (McClelland *et al*, 1998). MRNA expression of ZIP6 showed a highly significant association with the spread of breast cancer to the regional lymph nodes (manning *et al*, 1994). An explanation for the role of ZIP6 in cancer spread is that ZIP6 is the downstream target of STAT3 and thus essential for the nuclear localization of another transcription factor, SNAIL, which causes loss of cell adhesion by reducing expression of adherence genes such as E-cadherin (Peinado *et al*, 2007).

ZIP10 has also been shown to be associated with the metastasis of breast cancer to the lymph node and thus is involved in the invasive behaviour of breast cancer cells. Kagara *et al* have demonstrated that ZIP10 mRNA expression was higher in the invasive and metastatic breast cancer cell lines MDA-MB-231 and MDA-MB-435S than in less metastatic breast cancer cell lines, such as MCF7 and T47D. In support of this, the depletion of ZIP10 in MDA-MB-231 and MDA-MB-435S cells *in vitro* inhibited cell migration (Kagara *et al*, 2007) confirming the essential role of ZIP10 in metastatic breast cancer and providing a possible future treatment strategy.

Interestingly, a ZnT zinc efflux transporter has also been associated with breast cancer. Expression of ZnT1 is reduced by 55% in the MNU induced tumours in rats compared with normal tissue. Furthermore, zinc is also increased 12 times in same rat model compared to normal tissue (Lee *et*

al, 2003). In the same study, The mRNA and protein levels of metallothionein in tumours were 1.3 and 3.5 times of that in normal tissue, respectively (Lee *et al*, 2003). This suggests that aberrant expression of ZnT1 may contribute to breast cancer progression.

1.10.9 The zinc transporter ZIP7 in TamR (tamoxifen resistant MCF-7) cells

The zinc transporter ZIP7 (SLC39A7) has been shown to be located on the endoplasmic reticulum and transports zinc from intracellular compartments into the cytoplasm (Taylor *et al*, 2004). TamR cells have double the levels of intracellular zinc compared to the hormone responsive MCF-7 cells (Taylor *et al*, 2008). Removal of ZIP7 using siRNA in TamR cells led to a reduction in EGFR, IGF-IR, Src and Akt (Taylor *et al*, 2008) all of which have been shown to contribute to the aggressive phenotype of TamR cells with increased growth, proliferation and cell motility (Knowlden *et al*, 2005; Gee *et al*, 2005; Nicholson *et al*, 2007; Morgan *et al*, 2006). This ZIP7 reduction by siRNA treatment also led to a decrease in zinc. In further support of this observation, transfecting wild type tamoxifen responsive MCF-7 cells with a construct expressing recombinant ZIP7 led to an increase in EGFR, IGF-IR and Src (Taylor *et al*, 2008). There was also a corresponding increase of zinc.

A mechanism of ZIP7 action has been proposed (Taylor, 2008b) based upon the above involvement of ZIP7 in TamR cells; the presence of a calcium-dependent zinc wave in mast cells that leads to the inhibition of phosphatases by zinc that originates in the endoplasmic reticulum (Yamasaki *et al*, 2007)

where ZIP7 resides (Taylor *et al*, 2004); and also a proposed model for zinc management in cells whereby intracellular zinc is associated with a muffler in the cytoplasm, that allows zinc buffering and storage in the endoplasmic reticulum, before being released into the cytoplasm (Colvin *et al*, 2008). The role proposed for ZIP7 in intracellular zinc homeostasis is shown in figure 1.9 where zinc enters the cell from outside and is buffered and absorbed within a muffler before being transferred to the endoplasmic reticulum. Zinc that enters the cytoplasm is transported by ZIP7 and released in the form of a zinc wave, leading to inhibition of phosphatases (Taylor, 2008b).
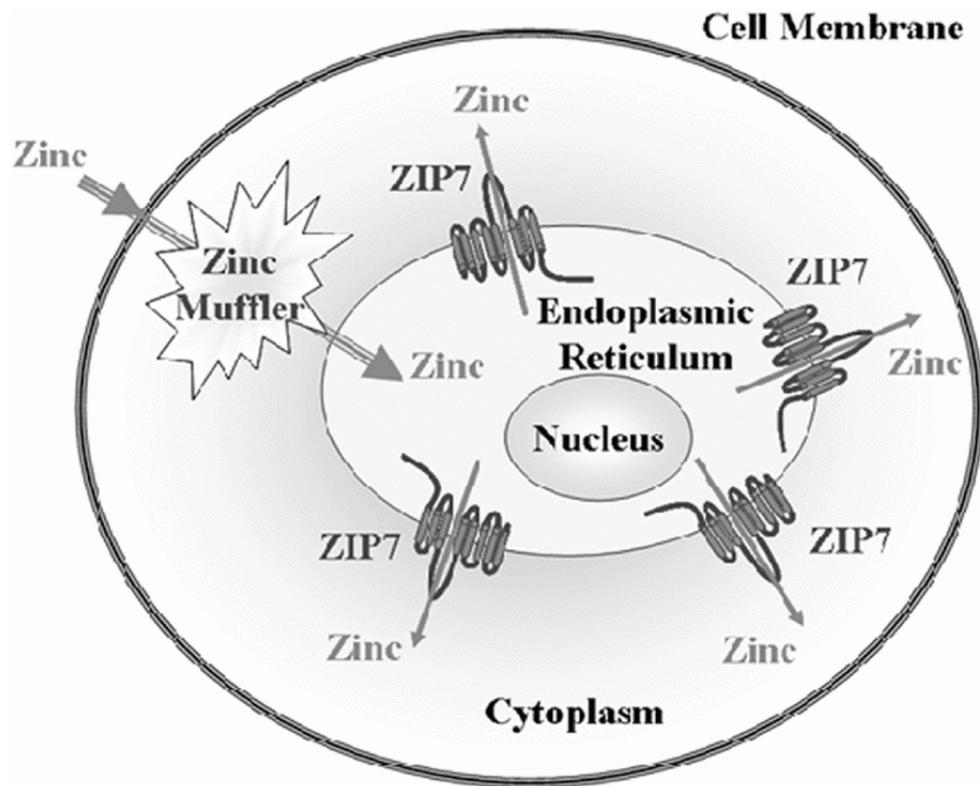
Figure 1.9: The predicted function of ZIP7. Reproduced from Taylor

2008b: A distinct role in breast cancer for two LIV-1 family zinc transporters.

1.11 Aims

The aim of this thesis is to identify novel therapeutic targets for the treatment of tamoxifen resistant breast cancer using the TamR model of tamoxifen resistance developed at the Tenovus Centre for Cancer Research.

In order to achieve this, the following aims were pursued:

- The use of affymetrix data analysis to identify tyrosine kinase genes that were up-regulated in TamR cells compared to MCF-7 cells as potential gene targets.

- The confirmation of affymetrix data by PCR and subsequent selection of a short list of genes.

- The identification of a single gene target and investigation into the role of the gene target in proliferation, apoptosis, migration and invasion of TamR cells using siRNA technology to silence gene translation.

Chapter 2: Materials and Methods

2. Materials and Methods

MATERIAL	MANUFACTURER, LOCATION
0.2µm Supor membrane VacuCap 60 filter unit	Gellman Laboratory Pall, Ann Arbour, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, Poole, Dorset, UK
Acrylamide/bis-acrylamide (30% solution (v/v), 29:1 ratio)	Sigma-Aldrich, Poole, Dorset, UK
Activated charcoal	Sigma-Aldrich, Poole, Dorset, UK
Agarose	Bioline Ltd, London, UK
Alpha Digidoc RT Densitometry Software	Alpha Innotech Corp. California, USA
Ammonium persulphate (APS)	Sigma-Aldrich, Poole, Dorset, UK
Amphotericin B (Fungizone)	Invitrogen, Paisley, UK
Antibiotics (penicillin/streptomycin)	Invitrogen, Paisley, UK
Anti-rabbit/Anti-mouse EnVision™+ System, Peroxidase (DAB) kits	DAKO, Cambridgeshire, UK
Aprotinin	Sigma-Aldrich, Poole, Dorset, UK
Aspiration pump	Gardner Denver Alton Ltd, Alton, UK
Bijou tubes (5ml)	Bibby Sterilin Ltd., Stone, UK
Bio-Rad DC Protein Assay (Reagents A, B and S)	Bio-Rad Laboratories Ltd, HERTS, UK
Bovine serum albumen (BSA)	Sigma-Aldrich, Poole, Dorset, UK
Bovine Trypsin	Lorne Laboratories Ltd, Reading, UK
Bromophenol blue (BPB)	BDH Chemicals Ltd, Poole, UK
Cecil CE 2041 Spectrophotometer	CECIL, Cambridge, UK
Cell culture medium: RPMI 1640 and Phenol-red-free RPMI 1640	Invitrogen, Paisley, UK

Cell scrapers	Greiner Bio-One Ltd, Gloucestershire, UK
Class II biological safety cabinet	MDH Intermed Airflow from Bioquell, Andover, UK
Corning Standard Transwell® inserts (6.5mm diameter, 8µm pore size)	Fisher Scientific, Leicestershire, UK
Coulter Counter counting cups and lids	Sarstedt AG and Co., Nümbrecht, Germany
Coulter Multisizer II	Beckman, High Wycombe, UK
Crystal violet	Sigma-Aldrich, Poole, Dorset, UK
Denly BA852 Autoclave	Thermoquest Ltd, Basingstoke, UK
DharmaFECT Transfection Reagents 1-4	Thermo Scientific, Loughborough, UK
Di-butylpthalatexylene (DPX)	Raymond A Lamb Ltd, Eastbourne, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Poole, Dorset, UK
Di-potassium hydrogen orthophosphate anhydrous (K_2HPO_4)	Fisher Scientific UK Ltd, Loughborough, UK
Disposable Cuvettes	Fisher Scientific UK Ltd, Loughborough, UK
Di-thiothreitol (DTT)	Sigma-Aldrich, Poole, Dorset, UK
dNTPs (dGTP, dCTP, dATP, dTTP; 100mM)	Amersham, Little Chalfont, UK
Eppendorf tubes	Eppendorf, Hamburg, Germany
Ethidium bromide (EtBr)	Sigma-Aldrich, Poole, Dorset, UK
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Fibronectin (from Human Plasma; 1mg/ml in 0.05M TBS; pH 7.5)	Sigma-Aldrich, Poole, Dorset, UK
Filter Paper (No. 4), Filter Paper (grade 3; 460 X 370mm)	Whatman, Maidstone, UK

Foetal calf serum (FCS)	Invitrogen, Paisley, UK
GeneQuant RNA/DNA Calculator	Biochrom Ltd Cambridge, UK
General laboratory glass- and plasticware	Fisher Scientific UK Ltd, Loughborough, UK
Gilson Pipettes (1-10µl, 5-50µl, 20-200µl, 100µl-1000ml and 500µl-5ml)	Gilson, Luton UK
Glacial Acetic Acid	Fisher Scientific UK Ltd, Loughborough, UK
Glass coverslips (thickness no. 2, 22mm2)	BDH Chemicals Ltd, Poole, Dorset, UK
Glass slides	Fisher Scientific UK Ltd, Loughborough, UK
Glycerol	Fisher Scientific UK Ltd, Loughborough, UK
Glycine	Sigma-Aldrich, Poole, Dorset, UK
Hamamatsu C4742-96 digital camera	Hamamatsu Photonics UK Ltd, HERTS, UK
Hoffman Condenser	Leica Microsystems Imaging Solutions Ltd, Cambridge, UK
HyperCassette™ developing cassette	Amersham, Little Chalfont, UK
Hyperladder™ I and Hyperladder™ IV	Bioline Ltd, London, UK
IBM Personal Computer	IBM, UK
IEC Micromax RF Micro-centrifuge	Thermo Electron Corporation, Hampshire, UK
Improvision OpenLab V4.04 software	Improvision, Coventry, UK
Isoton® II azide-free balanced electrolyte solution (sodium chloride at 7.9g.l-1, disodium hydrogen orthophosphate at 1.9g.l-1, EDTA disodium salt at 0.4g.l-1, sodium dihydrogen orthophosphate at 0.2g.l-1 and sodium fluoride at 0.3g.l-1)	Beckman Coulter Ltd, High Wycombe, UK

Jouan C312 Centrifuge	Thermo Fisher Scientific Inc., MA, USA
Labconco Purifier PCR Enclosure	GRI, Rayne, UK
Leica DM-IRE2 inverted microscope	Leica Microsystems Imaging Solutions Ltd, Cambridge, UK
Leupeptin	Sigma-Aldrich, Poole, Dorset, UK
L-glutamine	Invitrogen, Paisley, UK
Lipofectamine 2000 Transfection reagent	Invitrogen, Paisley, UK
Lipofectamine RNAiMAX	Invitrogen, Paisley, UK
Liquid DAB+ substrate chromogen system	DAKO, Cambridgeshire, UK
Lower buffer for SDS-PAGE Gels (Tris 1.5M, pH 8.8)	Bio-Rad Laboratories Ltd, HERTS, UK
Magnesium chloride (MgCl2)	Sigma
Magnetic Stirrer	Fisher Scientific UK Ltd, Loughborough, UK
Matrigel Basement Membrane Matrix	BD Biosciences, Oxford, UK
Methyl green	Sigma-Aldrich, Poole, Dorset, UK
Micro-centrifuge tubes (0.5ml and 1.5ml)	Elkay Laboratory Products, Basingstoke, UK
Mini-Protean ® 3 electrophoresis apparatus	BioRad Laboratories Ltd (Hertfordshire, UK)
Molony-murine leukaemia virus (MMLV) reverse transcriptase	Invitrogen, Paisley, UK
MXB Autoradiography Film (Blue Sensitive; 18 X 24 cm)	Genetic Research Instrumentation (GRI), Rayne, UK
N,N,N',N'-tetramethylene-diamine (TEMED)	Sigma-Aldrich, Poole, Dorset, UK
Nikon Eclipse TE200 Phase Contrast Microscope	Nikon, Kingston-upon Thames, UK
Nitrocellulose membrane BA85(0.45µM)	Schleicher and Schuell, Dassell,

	Germany
Olympus 8mp Digital Camera	Olympus, Oxford, UK
Olympus BH-2 phase contrast microscope	Olympus, Oxford, UK
Olympus DP-12 digital camera system	Olympus, Oxford, UK
Perbio Chemiluminescent Supersignal® West Pico, Dura and Femto	Pierce and Warriner Ltd, Cheshire, UK
Phenol/Chloroform/Isoamyl (25:24:1)	Sigma-Aldrich, Poole, Dorset, UK
Phenylarsine oxide	Sigma-Aldrich, Poole, Dorset, UK
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Poole, Dorset, UK
Pipette Tips	Greiner Bio-One Ltd, Gloucestershire, UK
Platform Rocker STR6	Stuart Scientific, Bibby Sterilin Ltd. (Stone, UK)
Polyoxyethylene-sorbitan monolaurate (Tween 20)	Sigma-Aldrich, Poole, Dorset, UK
PowerMAC G5 computer	Apple Computer Inc., CA, USA
Powerpac 1000 power pack	Bio-Rad Laboratories Ltd, HERTS, UK
Powerpac Basic™ power pack	BioRad Laboratories Ltd (Hertfordshire, UK)
Proteinase-K	Sigma-Aldrich, Poole, Dorset, UK
PTC-100 thermocycler	MJ Research Ltd, Massachusetts, USA
Rainbow protein size markers (10-250 kDa)	Amersham, Little Chalfont, England
Random hexamers (RH)	Amersham, Little Chalfont, UK
RNase-free H2O	Sigma-Aldrich, Poole, Dorset, UK
RNasin® ribonuclease inhibitor	Promega, Southampton, UK
Roller Platform	Stuart Scientific, Bibby Sterilin Ltd. (Stone, UK)

Sanyo 950W Microwave	Sanyo Europe, Loughborough, UK
Sanyo MCO-17AIC incubator	Sanyo E&E Europe BV, Loughborough, UK
SMARTpool siRNA	Thermo Scientific, Loughborough, UK
Sodium Acetate (NaOAc)	Sigma-Aldrich, Poole, Dorset, UK
Sodium chloride (NaCl)	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Poole, Dorset, UK
Sodium fluoride (NaF)	Sigma-Aldrich, Poole, Dorset, UK
Sodium molybdate (Na2MoO4)	Sigma-Aldrich, Poole, Dorset, UK
Sodium orthovanadate (NaVO4)	Sigma-Aldrich, Poole, Dorset, UK
Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol)	Fisher Scientific UK Ltd, Loughborough, UK
Sterile disposable pipettes (5ml, 10ml and 25ml), Falcon tubes (50ml), Coulter Counter lids and cups	Sarstedt AG and Co., Nümbrecht, Germany
Sterile Falcon tubes (15ml and 50ml)	Sarstedt AG and Co., Nümbrecht, Germany
Sterile phosphate buffered saline (PBS)	Invitrogen, Paisley, UK
Sterile Syringe Needles (BD microbalance™ 3 characteristics: 25 G5/8 (0.5 X 16)	Becton Dickinson (BD) Biosciences Ltd, Oxford, UK
Sterile universal containers (30ml)	Greiner Bio-One Ltd, Gloucestershire, UK
Sterile, disposable serological pipettes (5ml, 10ml and 25ml)	Sarstedt AG and Co., Nümbrecht, Germany
Sub-cell® Agarose Electrophoresis System	Bio-Rad Laboratories Ltd, HERTS, UK
Sucrose	Fisher Scientific UK Ltd, Loughborough, UK

Syringes (5ml and 10ml)	Sherwood Medical Davis and Geck, Gosport, UK
Tissue culture plasticware (24, 96-well plates, filter flasks, 35mm, 60mm and 100mm dishes)	Nunc Int., Roskilde, Denmark
TRI-Reagent	Sigma-Aldrich, Poole, Dorset, UK
Tris HCl	Sigma-Aldrich, Poole, Dorset, UK
Triton X-100	Sigma-Aldrich, Poole, Dorset, UK
Trizma (Tris) base	Sigma-Aldrich, Poole, Dorset, UK
Upper buffer for SDS-PAGE Gels (Tris 0.5M, pH 6.8)	Bio-Rad Laboratories Ltd, HERTS, UK
UV Transilluminator	Alpha Innotech Corp. California, USA
Vacuum flask	Gardner Denver Alton Ltd, Alton, UK
VectorShield Mounting Medium	Vector Laboratories Inc, Peterborough, UK
Western Blocking Reagent	Roche Diagnostics, Mannheim, Germany
White 96 well qPCR plates and caps	Bio-Rad Laboratories Ltd, HERTS, UK
X-O-graph Compact X2 x-ray developer	X-0-graph Imaging System, Tetbury, UK

2.1 Maintenance of cell lines

Wild-type (tamoxifen responsive) MCF-**7** breast cancer cells, a gift from

AstraZeneca (Macclesfield, UK), were cultured in phenol-red RPMI with 5%

(v/v) fetal calf serum plus 200 mM glutamine, 10 U/ml penicillin, 10 µg/ml

streptomycin, and 2.5 µg/ml Fungizone. Tamoxifen resistant (TamR) cells

were cultured in phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal

calf serum plus 200 mM glutamine, 10 U/ml penicillin, 10 µg/ml

streptomycin, 2.5 µg/ml Fungizone and 100nM 4-hydroxytamoxifen.

TAM/TKI-R (gefitinib resistant TamR) cells were cultured in phenol-red-free

RPMI with 5% (v/v) charcoal stripped fetal calf serum plus 200 mM

glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 2.5 µg/ml Fungizone,

100nM 4-hydroxytamoxifen and 1µM gefitinib. Cells were maintained for a

maximum of 25 passages before being replaced by cells with a lower passage

number from frozen stocks. Cell-culture was conducted in a MDH Class II

laminar-flow safety cabinet (BIOQUELL UK) under sterile conditions. The

cell culture equipment and consumables were sterilized using an autoclave or

purchased already sterile. Cells were stored and maintained in 75cm^2 flasks at

$37^{\mathbf{o}}$C in a humidified 5% CO_2 atmosphere. Medium was replaced every 3-4

days and cells were passaged by trypsinisation at 80-90% confluency as

determined using a Nikon Eclipse TE200 phase-contrast microscope.

Passaging cells in a 75cm^2 flask involved the removal of growth medium and

addition of 10ml of 0.05% trypsin/0.02% EDTA in PBS for 5 minutes at 37°C

to disrupt the cell monolayer and subsequent neutralisation of the trypsin using

10ml of medium containing serum. The cells were then pelleted by

centrifugation at 1000rpm for 5 minutes, the supernatant decanted and the cell pellet re-suspended in the relevant growth medium. For routine cell maintenance, the cells were seeded into new $75cm^2$ flasks at a 1:10 dilution. To accurately seed cells for experimental work, the cell number was counted using a Coulter counter (Beckman Coulter UK Ltd, UK) by adding a 50µl aliquot of cell suspension to 10ml of Isoton solution prior to seeding the required number of cells onto new plates or dishes.

2.2 Generation of TamR cells

TamR cells were generated in-house at the Tenovus Centre for Cancer Research following long-term exposure of MCF-7 cells to tamoxifen. MCF-7 cells were cultured in phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal calf serum plus 200 mM glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin, 2.5 µg/ml Fungizone and supplemented with 100nM 4-hydroxytamoxifen. The cells were continuously exposed to this treatment regimen for 6 months, with the medium being replaced every 3-4 days and the cells passaged by trypsinisation after 80-90% cell confluency was reached (approximately once a week). At first, the MCF-7 cell growth rates were significantly reduced, but after 2 months' exposure to the medium, cell growth gradually increased, indicating the development of a cell line resistant to the growth-inhibitory properties of 4-hydroxytamoxifen. The TamR cells line was maintained for a further 4 months prior to any cell characterisation studies beginning (Knowlden *et al*, 2003).

2.3 Generation of gefitinib resistant TamR cells (Tam/TKI-R)

The TamR cell cultures were washed with PBS and continuously exposed to gefitinib (1µM) and 100nM 4-hydroxytamoxifen in phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal calf serum plus 200 mM glutamine, 10 U/ml penicillin, 10 µg/ml streptomycin and 2.5 µg/ml Fungizone which was replaced every 4 days. Initially, TamR cell numbers were dramatically reduced and during the following 3 months the surviving cells were passaged approximately every 14 days with a seeding ratio of 1:2. Cell proliferation slowly increased to passage every 10 days with the seeding ratio increasing 1:4 over the next 2 months. A stable growth rate was reached after a total of 6 months with routine maintenance of the TAM/TKI-R cells involving passage every 7 days with a seeding ratio of 1:10 of the confluent cell number (Jones *et al*, 2004).

2.4 Treatment of TamR cells with growth factors or growth inhibitors

Where TamR cells were treated with growth factors or growth inhibitors, the concentrations used are shown in table 2.1. Since duration of treatment varied between experiments from 24 hours to several days, this information has been included in the figure legends of the individual experiments.

Table 2.1: Details of treatments and concentrations used

Treatment	Concentration	Target
17β-Oestradiol	1nM	ER
4-hydroxytamoxifen	100nM	ER
Fulvestrant	100nM	ER
Gefitinib	1µM	EGFR
SU6656	5µM	Src and Lyn

2.5 Experimental cell culture and cell treatments with siRNA

TamR cells were seeded into the appropriate cell culture dishes at $2 \times 10^5 /cm^2$

and maintained in phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal

calf serum plus 200 mM glutamine, 10 U/ml penicillin, 10 µg/ml

streptomycin, 2.5 µg/ml Fungizone and supplemented with 100nM 4-

hydroxytamoxifen until **7**0% confluent. The use of SMARTpool siRNA meant

that each siRNA preparation was in fact a pool of 4 distinct siRNA

preparations each targeting the same gene. Since it is possible that silencing

with siRNA may produce unwanted off-target effects, using a pool of siRNA

minimises the effect that this will have since it is unlikely that all 4 will cause

the same off-target effect. For each siRNA transfection, both the siRNA (final

concentration 100nM) and the transfection lipid were diluted in phenol-red-

free RPMI medium containing 200mM glutamine and 4-hydroxytamoxifen

without serum, antibiotics or fungizone. The diluted siRNA and transfection

lipid were mixed together within 5 minutes of dilution and left for an initial

serum free incubation period of 20 minutes at room temperature that allowed

sufficient time for the siRNA and transfection lipid to form complexes.

Phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal calf serum plus

200 mM glutamine and 100nM 4-hydroxytamoxifen without antibiotics and

Fungizone was added after to the cells after 20 minutes and the complex of

siRNA and transfection lipid was then added drop-wise to the cells whilst very

gently moving the plate to ensure even distribution of the complex. The

amount of lipid and siRNA used varied according to the size of cell culture

dishes used, and is detailed in table 2.2. An initial comparison of six

transfection lipids (section 3.5.1) resulted in the selection of Lipofectamine

RNAiMAX for subsequent experiments. The same concentration of reagents

was used regardless of type of transfection lipid.

Table 2.2: Variation of lipid and siRNA for cell culture dish.

Type of cells culture dish used	Chamber Slides	12-well plates	35 mm dishes/ Coverslips/ 6 well plates	60mm dishes
SiRNA (tube A)	20pmol (1µl)	100pmol (5µl)	200pmol (10µl)	550pmol (27.5µl)
Serum free medium (tube A)	25µl	125µl	250µl	687µl
Transfection lipid (tube B)	0.4µl	2µl	4µl	11µl
Serum free medium (tube B)	25µl	125µl	250µl	687µl
Medium containing serum added to cells	150µl	750µl	1.5ml	4.125ml
Final concentration of siRNA on cells	100nM	100nM	100nM	100nM

2.6 RNA extraction

Medium was removed from cells in a tissue culture dish and the cells were washed 3 times in ice cold PBS. 1ml of TRI® Reagent RNA Isolation Reagent per $10cm^2$ area of culture dish was added, cells were incubated for 5 minutes before scraping into 1.5ml centrifuge tubes. The cells were then stored at -$80^{o}C$ overnight (in order to increase RNA yield) before addition of 0.2ml per $10cm^2$ of chloroform agitating for 15 seconds and incubating at room temperature for 15 minutes. Centrifuging at 12,000 x g for 15 minutes at $4^{o}C$ caused the mixture to separate into a (lower) phenol-chloroform phase and an

(upper) aqueous phase. The RNA was contained in the upper phase which was removed into a centrifuge tube before 0.5ml of iso-propanol per 1ml of isolation reagent was added and the solutions mixed and incubated at room temperature for 5-10 minutes. This was then centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was removed, and the pellet washed in 1ml of 70% ethanol per 1ml of isolation reagent before centrifuging at 7,500 x g for 5 minutes at 4°C. The ethanol was removed and the pellet was air-dried for 10 minutes (taking care not to over-dry which makes resuspending difficult) before re-suspending in sterile RNAse free water.

2.7 RNA quantitation

RNA was diluted 4µl in 1ml of sterile RNAse free water and quantified on a spectrophotometer at 260nm and 280nm. 260nm is the wavelength for RNA and 280nm is the wavelength for DNA. The ratio of RNA to DNA (260nm:280nm) was calculated in order to ascertain if the RNA was contaminated with DNA. Total RNA has a ratio of 1.6-1.8. A sample with a ratio outside of these values would be discarded.

RNA yield was calculated using the formula:

Absorbance (260nm) X Dilution (250) X 40 = µg/ml

2.8 Identification of tamoxifen induced tyrosine kinase genes using Affymetrix microarrays

Triplicate preparations of RNA were isolated from experimental cultures of wild-type MCF-7 cells, tamoxifen resistant (TamR) cells (see 2.2) and

gefitinib resistant TamR cells (Tam/TKI-R (2.3)) using the RNA extraction

method and qualified/quantified as described in section 2.8. To ensure the

absence of genomic DNA contaminants the RNA was treated with DNase1 for

30 minutes and passed through RNeasy Mini Columns. The triplicate samples

were sent to the Cardiff University Central Biotechnology Services and used

to generate biotinylated cRNA which was then hybridised to cDNA

oligonucleotides on an Affymetrix U133A GeneChip. The U133A GeneChip

contains probes representing 14,500 well-characterized human genes and also

array controls. A separate cell sample was used for each GeneChip. The arrays

were then scanned and Microarray Suite 5.0 software (Affymetrix, UK) used

to generate fluorescence output data per probe. The RNA expression data was

formatted to allow analysis using Microsoft Excel software. Data for each cell

line was uploaded into the software package GeneSifter (www.genesifter.net)

then normalised and log transformed using the software to allow accurate

analysis. The Affymetrix probe set ID number for each of the 86 human

tyrosine kinase genes on the U133A chip numbers were inputted into

GeneSifter to enable a series of comparisons of tyrosine kinase gene

expression data between the three cell lines. The data output from GeneSifter

is in the form of a heat map where red represents gene up-regulation, black

represents no change in gene expression and green represents down-regulation

compared to the control. A number of genes had more than one probe on the

U133A chip. In this instance, the probe data for each gene was compared to

ensure that the expression profiles were reproducible. If they were not

reproducible, that particular gene was discounted from further analysis.

2.9 Reverse Transcription (RT)

RNA is reverse transcribed into its complementary DNA (cDNA) that is necessary for the subsequent amplification by PCR.

$1\mu g$ of RNA was used per reaction in a total volume of $7.5\mu l$. This was added to $11\mu l$ of a master mix solution containing $625\mu M$ each of dNTPs (dGTP, dCTP, dATP and dTTP), 1 X PCR reaction buffer (Bioline), 2.5mM $MgCl_2$, 10mM DTT (Dithiothreitol) and $10\mu M$ Random Hexamers. The solution was denatured at $95^{o}C$ for 5 minutes in a PCR machine then cooled rapidly on ice for 5 minutes. $1\mu l$ of MMLV (reverse transcriptase enzyme) and $0.5\mu l$ RNAse inhibitor was added to give a final volume of $20\mu l$.

RNA was transcribed using PCR programme:

Room temperature for10 minutes $(22^{o}C)$, reverse transcribed at $42^{o}C$ for 42 minutes then the reaction was terminated by $95^{o}C$ denaturation for 5 minutes.

2.10 PCR (per reaction)

The cDNA generated by reverse transcription is amplified by PCR.
$1\mu l$ of cDNA was added to a master mix solution made of 1X PCR reaction buffer (Bioline), 2.5mM $MgCl_2$, 0.1mM dNTPs, 10 pmol forward primer, 10 pmol reverse primer and 0.125U of Taq polymerase made up to a total volume of $24\mu l$ using RNAse/DNAse free water.

The details of the forward and reverse primers used and number of cycles used are listed in table 2.3. Where actin was used, 2 pmol of each primer was also added to the mix.

 cDNA was amplified in a PCR machine using the programme:

Denature for 2 minutes at 95°C, amplification step of 95°C for 1 minute, 60°C

for 30 seconds and 72°C for 1 minute for 28 cycles, before a final denaturation

at 94°C for1 minute then final extension time of 72°C for 5 minutes.

Table 2.3: Details of primers used for PCR reactions.

PRIMER	PRIMER SEQUENCE	No. Of Cycles	SIZE(bp)
STAT3 FWD	5'- AGACCGAGGTGTATCACCAAGGTCTCAAGA-3'	32	311
STAT3 REV	5'- TGTGATCTGACACCCTGAGTAGTTCACACC-3'	32	
EGFR FWD	5'- CAACATCTCCGAAAGCCA-3'	35	636
EGFR REV	5'- CGGAACTTTGGGCGACTAT-3'	35	
LAMIN FWD	5'-CCTCTCACTCATCCCAGACACAGG-3'	27	429
LAMIN REV	5'-ACTTGCCAATTGCCCATGGACTGG-3'	27	
EPHB3 FWD	5'- ACATCTCATCCAGAAAGTGG-3'	28	237
EPHB3 REV	5'- GAAGAGGTTGAAGGTCTCCT-3'	28	
PTK2B FWD	5'- CTGGGAAGAACTTCAAACTG-3'	30	241
PTK2B REV	5'- TGAAGGTCATACCTCCACTC-3'	30	
PTK7 FWD	5'-CTACCAATGGTTCCGAGATG-3'	30	378
PTK7 REV	5'-GACCCGTTGGCAAACACTGTG-3'	30	
LYN FWD	5'-CTTGAGTGACGATGGAGTAG-3'	29	230
LYN REV	5'-CGTGGAGAGATGTAATAGCC-3'	29	
TYRO3 FWD	5'-GAACCTGTTACCATTGTCTGG-3'	35	463
TYRO3 REV	5'-CTAGACCCTTGGTCTGAAAGG-3'	35	
SRC FWD	5'-CAGTGTCTGACTTCGACAAC-3'	33	433
SRC REV	3'-CTCCTCTGAAACCACAGCAT-5'	33	
β-actin FWD	5'-GGAGCAATGATCTTGATCTT-3'	27	204
β-actin REV	3'-CCTTCCTGGGCATGGAGTCCT-5'	27	

2.11 Agarose Gel Electrophoresis

A 2% (w/v) agarose gel was made supplemented with 1µl ethidium bromide (10mg/ml stock solution) in TAE buffer (Appendix I). A 20 well plastic comb was inserted and allowed to set for approximately 20 minutes. The agarose gel was assembled into a Bio-Rad gel electrophoresis kit and the tray filled with TAE buffer. 10µl of PCR amplification product was mixed with 5µl of 2X bromophenol blue loading buffer (Appendix II) and pipetted into individual wells. 10µl of a Hyperladder IV 1Kb marker (Bioline) was also added to one well. The gel was run for 50 minutes at 90V or until the Hyperladder IV 1Kb marker had separated sufficiently to allow accurate determination of band size. The gel was visualised under ultraviolet light using a trans-illuminator and the fluorescent image captured with a camera for densitometry. The intensity of each band was determined by multiplying band intensity (OD) with area (mm^2).

2.12 Cell lysis for Western blotting

Cells were washed three times in ice cold PBS, scraped into centrifuge tubes and lysed for 1 hour at 4^oC using ice cold lysis buffer (5.5mM EDTA/0.4% Nonidet P40/ 10% mammalian protease inhibitor cocktail (Sigma-Aldrich) in Krebs-Ringer HEPES buffer) (Taylor 2003). Cells were centrifuged at 13,000rpm for 15 minutes at 4^oC and the supernatant containing protein was removed into a new centrifuge tube and the pellet was discarded. Protein was

quantified using the DC assay kit (Bio-Rad Laboratories) which is a
colorimetric assay based on the Lowry protocol (Lowry OH et al, 1951). A
standard curve of known BSA concentrations between 0 and 1.45mg/ml was
used to calculate the concentration of protein samples. Protein was
subsequently aliquoted into 30µg ready for Western blotting and stored at -
80°C.

2.13 SDS-PAGE

The Bio-Rad Mini-Protean III apparatus (Bio-Rad Laboratories Ltd, HERTS,
UK) was used following the instructions provided by the manufacturer. Glass
plates provided with the apparatus were cleaned thoroughly with 70% ethanol
and assembled according to instructions. A 12% (w/v) SDS-polyacrylamide
resolving (lower) gel was prepared according to the recipe in table 2.4 and the
TEMED was added just prior to pouring the gel carefully between the glass
plates, leaving approximately 2cm for the stacking gel. A thin layer of water
was added by pipette to prevent the gel from drying out, before being left to
set at room temperature for approximately 30 minutes. The layer of water was
then carefully removed using blotting paper and a 4% (w/v) SDS-
polyacrylamide stacking (upper) gel (table 2.5) was mixed and poured on top
of the resolving gel. A plastic comb was immediately inserted between the
glass plates into the stacking gel and the gel was left to set for approximately
45 minutes at room temperature. 2X loading buffer (Appendix III) was added
to 30µg total protein and this was denatured at 100°C for 5 minutes in a
heating block. The comb was removed from the stacking gel, the apparatus

assembled with SDS-PAGE running buffer (Appendix IV) and the samples

loaded into the wells along reserving one lane for a protein molecular weight

marker. The proteins were separated by electrophoresis at 180V for

approximately 1 hour or until the loading buffer dye had reached the required

distance towards the bottom of the gel.

Table 2.4: 12% resolving gel recipe

REAGENT	VOLUME
Lower Buffer	3.75ml
Sterile water	6ml
30% acrylamide	4.95ml
SDS (10% in water)	150µl
APS (10% in water)	150µl
TEMED	15µl

Table 2.5: 4% stacking gel recipe

REAGENT	VOLUME
Upper Buffer	2.5ml
Sterile water	6.1ml
30% acrylamide	1.3ml
SDS (10% in water)	100µl
APS (10% in water)	50µl
TEMED	10µl

2.14 Western blotting

In order to perform Western blotting after SDS-PAGE, the apparatus was disassembled, the glass plates were separated and the gel was assembled into the plastic cassette according to the manufacturer's instructions. Briefly, 2 pieces of blotting paper, 1 piece of 0.45µM pore size nitrocellulose membrane and 2 Teflon sponges per polyacrylamide gel were soaked in transfer buffer (Appendix V) and then assembled according to the diagram in figure 2.2. taking care to minimise the occurrence of air bubbles since this inhibits the transfer onto blotting paper. The plastic housing was then placed into the apparatus along with an ice block to prevent over-heating and filled with transfer buffer and transferred at 100V for 1 hour.

| Clear side of plastic housing |
| Teflon sponge |
| Blotting paper |
| Nitrocellulose |
| Polyacrylamide Gel |
| Blotting paper |
| Teflon sponge |
| Black side of plastic housing |
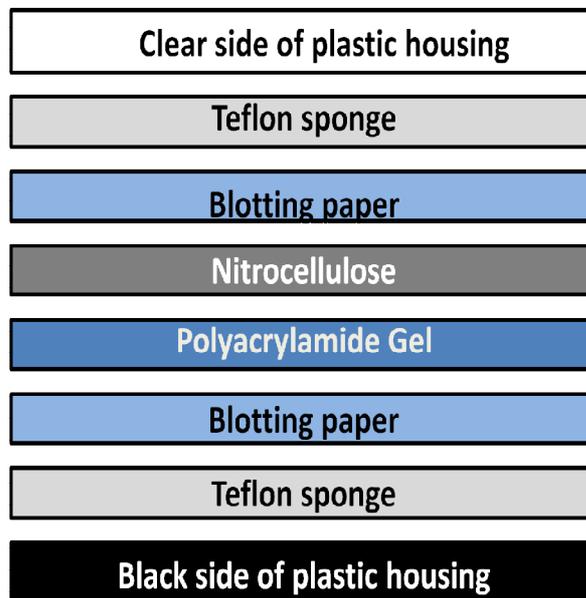
Figure 2.2: Assembly of Western blotting apparatus

The nitrocellulose was carefully removed from the apparatus and the protein bands were visualised using Ponceau S solution (0.1% in 5% acetic acid) and washed off using water before the available proteins were blocked for 1 hour in 5% (v/v) skimmed milk in Tris-buffered saline with tween (TBST- 10mM Tris Base, 100mM NaCl, 0.1% Tween20). The nitrocellulose was washed in TBST and incubated for at least 3 hours in primary antibody made in TBST containing 1% (v/v) skimmed milk. If primary antibody was to be kept at $4^{o}C$ and reused for a period of time then 0.05% (w/v) (final concentration) sodium azide was added to prevent the milk spoiling. Primary antibodies and the concentrations used are listed in table 2.5. The nitrocellulose was washed five times for 5 minutes each in TBST by gentle rocking before being incubated in either mouse or rabbit (see table 2.5 for the correct animal for each primary antibody) horseradish-peroxidase-linked secondary antibody 1/10,000 in TBST containing 1% (v/v) skimmed milk for 1 hour. The nitrocellulose was washed a further eight times for 5 minutes in TBST before protein detection by luminol/peroxide based chemiluminescence reagent (Super Signal West Dura, Femto or Pico chemiluminescent substrate). 250µl of chemiluminescence reagent made according to manufacturer's instructions was applied to the nitrocellulose for five minutes and then exposed to X-ray film for 10 seconds to overnight exposure depending on the signal strength or amount of a particular protein present.

Table 2.5: Details of primary antibodies used for Western blotting

Epitope	Concentration	Animal
Lyn Y396	1/1000	Rabbit
T-Lyn	1/5000	Mouse
Src Y418	1/2000	Rabbit
T-Src	1/1000	Rabbit
EGFR Y1068	1/1000	Rabbit
T-EGFR	1/1000	Rabbit
ERK 1/2 T202/Y204	1/1000	Rabbit
T-ERK 1/2	1/1000	Rabbit
AKT S473	1/2000	Rabbit
FAK Y397	1/1000	Rabbit
T-FAK	1/1000	Rabbit
T-AKT	1/1000	Rabbit
T-ZIP7	1/10,000	Rabbit
PARP	1/1000	Rabbit
β-actin	1/20,000	Mouse

2.15 Determination of MIB-1 proliferation index

Cells were seeded and grown onto cover slips, these were fixed using acetone at $-10^{o}C$ to $-30^{o}C$ for 10 minutes then allowed to air dry. 10% normal goat serum in phosphate buffer solution (PBS) was applied to cover slips for 10 minutes and the excess removed before addition of primary antibody DAKO Ki67 antibody (1/100 in PBS) for 45 minutes at room temperature. Cover slips were washed 3 times in PBS before application of secondary antibody Dako Goat anti-mouse immunoglobulins (1/25 in PBS) for 30 minutes. Cover slips were again washed 3 times in PBS. Dako Mouse PAP 1/250 in PBS was added for 30 minutes and cover slips were washed 3 times in PBS. Chromagen (Dako DAB) was applied for 8 minutes and then washed in distilled water 3 times before final addition of 0.5% aqueous methyl green for 5 minutes or longer to ensure counterstaining of cells. Cover slips were washed in distilled water to remove excess methyl green solution before air drying and mounting onto glass slides using DPX mounting medium and allowed to set at room temperature for 12 hours. Cells in 10 randomly selected fields of view were assessed as positive (showing MIB-1 staining) or negative (no staining for MIB-1). The number of cells positively stained for MIB-1 was expressed as a percentage of the total cell count in order to give the MIB-1 proliferation index.

2.16 FACS (Fluorescence-activated cell sorting)

Cell cycle stage was assessed using CycleTEST^{TM} PLUS Reagent Kit (Becton Dickinson). Solution A, B, C and buffer used were provided in the reagent kit.

Cells were treated with 0.05% trypsin/0.02% EDTA in PBS for 5 minutes at

37°C to disrupt the cell monolayer and the trypsin was then neutralised by the

addition of an equal amount of medium containing serum. The cells were then

pelleted by centrifugation at 300 x g for 5 minutes, the supernatant decanted,

1ml of buffer solution was added and the cells resuspended by gentle

agitation. The process of pelleting and resuspending in buffer was repeated

twice. $5x10^{5}$ cells were used per reaction as measured by Coulter counter as

described below. Once counted and aliquoted to give the correct number, cells

were pelleted by centrifugation at 400 x g for 5 minutes and the supernatant

removed. 250µl of solution A (trypsin) was added to the cell pellet, it was

mixed gently and left at room temperature for 10 minutes. 200µl of solution B

(RNAse A and trypsin inhibitor) was added, mixed gently and left at room

temperature for 10 minutes. 200µl of cold (4°C) solution C (propidium iodide)

was added and left for 10 minutes on ice in the dark. Samples were then

filtered through 35µm cell strainer caps into appropriate tubes for FACS

analysis. Analysis was performed on a FACS Calibur machine connected to an

Apple Macintosh computer and data was analysed using CellQuest software.

2.17 Determination of cell number by Coulter counter

Cells were treated in a 24 well plate. The medium was removed and

trypsin/EDTA was added for 5 minutes at 37°C. 1ml of Isoton II solution was

added and cells were drawn up into a syringe through a 25G needle. The wells

of the plate were then washed with a further 2ml of Isoton II solution which

was taken up into the syringe. The cell-isoton mixture was added to a counting

cup containing 6 ml of Isoton II solution to give a total volume of 10ml.

Cells were counted twice using a Coulter counter (Luton, UK) and the

average reading taken. Each condition was measured in triplicate and the cell

number was then expressed as mean cells per well.

2.18 Migration studies

TamR cells underwent treatment before being grown for 24 h in phenol red

free DCCM medium with 4-hydroxytamoxifen. Trypsin dispersion of cell

monolayers and cell counting using a Coulter counter (Luton, UK) followed.

All experiments were performed in triplicate. The migratory capacity of the

cells following siRNA knockdown was measured using a modified Boyden

chamber as previously described by Hiscox et al (Hiscox *et al*, 2006). Briefly,

a porous membrane was coated with fibronectin (10μg/ml in phenol red free

RPMI (no supplements)) by submerging the inserts in the fibronectin solution

and incubating for two hours at 37°C and allowing to air-dry before seeding

5×10^4 TamR cells in phenol-red-free RPMI with 5% (v/v) charcoal stripped

fetal calf serum plus 200 mM glutamine, without fungizone or antibiotics and

supplemented with 100nM 4-hydroxytamoxifen onto each insert. The cells

were allowed to migrate through the porous membrane for 24 hours at 37°C.

The medium was removed from the inserts and cells that had not migrated

through the porous membrane were removed using a cotton bud in order to

prevent staining of non-migratory cells. Cells that had migrated through the

membrane were fixed in 3.7% (v/v) formaldehyde for 10 minutes and then

washed in PBS before staining with crystal violet and counting using a X10

microscope lens on a Leica DM-IRE2 fluorescent microscope at 320nm. 10

fields of view were counted and the mean of these taken.

2.19 Invasion studies

Using a modified Boyden chamber, the invasive capacity of TamR cells was

measured. The inside of the inserts were coated with 50µL Matrigel (12mg/ml

diluted 1 in 3 with ice cold phenol-red-free RPMI), which mimics the

intracellular matrix and the inserts were placed at $37^{o}C$ for 2 hours to allow the

Matrigel to set. The cells were seeded onto the inserts at a density of 5 $X10^{4}$

TamR cells in phenol-red-free RPMI with 5% (v/v) charcoal stripped fetal

calf serum plus 200 mM glutamine, without fungizone or antibiotics and

supplemented with 100nM 4-hydroxytamoxifen and allowed to invade through

the porous membrane for 72 hours. Non-invasive cells were removed with a

cotton bud and those cells that had invaded through the membrane were fixed

in 3.7% (v/v) formaldehyde for 10 minutes, washed in PBS, and the porous

membrane containing the invaded cells removed from the plastic insert using a

scalpel and mounted onto a microscope slide using Vectashield mounting

medium (Vector laboratories) with 1.5 μg/ml DAPI (4, 6- diamidino-2-

phenylindole) to stain the nucleus and counted at X20 on a Leica DM-IRE2

fluorescent microscope at 320nm.

2.20 Fluorescent microscopy

0.17mm cover slips containing cells were fixed in 3.7% formaldehyde in PBS for 15 minutes, washed twice in PBS and permeablised in BSA buffer (1% bovine serum albumen with 0.4% saponin in PBS) for 15 minutes if cell permeablisation was required. Cover slips were blocked in 10% goat serum for 15 minutes, incubated in primary antibody (1/1000) for 1 hour then washed three times in BSA buffer and incubated in 1/10,000 secondary antibody conjugated to Alexa Fluor 594 or 488 for 30 minutes. For zinc imaging, the cells were loaded with 5µM Fluozin-3 and 25µM Zinquin for 30 min at 37°C in darkness and kept in dark conditions from then on prior to fixing with 3.7% formaldehyde in PBS. Cover slips were then assembled onto slides using Vectashield (Vector laboratories) mounting medium with 1.5 μg/ml DAPI (4, 6- diamidino-2-phenylindole) to stain the nucleus. The cover slip edges were sealed using clear nail varnish and these were stored in the dark at 4°C. All fluorescent images were captured using a Leica DM-IRE2 inverted microscope (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK) and a 63x oil immersion lens. Images were acquired using a multiple bandpass filter set to the appropriate wavelength for the reagent being visualised i.e. DAPI, 488nm (green) or 594nm (red). The microscope was fitted with a Hamamatsu C4742-96 digital camera. Three pictures were taken at each wavelength (0.5µm apart) so that one round of deconvolution could be performed to minimise background. The camera was attached to an Apple MAC computer running OpenLab software (Improvision). Further image

analysis was performed using Paint Shop Pro (Corel) to enhance image

contrast and brightness.

2.21 Statistical analysis

Analysis of the statistical significance of data was performed using Anova and

student t-tests. Data were considered statistically significant when P value was

less than 0.05. Error bars are standard deviation with at least n = 3 different

experiments.

Chapter 3: Results

Tyrosine kinase profiling of tamoxifen resistant cells

3.1 Identification of potential gene targets for siRNA knockdown

The aim of this stage of investigations was to identify a gene target that could be carried forward for siRNA knockdown. In order to achieve this, a high throughput microarray technique was utilised, focusing on the tyrosine kinase (TK) family.

The decision to focus the microarray investigation and comparison of cell lines on tyrosine kinases was taken for a number of reasons. It was necessary to limit the number of genes assessed and focusing on a particular family of genes was a way of achieving that. Over-expression of tyrosine kinases has been implicated in the development of almost all cancers. Of particular note within the family are the Ephrins and Eph receptors which have been shown to be over-expressed in many cancers including lung, colon, head and neck and breast cancers. The complex relationships between Ephrins and their receptors and a summary of their role in various cancers are reviewed in detail by Pasquale (Pasquale, 2010). Significantly, several have been implicated in the transition of breast cancer from a tamoxifen responsive to a tamoxifen resistant state via up-regulation of growth factor receptors including IGF-IR (Knowlden *et al*, 2005), HER2 and EGFR, and thus their downstream kinases such as PI3K/Akt and MAP kinases (Gee *et al*, 2005; Nicholson *et al*, 2007). Based on the well established role of these genes in both breast cancer and specifically tamoxifen resistance, it was felt that focusing on the tyrosine kinase family would prove productive.

The size of the human tyrosine kinase family (91 known human tyrosine kinase genes) and also the complexity of relationships between family

members and sub-families within the group meant that analysis of affymetrix

data was utilised as a means of identifying a number of robust gene targets to

be carried forward for further investigation. The U133A Gene Chip contained

a total of 139 tyrosine kinase probes that represented 86 of the 91 human

tyrosine kinase genes. This meant that some genes were represented multiple

times on the array by distinct probes.

There were a number of advantages to using a high throughput

microarray technique. These included the assessment of gene behaviour of

thousands of genes simultaneously since the U133A Gene Chip contains

14,500 human genes. This technique also allowed gene expression analysis to

be performed across several different cell lines for comparison. Even

focussing on the 86 tyrosine kinase genes, as opposed to the 14,500 human

genes on the U133A Gene Chip, it would not have been possible to compare

gene expression across several cell lines using other techniques with the same

speed as was afforded by microarray use.

The preparation of samples and hybridisation to the GeneChip is

described in chapter 2. The arrays were scanned and Microarray Suite 5.0

software (Affymetrix, UK) used to generate fluorescence output data for each

probe. The RNA expression data was formatted to allow analysis using

Microsoft Excel software. Data for each cell line was uploaded into the

software package GeneSifter (www.genesifter.net) then normalised and log

transformed. GeneCard software was used to identify the Affymetrix probe set

ID number for each of the 86 tyrosine kinase genes present on the U133A

array. These ID numbers were then entered into GeneSifter to allow pair-wise

comparisons of the tyrosine kinase genes in the cell lines investigated. The cell lines studied compared in this study were wild type hormone responsive breast cancer cells (MCF-7), tamoxifen resistant MCF-7 cells (TamR), and gefitinib resistant TamR cells (TAM/TKI-R).

3.1.1 Identification of genes showing increased expression in tamoxifen resistant breast cancer cells compared with tamoxifen responsive cells.

A pair-wise comparison of MCF-7 vs. TamR gene expression of all the tyrosine kinase gene probes present on the GeneChip was conducted and the resultant heat map is displayed in figure 3.1. In total, 50 probes were increased in TamR cells compared to MCF-7 cells, 29 probes were unchanged in TamR cells compared to MCF-7 cells and 60 probes were down-regulated in TamR cells compared to MCF-7 cells. Since the aim of this project was to investigate the role of selected tyrosine kinase genes in TamR cells by using siRNA knockdown, the work presented focuses on the 50 probes that were induced in TamR cells compared to MCF-7 cells. These probes represented 42 tyrosine kinase genes that were induced in TamR cells. To simplify data, interpretation where multiple probes represent the same gene, each of the 50 probes has been designated a number from 1 to 50 as detailed in table 3.1

Table 3.1. The 50 probes that are upregulated in TamR compared to

MCF-7 along with affymetrix probe number

	Gene name	Probe number		Gene name	Probe number
1	RET	211421_s_at	26	TYRO3	211431_s_at
2	TEK	206702_at	27	TEC	206301_at
3	PDGFRA	203131_at	28	EPHB1	211898_s_at
4	TYRO3	211432_s_at	29	MUSK	207632_at
5	EPHA7	206852_at	30	LCK	204890_s_at
6	LYN	202625_at	31	EPHA1	205977_s_at
7	PTK7	207011_s_at	32	ZAP70	214032_at
8	PTK2B	203110_at	33	RYK	214172_x_at
9	EGFR	201984_s_at	34	MERTK	206028_s_at
10	BLK	206255_at	35	JAK3	207187_at
11	YES1	202933_s_at	36	PTK2	208820_at
12	LMTK2	206223_at	37	MATK	206267_s_at
13	STYK1	221696_s_at	38	EPHA5	215664_s_at
14	LYN	210754_s_at	39	TIE1	204468_s_at
15	FGFR4	211237_s_at	40	HCK	208018_s_at
16	MUSK	207633_s_at	41	CSK	202329_at
17	ROR1	205805_s_at	42	ERBB3	202454_s_at
18	EPHB3	1438_at	43	PTK2B	203111_s_at
19	EPHB3	204600_at	44	ERBB4	206794_at
20	KIT	205051_s_at	45	JAK2	205842_s_at
21	TTK	204822_at	46	RYK	216976_s_at
22	FGFR3	204379_s_at	47	FGFR1	211535_s_at
23	EGFR	201983_s_at	48	AXL	202685_s_at
24	SRC	221284_s_at	49	MERTK	211913_s_at
25	TNK2	203838_s_at	50	LYN	202626_s_at

The 50 probes that were induced in TamR cells compared to MCF-7 cells and

U133A GeneChip probe number. Each probe was assigned a number from 1 to

50 with 1 being the most induced and 50 the least induced.

Figure 3.1: Regulation of 139 tyrosine kinase probes in TamR cells

compared to MCF-7 cells.

	MCF-7	TamR	Gene name	Description
1			RET	ret proto-oncogene
2			TEK	TEK tyrosine kinase, endothelial
3			PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
4			TYRO3	TYRO3 protein tyrosine kinase
5			EPHA7	EPH receptor A7
6			LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
7			PTK7	PTK7 protein tyrosine kinase 7
8			PTK2B	PTK2B protein tyrosine kinase 2 beta
9			EGFR	epidermal growth factor receptor
10			BLK	B lymphoid tyrosine kinase
11			YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
12			LMTK2	lemur tyrosine kinase 2
13			STYK1	serine/threonine/tyrosine kinase 1
14			LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
15			FGFR4	fibroblast growth factor receptor 4
16			MUSK	muscle, skeletal, receptor tyrosine kinase
17			ROR1	receptor tyrosine kinase-like orphan receptor 1
18			EPHB3	EPH receptor B3
19			EPHB3	EPH receptor B3
20			KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
21			TTK	TTK protein kinase
22			FGFR3	fibroblast growth factor receptor 3
23			EGFR	epidermal growth factor receptor
24			SRC	v-src sarcoma
25			TNK2	tyrosine kinase, non-receptor, 2
26			TYRO3	TYRO3 protein tyrosine kinase
27			TEC	tec protein tyrosine kinase
28			EPHB1	EPH receptor B1
29			MUSK	muscle, skeletal, receptor tyrosine kinase
30			LCK	lymphocyte-specific protein tyrosine kinase
31			EPHA1	EPH receptor A1
32			ZAP70	zeta-chain (TCR) associated protein kinase 70kDa
33			RYK	RYK receptor-like tyrosine kinase
34			MERTK	c-mer proto-oncogene tyrosine kinase
35			JAK3	Janus kinase 3
36			PTK2	PTK2 protein tyrosine kinase 2
37			MATK	megakaryocyte-associated tyrosine kinase
38			EPHA5	EPH receptor A5
39			TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1

Figure 3.1: Regulation of 139 tyrosine kinase probes in TamR cells compared to MCF-7 cells continued.

40			HCK	hemopoietic cell kinase
41			CSK	c-src tyrosine kinase
42			ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
43			PTK2B	PTK2B protein tyrosine kinase 2 beta
44			ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)
45			JAK2	Janus kinase 2 (a protein tyrosine kinase)
46			RYK	RYK receptor-like tyrosine kinase
47			FGFR1	fibroblast growth factor receptor 1
48			AXL	AXL receptor tyrosine kinase
49			MERTK	c-mer proto-oncogene tyrosine kinase
50			LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
51			LTK	leukocyte tyrosine kinase
52			TNK1	tyrosine kinase, non-receptor, 1
53			JAK3	Janus kinase 3 (a protein tyrosine kinase, leukocyte)
54			LCK	lymphocyte-specific protein tyrosine kinase
55			EGFR	epidermal growth factor receptor
56			EPHB4	EPH receptor B4
57			ITK	IL2-inducible T-cell kinase
58			YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
59			KDR	kinase insert domain receptor
60			EPHB1	EPH receptor B1
61			INSR	insulin receptor
62			SYK	spleen tyrosine kinase
63			FYN	FYN oncogene related to SRC, FGR, YES
64			MET	met proto-oncogene (hepatocyte growth factor receptor)
65			FYN	FYN oncogene related to SRC, FGR, YES
66			FGFR2	fibroblast growth factor receptor 2
67			NTRK1	neurotrophic tyrosine kinase, receptor, type 1
68			ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
69			ROS1	v-ros UR2 sarcoma virus oncogene homolog 1
70			TNK2	tyrosine kinase, non-receptor, 2
71			CSF1R	colony stimulating factor 1 receptor
72			RET	ret proto-oncogene
73			PDGFRB	platelet-derived growth factor receptor, beta polypeptide
74			FGFR2	fibroblast growth factor receptor 2
75			MET	met proto-oncogene (hepatocyte growth factor receptor)
76			SYK	spleen tyrosine kinase
77			DDR1	discoidin domain receptor family, member 1
78			FGFR4	fibroblast growth factor receptor 4
79			DDR	discoidin domain receptor family, member 1

Figure 3.1: Regulation of 139 tyrosine kinase probes in TamR cells compared to MCF-7 cells continued.

#			Symbol	Description
80			PTK2	PTK2 protein tyrosine kinase 2
81			FGFR3	fibroblast growth factor receptor 3
82			EPHB2	EPH receptor B2
83			NTRK2	neurotrophic tyrosine kinase, receptor, type 2
84			AXL	AXL receptor tyrosine kinase
85			ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
86			RET	ret proto-oncogene
87			MST1R	macrophage stimulating 1 receptor
88			DDR1	discoidin domain receptor family, member 1
89			FYN	FYN oncogene related to SRC, FGR, YES
90			INSRR	insulin receptor-related receptor
91			FGFR1	fibroblast growth factor receptor 1
92			STYK1	serine/threonine/tyrosine kinase 1
93			FES	feline sarcoma oncogene
94			DDR1	discoidin domain receptor family, member 1
95			EPHB6	EPH receptor B6
96			EPHB2	EPH receptor B2
97			FER	fer (fps/fes related) tyrosine kinase
98			RYK	RYK receptor-like tyrosine kinase
99			NTRK3	neurotrophic tyrosine kinase, receptor, type 3
100			BTK	Bruton agammaglobulinemia tyrosine kinase
101			EGFR	epidermal growth factor receptor
102			FGFR2	fibroblast growth factor receptor 2
103			ALK	anaplastic lymphoma kinase (Ki-1)
104			NTRK3	neurotrophic tyrosine kinase, receptor, type 3
105			FLT1	fms-related tyrosine kinase 1
106			SRC	v-src sarcoma
107			NTRK3	neurotrophic tyrosine kinase, receptor, type 3
108			NTRK3	neurotrophic tyrosine kinase, receptor, type 3
109			ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2
110			EPHA3	EPH receptor A3
111			ROR2	receptor tyrosine kinase-like orphan receptor 2
112			EPHB2	EPH receptor B2
113			BMX	BMX non-receptor tyrosine kinase
114			EPHB4	EPH receptor B4
115			DDR2	discoidin domain receptor family, member 2
116			ALK	anaplastic lymphoma kinase (Ki-1)
117			NTRK3	neurotrophic tyrosine kinase, receptor, type 3
118			ABL1	v-abl Abelson murine leukemia viral oncogene homolog 1
119			LMTK1	lemur tyrosine kinase 1

Figure 3.1: Regulation of 139 tyrosine kinase probes in TamR cells compared to MCF-7 cells continued.

120		TNK1	tyrosine kinase, non-receptor 1
121		FLT3	fms-related tyrosine kinase 3
122		TYK2	tyrosine kinase 2
123		FLT4	fms-related tyrosine kinase 4
124		EPHA3	EPH receptor A3
125		MET	met proto-oncogene (hepatocyte growth factor receptor)
126		FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
127		FRK	fyn-related kinase
128		EPHA2	EPH receptor A2
129		FGFR2	fibroblast growth factor receptor 2
130		JAK2	Janus kinase 2
131		INSR	insulin receptor
132		RAVER2	ribonucleoprotein, PTB-binding 2
133		LTK	leukocyte tyrosine kinase
134		TXK	TXK tyrosine kinase
135		IGF1R	insulin-like growth factor 1 receptor
136		IGF1R	insulin-like growth factor 1 receptor
137		PTK6	PTK6 protein tyrosine kinase 6
138		FGFR2	fibroblast growth factor receptor 2
139		EPHA4	EPH receptor A4

Heat map of all available tyrosine kinases showing up/down regulation of genes in TamR cells compared to wild type MCF-7 cells. The 50 up-regulated genes are summarised in table 3.1.

MCF-7   TamR

= Up-regulated in TamR vs. MCF-7

= Down regulated in TamR vs. MCF-7

Among the 42 genes that were identified as induced in TamR cells compared

to MCF-7 were EGFR and Src kinase. Their role in tamoxifen resistance has

been well documented in the literature (see Chapter 1). This result therefore

adds some weight to the gene screening method used, since one would expect

to see these genes up-regulated in TamR cells. It is also noteworthy that when

the 42 induced genes are mapped to the tyrosine kinase family tree (a

phylogenetic tree that shows how kinases relate to each other) as in figure 3.2,

a distinct cluster of induced genes is seen around the Src family, with five out

of the seven family members (Src, Yes-1, Lyn, Lck and Hck) induced in

TamR cells compared to MCF-7 cells.

Figure 3.2: Phylogenetic tree of the 86 tyrosine kinase genes represented on the U133A Gene Chip.
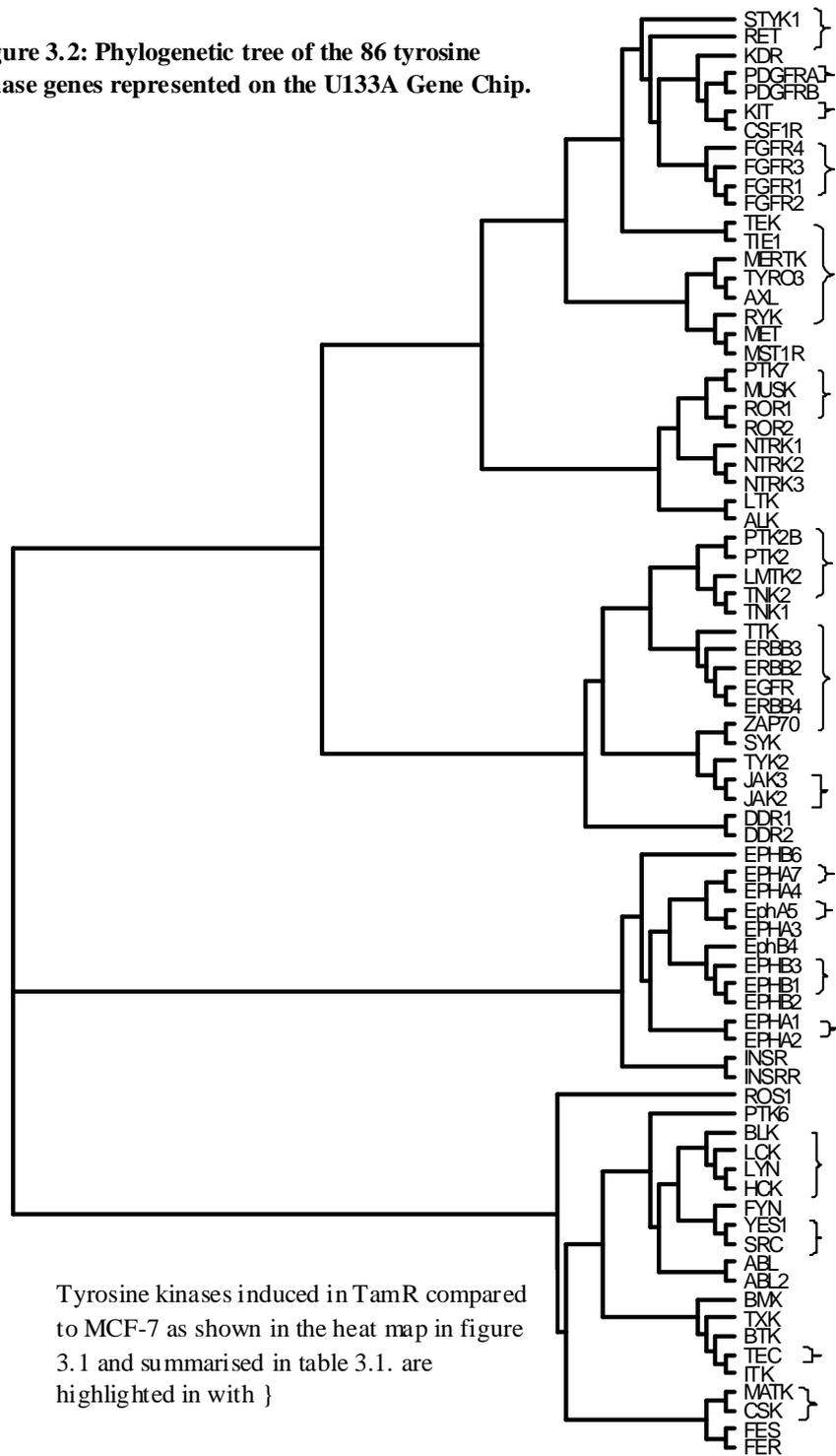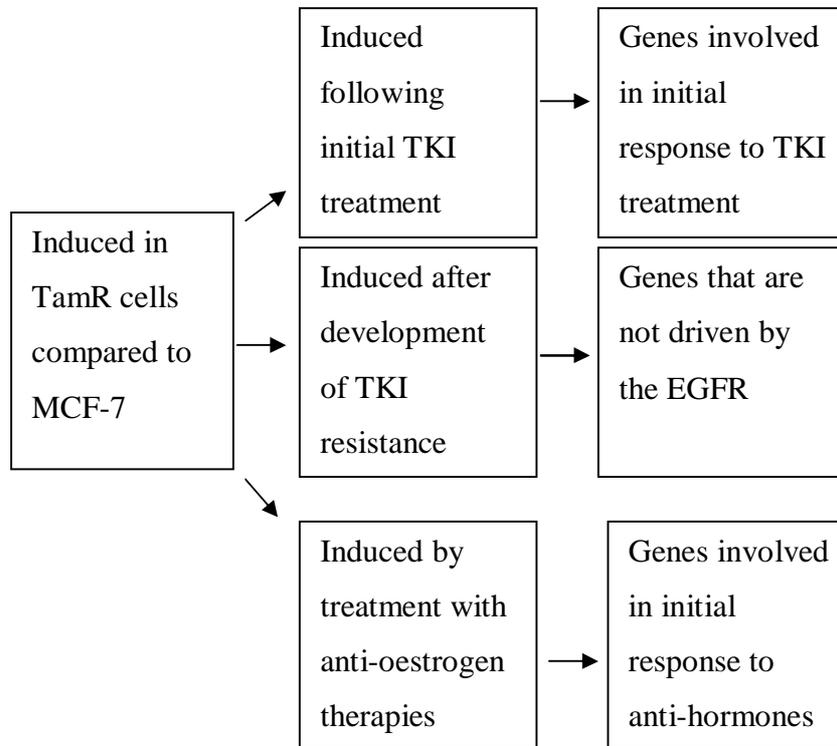

Tyrosine kinases induced in TamR compared to MCF-7 as shown in the heat map in figure 3.1 and summarised in table 3.1. are highlighted in with }

3.1.2 Further selection of genes induced in tamoxifen resistance

When considering a gene suitable for siRNA removal in TamR cells, an ideal target would be a gene whose expression is not driven by the EGFR, and is induced following anti-hormone treatment. The reasons for this 'ideal' profile are two-fold: A number of agents have been developed in recent years that target the Erb family (including EGFR) and are being trialled in the clinic. These include the monoclonal antibody to HER2 (trastuzumab), the small-molecule TK inhibitors (TKIs) of the adenosine triphosphate binding site of EGFR (gefitinib), and the dual TKI of both EGFR and HER2 (lapatinib) (Cleator *et al*, 2009). Studies are also in progress investigating the use of these agents in conjunction with tamoxifen (Osborne *et al*, 2011). As EGFR inhibitors are currently in use in the clinic, the selection of a non-EGFR driven target was an attempt to select a novel therapeutic target that was not currently targeted by available therapies. In addition, treatment with gefitinib in lung cancer can lead to initial poor response in the clinic implying mechanisms exist that lead to *de novo* resistance to gefitinib (Fukuoka *et al*, 2002) or the acquisition of resistance after an initial period of growth suppression and increased reliance on IGF-1R for growth (Jones *et al*, 2004). These data implied that alternative, non-EGFR driven pathways contribute to the growth of tamoxifen resistant breast cancer that have yet to be discovered and targeting said pathways may provide an improved therapy following acquisition of tamoxifen resistance in breast cancer. In order to identify genes that could be are involved in the initial response to anti-hormone therapy

genes that were induced following early anti-hormone treatment were selected.

Thus they could be targeted at the point of initial anti-hormone therapy in a

dual treatment, as opposed to a gene that is only induced following the

development of resistance to anti- hormone therapies.

In order to identify a gene displaying the ideal profile, a three-pronged

strategy of identification was taken as summarised in figure 3.3

Figure 3.3: Summary of gene identification strategy

Induced in TamR cells compared to MCF-7	→	Induced following initial TKI treatment	→	Genes involved in initial response to TKI treatment
	→	Induced after development of TKI resistance	→	Genes that are not driven by the EGFR
	→	Induced by treatment with anti-oestrogen therapies	→	Genes involved in initial response to anti-hormones

By studying the response of the 42 up-regulated genes to treatment with the
EGFR inhibitor gefitinib for 10 days and also their expression in Tam/TKI-R
cells (dual tamoxifen/gefitinib resistant cells) it was possible to identify genes
that were up-regulated following acquisition of tamoxifen resistance, and
whose growth was independent of the EGFR.

A project was created in GeneSifter to facilitate the side-by-side comparison
of the induced TamR cells (control), gefitinib treated TamR cells and the
Tam/TKI-R cells (figure 3.4). Those genes whose growth is not EGFR driven
should be induced following gefitinib treatment. If these genes continue to be
induced in the Tam/TKI-R cells, this shows that the genes continue to be
involved in long term gefitinib resistance and thus are not driven by EGFR. 6
genes (TYRO3, EPHB3, LCK, RYK (2 probes), PTK2, LYN) were induced in
both gefitinib treated TamR cells and also the Tam/TKI-R cells. This result
indicates that their initial growth is not driven by EGFR and they are not
involved in gefitinib resistance. 7 genes (TYRO3, LYN (2 probes), TTK,
FGFR3, EGFR, ERBB3, JAK2 were induced in gefitinib treated cells but
showed no change or were down regulated in the Tam/TKI-R cells. This
suggests that their growth is not EGFR driven. 5 genes showed no change with
gefitinib treatment, of which 3 were up-regulated (PTK2B, ERBB4, AXL), 1
was unchanged (TNK2) and 1 was down regulated (PDGFRA) in the
Tam/TKI-R cells. 6 genes were down regulated in gefitinib treated cells and
subsequently up-regulated in the TamR/TKI-R cells (TEK, PTK7, PTK2B,
FGFR4, EPHB3 (2 probes), MATK) suggesting they are involved in long term
resistance to gefitinib. 1 gene, KIT, was down regulated in gefitinib treated

cells and showed no change in the Tam/TKI-R cells, suggesting that it is driven by EGFR. All other probes were down regulated in both the gefitinib treated cells and also the TamR/TKI-R cells. This indicates that their growth is driven by EGFR.

The response of the TamR induced genes to 10 days of anti-hormone treatment was studied in order to ascertain if the gene induction begins early during response or whether it only occurs at the time of resistance. The 42 induced genes were studied by looking at affymetrix results for oestradiol, tamoxifen and fulvestrant treatment and comparing them to the 10 day MCF-7 cells (-E_2). The heat maps of the genes induced in TamR cells with early anti-hormone (tamoxifen and fulvestrant) and E_2 treatments, are shown in figure 3.5 with a summary of the heat map data in table 3.2.

Using this identification strategy, only one gene, namely Lyn was identified as fulfilling the 'ideal' gene profile. It was felt that limiting gene selection to this extent was at this early stage was not the best strategy, and so a short-list of 5 genes was carried forward: EphB3, PTK2B, TYRO3, PTK7 and LYN. These all fulfilled the following criteria:

- A tyrosine kinase
- Increased in tamoxifen resistant breast cancer cells compared to tamoxifen responsive breast cancer cells
- Not induced by the EGFR

Figure 3.4: Response of genes induced in TamR compared to MCF-7 to
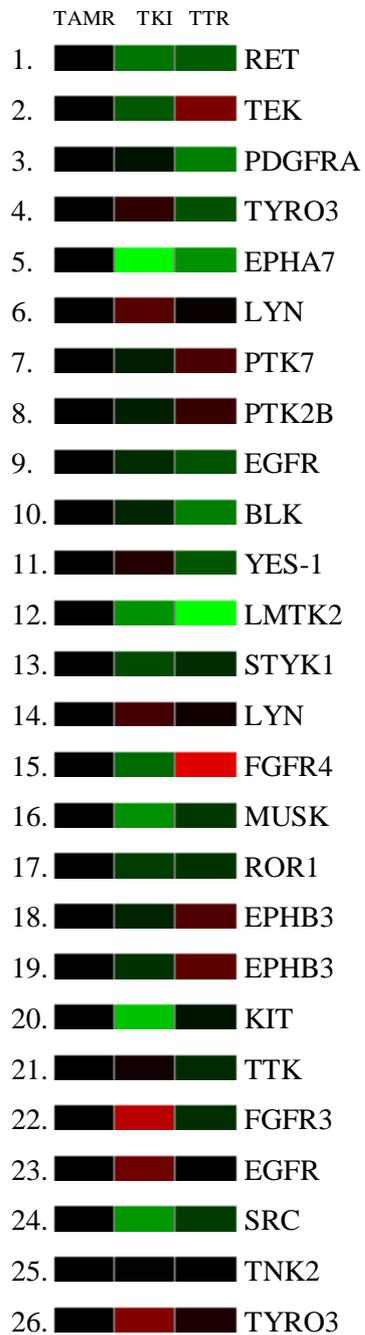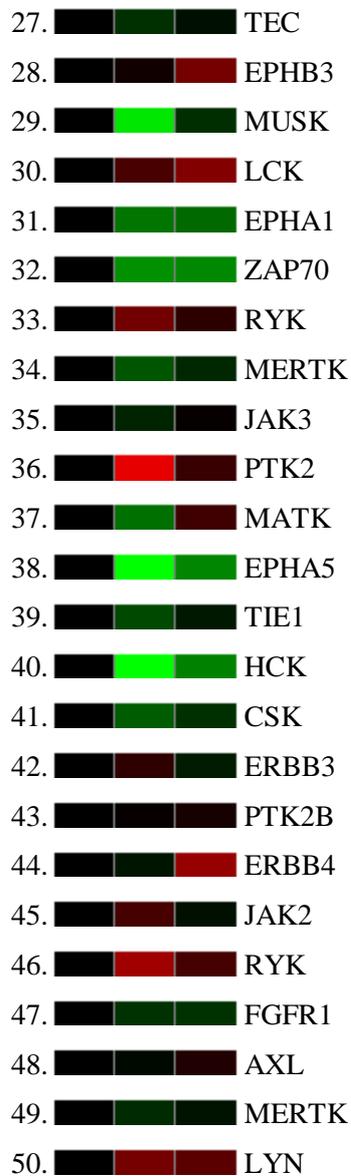
TKI treatment and development TKI resistance.

Figure 3.4: Response of genes induced in TamR compared to MCF-7 to

TKI treatment and development TKI resistance continued.

27. ███████████ TEC
28. ███████████ EPHB3
29. ███████████ MUSK
30. ███████████ LCK
31. ███████████ EPHA1
32. ███████████ ZAP70
33. ███████████ RYK
34. ███████████ MERTK
35. ███████████ JAK3
36. ███████████ PTK2
37. ███████████ MATK
38. ███████████ EPHA5
39. ███████████ TIE1
40. ███████████ HCK
41. ███████████ CSK
42. ███████████ ERBB3
43. ███████████ PTK2B
44. ███████████ ERBB4
45. ███████████ JAK2
46. ███████████ RYK
47. ███████████ FGFR1
48. ███████████ AXL
49. ███████████ MERTK
50. ███████████ LYN

Heat maps of the gefitinib treated and Tam/TKI-R with TamR as the control

where TKI = gefitinib treated cells and TTR= Tam/TKI-R. Legend:

-2   0   2

Table 3.2 Response of genes induced in TamR cells to anti-hormone or

oestradiaol treatment.

	TREATMENTS		
	OESTRADIOL	TAMOXIFEN	FULVESTRANT
RET	U	U	U
YES1	U	U	U
TTK	U	U	U
RYK	U	U	U
PTK2	U	U	U
HCK	U	U	U
JAK2	U	U	U
MATK	U	U	D
RYK	U	U	-
TEK	U	D	U
BLK	U	D	U
SRC	U	D	U
PDGFRA	U	D	D
MUSK	U	D	D
FGFR4	U	D	-
PTK2B	U	-	-
LCK	U	-	-
AXL	U	-	-
LYN	D	U	U
LYN	D	U	D
LYN	D	U	-
EGFR	D	D	U
EPHB1	D	D	U
EPHA5	D	D	U
PTK7	D	D	D
EGFR	D	D	D
ROR1	D	D	D

Table 3.2 Response of genes induced in TamR cells to anti hormone or

oestradiol treatment continued.

	TREATMENTS		
	OESTRADIOL	TAMOXIFEN	FULVESTRANT
KIT	D	D	D
FGFR3	D	D	D
TNK2	D	D	D
TYRO3	D	D	D
EPHA1	D	D	D
JAK3	D	D	D
ERBB3	D	D	D
ERBB4	D	D	D
EPHB3	D	D	-
ZAP70	D	-	D
STYK1	D	-	-
EPHA7	-	U	U
LMTK2	-	U	U
MUSK	-	D	U
TYRO3	-	D	D
TEC	-	D	D
TIE1	-	D	D
PTK2B	-	D	D
FGFR1	-	D	D
EPHB3	-	D	-
MERTK	-	D	-
CSK	-	-	-
MERTK	-	-	-

Summary of response of 42 genes induced in TamR cells to 10 days oestradiol

or anti-hormone treatment (tamoxifen or fulvestrant) as seen in the heat maps

of figure 3.1.3.

Legend: U=up-regulated D=down-regulated, - = no change

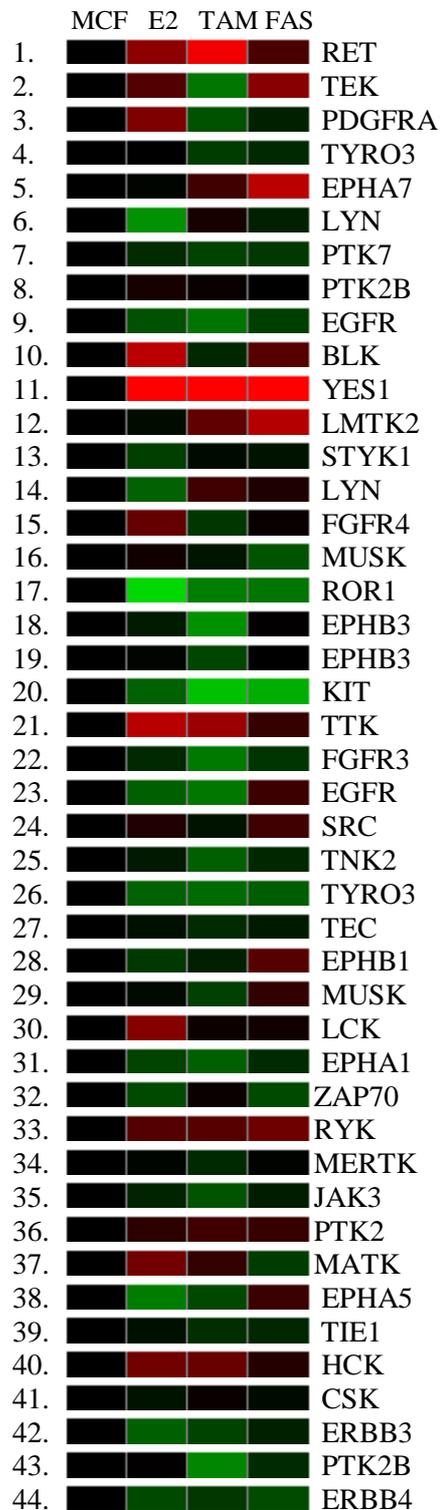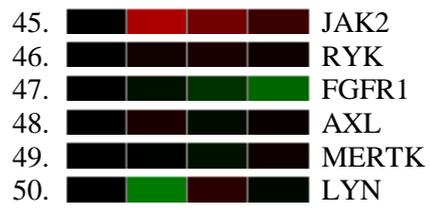**Figure 3.5 Response of TamR induced genes to early anti-hormone or**

oestradiol treatment.

	MCF	E2	TAM	FAS	
1.					RET
2.					TEK
3.					PDGFRA
4.					TYRO3
5.					EPHA7
6.					LYN
7.					PTK7
8.					PTK2B
9.					EGFR
10.					BLK
11.					YES1
12.					LMTK2
13.					STYK1
14.					LYN
15.					FGFR4
16.					MUSK
17.					ROR1
18.					EPHB3
19.					EPHB3
20.					KIT
21.					TTK
22.					FGFR3
23.					EGFR
24.					SRC
25.					TNK2
26.					TYRO3
27.					TEC
28.					EPHB1
29.					MUSK
30.					LCK
31.					EPHA1
32.					ZAP70
33.					RYK
34.					MERTK
35.					JAK3
36.					PTK2
37.					MATK
38.					EPHA5
39.					TIE1
40.					HCK
41.					CSK
42.					ERBB3
43.					PTK2B
44.					ERBB4

Figure 3.5 Response of TamR induced genes to early anti-hormone or

oestradiol treatment continued.

45. ▪▪▪▪▪ JAK2
46. ▪▪▪▪▪ RYK
47. ▪▪▪▪▪ FGFR1
48. ▪▪▪▪▪ AXL
49. ▪▪▪▪▪ MERTK
50. ▪▪▪▪▪ LYN

Heat maps showing response of TamR induced genes to treatment with either

oestradiol, tamoxifen or fulvestrant. Ideally, genes would be induced

following anti-hormone treatment and down regulated following oestradiol

Legend:

-2   0   2

3.2 Selection of gene target

3.2.1 PCR verification of genes identified by analysis of affymetrix data.

Following identification of the five potential gene targets EphB3, PTK2B, PTK7, LYN and TYRO3 by analysis of affymetrix data, primer pairs were designed to target the short-listed genes and PCR was used to further investigate the genes in TamR cells compared to MCF-7 cells. Fresh mRNA was obtained as a result of cell culture and RNA extraction as detailed in chapter 2. Triplicates of MCF-7 and TamR mRNA were obtained from three distinct cell preparations and each extraction was performed using cells of a different passage number. The aim was to confirm that the results obtained during affymetrix analysis were reproducible using new preparations of mRNA before proceeding with further analysis of each of the five genes.

3.2.1.1: EphB3

EphB3 mRNA (Figure 3.6A), was significantly increased in TamR cells compared to MCF-7 cells. Levels of EphB3 in TamR cells showed a greater than two-fold increase compared to those seen in MCF-7 when the of the mean values derived from the triplicate preparations was measured. This densitometric representation is shown in figure 3.6B.

3.2.1.2: PTK2B

Despite not showing as great a difference as EphB3, PTK2B mRNA was also significantly increased in TamR cells compared to MCF-7 cells (Figure 3.7A). The triplicate densitometric values were normalised to β-actin and the mean for each cell line is displayed in figure 3.7B. This shows a 50% increase in PTK2B mRNA in TamR compared to MCF-7.

3.2.1.3: PTK7

The primer designed to PTK7 mRNA amplified 2 variants of PTK7 hence the double band seen in figure 3.8A. It is noteworthy that amplification of the larger band is only seen in MCF-7 and not TamR. PTK7 was significantly increased by 100% in TamR cells compared to MCF-7 cells as represented by figure 3.8B showing the mean levels of PTK7 mRNA in both cell lines.

3.2.1.4: Lyn

Lyn (Figure 3.9) was confirmed to be significantly increased in TamR cells compared to MCF-7 cells following PCR analysis. Whilst the 35% increase in TamR cells was not as high as the previous three genes, it was still statistically significant and so Lyn kinase remained a viable gene target for further investigation. Interestingly, Lyn kinase displayed the greatest increase in TamR compared to MCF-7 cells of all five genes in this section following analysis of the affymetrix data (section 3.1), showing that the PCR analysis in this section does not necessarily exactly echo the results seen with the affymetrix.

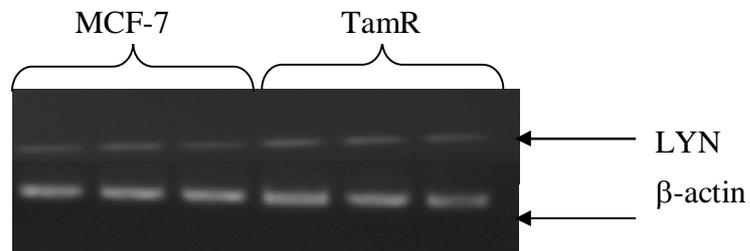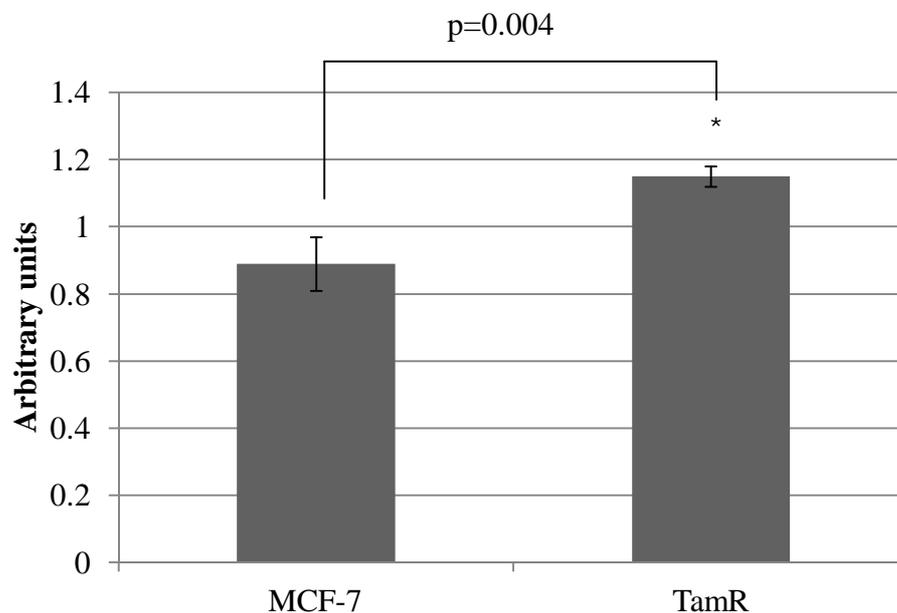**Figure 3.6 EphB3 is increased in TamR cells compared to MCF-7 cells**
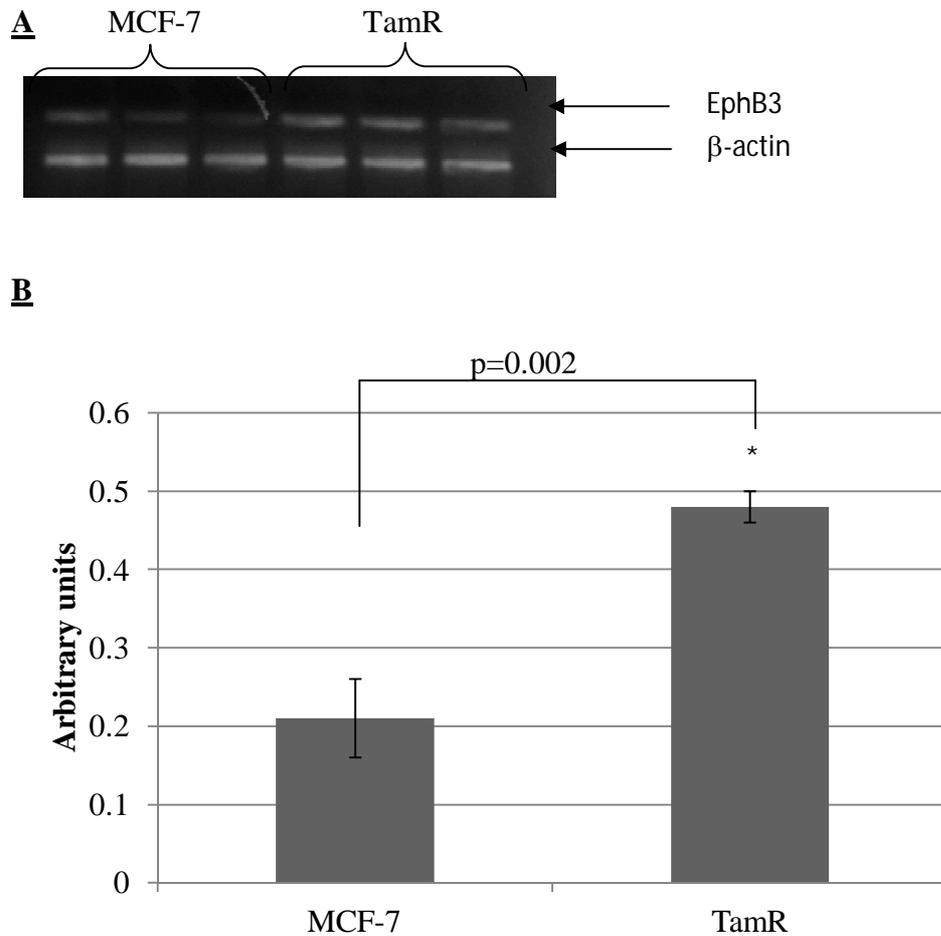
A: EphB3 was amplified in triplicate preparations of MCF-7 and TamR mRNA by PCR. β-actin was included as a reference gene allowing densitometric analysis

B: Levels of MCF-7 cells compared to TamR cells as determined by densitometry. Blots were normalised to β-actin levels. Statistical significance was determined using Student's t-test (n=3).

Figure 3.7 PTK2B is increased in TamR cells compared to MCF-7 cells

<u>A</u>
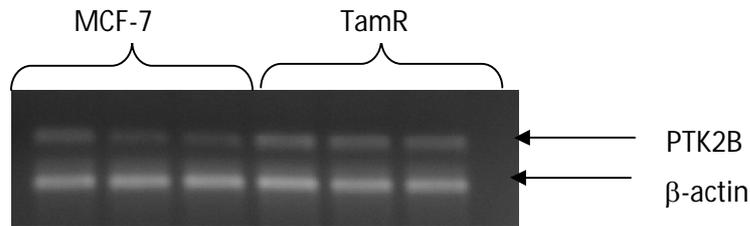
<u>B</u>
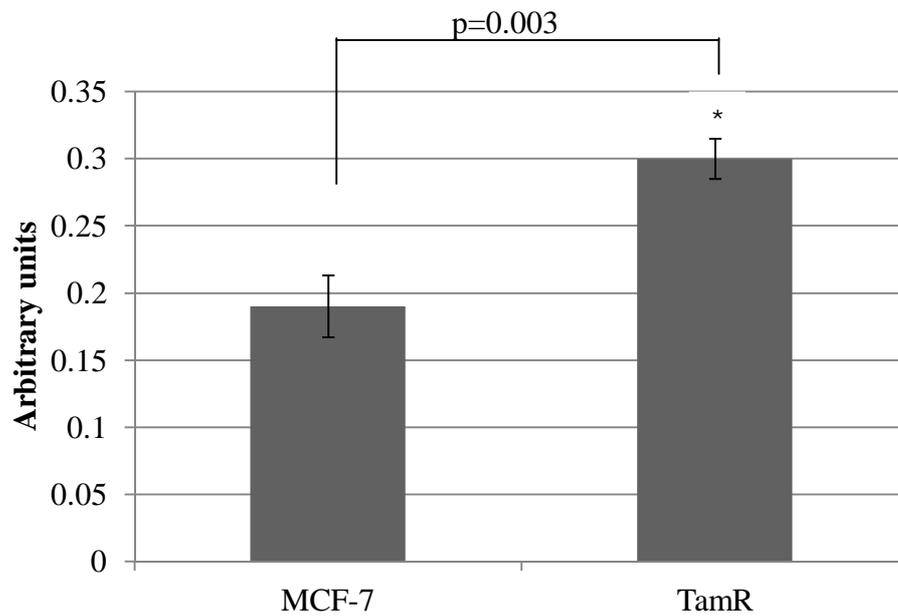
A: PTK2B was amplified in triplicate preparations of MCF-7 and TamR
mRNA by PCR. β-actin was included as a reference gene allowing
densitometric analysis

B: Levels of MCF-7 cells compared to TamR cells as determined by
densitometry. Values were normalised to β-actin levels. Statistical significance
was determined using Student's t-test (n=3).

Figure 3.8 PTK7 is increased in TamR cells compared to MCF-7 cells

<u>A</u>
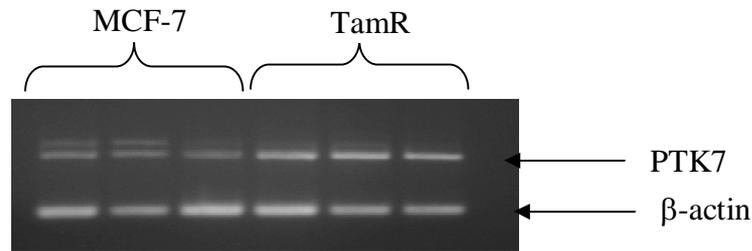
<u>B</u>
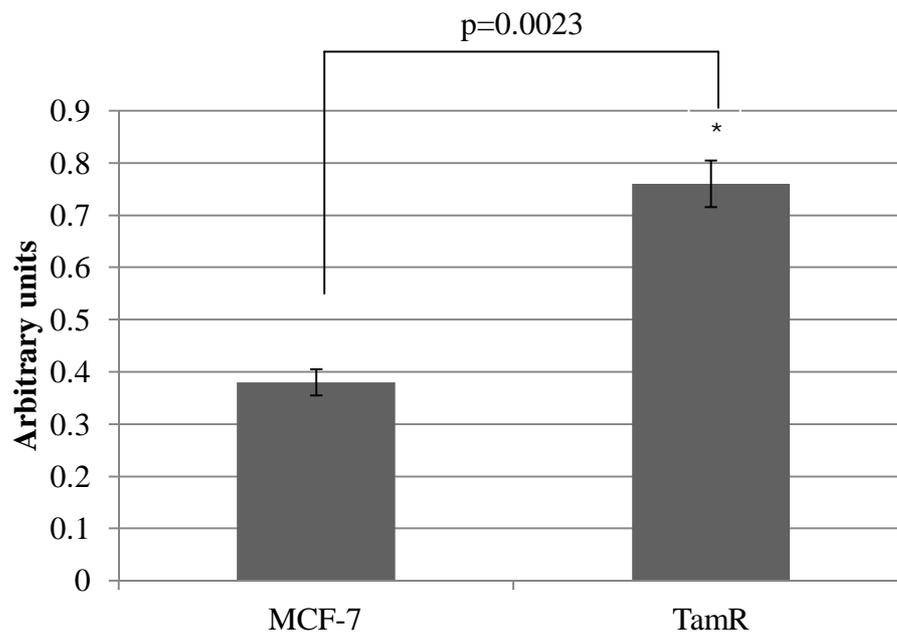
A: PTK7 was amplified in triplicate preparations of MCF-7 and TamR mRNA by PCR. β-actin was included as a reference gene allowing densitometric analysis.

B: Levels of MCF-7 cells compared to TamR cells as determined by densitometry. Densitometric values were normalised to β-actin levels. Statistical significance was determined using Student's t-test (n=3).
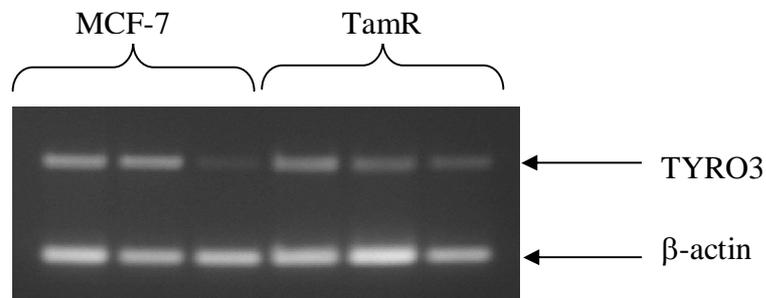
Figure 3.9 Lyn is increased in TamR cells compared to MCF-7 cells

<u>**A**</u>

<u>**B**</u>

A: Lyn was amplified in triplicate preparations of MCF-7 and TamR mRNA by PCR. β-actin was included as a reference gene allowing densitometric analysis.

B: Levels of MCF-7 cells compared to TamR cells as determined by densitometry. Densitometric values were normalised to account for β-actin levels. Statistical significance was determined using Student's t-test (n=3).

3.2.1.5: TYRO3

The final gene, TYRO3 did not show a significant increase (figure 3.10). In fact, TYRO3 levels in TamR cells were repeatedly lower or equal to those in MCF-7 cells though this difference was not statistically significant. This was despite extensive PCR optimisation using multiple primer pair and PCR conditions. Figure 3.10 is representative of results obtained with all primer pairs/ conditions. Therefore TYRO3 was not carried forward as a viable target to the next stage of analysis. This result demonstrates the importance of confirming the data obtained by affymetrix before proceeding with the gene analysis.

Summary of section

This step of PCR verification of the five selected genes led to the discounting of one of the genes: TYRO3 since its increase in TamR cells compared to MCF-7 cells could not be verified by PCR despite a promising affymetrix profile. The other four genes: EphB3, PTK2B, PTK7 and Lyn remain as viable targets and so will be investigated further.

Figure 3.10 TYRO3 levels do not change significantly in TamR cells

compared to MCF-7 cells

A

B
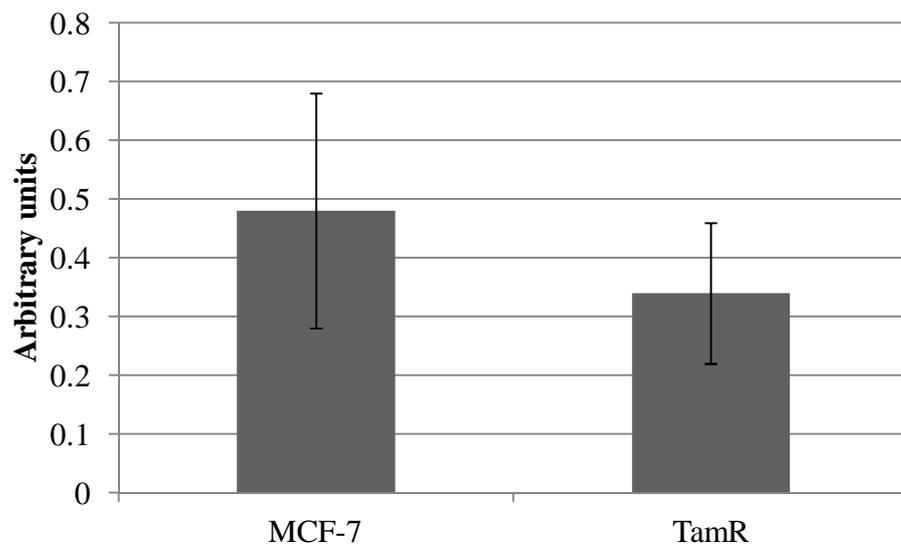
A: TYRO3 was amplified in triplicate preparations of MCF-7 and TamR
mRNA by PCR. β-actin was included as a reference gene allowing
densitometric analysis as shown in
B: Levels of MCF-7 cells compared to TamR cells as determined by
densitometry. Densitometric values were normalised to account for β-actin
levels. Statistical significance was tested using Student's t-test (n=3).

3.3 Review of available literature of four remaining gene targets

With four genes remaining as possible targets for knockdown in TamR cells:

(EphB3, PTK2B, PTK7 and Lyn), a review of available literature was

conducted to determine whether there was any prior knowledge of the role of

the genes, with particular emphasis on established roles in cancer, and also

roles in metastatic disease. The aim in conducting this review was to assist in

the selection of one of the remaining four genes for further analysis of its role

in tamoxifen resistant breast cancer in conjunction with the affymetrix data

obtained in chapter 3.1.

3.3.1 EphB3

Eph receptors (Erythropoietin-producing human hepatocellular carcinoma)

represent the largest family of PTKs with 16 members (Heroult *et al*, 2006).

Eph receptors and their corresponding ephrin (Eph family receptor interacting

proteins) are classified into A and B subfamilies (Gale *et al*, 1996). Expression

of this family has been associated with the development of many types of

human cancer, including breast, lung and prostate cancers, melanoma and

leukaemia.

EphB3 binds to both ephrin B1 and ephrin B2 and is widely expressed

in tissues. A particular role for EphB3 has been described in the development

of the vascular system, with EphB3 knockout mice displaying deficiencies in

vascular development (Wang *et al*, 1998). A number of studies have identified

increased EphB3 expression in cancer, including breast cancer.

Hafner *et al* studied the differential expression of Eph receptors in four human cancers: lung, liver, colon and kidney compared to benign tissue. EphB3 was increased 1.8 fold in lung cancer, 2.9 fold in liver cancer and 1.5 fold in colon cancer. It was down-regulated 3.4 fold in kidney cancer (Hafner *et al*, 2004).

The role of EphB3 in invasive breast cancer was investigated using MCF-10A cells to represent normal breast tissue, MCF-7 cells to represent non invasive breast cancer and MDA-MB-231 cells to represent invasive cancer. EphB3 was found to be down-regulated in both MCF-7 and MDA-MB-231 cells compared to MCF-10A cells (Fox and Kandpal, 2004). This suggested that down-regulation of EphB3 contributes to the invasiveness of breast cancer cells.

EphB3 was over-expressed in eight colon carcinoma cell lines compared to HUVEC (human umbilical vein endothelial cells). It was also present in colon cancer patient specimens suggesting that EphB3 plays a role in the progression of colon carcinoma (Liu *et al*, 2002). Conversely, other studies have indicated that EphB3 is down-regulated in colon cancer. Batlle *et al* showed that EphB3 is silenced in colorectal cancer suggesting a tumour suppressor role (Batlle *et al*, 2005). EphB3 over-expression in HT-29 human colon cancer cells led to growth inhibition *in vitro*. The cells also displayed changes consistent with MET (mesenchymal-to-epithelial transition) (Chiu et al, 2009).

3.3.2 PTK7

PTK7 is also known as CCK4 (colon cancer kinase 4) and is a pseudokinase. Pseudokinases form approximately 10% of the tyrosine kinase family and were originally predicted to be catalytically inactive since they were unable to phosphorylate ligands, however research suggests that many pseudokinases play important roles in cellular processes (Boudeau *et al*, 2006). Four splice variants of PTK7 have been identified (Jung *et al*, 2002). PTK7 is thought to be involved in the signalling mechanisms during developmental stages since mouse embryos expressing a truncated form of PTK7 die perinatally (Lu *et al*, 2004).

PTK7 expression has been shown to be increased in mouse foetal colon but not in human adult colon tissues suggesting a role in colon development but subsequent down-regulation in normal adult colon (Kobus and Fleming, 2005). It was first identified in colon cancer cell lines in 1995 (Mossie *et al*, 1995). Serial analysis of gene expression (SAGE) technology was used to identify expression of mRNA corresponding to genes in colorectal cancer. Two gene libraries of normal colonic tissues, two of primary colorectal cancers and one of metastatic colorectal cancer were compared. PTK7 was increased in the metastatic colorectal cancer tissues compared to both the normal colonic tissue and also the primary colorectal cancer (Saha, S *et al*, 2001). Since the primary cause of death from colorectal cancer is metastasis to the liver, this suggested that PTK7 is involved in cell migration and may also be a marker for worsened prognosis in colon cancer.

In melanoma cells lines, PTK7 expression progressively decreased or was lost in advanced melanomas. Of the melanoma cell lines tested, PTK7 was undetectable in 50% of lines and the cell lines that did express PTK7 were those from early melanoma lines (Easty *et al*, 1997). This suggests that loss of PTK7 expression might lead to tumour progression in melanoma.

3.3.3 PTK2B

PTK2B is also known as PYK2 (proline rich tyrosine kinase 2) and FAK2 (focal adhesion kinase 2). Along with FAK (focal adhesion kinase), PTK2B belongs to a unique subfamily of tyrosine kinases. While FAK is expressed in most tissues, PTK2B is highly expressed in the nervous system and hematopoietic cells and is therefore tissue specific. FAK and PTK2B share high sequence homology. PTK2B has been shown to be involved in cell adhesion (Litvac *et al*, 2000) and cytoskeletal reorganisation at focal adhesion sites (Avraham et al, 2000).

PTK2B expression in prostate cancer was inversely correlated with the degree of malignancy exhibited meaning it progressively disappeared in increasingly high grade adenocarcinoma until disappearing completely in anaplastic undifferentiated cancer (Stanzione *et al*, 2001). This indicated that PTK2B acts as an onco-suppressor gene in prostate, therefore loss of PTK2B activity correlates with a more aggressive phenotype in prostate cancer. Loss of PTK2B was also been linked to increased motility in prostate cells. It was proposed that PTK2B recruits Src which in turn activates ERK1/2 leading to increased motility (De Amicis *et al*, 2006).

In the T47D human breast cancer cell line, PTK2B phosphorylation by heregulin stimulation lead to the formation of a complex consisting of PTK2B, c-Abl, paxillin, p130cas and p190 RhoGAP. These proteins are associated with functions such as cytoskeleton reorganisation and cell migration. Over-expression of wild-type PTK2B in T47D cells led to an increase of approximately 65% in cell migration. In addition, over-expression of two different catalytically inactive mutants of PTK2B inhibited the T47D cell migration suggesting that formation of this complex in breast cancer cell lines contributes to cell migration (Zrihan-Licht *et al*, 2004).

3.3.4 Lyn

Lyn is a member of the Src family tyrosine kinases. It has been shown to have a broad range of functions ranging from cytoskeletal reorganisation to the induction of apoptosis. It is predominantly expressed in haematopoietic cells (Yamanashi *et al*, 1987) and is most often associated with B- and T-cell immune responses The Lyn kinase domain is activated by phosphorylation of Tyr^{396} and inhibited by phosphorylation of Tyr^{508}.

Most of the current available research centres on Lyn's role in leukaemia. In chronic myeloid leukaemia (CML), the Bcr-Abl1 gene is expressed. Lyn kinase forms signalling complexes with Bcr-Abl1 and is subsequently activated. Historically, Imatinib, the Abl tyrosine kinase inhibitor has been used to treat CML, however resistance to Imatinib is a serious problem in the clinical setting. Using siRNA to achieve a 90% knockdown of Lyn at the mRNA level in a myeloid cell line lead to apoptosis

of primary CML blast cells while leaving normal hematopoietic cells
unaffected (Ptasznik *et al*, 2004). This suggested that targeting Lyn in CML
may be advantageous, particularly in Imatinib resistant leukaemia. A dual Bcr-
Abl/Lyn tyrosine kinase inhibitor was designed called NS-187. This was
shown to be 10 times more effective than Imatinib at inhibiting tumours
positive for Bcr-Abl1 (Kimura *et al*, 2008). These results suggest that
targeting Lyn as a first line therapy may be advantageous in treating CML.

CD44 is over-expressed in colon cancer compared to normal colon
tissues and has been associated with aggressive tumour behaviour. In the colon
cancer cell line LIM 1863, CD44 was shown to trigger a signalling pathway
that lead to resistance to chemotherapy. Activation of this signalling pathway
involved the recruitment of Lyn by CD44 and subsequent activation of
PI3K/Akt which has been well documented as a mediator of cell survival
(Bates *et al*, 2001). This interaction between CD44 and Lyn and subsequent
activation of Akt has also been documented with regard to cell
motility/migration in another cell line called SW620 where a model has been
proposed where the association of CD44 and Lyn leads to the up-regulation of
cofilin and thus cell migration (Subramaniam *et al*, 2007).

In pancreatic acinar cells (secretory cells), Lyn has been shown to play
a central role in signalling by many gastrointestinal hormones and growth
factors that alter cell functions (Pace *et al*, 2006). In particular, a role for Lyn
in the activation of MAP kinase by the digestive hormone CCK
(cholecystokinin) has been suggested. In PANC-1 pancreatic cells, cell growth
and invasion was inhibited by the negative regulation of Lyn kinase by CHK

(Csk homologous kinase) phosphorylation of Tyr507 (the inhibitory tyrosine) of Lyn (Fu *et al*, 2006). Thus inhibition of Lyn kinase suppressed pancreatic cell invasion.

Lyn is over-expressed in 95% of human prostate cancers compared to normal prostate epithelial cells. Lyn removal by siRNA in prostate cancer cell lines *in vitro* led to a dramatic loss of cell proliferation (Park *et al*, 2008) suggesting a role for Lyn in the growth of prostate cancer. Inhibition of Lyn by a peptide inhibitor in hormone refractory prostate cancer (HRPC) cell lines led to inhibition of cell proliferation *in vitro. In vivo,* treatment of nude mice bearing explants of HRPC tumours with the Lyn inhibitor led to tumour regression and apoptosis (Goldenburg-Furmanov *et al*, 2004). This result was particularly significant since early prostate cancer can respond to hormone therapies, however most metastatic prostate cancers become resistant (hormone refractory) (Gopalkrishnan *et al*, 2001). This HRPC eventually results in death since there is no effective treatment.

Summary of chapter 3

Following appraisal of the roles of the four remaining genes in human disease and particularly cancer, it was clear that any of them could have been legitimately carried forward as a siRNA target in TamR cells. Each has been shown to play key roles in the development and progression of human cancer. Lyn kinase was eventually selected as a likely candidate for further investigation due to a number of factors. Firstly, when the genes that were induced in TamR cells were mapped to a phylogenetic tree of the tyrosine

kinase family tree, a distinct cluster of induced genes was seen around the Src family, with five out of the seven family members (Src, Yes-1, Lyn, Lck and Hck) induced in TamR cells compared to MCF-7 cells. TamR cells have been shown to have significantly greater levels of Src activity (as measured by phosphorylation at Y419) compared to MCF-7 cells (Morgan *et al*, 2006) that was not due to increases in Src protein or mRNA. Furthermore, treatment of TamR cells with the Src inhibitor AZD0530 led to decreased invasion suggesting that Src plays a role in the invasive behaviour seen in TamR cells (Morgan *et al*, 2006). Since Src has been shown to play such a significant role in the tamoxifen resistant phenotype, whether another family member, namely Lyn, was also associated with the tamoxifen resistant phenotype was of interest.

Also of particular relevance with regards to Lyn kinase was its over-expression in 95% of human prostate cancers compared to normal prostate epithelial cells. Breast and prostate cancers share many biological similarities such as hormone dependent growth (Risbridger *et al*, 2010) and as such both can be treated successfully with anti-hormone therapies but unfortunately both prostate and breast cancer patients treated with these therapies will eventually develop resistance resulting in the recurrence of tumour growth. Numerous studies have shown a correlation between Lyn expression and tumour cell proliferation in prostate cancer. SiRNA removal of Lyn in prostate cancer cell lines *in vitro* led to a dramatic loss of cell proliferation (Park *et al*, 2008) and also inhibition of Lyn by a peptide inhibitor in hormone refractory prostate cancer cell lines led to inhibition of cell proliferation *in vitro* (Goldenburg-

Furmanov *et al*, 2004). *In vivo,* treatment of nude mice bearing explants of hormone refractory prostate cancer tumours with the Lyn inhibitor led to tumour regression and apoptosis (Goldenburg-Furmanov *et al*, 2004). This result was particularly significant since early prostate cancer can respond to hormone therapies, however most metastatic prostate cancers become resistant (hormone refractory) (Gopalkrishnan *et al*, 2001) as is also seen in breast cancer. This hormone refractory prostate cancer eventually results in death since there is no effective treatment. Assessing the similarities between both breast and prostate cancer it is therefore conceivable that Lyn may also play a significant role in hormone resistant breast cancer.

Chapter 4: Results

Characterisation of Lyn in TamR cells

4.1 Lyn kinase protein is increased in TamR compared to MCF-7 cells.

Analysis of microarray data has provided an accurate and fast way to identify genes of interest, in our case genes that are up-regulated in tamoxifen resistant cells compared to tamoxifen responsive cells. However all of the data gathered up to this point has been at the RNA level. In order to be able to study and elucidate the effects these interactions may have within TamR cells, it is essential that these differences between TamR and MCF-7 cells are also seen at the protein level. Lyn kinase exists in two isoforms: p56 and p53 (sometimes referred to as lynA and lynB respectively). They were first identified in 1991 by Yi in mouse haematopoietic cells (Yi *et al*, 1991) and are due to alternative splicing of a 21 amino acid region located in the unique region of Lyn. Figure 4.1 shows the 63 base pair difference between the p56 and p53 isoforms. It also shows the location of the two Lyn phosphorylation sites: Phosphorylation at Y396 activates Lyn kinase activity whereas phosphorylation at Y507 represses Lyn kinase activity.

Figure 4.1: Schematic of Lyn kinase domains showing differences between p56 and p53 and also the location of Lyn phosphorylation sites.
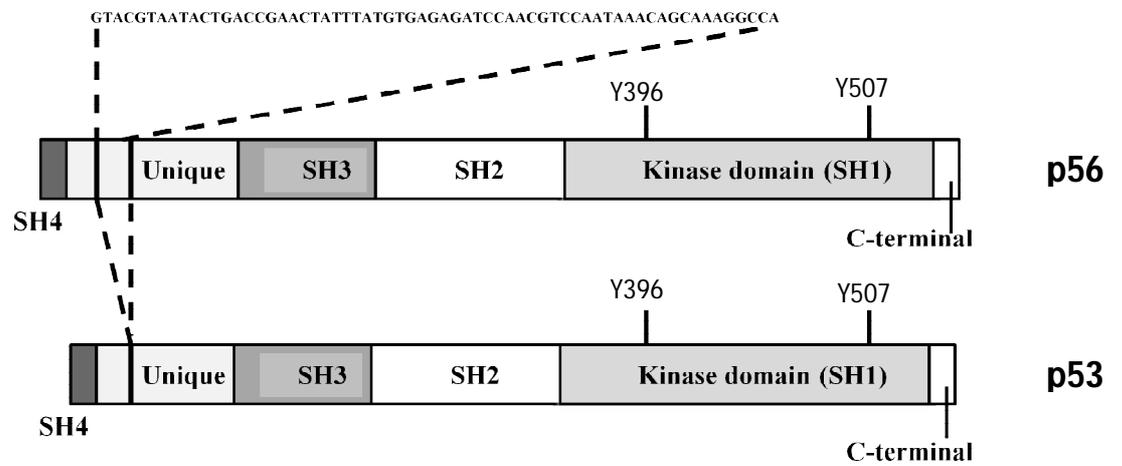
4.1.1: Total Lyn kinase protein is increased in TamR cells

Protein was lysed from both TamR cells and MCF-7 cells and probed for un-phosphorylated (total) Lyn protein using SDS-PAGE and Western blotting in figure 4.2.A Un-phosphorylated Lyn was significantly increased two-fold in TamR compared to MCF-7 cells. The double band obtained by the total Lyn antibody was due to recognition of both the p53 and p56 isoforms of Lyn.

4.1.2: Activated Lyn kinase protein is increased in TamR cells

Protein was lysed from both TamR cells and MCF-7 cells and probed for activated Lyn (Y396) using SDS-PAGE and Western blotting. Y396 is located in the kinase domain of Lyn, SH1. Lyn (Y396) was significantly increased in TamR cells compared to MCF-7. Again, both isoforms of Lyn were recognised (figure 4.2B).

A significant increase in both un-phosphorylated Lyn and activated Lyn (Y396) in TamR cells echoes the data previously obtained by the analysis of microarray data and also that seen following mRNA extraction and subsequent PCR. Lyn therefore remains a viable target for further study in tamoxifen resistance.

Figure 4.2. Comparison of total Lyn and activated Lyn (Y396) levels in TamR and MCF-7 cells.

A
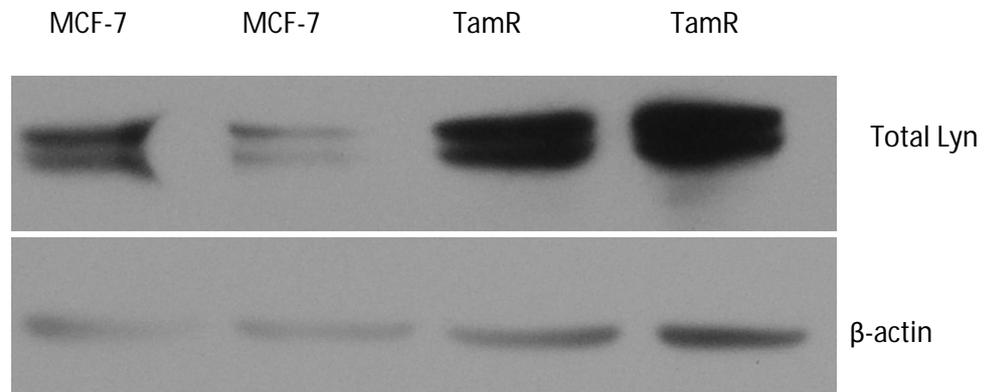
B
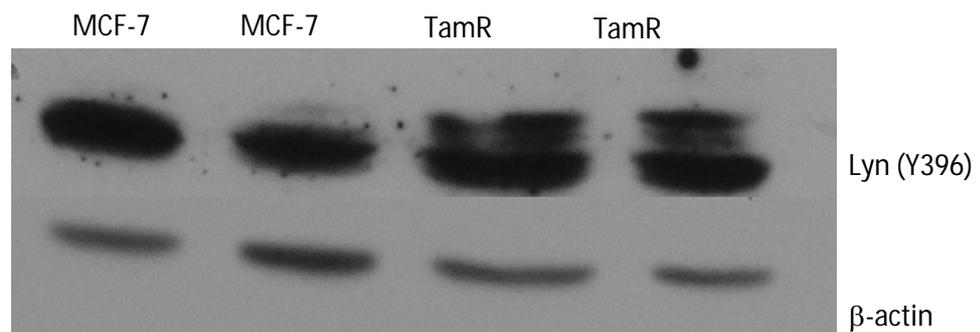
A: Duplicate MCF-7 and TamR protein lysates run on SDS-PAGE and probed for total-Lyn and actin to show equal loading.

B: MCF-7 and TamR cells lysed in duplicate run on SDS-PAGE and probed for phosphorylated Lyn (Y396) and actin to show equal loading.

4.2: Optimisation of a protocol for siRNA knockdown of Lyn.

Following the identification of Lyn as a possible gene of interest in tamoxifen resistant breast cancer cells, further research into its role in TamR cells was required. In order to begin to explore the role that Lyn was playing in TamR cells, a strategy to remove Lyn from TamR cells using siRNA and study the effects was devised. In order to achieve the highest siRNA knockdown of Lyn possible, the manufacturer's protocol for transfection of cells was optimised specifically for Lyn removal. A number of different conditions were optimised to specifically target Lyn including choice of transfection lipid and harvest time for RNA.

4.2.1 Comparison of lipids for siRNA transfection.

In order to deliver siRNA into a cell, a cationic (positively charged) transfection lipid is required. The cationic lipid consists of a positively charged head group and two hydrocarbon chains (Liu *et al*, 2003). It is this positive charge that allows the formation of a siRNA/lipid complex. The lipid facilitates the uptake of siRNA into the cell in two ways. The positive charge of the complex interacts with the phosphate backbone of the nucleic acid, leading to DNA condensation and also allows the siRNA to interact with the negatively charged cell membrane and enter the cell by endocytosis (Rao, 2010).

A number of problems exist with using transfection lipid delivery of the siRNA to the cell, most notably cytotoxicity. The transfection lipids

selected were however shown by the manufacturers to be compatible with the

parental MCF-7 cells from which the TamR cells are derived.

Four DharmaFECT transfection reagents manufactured by Dharmacon were

investigated. The remaining two reagents were manufactured by Invitrogen.

These were Lipofectamine 2000 transfection reagent which had been used to

successfully transfect TamR cells at the Tenovus Centre for Cancer Research

for a number of years, however had not been formulated for use with siRNA

and Lipofectamine RNAiMAX, which had been designed specifically for use

with siRNA and claimed to lead to more successful gene knockdown with less

off target effects than Lipofectamine 2000.

In order to compare the Lyn kinase knockdown achieved using the

different transfection reagents, TamR cells were transfected with siRNA

targeting Lyn (siLyn) for 48 hours using each transfection reagent, in

triplicate, with cells left untreated as a control. The mRNA was then extracted,

quantified, reverse transcribed and a PCR for Lyn performed.

Figure 4.2 shows the knockdown of Lyn by siRNA using the six

different transfection reagents discussed above. The levels of Lyn knockdown

were expressed as % of Lyn mRNA compared to the untreated control cells.

A significant reduction of between 68% (Lipofectamine 2000) and 91%

(Lipofectamine RNAiMAX) Lyn mRNA was seen compared to the untreated

control. The four DharmaFECT reagents led to a reduction of between 73%

and 89% of Lyn kinase mRNA with DharmaFECT 2 giving the greatest

reduction. All six transfection reagents produced a statistically significant

reduction of Lyn kinase, with Lipofectamine RNAiMAX and DharmaFECT 2

treatment both resulting in a knockdown that was significantly lower than the

established transfection lipid Lipofectamine 2000. Since cytotoxicity is a

significant problem when using lipids to transfect oligonucleotides into cells

and it was noted prior to mRNA extraction after 48 hours that cell apoptosis

was higher in cells treated with DharmaFECT 2 compared to Lipofectamine

RNAiMAX treated cells. This resulted in much lower yields of mRNA,

therefore the Lipofectamine RNAiMAX reagent was selected and used for all

subsequent transfections.

Figure 4.2 Comparison of Lyn siRNA knockdown achieved using

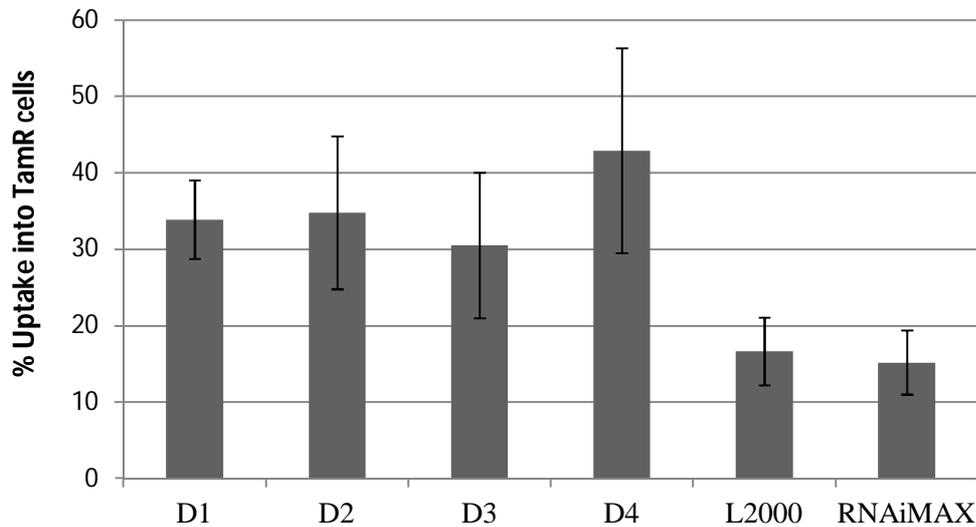**different transfection reagents**
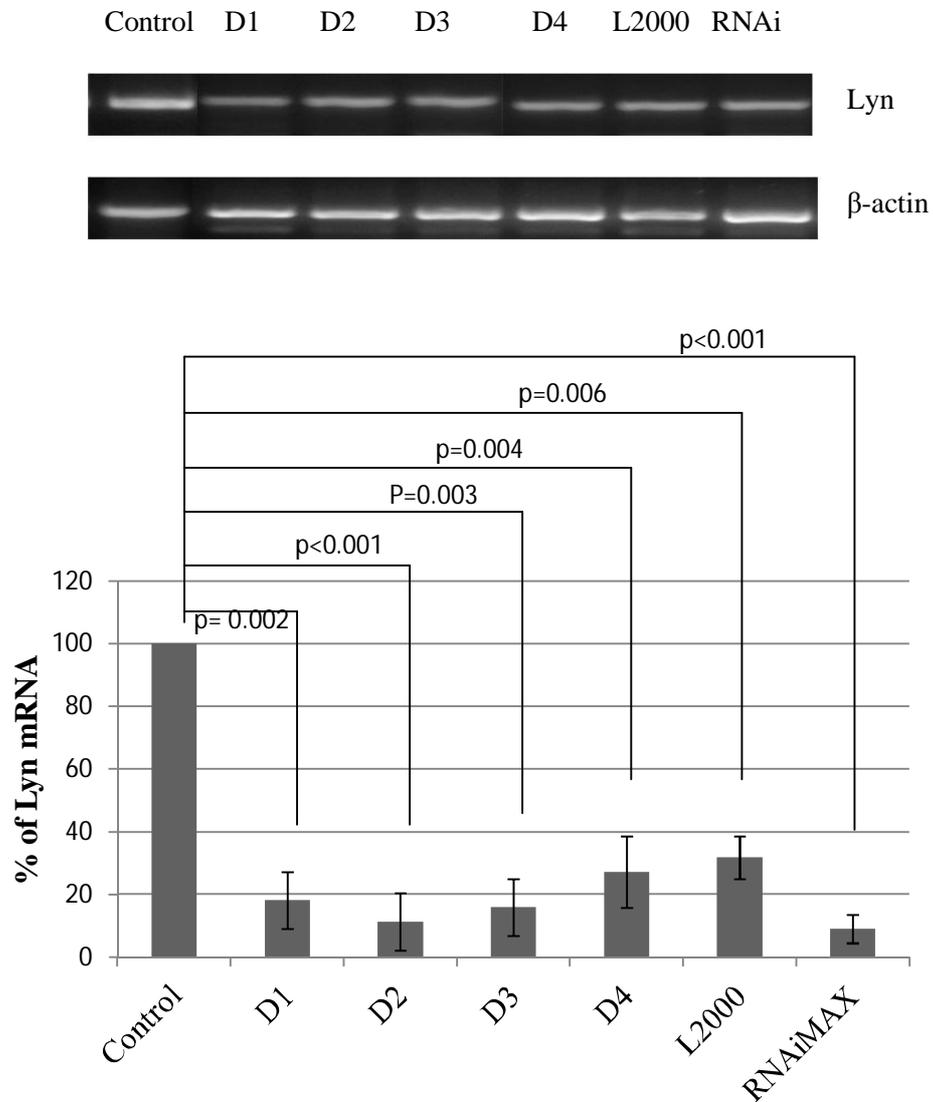
Lyn kinase mRNA expressed as % of Lyn mRNA following 48hr Lyn siRNA treatment using various transfection lipids. Where control=untreated TamR cells, D1-D4=Dharmafect lipid 1-Dharmafect lipid 4, L2000=Lipofectamine 2000 and RNAiMAX= Lipofectamine RNAiMAX. Statistical significance was determined using a one-way Anova test (n=3).

4.2.2 Does siRNA uptake affect gene knockdown in TamR cells?

To investigate whether the differences between lipids seen in Figure 4.1 were due to differences in uptake of siRNA, TamR cells were treated for six hours using each different transfection lipid and siGlo Lamin A/C siRNA, an siRNA targeting the filament protein lamin, a component of cell nuclei. This was tagged with a red fluorescent label to allow visualisation of lamin siRNA uptake into the cell using a fluorescent microscope. Transfections were performed at 37°C in dark conditions in order to maintain fluorescence. Cells were fixed using 3.7% formaldehyde (v/v) in PBS and the nuclei stained with DAPI as described in chapter 2. Data were expressed as % uptake calculated as number of cells positive for siGlo uptake/ total number of DAPI stained cells. Figure 4.3 demonstrates that the four DharmaFECT transfection lipids showed significantly increased uptake of siRNA compared to Invitrogen's Lipofectamine RNAiMAX and Lipofectamine 2000. This was interesting because in section 4.1 it was demonstrated that RNAiMAX and DharmaFECT 2 yielded the highest levels of Lyn knockdown in TamR cells. This result suggests that the uptake of siGlo (siRNA) into TamR cells does not affect the ability for Lyn mRNA knockdown.

Since 6 hour uptake of siRNA into cells does not affect subsequent mRNA knockdown, the decision to use Invitrogen's Lipofectamine RNAiMAX in subsequent experiments was based on figure 4.3 where RNAiMAX transfection led to the greatest removal of Lyn mRNA whilst causing the least amount of cell apoptosis.

Figure 4.3 Uptake of siGlo into TamR cells following treatment with transfection reagents


SiRNA uptake following 6hr siGlo fluorescent siRNA treatment using different transfection lipids. TamR cells were treated with siGlo: a red tagged siRNA targeting Lamin for 6 hours at 37°C in dark conditions Cells were fixed using 3.7% (v/v) formaldehyde in PBS, DAPI stained and visualised under a fluorescent microscope. Data was expressed as % uptake calculated as number of cells positive for siGlo/ total DAPI stained cells Where D1-D4=Dharmafect lipid 1- 4, L2000=Lipofectamine 2000 and RNAiMAX= Lipofectamine RNAiMAX.

4.2.3 Optimal harvest time for siRNA transfection

Once RNAiMAX had been identified as the most effective transfection reagent for knockdown of Lyn kinase, the optimal time to harvest the cells to maximise gene knockdown was investigated. A time-course between 24 and 120 hours was investigated, then mRNA was extracted and Lyn mRNA levels were measured by PCR. Future experiments may require a varying transfection period in order to investigate the role of Lyn in TamR cells. This provided a further reason for investigating siRNA transfection and subsequent knockdown over a period time.

Figure 4.4 demonstrates that a harvest time of between 48 and 72 hours produces the highest gene knockdown. It is encouraging that a knockdown of Lyn kinase mRNA is seen at all time-points compared to the controls. At 24 hours there is a 50% reduction in Lyn mRNA, increasing to 85% and 90% at 72 hours and 48 hours respectively. A 75% reduction is apparent at 96 hours post-transfection and after 120 hours, a 60% reduction in Lyn mRNA is still achieved. These results mean that siRNA could confidently be used to study endpoints of up to 5 days.

Figure 4.4 Lyn siRNA treatment time course
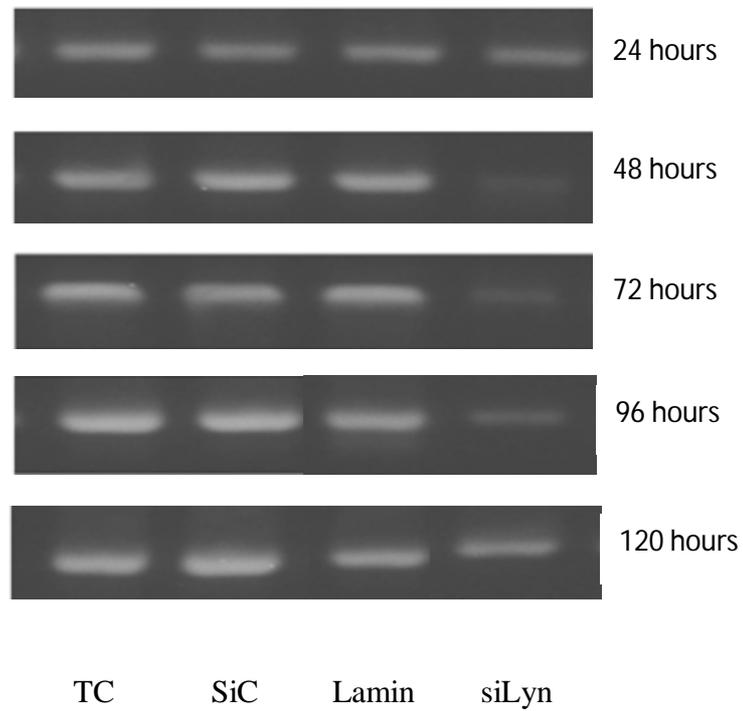
Cells were treated with siRNA targeting Lyn kinase for between 24-120hrs and then Lyn mRNA levels were assessed by PCR. TC=lipid control, SiC=non-targeting siRNA control, Lamin=siRNA targeting housekeeping gene lamin and siLyn=siRNA targeting Lyn kinase.

4.2.4 Removal of Lyn protein using siRNA.

Following optimisation of Lyn mRNA knockdown using siRNA, it was investigated whether a similar reduction in Lyn protein was achievable using RNAiMAX lipid in TamR cells. Cells were transfected with a complex of RNAiMAX and siRNA targeting Lyn kinase for 72 hours. The cells were then lysed for protein and Lyn kinase protein levels were measured using SDS-PAGE and Western blotting.

Figure 4.5 shows a significant 70% reduction in un-phosphorylated (total) Lyn protein following 72 hour treatment with siRNA compared to the controls. This result demonstrated that siRNA can be used to successfully knockdown Lyn protein in TamR cells.

Figure 4.6 demonstrated that a 95% reduction in active Lyn kinase protein (Y396) was achieved using RNAiMAX lipid and a harvest time of 72 hours, whilst there was no effect on either the transfection control or the siRNA control. This reduction was statistically significant compared to the controls.

4.2.5 Is Lyn siRNA specific?

Lyn is a member of the Src family. The family consists of eight members which all share significant homology both in sequence and structure, especially in the region of the active site (Boggon and Eck, 2004). Since accurate removal of Lyn kinase was critical for the completion of further experiments into the role Lyn plays in tamoxifen resistance, it was important

that a robust method of Lyn kinase removal was established. It was therefore

necessary to ascertain that Lyn kinase could be targeted specifically without

affecting the other family members so that observed changes or effects

following Lyn knockdown could be attributed to Lyn alone.

In order to investigate Lyn siRNA specificity, cells were treated for 48

hours with siRNA targeting either Lyn or Src kinase. The levels of the Src

family members Fyn and Yes-1 were assessed by PCR following mRNA

extraction and are shown in Figure 4.7. Treatment with siRNA targeting Lyn

or Src kinase only led to a reduction in Lyn and Src respectively, it did not

lead to a reduction in either of the other two Src family members. Thus despite

family members showing a great deal of similarity, siRNA provides an

effective, robust but also specific down regulation of Lyn kinase.

Figure 4.5 Knockdown of total Lyn protein in TamR cells
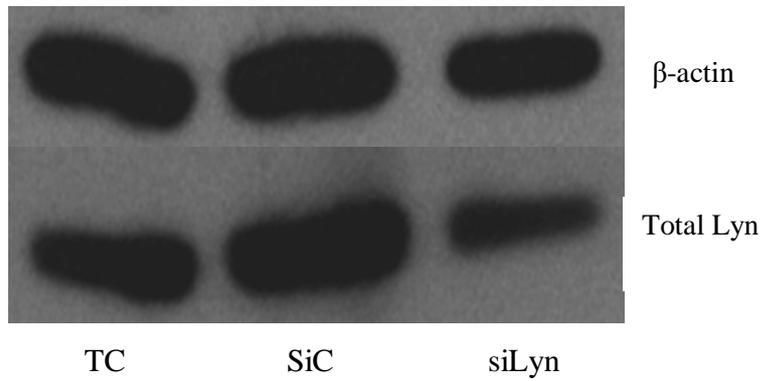
TC   SiC   siLyn

Cells were treated with siRNA targeting Lyn kinase for 72hrs, protein harvested and SDS-PAGE and Western blotting using total Lyn antibody and β-actin as a loading control.   TC=lipid control, SiC=non-targeting transfection control, siLyn=siRNA targeting Lyn kinase.

Figure 4.6 Knockdown of active Lyn protein (Y396) in TamR cells
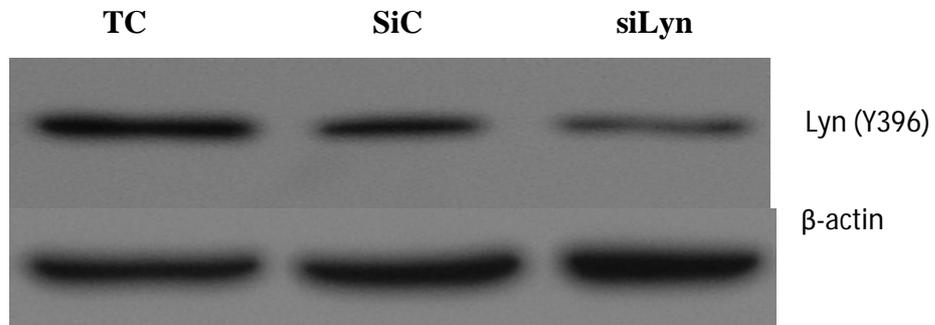
Cells were treated with siRNA targeting Lyn kinase for 72hrs, protein harvested and SDS-PAGE and Western blotting using p-Lyn (Y396) antibody and β-actin as a loading control. TC=lipid control, SiC=non-targeting transfection control, siLyn=siRNA targeting Lyn kinase.

Figure 4.7 Is Lyn siRNA specific?
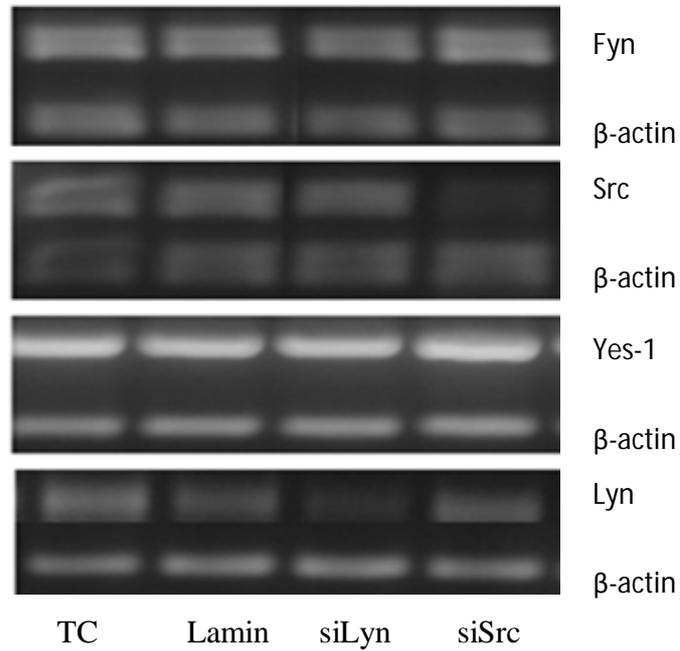
TamR cells were treated with siRNA targeting Lyn (siLyn), Src (siSrc), or Lamin for 48hrs. mRNA was amplified using primers targeting Fyn, Src, Yes-1 and Lyn kinase. TC=lipid control, SiC=non-targeting transfection control.

4.3 Effect of siRNA knockdown of Lyn and Src in TamR cells.

This results chapter has so far focussed on identifying a viable tyrosine kinase gene target for siRNA knockdown in TamR cells. It has been established previously that Lyn kinase is a viable target for further study, following array analysis (chapter 3) and subsequent confirmation of this at the mRNA level. The two-fold increase in both total and activated Lyn protein in TamR cells compared to MCF-7 cells also confirms that Lyn is a good candidate for further study. The optimisation of a protocol for the specific targeting and knockdown of Lyn at both the mRNA and the protein level by siRNA demonstrated that siRNA is both an effective and accurate tool for Lyn down-regulation in TamR cells.

Since Lyn kinase can now be accurately removed from TamR cells it was possible to begin to study the effects of removing Lyn from TamR cells on clinically important endpoints including growth, apoptosis and invasion. In this section siRNA targeting both Lyn and Src were used. This was in order to identify if there are any significant advantages to targeting Lyn instead of Src in tamoxifen resistance, especially in view of the roles Src is known to play in tamoxifen resistance (Chapter 1). Despite demonstrating that 72 hour siRNA treatment provided the greatest knockdown of Lyn mRNA, an extra day of siRNA treatment was utilised in order to make sure the full effects of the knockdown could be measured since these series of experiments were looking at functional endpoints. As established in figure 4.4, 96 hour treatment still provided a significant 75% knockdown.

4.4 Cell growth following siRNA knockdown

The transition of tamoxifen responsive MCF-7 cells to tamoxifen resistant

TamR cells was accompanied by a 200% increase in growth rate (Knowlden *et*

al, 2003). Increased growth rate was therefore an important feature in the

development of tamoxifen resistance. In order to begin to identify a role for

Lyn kinase in the growth of TamR cells, a number of growth endpoints were

measured following Lyn knockdown including the proliferation marker MIB-

1, growth by Coulter counter and DNA synthesis. In addition to removing Lyn

using siRNA, Src was also removed since its role in TamR cells has been well

characterised at the Tenovus Centre for Cancer Research. Src was also

included in order to ascertain if Lyn might prove a more worthy gene target

than Src.

4.4.1 Assessment of TamR proliferation using MIB-1.

The proliferation marker MIB-1 was used in order to see whether removal of

Lyn kinase affected the proliferation of TamR cells. The MIB-1 antibody

recognises the Ki-67 protein which is only present in proliferating cells. Tam-

R cells were treated with siRNA targeting Lyn or Src for 4 days. They were

fixed with 3.7% (v/v) formaldehyde in PBS, stained for the proliferation

marker MIB-1 and counter stained with methyl green as seen in Figure 4.8A.

MIB-1 positive cells were counted and expressed as % of cells positive for

MIB-1 (Figure 4.8B).

A significant decrease in MIB-1 was observed following removal of

both Lyn kinase ($p=0.004$) and Src kinase ($p=0.009$) compared to the

transfection control. A slight increase in the siRNA control (SiC) occurred, however this was not statistically significant. This decrease in proliferation following Lyn knockdown suggests that it plays a role in the growth of TamR cells.

Figure 4.8 Assessment of TamR proliferation using MIB-1

<u>**A**</u>

MCF-7   TamR TC   TamR SiC

TamR SiLyn   TamR SiSrc

<u>**B**</u>
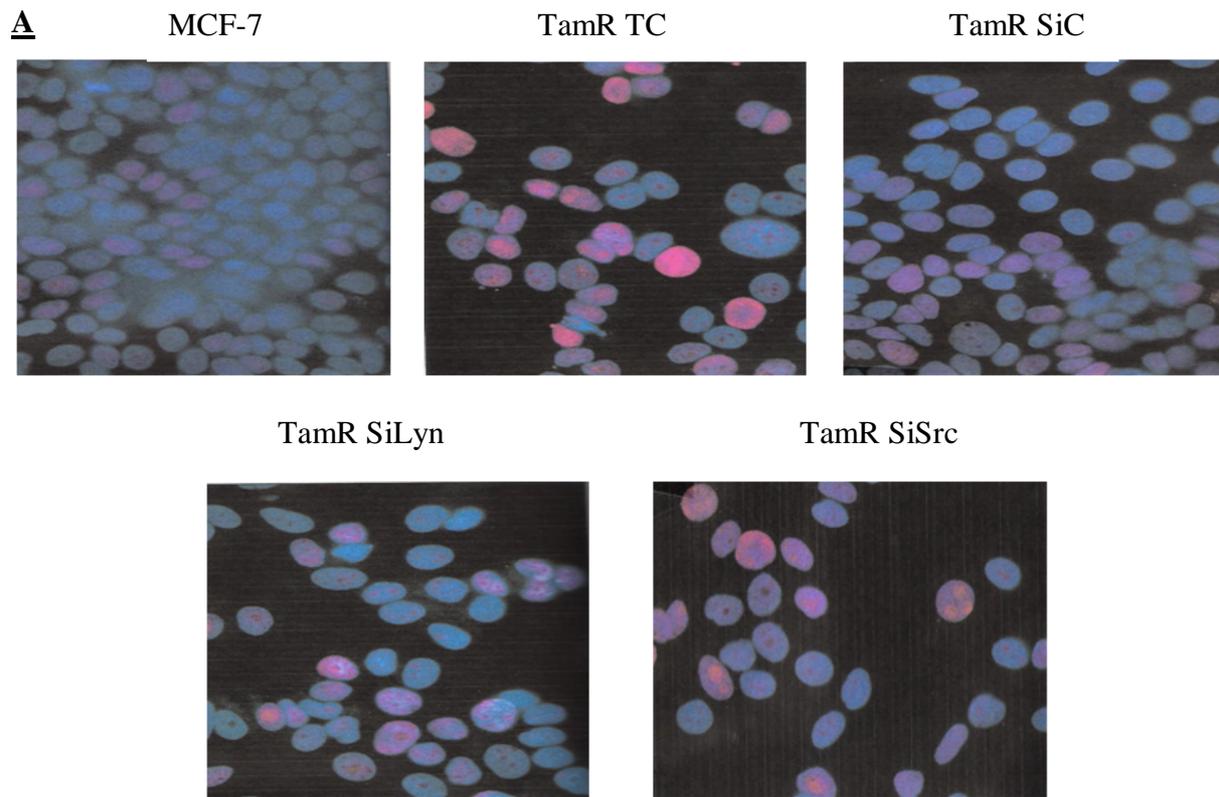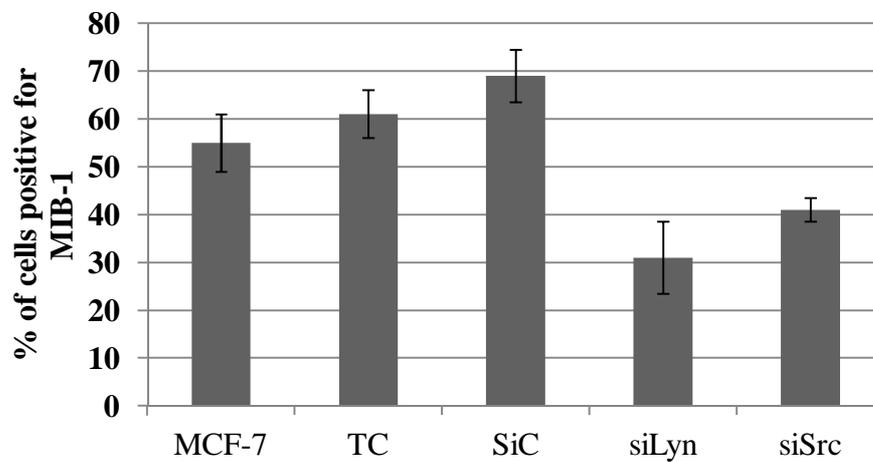
MCF-7 and Tam-R cells were treated with siRNA targeting Lyn or Src for 4 days, fixed and stained for the proliferation marker MIB-1 and GAR594, and 1/10 DAPI and positive cells counted and expressed as % of cells positive for MIB-1. TC=lipid control, Lamin=siRNA targeting housekeeping gene Lamin, siLyn=siRNA targeting Lyn kinase and siSrc=siRNA targeting Src kinase. Error bars represent standard deviation. Statistical significance was determined using a one-way Anova test (n=4).

4.4.2 Cell growth measured by Coulter counter

Next the effect of Lyn and Src siRNA treatment on cell growth was

investigated by Coulter counter. TamR cells were treated with siRNA for 4

days then trypsinised and counted by Coulter counter and expressed as number

of cells per well in Figure 4.9.

Both Lyn and Src kinase removal led to a significant decrease in cell

growth with a decrease of 39% (p=0.002) and 31% (p=0.0035) respectively

compared to the transfection control. Transfection of the cells with an siRNA

targeting the housekeeping gene lamin for the same period did not result in a

change in cell growth by Coulter counter. Whilst removal of Lyn kinase

caused a greater decrease in cell growth than removal of Src kinase, this

difference was not significant suggesting that Lyn and Src kinase may be

inhibiting growth via the same mechanism.

4.4.3 Effect of Lyn and Src on DNA synthesis

Cell cycle stage was assessed by FACS analysis following Lyn kinase and Src

kinase removal. The percentage of cells in S (synthesis) phase was taken as a

measure of cell growth since it is during S phase in the cell cycle that DNA

replication occurs. TamR cells were treated with siRNA for 4 days, then the

number of cells in S phase at the time of harvest was measured using the

cycleTEST Plus kit (BD Biosciences) by FACS.

Removal of Lyn and Src in TamR cells (Figure 4.10) led to a significant

decrease of 52% (p=0.02) and 50% (p=0.02) respectively in DNA synthesis

whereas removal of Lamin as a control did not affect DNA synthesis. This

result suggests that Lyn and Src do play a role in DNA replication in TamR

cells.

Figure 4.9 Growth of TamR cells
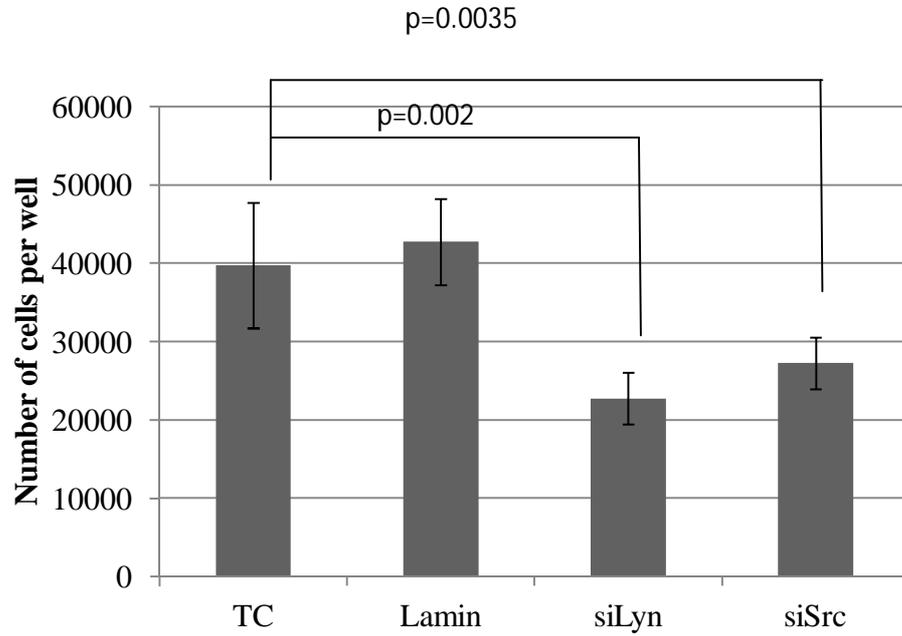
Tam-R cells were treated with siRNA for 4 days then trypsinised and counted by Coulter counter and expressed as number of cells per well. TC=lipid control, Lamin=siRNA targeting housekeeping gene Lamin, siLyn=siRNA targeting Lyn kinase and siSrc=siRNA targeting Src kinase Statistical significance was determined using Student's t-test (n=4).

Figure 4.10 Number of cells in S phase following siRNA treatment

Tam-R cells were treated with siRNA for 4 days, then the number of cells in S phase at the time of harvest was measured using 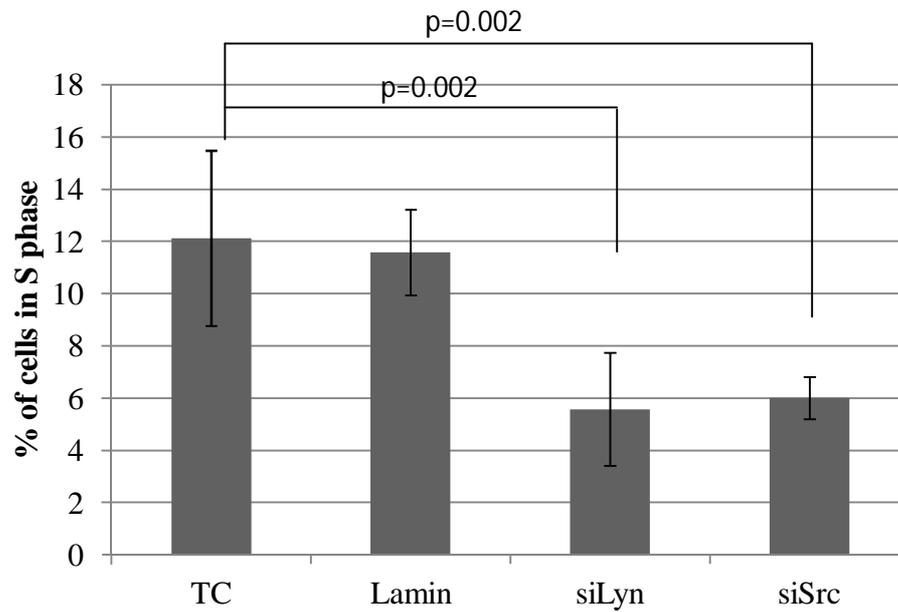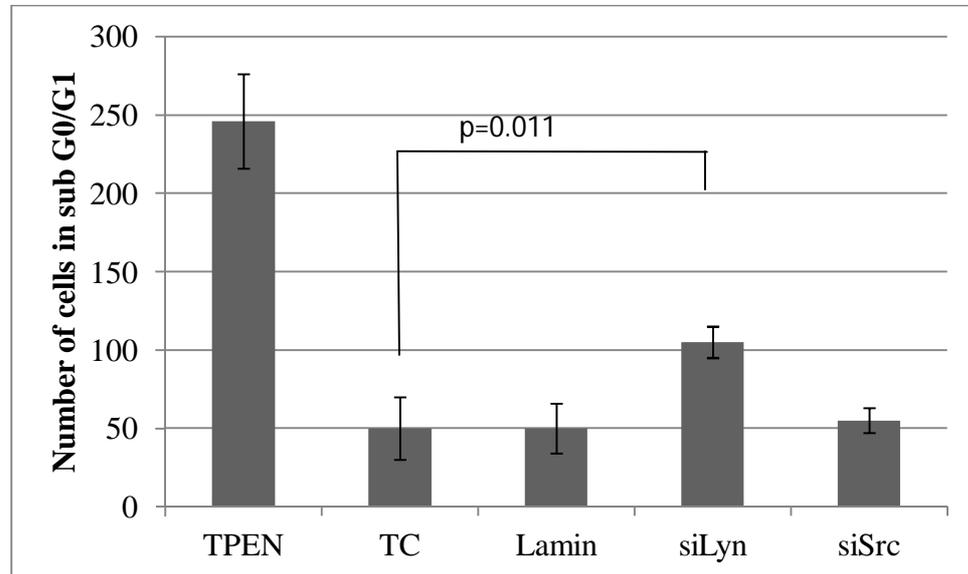the cycleTEST Plus kit (BD Biosciences) by FACS. Output data was then analysed using the software Winmidi and cyclered. TC=lipid control, Lamin=siRNA targeting housekeeping gene Lamin, siLyn=siRNA targeting Lyn kinase and siSrc=siRNA targeting Src kinase (n=4).

4.5 The effect of Lyn and Src knockdown on apoptosis in TamR cells

Apoptosis is a type of genetically controlled programmed cell death. Defects in the apoptotic pathways have been associated with many types of cancer (Thompson, 1995). These defects can include loss of gene function or mutations in the genes that control apoptosis. The effect of Lyn and Src removal on apoptosis of TamR cells was therefore an important factor for investigation. Apoptosis was assessed by a number of means including measuring the number of cells in sub G0, and mitochondrial membrane potential.

4.5.1 Effect of Lyn and Src on cells in sub G0/G1

Cells enter the sub G0/G1 phase of the cell cycle when they are not in a position to progress beyond G1 phase into S phase to divide, for example due to a lack of growth factors or nutrients. This phase is characterised by a loss of DNA in cells. Sub G0/G1 measured by FACS analysis identifies cells with a late stage cell death phenotype. This measurement was used to determine identify TamR cells undergoing apoptosis. The TamR cells were treated with siRNA for 4 days, then the number of cells in sub G0/G1 phase at the time of harvest was measured using the cycleTEST Plus kit (BD Biosciences) by FACS in Figure 4.11 TPEN was included as a positive control for apoptosis.

Figure 4.11 Effect of Lyn and Src on cells in sub G0/G1

Tam-R cells were treated with siRNA for 4 days, then the number of cells in sub G0/G1 phase at the time of harvest was measured using the cycleTEST Plus kit (BD Biosciences) by FACS. Output data was then analysed using the software Winmidi and cyclered and expressed as % of transfection control. TC=lipid control, Lamin =siRNA to Lamin, siLyn=siRNA targeting Lyn kinase and siSrc=siRNA targeting Src kinase (n=4).

No statistical difference (as measured using Student's t-test) in number of cells in sub G0/G1 was observed following removal of Src or the control gene lamin. Removal of Lyn led to a significant 78% increase in cells in sub G0/G1 (p=0.011) suggesting that cells that had Lyn removed were unable to progress further into the cell cycle and undergo cell division.

4.5.2 Mitochondrial membrane potential following Lyn knockdown

Mitochondria are the primary source of energy in human cells. They produce ATP via oxidative phosphorylation and the citric acid cycle. Mitochondrial membrane potential was therefore used as a measure of cellular viability, since it was indicative of hydrogen ions crossing the cell membrane during oxidative phosphorylation. Mitotracker is a mitochondrial stain which localises to mitochondria in live cells and its accumulation is dependent upon mitochondrial membrane potential (as measured by intensity of fluorescent staining). Live cells stain highly with mitotracker (intense red) whereas dead/dying cells will display lower intensity of staining. Cells were treated with siRNA targeting Lyn or Src for 72 hours, incubated with the mitochondrial dye mitotracker (592nm) for 30 minutes, fixed with 3.7% (v/v) formaldehyde in PBS. The nuclei were stained using DAPI and Lyn was stained using pLyn (Y396) and Alexafluor goat anti-rabbit 488 secondary antibody. The cells were then visualised using a Leica microscope.

Following Lyn and Src removal a decrease in mitotracker intensity was observed compared to the controls (Figure 4.12). A greater decrease in

mitochondrial membrane potential was observed following Lyn removal

compared to Src. This result indicated that both Lyn and Src kinase play a role

in cell death.

Figure 4.12 Mitochondrial membrane potential following Lyn knockdown
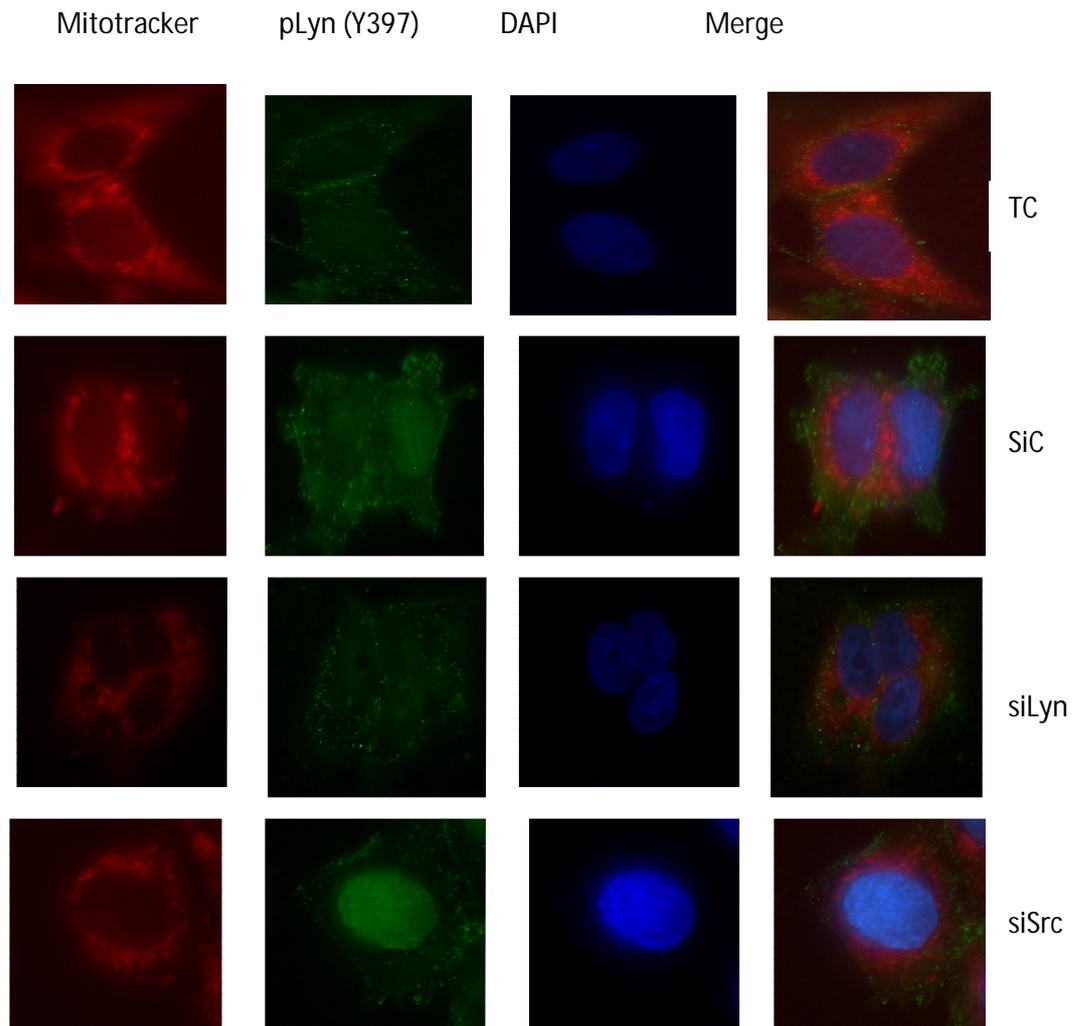
Cells were treated with siRNA targeting Lyn or Src for 4 days, incubated with the mitochondrial dye mitotracker (592nm) for 30 minutes, fixed and stained using pLyn (Y396) and Alexafluor GAR 488 and 1/10 DAPI then visualised using a Leica microscope.

following Lyn removal compared to Src suggests that Lyn may play a greater

role in apoptosis in TamR cells than Src.

4.6 Cell migration following siRNA knockdown.

Migration levels following removal of Lyn kinase and Src kinase were

investigated by measuring the migration of cells across a modified Boyden

chamber coated in fibronectin. Src had been shown previously to play an

important role in cell migration, but Lyn kinase had not been investigated.

TamR cells were treated with siRNA for 72 hours or SU6656 for 24 hours

then seeded onto a porous membrane coated with fibronectin and the cells

were allowed to migrate through the porous membrane for 24 hours. The cells

that had migrated through the membrane were stained using crystal violet and

counted using a X20 microscope lens. The combined period of siRNA

transfection and migration through the porous membrane was 96 hours which

was longer than previous experiments. Since Lyn knockdown of 75% was

detected 96 hours post-transfection , this extended experimental time was not a

concern. Representative pictures showing the fluorescent stained migratory

cells are shown in Figure 4.13.

Removal of Src kinase led to a significant reduction of over 50% in the

migratory capacity of TamR cells (p=0.003). Lyn kinase removal gave a 65%

reduction in migratory capacity (p=0.001); however the difference between

Lyn kinase and Src kinase was not statistically significant. Cells treated with

SU6656 showed a 61% decrease in migration (p=0.001). The low number of

migratory cells seen, even under control conditions can be explained in part by

the effect of siRNA transfection reagents on TamR cells. Following siRNA

transfection, a lag period of approximately 2 days is seen before the cells

begin to grow again.

4.7 The effect of siRNA knockdown on the invasive capacity of the cells.

As with migration, the important role of Src on the invasive capacity of TamR

cells has been well documented but a similar role for Lyn has not been

investigated. The invasive capacity of cells was investigated by measuring the

invasion of cells across a modified Boyden chamber coated in Matrigel (an

artificial basement membrane). TamR cells were treated with siRNA for 72

hours to ensure a sufficiently high level of gene silencing or SU6656 for 24

hours then seeded onto a porous membrane coated with Matrigel. The cells

were allowed to invade through the porous membrane for 72 hours then the

cells that had invaded through the membrane were fixed using 3.7% (v/v)

formaldehyde in PBS. The nuclei were stained using DAPI and counted at

X20 on a fluorescent microscope at 320nm. These conditions meant that the

total length of the experiment was 6 days. This period extends beyond those

investigated previously however 5 days post-transfection, an siRNA

knockdown of 60% is still observed, therefore it was felt that the 24 hour

extension was justified to allow the cells sufficient time to migrate.

Representative pictures of a DAPI stained control and a siLyn treated well are

shown in Figure 4.14. Treatment with the non-targeting siRNA control SiC led

to a significant decrease in the invasive capacity of the cells compared to the

TC. This showed that an off-target effect from siRNA transfection may have

occurred. A significant decrease of over 90% in the invasive capacity was observed following Lyn kinase ($p < 0.001$) and Src kinase ($p < 0.001$) removal and also treatment with SU6656 ($p < 0.001$) compared to the transfection control (TC). Invasion of Lyn siRNA treated, Src siRNA treated, and SU6656 treated cells were still significantly decreased compared to the SiC ($p < 0.001$). This result demonstrates the importance of running several controls when using siRNA. Very similar levels of inhibition of invasion were observed following Lyn and Src siRNA treatment and also SU6656 treatment, suggesting that the Src family members may all play a similar role in invasion within TamR cells.

Figure 4.13 Cell migration following siRNA knockdown

Tam-R cells were treated with siRNA for 4 days or SU6656 for 24 hours then seeded onto a porous membrane coated with fibronectin. The cells were allowed to migrate through the porous membrane for 24 hours then the cells that had migrated through the membrane were stained using crystal violet and counted using a X20 microscope lens. SiC= a control non-targeting siRNA, siLyn= siRNA to Lyn kinase, siSrc= siRNA to Src kinase and SU= Src inhibitor SU6656 (5µM) (n=4).

Figure 4.14 Cell invasion following siRNA knockdown
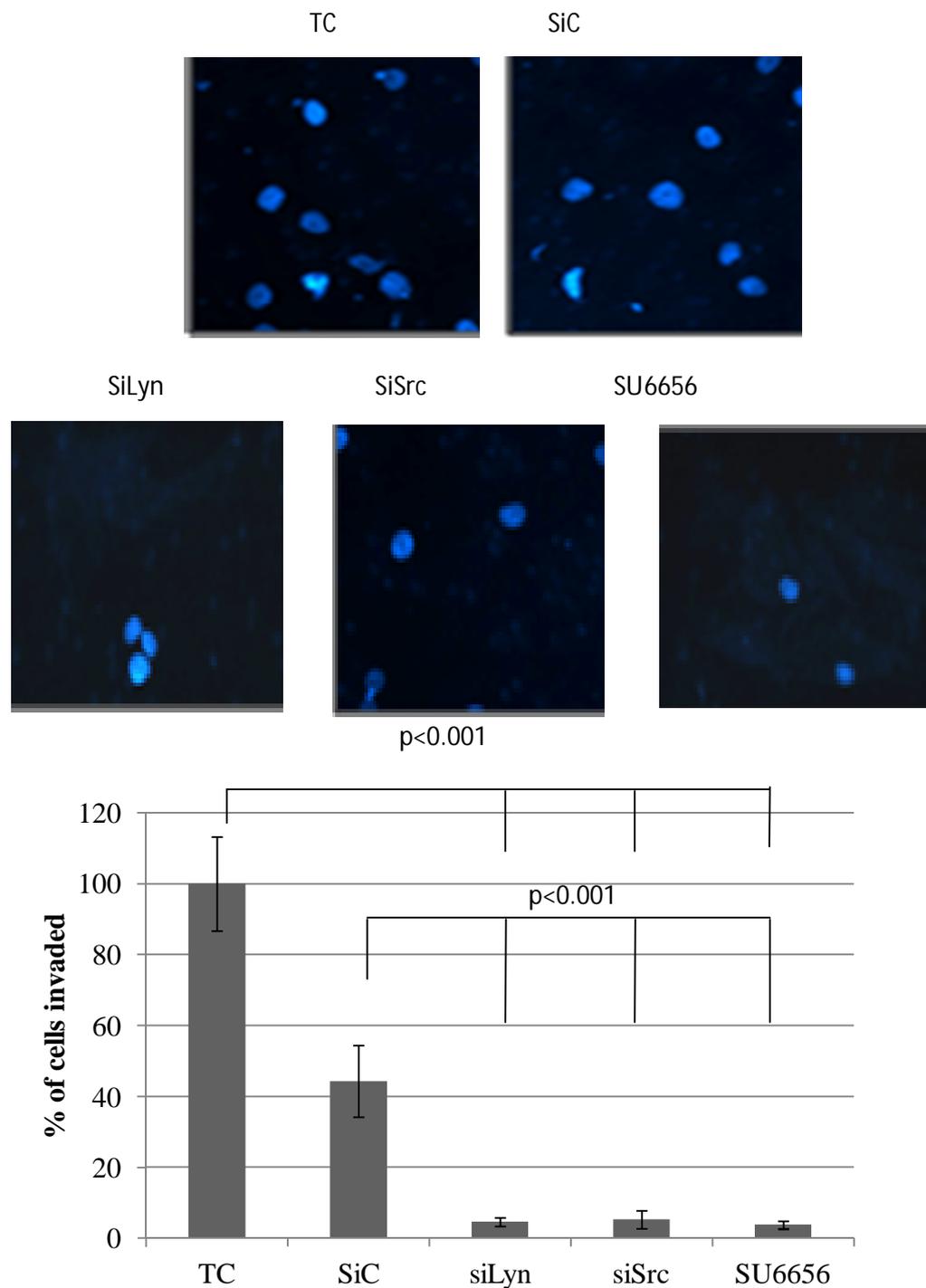
Tam-R cells were treated with siRNA for 4 days or SU6656 for 24 hours then seeded onto a porous membrane coated with matrigel. The cells were allowed to invade through the porous membrane for 72 hours then invaded cells were fixed, the nuclei stained using DAPI and counted at X20 on a fluorescent microscope at 320nm. TC= transfection control, SiC= a control non-targeting siRNA, siLyn= siRNA to Lyn, siSrc= siRNA to Src and SU= Src inhibitor SU6656 (5µM) (n=4).

Summary of Chapter

In this chapter it was demonstrated that removal of Lyn kinase and Src kinase by siRNA leads to significant decreases in TamR cell growth, migration and invasion, and leads to an increase in cell apoptosis. Significantly removal of Lyn kinase proved to have a greater anti-proliferative effect on TamR cells than Src kinase removal. Also observed when Lyn kinase was removed was a significant increase in apoptosis compared to controls whilst apoptosis following Src removal remained at levels consistent with the controls. Since these data suggest that Lyn and Src may be acting via different signalling pathways, further characterisation of the Lyn pathway was required.

Chapter 5: Results

A role for Lyn in zinc transport in TamR cells

Chapter 5: A role for Lyn in zinc transport in TamR cells?

TamR cells have increased intracellular zinc compared to the hormone responsive MCF-7 cells and also show an increase in expression of the zinc transporter ZIP7 (Taylor *et al*, 2008). ZIP7 is located on the endoplasmic reticulum (Taylor *et al*, 2004) and has been shown to be capable of increasing intracellular zinc levels. The important role that zinc and zinc transporters play in breast cancer and specifically anti-hormone resistant breast cancer is becoming increasingly apparent. A calcium-dependent zinc wave in mast cells that leads to the inhibition of phosphatases by zinc has been described. (Yamasaki *et al*, 2007). It has been proposed that a similar phenomenon occurs in TamR cells with the ZIP7 zinc transporter playing a key role (Taylor, 2008b).

Mast cells play a central role in the development of allergic inflammatory reactions (Kopec *et al*, 2006), and play an essential role in regulating innate and adaptive immune responses (Galli et al, 2005). Mast cells express a high-affinity receptor (FcεRI) for the Fc region of IgE (Prussin and Metcalfe, 2003) and are activated by the interaction of an allergen with the specific IgE bound to the FcεRI expressed on the surface of mast cells (Kopec *et al*, 2006). This initiates a signalling cascade that includes activation of tyrosine kinases. Several Src family kinases including Lyn, Fyn, and Hck are highly expressed and have been shown to have distinct functions in this signalling cascade (Lee *et al*, 2011). Lyn was the initial focus of early studies of the Src family kinases in mast cells since it was found to be associated with FcεRI, and thus numerous studies have focussed on Lyn's role in mast cell

activation. This role of Lyn in mast cell signalling is further supported by the observation that cells that express a mutant form of Lyn display impaired function (Poderycki et al, 2010). Lyn negatively regulates mast cell growth and maturation in part by regulating the activation of PI3K/AKT via the p85α regulatory subunit of PI3K (Ma *et al*, 2011). Lyn kinase is activated by stimulation of the IL-3 receptor (Torigoe et al, 1992).

The activation of these signalling pathways leads to mast cell degranulation, the process by which mast cells release antimicrobial cytotoxic molecules including histamine, proteases, and proteoglycans from secretory vesicles called granules (Yamasaki *et al*, 2005). This mast cell degranulation occurs in two ways: the calcium dependent pathway where granules translocate to the plasma membrane in which Lyn has been shown to play a crucial role and the calcium independent pathway that includes fusion of the granule with the plasma membrane and exocytosis (Nishida *et al*, 2005).

Lyn kinase is crucial to the calcium dependent activation of mast cells (Yamasaki *et al*, 2005) and we have shown that it is significantly increased in TamR cells. Since a similar pathway has been proposed for the release of zinc from the endoplasmic reticulum in TamR cells, which contain increased zinc and ZIP7 (Taylor et al, 2008b), a hypothesis was proposed whereby Lyn was also involved in the release of zinc from the endoplasmic reticulum in tamoxifen resistant breast cancer cells in a role similar to that which it plays in mast cells with the ZIP7 zinc transporter playing a key role (Taylor, 2008b).

5.1: ZIP7 knockdown in TamR cells

Since the method for siRNA knockdown in TamR cells was optimised in chapter 4 using Lyn, it was essential to demonstrate that it was also a robust method for the removal of ZIP7 in TamR cells since all subsequent experiments in this section are reliant on the down regulation of ZIP7. TamR cells were treated with transfection lipid alone (TC), siRNA targeting the housekeeping gene lamin or siRNA targeting ZIP7 (siZIP7) for 48 hours and the mRNA was harvested, reverse transcribed and amplified by PCR. The two controls, TC and Lamin showed no change in ZIP7 levels whereas the siZIP7 showed a large, statistically significant decrease in ZIP7 ($p=0.063$). A representative gel is shown in figure 5.1.

5.2: Lyn does not decrease following treatment with siZIP7

To see if ZIP7 removal influenced Lyn, TamR cells were treated with siRNA targeting ZIP7 (siZIP7), or Lyn (siLyn) for 48hrs and treated with 20µM zinc (including siRNA treated cells) for 20 minutes prior to harvest (figure 5.2). The addition of zinc was in order to initiate a zinc wave in the endoplasmic reticulum. It has also been shown, however that the addition of EGF and ionophore can also trigger this zinc release without the addition of exogenous zinc (Yamasaki *et al*, 2007) and so an EGF treatment arm was also included. MRNA was amplified using primers targeting Lyn.

Both EGF and zinc treatment led to an increase in Lyn mRNA compared to basal levels of Lyn in TamR cells. To perform densitometry on agarose gels, it is necessary to produce a figure that is not saturated in order to accurately measure mRNA amounts. Therefore to achieve a quantifiable band

following zinc treatment, the large increase in Lyn following zinc treatment

makes the basal levels of Lyn appear very low compared to those observed in

chapter 4. Following dual Lyn knockdown and zinc treatment, a decrease of

90% of Lyn was observed, to a level below the basal level, suggesting that

Lyn may be needed for the effect seen by zinc treatment. Removal of ZIP7

using siRNA does not appear to influence Lyn mRNA levels in TamR cells

with levels remaining comparable to zinc treated control cells. Since ZIP7

removal does not affect Lyn kinase but Lyn removal affects ZIP 7, this implies

that Lyn may be upstream of ZIP7 in the initiation of the zinc wave from the

endoplasmic reticulum.

## 5.3: ZIP7 decreases following treatment with siLyn

To see if Lyn removal influenced ZIP7 levels, TamR cells were treated with

siRNA targeting Lyn or ZIP7 for 72hrs (figure 5.3).

Zinc treatment led to an increase in ZIP7 protein levels compared to basal

levels of ZIP7 in TamR cells. A decrease in ZIP7 following siZIP7 treatment

is observed thus the siRNA knockdown method is also effective in removing

ZIP7 protein in addition to mRNA as displayed in figure 5.1. Following Lyn

knockdown, a decrease of 60% of ZIP7 protein is observed, a greater decrease

than that observed with siZIP7. As with siZIP7, the levels are greater than

those observed in non-zinc treated cells. The combined data shown in figures

5.2 and 5.3, indicating that Lyn removal leads to a decrease in ZIP7, however

ZIP7 removal does not affect Lyn suggests that Lyn may be upstream of ZIP7

in the zinc wave.

Figure 5.1 siRNA can remove ZIP7 in TamR cells

<u>**A**</u>

<u>**B**</u>
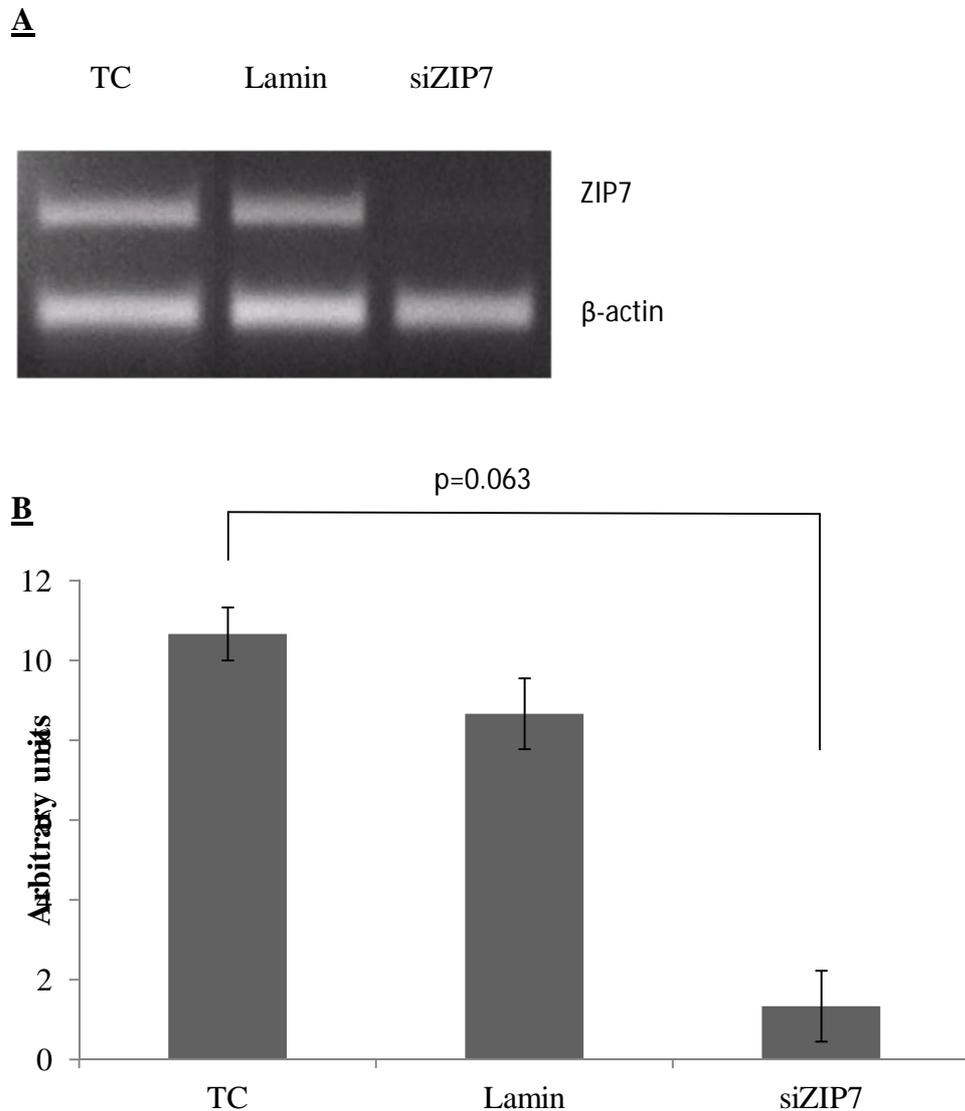
A: TamR cells were treated with siRNA targeting ZIP7 (siZIP7), for 48hrs and mRNA was amplified using primers targeting ZIP7. TC= Lipid control, Lamin=positive control targeting housekeeping gene Lamin, siZIP7=siRNA targeting ZIP7. β-actin was included as a loading control allowing densitometric analysis

B: Levels of ZIP7 in TamR cells treated with siRNA as determined by densitometry. Densitometric values were normalised to account for β-actin levels. Statistical significance was determined using Student's t-test (n=3).

Figure 5.2 Lyn does not decrease following treatment with siZIP7

A

20μM zinc treatment

| Basal | 20μM EGF | SiC | siLyn | siZIP7 |

Lyn

β-actin

B

20μM zinc treatment
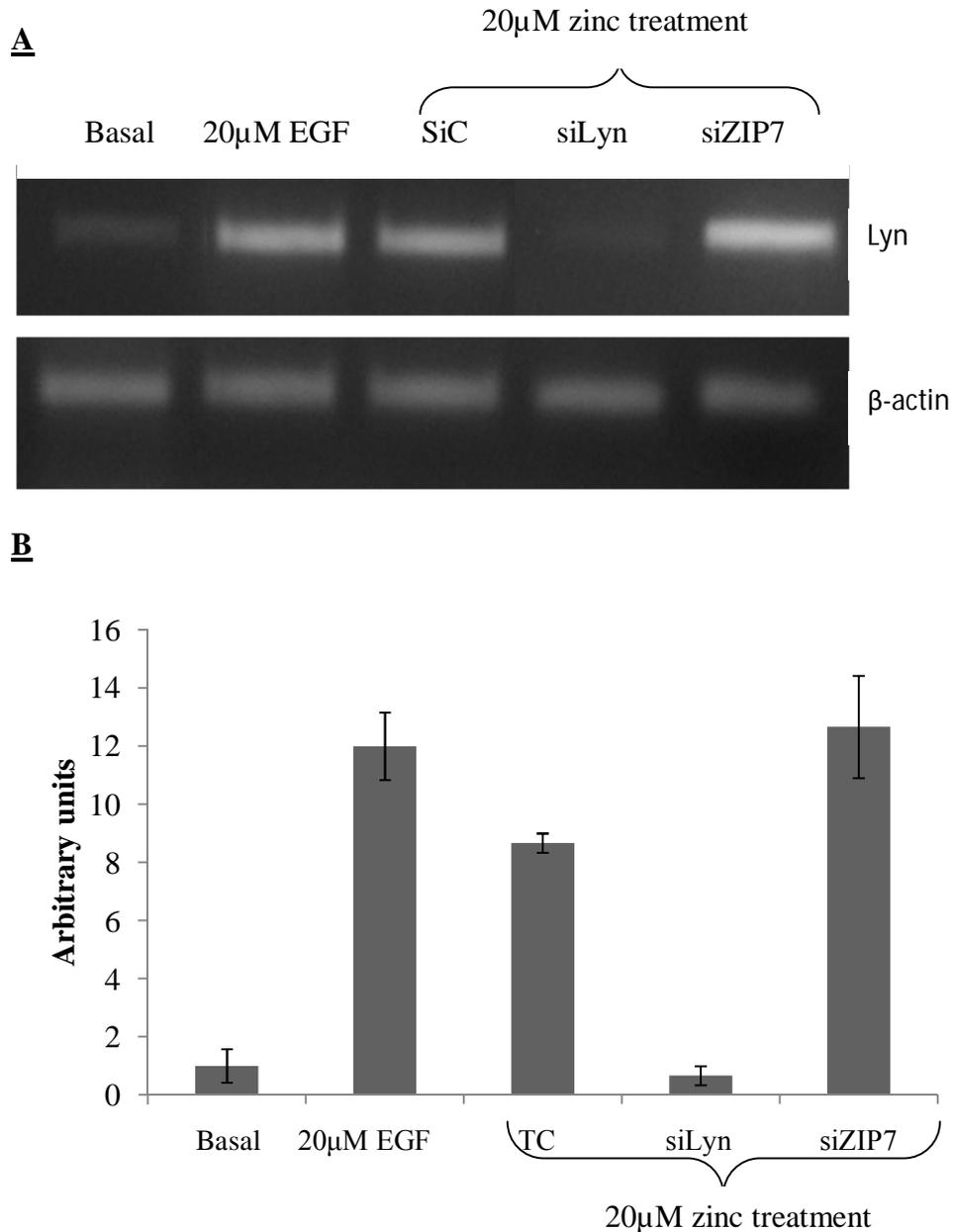
A: TamR cells were treated with siRNA targeting ZIP7 (siZIP7), or Lyn (siLyn) for 48hrs and 20μM zinc (including siRNA treated cells) for 20 minutes prior to harvest. mRNA was amplified using primers targeting Lyn. SiLyn= siRNA targeting Lyn, siZIP7=siRNA targeting ZIP7. β-actin was included as a loading control allowing densitometric analysis

B: Levels of ZIP7 in TamR cells treated with siRNA as determined by densitometry. Densitometric values were normalised to account for β-actin levels. Statistical significance was determined using Student's t-test (n=3).

5.4 Zinc activates Lyn in TamR cells

To begin to dissect the relationship between Lyn and zinc in TamR cells, cells were treated with 20μM zinc and 40μM sodium pyrithione (ionophore) or left untreated for 20 minutes and then fixed and stained with either total Lyn or pLyn (Y396) antibody conjugated to Alexa Fluor 594 then visualised at X63 on a fluorescent microscope at 594nm (figure 5.4).

An increase in activated Lyn as measured by fluorescent staining of Lyn following 20 minutes zinc treatment was observed. The total Lyn antibody also showed an increase in Lyn following zinc treatment. Whilst inactivate Lyn should not be increased following zinc treatment, it is due to the total Lyn antibody also picking up the activated Lyn. The same increase in activated Src has also been reported in response to the zinc wave (Taylor *et al*, 2008). This increase is likely to be due to the inhibition of phosphatases by zinc release and subsequent activation of tyrosine kinases including Lyn and Src. The activated Lyn is located on the plasma membrane. A corresponding western blot confirming these fluorescent microscopy data is shown in figure 5.5. Whilst activated Lyn kinase shows a greater increase following zinc treatment, levels of total Lyn are also shown to be increased.

5.5 Lyn activation following zinc treatment

Following a 4 day siRNA treatment, TamR cells were exposed to 20μM zinc and 40μM sodium pyrithione for either 5 or 20 minutes then

stained using p-Lyn (Y396) and Alexa Fluor 594 and visualised at X63 briefly

to avoid bleaching on a Leica microscope (figure 5.6).

Zinc treatment led to an increase in Lyn activation within 5 minutes as seen in

the transfection control (TC). Lyn removal meant that there was no increase in

Lyn activation by zinc at both the 5 and 20 minute time points. Src removal

did not affect this zinc mediated activation of Lyn, suggesting that Src does

not play a role in this mechanism. A reduction in Lyn activation was observed

following ZIP7 removal at 20 minutes and to a lesser extent 5 minutes zinc

treatment. This later influence of ZIP7 removal on zinc mediated activation of

Lyn implies that Lyn's role may be in initiating the zinc wave in TamR cells

upstream of zinc release from the endoplasmic reticulum. It is this zinc release

that would then inhibit phosphatases resulting in tyrosine kinase activation.

5.6 Lyn is required for zinc wave

Lyn, Src and ZIP7 were removed from TamR cells for 4 days using siRNA.

The cells were then exposed to 20µM zinc and 40µM sodium pyrithione for

20 minutes and either 30 minutes Fluozin-3 (figure 5.7A) or 30 minutes

zinquin (figure 5.7B) and visualised at X63 with care to avoid bleaching on a

Leica microscope. Fluozin-3 and zinquin are zinc specific fluorescent dyes

and both of these dyes were utilised since Fluozin-3 has a high affinity for zinc

with a Kd of approximately 15nM whereas zinquin has a Kd of approximately

100nM for zinc and may therefore not detect more subtle changes in zinc.

Figure 5.3 ZIP7 decreases following treatment with siLyn
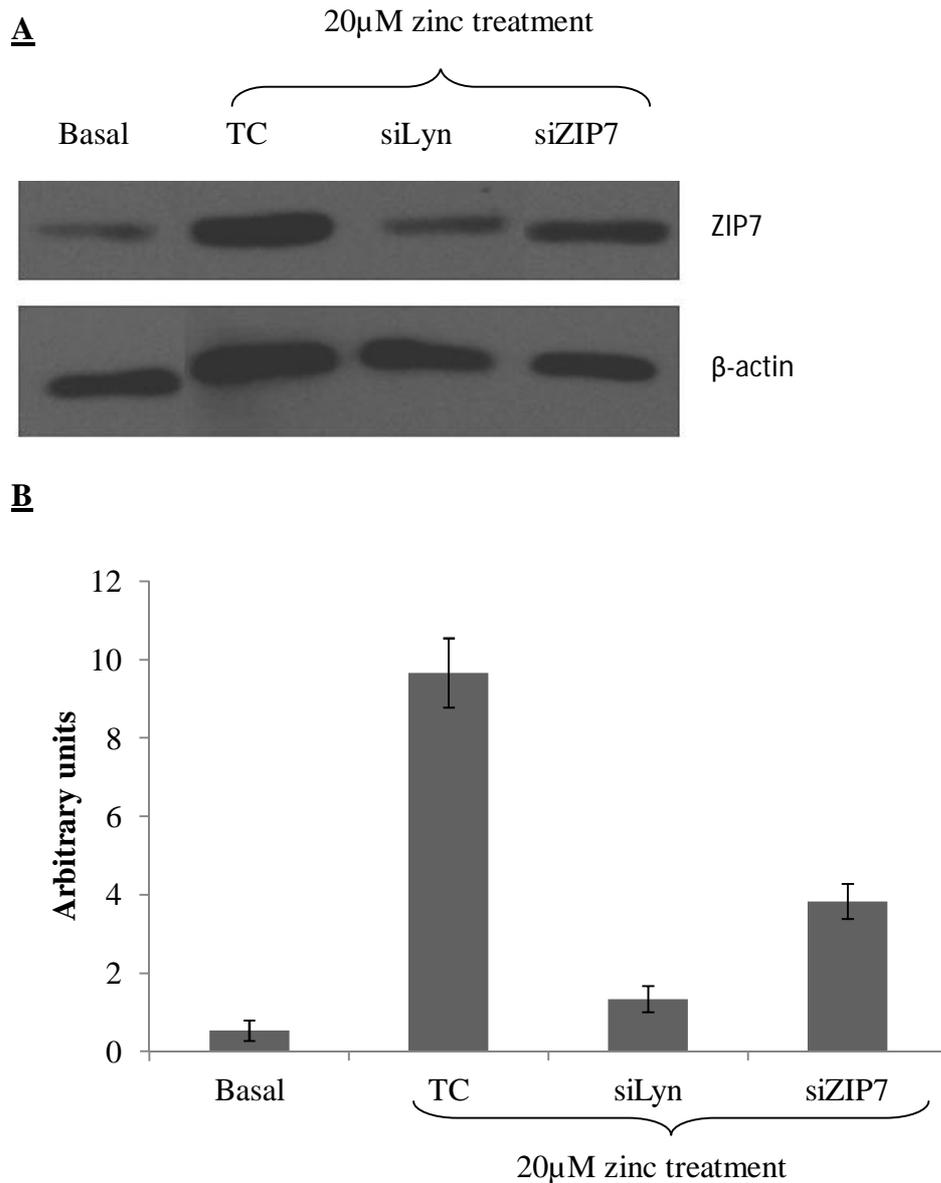
A: Cells were treated with siRNA targeting Lyn or ZIP7 for 72hrs, treated 20µM zinc (including siRNA treated cells) for 20 minutes prior to protein lysis and SDS-PAGE and Western blotting using ZIP7 antibody and β-actin as a reference gene allowing densitometric analysis

B: Levels of ZIP7 in TamR cells treated with siRNA as determined by densitometry. Densitometric values were normalised to account for β-actin levels. Statistical significance was determined using Student's t-test (n=3).

Figure 5.4 Zinc treatment increases Lyn in TamR cells
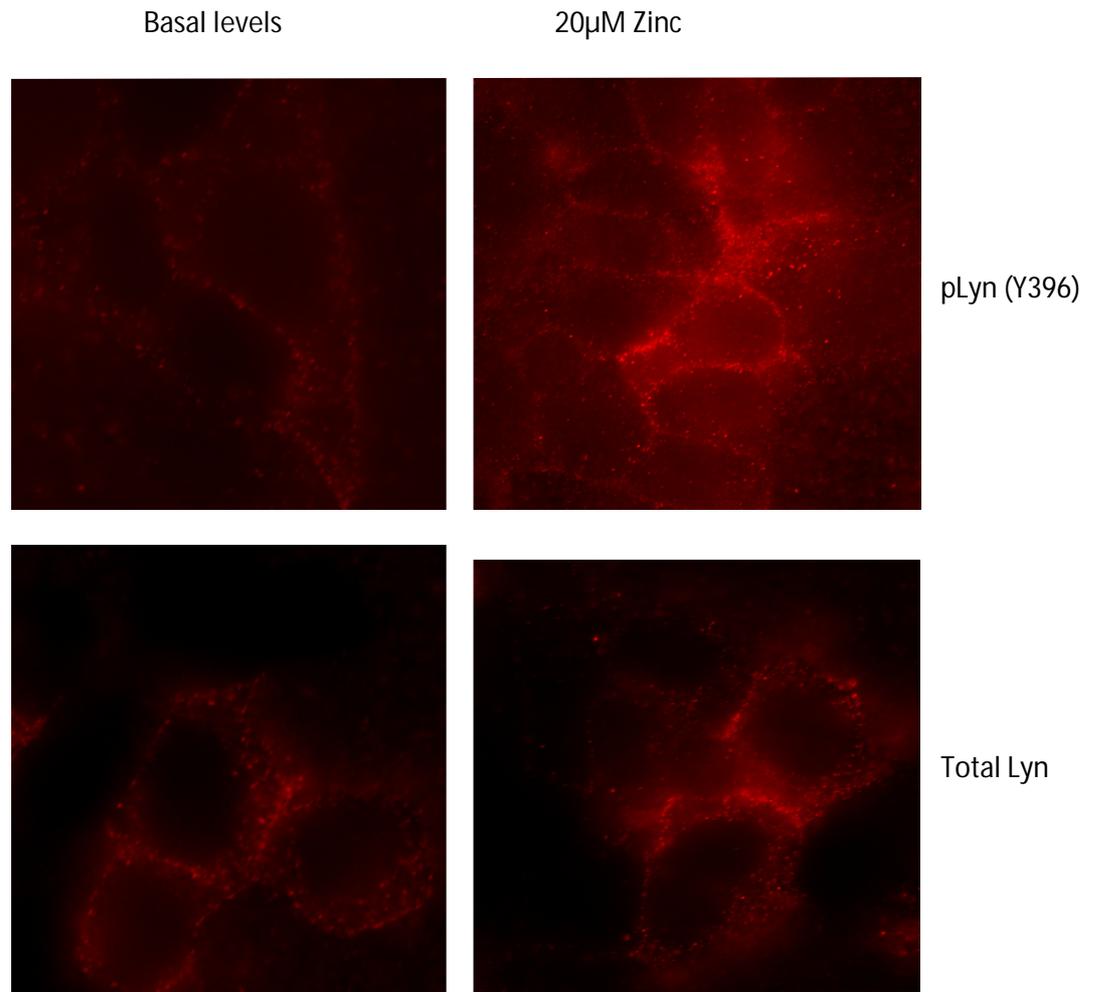
Basal levels   20µM Zinc

pLyn (Y396)

Total Lyn

TamR cells were treated with 20µM zinc or left untreated for 20 minutes and

then fixed and stained with total or pLyn (Y396) antibody and Alexafluor 594

then visualised at X63 on a fluorescent microscope at 594nm.

5.5 Zinc treatment increases Lyn protein in TamR cells.
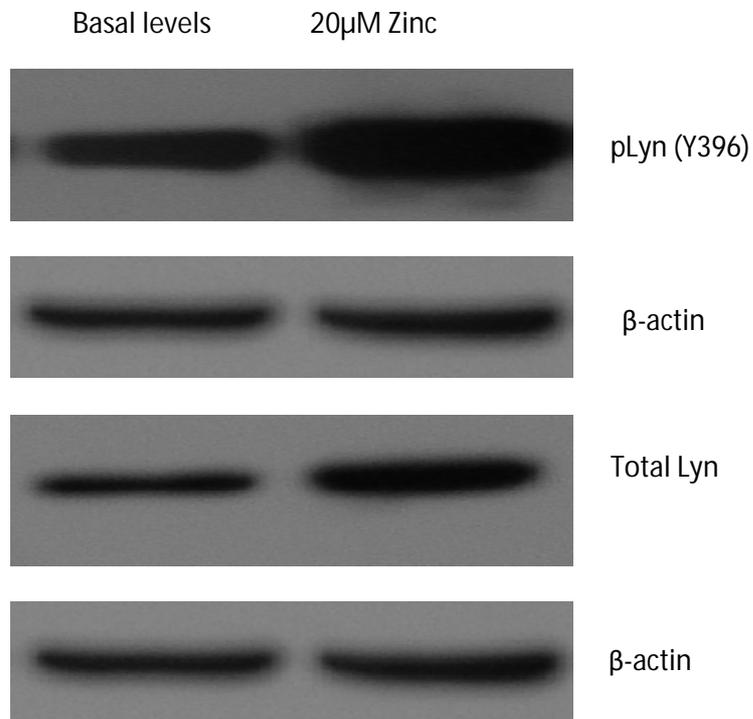
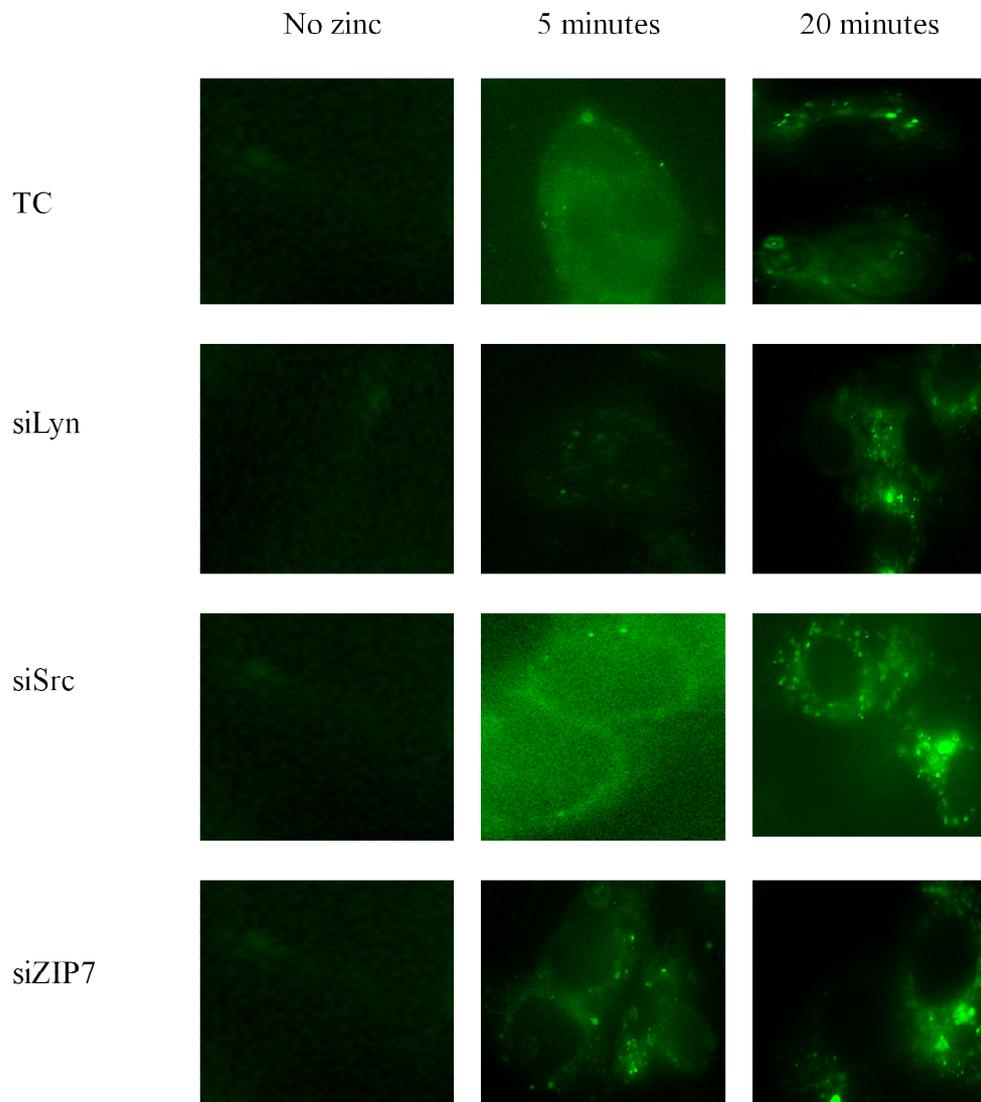TamR cells were treated with 20μM zinc or left untreated for 20 minutes prior to protein lysis and SDS-PAGE and Western blotting using activated or total Lyn antibody and β-actin as a reference gene.

Zinc treatment led to a large increase in zinc in TamR cells at 5 and 20 minutes in the transfection lipid control as measured by Fluozin-3 due to the ZIP7 mediated zinc release from the endoplasmic reticulum. Cells where Lyn had been removed by siRNA treatment did not show this same increase in zinc. The increase in zinc was also prevented, but to a lesser extent by removal of ZIP7 from cells and was more prominent following 20 minutes compared to 5 minutes. Removal of Src did not prevent the increase in zinc following treatment, showing comparable levels to the control. This result again suggests that Src does not play a role in the generation of a zinc wave in TamR cells in contrast to Lyn. The higher zinc levels following ZIP7 removal compared to Lyn removal can be explained by the presence of Lyn in the siZIP7 arm, meaning that activation of the zinc wave still occurred but to a lesser extent than the control. This is further evidence that Lyn is upstream of ZIP7 in the zinc wave suggesting a distinct role for Lyn in TamR cells that has not been previously reported. The high level of Fluozin-3 zinc staining seen in the 20 minute TC arm, suggests that an excess of zinc was present at that time point. The two zinc dyes both shown the same pattern of zinc release following treatment. Subtle differences observed between the two can be explained by the difference in the affinity of each compound for zinc since Fluozin-3 can detect zinc in much smaller amounts than is possible using zinquin and so more subtle changes in zinc levels may be observed.

Figure 5.6 Effect of siRNA and zinc on Lyn activation
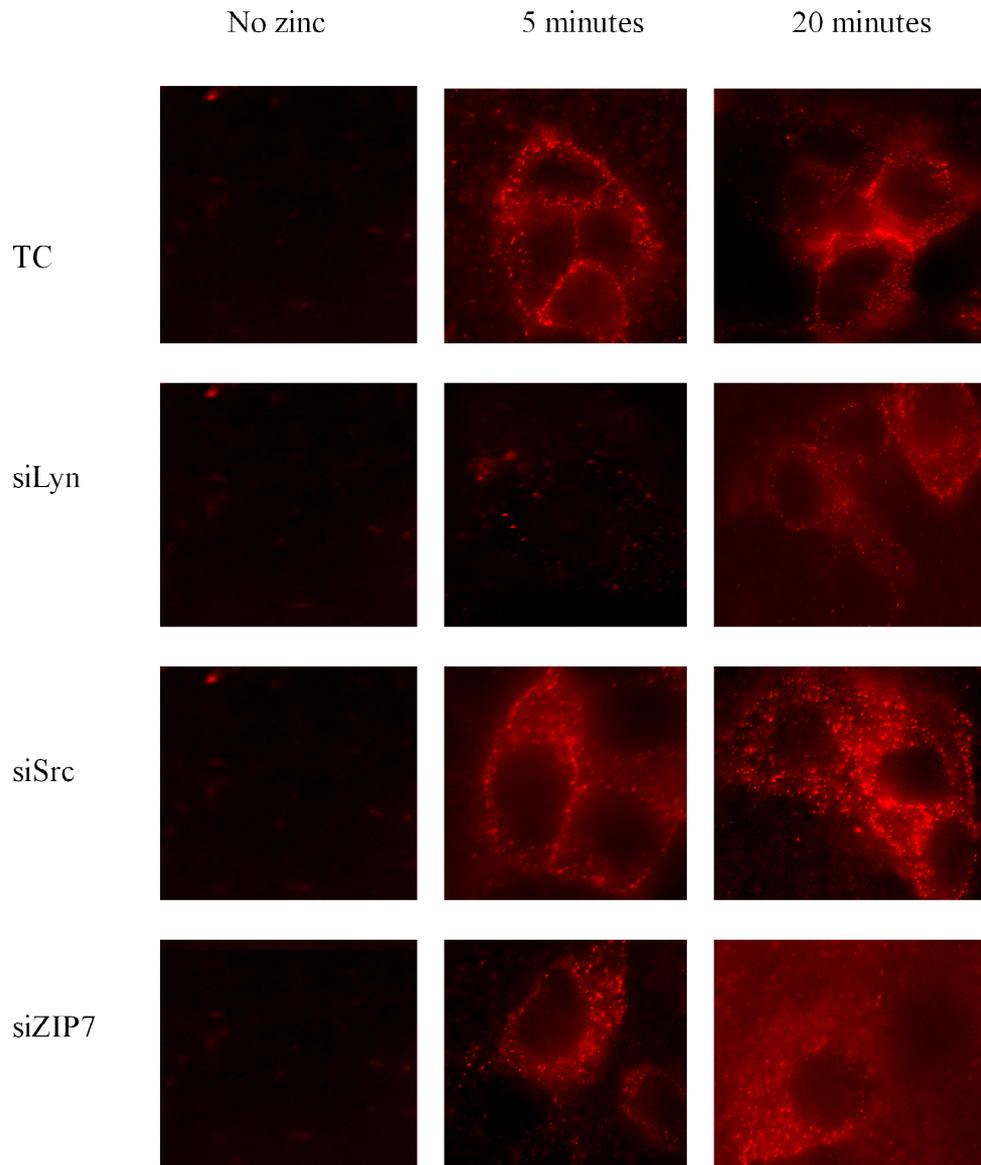
TamR cells were treated with transfection lipid (TC), siLyn, siSrc or siZIP7

for 4 days, exposed to 20μM zinc and 40μM sodium pyrithione for 20 minutes

then stained using p-Lyn (Y396) and Alexafluor 594 and visualised at X63 on

a Leica microscope

Figure 5.7 A Zinc levels following siRNA and 5 or 20 minute zinc

treatment measured by Fluozin 3 .

	No zinc	5 minutes	20 minutes
TC			
siLyn			
siSrc			
siZIP7			

TamR cells were treated with lipid only (TC), siLyn, siSrc or siZIP7 for 4

days, loaded with Fluozin-3 for 30 minutes then exposed to 20µM zinc and

40µM sodium pyrithione for 20 minutes and visualised at X63 on a Leica

microscope.

Figure 5.7 B Zinc levels following siRNA and 5 or 20 minute zinc

treatment measured by zinquin.
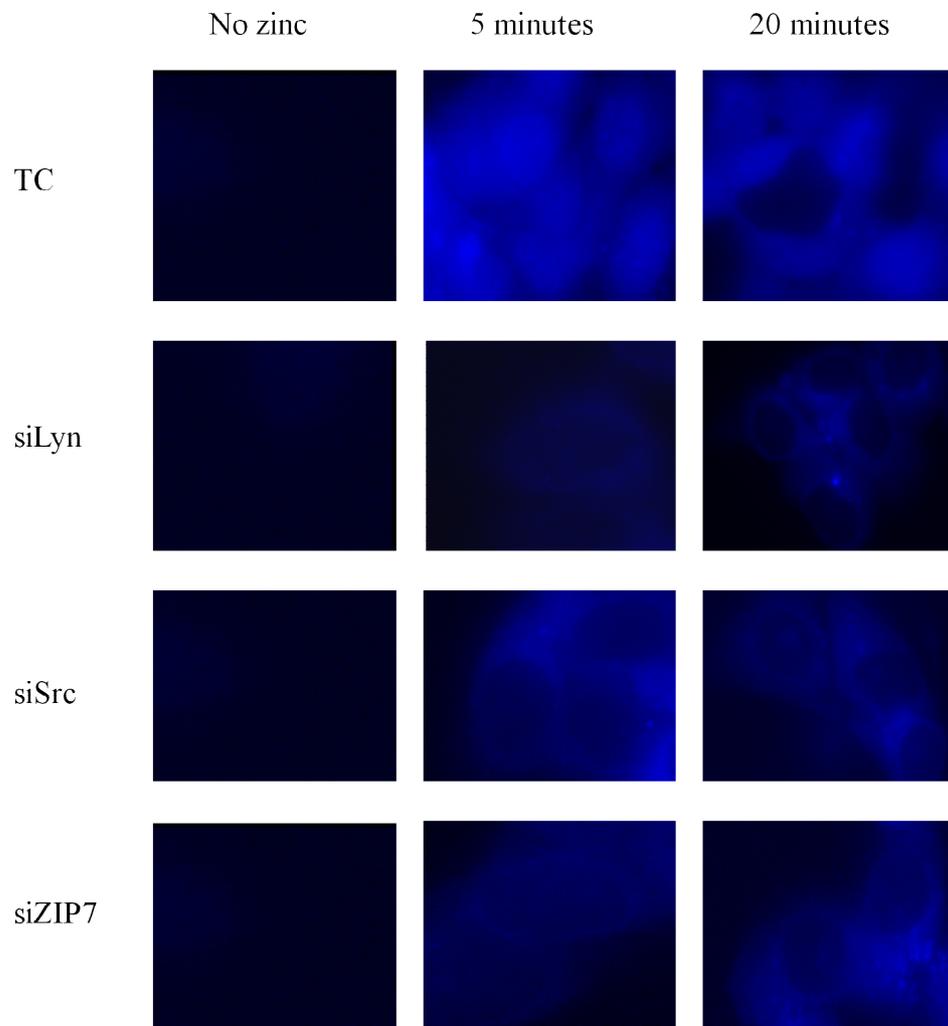
| | No zinc | 5 minutes | 20 minutes |

TamR cells were treated with transfection lipid (TC), siLyn, siSrc or siZIP7

for 4 days, loaded with zinquin for 30 minutes then exposed to 20µM zinc and

40µM sodium pyrithione for 20 minutes and visualised at X63 on a Leica

microscope.

Summary of chapter

Initial experiments using fluorescent microscopy revealed that Lyn kinase is located on the plasma membrane and activated following treatment with zinc. Src kinase was also activated, mirroring what had been published previously in response to the zinc wave (Taylor *et al*, 2008b). The likely conclusion from this result is that zinc release leads to the inhibition of phosphatases and thus activation of tyrosine kinases.

Removal of Lyn by siRNA led to a decrease in the zinc activation of Lyn that was previously witnessed. A reduction in Lyn activation was observed following ZIP7 removal at 20 minutes and to a lesser extent 5 minutes zinc treatment. This later influence of ZIP7 removal on zinc mediated activation of Lyn implies that Lyn kinase's role may be in initiating the zinc wave in TamR cells upstream of zinc release from the endoplasmic reticulum. It is this zinc release that would then inhibit phosphatases resulting in tyrosine kinase activation.

Src removal did not affect this zinc mediated activation of Lyn, suggesting that Src does not play a role in this mechanism. It also adds weight to the theory that the results seen are not due to zinc inactivation of phosphatases, since all tyrosine kinases including Src would be affected were this occurring. This alternative mechanism displayed may also explain the differences seen between Src and Lyn in the previously studied endpoints in chapter 4.

Chapter 6: Discussion

Chapter 6: Discussion

The problem of tamoxifen resistance

Tamoxifen has made a significant contribution in decreasing breast cancer related deaths for over 30 years and until recently was the gold standard for treatment of ER positive breast cancer (Fisher *et al*, 1998). It still remains the most widely used anti-oestrogen drug (Ring and Dowsett, 2004). Resistance to tamoxifen is however a considerable issue with cells utilising a number of molecular mechanisms to bypass the growth inhibition caused by blocking ER activity. This move towards an anti-hormone resistant state from an anti-hormone responsive state is associated with the transition to a much more aggressive phenotype including increased proliferation and also invasiveness. Thus unfortunately, acquisition of tamoxifen resistance is not only associated with a recurrence of breast cancer, and this cancer is also much more aggressive in nature with fewer treatment options available than the initial cancer. Acquisition of resistance has also proved to be a significant concern with subsequent anti oestrogen therapies such as fulvestrant and aromatase inhibitors

EGFR has been shown to mediate the alternative growth of tamoxifen resistant cells in both our TamR model (Knowlden *et al*, 2003) and also other models of tamoxifen resistance (Vendrell et al, 2005; Cleator et al, 2009). Vendrell et al have developed two tamoxifen resistant cell lines derived from the MVLN human breast carcinoma cell line called CL6.8 and CL6.32 (Vendrell et al 2005). Both tamoxifen resistant cell lines displayed the development of

agonist activity of tamoxifen on cell proliferation and also resistance to tamoxifen induced apoptosis as is seen in our model of tamoxifen resistance. They also displayed an increase in MAPK activity, and over-expression of several genes coding for EGFR, and numerous ErbB-specific ligands and also increased MAP kinase activity (Vendrell et al, 2005). This suggests the involvement of such events in the development of tamoxifen resistance, again supporting that which is observed in our TamR cells. A number of molecular differences were observed between the two tamoxifen resistant cell lines despite being developed at the same time. This further highlights the complexity of tamoxifen resistance development.

Interestingly, inhibition of EGFR, shown to mediate the alternative growth of tamoxifen resistant cells, using the EGFR specific tyrosine kinase inhibitor gefitinib, lead to an initial decrease in proliferation, however cell growth recommences within months (Knowlden *et al*, 2005). The IGFR (insulin like growth factor receptor) signalling also plays an important role in tamoxifen resistance, facilitating EGFR signalling (Gee *et al*, 2005). An added shortfall was that many patients display intrinsic resistance to gefitinib and so cannot gain any initial benefit from treatment (Bianco *et al*, 2005).

**Advances in treatment options for breast cancer**

In addition to endocrine therapies, including tamoxifen, fulvestrant and aromatase inhibitors, detailed in chapter 1, a number of other treatment options are currently being investigated for the treatment of ER positive breast cancer. These may be used as single agents or used in combination with other

therapies to provide improved treatment options. These include growth factor inhibitors, such as gefitinib, the small molecule EGFR inhibitor as mentioned above (Gee *et al*, 2003). Despite disappointing results when used as a single agent, it is currently being investigated as part of a dual therapy, to see if it can improve outcomes when combined with aromatase inhibitors (Davies and Hiscox, 2011). Another treatment option currently being investigated is the use of mTOR inhibitors. Mammalian target of rapamycin (mTOR) is a kinase that functions as a master switch between catabolic and anabolic metabolism (Faivre *et al*, 2006). It has been identified as a key kinase acting downstream of the activation of PI3K. The phosphatidylinositol 3-kinase (PI3K)/AKT kinase cascade, is responsible for cell growth and proliferation. Additionally, mTOR has been shown to regulate apoptotic cell death, which is dictated by the downstream targets including p53. The PI3K/Akt pathway is often up regulated in cancers and as such mTOR is a target for the design of anticancer drugs, with rapamycin having initially improved the survival of patients with advanced renal cancer (Faivre *et al*, 2006). In ER positive breast cancer, later incarnations of rapamycin, particularly everolimus (Satheesha *et al*, 2011) are being investigated both as single agents and also in combination with other therapies (Davies and Hiscox, 2011). Unfortunately, as with other therapies, such as EGFR inhibitors, mTOR inhibitors have thus far proved less successful in cancer clinical trials than might be hoped from the importance of the molecular pathways involved due to either intrinsic or acquired resistance (Satheesha *et al*, 2011). Thus a deficit remains in therapeutic options for the treatment of ER positive breast cancer, reinforcing the need for further

characterisation of anti hormone resistant breast cancer and identification of novel therapeutic targets.

Identification of new therapeutic targets

This deficit in available treatment for tamoxifen resistant breast cancer in the clinical setting despite a significant contribution to the resistant phenotype meant that there was an opportunity for the identification of novel gene targets to facilitate the development of new therapies. Thus a strategy was devised using available microarray data in order to identify novel gene that could potentially be targeted either alone or in conjunction with other therapies and is summarised in figure 6.1.

Figure 6.1: Summary of gene identification strategy
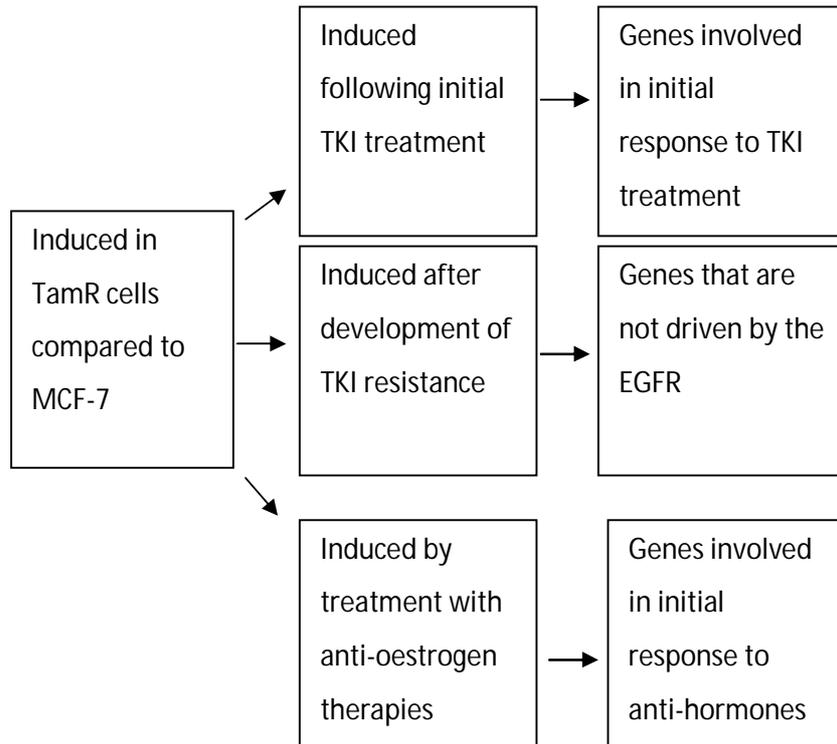
A problem of this method of selection using affymetrix that became apparent during later experiments was that the growth of the genes selected may still be driven by the EGFR. This became clear in chapter 6, when treatment with the epidermal growth factor (EGF) was able to induce Lyn expression. A more suitable method at this stage of selection would have been to select genes that were unchanged following EGFR treatment and also subsequently unchanged in the tamoxifen/gefitinib model rather than genes that were induced following treatment. An additional step of repeating the treatments used on the microarray samples and subsequent Western blotting would have provided a more robust method of identifying genes whose growth was truly independent of the EGFR. Despite this, microarray analysis allowed the identification of Lyn kinase as a potential therapeutic target in tamoxifen resistant breast cancer.

Lyn kinase in tamoxifen resistant models

In chapter 4 we demonstrated that removal of Lyn kinase affects the growth, cell death, invasive and migratory capacity of TamR cells. Of particular significance is the fact that Lyn removal and Src removal yielded different endpoints, suggesting that they may be playing distinct roles in TamR cells. This agrees with work carried out in mast cells, where members of the Src family have been shown to have unique and distinct roles in activation (Ma *et al*, 2011). With unique roles emerging for Src family members in specific tissues and also other cancers (Saito *et al*, 2010; Bilal *et al*, 2011), it is

possible that results were due to other Src family members including Lyn

kinase.

Lyn kinase and zinc signalling

A calcium dependent zinc wave has been described in mast cells that leads to

the inhibition of phosphatases and thus activation of tyrosine kinases

(Yamasaki *et al*, 2007). It was proposed that this zinc wave initiates in the

endoplasmic reticulum and is therefore likely to be due to ZIP7 mediated

release of zinc. In addition, a model for zinc handling within cells has been

proposed where intracellular zinc is associated with a muffler in the

cytoplasm such as metallothionein that allows it to be strongly buffered and

subsequently distributed into a deep store to be released into the cytoplasm

(Colvin *et al*, 2008). In TamR cells, a similar phenomenon based upon these

two models has been proposed with the ZIP7 zinc transporter playing a key

role (Taylor *et al*,

2008).

Figure 6.2: The predicted function of ZIP7. Reproduced from Taylor

2008b: A distinct role in breast cancer for two LIV-1 family zinc transporters.
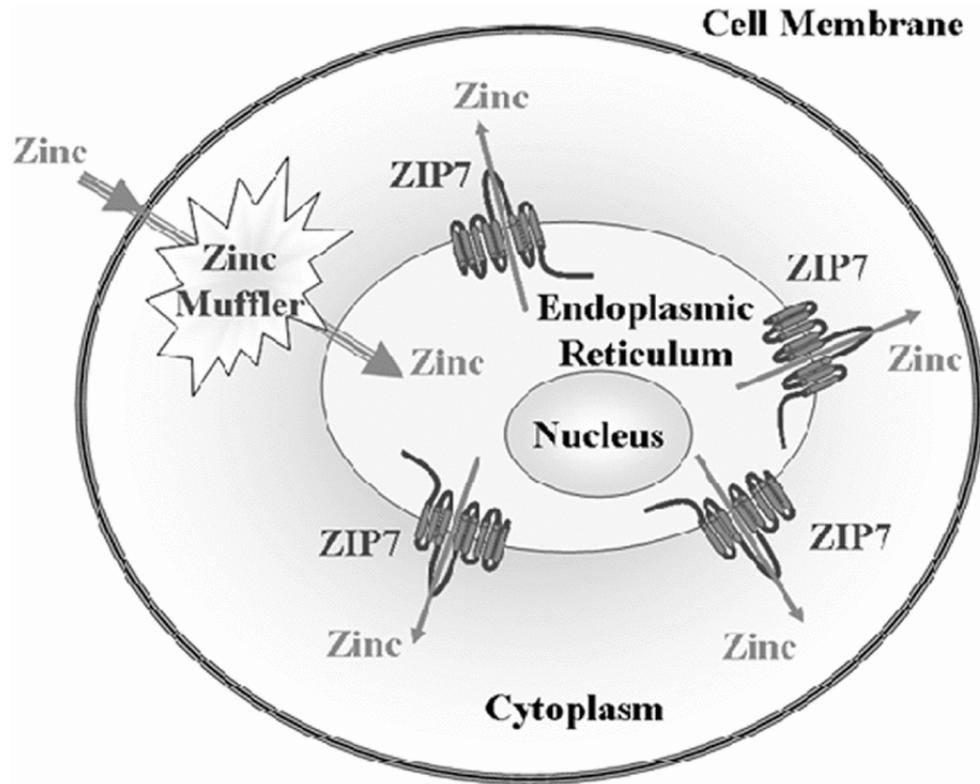
Lyn kinase and not Src kinase has been identified as playing a crucial role in the calcium dependent activation of mast cells (Nishida *et al*, 2005). With zinc signalling the new emerging mechanism driving cell growth in TamR cells a role for Lyn has been suggested due to its role in mast cells. The activation of EGFR by zinc in tamoxifen-resistant breast cancer cells was demonstrated to be Src dependent and zinc levels as low as 20 μM were able to partially reverse the inhibition of invasion by use of the Src inhibitor SU6656 which also inhibits Lyn (Taylor *et al*, 2008b). It is therefore possible that this activation of EGFR by zinc in tamoxifen-resistant breast cancer cells may have been due to Lyn inhibition, especially since this study has demonstrated that ZIP7 removal using siRNA did not affect Src kinase activation, it does however affect Lyn kinase activation. This difference could also explain the differences seen in apoptosis following Lyn and Src removal and suggest a distinct role for Lyn compared to Src. In chapter 5 (figures 5.2 and 5.3) Lyn removal by siRNA lead to a decrease in ZIP7, however ZIP7 removal did not affect Lyn. This observation suggests that Lyn kinase is upstream of ZIP7 in the zinc wave in TamR cells. A proposed position for Lyn in relation to the zinc wave is presented in figure 6.3

Figure 6.3 Proposed hierarchical position of Lyn and ZIP7
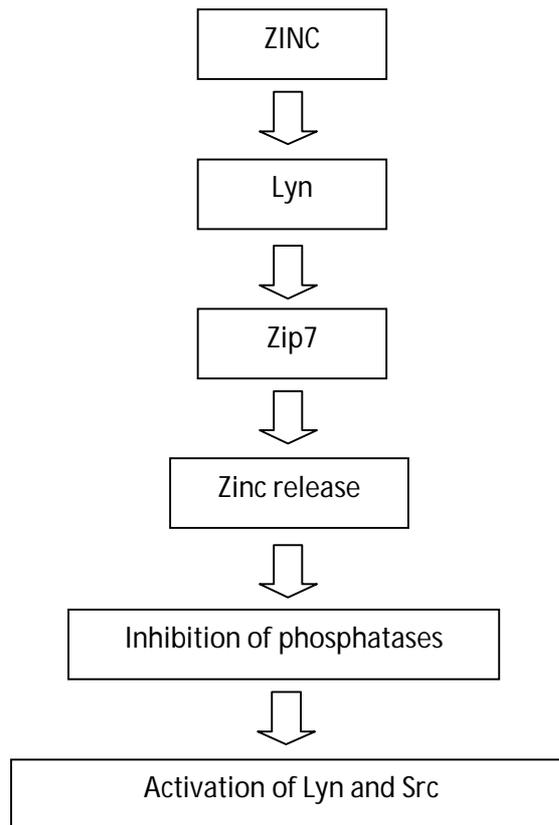
Despite Lyn being thought to be expressed primarily in haematopoietic cells, it is not unprecedented for tissue specific genes to be activated in cancers. For example, Yes-1, another member of the Src kinase family which is usually only expressed in epithelial cells of the renal proximal tubules in human adults (Krueger et al, 1991), is over-expressed in basal-like breast cancers (Bilal et al, 2010). This fact demonstrates a flaw in the initial selection process of potential target genes, since some genes were discounted for further investigation due to their tissue specificity, whereas genes have been shown to be over-expressed in cancers despite not being expressed ubiquitously in normal cells. Despite the exact role of Lyn in tamoxifen resistance not being clear, targeting Lyn kinase may be a means of specifically targeting zinc levels, particularly since it is not ubiquitously expressed in normal (non-cancerous) cells.

Excitingly, recent discoveries have led to a much greater understanding of the role of zinc signalling in breast cancer. CK2 is a protein kinase that has been shown to regulate multiple oncogenic pathways including EGFR-regulated pathways, Akt and WNT signaling cascades, NF-κB transcription, angiogenesis, Hsp90 chaperone pathway and the DNA damage response (Siddiqui-Jain *et al*, 2010). Elevated levels of protein kinase CK2 have long been associated with increased cell growth and proliferation both in normal and cancer cells (Trembley *et al*, 2009). It can also act as a suppressor of apoptosis thus playing an important role in many cancers since deregulation of both cell proliferation and apoptosis are among the key features of cancer cell biology (Trembley *et al*, 2009). Down regulation of CK2 leads to induction of

apoptosis in cancer cell models, hence the development of CK2 inhibitors for use in clinical trials. CX-4945, a potent and selective orally bioavailable small molecule inhibitor of CK2 has been developed (Siddiqui-Jain *et al*, 2010). CX-4945 displayed anti-proliferative activity and led to cell-cycle arrest. It also selectively induced apoptosis in cancer cells relative to normal cells and caused a decrease in PI3K/Akt signalling (Siddiqui-Jain *et al*, 2010). CX-4945 is currently in clinical trials for the treatment of cancer (Pierre *et al*, 2011).

It has been demonstrated that ZIP7 can be activated by phosphorylation by CK2 (Taylor *et al*, 2012). CK2 phosphorylates ZIP7 leading to the release of zinc ions into the cytosol. This then activates the kinases Akt and Erk 1/2 that are involved in cell proliferation and growth (Taylor et al, 2012). A schematic of this, reproduced from Taylor *et al*, (Taylor *et al*, 2012b) is shown in figure 6. 4. Previous studies using the CK2 inhibitor DMAT led to the caspase-mediated killing of tamoxifen resistant breast cancer cells while failing to kill parental MCF-7 cells (Yde *et al*, 2007). This in combination with the discovery of a potential role for CK2 in the control of ZIP7 in tamoxifen resistant breast cancer provides a novel means of targeting zinc in breast cancer.

Figure 6.4: Schematic of second messenger signalling pathway for zinc.

Reproduced from Taylor *et al,* 2012b
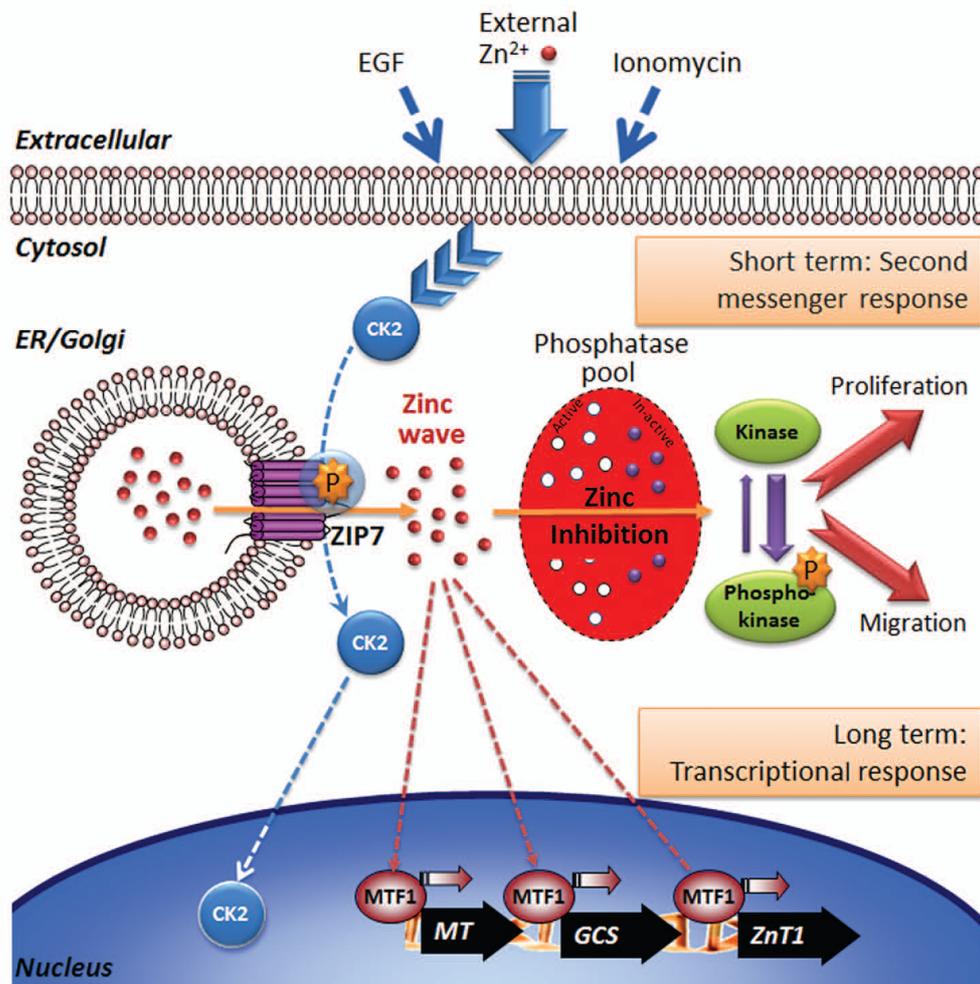
Schematic illustration of the second messenger signaling pathway proposed for Zn2+. Various external stimuli cause protein kinase CK2 to phosphorylate endoplasmic reticulum (ER)-located zinc transporter ZIP7, which causes the gated release of Zn2+ stored within the ER through the ZIP7 channel to create a cytosolic "zinc wave." After phosphorylating its target, CK2 dissociates from ZIP7 and may move into the nucleus. We postulate that elevated Zn2+ within the cytosol may selectively inhibit tyrosine phosphatases, leading to prolonged tyrosine kinase activation and an observed downstream increase in pERK and pAKT, resulting in cell proliferation and migration. These events, representing a short-term second messenger activity for Zn, completing within a 20 min time period, are thus temporally separated from established transcriptional impact of zinc ions, which is mediated by metal transcription factor-1 (MTF-1) and is known to regulate metallothionein (MT), glutamylcysteine synthetase (GCS) and ZnT1 (SLC30A1).

Lyn in basal-like breast cancer

Since commencement of this research and selection of Lyn kinase as a potential therapeutic target for further study, a number of other groups have also identified Lyn as potentially important in breast cancer. Of particular significance is the identification of Lyn as a key player in the basal-like breast cancer phenotype (triple negative- ER, PR and HER2 negative). Unfortunately, fewer treatment option are available for basal-type breast cancers, with fewer targeted therapies available (Hochgrafe et al, 2010). The therapies available include poly (ADP-ribose) polymerase (PARP) inhibitors. PARP is responsible for the regulation of the DNA base-excision–repair pathway. Use of PARP inhibitors in combination with chemotherapy are currently in phase III trials for the treatment of metastatic triple negative breast cancer (O'Shaughnessy *et al*, 2011). mTOR inhibitors as mentioned earlier in this chapter are being trialled as a combination therapy for the treatment of triple negative breast cancer (Davies and Hiscox, 2011). In addition the BCR-Abl and Src inhibitor Dasatinib is investigated as a single therapy and also as a combination therapy in triple negative breast cancer. Dasatinib is a small molecule tyrosine kinase inhibitor. Among the most sensitive dasatinib targets are ABL, the SRC family kinases (SRC, LCK, HCK, FYN, YES, FGR, BLK, LYN, and FRK), and the receptor tyrosine kinases c-KIT, platelet-derived growth factor receptor (PDGFR) a and b, discoidin domain receptor1 (DDR1), c-FMS, and ephrin receptors (Montero et al, 2011).

In an independent study, Lyn has been identified as a mediator of EMT (epithelial-mesenchymal transition) (discussed previously), which leads to cell

metastasis in breast cancer cell lines (Choi *et al*, 2010). It was associated with much shorter overall survival in the clinic and correlated with the basal-like phenotype (Choi et al, 2010). Basal breast cancer cells are characterized by elevated tyrosine phosphorylation of Met, Lyn, EphA2, epidermal growth factor receptor (EGFR), and FAK. RNAi knockdown of Lyn in these triple negative basal type cell lines blocked invasion but not cell proliferation (Choi *et al*, 2010). It is studies such as these which identify new therapeutic targets that lead to more treatment options for breast cancer patients with the triple negative phenotype.

Treatment of basal-type breast cancer cell lines with dasatinib also blocked invasion but not proliferation as with siRNA targeting Lyn treatment (Choi et al, 2010). In hormone receptor–positive breast cancer, trials using combinations of dasatinib with anti-hormonal therapies are ongoing (Montero et al, 2011). The role of Lyn kinase in EMT as described by Choi *et al* may explain the significant decrease in migration/invasion observed following Lyn kinase removal by siRNA in TamR cells, however the observation that proliferation is unchanged following siRNA or dasatinib treatment is in contrast to our findings in TamR cells. This suggests a different or additional role for Lyn kinase in TamR cells. Lyn kinase has been identified as a viable therapeutic target for the treatment of tamoxifen resistant breast cancer. Our findings are also supported by the observations of other groups of Lyn kinase playing a key role in the progression of breast cancer (Choi *et al*, 2010, Montero *et al*, 2011). It is this progression of breast cancer to a more invasive state that leads to metastasis in the clinic with Lyn kinase shown to play a key

role. Metastasis to other sites in the body is ultimately responsible for fatalities due to breast cancer and so being able to block its action is key to treating breast cancer in the clinic. Therefore identifying Lyn kinase as a gene target that leads to the advancement of breast cancer to a more aggressive state provides a powerful tool for treating breast cancer in the clinic.

This study is unique in TamR cells in that it is the first time, that the roles of individual Src kinase family members have been investigated which may ultimately lead to a better understanding of the complex acquisition of tamoxifen resistance and also improved treatment due to more specific gene targeting.

Conclusion

In this work we have shown that Lyn plays an important role in the phenotype associated with resistance to tamoxifen in breast cancer. Lyn removal lead to a decrease in cell migration, invasion and proliferation. Of particular significance were the differences observed between Lyn and Src following gene knockdown on apoptosis in TamR cells. Lyn removal and not Src removal led to a significant reduction in apoptosis, suggesting that Lyn and Src operate via different mechanisms in tamoxifen resistance. It is significant that we have shown a link between Lyn kinase activity and the release of zinc from the endoplasmic reticulum in TamR cells, based upon a similar role played by Lyn in the calcium dependent activation of mast cells. The vital role played by zinc and the zinc transporter ZIP7 in TamR cells is becoming increasingly apparent. Thus therapies that are able to target the zinc release

and subsequent increase in phosphatase activity leading to tyrosine kinase

inhibition in TamR cells provide an exciting, novel opportunity for therapeutic

drug development. Our data proposes that Lyn lies upstream of ZIP7 and the

zinc release from the endoplasmic reticulum suggesting that targeting Lyn as a

therapy for breast cancer may prove productive. This could either be

following acquisition of tamoxifen resistance or alternatively in conjunction

with anti-hormone therapies such as tamoxifen or aromatase inhibitors as an

upfront dual therapy for breast cancer.

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Chapter 8: Appendices

6.1 Appendix I: 50X Tris-Acetate-EDTA (TAE) Buffer

	Per litre	Final concentration
Tris Base	242g	2M
Glacial acetic acid	57.1ml	1M
EDTA (0.5M, pH 8.0)	100ml	0.05M

- Adjust pH to 8.3
- Make up to 1 litre with distilled H_2O
- Dilute 1:50 with distilled H_2O for use

6.2 Appendix II: Loading buffer for gel electrophoresis

	To give 10ml	Final concentration
Sucrose	6g	60% (w/v)
Bromophenol blue	0.025g	0.25% (w/v)

- Make up to a volume of 10ml with distilled, RNase free H_2O
- Filter in a 0.2μM syringe to remove any remaining bromophenol blue crystals

6.3 Appendix III: 3X loading buffer

	For 10ml	Final concentration
SDS	0.6g	2% (w/v)
Glycerol	3ml	10% (v/v)
Tris base	3.6ml (0.5M stock)	60mM
H_2O	To give 10ml	
Bromophenol blue	0.003g	0.001% (w/v)

6.4 Appendix IV: SDS-PAGE running buffer

	For 1 litre	Final concentration
Tris base	3.03g	0.25M
Glycine	14.4g	1.92M
SDS	1g	0.1% (w/v)
H_2O	1 litre	

- Use 5M HCl to adjust pH to 8.3 before use

6.5 Appendix V: Western blot transfer buffer

	For 1 litre	Final concentration
Tris base	3.03g	0.25M
Glycine	14.4g	1.92M
Methanol	200ml	20% (v/v)
H_2O	800ml	