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Combination of Post-transcriptional and Post-translational down-regulation of the Oestrogen Receptor in Breast Cancer.

A thesis presented for the degree of
Doctor of Philosophy at Cardiff University by

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Summary.

Greater concentrations of fulvestrant are being employed within the clinic due to increased oestrogen receptor (ER) down-regulation and greater clinical benefit in ER+ breast cancer. However, complete ER down-regulation has not been achieved. The importance of residual ER is unknown and could allow cells to survive initial anti-hormone impact and progression to hormone insensitivity. This project aims to go further than current clinical therapy, using the MCF-7 cell model to target and assess the importance of residual ER.

Cells were treated with fulvestrant aiming to achieve maximal ER down-regulation. The effect of any residual ER on signalling and growth was subsequently assessed. An alternative model for ER loss, ER siRNA, was employed to see whether this had a greater anti-ER effect. Finally, fulvestrant and ER siRNA were employed in combination to assess whether these agents work synergistically to give greater ER down-regulation and increased anti-tumour effect.

With fulvestrant at 10^{-7}M, ER levels were markedly reduced, although residual ER was observed that remained with increasing drug concentrations. There was significant reduction of ER signalling, proliferation and growth, but the inhibition was incomplete. When ER siRNA was assessed, similar results were obtained, with comparable and incomplete ER loss, residual signalling and growth. Following combination treatment of fulvestrant and ER siRNA, residual ER was almost undetectable, though this did not correspond to greater loss of ER signalling or growth when compared to either agent alone.

While this work showed greater ER loss than previously recorded by targeting both protein and mRNA together, no greater anti-tumour activity was observed. Thus, while the mechanism underlying residual growth warrants future investigation (along with longer exposure), targeting ER alone, no matter how successfully, may not be the best treatment regimen and a combination of targets may be required as the optimum strategy to treat ER+ breast cancer.

Dedication.

This thesis is dedicated to the memory of my mum, Jacqueline Pamela Longman.

-Fondly remembered, forever loved, and so sorely missed.

Acknowledgements.

There are so many people deserving of thanks, responsible for me standing where I am today, that it would fill a second volume this size to mention you all by name.

However, particular thanks must go to Professor David Lloyd, for originally encouraging me to apply for a PhD. I would also like to thank both my supervisors, Professor Robert Nicholson and Dr Iain Hutcheson, for their guidance, insights, and continued support throughout some incredibly difficult periods of my life.

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Thank you for turning a young and naïve graduate into the scientist I am today.

Special thanks go to my family, my Mum and Dad, my two sisters Samantha and Kimberley, and my brother Andrew, as well as my grandparents, aunts, uncles and cousins. You all believed in me, even when I didn't believe in myself and words can not express how much your support has meant to me over the years.

I would also like to thank my niece and nephew, Tiffany and Caleb who may one day read these words. Your innocent, unbiased and unconditional love for your 'uncle mike' is a blessing I am undeserving of.

Thank you all, for so much.

Michael.

Declarations.

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Statement 1.

This thesis is the result of my own investigation except where otherwise stated. Other sources are cited in the text, and a list of references appended.

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N.B. Presenter underlined, available abstracts in appendices section.*Awarded prize.

Abbreviations.

- μ - Micro, when used as a prefix.
- ADP – Adenosine di-phosphate.
- AF – Activator function, AF-1 or AF-2.
- AGO2 - Argonaute 2.
- AI – Aromatase inhibitor.
- AKT – Protein kinase B.
- AMD - Age-related macular degeneration (wet-form).
- APS – Ammonium persulphate.
- AP-1 - Activating protein 1.
- ATP – Adenosine tri-phosphate.
- bp – Base pair.
- BSA – Bovine serum albumin.
- CA9 - Carbonic anhydrase IX.
- CB – Clinical benefit.
- cDNA – complementary DNA.
- CHS - Chalcone synthase.
- CoR – Co-repressor.
- C-terminal – Carboxy-terminal/COOH.
- DAB – 3.3'-diaminobenzidine.
- DAPI – 4'6-diaminidio-2-phenylindole-2HCL.
- DBD - DNA binding domain.
- DCCM – Defined cell culture medium.
- DCIS - Ductal carcinoma in situ.
- DMSO - dimenthyl sulphoxide.
- DNA – Deoxyribonucleic acid.
- DNMTI - DNA methyltransferase.
- dNTP – Deoxynucelotide tri-phosphate.
- DPX – Di-butylpthalatexylene.

- DTT – Di-thiothreitol.
- E – Oestrogen.
- E1- Estrone.
- E2 - 17β-oestradiol.
- E3 – Estriol.
- EDTA – Ethylene diamine tetraacetic acid.
- EGFR – Epidermal growth factor receptor.
- ER – Oestrogen receptor.
- ERα – Oestrogen receptor Alpha.
- ERβ – Oestrogen receptor Beta.
- ErbB – Epidermal growth factor receptor family.
- ERE – Oestrogen response element.
- ERICA – Oestrogen receptor immunocytochemical assay.
- ERK – Extracellular-signal regulated kinase.
- FCS – Foetal calf serum.
- Fulv – Fulvestrant.
- g – Gram.
- GLUT-1 – Glucose transporter 1.
- H_2O – Water.
- HAT - Histone acetyltransferase.
- HCL – Hydrochloric acid.
- HD – High dose.
- HDAC - Histone deacetylase.
- HER – Human epidermal growth factor receptor.
- HGF – Human growth factor.
- HRP – Horse-radish peroxidise.
- HSP – Heat shock protein.
- ICC – Immuno-cytochemistry.
- IGF-1 - Insulin-like growth factor ligand.
- IGF-1R - Insulin-like growth factor receptor.

- KDa – Kilo Daltons.
- KRT- Keratin.
- L – litre.
- LBD - ligand binding domain.
- LD – Low dose.
- LTED - Long term oestrogen deprivation.
- M – Molar.
- m – Milli, when used as a prefix.
- MAPK – Mitogen-activated protein kinase.
- MCF – Michigan Cancer Foundation.
- MEK – MAP-kinase extra-cellular signal-regulated kinase.
- $MgCl_2$ – Magnesium chloride.
- MMLV-RT – Molony-murine leukaemia virus reverse transcriptase.
- mRNA – Messenger RNA.
- mTOR – Mammalian target of rapamycin.
- MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.
- Na_2HPO_4 – Di-sodium hydrogen orthophosphate anhydrous.
- Na_2MoO_4 – Sodium molybdate.
- NaCl – Sodium chloride.
- NaF – Sodium fluoride.
- NaH_2PO_4 – Sodium di-hydrogen orthophosphate.
- NaOH – Sodium hydroxide.
- $NaVO_4$ – Sodium orthovanadate.
- NCoA - Nuclear-receptor co-activator.
- N-terminal – Amino terminal/NH_2.
- NHS – National Health Service.
- NLS - Nuclear localisation sequence.
- NR - Nuclear receptor.
- OD – Optical density.
- PAGE – polyacrylamide gel electrophoresis.
- PBS – Phosphate-buffered saline.

- PCR – Polymerase chain reaction.
- PI3K – Phosphatidylinositol-3-kinase.
- PKC – Protein kinase C.
- PKR - Serine/threonine protein kinase.
- PMSF – Phenylmethylsulfonyl fluoride.
- PR – Progesterone receptor.
- PSMA - Prostate specific membrane antigen.
- RH – Random hexamers.
- RIP140 - Nuclear receptor-interacting protein 1.
- RiSC - RNA-induced silencing complex.
- RNA – Ribonucleic acid.
- RNAi – RNA interference.
- RNase – Ribonuclease.
- rpm – Revolutions per minute.
- RPMI – Roswell Park Memorial Institute.
- RT-PCR – Reverse transcription-polymerase chain reaction.
- SARS – Severe acute respiratory syndrome.
- SDS – Sodium dodecyl sulphate.
- SERD – Selective oestrogen receptor down-regulator.
- SERM – Selective oestrogen receptor modulator.
- SFCS – Charcoal-stripped foetal calf serum.
- SiC – siRNA control.
- siRNA – Small interfering RNA.
- SNALP - Stable nucleic acid-lipid particle.
- SP-1 - Specificity protein 1.
- SRA - steroid receptor RNA-activator.
- STMN1 – Stathmin.
- TAE – Tris-acetate-EDTA buffer.
- Taq - *Thermus aquaticus.*
- TBS – Tris-buffered saline.
- TBS-Tween – Tris-buffered saline containing 0.05% v/v tween-20.

- TEMED – N,N,N',N'-tetramethylene-diamine.
- TESPA – 3-aminopropyltriethoxysilane.
- TGFα – Transforming growth factor alpha.
- TLR - Toll-like receptor.
- Tris – Tris(hydroxymethyl)aminomethane.
- TTP – Time to progression.
- Tween-20 – Polyoxyethylene-sorbitan monolaurate.
- UV – Ultra violet.
- V – Volts.
- VEGF - Vascular endothelial growth factor.
- VIM – Vimentin.
- v/v – Volume per volume.
- w/v – Weight per volume.

Chapter 1. Introduction Section.

1.1. Cancer.

Cancer is probably the most feared of all medical diagnoses, a group of diseases which are typified by the uncontrolled division of cells, leading to tumour formation and subsequent local tissue degradation, and invasion into surrounding tissues (metastases). This aberration of normal cell behaviour, increased proliferation and unbalanced tissue homeostasis is due to alteration by inappropriate activation or suppression of components within cell regulatory systems. Occurrence of cancer is not confined to a specific area within the body, and over two-hundred different forms have been characterised, in all major tissues and organs, most commonly named after the site of original tumorigenesis.

Cancer is also not a recent discovery, since reference to the phenomena of a tumour of the breast was discovered on Egyptian papyrus dated roughly 3500 years ago. The most common term for cancer is Carcinoma and was coined by the "Father of medicine" the Greek physician Hippocrates, who determined the difference between benign and malignant tumours around 2400 years ago, due to the crab-like (Carcino) swelling (Oma) he observed in malignant tissue (Hippocrates 400B.C.).

Today cancer is a major world-wide disease, estimations determine that in 2008 there were over 7.6 million cancer related deaths worldwide and 12.7 million new cases of cancer diagnosed in this period (Jemal *et al* 2011). This means globally, nearly 21,000 people die from cancer and 35,000 people are newly diagnosed with cancer daily and the incidence rate is rising.

Globally the most commonly diagnosed cancers in males are that of the lung, equating to 13% (1.6 million) of new cases and 18% (1.4 million) of cancer related deaths. Breast cancer is the most common form of cancer in females equating to 23% (1.38 million) of all new cancer cases in 2008 and 14% (458,400) of total cancer deaths (Jemal *et al* 2011).

In the United Kingdom there is a 1 in 3 risk of developing cancer over a lifetime. Between the years 2004-2006 an average of 147,000 males and 146,000 females were diagnosed with cancer each year, and approximately 80,000 males and 74,000 females died from cancer each year during this period. In 2004 in the UK, cancer accounted for 29% of total male death, and 25% of total female death. Of these incidences breast cancer was most common, and accounted for a third of all new cases in 2006 (Office for National Statistics 2009).

Due to increases in health care there has been a decline in number of deaths caused by the other major European killers (infectious disease, heart disease and stroke). This means the proportion of cancer-related deaths has increased. However, while the incidence of cancer cases has increased by 26% between 1979 and 2008, with 309,500 new cases now diagnosed annually. Cancer-related mortality in the UK has actually decreased by 20% between 1979 and 2008 with 156,000 deaths from cancer in 2008 and lung, bowel, breast and prostate cancer all showing significantly reduced mortality rates, especially within the last decade (Cancer Research UK Statistical Information Team 2011). The improvement in cancer-related mortality is due primarily to earlier detection and greater availability of therapeutic options. However even

without new therapeutic options it is believed that approximately 50% of all cancer cases could be prevented if significant lifestyle changes were implemented worldwide, attitudes on tobacco, alcohol, physical activity and sun exposure being the most important of these (Stein & Colditz 2004).

Today cancer still claims a large number of patients and understanding the biology of cancer is of paramount importance. Cancer development is complicated, and now known to be due to a cellular evolutionary process caused by genomic instability (Schneider and Kulesz-Martin 2004). It has been discovered for example, that more than half of all cancers show mutations within the TP53 gene. This gene encodes for the p53 tumour suppressor protein, which usually plays a protective role against DNA damage, thus these cancers lack this pro-apoptotic function and become tumours rather than undergoing apoptosis (Vogelstein *et al* 2000, Hanahan and Weinberg 2000). Cancers have six commonalities, various aberrant mechanisms that contribute to their behaviour, and these are;

1. Self-sufficient growth signalling.
2. Insensitivity to growth inhibition signals.
3. Resistance/evasion of apoptosis (programmed cell death).
4. Limitless replication potential.
5. Sustained angiogenesis.
6. Metastasis.

These processes are all still important areas of research, able to deliver real clinical benefit to patients by discovery and manipulation of novel therapeutic

targets within these systems. However despite the best effort of science, currently 1 in 4 people will still die of cancer.

1.2. Breast cancer.

1.2.1. UK and Worldwide facts and figures.

Breast cancer is the most common form of female cancer in the UK, with a lifetime risk of suffering from the disease now being roughly 1 in 8. This disease accounts for more than 30% of all female cancer (Quinn *et al* 2001) with approximately 45,000 new cases in the UK (Cancer Research UK 2009), and 1 million new cases diagnosed worldwide annually (Jemal *et al* 2007). 4.4 million cases have been diagnosed within the last five years (Parkin and Fernandez 2006), this representing by far the most common form of cancer in women worldwide (Boyle *et al* 2005).

In the UK 12,082 people (11,990 women and 92 men) died from breast cancer in 2007, making it the second most common cause of death from cancer (after lung) in women (Cancer Research UK 2009). Worldwide, 465,000 people died from breast cancer in 2007, again making it the second most common form of cancer worldwide when both sexes are considered (Parkin and Fernandez 2006), highlighting the importance of breast cancer as a major health issue (Irvin and Carey 2008).

Approximately 80% of UK cases occur in postmenopausal women who are 50 years plus in age, this is roughly 36,000 of the new cases each year (Cancer Research UK 2009). Despite increases in survival rates, breast cancer still claims an average of 12,500 lives a year in the UK alone. This equates to 17% of all female cancer related deaths.

More than half of all breast cancer cases occur in the 'developed' world, with the highest incidence rate being in the USA, with approximately 180,000 new cases expected in 2007 (Jemal *et al* 2007). The increased incidence rate is believed to be due in part to better access to screening programmes in these areas (Parkin and Fernandez 2006). The lowest incidence rates are in African and Asian populations, who have a five –fold lower risk (Key *et al* 2001), though the incidence there is also on the increase (Parkin and Fernandez 2006, McPherson *et al* 2000). The increasing incidences in developing countries are thought to be on the rise as a westernised lifestyle becomes adopted, adopting as well the related risk factors associated with breast cancer. It has also been noted that people moving from low to high risk countries acquire the same level of risk as natives of the host country within two generations (Key *et al* 2001), illuminating the importance of life-style choice, socio-economic status as well as environmental factors in the development of breast cancer (Parkin and Fernandez 2006, McPherson *et al* 2000). In the UK between 1981 and 2005, the incidence rate increased by 57%, due mainly to the introduction of a national screening programme by the NHS in 1988 (Hery *et al* 2008). This lead to an increase in breast cancer incidence as early undiagnosed cancers were detected in women aged 50-64

(Quinn and Allen 1995), with an estimated 1400 UK women being saved annually by this programme.

Unfortunately while there are large differences in the incidence rates between countries, the difference in mortality rates is far smaller, meaning that in the less developed world more breast cancer sufferers die from the disease (Parkin and Fernandez 2006). However, encouragingly in the UK there has been a steady decrease in death rate since the 1980's believed to be due to the improved screening, and the development and application of more effective treatments including anti-hormonal measures of which tamoxifen has been the principal agent (Peto *et al* 2000).

The high incidence rate of breast cancer and the fact that the disease claims over 400,000 lives globally every year, shows the worldwide health threat that this disease presents, and also shows the importance of the ongoing research into breast cancer. The understanding of this disease with onus on new methods to reduce mortality and incidence rates should be a global goal, which would benefit millions worldwide.

1.2.2. Risk factors in breast cancer.

There are many factors which can increase a person's chance of developing breast cancer; both environmental and genetic factors have been associated with an increased risk of being diagnosed. Some modifiable risk factors associated with Western lifestyle choices, which increase the chance of

developing breast cancer are; obesity (Van den Brandt *et al* 2000), alcohol consumption (Hamajima *et al* 2002) and a lack of exercise (Key *et al* 2001) although the extent of the risk is not fully determined.

One of the greatest risk factors (after gender) leading to an increased chance of breast cancer development is age, with over 80% of breast cancer cases in women over 50 years of age, which arise postmenopausally (McKeage *et al* 2004). Increasing age is an important risk factor in both pre and post-menopausal women, though the risk is increased following menopause (Barlow *et al* 2006). Breast cancer incidence doubles roughly every 10 years until the menopause, so that the risk of developing breast cancer by the age of twenty nine is estimated at 1 in 2,300, but by the age of forty nine, the chance has increased to 1 in 52 (Cancer research UK 2009).

As women account for over 99% of all breast cancer cases (Brekelman 2003), many breast cancer risk factors are associated with increased life-time exposure to female steroid hormones, in particular oestrogens, and exposure to both endogenous and exogenous oestrogens are major factors in development of breast cancer (McPherson *et al* 2000). Some exogenous oestrogens present in the environment identified as having oestrogen agonistic qualities can include xenoestrogens, phytoestrogens and mycoestrogens and are either synthetic, derived from plants, or from fungi, respectively, and are not always steroidal in structure (Fang *et al* 2001). However major risk factors associated with increased endogenous oestrogen exposure include, early menarche (before age 11) and late menopause (after age 54) (Kelsey *et al* 1993). A

woman's later age at first full-term pregnancy and parity (Layde *et al* 1989), as well as choice not to breast feed (Lipworth *et al* 2000) can all increase risk of developing breast cancer. Use of oral contraceptives (McPherson *et al* 2000) and hormone replacement therapy (HRT) (Beral 2003) have been shown to slightly increase the risk. The level of risk in these cases depend on duration and type of therapeutic used, and the risk will return to previous levels after treatment is stopped, though this can take up to a decade (Veronesi *et al* 2005).

While most breast cancers are 'sporadic', caused by accumulation of mutations over a lifetime, leading to incomplete DNA repair, loss of apoptotic triggers, uncontrolled proliferation and an increased migratory capacity in these cells (Pisano *et al* 2008, Finkel 1999), some breast cancers are linked to inheritance of mutated genes. Women with family history of the disease are responsible for up to 10% of breast cancers in the western world (McPherson *et al* 2000), suggesting involvement of hereditary genetic factors. Indeed several genes have been identified which are believed to be responsible for an inherited pre-disposition to developing breast cancer. Of these the tumour suppressor genes *BRCA1* and *BRCA2* are the most understood and clinically relevant. Carrying a mutated form of *BRCA1* or *BRCA2* is believed to account for up to 10% of patients diagnosed breast cancer (McPherson *et al* 2000) and up 45% in families diagnosed with multiple cases (Evans *et al* 1994). Carrying a mutated form of *BRCA1*significantly increases the chance of developing breast cancer (Telli and Ford 2010). Encouragingly though, the survival rates for patients with such tumours are similar to that of patients with sporadic

tumours (Chang and Elledge 2001) despite *BRCA1* expressing cancers being associated with more aggressive disease (Evans *et al* 1994).

1.2.3. Breast Cancer stages and treatments.

Breast cancer is classified into grades and stages and treated accordingly, depending whether the tumour is classed as non-invasive (confined to the breast ducts without the ability to spread, also termed Ductal carcinoma *in situ* (DCIS)) or invasive, what is generally meant when the term 'breast cancer' is used . Invasive ductal carcinoma describes a tumour that has spread into the breast tissue and is also able to spread to other parts of the body. Tumours are graded following histological assessment, looking at cellular appearance to give an indication of growth rate and metastatic potential, with the grade increasing in direct correlation. Invasive breast cancer is split into four stages depending on the spread and size of the tumour, known as the tumour, node, metastasis system (McGuire 1991);

- Stage 1. The tumour is less than 2cm with no sign of spread.
- Stage 2. The tumour is 2-5cm with/out lymph node spread and no spread to other parts of the body.
- Stage 3. The tumour is larger than 5cm but is fixed to chest wall, muscle or skin.
- Stage 4. The tumour is any size, with/out lymph node involvement but has spread to other parts of the body (metastases).

Surgery is the most common strategy to treat most stages of cancer; this can be combined with chemotherapy or radiotherapy therapy either prior or post surgery (Bundred 2001). Radiotherapy is commonly given after surgery to destroy cancerous cells not removed during the operation. Chemotherapy can also be used prior to surgery to shrink the size of the tumour or afterwards to destroy any cells that may have spread, or used in patients unable to undergo surgery (Veronesi *et al* 2005). The surgical operation performed is dependant on how advanced the cancer is. The operations range from a lumpectomy (removal of the tumour with some surrounding breast tissue) to the more extreme modified radical mastectomy (complete breast removal and some muscle tissue removal from the chest wall). Lymph nodes can also be removed as a measure of cancer spread beyond the breast (Axelsson *et al* 1992), although more recently sentinel node biopsy has been employed as a measure of detecting cancer spread, with a negative sentinel node biopsy giving only a 0.3% change of axillary re-occurrence (Van der Ploeg *et al* 2010).

Roughly half of the patients treated with surgery and radiotherapy will relapse and die from metastatic disease and it is believed that in these cases the cancer must have spread undetected before the surgery is performed (Richards and Smith 1994). As the risk of relapse and spread is likely, additional (adjuvant) chemotherapy or anti-hormone therapy may be given post surgery. The use of adjuvant systemic therapy has been shown to save an additional 10 or 12 lives per 100 patients treated (Early Breast Cancer Trialists Collabritive Group 2002).

The choice of which adjuvant therapy to use depends on the tumours endocrine responsiveness. This is indicated by histological staining of the tumour for the presence of steroid hormone receptors, the presence of either the progesterone receptor (PR) or the oestrogen receptor (ER) being a good indication that endocrine therapy will be effective (Veronesi *et al* 2005) and testing for these receptors is now standard following surgery. In hormone receptor positive cancer endocrine therapy is preferable to chemotherapy due to higher success rates, and patient toleration as it is associated with less severe side-effects. In pre-menopausal cases ovary function is ablated either surgically or therapeutically, removing the primary oestrogen source in these patients, and can be accompanied with anti-oestrogen therapy. However in post-menopausal cases, patients are offered a variety of anti-hormone therapies; these are discussed in depth in a following section (see section 1.5.).

Patients with no indication of PR or ER are offered courses of chemotherapy to prevent disease development (Veronesi *et al* 2005). Chemotherapy is the administration of various cytotoxic compounds, tailored for best responses in individual cases. These are usually administered every 3-4 weeks during a 4-6 month period (6 courses in total), but unfortunately this is usually accompanied by severe side-effects, including infertility, chronic fatigue, nausea and hair loss despite its anti-tumour efficacy.

1.3. Oestrogens and their link to breast cancer.

1.3.1. Oestrogens.

Oestrogens, the primary ligand for the oestrogen receptor (ER) play an important role in development of the secondary sexual characteristics of females during puberty, and are vital in the maintenance of reproductive function. Oestrogens also perform a variety of other functions, with roles in cardiovascular, musculoskeletal, immune and central nervous systems in both males and females (Gustafsson 2003). They are a unique family of aromatic steroids being produced in the body by conversion of cholesterol to androstenedione and then testosterone. Testosterone is finally converted to 17β-estradiol (E2) which is catalysed by the cytochrome p450 enzyme aromatase. Primarily oestrogens are produced in the ovaries, but secondary sources of oestrogens include the brain, liver and adipose tissue (Dowsett *et al* 2005) which become the main source of this ligand following menopause. While E2 is the most biologically important oestrogen in females between menarche and menopause, it has two metabolites, oestrone (E1) and oestriol (E3) with more E1 than E2 present following menopause. However, E1 has far less of an agonistic effect than E2 on the ER despite high affinity binding (Kuiper *et al* 1997).

1.3.2. Oestrogen function in breast cancer.

Many breast cancer risk factors (described in section 1.2.2.) relate to increased lifetime oestrogen exposure, and both clinical and experimental evidence of the link between oestrogens and breast cancer development has been documented for over 150 years. However, the greatest breakthrough came in 1896 when George Beatson demonstrated that breast tumour regression could occur following oophrectomy (removal of ovaries) in patients (Beatson 1896). The biological link between oestrogen ablation and tumour regression came over 50 years later with the discovery by Elwood Jenson of an oestrogen binding protein found in a rat uterus, now recognised as ER-alpha (Jenson 1962). Several years later the first ER assay was developed and was subsequently used to determine whether patients would respond to either oopherectomy (pre-menopausal patients), or adrenalectomy (post menopausal patients) (Jenson *et al* 1971).

It is now widely accepted that the presence of functional estrogen receptor within a tumour indicates its hormone responsiveness (Bundred 2001 and Veronesi *et al* 2005), and roughly 60-70% of breast cancers are termed hormone responsive and as such are reliant on oestrogen for growth and may respond to anti-oestrogen therapy (discussed further in section 1.6.).

1.4. Oestrogen receptors (ER).

1.4.1. Oestrogen receptor discoveries and genetic structure.

Following the discovery of the oestrogen binding protein (ERα) by Jacobson and colleagues, the gene encoding this receptor was eventually cloned from the MCF-7 human breast cancer cell line and designated ESR1 (Green *et al* 1986). ESR1 is a large gene, 322kb in size and located on the long arm of the sixth chromosome (MacGregor and Jordan 1998). This gene contains a large promoter region (over 150kb) and a protein coding region of approximately 140kb, containing 8 exons and 7 introns (Gosden *et al* 1986) which encodes for a complete protein of 595 amino acids with a molecular weight of 66kD (MacGregor and Jordan 1998).

A second distinct oestrogen receptor (ERβ) was later discovered and cloned from rat prostate (Kuiper *et al* 1996) and is encoded by the ESR2 gene. The ESR2 gene is 235kb in size and located on the fourteenth chromosome, encoding for a protein of 485 amino acids with a molecular weight of 55kD (MacGregor and Jordan 1998).

1.4.2. Oestrogen receptor protein structure.

ERα and ERβ are both members of the nuclear receptor superfamily (NR), a family of hormone activated transcription factors able to initiate or enhance

transcription of genes containing hormone responsive elements. Other members of this family include the progesterone receptor (PR), thyroid hormone receptor, retinoid and vitamin-D receptors (Mangelsdorf *et al* 1995). Both ER proteins are divided into six structural domains, dedicated A-F from the Amino (NH_2) terminus to the carboxyl (COOH) terminus and share some homology between domains, especially in the DNA binding domain (figure 1.1.).

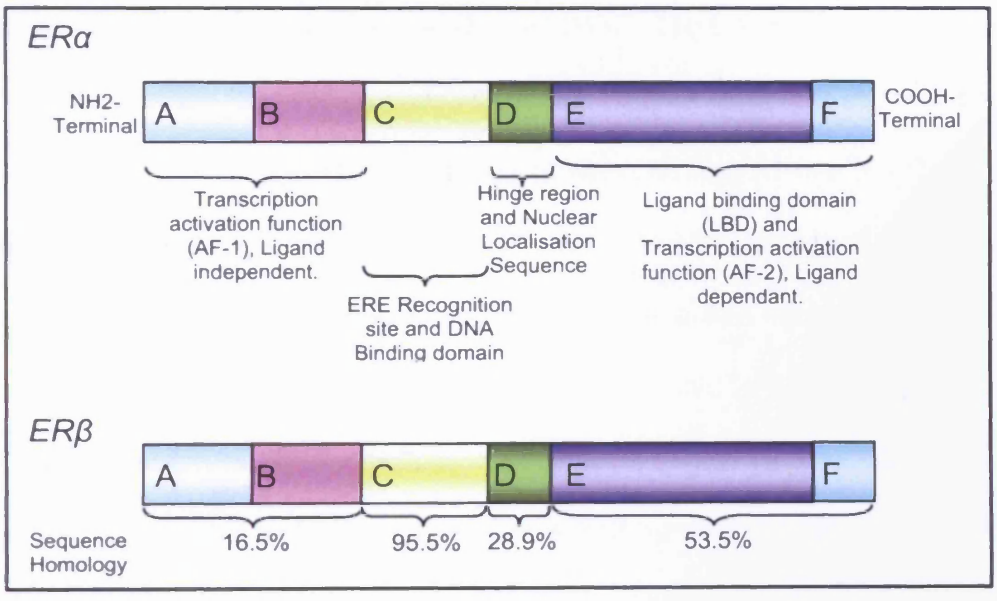


Figure 1.1. Diagram of ERα and ERβ proteins showing A-F domains, percentage homology, and functional structures.

The A and B domains present at the amino terminus are poorly conserved and contain the transcription activation function (AF-1) site, a ligand-independent transcriptional activation function domain which is constitutively active (Campbell *et al* 2001).The C domain contains the highly

conserved DNA binding domain (DBD). The DBD comprises two zinc-fingers able to fold into helical structures, these are involved in the recognition of oestrogen responsive elements (ERE) on the DNA, one helix binds directly to the DNA and the second supports this structure (Schwabe *et al* 1993).

The D domain is termed the hinge region, between the DBD and the ligand binding domain (LBD) (Osborne *et al* 2000). This domain also contains nuclear localisation sequences (NLS), and sites important for receptor dimerisation, which is required for full transcriptional activity, as well as sites important for co-factor interaction (Klinge 2001). The C terminus region is comprised of the E and F domains which also contain sequences important for receptor dimerisation, co-factor interaction sites and sites for binding of chaperone proteins, such as HSP90 (Parker *et al* 1993). This region also contains the ligand binding domain (LBD) and the second transcription activation function (AF-2) domain (Nicholson *et al* 2002). The LBD is a structure comprised of 12 α-helices. Five of these helices form a hydrophobic pocket to allow ligand binding. The binding between E2 and ER results in an alteration in receptor conformation, with helix 12 (H12) forming a 'lid' over the binding pocket, securing the ligand and with interactions between helices 3-5 promoting co-factor recruitment and transcriptional activity (Brzozowski *et al* 1997). In the presence of an oestrogen antagonist, which usually contain a bulky side chain (figure 1.2.) H12 is displaced preventing co-activator recruitment to this domain and so inhibiting AF-2 driven transcription (White and Parker 1998).

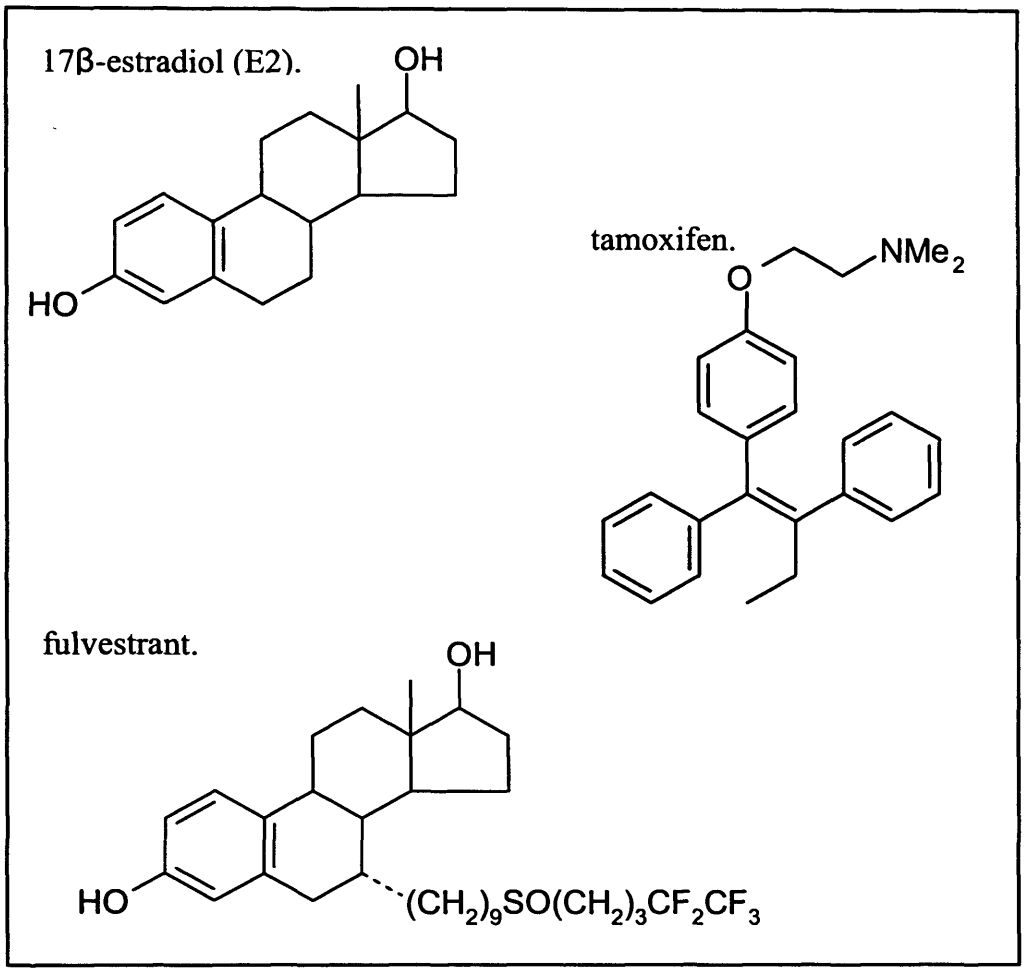


Figure 1.2. Structure of 17β-estradiol and the common anti-oestrogens, tamoxifen and fulvestrant.

1.4.3. Activator function domains of oestrogen receptors.

AF-1 is constitutively active and does not require the presence of a ligand to function, however its activity can be increased following phosphorylation at multiple sites. One example would be phosphorylation of the serine 118 residue, required for maximal activity of the AF-1 domain (Nicholson *et al* 2002). AF-2 is dependant on ligand binding to the LBD for full transcriptional activity.

While activation of both AF-1 and AF-2 is usually required for maximal ER transcriptional activity, not all genes need both AF-1 and AF-2 to be active for successful transcription. The fact that both domains are able to function independently has implications for some forms of anti-oestrogen therapy (Osborne *et al* 2000).

1.5. Oestrogen receptor signalling.

In the absence of E2 the ER exists in an inactive, monomeric form most commonly within the nucleus, bound in a large protein complex containing stabilising chaperone proteins such as the heat-shock proteins HSP90, HSP70 and heat-shock interacting protein p23 (Pratt and Toft 1997). Following E2 exposure there are three mechanisms of oestrogen receptor signalling, 'classical' and 'non-classical' genomic signalling and non-genomic signalling. These processes are illustrated in figure 1.3. and explained in more detail in the following sections, briefly however 'classical' genomic ER signalling occurs when E2 binds to the nuclear ER, the receptor forms a dimer which binds to oestrogen responsive elements (EREs) on gene promoters, which subsequently recruits and drives the transcription machinery. In non-classical genomic signalling, E2 binds to the nuclear ER and dimerisation occurs but the E2-ER complex interacts with other transcription factors to drive transcription at alternative promoters. Finally non-genomic ER signalling occurs rapidly, where cytoplasmic or membrane associated ER binds with E2

and forms direct protein-protein interactions which activate growth signalling pathways.

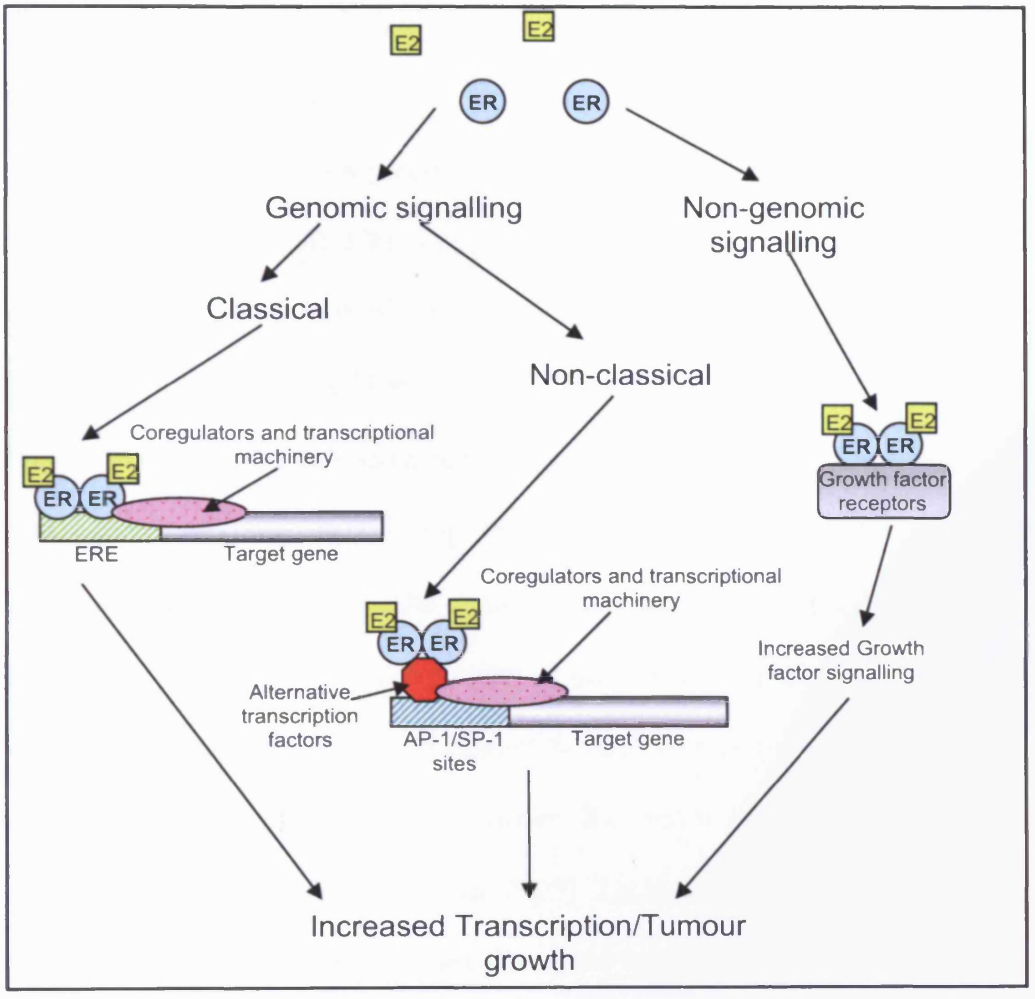


Figure 1.3. Mechanism's of Estrogen receptor signalling (adapted from Schiff et al 2003).

1.5.1. 'Classical' genomic ER signalling.

E2 is steroidal and lipid soluble in nature and thus able to pass through the cell plasma membrane and into the nucleus where it binds at the LBD within ER (section 1.4.2.). This causes a conformational change in the receptor leading to

phosphorylation at multiple serine and tyrosine residues occurring and its dissociation from the HSP90-based chaperone complex (Nicholson *et al* 1999), permitting maximal AF-1 activity (Osborne and Schiff 2005). The now active ligand-receptor complex rapidly homo or hetero-dimerises and binds to specific sequences of DNA present in the gene promoters known as EREs (White and Parker 1998). EREs are repeats of a palindromic sequence of GGTCA split by three variable nucleotides, though most oestrogen-regulated genes do not have perfect ERE sequences (Driscoll *et al* 1998). The ERE bound ER complex is able to recruit numerous co-regulatory proteins, able to either enhance (known as co-activators) or suppress transcription (co-repressors) and depending on the ratios of these co-regulators present in the tissue, The ER/DNA/co-regulator complex promotes or represses transcription of the gene. Many of the genes regulated by oestrogen in this way are involved in processes such as increased cell proliferation, inhibition of apoptosis or regulation of invasion (Osborne *et al* 2005). The interactions between the ER and these co-regulators are complex due to the fact over 200 have currently been identified, and new ones are still being discovered (Lonard and O'Malley 2006).

Co-activators have been shown to contain an 'nuclear receptor box' (NR box), comprised of a conserved 5 amino acid motif containing three leucines and two other amino acids in an α-helical LxxLL structure. Within the NR box the leucines create a hydrophobic surface that is able to bind to the major groove of the receptor's AF-2 domain (McKenna and O'Malley 2002, 2002a, Hall and McDonnell 2005). AF-1-interacting co-activators have also

been described, including steroid receptor RNA-activator (SRA) and p68RNA helicase (Lanz *et al* 1999, Endoh *et al* 1999). The most common function of most co-activators is histone acetyltransferase (HAT) activity.

The common compact chromatin state is due to attraction between positively charged lysine residues present on histones and the negatively charged DNA phosphate backbone which wraps around these, limiting access to the transcriptional machinery, and thus the efficiency of gene activation. HAT enzymatic function catalyzes the acetylation of these lysine residues in histone tails, removing the positive charge resulting in a less condensed chromatin structure which, in parallel with other DNA modifications, results in local decondensation of the chromatin necessary for gene activation (Tsukiyama and Wu 1997, Urnov and Wolffe 2001). The resulting remodelling of the local chromatin structure allows recruitment of the transcriptional machinery, facilitating gene transcription. (Histone deacetylase's (HDAC's) are able to remove these acetyl groups from the lysine residues, reversing this process and reducing gene activation, see below).

The most established AF-2 interacting co-activators are the three members of the p160 family, nuclear-receptor co-activator 1 (NCoA1 or SRC1), NCoA2 (TIF2 or GRIP1) and NCoA3 (P/CIP, ACTR, AIB-1, RAC3 or TRAM1) (McKenna *et al* 1999). While these contain intrinsic HAT activity their primary function is believed to be recruitment of other co-activators which contain NR boxes and HAT activity such as the SWI/SNF complexes

(Sudarsanam and Winston 2000), p300/CBP-associated factor (PCAF) (Vo and Goodman 2001), and the CREB binding protein.

Co-repressors play an equally important role in regulation of ER-mediated gene expression by repressing transcription by interacting with unliganded or antagonist-bound receptors (Dobrzycka *et al* 2003). Co-repressors are able to repress transcription via a number of mechanisms such as competitive binding versus co-activators, localisation of ER to the cytoplasm, and can interfere with DNA binding by condensing chromatin via intrinsic HDAC activity or recruitment of HDAC's (Dobrzycka *et al* 2003).

The first studied co-repressors were the nuclear receptor co-repressor (NCoR) (Horlein *et al* 1995) and the silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Chen and Evans 1995). These co-repressors bind to the LBD of ER as they contain two domains termed CoR-NR boxes which are similar to the NR boxes found in co-activators (Hu and Lazar 1999). NCoR and SMRT are both able to suppress transcriptional activation of ER in the absence of ligand (though they dissociate upon agonist binding) by containing intrinsic silencing domains (Horlein *et al* 1995, Chen and Evans 1995) and by the recruitment of other co-repressors, such as mSin3 which associate with HDACs (Hu and Lazar 2000). Other ER co-repressors, such as nuclear receptor-interacting protein 1 (RIP140), associate with ligand bound ER, preventing access to AF-2 for co-activators, and interact with HDAC complexes (Smith and O'Malley 2004), to bring about one mechanism of oestrogen-repressed events.

New research suggests that although they have opposite functions, both co-activators and co-repressors are both associated with ER in the same multi-protein complex. Oestrogen binding to the receptors results in a rapid reorganisation of these co-factors, the balance and activity of which regulate the expression of the nuclear receptor target genes (Acconcia and Kumar 2006).

1.5.2. 'Non-classical' genomic ER signalling.

'Non-classical' genomic ER signalling utilises protein-protein interactions (also known as tethering) between ER and other DNA-bound transcription factors to regulate transcription of genes without traditional ERE sequences. Signalling in this manner, the ER itself acts as a co-activator for alternative transcription factors, strengthening DNA binding and recruiting other co-activators to the transcription-factor complex to promote gene expression (Shupnik 2004). Through this mechanism ER is able to facilitate transcription of a wider variety of genes by tethering, for example to AP-1 (activating protein 1) transcription factor, which is comprised of a heterodimer of c-jun and c-fos and driving transcription at AP-1 binding sites. Genes such as cyclin D1 and Insulin-like growth factor ligand (IGF-1) are regulated by E2 in this way (Cheung *et al* 2005, Osborne *et al* 2005). As well as mediating transcription at AP-1 binding sites, activated ER is also able to bind with the Specificity protein 1 (SP-1) transcription factor which regulates transcription of genes controlled by GC-rich promoters (Kim *et al* 2005). Other promoter

sequences that are influenced by non-classical genomic ER signalling also include the STAT and NF-κB response elements (Nicholson *et al* 2002) which can lead to the regulation of proteins important for cell proliferation and survival, such as insulin-like growth factor receptor (IGF-1R) and Bcl-2 (Osborne *et al* 2005).

1.5.3. Non-genomic ER signalling.

Genomic ER signalling as previously described, takes hours to occur, however recently, evidence of more rapid events occurring within minutes of exposure to steroid hormones, which can not be attributed to transcriptional activation of genes by ER have been reported (Song and Santen 2006). Non-genomic signalling is thought to take place at either the plasma membrane or in the cytoplasm and can require translocation of ER from the nucleus, or ER previously present at these positions (Shupnik 2004). Upon activation by E2 these membrane/cytoplasmic ER are able to rapidly (within seconds), activate signalling molecules such as IGF-IR, EGFR, Raf, AKT, Protein Kinase C and MAPK (Levin 2005, Song and Santen 2006). ER phosphorylation forms a docking site for SH2-domain containing proteins (Barletta *et al* 2004) allowing a formation of a large signalling protein complex which allows direct, physical associations between the ER and molecules such as Shc, Src and PI3K (Shupnik 2004). These protein complexes can then activate EGFR and IGF-1R through these interactions. (Song and Santen 2006).

Downstream activation of MAPK and AKT signalling pathways in this manner, can cause up-regulation of genomic ER transcription through phosphorylation of serine residues (118, and 116) on the AF1 domain of ER present within the nucleus (Campbell *et al* 2001). Non-genomic ER signalling via MAPK and AKT can also promote phosphorylation of AP-1 and SP-1 transcription factors tethered to E2 bound ER further increasing their transcription activity (Levin 2005). Thus, non-genomic ER signalling has the capability to exert rapid control over ER-responsive genes, via this impact on nuclear ER function (Campbell *et al* 2001), as well as via direct growth factor signalling (kinase) mechanisms.

1.6. Anti-hormone therapy.

As oestrogen, via oestrogen receptor signalling is able to increase proliferation, as well as decrease apoptosis and increase cell survival in ER positive breast cancers various therapies based on inhibiting this phenomenon have been developed. These treatments include the anti-oestrogenic ER inhibitors known as selective oestrogen receptor modulators (SERM's) and selective oestrogen receptor down-regulators (SERD's), which work by competitively binding to the ER to block its function and in the case of SERD's promote degradation of the receptor. An alternative class of anti-hormones are the aromatase inhibitors (AI's) which prevent the formation of endogenous oestrogens by inhibition of a vital step in oestrogen synthesis. The

mechanism of these compounds, their use in the clinic and complications that can arise from their application will be discussed in the following sections.

1.6.1. Selective oestrogen receptor modulators.

Selective oestrogen receptor modulators (SERM's) are synthetic non-steroidal anti-oestrogens. They are termed SERM's as they have the ability to act as both ER antagonist and agonist in a tissue specific manner (Lewis and Jordan 2005). The most commonly known and used molecule in this class is tamoxifen, a non-steroidal triphenylethylene derivative. Tamoxifen was originally developed as a contraceptive, as anti-oestrogens were shown to prevent ovulation in animals, however this did not translate into human studies and treatment actually induced ovulation (Jordan 2006). After evaluation in the breast cancer context, in the 1970's the drug tamoxifen was ''reinvented'' as the first adjuvant therapy for ER positive breast cancer, shown to be inhibitory in breast cancer and so becoming the most commonly administered form of endocrine therapy. It emerged as the gold standard treatment for over 25 years with 400,000 women believed to be alive today as a direct result of its use (MacGregor and Jordan 1998 and Jordan 2003, 2003a, 2006).

Tamoxifen competitively binds to the ER at the LBD, thus, preventing oestrogen from doing so. Similar to E2 binding the tamoxifen-ER complex induces a conformational change in the receptor, causing chaperone dissociation, receptor dimerisation and translocation of the tamoxifen-ER dimer to promoters of genes regulated by oestrogen (Nicholson *et al* 2002).

However due to the shape of tamoxifen with its side chain, H12 of the LBD is in an alternative position than when E2 is bound and, thus, inhibits the ligand dependent AF-2 domain (Pearce *et al* 2003). This prevents full activation of the ER and inhibits AF-2 mediated gene transcription, disrupting 'normal' regulatory signalling controlling tumour proliferation and to some extent cell survival (Osbourne *et al* 2000). This conformational change in the tamoxifen-ER dimer can also further inhibit gene transcription by preferential recruitment of co-repressors such as NCoR and SMRT to the gene promoters bound by this complex (Webb *et al* 2003, Lewis and Jordan 2005).

Unfortunately, tamoxifen inhibition of ER signalling is incomplete and genes that require the AF-1 domain for enhanced transcription are unaffected by use of tamoxifen, indeed, some genes show increased expression due to the partial agonist properties of tamoxifen (Howell 2006 and Osborne *et al* 2000). The oestrogenic activity of tamoxifen through AF-1 mediated gene transcription can be further increased due to receptor phosphorylation of ER at serine residues 118 and 167 by growth-factor signalling pathways promoting ligand independent gene transcription and cell growth (Ring and Dowsett 2004, Shah and Rowan 2005). Binding of tamoxifen to ER can also promote non-genomic ER signalling which can in turn promote the activation of these growth factor signalling pathways, further enhancing tamoxifen agonist activity on genomic ER signalling (Fan *et al* 2007). Tamoxifen has also been shown to act in agonistic manner through non-classical genomic oestrogen receptor signalling particularly in interaction with AP-1 sites (Osborne *et al* 2000).

Although well tolerated in the clinic, the incomplete inactivation of ER activity by tamoxifen can lead to resistance and due to its oestrogen agonistic activity in other tissues. Long term tamoxifen use can lead to increased incidences of unwanted side-effects such as stroke and endometrial cancer as well as recurrence (Fisher *et al* 1998). As such tamoxifen treatment is often limited to 5 year maximum (Jones and Buzdar 2004). However, recent studies such as the ATLAS trial which explored 10 vs. 5 years tamoxifen have re-visited this concept and interesting have shown that there may be some benefit to continuing tamoxifen treatment beyond this period (Peto *et al* 2007).

1.6.2. Aromatase inhibitors.

Following the complications associated with long term tamoxifen use and the possibility of acquired resistance in the clinic, a new class of drug was designed to treat oestrogen receptor positive breast cancer. These are called aromatase inhibitors (AI's), of which the third generation compound anastrozole (Arimidex) is the most commonly used in post-menopausal women. As stated previously, the main sites for oestrogen synthesis following menopause is adipose and muscle tissue, while some breast tumours themselves can also be sources of oestrogen production (Green and Furr 1999). The AI's are compounds designed to prevent the synthesis of oestrogen by interfering with the precursor conversion of androstenedione to oestrone by the inhibition of the cytochrome p450 enzyme aromatase. This treatment

reduces the level of oestrone available for conversion to E2 by 17β-hydroxysteroid dehydrogenase (Green and Furr 1999). The effect of this treatment is a reduction in the levels of circulating oestrogens within the body, removing it as a stimulus for oestrogen dependent tumours, oestrogen synthesis being disrupted in a similar manner to oopherectomy in pre-menopausal women (Nicholson and Johnston 2005). Zoladex (chemical castration) is used to bring about E2-deprivation in pre-menopausal women as AI's are not appropriate in this setting.

While the original first generation AI's had poor specificity and were not well tolerated due to high toxicity, the current third generation AI's including Anastrozole (as well as Letrozole and Exemestane) have proven highly effective at reducing estrogen levels (by 85-92%) (Nicholson and Johnston 2005) and oestrogen driven ER effects on cancer growth, as well as having high selectivity and a reduced toxicity profile (Jones and Buzdar 2004).

The ATAC trial comparing Anastrozole to tamoxifen (alone or in combination) in the clinic has shown that Anastrozole significantly prolonged disease-free survival, time to disease recurrence and reduced risk of distant metastases (and even some evidence of fewer deaths following recurrence of disease in the Anastrazole arm which may become more significant after long-term follow up) when compared to tamoxifen at all time points including the median end point of 120 months (Cuzick *et al* 2010). AI's were also better tolerated than tamoxifen, with fewer associated risks and side-effects such as endometrial cancer and thrombolytic events (Cuzick *et al* 2010). There were however some associated side-effects following AI treatment, such as an

increased number of fractures in this group. The fractures were attributed to loss of bone mineral density, though this can be countered by co-treatment with compounds such as bis-phosphonates which reduce bone demineralisation (Cuzick *et al* 2010). Despite some side-affects these data led to the recommendation that Anastrazole replace tamoxifen as the adjuvant therapy in post-menopausal ER positive cancer (Howell *et al* 2005) though further longer term studies into survival rates of patients using prolonged AI therapy need to be conducted (Nicholson and Johnston 2005).

The reason for AI's ability to out perform tamoxifen in this study maybe due to the ability of AI's to inhibit both oestrogen dependant genomic, and non-genomic ER signalling, where as tamoxifen has some weak agonistic activity, and may stimulate non-genomic ER signalling (Osbourne and Schiff 2005). Interestingly however the combined arm of tamoxifen and Anastrozole gave no clinical benefit over the tamoxifen condition alone and was stopped prior to the studies completion (Howell *et al* 2005). These data are not surprising as again tamoxifen is a weak oestrogen agonist at AF-1 and able to activate non-genomic functions of ER, promoting growth-factor-receptor and oestrogen independent ER signalling pathways (Staka *et al* 2005). Thus its addition prevented the impact of oestrogenic deprivation (Osborne and Schiff 2005).

AI's have been used successfully in the clinic, however there is scope for acquisition of resistance to AI's to develop in the clinic (Nicholson and Johnston 2005), and this has been indicated via generation of MCF-7 cell

models which have become resistant to oestrogen deprivation (Staka *et al* 2005).

1.6.3. Selective oestrogen receptor down-regulators.

Due to the side effects and risk of development of resistance, which can be a serious issue in the otherwise well received use of tamoxifen, work was also undertaken to discover compounds with equivalent (or improved) levels of success in ER positive breast cancer treatment, compounds with complete, rather than partial ER antagonistic effects (Morris and Wakeling 2002).

The search for a true ER antagonist able to block all ER signalling prompted the synthesis of a series of steroidal 7α-alkylamide oestradiol analogues. The original 'pure' oestrogen antagonist of this steroidal class to be investigated was ICI 164,384. This compound was first described by Wakeling and Bowler; their research showed this compound was able to completely block the action of both oestradiol and tamoxifen in rat uterus (Wakeling and Bowler 1987). Later work developed a far more potent pure anti-oestrogen, ICI 182,780 which is now known as fulvestrant (Faslodex) (Wakeling *et al* 1991). Fulvestrant is a 7α-alkylsulphinyl analogue of 17β-oestrdiol and like E2 is a steroidal compound, with a similar structure to E2. Crucially however, fulvestrant contains a long side chain present on the seventh carbon, vital for fulvestrant's anti-oestrogen properties determined by the length, flexibility and position of this chain (Bowler *et al* 1989, Howell 2006). The chemical structure of fulvestrant differs from tamoxifen and accounts for the differences

in function between these two compounds. Being based on the structure of E2 fulvestrant has a much closer ER binding affinity to the ER than tamoxifen, 89% of E2 (Wakeling and Bowler 1987). Fulvestrant was termed a 'pure' anti-oestrogen as it shows no oestrogen agonistic properties (Howell *et al* 2000) in any tissues (Osborne *et al* 2000). Fulvestrant was classed as a selective oestrogen receptor down-regulator (SERD) (Nicholson *et al* 2002) due to it possessing the property of being able to cause down-regulation of ER protein, reducing its cellular levels (Howell *et al* 2000).

Fulvestrant competitively binds to the ER at the LBD and by binding in this manner induces a serious conformational change (Osborne *et al* 2004, Morris and Wakeling 2002) in the receptor allowing dissociation of chaperonins. Once fulvestrant is bound, the H12 of LBD is disorientated and does not fit over the ligand, blocking transcriptional activity. The interference of the side-chain means the fulvestrant-ER complex is unable to homodimerise effectively and energy-dependant shuttling of ER between the cytoplasm and the nucleus is prevented, blocking ER nuclear localisation (Fawell *et al* 1990, Dauvois *et al* 1992). In addition to this fulvestrant exposure and subsequent ER binding causes a decrease in cellular ER levels via several mechanisms. The fulvestrant-ER complex is unstable in the cytoplasm and rapidly degraded by the Ubiquitin-proteasome pathway, reducing ER half-life, increasing ER turnover and reducing ER protein levels (Fawell *et al* 1990, Dauvois *et al* 1992, Wijayaratne *et al* 1999). As opposed to tamoxifen action, fulvestrant binding blocks the activity of both the AF-1 and AF-2 domains, thus although any remaining fulvestrant-ER complexes within the nucleus can bind to DNA,

ER regulated gene transcription is suppressed (Wijayaratne *et al* 1999). Fulvestrant not only suppresses transcription at ER mediated ERE's by ER protein down-regulation and preventing co-activator association but also suppresses non-classical genomic signalling, for example at AP-1 sites (Webb *et al* 2003). Fulvestrant also has been shown to suppress non-genomic signalling, with MAPK pathway activation via membrane associated ER ablated in the presence of fulvestrant *in vitro* (Improta-Brears *et al* 1999). In fact, when microarray analysis was performed by Frasor and colleagues on oestrogen regulated genes, fulvestrant was shown to suppress transcription of 95% of E2 up-regulated genes and to induce 91% of the E2 down-regulated genes studied (Frasor *et al* 2003). This was in stark contrast to the activity of tamoxifen which suppressed transcription of only 47% and induced 26% of E2 up-regulated and down-regulated genes respectively. Tamoxifen also showed agonist-like activity on 23% and 31% up-regulated and down-regulated genes respectively (Frasor *et al* 2003).

Fulvestrant exposure has been shown to have greater anti-tumour activity than Tamoxifen in both ER positive breast cancer cell models and animal studies. Preclinical use of fulvestrant resulted in a reduction of ER protein levels (but not mRNA) as well as decreased expression of ER-regulated genes such as progesterone receptor (PR) and pS2 in the MCF-7 breast cancer cell line (McClelland *et al* 1996). In MCF-7 xenograft models this was also the case, and reduced levels of other oestrogen-regulated genes such as LIV1 were also observed (Osborne *et al* 1994, 1995). This xenograft model is the transplantation of MCF-7 cells mixed with Matrigel via

subcutaneous injection into an animal model, most commonly a nude mouse, and is used as a model to mimic tumour growth and development within the clinic. Fulvestrant was also shown to inhibit growth of the MCF-7 cell line with greater efficacy than tamoxifen, under the same conditions in even the earliest experiments (Wakeling and Bowler 1987). *In vivo* studies, using either MCF-7 or Br10 xenografts in nude mice, showed a complete growth arrest of tumours that had been continuously treated with E2 for four weeks following a single injection of fulvestrant (Wakeling *et al* 1991). A further MCF-7 xenograft study showed that fulvestrant treatment suppressed tumour growth for twice as long than tamoxifen treatment (Osborne *et al* 1995).

Due to the evidence gained from preclinical studies, the value of fulvestrant as an ER positive breast cancer therapy has been assessed clinically in a variety of ways. Clinical use of fulvestrant was shown to reduce ER levels beginning with a study from DeFriend (DeFriend *et al* 1994). Further study showed that in ER positive breast tumours a significant ER protein down-regulation after short term treatment with fulvestrant was observed compared with only a small effect on ER protein level when tamoxifen was used (Robertson 2001, Robertson *et al* 2001). Furthermore, clinical use of fulvestrant has shown evidence of reduced ER signalling as treatment was able to reduce oestrogen regulated genes in a concentration dependent manner, with reductions in PR and also Ki67 proliferation marker levels being significantly greater following 250 mg/month of fulvestrant when compared to a treatment of 20 mg/day of tamoxifen (Robertson *et al* 2001). Indeed, PR

levels were actually increased with tamoxifen due to its agonistic effects (Robertson *et al* 2001).

Fulvestrant has also been assessed as a first line therapy for advanced breast cancer in postmenopausal women. Trial 0025 compared 250mg/month fulvestrant and 20mg/day tamoxifen (Howell *et al* 2004). Disappointingly however there was no significant difference observed between the treatments in time to progression (TTP) of disease. The drugs were tolerated equally well, though tamoxifen patients suffered statistically more hot flushes than fulvestrant treated patients. Gastrointestinal disturbances, vaginitis and thromboembolic disease were reported equally between groups. Tamoxifen treatment also showed a significantly higher clinical benefit (CB), with CB rate of 62% to 54.3% for tamoxifen and fulvestrant, respectively. Time to treatment failure, (fulvestrant 5.9 months, tamoxifen 7.8 months) and overall survival (fulvestrant 36.9 months to 38.7 months for tamoxifen) were also similar, and unfortunately fulvestrant treatment in this manner did not meet the requirement of tamoxifen non-inferiority when used as a first line therapy (Howell *et al* 2004). However, in this study approximately 20% of patients had unknown ER status. Re-analysis of these data looking at ER and/or PR positive breast cancer patients (thus those most likely to respond to endocrine therapy) showed a TTP of 8.2 and 8.3 months with fulvestrant and tamoxifen treatment, respectively. The CB and overall survival rates were similar as well indicating tamoxifen and fulvestrant had similar efficacy (Howell *et al* 2004). Further analysis of both ER and PR positive patients showed similar TTP, CB and survival rates between the two treatments and prompted further studies of

using fulvestrant in a first line setting (Howell *et al* 2004). Developing the optimum treatment strategy using therapeutic fulvestrant has also been investigated, with trials still ongoing.

As endocrine therapy suppresses hormone-responsive tumour growth, rather than being directly cytotoxic, the time to treatment response maybe longer than for chemotherapy (Torrisi *et al* 2004). Fulvestrant patients may actually see some tumour progression in the first two months of treatment before an objective response is seen. This is most likely due to time taken for the agent to reach therapeutic levels. Reaching the therapeutic plasma concentrations of fulvestrant can take 3-6 months to reach steady-state levels at 250mg/month dosages (Robertson *et al* 2004), showing scope for improvement by using a loading dose (LD) or high dose (HD) treatment regimens. Indeed phase III clinical trials 0020 and 0021 showed that repeated rather than single doses took less time to reach steady state fulvestrant levels, and these trials mirrored the pharmokinetic predictive models (Robertson *et al* 2004). As such, further pharmokinetic models were used to evaluate both LD and HD models. A LD model of 500mg fulvestrant on day 0 plus 250mg/month, and a HD model of 500mg monthly plus 500mg on day 0 and 14 of month 1 both showed a steady fulvestrant plasma concentration achieved within one month. The HD model actually showed roughly twice the fulvestrant plasma concentration when compared to usual treatment of 250mg/month fulvestrant (Robertson 2007). While reducing the time taken to attain a steady state has the potential to reduce time to reach therapeutic levels, in trials 0020 and 0021 the 250mg/month fulvestrant dose showed similar time

to response and time to progression to that of the AI anastrazole at 1mg/day (Robertson *et al* 2003, Robertson *et al* 2004), which only takes 7 days to reach a steady state and maximal oestrogen suppression in 2-4 days (Buzdar *et al* 2002), indicating a possible improvement over AI's if a therapeutic steady state level of fulvestrant could be achieved at an earlier point during treatment.

However, increased concentrations of fulvestrant have been under evaluation to see whether they confer a greater anti-tumour response in ER positive breast cancer patients. Increased fulvestrant concentrations have previously been demonstrated to increase ER down-regulation in a dose dependent manner. For example, in clinical trial 0018 a single fulvestrant dose of either 50mg, 125mg or 250mg was given prior to surgery, and ER and PR levels were assessed. This study showed that there was a dose dependant, significant down regulation of both ER and PR when compared to placebo, though the level of ER reduction was incomplete (70% down regulation at 250mg fulvestrant) (Robertson *et al* 2001, Nicholson *et al* 2001). Other biopsy studies have also confirmed clinical residual ER (up to 50%), even after six months of fulvestrant treatment (Gutteridge *et al* 2004). Dose dependent ER down regulation by fulvestrant was also seen in a clinical trial using daily injections of short acting fulvestrant, with 6mg/day significantly down regulating ER and PR expression, but not as significantly as the higher 18mg/day concentration. (DeFriend *et al* 1994)

These dose dependant fulvestrant effects are thought to be due to the fulvestrant plasma concentrations, with single doses of 50mg, 125mg, and 250mg giving plasma fulvestrant levels of 1ng/ml, 2.5ng/ml and 5.0ng/ml,

respectively. 18mg/day of short-acting fulvestrant gave fulvestrant plasma levels of 23ng/ml compared to 7ng/ml as seen in the 6mg/day arm. Indirect comparisons of ER expression showing a mean ER H-score of 60 at 1ng/ml fulvestrant plasma levels and a predicted fulvestrant plasma level of 20ng/ml and an ER H-score of 30 when a 500mg fulvestrant dose is used. This Suggests higher fulvestrant dosage equates to a higher fulvestrant plasma concentrations, leading to greater ER down regulation and subsequently ER signalling hopefully leading to clinical benefit (Robertson 2001). It also suggests that a steady state fulvestrant level will be achieved more quickly. However, currently there has of yet been no direct relationship between fulvestrant plasma levels, ER down regulation and clinical efficacy defined (Robertson 2001).

In the NEWEST (Neoadjuvant Endocrine therapy for Women with Estrogen-Sensitive Tumors) trial, a phase II clinical trial comparison of monthly 500mg and 250mg fulvestrant treatment showed that the higher 500 mg dose results in (higher fulvestrant plasma levels and) significantly greater down regulation of ER, PR and Ki67 than the approved 250 mg dose (Kuter *et al* 2008) and so a higher fulvestrant concentration may give better clinical benefit in a first line setting.

In fact in the last few months the initial results from the CONFIRM (COmparisoN of Faslodex In Recurrent or Metastatic breast cancer) trial have been presented at the San Antonio Breast Cancer Symposium. This international phase III trial compared the usual 250mg/month fulvestrant regimen with 500mg/month fulvestrant (plus 500mg on day 14 of month 1) in

patients with ER-positive advanced breast cancer who have relapsed or progressed on previous endocrine therapy. This was a double blind, double dummy study of 736 women (362 and 374 taking 500mg/month or 250mg/month fulvestrant, respectively). This study showed the 500mg fulvestrant regimen significantly prolonged TTP compared to fulvestrant 250 mg, with a median TTP of 6.5 months to 5.5 months, respectively. There was also a 16% reduction in the risk of mortality for patients receiving 500mg fulvestrant when compared with 250mg fulvestrant (though this was not significant). The objective response rates were also similar, with 13.8% at 500mg and 14.6% at 250mg, respectively. The rate of clinical benefit was 45.6% and 39.6% for the 500 mg and 250 mg arms. Importantly however the safety profile was the same for both groups and the duration of clinical benefit was 16.6 months at the 500mg/month fulvestrant dose compared with 13.9 months for 250mg/month fulvestrant (Di Leo *et al* 2009). These data all lead to Dr Angelo Di Leo (the CONFIRM principal investigator) announcing:

"We believe that based on the results of this (the CONFIRM) study, treatment and practice should change. Patients should routinely receive the 500mg (fulvestrant) dose". This subsequently resulted in 500mg/month fulvestrant being the new recommended dose (Di Leo *et al* 2010).

Fulvestrant was the first of the 'pure' anti-oestrogens to enter clinical development (Nicholson and Johnston 2005). Although the true potential as a first-line therapeutic and its effectiveness with respect to AI's and tamoxifen are still being assessed, it is currently licensed as a second line therapy following disease progression from other anti-hormone therapies (such as

Tamoxifen and AI's), as further clinical benefit can be gained from the drug when used in this manner (Howell *et al* 2002, Ingle *et al* 2006). Due to the lack of ER agonist effects from use of fulvestrant there are less risks of endometrial cancers and stroke than tamoxifen use and it is well tolerated in clinic with only minor side-affects reported (Howell 2006). The ability of fulvestrant to confer clinical benefit on disease that has progressed from tamoxifen therapy means it is invaluable as a second line therapy (Gradishar 2004, Gradishar and Morrow 2002) due to its lack of cross-resistance with other endocrine therapies (Johnston 2006). As well as dose-related studies, there are further first-line studies comparing 500mg/month fulvestrant versus aromatase inhibitors including the FIRST study, comparing fulvestrant vs. Anastrazole alone, which interestingly demonstrated that high dose fulvestrant treatment was associated with a significantly longer TTP than the AI (Robertson *et al* 2009). While AI's and tamoxifen gave no greater clinical benefit in combination, AI's combined with alternative 'pure' anti-oestrogens may feasibly have a greater clinical benefit, as a combined treatment of AI's with fulvestrant for example could potentially allow for inhibition of any further oestrogen independent ER activity still active following AI treatment alone (Osborne and Schiff 2005). However preliminary results from the FACT phase III clinical trial suggest that while the combination treatment is well tolerated (with only a slightly higher incidence of side-effects when compared to anastrozole alone) there may be no additional benefit of such combination therapy. This showed no improvement over AI alone in hormone-responsive

post-menopasual patients who had relapsed from disease despite the promising pre-clinical evidence (Bergh *et al* 2009).

1.6.4. Endocrine resistance.

While anti-hormone therapies are tolerated well in the clinic, and responsible for much of the recent improved breast cancer survival rates, the story is not a complete success. Resistance to therapy is observed *in vitro* (McClelland *et al* 2001) and subsequent disease relapse is also a major clinical problem (Howell 2006, Robertson 2007). Through their mechanisms of action, anti-hormone therapy is only beneficial to patients with ER positive tumours (60-70% of patients) (Bundred 2001). The ER negative tumours are intrinsically (or *de novo*) resistant to this type of therapy due to lacking the target receptor, the major cause of this type of resistance. Patients presenting with ER positive tumours may also be unresponsive to one or more endocrine therapies, with up to a third also showing *de novo* resistance (Johnston 2005). However, roughly two thirds of patients with ER positive tumours will initially respond to anti-hormone treatment. Unfortunately, of the tumours which do respond to endocrine therapy at the outset many will relapse and acquire resistance during the course of treatment (Clarke *et al* 2003, Nicholson *et al* 2005, McClelland *et al* 2001 and Santen *et al* 2005). Therefore, understanding mechanisms of both *de novo* and acquired endocrine resistance is vital to our understanding of the disease and therapeutic improvement therein, as resistance often results in the aggressive recurrence of the disease and a higher mortality rate (Ring and

Dowsett 2004) and is expected to effect 5 million women within the next decade (Jemal *et al* 2005).

Some mechanisms proposed and investigated in both *de novo* and acquired endocrine resistance include;

1. Alteration or loss of ER function or loss of ER expression.
2. Redistribution of ER from the nucleus to cytoplasm.
3. Alteration in expression or function of ER co-regulators.
4. Increased metabolism of the endocrine agent.
5. Increased growth factor signalling.
6. Undefined actions of ERβ.

The mechanisms behind acquisition of resistance to endocrine therapy are not yet fully understood. The transition of a clinical tumour or cancer cell model from an initial hormone and endocrine therapy sensitive phenotype to an anti-hormone resistant and/or hormone independent phenotype is complicated and multi-factorial. Mechanisms underlying acquisition of endocrine resistance are also contextual, depending on original presentation of cancer genotype and phenotype, exogenous environmental factors (such as culture conditions, levels of hormones and other growth factors) and duration, intensity and sequence of endocrine therapies the cells are exposed to, the pressure exerted by the treatment itself being the major factor driving acquisition of resistance in almost all cases. Tumours are highly plastic and capable of remodelling their cell populations in response to host immunity or endocrinology, or administration of therapies (both local and systemic). This

can occur by selection between different cells within the population (different populations die out, others become more dominant) and by adaptation of individual cells alteration of their transcriptomes/proteosomes (Clarke *et al* 2003).

While acquisition of resistance is not yet completely understood, various mechanisms responsible for this form of endocrine resistance in ER positive breast cancers have been proposed and investigated. The insights provided into acquired resistance are largely due to the generation of *in vitro* cell models resistant to hormone therapy. These resistant cell models are generally of two kinds, created either by continuous culture of hormone sensitive breast cancer cell lines (usually MCF-7 based) with sub-toxic levels of anti-oestrogens until growth is recovered and cells are no longer growth inhibited by these agents (producing either tamoxifen or fulvestrant resistant cells), or produced by long term culture within an environment low or devoid of oestrogens (also known as long term oestrogen deprivation or LTED). This is to mimic the activity of AI's until growth is recovered, producing a model of AI resistant breast cancer.

These models of resistance and models resistant to multiple endocrine therapies differ in their phenotype, protein expression, growth rate, invasiveness, migratory capacity and favoured signalling pathways, and there is also differentiation both between different therapies and between other resistant models to the same endocrine therapy generated by different groups. However despite the differences there is also some commonality between models. In the broadest terms, endocrine therapy drives a transition in

signalling within these cells from oestrogen/ER driven cell signalling and growth to alternative growth factor dependant signalling and growth (Nicholson *et al* 2003, 2004).

Due to a lack of ER being the major cause of *de novo* endocrine resistance it has subsequently been studied in relation to acquisition of resistance. There has been some evidence that continued use of anti-hormone agents can lead to a loss of ER expression by a variety of means under certain circumstances with both *in vitro* and *in vivo* examples. In the clinic, up to 30% of ERα positive patients with metastatic breast tumours that acquire resistance to tamoxifen have shown loss of ER expression during treatment with this agent (Oh *et al* 2001). Further clinical evidence shows that ER loss can also occur when aromatase inhibitors are used in clinical disease; this transition to an ER negative phenotype can occur during therapy and is associated with poorer prognosis. Clinical studies have shown that 10% of ER positive patients undergoing neo-adjuvant treatment with the AI Letrozole became ER negative and this was associated with elevated mortality and relapse (Ellis *et al* 2008, 2008a).

Methods that may contribute to ER loss include promoter hypermethylation and subsequent transcriptional loss or acquired mutations within the ESR1 gene itself. While hypermethylation at CpG islands within the ER promoter has been reported in approximately 25% of ER negative tumours *in vivo* (Lopez-Tarruella and Schiff 2007), the link between methylation and ER status remains unclear. It has been reported by both Falette and colleagues and Hori and colleagues that there was no association

between ER status in breast tumours and ESR1 gene methylation at specific sites of the ER gene promoter (Falette *et al* 1990, Hori *et al* 1999). However, other studies have provided indirect data showing loss of ER due to promoter methylation. Evidence has shown that ER expression can be reversed through exposure to 5-Azacytidine, a de-methylating agent as well as the Histone deacetylase (HDAC) inhibitor Trichostatin A (Bovenzi and Momparler 2001, Ferguson *et al* 1995). Subsequent studies have shown the presence of both HDAC and DNA methyltransferase (DNMTI) at the promoters of ER and that they were responsible for silencing ERα in some ER negative breast tumours (Macaluso *et al* 2007). This silencing was shown to be reversible by use of 5-Azacytidine, which inhibited DNMTI (Yang *et al* 2001).

As well as hypermethylation as a cause of ER loss, alternative research has suggested that natural mutations in the ER occurring during cancer progression could contribute to ER negativity in some tumours (Behbod and Rosen 2005). While various natural single point mutations have been observed in human clinical samples, such as the missense A86V mutation (Herynk and Fuqua 2004), and this correlates with lower levels of ER protein, there is no conclusive data that this mutation has a significant correlation with ER loss in clinical breast tumours (Herynk and Fuqua 2004). It is believed these types of mutations are probably too rare in occurrence to contribute to a widespread reason for ER negativity in general within the clinic (Chu *et al* 2007).

Similarly, a variety of ER splice variants have been reported in breast tumours (Herynk and Fuqua 2007). Usually un-translated ERα exons have been discovered and alternate splicing of these exons has been shown to affect

the subsequent activity and function of the ER. There is however no clear evidence that presence of splice variants within a tumour is associated with the development of an ER negative phenotype. While an exon 5 deleted splice variant of ER has been observed in some ER negative and PR positive breast cancers (Herynk and Fuqua 2004), there is no recorded data of exon 5 having any association with clinical ER or PR status (Zhang *et al* 1996).

The rarity of these epigenetic events and presence of mutations indicate that there are other more important mechanisms of acquired endocrine resistance. In fact while loss of ER levels and activity can lead to endocrine insensitivity and resistance to anti-hormone agents, many tumours with acquired tamoxifen resistance still respond to second line anti-oestrogens such as fulvestrant (Hutcheson *et al* 2003), and 75% of tumours with acquired tamoxifen resistance still express levels of ER equal to those prior to treatment (Clarke *et al* 2003). As such in most cases of acquired resistance ER loss can not be the primary resistance mechanism. For example, our in-house tamoxifen resistant MCF-7 cell model still expresses ER (Hutcheson *et al* 2003).

Alternative evidence from *in vitro* studies have shown elevated expression of the ER co-activators CBP, P300 and AIBI, correlating with HER2 over-expression implying a link with acquired endocrine resistance, and AIBI and HER2 co-expression has been associated with poor clinical outcome (Johnston 2006, Osborne *et al* 2003). Furthermore a decrease in recruitment of the ER co-repressors NCoR and SMRT was found in mouse models of acquired tamoxifen resistance compared to the tamoxifen sensitive models

(Lavinsky *et al* 1998, Herynk and Fuqua 2004), suggesting a link between mismanaged co-activator recruitment and endocrine resistance.

Despite the above, the apparent primary mechanism in most forms of acquired endocrine resistance seems to be due to increased growth factor signalling (Nicholson and Johnston 2005). This can occur either by the up-regulation of growth factor receptors, or by an increased supply of growth factors by auto- or paracrine means. The epidermal growth factor receptor (or ErbB) family as well as the insulin-like growth factor receptors, have been shown to be responsible in multiple cases of endocrine resistance (Nicholson *et al* 2004). The ErbB family is a group of receptor tyrosine kinases containing 4 members, EGFR (ErbB1/HER1), HER2 (ErbB2/NEU), ErbB3 (HER3) and ErbB4 (HER4). These receptors can be activated by various growth factor ligands such as Epidermal growth factor (EGF), Transforming Growth Factor (TFGα) and the Heregulins (Nicholson and Johnston 2005). These receptors are able to homodimerise or heterodimerise with one another to activate growth factor signalling pathways leading to growth and survival via AKT or ERK/MAPK activation. Much previous research has shown activation of MAPK and AKT provide proliferative and survival signals in anti-oestrogen resistant cells (Staka *et al* 2005, Nicholson *et al* 2004, and Knowlden *et al* 2003).

Over expression of the EGFR and HER2 (as well as increased expression of TGFα and activation of ERK) has been found to be responsible for acquired resistance to fulvestrant in cell models (Nicholson *et al* 2003) and also in patients with *de novo* tamoxifen resistant ER positive breast tumours

(Gee *et al* 2005). The FasMCF-7 model of acquired fulvestrant resistance developed by the Tenovus Centre of Cancer Research shows increased EGFR and HER2 expression (McClelland *et al* 2001), and the ER positive cell line BT-474 which is *de novo* tamoxifen resistant expresses high levels of these receptors (Gee *et al* 2005). Also artificial over-expression of HER2 in MCF-7 cells was also able to confer *de novo* tamoxifen resistance in *in vitro* and xenograft studies as it increased the agonist qualities of the drug (Shou *et al* 2004). Interestingly increased EGFR/HER2 dependence is important as it is thought to allow survival of initial anti-hormone insult during acquisition of resistance to both fulvestrant and tamoxifen (McClelland *et al* 2001, Gee *et al* 2003), and inhibition of EGFR signalling was able to overcome development of resistance in both cases in MCF-7 cells. This shows induction of alternative compensatory signalling occurs during fulvestrant or tamoxifen treatment, with hormone responsive cells switching from preferred cross-talk between E2 and Insulin-like growth factor receptor signalling (Fagan and Yee 2008) to dependence on alternative (erbB) growth factor driven pathways (Gee *et al* 2003).

While there is little clinical information on acquired fulvestrant resistance, the importance of increased growth factor signalling and its downstream effects has been shown in multiple cell models of acquired fulvestrant resistance. This includes the FasMCF-7 model of acquired fulvestrant resistance mentioned previously. This model was based on the MCF-7 cell model and was generated after 3-4 months of continuous fulvestrant exposure *in vitro*. In this model termed FasMCF-7, elevated EGFR

and MAPK signalling was observed, and cells were subsequently growth inhibited by use of gefitinib (an EGFR inhibitor) (McClelland *et al* 2001). IGF1-R signalling has also shown to be deregulated *in vitro* in some models of fulvestrant resistance (Frogne *et al* 2005, Campbell *et al* 2001).

Other models of acquired fulvestrant resistance generated by *in vitro* or *in vivo* methods include the MCF-7/182-R (and 164-R) cell lines, MCF7/LCC9, MCF7/F, MCF7/HER2/*neu-18*, MCF7/HER2-18 and ICI-R. Most of these fulvestrant resistant models, like the McClelland FasMCF line, retain ER protein expression, detectable ER mRNA (Brunner *et al* 1997, Dumont 1996, Fan *et al* 2009, Jensen *et al* 1999, and Larsen *et al* 1997) and show dependence on increased growth factor signalling. The MCF-7/182-R and 164-R series of cell lines shows increased expression of EGFR as well as increased activation of HER3, AKT and MAPK and is preferentially sensitive to the inhibitory effects of gefitinib, compared to parental cells (Sonne-Hansen *et al* 2010, Frogne *et al* 2009). In models of fulvestrant resistance, changes in the growth factor receptor pathways have been observed at the receptor level, ligand level and downstream targets which could further enhance activation of these pathways (McClelland *et al* 2001, Atlas *et al* 2003, Sommer *et al* 2003, Frogne *et al* 2005 and Fan *et al* 2006). There has also been evidence of increased growth inhibition in cells with acquired fulvestrant resistance by combination inhibition of ErbB family members, either by combining individual signal transduction inhibitors or by use of a pan-ErbB inhibitor such as CI-1033. However there seems to be some reversibility, and an adaptability to switch between ER and growth factor signalling dependant on the pressure

provided by inhibition (Sonne-Hansen *et al* 2010), and interestingly in the FasMCF-7 model, similar to the other models mentioned above, the low ER expression and function was found to be reversible after a period of anti-hormone removal (Nicholson *et al* 2005, McClelland *et al* 2001).

Further evidence of the important induction of compensatory signalling following anti-hormone treatment was demonstrated when both fulvestrant and gefitinib were used in combination in MCF-7 cells, leading to a significant anti-tumour effect (Hutcheson *et al* 2003, Knowlden *et al* 2003), and lack of resistance to either molecule developing following long-term exposure (Nicholson *et al* 2005, Gee *et al* 2003, Nicholson *et al* 2004). Interestingly, while co-treatment with fulvestrant and growth factor inhibitors can delay the onset of resistance to fulvestrant in cell models, activation of HER2 by Heregulin reduces the time taken to acquire resistance. This Heregulin mediated rescue of fulvestrant induced anti-tumour effect can be stopped by HER2 inhibition, indicating that the effect was mediated through ErbB activation, likely by HER3/4 dimerisation with HER2 (Sonne-Hansen *et al* 2010). The inhibitory effect of CI-1033 on the MCF-7/182-R series was also more pronounced in the presence of fulvestrant as cells were prevented from utilising ER signalling due to the anti-oestrogen effect (Sonne-Hansen *et al* 2010).

The cross-talk between ER and growth factor receptors is clearly more important and more common in development of endocrine resistant than first thought, and the ability to switch between signalling networks is a major factor in acquisition of endocrine resistance. Work has led to the discovery of

a bidirectional cross-talking loop between ER and EGFR signalling in tamoxifen resistant cells (Nicholson *et al* 1999, 2004).

Increased activation of EGFR by TGFα and amphiregulin, leads to activation of ERK, and AKT signalling, increasing the phosphorylation of the ER at serine residues 118 (Kato *et al* 1995) and 167 respectively (Campbell *et al* 2001). While these sites are normally required for maximal AF-1 and AF-2 mediated transcription, in the presence of tamoxifen this leads only to an up-regulation of AF-1 transcriptional activity, causing an increase in hormone independent ER transcription (Ring and Dowsett 2004) and increased growth in the presence of the anti-hormone. Furthermore, increased EGFR dependent activation of ERK (Knowlden *et al* 2003) and AKT (Jordan *et al* 2004), in addition to elevated ER phosphorylation (Britton *et al* 2006) has been demonstrated in *in vitro* models of acquired tamoxifen resistance. This has also been demonstrated in some fulvestrant resistant cell lines (Sonne-Hansen *et al* 2010).

Significantly, EGFR can also interact with insulin-like growth factor receptor 1 (IGF-1R) signalling to aid resistant growth with IGF-II promoting IGF-1R driven activation of c-SRC, which in turn causes phosphorylation of tyrosine 845 on EGFR to up-regulate EGFR activity (Knowlden *et al* 2005).

Additionally, EGFR may also promote further gene transcription by increasing activation of ER co-activators, and decreasing activation of ER co-repressors (Ring and Dowsett 2004). This bi-directional cross-talk in total is believed to allow ligand independent ER driven gene transcription in the

presence of tamoxifen leading to increased expression of growth factors, such as TGFα and amphiregulin (Nicholson *et al* 2003, Britton *et al* 2006).

As well as increases in EGFR/HER2 activity following ER promoted genomic signalling in this autocrine signalling 'loop', non-genomic ER signalling is also suggested to be able to stimulate growth factor signalling at the plasma membrane in some models of endocrine resistance (Schiff *et al* 2004, Chung *et al* 2002, and Massarweh *et al* 2008). For example, ER has been shown to promote EGFR signalling via direct interactions with adaptor molecules such as SHC and c-SRC (Fan *et al* 2007, Yue *et al* 2007).

De-regulated growth factor signalling as a mechanism of resistance has also been reported in models resistant to severe E2 deprivation in vitro, with elevated levels of IGF-R1, EGFR, HER2 and MAPK and P13K/AKT signalling observed. These signalling molecules are again subsequently able to interact with ER via both genomic and non-genomic ER signalling (Santen *et al* 2005, Martin *et al* 2005).

While the ER dependant bi-directional loop with growth factor signalling elements holds true for some models of endocrine resistance, in others (primarily including models of fulvestrant resistance) there are also mechanisms of ER-independent activation of growth factor receptors. Activation of ErbB family members have been shown to repress ER expression, making cells less sensitive to endocrine therapy (Liu *et al* 1995, Stoica *et al* 2000, and Oh *et al* 2001, Bayliss *et al* 2007) and vice-versa (Sonne-Hansen *et al* 2010). This is supported by clinical evidence showing the inverse relationship between ER and EGFR/HER2 with the over-expression of

the latter leading to decreased anti-oestrogen sensitivity (Housten *et al* 1999, De Laurentis *et al* 2005). This is likely due in part to increased activation of ERK, a downstream element of EGFR/HER2 signalling which confers resistance to tamoxifen and poor patient prognosis in the clinic (Gee *et al* 2001).

There is further clinical evidence of increased growth factor signalling in anti-hormone resistance. Elevated growth factor signalling has been associated in the clinic with both *de novo* tamoxifen resistance as well as acquired resistance (Gee *et al* 2005, Gee *et al* 2001) and correlates with poorer patient prognosis (Gee *et al* 2001). Increased HER2 and p38 MAPK signalling has also been observed in clinical tamoxifen relapse samples (Gutierrez *et al* 2005). Also in ER positive, pre-menopausal breast cancer patients who have undergone anti-hormone treatment who also had high levels of AKT activity were shown to have an increased likelihood of relapse on treatment and a greater risk of metastases (Perez-Tenorio *et al* 2002). Increased levels of EGFR and HER2 have also been associated with a decreased ability of anti-hormones to lower tumour proliferation compared to those without (Dowsett *et al* 2005, Miller *et al* 2005). Other clinical studies have shown patients with phosphorylated HER2 or those over-expressing EGFR had the shortest survival time (DiGiovanna *et al* 2005). Evidence for ER/growth factor cross-talk during acquisition of resistance has also been obtained in the clinic. Thus fulvestrant can work effectively as a second line agent in two thirds of ER+ patients following tamoxifen relapse (Howell 2006), while clinical response to

anti-growth factor blockade (e.g. gefitinib) was reported in some ER positive patients following tamoxifen relapse (Gutteridge *et al* 2010).

It is important also to note that while most models of acquired fulvestrant resistance still display ER, long-term culture (for over two years) has been shown to produce a completely irreversible ER-negativity in a fulvestrant resistant cell model, produced in the Tenovus Centre for Cancer research (Nicholson *et al* 2005, Hiscox *et al* 2006). Only one other model, the MCF7-F model (Liu *et al* 2006) shows similar characteristics to this FasR cell line in that it also demonstrates complete and irreversible loss of ER protein and mRNA. However the resistance mechanism is less clearly understood in these ER-negative MCF-7 models of late fulvestrant resistance, though there is increased expression of various signalling molecules, including the c-Met receptor and exposure to HGF can increase these cells invasive capacity (Hiscox *et al* 2006a). Growth factor receptors and their downstream kinases have also been implicated in other models of acquired resistance to both anti-hormones, and E2 deprivation strategies (McClelland *et al* 2001, Brodie *et al* 2007). The long-term activation of these growth factors and the subsequent constituent AKT and MAPK signalling may similarly lead to acquisition of an ER negative, endocrine resistant phenotype in some instances.

1.7. RNA interference.

1.7.1. Discovery of RNA interference.

The phenomenon known as RNA interference (or RNAi) was only first documented in the 1990's by plant scientists, Napoli, Jorgensen and colleagues (Napoli *et al* 1990, Jorgensen *et al* 1996). The initial research was interested in the development of a deeper purple colouration in the petals of petunia plants. In petunias as in many plants, the anthocyanin biosynthesis pathway is responsible for pigmentation. Chalcone synthase (CHS) is a key enzyme in this pathway, and Napoli and Jorgensen believed it to be the rate-limiting enzyme in anthocyanin biosynthesis. Thus, in an attempt to generate dark-violet petunias, Napoli and Jorgensen over-expressed CHS in petunias, by artificially inserting multiple copies of double-stranded (ds)RNA specific for this gene. This experiment however, unexpectedly resulted in pale purple and even white petunias. When investigated further, the levels of CHS (both endogenous and introduced) in the experimental plants were found to be 50-fold lower than in the wild-type petunias. This led to the hypothesis that the introduced dsRNA was somehow able to suppress the endogenous CHS gene (Napoli *et al* 1990).

In the following years, similar phenomenon was recorded in other organisms such as a red bread mould, *Neurospora crassa* (Romano and Macino 1992), and in the nematode worm *Caenorhabditis elegans* (Guo and

Kemphues 1995). It was at this time noted that introduction of either sense or antisense RNA resulted in degradation of the specific mRNA (Guo and Kemphues 1995) though this was later proved to be due to contamination of the antisense RNA preparation with the sense strand (Fire *et al* 1998). The publishing of Fire and colleagues paper showed the trigger for gene silencing was double-stranded rather than single stranded (ss)RNA in their experiments in *C.elegans*, with ssRNA (sense or antisense) being 10-100 fold less effective than dsRNA at targeting the same mRNA (Fire *et al* 1998). The fact dsRNA exposure caused systemic silencing (Voinnet and Baulcombe 1997), and could be passed on to future generations (Grishok *et al* 2000) led to belief that there was a stable intermediate involved. While it was thought the antisense strand was the one that bound to the mRNA, the full-length strand could never be detected. Short fragments of roughly 25 nucleotides were eventually discovered (Hamilton and Baulcombe 1999) and these small interfering (si)RNA's were shown to be necessary for RNAi to function.

The definitive papers on the subject came when artificially synthesised siRNA's were shown to silence both heterologous and endogenous genes in *Drosophilla,* and for the first time RNAi was demonstrated in mammalian cells (Elbashir *et al* 2001, 2001a). While a role for using siRNAs as a gene silencing tool had now been established in animal cells, questions remained about the precise mechanism of RNAi. Eventually however the enzymes responsible for the first RNAi step of conversion of dsRNA to siRNAs, and the second step of cleavage of target mRNA were discovered (Hammond *et al*

2000, Bernstein *et al* 2001). The mechanism of RNAi will be discussed in the following section (and shown in figure 1.4.).

1.7.2. Mechanism of RNAi action.

Once dsRNA is introduced into the cell it is cleaved by a type III RNase III enzyme called Dicer, an endoribonuclease which contains two RNase III motifs and an amino-terminal helicase domain, able to cleave dsRNA into siRNA's of 20-25 nucleotides in length (Bernstein *et al* 2001). Dicer homologues have been discovered in all organisms which exhibit RNAi (Sontheimer 2005). The double stranded siRNA duplexes formed become associated with a multi-enzyme complex including an RNA recognition site, an exonuclease component, and an endonuclease component in the form of the enzyme Argonaute 2 (AGO2). This RNA-induced silencing complex (RiSC) then discards and cleaves the siRNA sense strand (Matranga *et al* 2005). This siRNA-RiSC protein complex becomes activated by adenosine tri-phosphate (ATP), and once activated it recognises mRNA sequences complementary to its bound siRNA strand and is able to bind to them. The bound mRNA is then degraded by the endonuclease activity of AGO2, cleaving the mRNA strand between complementary nucleotides 10 and 11 of the siRNA (from 5' end). Once degraded the siRNA-protein complex is free to bind another complementary mRNA in a catalytic manner, and as such this process is able to reduce specific mRNA to very low levels, rapidly (Hutvagner and Zamore

2002). This loss of mRNA causes gene silencing by allowing down regulation of the protein to low levels (with the time taken depending on the half life of the protein) with no further protein being translated. In addition to full RNAi activity by introduction of dsRNA for a gene, it is possible to use this phenomenon as a tool for genetic knockdown studies in mammalian cells by introduction of synthetic siRNA to a gene of interest, since previously the introduction of long dsRNA into mammalian cells was shown to elicit an interferon response that caused a general inhibition of translation leading to loss of the RNAi specificity (Elbashir *et al* 2001a). This process works in the previously described manner excepting that it by passes the Dicer step as they are already processed into small RNA of 20-25 nucelotides in length. These siRNA have been shown to be effective at nanomolar (or lower) concentrations (De Fougerolles *et al* 2007).

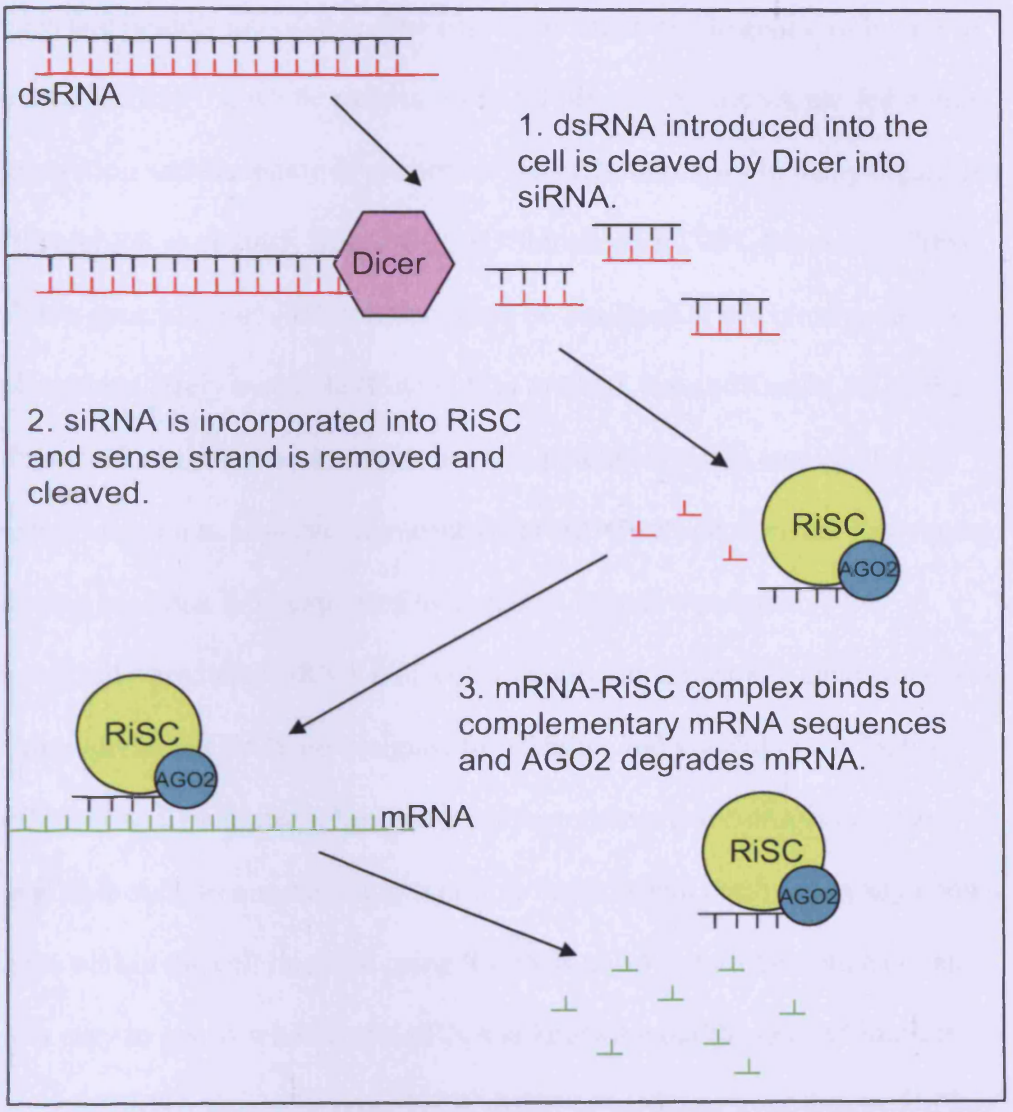


Figure 1.4. Mechanism of RNAi action.

1.7.3. Applications of siRNA in scientific research.

In recent years the RNAi pathway and use of siRNA's has become the major means of assessing loss of gene function in many organisms. Conventional mutagenesis requires production of single mutants and for double mutants two single mutants then need to be crossed, this is inefficient, and in some cell

lines and models impossible. The ease with which single genes, or by use of multiple siRNA's, whole pathways can be silenced by siRNA has led to the generation and screening of genome-wide siRNA libraries in many organisms (Westbrook *et al* 2005, Sen *et al* 2004, Shirane *et al* 2004, Berns *et al* 2004). While generation of siRNA librarys can be designed *in silico* using various algorithms freely available (Reynolds *et al* 2004, Pei and Tuschi 2006) the only tried and true way to select the most potent siRNA is empirically, by experimentation. However in recent years siRNA production and subsequent testing has been fully exploited by industry. It is now possible to buy artificially produced siRNA molecules specific to almost any gene you desire. Commercial siRNA's are designed for stability and knockdown is highly efficient and guaranteed. Various positive and negative controls are also available such as non-targeting scramble controls that don't target any known gene within the cell line, and using RT-PCR and Western blotting techniques it is easy to assess whether the siRNA is knocking out the gene of interest.

SiRNA due to its remarkable ability to modulate gene expression has become an invaluable tool in gene mapping; pathway dissection and gene function analysis and may lead to finding the initial and further functions of the 25,000 human genes and pseudogenes we don't yet fully understand.

1.7.4. Clinical uses of siRNA.

After initial discovery the possible therapeutic applications of siRNA technology quickly became apparent. The ability of siRNA technology to

down regulate a gene (or genes) of interest has lead to research into its development as clinical therapy for a number of diseases where inhibition of specific genes could be a valid druggable target in the disease setting, especially where small molecule inhibitors are not a valid option (reviewed in De Fougerolles *et al* 2007 and Aagaard and Rossi 2007). Research has been conducted into a wide range of diseases and delivery methods, from SARS treatment with an intranasal preparation to intra-ventricular preparations designed to target genes in diseases of the central nervous system. There has also been some work in preparations designed to target tumours, though effective delivery is still an issue new preparations are being devised all the time (De Fougerolles *et al* 2007). RNAi has moved from research into clinical trials rapidly. Initial trials unsurprisingly focused on well known therapeutic targets such as the vascular endothelial growth factor (VEGF) pathway to treat the wet form of age-related macular degeneration (AMD). This was the first clinical trial of an siRNA based therapeutic (Sah 2006). As of February 2008, there were five ongoing clinical trials of siRNA therapeutics. Of these therapeutics, Bevasiranib and AGN-745 are both targeted to the VEGF receptor, all its isoforms or VEGF-1R respectively to treat wet AMD. RTP801i-14 has been designed to work synergistically with either of these agents by targeting the hypoxia-inducible gene RTP801. The forth therapeutic, ALN-RSV01 targets the viral nucleocapsid (N) gene to treat respiratory syncytial virus infection. An siRNA based therapeutic targeting the p53 gene is undergoing trials to treat acute renal failure. However as successful delivery is still an issue, these drugs are all uncomplexed, or saline-based formulations

and are designed for direct delivery. However new siRNA therapies are currently under development, using lipid-nanoparticles and conjugated siRNA's to allow for systemic delivery for use in a wider variety of diseases (reviewed by De Fougerolles 2008).

1.7.5. Delivery of siRNA.

As siRNAs are relatively large, negatively charged molecules they do not easily pass through the cell plasma membrane into cells. While some types of cells; such as those present in eyes (Reich *et al* 2003, Shen *et al* 2006), lungs (Bitko *et al* 2005, Li *et al* 2005), and the central nervous system (Makimura *et al* 2002, Dorn *et al* 2004) have been shown to uptake uncomplexed siRNA molecules directly into the cytoplasm many cell types will not, and the mechanism behind this is not well understood. Most siRNA need to be artificially introduced into cell models, and effective siRNA delivery is the most important limiting factor in RNAi technology, and is even more of a barrier in developing siRNA based drugs and *in vivo* studies. Because of this various methods of siRNA modification and packaging have been developed. siRNA modification can take the form of conjugate with a cholesterol molecule, which was initially used as a conjugate to anti-sense oligonucleotides to for hepatic delivery (Biessen *et al* 1999), other lipophillic molecules, proteins, short-peptides; such as penetratin and transportan (Muratovska and Eccles 2004), transferrin, folate, or Arg-Gly-Asp peptides (Hu-Lieskovan *et al* 2005, Kim *et al* 2005, Schiffelers *et al* 2004 respectively).

Antibodies and aptamers have also been conjugated with siRNA for improved delivery (De Fougerolles *et al* 2007). The use of siRNA conjugates is an interesting method for development of future siRNA based delivery technology for two reasons; initially from a technical point of view, as only the antisense siRNA strand is required for gene knockdown, this leaves the passenger or sense strand as a perfect candidate for conjugation with no detriment to the effectiveness of the siRNA. Secondly from a drug design issue, the conjugate could theoretically be cell or tissue specific, for example siRNAs conjugated to an RNA aptamer specific for the prostate specific membrane antigen (PSMA) which is over-expressed in prostate cancer cells and tumour vasculature were recently used both *in vitro* (Chu *et al* 2006) and *in vivo* (McNamara *et al* 2006) with some success. These PMSA aptamers were conjugated to siRNA specific for the survival genes PLK1 and BCL2 and were uptaken into cells leading to RNAi mediated cell death *in vivo* (McNamara *et al* 2006). Though useful for local delivery these aptamer-siRNA conjugates can be rapidly removed by normal kidney function, and have a half-life unsuitable for systemic therapy without further formulation (Nimjee *et al* 2005).

While siRNA can also be introduced into cells by prior packaging into viral capsules, the most common form of siRNA delivery is with a lipid transfection reagent, forming a lipid nanoparticle (De Fougerolles 2008). The lipid complex is usually comprised of a phospholipid bilayer, surrounding an aqueous compartment, able to transport substances into cells by fusion with cell membranes to deliver its load. When complexed with siRNA they are

termed lipoplexes or Liposomes/SNALPs (stable nucleic acid-lipid particles) (Judge *et al* 2005). Liposomes are particles with stable physiochemical characteristics, making them suitable drug delivery systems (De Fougerolles 2008). Liposomes for siRNA delivery tend to be comprised of multiple lipids, each with a specific function within the liposome, (Torchilin 2006, Li and Szoka 2007) outlined in the following table (see table 1.1.).

Type of lipid.	Function within liposome.
Cationic lipids	Aids formulation, cellular uptake and endosomal release.
Fusogenic lipids	Facilitates endosomal release.
Polyethylene glycosolated lipids (PEGs)	Stabilise the lipoplex, and have fusogenic properties.
Cholesterol	Stabilises the lipoplex.

Table 1.1. Lipids and their functions within an siRNA liposome.

In contrast lipoplexes are formed spontaneously on combination between siRNA and commercially available cationic transfection lipids. These amorphous siRNA lipoplexes tend to be less structurally stable, more heterogenous in nature, and can rapidly aggregate. Lipoplexes are therefore prepared immediately prior to application (De Fougerolles 2008).

1.7.6. Limitations of siRNA.

As stated previously effective delivery is the greatest limiting factor in use of siRNA, however other limitations of the technology need to be taken into account.

Specificity of siRNA's.

While RNAi technology can be used to silence gene expression with high specificity, even able to specifically silence alleles containing a single nucleotide polymorphism (Schwarz *et al* 2006), there is still the possibility of 'off-target' effects. An off-target effect is when the siRNA used is able to interfere with alternative mRNA expression, most common in mRNAs sharing partial homology with target mRNA. The degradation of other undesired mRNA's may have its own effects on cell behaviour. These off-targets however tend to be three-fold lower than the gene of interest (Jackson *et al* 2003, Lin *et al* 2005 and Qui *et al* 2005), however to accurately assess all possible off-targets from use of an siRNA would require either detailed proteomic analysis or Affymetric gene expression evaluation (Jackson *et al* 2003, Lin *et al* 2005 and Qui *et al* 2005).

Activation of innate immune-systems.

A further unwanted side-effect of siRNA use can be the undesired activation of the serine/threonine protein kinase (PKR) pathway, this is usually a defence against viral infection, but can recognise dsRNA greater than 30 nucleotides in length, though at higher concentrations shorter siRNA maybe able to activate this pathway, resulting in cell death (Schlee *et al* 2006). Another concern is the possible activation of Toll-like receptors (TLRs) which trigger production of type one interferons, pro-inflammatory cytokines and can induce NF-kB activation (Hornung *et al* 2005), though immunostimulation by siRNA's can be circumvented by sequence choice, for example preventing TLR activation by choosing siRNA sequences that are not Guanine and Uracil-rich as TLR-7 and 9 binding has been shown to be GU specific (Hornung *et al* 2005, Judge *et al* 2006).

siRNA stability.

Though not studied comprehensively in various fluids, uncomplexed or 'naked' siRNA has a half life of minutes in human plasma (Layzer *et al* 2004, Choung *et al* 2006), degraded rapidly by the high level of endo and exonuclease activity, but by chemical modification of the siRNA this can be overcome. Introduction of a phosphorothioate linkage at the 3' end of the siRNA and modification of sugars by adding methyl or fluoro groups can confer exo and endonuclease resistance respectively (De Fougerolles *et al*

2005, Layzer *et al* 2004, and Choung *et al* 2006) and this can be performed without loss of silencing ability. The inclusion of siRNA into various carrier systems can also give additional protection from nuclease digestion.

While these points are slight weaknesses in the technology rather than unassailable problems and research is ongoing currently to improve on these areas, and although still a relatively new technology, it has been rapidly utilised with good degrees of success, answering previously unanswerable questions.

1.8. Aims and hypothesis of PhD project.

There is much evidence, both in pre-clinical models and in the clinic, that the ER plays a vitally important role in the majority of breast cancers. The most successful current breast cancer therapies are based around inhibiting ER signalling (tamoxifen and fulvestrant), or lowering levels of oestrogens (AI's). However none of these therapies have yet been shown to be able to completely ablate ER action, and an ER positive phenotype has been shown to remain for a considerable period into disease progression. This can occur even following clinical use of fulvestrant, the most potent ER down-regulator available, and the only one currently sanctioned for clinical use. It has also been shown that there is a role of continued ER activity during acquisition of some forms of endocrine resistance, with both model and clinical evidence of acquired tamoxifen, AI or fulvestrant resistance often showing an anti-tumour response to further endocrine agents such as fulvestrant. Emerging clinical evidence

shows that increasing concentrations of fulvestrant are able to further reduce ER levels and function, and to contribute to better anti-proliferative and patient response. However, it remains unknown if maximal depletion of ER during the hormone responsive phase could subvert residual cell proliferation (and thus potentially acquisition of resistance).

This thesis aims to assess the biological importance of this residual ER in breast cancer, by attempting to produce a complete loss of ER levels and assessing subsequent signalling activity and anti-tumour effect. This will be achieved by the use of the following three methodologies to manipulate ER levels;

1. The use of short-term, high-concentration fulvestrant exposure, in our MCF-7 cell model to assess the maximum ER down-regulation, and signalling inhibition achievable by targeting the ER protein using this drug, and its effect on tumour growth and proliferation.
2. By targeting translation of ER mRNA, utilising the novel mechanism of RNAi to inhibit ER mRNA, to provide an alternative means to subsequently reduce ER protein levels, signalling, and its tumour activity. This mechanism will be assessed as a model to see whether ER mRNA targeting could be an improved therapeutic strategy in treatment of ER positive breast cancer.
3. Finally, both strategies for targeting ER protein or ER mRNA will be used in conjunction, to see whether there is any

synergistic action of these in combination, and whether this can achieve complete ER ablation. The project will address whether a complete/greater ER loss than previously recorded in cell models or within the clinical setting can be achieved, and if this shows greater anti-tumour activity in our cell-models. This should help address if a greater improvement in ER targeting could be of future therapeutic benefit to patients presenting with ER positive breast tumours beyond other current therapeutic regimens.

Chapter 2. Materials and Methods Section.

Section 2.1. Materials.

Below is a complete list of all materials used in subsequent experiments and the companies they were purchased from. Please see section 2.2. For their use.

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), from Sigma-Aldrich, Poole, Dorset, UK.
- 3-(aminopropyl)triethoxy-silane (TESPA), from Sigma-Aldrich, Poole, Dorset, UK.
- Acrylamide/bis-acrylamide (30% solution (v/v), 29:1 ratio), from Sigma-Aldrich, Poole, Dorset, UK.
- Activated charcoal, from Sigma-Aldrich, Poole, Dorset, UK.
- Agarose, Bioline Ltd, London, UK.
- Ammonium persulphate (APS), from Sigma-Aldrich, Poole, Dorset, UK.
- Amphotericin B (Fungizone), from Invitrogen, Paisley, UK.
- Antibiotics (penicillin/streptomycin), from Invitrogen, Paisley, UK.
- Anti-mouse horseradish-peroxidase-linked IgG (source: sheep), from Amersham, Little Chalfont, UK.
- Anti-rabbit horseradish-peroxidase-linked IgG (source: donkey), from Amersham, Little Chalfont, UK.
- Anti-rabbit/Anti-mouse EnVision™+ System, Peroxidase (DAB) kits, from Cytomation, California, USA.

- Bovine serum albumin (BSA), from Sigma-Aldrich, Poole, Dorset, UK.
- Cell culture medium: RPMI 1640 and Phenol-red-free RPMI 1640, from Invitrogen, Paisley, UK.
- Cell culture medium: Phenol-red-free DCCM, from Biological Industries Ltd, Israel.
- Cell scrapers, from Greiner Bio-One Ltd, Gloucestershire, UK.
- Chemiluminescent Supersignal® West HRP Substrate (Pico, Dura and Femto), from Pierce and Warriner Ltd, Cheshire, UK.
- Coulter Counter counting cups and lids, from Sarstedt AG and Co., Nümbrecht, Germany.
- Di-butylpthalatexylene (DPX), from Sigma-Aldrich, Poole, Dorset, UK. .
- Dimethyl sulphoxide (DMSO), from Sigma-Aldrich, Poole, Dorset, UK.
- Disposable Cuvettes, from Fisher Scientific UK Ltd, Loughborough, UK.
- DNase free, DNA free, RNase free and RNA free H_2O, from Sigma-Aldrich, Poole, Dorset, UK.
- dNTPs (dGTP, dCTP, dATP, dTTP; 100mM), from Amersham, Little Chalfont, UK.
- Dual-Luciferase Reporter Assay system, from Promega Ltd, WI, USA.
- Ethidium bromide (EtBr), from Sigma-Aldrich, Poole, Dorset, UK.

- Ethylene diamine tetraacetic acid (EDTA), from Sigma-Aldrich, Poole, Dorset, UK.
- Ethylene glycol-bis(2-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA), from Sigma-Aldrich, Poole, Dorset, UK.
- Filter paper (grade 3), from Whatman, Maidstone, UK.
- Foetal calf serum (FCS), from Invitrogen, Paisley, UK.
- General laboratory glass- and plastic ware, from Fisher Scientific UK Ltd, Loughborough, UK.
- Glass coverslips, from Fisher Scientific UK Ltd, Loughborough, UK.
- Glass slides, from Fisher Scientific UK Ltd, Loughborough, UK.
- Glycerol, from Fisher Scientific UK Ltd, Loughborough, UK.
- Glycine, from Sigma-Aldrich, Poole, Dorset, UK.
- Hydrochloric acid (HCl; 5M), from Fisher Scientific UK Ltd, Loughborough, UK.
- Hyperladder™ I and Hyperladder™ IV, from Bioline Ltd, London, UK.
- Isoton, from Coulter Beckman, UK.
- Kodak Medical X-ray film, from Genetic Research Instrumentation (GRI), Rayne, UK.
- LB-Agar EZMix™ powder and LB-Broth EZMix™ powder , from Sigma-Aldrich, Poole, Dorset, UK.
- Leupeptin, from Sigma-Aldrich, Poole, Dorset, UK.
- L-glutamine, from Invitrogen, Paisley, UK.

- Lower buffer for SDS-PAGE Gels (Tris 1.5M, pH 8.8), from Bio-Rad Laboratories Ltd, HERTS, UK.
- Magnesium chloride ($MgCl_2$), from Sigma-Aldrich, Poole, Dorset, UK.
- Methyl green, from Sigma-Aldrich, Poole, Dorset, UK.
- Micro-centrifuge tubes (0.5ml and 1.5ml), from Elkay Laboratory Products, Basingstoke, UK.
- Molony-murine leukaemia virus (MMLV) reverse transcriptase, from Invitrogen, Paisley, UK.
- mRNA primers for ER, pS2, PR and Actin, from MGW biotech, Germany.
- N,N,N',N'-tetramethylene-diamine (TEMED), from Sigma-Aldrich, Poole, Dorset, UK.
- Nitrocellulose transfer membrane (Protran® BA85; 0.45µm pore size), from Schleicher and Schuell, Dassell, Germany.
- pH calibration buffer tablets (pH 4, 7 and 10), from Fisher Scientific UK Ltd, Loughborough, UK.
- Phenylarsine oxide, from Sigma-Aldrich, Poole, Dorset, UK.
- Phenylmethylsulfonyl fluoride (PMSF), from Sigma-Aldrich, Poole, Dorset, UK.
- Phosphate buffered Saline (PBS), from Sigma-Aldrich, Poole, Dorset, UK.
- Phospho-EGFR antibody (tyr1068), from Cell Signalling Technology, Danvers, MA, USA.

- Phospho-ERK antibody (E-4) sc-7383, from Santa Cruz Biotechnology, Heidelberg, Germany.
- Phospho-IGF-1R antibody (Y1131), from Cell Signalling Technology, Danvers, MA, USA.
- Pipette tips, from Greiner Bio-One Ltd, Gloucestershire, UK.
- Polyoxyethylene-sorbitan monolaurate (Tween 20), from Sigma-Aldrich, Poole, Dorset, UK.
- Ponceau S solution (0.1% [w/v] in 5% acetic acid), from Sigma-Aldrich, Poole, Dorset, UK.
- Potassium chloride (KCl), from Sigma-Aldrich, Poole, Dorset, UK.
- Random hexamers (RH), from Amersham, Little Chalfont, UK.
- RNase-free H_2O, from Sigma-Aldrich, Poole, Dorset, UK.
- RNasin® ribonuclease inhibitor, from Promega, Southampton, UK.
- siGenome ER siRNA *smart*pool, individual siGenome ER siRNA, Scrambled siRNA, On-Target ER siRNA *smart*pool, siTOX siRNA and Dharmafect #1 transfection lipid, from Dharmacon, CO,USA.
- Sodium azide, from Sigma-Aldrich, Poole, Dorset, UK.
- Sodium chloride (NaCl), from Sigma-Aldrich, Poole, Dorset, UK.
- Sodium dodecyl sulphate (SDS), from Sigma-Aldrich, Poole, Dorset, UK.
- Sodium fluoride (NaF), from Sigma-Aldrich, Poole, Dorset, UK.
- Sodium hydroxide (NaOH; 5M), from Fisher Scientific UK Ltd, Loughborough, UK.

- Sodium molybdate (Na_2MoO_4), from Sigma-Aldrich, Poole, Dorset, UK.
- Sodium orthovanadate ($NaVO_4$), from Sigma-Aldrich, Poole, Dorset, UK.
- Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol), from Fisher Scientific UK Ltd, Loughborough, UK.
- Sterile bijou vials (5ml), from Bibby Sterilin Ltd, Stone, UK.
- Sterile cell culture plasticware (i.e. flasks, Petri-dishes, 12-, 24- and 96-well plates), from Nunc Int., Roskilde, Denmark.
- Sterile Falcon tubes (15ml and 50ml), from Sarstedt AG and Co., Nümbrecht, Germany.
- Sterile phosphate buffered saline (PBS), from Invitrogen, Paisley, UK.
- Sterile syringe filters (0.2μm), from Corning Inc., Corning, NY, USA.
- Sterile syringe needles (BD Microbalance™ 3; 25G x 5/8"), from Becton Dickinson (BD) UK Ltd, Oxford, UK.
- Sterile syringe needles (Sherwood Medical Monoject; 21G x 1½"), from Sherwood - Davis & Geck, Gosport, Hampshire, UK.
- Sterile syringes (BD Plastipak™; 1ml, 5ml and 10ml), from Becton Dickinson (BD) UK Ltd, Oxford, UK.
- Sterile universal containers (30ml), Greiner Bio-One Ltd, Gloucestershire, UK.
- Sterile, disposable serological pipettes (5ml, 10ml and 25ml), from Sarstedt AG and Co., Nümbrecht, Germany.

- Taq DNA polymerase (BioTaq™; 5U/μl), from Bioline Ltd, London, UK.
- Total Beta actin antibody (Sigma clone AC-15), from Sigma-Aldrich, Poole, Dorset, UK.
- Total EGFR antibody (1005) sc-03, from Santa Cruz Biotechnology, Heidelberg, Germany.
- Total ER antibody clone 6F11, from Vector, CA, USA.
- Total ERK antibody (9101) p44/p42 MAPkinase, from Cell Signalling Technology, Danvers, MA, USA.
- Total IGF-1R antibody (N-20) sc-712, from Santa Cruz Biotechnology, Heidelberg, Germany.
- Total HER2 antibody (2242), from Cell Signalling Technology, Danvers, MA, USA.
- Total IRS1 antibody (2382), from Cell Signalling Technology, Danvers, MA, USA.
- Total Ki67 antibody (M7240) mouse monoclonal anti-human Ki67 antigen MIB-1 clone, from DAKO, Denmark.
- Total PR antibody (VP-P976) mouse monoclonal clone 16, from Vector, CA, USA.
- Total pS2 antibody (NCL-pS2) rabbit polyclonal, from Novacastra, Newcastle, UK.
- TRI-Reagent, from Sigma-Aldrich, Poole, Dorset, UK.
- Tris HCl, from Sigma-Aldrich, Poole, Dorset, UK.
- Triton X-100, from Sigma-Aldrich, Poole, Dorset, UK.

- Trizma (Tris) base, from Sigma-Aldrich, Poole, Dorset, UK.
- Trypsin/EDTA 10x Solution, from Invitrogen, Paisley, UK.
- Upper buffer for SDS-PAGE Gels (Tris 0.5M, pH 6.8), from Bio-Rad Laboratories Ltd, HERTS, UK.
- Virkon, from Antec International Ltd, Suffolk, UK.
- Western Blocking Reagent, from Roche Diagnostics, Mannheim, Germany.
- X-ray film developer solution (X-O-dev) and X-ray film fixative solution (X-O-fix), from X-O-graph Imaging System, Tetbury, UK.

Section 2.2. Methods.

2.2.1. Cell culture.

Routine cell culture.

The hormone-sensitive MCF-7 wild type cell line (MCF-7) was a kind donation from AstraZeneca Pharmaceuticals (Macclesfield, Cheshire, UK), though was originally obtained from the American Type Culture Collection (ATCC #HTB-22). The oestrogen receptor positive T-47D, BT-474 and MDA-MB-361 cell models were also originally sourced from the American Type Culture Collection (ATCC #HTB-133, HTB-20 and HTB-27, respectively).

All cell-culture was carried out under sterile conditions in a MDH Class II laminar-flow safety cabinet (BIOQUELL UK Ltd, Andover, UK). All equipment and consumables were either purchased sterile for single use or were sterilized at 119°C using a Denley BA852 autoclave (Thermoquest Ltd, Basingstoke, UK).

The MCF-7 and T-47D cell lines used were routinely maintained in 75cm^2 flasks (T-75), containing a liquid medium comprised of RPMI 1640 (with phenol-red pH indicator) which contained 5% (v/v) foetal calf serum (FCS), and the antibiotics; penicillin (10 units/ml), and streptomycin (100μg/ml) and the anti-fungal agent amphotericin B at 2.5μg/ml.

The cells were grown in a Sanyo MCO-17AIC incubator (Sanyo E&E Europe BV, Loughborough, UK) at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was replaced with fresh media every 3-4 days and the cells were visually assessed during this culture using a Nikon Eclipse TE200 phase-contrast microscope (Nikon UK Ltd, Kingston-upon-Thames, UK). The cells were passaged approximately every 7 days (once reaching a confluency of approximately 80%) with a seeding ratio of one in ten (1:10).

Passaging of cells was performed by removal of media, followed by disruption of the cell monolayer by addition of 10ml of Trypsin/EDTA (0.05%/0.02% w/v) in Dulbecco's phosphate-buffered saline (PBS), and returned to the incubator for a period of 3-5 minutes until the cells were in suspension. The Trypsin/EDTA was neutralised by addition of 10mls phenol-red containing RPMI 1640 containing 5% (v/v) foetal calf serum (FCS). Cells were then pelleted by centrifugation at 1000rpm for 5 minutes, and followed by re-suspension of the cell pellet in 1ml phenol-red containing RPMI 1640 containing 5% (v/v) foetal calf serum (FCS). This suspension was drawn into and expelled from a pipette tip until no clumps of cells were visible, one tenth of this suspension was then added to a T-75 containing 15mls of routine culture media.

The BT-474 and MDA-MB-361 cell lines were cultured and passaged in the same manner as the MCF-7 and T-47D cell lines though were routinely cultured in a liquid medium comprised of RPMI 1640 (with phenol-red pH

indicator) which contained penicillin (10 units/ml), streptomycin (100μg/ml) and amphotericin B at 2.5μg/ml but 10% (v/v) FCS.

The fulvestrant-resistant FasMCF MCF-7 cell line was previously developed in-house following culture of wild-type MCF-7 cells in phenol-red free RPMI 1640 which contained 5% (v/v) charcoal stripped (steroid-depleted) foetal calf serum (SFCS) (See appendix 1 for charcoal stripping procedure), l-glutamine (4mM), penicillin (10 units/ml), and streptomycin (100μg/ml) and amphotericin B (2.5μg/ml). This media was supplemented with 10^{-7}M fulvestrant. Culture was maintained for a period of approximately 12 months, until after initial growth inhibition, out-growths of resistant cells were observed. For this thesis, the resultant FasMCF cells were maintained in a similar manner to MCF-7 cells, except they were routinely cultured in phenol-red free RPMI 1640 which contained 5% (v/v) 5% SFCS, l-glutamine (4mM), penicillin (10 units/ml), and streptomycin (100μg/ml) and amphotericin B (2.5μg/ml), supplemented with 10^{-7}M fulvestrant.

Experimental cell culture.

The seeding media used for the MCF-7, T-47D, BT-474 and MDA-MB-361 cell lines was either; phenol-red free RPMI containing 5% (v/v) SFCS, l-glutamine (4mM), penicillin (10 units/ml), and streptomycin (100μg/ml) and amphotericin B (2.5μg/ml) for the stripped serum conditions, or for the whole serum experimental condition the media was as follows; phenol-red free RPMI

containing 5% (v/v) FCS, l-glutamine (4mM), penicillin (10 units/ml), and streptomycin (100μg/ml) and amphotericin B (2.5μg/ml).

The seeding media used for the FasMCF cells was phenol-red free RPMI 1640 which contained 5% (v/v) SFCS, l-glutamine (4mM), penicillin (10 units/ml), and streptomycin (100μg/ml) and amphotericin B (2.5μg/ml), supplemented with 10^{-7}M fulvestrant.

Prior to seeding cells for experimentation, cell monolayers were washed twice in PBS, before dispersion, centrifugation and re-suspension as described previously. The suspension was then passed through a sterile 25G syringe needle to obtain a single-cell suspension. A 50μl aliquot of this suspension was added to 10mls of Isoton solution and cell number was calculated using a Coulter™ Multisizer II (Beckman Coulter UK Ltd, High Wycombe, UK). Once counted the desired cell number was then added to the correct amount of seeding media according to the number of conditions and type of experiment to be conducted (see table 2.1. for comprehensive figures for cell seeding densities into experimental culture apparatus for each experiment type and duration of experiments). Once cells were seeded out into desired culture apparatus, 24 hours were allowed for cells to adhere to culture surfaces then seeding media was removed and treatment media was added, and refreshed every 4 days during the time course of the experiment. Table 2.2 shows the composition of the various treatment media used in this thesis and the conditions used for each experiment are shown in figure legends.

Cell seeding densities for experimental work.

Plate size	Media used/well (Seeding or Treatment media)	Initial seeding density/ plate	Type of experiment.	Duration of experiment.
35mm Dish	1.5ml	$1x10^5$	Immunocytochemistry (1 dish per condition, at least 3 replicates)	Immunocytochemistry – 4 or 8 days)
6 well plate	1.5ml	$1x10^6$	Protein harvest. (1 well per condition, at least 3 replicates)	Protein harvest for Western blotting analysis – 4 or 8 days
12 well plate	1ml	$1x10^6$	RNA harvest. (1 well per condition, at least 3 replicates)	RNA harvest for RT-PCR – 4 days.
24 well plate	1ml	$1x10^6$	Total cell counting, (3 wells used per condition, at least 3 replicates). ERE reporter gene assay. (3 wells used per condition, at least 3 replicates)	Total cell counting – 8 days. ERE reporter gene assay – 4 days.
96 well plate	150µl	$1x10^6$	MTT assay. (8 wells used per condition, at least 3 replicates)	MTT assay – 8 days.

Table 2.1 Cell seeding densities, media amounts and culture apparatus used for each experiment type and its duration.

Treatment	Volume added to 1ml media (Containing whole or stripped serum)	Final treatment concentration
Media Control	No treatments	N/A
10^{-10}M, 10^{-9}M, 10^{-8}M, 10^{-7}M or 10^{-6}M Fulv	1µl of 10^{-7}M-10^{-3}M Fulvestrant stock (respectively) in Ethanol . NB. Vehicle control was 1µl Ethanol.	0.1nM-1000nM Fulvestrant respectively
10-7M Fulv	1µl of 10^{-4}M Fulvestrant stock in Ethanol	100nM Fulvestrant
Lipid (2nM-10nM)	0.1µl-0.5µl Dharmafect#1 transfection lipid as supplied	2nM-10nM Dharmafect respectively.
siRNA control/siC	5µl of stock siControl siRNA as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied.	100nM siRNA and 2nM Dharmafect
siTOX	5µl of stock siTOX siRNA as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied.	100nM siRNA and 2nM Dharmafect
siGenome siER/siG siER	5µl of stock siGenome siER siRNA (25% each of siER constructs 1-4) as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied.	100nM siRNA and 2nM Dharmafect
siER 1-4 constructs	5µl of stock siER siRNA constructs 1-4 respectively as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied.	100nM siRNA and 2nM Dharmafect
On-target siER/Ont siER	5µl of stock On-target siER siRNA as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied.	100nM siRNA and 2nM Dharmafect
siRNA control +Fulvestrant/siC+fulv	5µl of stock siControl siRNA respectively as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied. Also 1µl of 10^{-4}M Fulvestrant stock in Ethanol	100nM siRNA, 2nM Dharmafect and 100nM Fulvestrant.
Combination condition/On-target siER +Fulvestrant/ siER +Fulv	5µl of stock On-target siER siRNA as described in 2.2.2. combined with 0.1µl Dharmafect#1 transfection lipid as supplied. Also 1µl of 10^{-4}M Fulvestrant stock in Ethanol	100nM siRNA and 2nM Dharmafect and 100nM Fulvestrant.
N.B. Any +E_2 conditions additionally contained 1µl of 10^{-6}M 17β-oestradiol stock (in ethanol) per ml media for a final concentration 10^{-9}M E_2		

Table 2.2. Treatments added to either whole or stripped serum containing media to produce conditions used throughout the project.

2.2.2. SiRNA transfection.

This methodology was adapted from Dharmacon Technologies product information supplied with Dharmacon *smart*pool siGenome siRNA.

All the commercially obtained oestrogen receptor siRNA constructs (SiGenome ESR1 *smart pool*, SiGenome ESR1 siRNA constructs 1-4 and On-target ESR1 *smart pool*) and siRNA controls (On-target non-targeting pool - siControl and TOX transfection control- siTOX) were all treated in the same way and made into individual standard stock solutions using the 1x siRNA buffer provided. The 1x siRNA buffer was made up from a 5x solution by the addition of four times the volume of DNase and RNase-free H_2O (from Sigma). SiRNA buffer was added to the siRNA to make up to a final concentration 20μM(pmol/μl) and aliquoted in 20μl aliquots into sterile microcentrifuge tubes, to prevent degradation by multiple freeze-thaw cycles. The siRNA stock was stored at –20°c, until required. Dharmafect #1 transfection lipid was supplied ready for use at the concentration provided and stored at 1-4°c (Dharmacon transfection reagent 2002).

The amount of siRNA and transfection lipid required varied depending on media required for the plate sized used. The recommended concentrations of Dharmafect#1 transfection lipid according to the manufacturer's instructions were found to be highly cytotoxic. The protocol was then optimised to reduce cytotoxicity, (see table 2.3. for lipid amounts originally recommended and final optimised amounts. N.B. For both the optimised and

original siRNA use, the final siRNA concentration was the same shown in table 2.2. The original Dharmafect final concentration was 5-fold higher than table 2.2. indicates), and see section 3.2 for explanation of optimisation performed.

To perform siRNA transfection, cells for experimentation were harvested as previously described in section 2.2.1. and the desired number of cells were diluted into correct amount of seeding media per size and number of plates required (see table 2.1.). Cells were allowed to settle for 24 hours prior to treatment, in an incubator (37°C, in a humidified atmosphere of 5% CO_2 as previously described).

Transfection lipid and siRNA used for different sizes of culture apparatus.

Plate/Well	Stock siRNA (µl)	Optimised Dharmafect (µl)	Original Dharmafect (µl)	Media (mls)
60mm Dish	25	0.5	2.5	5
35mm Dish	5	0.1	0.5	1
6 well plate	10	0.2	1	2
12 well plate	5	0.1	0.5	1
24 well plate	2.5	0.05	0.25	0.5
96 well plate	0.75	0.015	0.075	0.15

Table 2.3. Amounts of siRNA stock and transfection lipid needed for each well size.

After 24 hours the seeding media was removed, just prior to removal the siRNA constructs was allowed to thaw on ice and then each pipetted at the desired amount into separate sterile micro-centrifuge tubes and the correct amount of Dharmafect transfection lipid required was then added to each micro-centrifuge tube containing siRNA constructs, additionally a further micro-centrifuge containing only lipid was used as a Dharmafect lipid control.

The Dharmafect lipid and siRNA were each then gently mixed up and down three times through a pipette tip. The mixture was then allowed to stand for 20 minutes to allow formation of micelles. After micelle formation these siRNA/lipid complexes (or lipid alone) were then added to the relevant treatment media (already containing the serum, glutamine and antibiotics as previously described and fulvestrant in the case of the combination and siControl +fulvestrant conditions, see table 2.2.). For example, for the siControl arm of an MTT experiment, 8 wells each containing 150µl of treatment media are required (Table 2.1), so 6µl of siControl siRNA stock would be added to 0.12 µl of Dharmafect which was then added to 1.2ml treatment media (see Table 2.2) , and 150µl of this completed treatment media was then added to each of the 8 wells.

These various media were added to the cells after removal of seeding media. Cultures were replenished with freshly made treatment medium every 4 days for duration of experiment containing freshly formed siRNA/lipid complexes, at 0 and 4 days for an 8 day experiment (such as MTT) or only on day 0 for a 4 day experiment (such as RNA harvest).

The experimental ER siRNA was always used with several controls to ensure that any observed effect was due to the use of the experimental siRNA of interest only. Dharmafect lipid alone and Dharmafect with a scrambled siRNA control (one designed so it does not target any known mRNA sequences) was deemed sufficient to determine whether the effect observed is due to knockdown of the gene of interest alone and not the process of transfection and siTox positive control was used to determine transfection efficiency.

2.2.3. Immunocytochemical methods (ICC).

2.2.3.1. Cell fixation.

Cells for experimentation were harvested as previously described in section 2.2.1. and $1x10^5$ cells were diluted into 1.5ml of experimental media per condition and time point required. Cells were then seeded onto sterile TESPA-coated coverslips placed into the bottom of 35mm culture dishes, cells were allowed 24 hours to settle and adhere to the coverslips prior to removal of seeding media and then the media containing treatments were added for the desired duration. After the desired time point was reached the cells were then fixed using either an ER-ICA fixation for total ER, PR and pS2 assays, or a formal-saline fixation for the Ki67 assay, prior to immunocytochemical staining for these proteins.

These assays are all standard assays within our laboratory and the decision to use the fixes for the assays below was based upon the expert advice, given by members of our ICC department. The phosphate-buffered saline (PBS) used in all immunocytochemical experiments was a 0.01M PBS (see appendix 2 for recipe).

Cell fixation by ER-ICA fixation.

The oestrogen-receptor immunocytochemical assay, or ER-ICA, fix was originally developed for the detection of the oestrogen receptor, but has also been found to be an effective fixation method, allowing for immunocytochemical staining of a number of proteins.

Firstly coverslips were placed in a rack and submerged in 3.7% formaldehyde solution at room temperature for 15 minutes (to make 300ml 3.7% formaldehyde solution add 30ml of 37% formaldehyde into 270ml PBS). Racks were removed and placed into PBS at room temperature for 5 minutes. Once the PBS wash was complete, racks were placed in 100% Methanol (between $-10^{\circ}C$ and $-30^{\circ}C$) for 5 minutes. Following that racks were placed in Acetone (between $-10^{\circ}C$ and $-30^{\circ}C$) for 3 minutes. Finally racks were placed in PBS at room temperature for 5 minutes. Coverslips were then removed from racks and placed into clean 35mm dishes. Coverslips could be stained immediately or filled with sucrose storage medium (SSM, see appendix 3 for recipe) and stored at $-20^{\circ}C$ prior to immunocytochemical staining.

Cell fixation by Formal-saline fixation.

Media was removed from coverslip dishes. Dishes were filled (approximately 1ml) with a formal-saline solution (comprised of 4.5g Sodium Chloride, 50ml 37% Formaldehyde solution and 450ml Tap Water) for 10 minutes at room temperature. Formal saline solution was replaced with 100% ethanol at room temperature for 5 minutes (followed by a quick 100% ethanol rinse). The ethanol was removed and dishes were rinsed quickly with PBS. Dishes were filled with PBS at room temperature for 5 minutes. Dishes were then quickly rinsed with PBS. The PBS was then removed and the coverslips were stained immediately or the dishes were filled with sucrose storage medium. The 35mm dishes were stored at –20°c prior to immunocytochemical staining.

2.2.3.2. Immunocytochemical staining.

All primary antibodies were selected on (i) the expert quality control advice of the Tenovus group technical staff for current ER and PR antibodies and (ii) for highest sensitivity in the case of ER in the monolayer cultures, to allow for greatest detection of any residual ER remaining following treatments.

Total oestrogen receptor staining.

The cells were fixed by ER-ICA fixation (as in 2.2.3.1.) and if previously stored at –20°c in sucrose storage media, the assay was started by washing the

coverslips of sucrose storage media by three, 3 minute washes in room temperature PBS. Then a 0.02% PBS tween solution (see appendix 4 for recipe) was applied to the coverslips for a further 3 minutes. The excess was then removed prior to addition of the primary antibody. Vector Total ER clone 6F11 (mouse) antibody was then applied at 1/75 in PBS for 90 minutes and 50µl of primary antibody was used per coverslip. Following the 90 minute incubation the coverslips were washed twice in 0.02% PBS tween for 5 minutes per wash. The excess was removed and DAKO Mouse Envision (1 drop per cover slip) was applied for 75 minutes. After the secondary antibody incubation, the coverslips are washed twice in 0.02% PBS tween for 5 minutes per wash. Dako DAB was then applied to the coverslips for 10 minutes (1 drop of DAB to 1ml of substrate) and 70µl per cover slip was used. Coverslips were then washed twice in dH_2O for 5 minutes per wash. After the excess was removed an aqueous solution of 0.5% methyl green was applied for 5 minutes as a counter stain. The coverslips were washed twice in distilled water for 2 minutes per wash. Finally coverslips were allowed to dry completely before being mounted onto slides using DPX mountant. The slides were then stored for assessment under a microscope for ER expression.

Total progesterone receptor staining.

The cells were fixed by ER-ICA fixation (As in 2.2.3.1.) and if previously stored at –20°c in SSM, the assay was started by washing the SSM from the coverslips by three, 3 minute washes in room temperature PBS. Then a 0.02%

PBS tween solution was applied to the coverslips for a further 3 minutes. The excess was then removed prior to addition of the primary antibody. Vector Total PR clone 16 (mouse) antibody was applied at 1/30 in PBS for 60 minutes and 50µl of primary antibody was used per coverslip. Following the 60 minute incubation the coverslips were then washed twice in 0.02% PBS tween for 5 minutes per wash. The excess was removed and DAKO Mouse Envision (1 drop per cover slip) was applied for 75 minutes. After the secondary antibody incubation, the coverslips were washed twice in 0.02% PBS tween for 5 minutes per wash. Dako DAB was applied to the coverslips for 10 minutes (1 drop of DAB to 1ml of substrate) and 70µl per coverslip was used. Coverslips were then washed twice in dH_2O for 5 minutes per wash. After the excess was removed an aqueous solution of 0.5% methyl green was applied for 5 minutes as a counter stain. The coverslips were washed twice in distilled water for 2 minutes per wash. Finally coverslips were allowed to dry completely before mounting them onto slides using DPX mountant. The slides could then be assessed under a microscope for PR expression.

Total pS2 staining.

The cells were fixed by ER-ICA fixation (As in 2.2.3.1.) and if stored at –20°c in sucrose storage medium, the assay was begun by washing the SSM from the coverslips by three, 3 minute washes in room temperature PBS. Then a 0.02% PBS tween solution was applied to the coverslips for a further 3 minutes. The excess was removed prior to addition of the primary antibody. NovoCastra

pS2 antibody (Rabbit) at 1/400 in PBS was applied for 90 minutes using 50µl of primary antibody per coverslip. Following the 90 minute incubation the coverslips were washed twice in 0.02% PBS tween for 5 minutes per wash. The excess was removed and Dako Rabbit Envision (1 drop per cover slip) was then applied for 60 minutes. After incubation with the secondary antibody, coverslips were washed twice in 0.02% PBS tween for 5 minutes per wash. The excess was removed and Dako DAB was applied to cover slips for 10 minutes (1 drop of DAB to 1ml of substrate) and 70µl per coverslip was used. Coverslips were then washed twice in dH_2O for 5 minutes per wash. The excess was removed and an aqueous solution of 0.5% methyl green was applied for 5 minutes as a counter stain. The coverslips were then washed twice in distilled water for 2 minutes per wash. Finally cover slips were allowed to dry completely before mounting them onto slides using DPX mountant. The slides could then be assessed under a microscope for pS2 expression.

Total Ki67 staining.

The cells were previously fixed by Formal saline fixation (As in 2.2.3.1.) and if stored at –20°c in SSM prior to the following assay, the assay was begun by washing SSM from the coverslips by three, 3 minute washes in room temperature PBS. 0.02% PBS tween was applied to the coverslips for 3 minutes but removed prior to addition of primary antibody. Dako MIB-1 (clone M7240) at 1/100 in 0.02%PBS tween was applied to coverslips for 60

minutes and 50µl of primary antibody was used per coverslip. After incubation with primary antibody, coverslips were washed twice in 0.02% PBS tween for 5 minutes per wash. Excess was removed prior to addition of secondary antibody. Dako Mouse Envision (1 drop per cover slip) was applied to the cover slips for 75 minutes. Once incubation with the secondary antibody was complete coverslips were washed with PBS for 3 minutes, followed by two five minute washes in 0.02% PBS tween. The excess was removed and Dako DAB was applied to coverslips for 10 minutes (1 drop of DAB to 1ml of substrate) and 70µl per coverslip was used. Coverslips were then washed twice in dH_2O for 5 minutes per wash. The excess was removed and an aqueous solution of 0.5% methyl green was applied for 5 minutes as a counter stain. The coverslips were then washed twice in distilled water for 2 minutes per wash. Finally cover slips were allowed to dry completely before mounting onto slides using DPX mountant. The slides could then be assessed under a microscope for the percentage of cells expressing Ki67.

ICC assessment.

Following fixation and staining, slides were assessed by H-score analysis (Nuclear and cytoplasmic cell expression for ER and pS2, nuclear, cytoplasmic and total cell expression for PR) or % nuclear positivity alone (for Ki67).

Each coverslip for a particular treatment and experiment was assessed by looking at six fields of view (under x20 magnification using a light

microscope, assessing approximately 2000 cells. For HScore analysis, in every field the percentage of cells within each staining intensity category (0= no visible staining, 1+ = weak staining, 2+ = moderate staining, 3+ = highly intense staining) was estimated and this was used in the following formula to calculate a field HScore: (% 1+ x1) + (%2+ x2) +(%3+ x3)

H-score values thus lay on a 0-300 scale, with means averaged over the six fields of view giving a H-score for the treatment in that experiment. The corresponding treatments from replicate experiments (at least 3) were also assessed in the same manner and the H-scores were averaged to give a final H-score which were then used in subsequent statistical analysis.

Percentage positivity was assessed in a similar manner, with cells being ranked as either positive (any detectable staining) or negative (no detectable staining) giving a % positivity out 100.

2.2.4. mRNA analysis.

RNA extraction and Quantification.

This procedure was adapted from the Sigma-Aldrich TRI REAGENT protocol (Product number T 9424 Technical Bulletin MB-205 August 99).

Cells for experimentation were harvested as previously described in section 2.2.1. and $1x10^6$ cells suspended into 12 ml of experimental medium for each 12 well plate required. Cells were then seeded into the 12 well plates

(1ml per well) and allowed to settle for 24 hours at 37°C in a humidified atmosphere of 5% CO_2. Following incubation, the seeding medium was removed and media containing treatments were added (1ml per well), these media were refreshed every 4 days. After the desired time point was reached the experimental media was removed, and cells were washed twice with 37°C sterile PBS.

After the PBS was removed 300µl of TRI-reagent (Sigma) was added to each well and left for 5 minutes. Using a cell scraper, cells were agitated from the bottom of each well and pipetted into separate sterile 1.5ml microcentrifuge tubes and were stored for at least 24 hours at -20°c (for short-term storage, up to a month) or at -80°C (for longer term storage, up to 6 months).

RNA isolation was continued by thawing of the samples on ice for 10-20 minutes, followed by standing at room temperature for 5 minutes. 100µl of 100% chloroform was then added to each sample, and samples were vortexed to mix (for 30 seconds). Samples were then allowed to stand at room temperature for 10 minutes, before centrifugation for 15 minutes at 13,000 rpm at 4°C in a microcentrifuge. After centrifugation, RNA was retained in the upper (aqueous) clear phase, with DNA sedimented at the interface and protein in the lower (aqueous) pink phase. The upper aqueous phase (about 200µl) was carefully removed from each sample and placed without disturbing the interface into a fresh microcentrifuge tube. 200µl of 100% isopropanol was added to each of these new samples. These samples were vortexed (10seconds) and then left to stand for 10 minutes at room temperature. To

precipitate down the RNA the samples were then centrifuged for 15 minutes at 13,000 rpm at 4°C, pelleting down the RNA as a whitish smear at the bottom of the tube. Supernatant was then carefully poured off; keeping the precipitate/pellet, 600µl of 75% ethanol was added to each sample. The samples were then vortexed (5 seconds) to wash, and then centrifuged for 15 minutes at 13,000 rpm. Supernatants were poured off carefully without disturbing the pellet. Samples were then air-dried on ice. Once samples were dried, 10µl of sterile RNase free H_2O (Sigma) was added to each sample. Finally samples were stored at -20°C (for short-term) or -80°C (for long-term) for later analysis.

Prior to storage the RNA was quantified by spectrophotometry. 2µl of RNA sample was diluted in 1ml of DNase free, RNase free H_2O. The optical density (OD) at 260nm (RNA value) and 280nm (DNA value) were then measured. The ratio between the two values was used to give a measure of the purity of the RNA (a ratio of >1.7 representing a pure nucleic acid solution). The RNA concentration was determined by multiplication of the optical density at 260nm by the RNA co-efficient (40) and then multiplied by the dilution factor.

Reverse Transcription Polymerase Chain Reaction.

RT-PCR is a method to amplify and identify specific sequences of mRNA that maybe present in a sample of RNA. It is performed in two steps, the first is Reverse Transcription, a process whereby all RNA molecules are replicated

into more thermally stable complementary DNA molecules (cDNA) . The second step of a Polymerase Chain Reaction (PCR) is an exponential amplification of a specific region of the cDNA (RNA) of interest through the use of specific oligonucleotide primers. All PCR reactions were performed in a Labconco Purifier PCR Enclosure (GRI, Rayne, UK) and sterile pipette tips and reaction tubes were used at all times. Prior to experimentation all equipment and work surfaces were wiped with 70% ethanol and allowed to air-dry.

Reverse Transcription.

Aliquots of RNA samples to be used for reverse transcription were diluted to a concentration of 1μg/7.5μl into DNase and RNase free H_2O. Following this, 7.5μl of each sample was added to 11μl of a Reverse Transcriptase master mix (see appendix 5.). The RNA in these samples were then denatured at 95°C for 5 minutes in a PTC-100 thermocycler (MJ Research Ltd, Massachusetts, USA), followed by rapid cooling on ice. The samples were pulse spun in a micro-centrifuge (IEC Micromax RF, Thermo Electron Corporation, Hampshire, UK) to recollect all the solution, and the samples were returned to the ice. 1μl MMLV-reverse transcriptase enzyme (100u/μl)and 0.5μl RNasin (an RNase inhibitor, 40u/μl) was added to each sample, making a total sample volume of 20μl. The samples were returned to the PTC-100 thermocycler and reverse transcribed for a single cycle under the following conditions:-

22°C for 10 minutes (annealing time)

42°C for 42 minutes (RT extension time)

95°C for 5 minutes (denaturing time)

The resulting cDNA can be used instantly or stored at –20°C until required.

Polymerase Chain Reaction.

To perform PCR on the previously obtained cDNA samples; 1µl of cDNA was added to 24µl of a PCR master mix (see appendix 6.). Table 2.4. shows primer sequences used and annealing temperatures and cycle numbers for each gene studied. A negative control in which cDNA was substituted with an equal volume of sterile H_20 was also run for each experiment. To prevent saturation of Actin for subsequent normalised densitometry (performed using Alpha digiDoc RT v.4.1.0 with AlphaEaseFC imaging system from Alpha Innotech) only genes with lower cycle numbers were co-amplified (PR and pS2).

Gene	Primer Sequence		Amplicon size	Annealing Temp(°C)	Cycle no.
β-Actin	For	5'- ggagcaatgatcttgatctt -3'	204	55	28
	Rev	3'- ccttcctgggcatggagtcct -5'			
ERα	For	5'- ggagacatgagagctgccaac -3'	432	55	30
	Rev	3'- ccagcagcagcatgtcgaagatc -5'			
PR	For	5'- ccatgtggcagatcccacaggagtt -3'	320	55	25
	Rev	3'- tggaaattcaacactcagtgcccgg -5'			
pS2	For	5'- catggagaacaaggtgatctg -3'	336	55	25
	Rev	3'- cagaagcgtgtctgaggtgtc -5'			

Table 2.4. Primer sequences, amplicon sizes, annealing temperatures and cycle numbers used for genes used in PCR.

The PCR reaction samples were vortexed in a micro-centrifuge (IEC Micro-max RF, Thermo Electron Corporation, Hampshire, UK) to mix, and were loaded into the PTC-100 thermocycler. The heated lid was set at 100°C and the PTC-100 thermocycler was set to the following programme (Continued overleaf);

- 2 minutes at 95°C, 1 minute at 55°C, 5 minutes at 72°C (initial denaturation).
- A cycle of 1 minute at 95°C (denaturation), then 30 seconds at 55°C (Annealing), followed by 1 minute at 72°C (Extension). These 3 steps

were repeated for appropriate number of cycles from primers used (shown in table 2.3).

- 1 minute at 94°C (a final denaturation step).
- 5 minutes at 72°C (final extension step).

The PCR products were stored at 4°C (or –20°C for long-term storage) until needed for agarose gel electrophoresis.

Agarose Gel Electrophoresis.

PCR amplified products were visualised using a 1% (w/v) agarose gel in Tris-Acetate-EDTA buffer (TAE, see appendix 7.) which contained 1µl of a 10mg/ml ethidum bromide solution per 50ml gel solution. Gels were cast and run using a horizontal gel Electrophoresis System (Bio-Rad) connected to a Powerpac 1000 power pack (Bio-Rad). 10µl of each PCR sample was added to 5µl loading buffer (see appendix 8.) and carefully pipetted into wells present on the gel. A DNA size marker (Hyperladder™ IV 100-1000bp; 5µl) was also added to a parallel lane to allow easier identification of gene fragment of interest. Once loaded the gel was run at 100volts (constant voltage) for approximately 40 minutes. Gels were visualised under UV light using a FOTODYNE 3-3002 UV trans-illuminator and photographed using the digidoc system (GRI) and alpha ease FC software to generate a digital photograph.

2.2.5 Protein analysis.

Cell lysis, Protein extraction and quantification.

Cells for experimentation were harvested as previously described in section 2.2.1. and seeded out at a density of $1x10^6$ cells per 6-well plate required (1.5ml media/well). Cells then were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours to allow adherence to the culture surface. Following incubation, the seeding medium was replaced with media containing desired treatments (1.5ml treatment medium per well), and was refreshed every 4 days. After the desired time point was reached (4 days unless stated) the medium was removed. The cultures were then washed three times with ice-cold sterile PBS. Excess PBS was then removed prior to addition of 200µl of ice-cold complete lysis buffer (see appendix 9. for recipe) to each well, and then left to stand on ice for 5 minutes. Using a sterile cell scraper the cells were removed from the growth surface, and the dishes were then inclined and left on ice for a further 5 minutes. Cell lysis was aided by pipetting several times, and the resulting lysate was transferred to a sterile, ice-cold microcentrifuge tube and incubated on ice for a further 10 minutes.

Cell lysates were then centrifuged at 13,000rpm for 15 minutes at 4°C to remove cell debris. The supernatants were dispensed into 50µl aliquots in sterile microcentrifuge tubes to prevent degradation of sample by multiple freeze-thaw cycles and stored at –20°C (or –80°C for long-term storage). Prior to storage the protein concentration in these samples was quantified.

Protein Quantification using the Bio-Rad system.

Using a method based on the Bradford assay, the protein concentration in each sample was measured by comparison with a standard curve using known concentrations of a Bovine Serum Albumin (BSA) solution (from 0-25μg/ml) prepared as follows, (see table 2.5.).

Concentration (μg/ml)	BSA (1mg/ml stock) (μl)	dH_2O (μl)
0	0	1000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975

Table 2.5. Table showing concentrations for standard curve.

Using plastic cuvettes, 200μl of Bio-Rad Dye concentrate (Bio-Rad) was added to 800μl of the BSA standards described in table 2.5, and these standards were produced in duplicate. The protein samples to be quantified were diluted by 1/200 also in duplicate. 4μl of protein sample was added to 796μl of dH_2O, to which was added 200μl of Bio-Rad Dye concentrate. The cuvettes were inverted several times to mix and a stable colour was allowed to develop for a minimum of 10 minutes prior to measuring absorbance.

The absorbance of the protein standards was measured spectrophotometrically, at a wavelength of 595nm, producing a standard curve. The test protein samples were also measured at this wavelength and were automatically plotted against the standard curve to give the protein concentration of each sample.

Sodium-Dodecyl-Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Protein lysates were resolved by SDS-PAGE using the Bio-Rad Mini-Protean® III system (Bio-Rad). The apparatus was assembled as described in the manufacturer's manual. A discontinuous system was used based on the method of Laemmli (Laemmli 1970) consisting of a 12% resolving Gel (see appendix 10. for details) overlaid with a 4.5% stacking gel (see appendix 11. for details) into which well-forming combs were set.

The polymerised gels were transferred to a gel dock and the central reservoir thus formed was filled with 1x Running Buffer (diluted with dH_2O from a 10x stock, see appendix 12.). Protein samples (equivalent to 50µg of protein) were mixed with an equal volume of 2x Laemmli sample loading buffer (see appendix 13.) and were denatured at 100°C for 5 minutes. These samples were then loaded into the wells, along with a final well containing 5µl of Rainbow molecular weight marker to verify the size of the protein signal. 1x Running buffer was then placed in the outer reservoir of the apparatus, so that both the top and bottom of the gel was in contact with the buffer ensuring that an even current would pass through the gel. Electrophoresis was

performed at 150 volts (constant volts) for approximately 1 hour (until the sample buffer dye had run the length of the gel).

Western Blotting.

Following SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane (0.45μm pore size). This was done by using the Mini-Protean® III Transblot gel apparatus powered by a Powerpac Basic™ power pack (Bio-Rad). This system takes two gel holder cassettes for electro-protein transfer of mini format gels.

Two Teflon sponge pads, two pieces of grade 3 filter paper, and one piece of Protran BA83 nitrocellulose membrane (0.45μm pore size) cut to the same size as the gel were all pre-soaked with Transfer buffer (see appendix 14. for details) prior to assembly. The SDS-PAGE gel was carefully removed from the glass plates and separated from stacking gel, and placed in transfer buffer to equilibrate for 30 seconds. The Western blotting transfer cassette was then assembled as follows (see figure 2.1. for diagram); placed on the black side of the cassette was a Teflon sponge, followed by filter paper, SDS-PAGE gel, the nitrocellulose membrane, a second piece of filter paper and finally the second sponge, the opaque side of the cassette was then closed over this. Prior to closure one of the glass plates was drawn across the assembled 'sandwich' to expel any air bubbles between the gel and nitrocellulose membrane (as air bubbles can prevent even protein transfer).

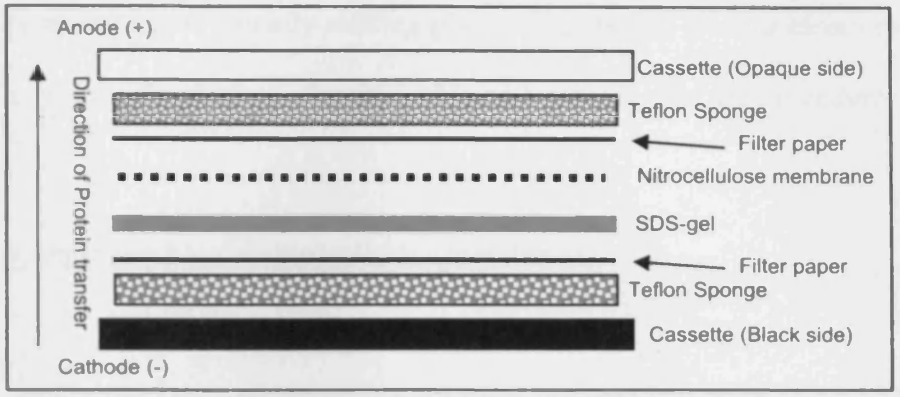


Figure 2.1. Assembly of Gel cassette for protein transfer.

The gel holder cassette was then inserted into the transfer apparatus with the white side facing the positive anode (this ensures the negatively-charged proteins migrate across to the nitrocellulose membrane). An ice pack and a magnetic stirring bar were added to the apparatus to prevent over-heating of the gel, and to maintain an even temperature throughout. The transfer apparatus was completely filled with transfer buffer and run at 100volts (constant volts) for 60 minutes.

Immunodetection of Proteins.

Once transfer was complete the nitrocellulose membrane was placed into a lidded sterile plastic container able to accommodate the membrane, and unreacted binding sites on the membrane were blocked by incubation with 5% Marvel milk (commercially obtained, powdered milk) solution (w/v) in TBS containing 0.05% (v/v) Tween 20 (TBS-Tween), for 1 hour at room

temperature on a gently rocking platform. This step was necessary to minimise non-specific binding of proteins during later stages of the procedure.

Immunoprobing of nitrocellulose membrane.

Following blocking the nitrocellulose membrane was washed with TBS-Tween (two 5 minute washes) then probed with primary antibody, prepared in either 1% (w/v) Marvel milk in TBS-Tween for total anti-bodies or 5% (v/v) Western blocking reagent in TBS-Tween for phospho-antibodies. The primary antibody dilutions employed and incubation conditions are outlined in table 2.6. Primary antibodies were selected on the expert advice of the Tenovus group technical staff and for highest sensitivity in the case of ER, to allow for greatest detection of any residual ER remaining following treatments.

Antibody (see section 2.1. for details of supplier).	Source.	Dilution.	Incubation conditions.
Total-β Actin	Mouse	1/100,000	2 hours, room temperature.
Total-ERα	Rabbit	1/10,000	Overnight, 4°C.
Phospho-EGFR (tyr 1068)	Rabbit	1/1,000	2 hours, room temperature.
Total-EGFR	Rabbit	1/1,500	2 hours, room temperature.
Total-HER2	Rabbit	1/1,500	Overnight, 4°C
Total-ERK	Rabbit	1/1000	Overnight, 4°C.
Phospho-IGF-1R (Y1131)	Mouse	1/1000	Overnight, 4°C
Total-IGF-1R	Rabbit	1/1000	Overnight, 4°C.
Total-IRS1	Rabbit	1/1000	Overnight, 4°C.

Table 2.6. Primary antibodies, concentrations and incubation conditions.

Once the primary incubation was finished the blot was washed with TBS-Tween (three washes of 10 minutes each), to remove any unbound antibody. The complementary secondary antibody (either mouse or rabbit depending on species of primary antibody), made up at 1/10,000 in a solution of 1% powdered milk (w/v) in TBS-Tween was then applied. The compensatory

secondary antibody was incubated with the nitrocellulose membrane at room temperature for 1 hour while being constantly rocked, followed by washing in TBS-Tween (five washes of 10 minutes each). Once incubation was complete five further washes of ten minutes in TBS-Tween were performed and the blot was then ready for development.

After washing, the bound antibody and thereby the protein of interest was visualised using a variety of commercially available chemiluminescent detection systems (Supersignal West Pico, Supersignal West Dura or Supersignal West Femto reaction). These were all antibody conjugated enzymes using a luminol/peroxide based system. Luminol (in the supersignal product) is oxidised by horse radish peroxidase (conjugated to the secondary antibody), in the presence of peroxide which produces an excited state product, which upon decaying releases photons of light which can be captured on x-ray film.

The protocol was the same for each detection system. For a single blot, 125µl of both the Luminol and peroxide reagents were mixed together in a microcentrifuge tube; this was then pipetted onto the nitrocellulose membrane which had been placed in a Kodak x-comet x-ray film cassette between the plush sheet, where a Kodak photographic film was placed over the blot for a set time.

The strength of the signal can vary, so that several different exposure times with film maybe required to obtain the optimum signal. X-ray films were developed using an X-O-graph Compact X2 x-ray developer (X-O-graph Imaging System, Tetbury, UK).

2.2.6 ERE reporter gene assay.

ERE construct, amplification and maintenance.

The ERE construct (an ERE-tk-luc(*firefly*) reporter) was a gift to the department from Professor Malcolm Parker. A structurally similar construct, tk-luc(*renilla*) reporter (Promega) was also used as a constitutively active control reporter . The plasmids were maintained within the *E.coli* strain DH5-alpha (Invitrogen). Bacteria for transformation were made competent and transformed by the method of Cohen (Cohen *et al* 1973). To obtain the plasmid constructs for transfection into breast cancer cell lines, several transformed *E.coli* colonies containing the plasmid were picked from 'streaked out' colonies grown on L-broth Agar containing ampicillin to use as an antibiotic marker to select for transformed colonies, and grown as 5ml starter cultures this was then used to inoculate 250ml overnight cultures. Reporter gene constructs were recovered by using these cells with a plasmid purification kit, the Qiagen Plasmid Maxi Kit (Qiagen, UK) as per instructions provided.

Dual-luciferase reporter gene assay.

Cells for experimentation were harvested as previously described in section 2.2.1. and $1x10^6$ cells were diluted in 25ml of seeding media per 24 well plate required, this gave a final dilution of $4x10^4$ cells/well. Cells were allowed to

settle for 24 hours in an incubator (37°C, in a humidified atmosphere of 5% CO_2 in air as previously described).

For each condition, three wells were used per time point. To prepare the reporter gene construct, excess DCCM with 2% (v/v) l-glutamine was prepared and warmed to 37°C. To a sterile microcentrifuge tube 30µl of this medium was added per well to be transfected, to this tube 1.5µl Dharmafect transfection lipid was added for each well to be transfected. To a second tube 30µl of the DCCM +l-glutamine was added per well to be transfected, to this 0.55µg of plasmid DNA was added. This 0.55µg comprised of 200ng of ERE construct, 75ng of REN construct and 275ng of 'junk' PCR script (a non-transcriptionally active plasmid which was used as carrier DNA, to make up the DNA concentrations to levels required for reliable transfection, obtained from Stratagene.). The first and second tubes were then gently shaken and each left to separately equilibrate at 37°C for 45 minutes.

During the incubation period a third tube was prepared containing 190µl DCCM+l-glutamine for each well to be transfected (to give a final volume of 250µl per well). To this tube 2.5µl molecular grade DMSO was added per well to be transfected (which has been shown in-house to improve transfection efficiency).

After the 45 minutes, the first and second tubes were gently mixed together, and returned to the incubator for 15 minutes, after which this combined mix was added to the third tube to give the final transfection media.

The original seeding media was removed from the wells to be transfected, and wells were washed with 250µl of DCCM and the 250 µl of

the final transfection media was applied to each well to be transfected. To obtain a background fluorescence value as a control, 3 wells were left untransfected, but treated the same in all other respects. The cells were then left to incubate at 37°C, in a humidified atmosphere of 5% CO_2 in air for 6 hours. After incubation the ERE transfection media was removed, and experimental media containing treatments was added for the duration of the experiment. For the siRNA treatment experiments, all treatment media was made up as indicated in table 2.2. After adhering, cells were transfected with the ERE constructs for 6 hours. This ERE transfection media was then removed and cells treated (with the treatment media containing faslodex, ER siRNA, co-treatment etc. as in Table 2.2) for a further 4 days in the same manner as for non-siRNA experiments.

After 4 days of treatment, the treatment medium was removed and the wells were washed twice with 1ml of room temperature PBS. The excess was removed and 100µl of 1x Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega UK Ltd) diluted from 5x stock with sterile H_2O was added to each well. The cells were then scraped from the wells and transferred to sterile microcentrifuge tubes. The microcentrifuge tubes were kept on ice until all wells were harvested, then stored at -70°C. Cells were stored in this manner so a single freeze-thaw would complete the lysis.

To determine reporter activity a Dual-Luciferase Reporter Assay System kit was used. To each thawed sample, 100µl of LAR II was added (resuspended firefly Luciferase Assay Substrate in Luciferase Assay buffer II, provided). This mixture was read for 10 seconds by a Lumat LB 9507

luminometer, which gave the ERE Luciferase reading. The sample was then removed and 100µl of Stop & Glo reagent was added (Stop & Glo substrate dissolved in Stop & Glo substrate solvent provided). This terminated the ERE luciferase activity, and initiated renilla luciferase activity, this sample was then read a second time. This was repeated for all samples to be read. To calculate the ERE activity, the constitutively active *Renilla* reported luciferase activity was used to normalise the ERE-transactivated firefly luciferase value.

2.2.7. Cell growth assays.

Coulter counter growth assay.

Cells for experimentation were harvested as previously described in section 2.2.1. and $1x10^6$ cells were diluted into 25ml of experimental media per 24 well plate required, this gave a final dilution of approximately $4x10^4$ cells/well. Cells were allowed to settle for 24 hours prior to treatment in an incubator (37°C, in a humidified atmosphere of 5% CO_2 as previously described). After 24 hours the media was removed and fresh media containing desired treatments were added (1ml per well). During the course of the experiment the media was removed and fresh media containing treatments were replaced every 4 days.

For each desired time point the media was removed from the required cells and the cells were incubated with 1ml of trypsin at 37°C for approximately 3 minutes (or until the cells were seen to be in suspension

under a microscope). Cells were then turned into a single cell suspension by passing through a BD.Microlance 25 gauge needle into a syringe, and passed back through the needle three times. To collect all the cells present, 1ml of isoton was added to the well and drawn up the syringe, this was repeated twice. The cells present in the 4mls now in the syringe (3mls isoton, 1ml trypsin) were then passed through the needle into a coulter counter cup containing 6mls of isoton. A Coulter Multisizer II was then used to count each cup twice, at a fixed volume of 500µl (counts were then multiplied by 20 to give total cell number per well). For each arm of the experiment three wells were treated and counted twice for each condition, and at each time point, with 3 wells used as a base count after the initial 24 hour incubation.

MTT growth assay.

The MTT assay was first described by Mosmann in 1983. It is a marker of cellular proliferation by utilisation of a mitochondrial dehydrogenase enzyme present in healthy cells, which is able to reduce a soluble yellow compound (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, also known as MTT) to form insoluble dark blue formazin crystals. When the cells are lysed by addition of Triton the formazin crystals can dissolve and the absorbance of the resultant solution can be read, with the absorbance value being proportional to cell number.

Cells are prepared as described in section 2.2.1. and diluted into 20mls of experimental media seeded out at $1x10^4$ cells/well (200µl media per well) per number of 96 well plates required. Cells were allowed to settle for 24 hours prior to treatment in an incubator (37°C, in a humidified atmosphere of 5% CO_2 as previously described). After 24 hours the media was removed and fresh media containing treatments (200µl media per well, and 8 wells used per condition) were added. For the duration of the experiment media and treatments were refreshed every 4 days. Once the desired time point was achieved the media was removed and the cells are washed twice with 37°C sterile PBS (200µl per well). Cells were then incubated with 200µl per well of 0.5mg/ml MTT solution (made up in 37°C phenol-red free RPMI 1640 with no additions) for 4 hours in the incubator. After incubation the MTT solution was then carefully removed, the wells are carefully washed twice with sterile PBS. Finally 100µl of Triton X-100 (made up to 10% v/v in PBS) was added to each well to lyse the cells. This was incubated at 4°C overnight. After incubation the plate was brought up to room temperature, gently agitated to mix the samples and read on a Multiskan® MCC/340 plate-reader (Titertek, USA) at a wavelength of 540nm.

2.2.8. Statistical analysis.

Where the data allowed, statistical analysis of the recorded values was performed, using SPSS v16 software, using either a Mann Whitney-U test or

analysis of variance (ANOVA) with a Dunnett post hoc test for confidence intervals of 95% ($p<0.05$) or greater.

Chapter 3. Results Section.

3.1. The Effect of the 'pure' anti-oestrogen fulvestrant on MCF-7 cells.

The ability of fulvestrant to act as an anti-oestrogen by blocking function and facilitating degradation of the oestrogen receptor (ER) protein, and thus reducing oestrogen driven growth of breast cancer cells, has been previously documented both in breast cancer cell lines and in the clinic (Robertson 2007, Nicholson *et al* 2001). This research showed ER degradation to be incomplete, and residual levels of ER were shown to be maintained until acquisition of resistance to fulvestrant in preclinical studies (McClelland *et al* 2001). ER negativity was reached only after a 2 year period of exposure to this pure anti-oestrogen (Nicholson *et al* 2005). The aim of this study was to investigate whether the residual ER observed following fulvestrant treatment provides a growth signal that allows the cells to tolerate the initial impact of the anti-oestrogen and, thus, whether targeting the residual ER improves fulvestrant response. Initial experiments were undertaken to discover the acute (4-8 days) effect of fulvestrant as a single treatment agent on ER expression and activity in our cell models. Special interest was paid to residual ER expression and functionality, to see how much scope there was for improving the action of fulvestrant in our model system.

3.1.1. Optimal concentration of fulvestrant for maximum ER down regulation and growth inhibition.

MCF-7 cells, which are widely used to examine ER function in breast cancer, were the principal cell model used in these studies. Initially in our MCF-7 cell model, two fulvestrant concentration response experiments were performed after 8 days treatment in order to determine the optimal concentration of fulvestrant to induce maximal down-regulation of ER protein expression and growth inhibition of MCF-7 cells. ER protein down-regulation was assessed by immuno-histochemical staining of the ER and subsequent nuclear H-score analysis. Anti-proliferative activity was assessed by cell counting using a Coulter counter.

ER down regulation.

Untreated MCF-7 cells were found to have a high basal ER expression, exclusively within the nucleus, with the cells showing intense staining in 90% of the population, and moderate staining in the remaining cells (figure 3.1.A.). Exposure of these cells to increasing concentrations of fulvestrant resulted in a decrease in ER protein expression in the cells, with moderate to low nuclear ER staining observed in 90% of the cells treated with 10^{-10}M fulvestrant (figure 3.1.A.). From a concentration of 10^{-9}M fulvestrant, substantial ER protein down regulation was achieved with 20% of cells being ER negative,

but low expression being observed in the remaining cells (figure 3.1.A.). Corresponding ER nuclear H-scores (figure 3.1.B.) also showed a concentration dependant loss of ER expression in response to fulvestrant, with significant ($p \leq 0.05$, n=3) down regulation being observed at concentrations as low as 10^{-10}M fulvestrant. The fulvestrant induced ER down regulation reached a plateau at 10^{-9}M fulvestrant with no further significant ER down regulation being observed beyond this concentration. A low level of residual ER staining was still observed even at the highest concentrations of fulvestrant (10^{-6}M) used in this study (figure 3.1.A. and enlargement images).

Growth response.

Fulvestrant exposure caused a concentration-dependant growth inhibition of MCF-7 cells with significant ($p \leq 0.05$, n=3) growth inhibition being achieved at 10^{-9}M fulvestrant, and no greater growth inhibition being achieved beyond 10^{-8}M fulvestrant (figure 3.1.C.) with a maximal 60% growth inhibition being observed at the highest concentration used. Growth inhibition was incomplete at all fulvestrant concentrations used. Although a statistically significant reduction in ER levels was observed at a concentration of 10^{-10}M fulvestrant, no significant growth inhibition was observed at this concentration. Indeed, maximal growth inhibition was only observed at a concentration of 10^{-8}M fulvestrant, a 100 fold higher concentration. A concentration of 10^{-7}M fulvestrant also maximally reduced both ER and cell growth and was consequently used in all further studies.

3.1.2. Influence of culture conditions on fulvestrant response in MCF-7 cells.

Initial studies were performed using MCF-7 cells cultured in experimental media containing stripped foetal calf serum (5% SFCS). MCF-7 cells cultured in this way experience an environment with low concentrations of exogenous oestrogens, a condition that provides reduced competition for fulvestrant binding to the ER, thus potentially maximising response to this agent. However this experimental design (with residual oestrogens of the order of 10^{-13}M), while approximately equivalent to the oestrogen deprivation achieved in patients taking aromatase inhibitors, does not encompass oestrogen levels in untreated postmenopausal women. To provide a greater understanding of the importance of residual ER across a such a cohort of patients, experiments were also performed in media containing 5% whole foetal calf serum (FCS) instead of stripped serum, which is approximately equivalent to post-menopausal oestrogen levels, and furthermore in stripped serum containing 10^{-9}M oestradiol, providing a highly oestrogenic environment, probably exceeding pre-menopausal levels. All the stripped and whole serum experiments were repeated in the MCF-7 cell line to allow direct comparison, and also for easier reference and comparison to most previous oestrogen receptor positive breast cancer *in vitro* studies.

3.1.3. The Effect of fulvestrant treatment on oestrogen receptor expression levels in MCF-7 cells, grown in both stripped and whole serum.

Western blotting analysis revealed that there was an impressive down regulation of total ER protein expression after 4 days of 10^{-7}M fulvestrant treatment in MCF-7 cells grown in either stripped or whole serum conditions while beta-actin, used as a control was equivalent (figure 3.2.A.). Despite using an optimal concentration of fulvestrant this down regulation of ER was not complete in either serum type with residual ER clearly detectable (figure 3.2.A.).

This residual ER protein expression following fulvestrant treatment was also apparent following assessment by immunocyto-chemical staining of fulvestrant treated MCF-7 cells cultured in both stripped and whole serum (figures 3.2.B.). The untreated MCF-7 cells showed high levels of oestrogen receptor expression in both serum conditions, with high staining intensity in 80% of the cells nuclei and moderate staining in the remaining nuclei. No detectable cytoplasmic or membrane ER was observed. In the fulvestrant treated MCF-7 cells, under both serum conditions, neither cytoplasmic or membrane ER was detected, and greatly reduced levels of nuclear staining was observed. However while approximately 5-10% of cells showed some moderate staining in either culture condition (figure 3.2.B.), 20% of cells were negative for nuclear ER expression, but the remaining cells all showed low but

detectable levels of residual ER expression (figure 3.2.B. enlargements). The decrease in ER expression was statistically significant ($p \leq 0.05$, n=3) under both experimental conditions, shown by subsequent nuclear H-score analysis (figure 3.2.C). When examined in FasMCF cells the residual levels of ER observed were maintained through to the acquisition of resistance to the pure anti-oestrogen, with similar low level expression being observed in MCF-7 cells treated with 10^{-7}M fulvestrant for either 8 days or in the 12 months FasMCF cells (figure 3.3.A. and enlargements). These resistant cells were able to grow despite the presence of the pure anti-oestrogen in the growth medium.

The residual ER protein observed in MCF-7 cells following fulvestrant exposure, was not unique to the MCF-7 cell model, as similar findings were observed in three alternative ER positive breast cancer cell lines (T-47D, BT-474 and MDA-MB-361 cells). Assessment of nuclear ER by H-score analysis revealed that following fulvestrant exposure, an incomplete down regulation of ER was observed in all three cell lines, with low levels of residual ER clearly apparent (figure 3.3.B.). Interestingly, while the initial ER levels were varied across the cell lines used the down-regulation caused by fulvestrant treatment was approximately equal, with all cell lines showing an approximate ER protein down-regulation of between 65-85% following fulvestrant exposure.

The mechanism of fulvestrant action is to bind to the ER protein at EREs, to prevent normal transcription and facilitate receptor down regulation. To determine that the observed down regulation of ER protein expression was due to mechanism of fulvestrant action and not an unrelated ER mRNA down

regulation, ER mRNA expression levels were assessed by RT-PCR. A short-term fulvestrant treatment of 4 days showed no effect on oestrogen receptor mRNA expression in MCF-7 cells grown in stripped serum or whole serum when compared to untreated controls (figures 3.3.C.).

3.1.4. The Effect of fulvestrant treatment on oestrogen signalling.

As fulvestrant treatment showed a significant but incomplete down regulation of ER protein expression, the next set of studies were designed to investigate whether this residual ER had any apparent genomic biological activity by assessment of ER transcriptional activity, pre and post fulvestrant treatment in MCF-7 cells.

Measure of fulvestrant effect on mRNA and protein expression of PR and pS2.

PR mRNA expression

The transcriptional activity of this residual ER was initially investigated by measurement of the expression of classic ER regulated genes, Progesterone receptor (PR) and pS2. A down regulation of PR mRNA expression was observed when MCF-7 cells, cultured in both stripped and whole serum were treated with fulvestrant. However the down regulation of PR at the mRNA

level was not complete, with residual PR mRNA expression still clearly being observed (figure 3.4.A.).

pS2 mRNA expression.

In the stripped and whole serum cultured MCF-7 cells, analysis of pS2 mRNA expression also showed that under both serum conditions, expression of this gene was reduced in the presence of fulvestrant (figure 3.4.B.). As with PR gene expression, the down regulation of pS2 mRNA expression was incomplete in both serum conditions again providing evidence for the presence of residual ER transcriptional activity.

Although fulvestrant repression of ER transcriptional activity on PR and pS2 transcription was incomplete at the gene level, the level of repression observed may be sufficient to inhibit the protein expression of these genes. The effect of fulvestrant on the protein expression of the PR and pS2 was therefore assessed by Immuno-cytochemistry.

PR protein expression.

In stripped serum the basal level of PR protein expression in MCF-7 cells was low (figure 3.5.A.), but observable expression was present, being both cytoplasmic (20% of cells) and nuclear (40% of cells) with the remainder being PR negative (blue cells). The fulvestrant treated cells revealed a reduction in PR expression with detectable staining in the cytoplasm of 15%

of the cells and 20% of cells showed some nuclear expression (figure 3.5.A. enlargement 1.); the remaining cells were PR negative. These observations were confirmed by the nuclear H-score analysis (figure 3.5.B.) which showed a significant ($p \leq 0.05$, n=3) down regulation of PR expression in the fulvestrant treated cells, but the presence of residual nuclear PR was also apparent in a small population (see table 3.2. for complete H-score breakdown).

Both cytoplasmic and nuclear PR protein expression was observed in MCF-7 cells cultured in whole serum (figure 3.5.A.), with high intensity staining being observed in the nucleus of 70% of the cells and lower expression levels observed in the remaining cells. There was also very high expression of cytoplasmic PR in 60% of the cells with the remaining cells showing moderate staining. Fulvestrant treatment greatly reduced PR protein expression but low level nuclear staining was still observed in 40-50% the cells (figure 3.5.A. enlargement 2.). Cytoplasmic staining was also decreased but low staining was observed in 40-50% of cells and no PR expression in the cytoplasm of the remaining cells. Furthermore, approximately 20% of the cells were negative for both nuclear and cytoplasmic PR combined in both culture conditions. Nuclear PR was used for the corresponding H-score assessment (figure 3.5.B. and table 3.2.) because PR function is mainly nuclear. Both a statistically significant ($p \leq 0.05$, n=3) reduction of PR protein expression, and a clear presence of residual nuclear PR expression was demonstrated following fulvestrant exposure under both culture conditions.

pS2 protein expression.

Looking at pS2 protein expression in MCF-7 cells cultured in stripped serum (figure 3.6.A.), a pattern of low pS2 expression exclusively within the cytoplasm was observed. Untreated MCF-7 cells showed moderately intense staining in the cytoplasm of 20% of the population, with pS2 negative cells comprising 15% of the population and very low pS2 expression observable in the cytoplasm of the remaining cells. Fulvestrant exposure caused a loss of all moderate intensity cytoplasmic staining with 20% of cells showing low pS2 expression and the remaining cells showing no pS2 expression. The corresponding cytoplasmic H-score analysis (figure 3.6.B. and table 3.3.) showed a significant ($p \leq 0.05$, $n=3$) down regulation in the presence of fulvestrant but also indicated the presence of very low residual pS2 protein expression in the fulvestrant exposed cells (enlargement 1. in figure 3.6.A.).

Untreated MCF-7 cells showed no nuclear staining for pS2 protein (figure 3.6.A.) when cultured in whole serum, however moderate cytoplasmic staining was observed in 60% of the population, low staining in 30% and no staining in 10% of cells. There was a clear reduction in pS2 protein levels across all the cells in the fulvestrant treated arm, and no nuclear staining was observed, but 20% of the cells demonstrated moderate to low cytoplasmic staining and the remainder were pS2 negative (figure 3.6.A.). Some of the pS2 expression observed following fulvestrant exposure was peri-nuclear, indicating the continued transcription of pS2 protein following fulvestrant treatment. The corresponding cytoplasmic H-score analysis (figure 3.6.B. and

table 3.3.) reflected these observations, showing a statistically significant ($p \leq 0.05$, n=3) down regulation in the fulvestrant exposed cells but still clear evidence of residual pS2 protein expression.

Effect of fulvestrant on ERE signalling in MCF-7 cells.

To assess quantitatively the level of oestrogen receptor genomic signalling activity remaining after fulvestrant treatment, an oestrogen responsive element (ERE) driven dual *luciferase* assay was performed. MCF-7 cells were transfected with an ERE driven firefly *luciferase* reporter gene construct alongside a constitutively active firefly *renilla* construct to act as a transfection control. The cells were transfected with both constructs and cultured with either stripped or whole serum for 4 days and treated with either fulvestrant, oestradiol as a positive control or vehicle control. Fulvestrant treatment caused a significant ($p \leq 0.05$, n=3) but modest 30% reduction in ERE transcriptional activity (figure 3.7.A.) in MCF-7 cells cultured in stripped serum. After fulvestrant treatment, residual ERE transcriptional activity levels were still clearly apparent, although it should be noted that these levels are quite low since under stripped serum conditions the level of ERE activity in the control condition is already minimal. This experiment also showed a statistically significant ($p \leq 0.05$, n=3) increase of 25 fold in ERE activity in the oestradiol stimulated arm, compared to the untreated MCF-7 cells.

In the MCF-7 cells cultured in whole serum (figure 3.7.B.) a statistically significant ($p \leq 0.01$, n=3) 80% reduction of ERE activity was

observed in the presence of fulvestrant, and the basal ERE was higher due to the higher oestrogenic environment in this culture condition. Though fulvestrant exposure decreased ERE transcriptional activity significantly, the residual level of ERE activity was still 20% of the control and ten-fold greater than the background fluorescence readings in cells not transfected with the ERE construct. Due to the higher basal ERE activity under whole serum conditions only a 6-fold increase in oestrogen receptor signalling activity was also observed in the presence of oestradiol when compared to the unstimulated MCF-7 cells.

Fulvestrant exposure in oestradiol stimulated cells.

As shown under whole serum culture conditions, the presence of moderate levels of oestradiol enhances ER signalling. However, fulvestrant was able to block this effect. Further studies were performed to examine the ability of fulvestrant to overcome high levels of exogenous oestradiol (10^{-9}M) as some breast carcinomas have been shown to produce high levels of oestrogens *in vivo* (Green and Furr 1999) and these studies could also better reflect the pre-menopausal situation observed within the clinic.

When MCF-7 cells grown in stripped serum were stimulated with the addition of 10^{-9}M oestradiol to the media the basal expression levels of both PR and pS2 protein were increased when compared to unstimulated cells in stripped serum control conditions. The oestradiol stimulated cells showed intense staining of PR in the nucleus of all of the cells, and high expression of

PR in the cytoplasm was also observed in 80% of the cells, with less intense staining also being seen in the remainder of the cells (figure 3.8.A.) Similarly the oestradiol-stimulated cells showed far stronger staining for pS2 across the MCF-7 population, with many cells showing intense cytoplasmic staining. A significant down regulation of oestradiol-induced PR expression was apparent, following exposure to fulvestrant in the presence of oestradiol, with 40% of cells showing reduced intensity staining in both the nucleus and the cytoplasm, and evidence of some PR negative cells. Oestradiol-treated MCF-7 cells exposed to fulvestrant showed less intense peri-nuclear pS2, with 10% of cells showing no expression of pS2 protein at all (figure 3.8.A.). Corresponding H-score analysis of nuclear PR and cytoplasmic pS2 staining supported these observations and both showed a statistically significant down regulation of PR and pS2 expression following fulvestrant treatment (figure 3.8.B. and table 3.2.). However both the immunocytochemical pictures (see enlargements) and the subsequent H-score analysis showed presence of residual PR and pS2 after fulvestrant exposure.

3.1.5. The effect of fulvestrant treatment on MCF-7 cell proliferation.

As the previous studies revealed that fulvestrant exposure produced an incomplete down regulation of ER expression and transcriptional activity, experiments were then performed to assess whether the growth inhibitory

activity of this agent was also incomplete. A number of parameters were assessed to examine the effect on MCF-7 cell growth, proliferation and viability:

A) Expression of Insulin-like growth factor (IGFR) signalling pathway components as IGF-1R and IRS1 are oestrogen regulated genes, as well as key growth regulators of MCF-7 cells, thus providing a means of showing treatment impact on genomic activity of ER relevant to proliferation.

B) Expression of the proliferation marker Ki67.

C) Assessment of cell viability by MTT assay.

D) Assessment of cell number by Coulter counter analysis.

IGF-1R signalling component analysis.

Insulin-like growth factor signalling component analysis (IGF-1R), showed that fulvestrant treatment caused a slight down regulation of total and phosphorylated IGF-1R and a greater down regulation of total IRS1 compared to the untreated MCF-7 cells in both stripped and whole serum culture conditions (figures 3.9.A. and B.). It should be noted however that the reduction was incomplete for both proteins, under both culture conditions.

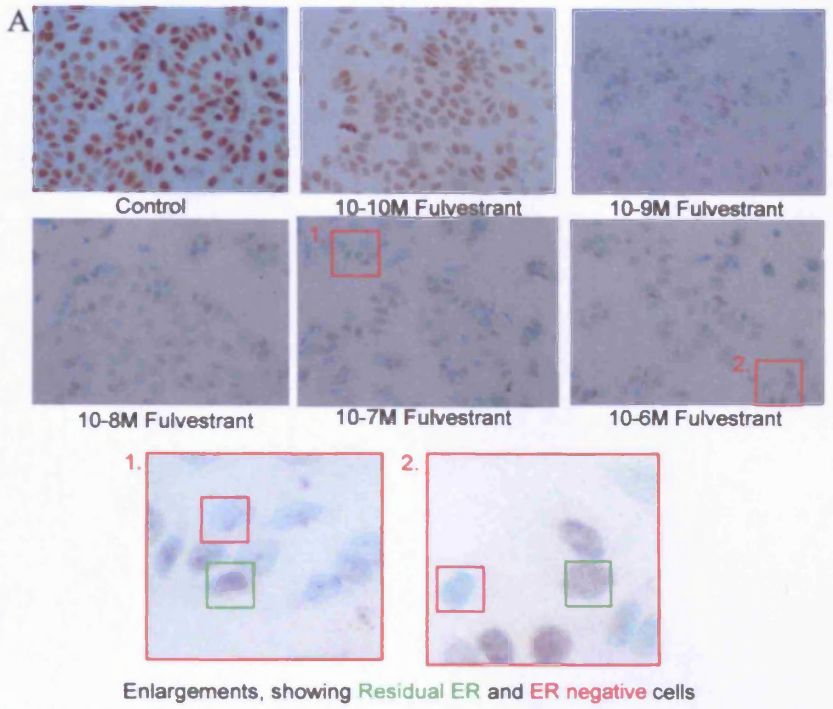
Ki67 analysis.

Untreated MCF-7 cells cultured with stripped serum demonstrated high levels of nuclear staining for the proliferation marker Ki67 in 30% of the population and less intense staining in a further third of the population. The remaining cells did not express the antigen (figure 3.10.A.). Untreated MCF-7 cells cultured with whole serum showed a higher percentage of cells expressing the antigen compared to the control MCF-7 cells grown in stripped serum, with 80% of the whole serum cultured control cells expressing nuclear Ki67 (figures 3.10.A. and B.). The total Ki67 positivity score was 65% and 80% for cells cultured with stripped and whole serum respectively and indicated that roughly two thirds and four fifths of the cells in these populations were actively proliferating (figure 3.10.B.). Fulvestrant exposure caused a significant decrease in Ki67 positivity under both culture conditions. The remaining staining was far less intense across the population compared to the untreated MCF-7 cells, with only a quarter of cells showing any presence of the antigen within the nucleus, in the stripped serum condition (see enlargements). In the whole serum condition the antigen was still present in the nuclei of 30% of cells following fulvestrant treatment (shown in enlargement 2. of figure 3.10.A.).

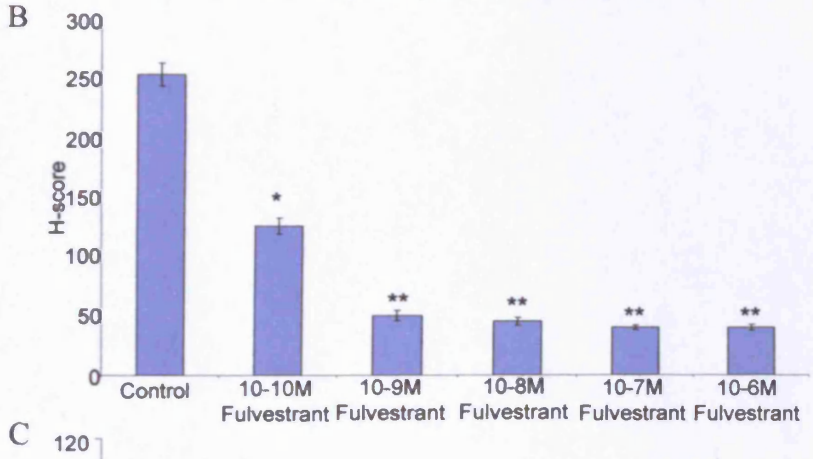
Viability assay and total cell count analysis.

MTT assays also revealed incomplete growth inhibition in response to fulvestrant. An experiment of 8 days fulvestrant exposure showed a small but significant ($p \leq 0.05$, n=3) drop in growth of 20% in stripped serum in response to fulvestrant exposure (figure 3.11.A.). A more significant ($p \leq 0.05$, n=3) growth inhibition of 70% by fulvestrant exposed was observed in cells cultured in whole serum, but levels of residual growth were still apparent in the presence of the anti-oestrogen (figure 3.11.A.). This incomplete decrease in growth rate was also reflected by the decrease in cell number observed following fulvestrant treatment when the total cell counts were assayed by Coulter counter analysis. In the cells cultured in stripped serum a significant ($p \leq 0.05$, n=3) decrease of 50% in total cell number was observed when the cells were exposed to fulvestrant (figure 3.11.B.), whereas studies conducted in whole serum revealed a statistically significant ($p \leq 0.05$, n=3) decrease in total cell number of 60% in response to fulvestrant treatment (figure 3.11.B.). Once again a greater response was observed in the whole serum conditions but growth inhibition was still incomplete. Coulter counter analysis also showed that the incomplete growth inhibition in response to fulvestrant was not unique to MCF-7 cells, with T47D, BT474 and MDA361 ER positive breast cancer cell models showing growth inhibitions of 35%, 20% and 50% respectively at day 7 (figure 3.11.C.).

The optimal fulvestrant concentrations for MCF-7 ER protein down regulation and growth inhibition.



Enlargements, showing Residual ER and ER negative cells



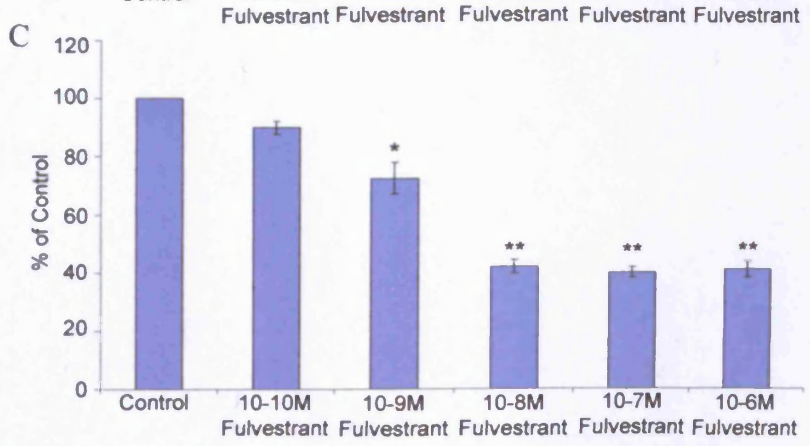


*Figure 3.1.(A) Immunocytochemical staining (enlargements, indicating residual staining and negative cells in green and pink respectively) (magnification x20) and (B) average nuclear ER H scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% charcoal-stripped steroid-depleted foetal calf serum (SFCS) and treated with increasing concentrations of fulvestrant (10-10M to 10-6M) for 8 days (n=3). (C) Effect of increasing concentrations of fulvestrant (10-10M to 10-6M) on basal MCF-7 cell growth cultured in phenol-red free RPMI containing 5% SFCS on day 8 from initial treatment and represented as percentage of untreated control after Coulter counting (n=3). N.B . *= P≤0.05. **= P≤0.01 vs Control.*

ER expression levels in MCF-7 cells treated with fulvestrant and cultured in stripped or whole serum.

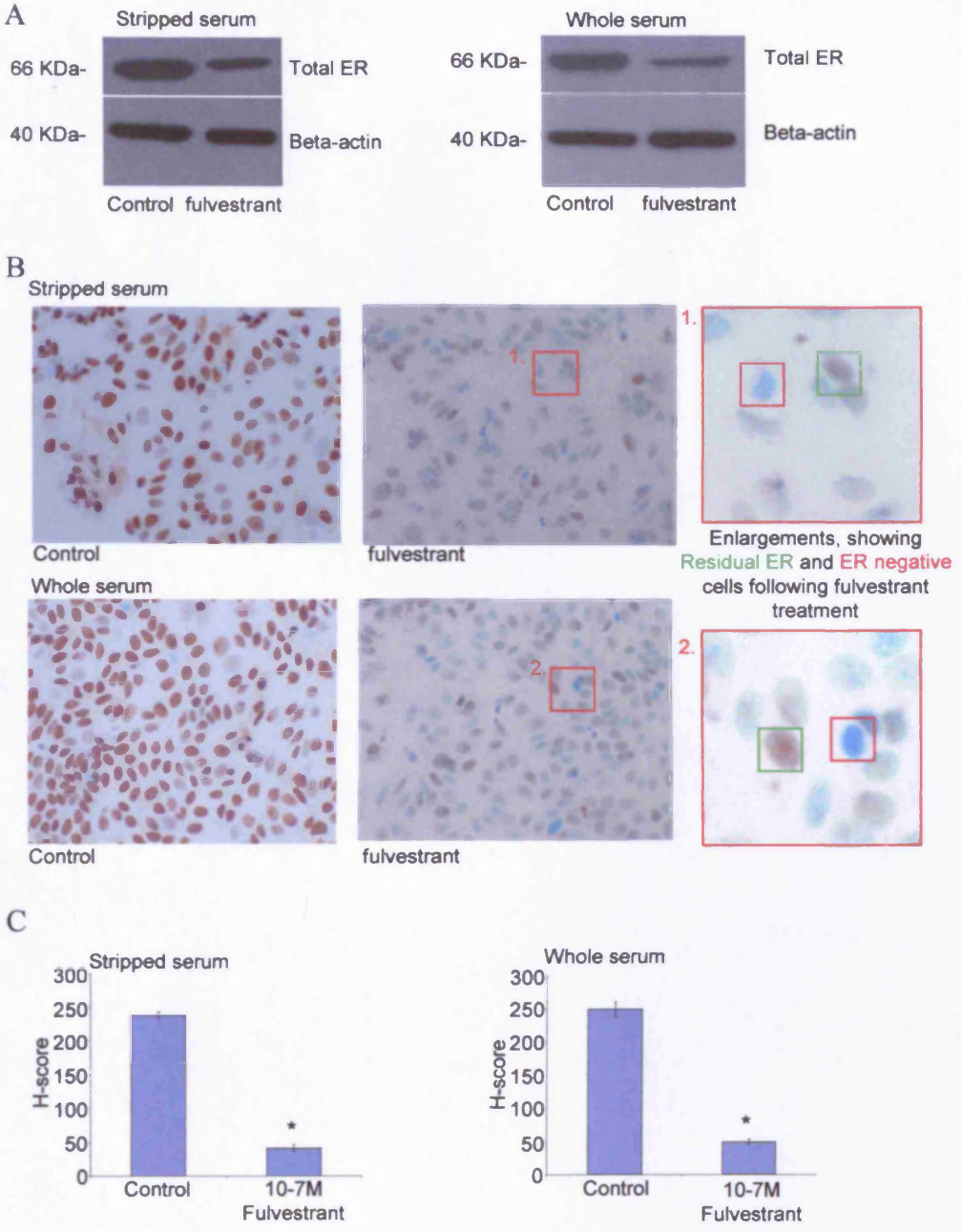


*Figure 3.2.(A) Western analysis of ERα and β-actin expression in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. (B) Immunocytochemical staining (enlargements, indicating residual staining and negative cells in green and pink respectively) (Magnification x20) and (C) average nuclear ER H scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05. vs Control.*

ER mRNA and protein expression levels of hormone responsive and anti-hormone resistant breast cancer cells after fulvestrant treatment.

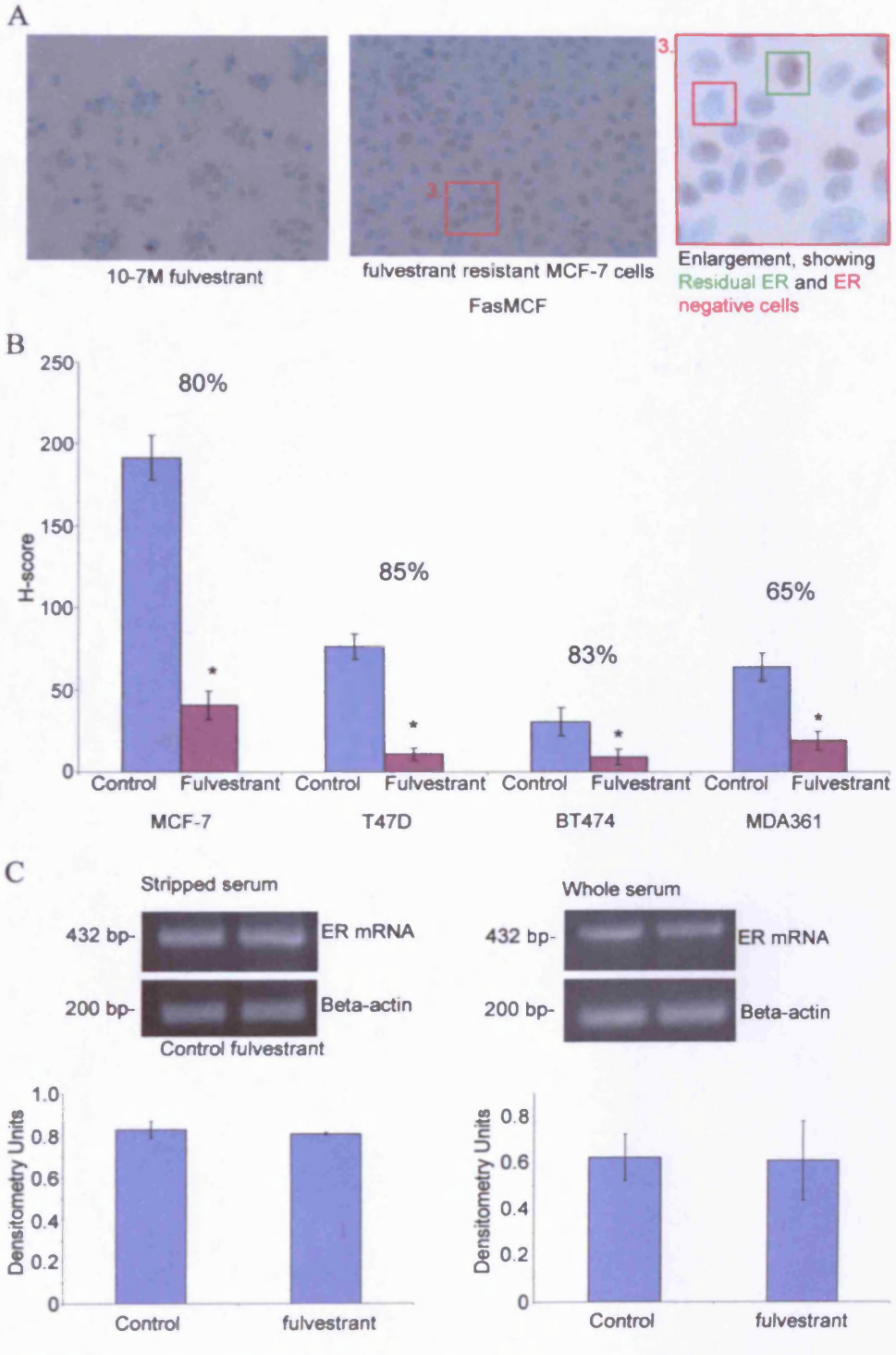


*Figure 3.3.(A) Immunocytochemical staining of ERα in MCF-7 cells and fulvestrant resistant MCF-7 cells (FasMCF) cultured in phenol-red free RPMI containing 5% SFCS treated with 10-7M fulvestrant, on day 4 from initial treatment (enlargement indicating residual staining and negative cells in green and pink respectively) magnification x20. (B) Average nuclear ER H-score (N=3) in MCF-7, T-47D, BT-474 and MDA-MB-361 cells cultured in phenol-red free RPMI containing 5% SFCS, treated with either 10-7M fulvestrant or vehicle control, on day 8 after initial treatment and % ER down-regulation. (C) Expression of ER α and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant or vehicle control on day 4 after initial treatment and normalised densitometry. N.B. *= P≤0.05 vs Control.*

PR and pS2 mRNA expression in MCF-7 cells treated with fulvestrant, cultured in stripped and whole serum.

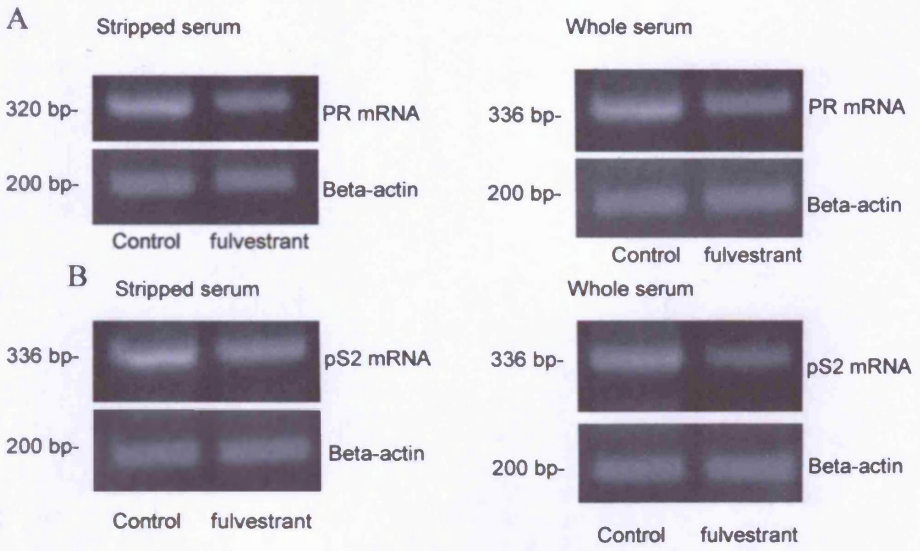


Figure 3.4.(A) Expression of PR and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing %5 SFCS or FCS, treated with either 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. (B) Expression of pS2 and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant or vehicle control, on day 4 from initial treatment.

PR protein expression in MCF-7 cells treated with fulvestrant and cultured in stripped or whole serum.

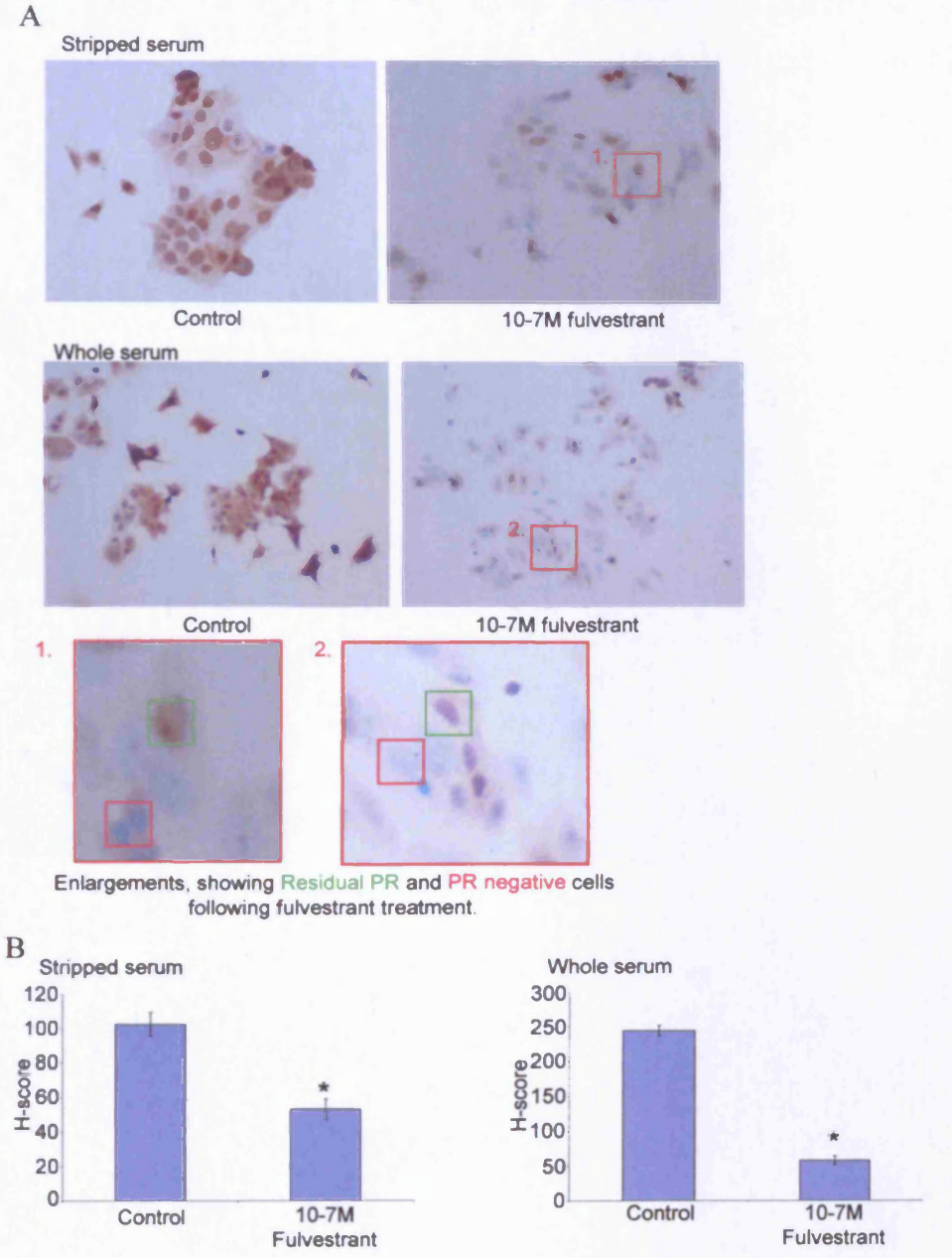


*Figure 3.5.(A) Immunocytochemical staining (enlargements, indicating residual staining and negative cells in green and pink respectively) magnification x20 and (B) average nuclear PR H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control.*

pS2 protein expression in MCF-7 cells treated with fulvestrant and cultured in stripped or whole serum.

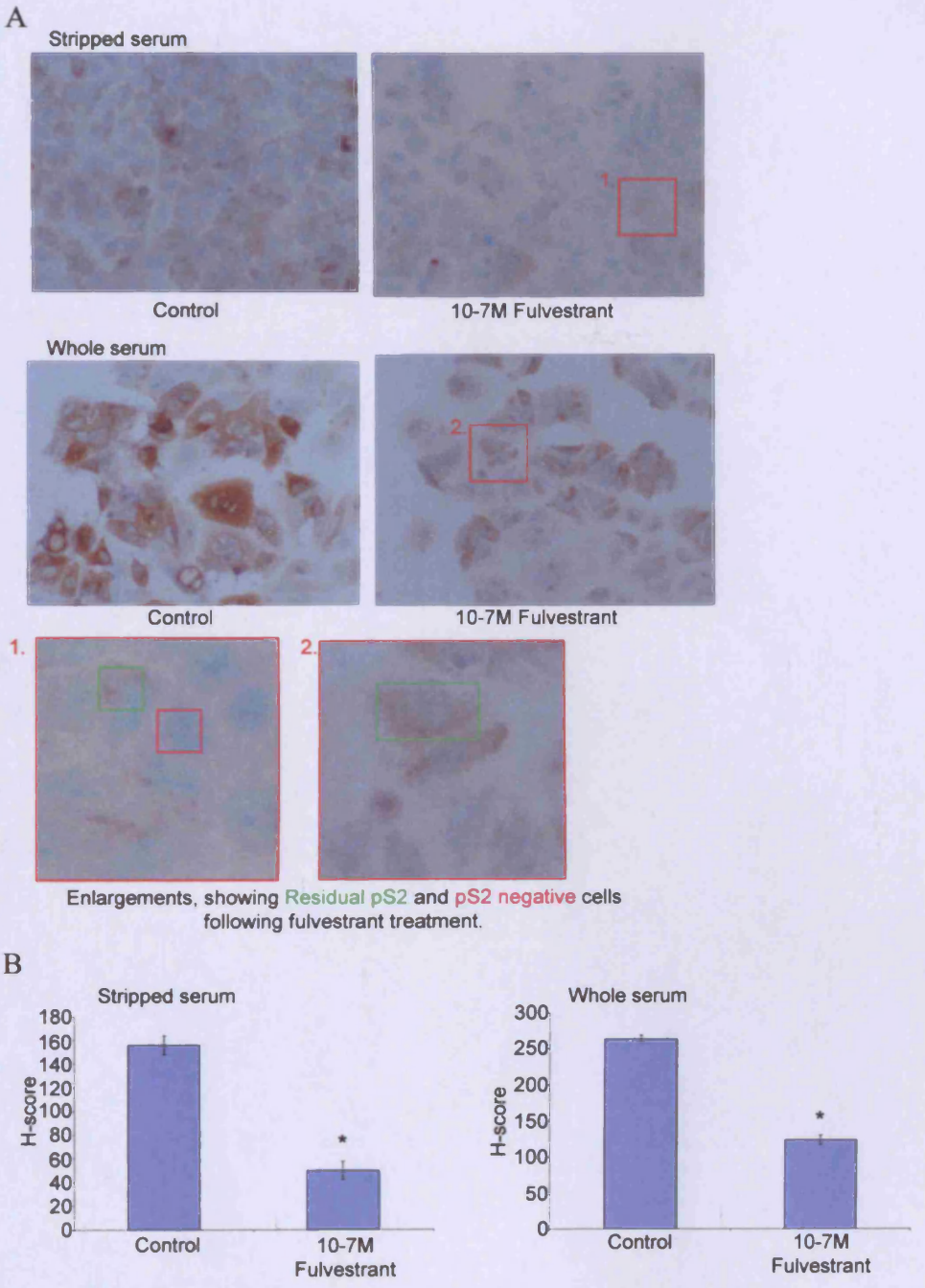


*Figure 3.6.(A) Immunocytochemical staining (enlargements, indicating residual staining and negative cells in green and pink respectively) magnification x20 and (B) average cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control.*

Level of ERE signalling in MCF-7 cells treated with fulvestrant or oestradiol, cultured in stripped or whole serum.

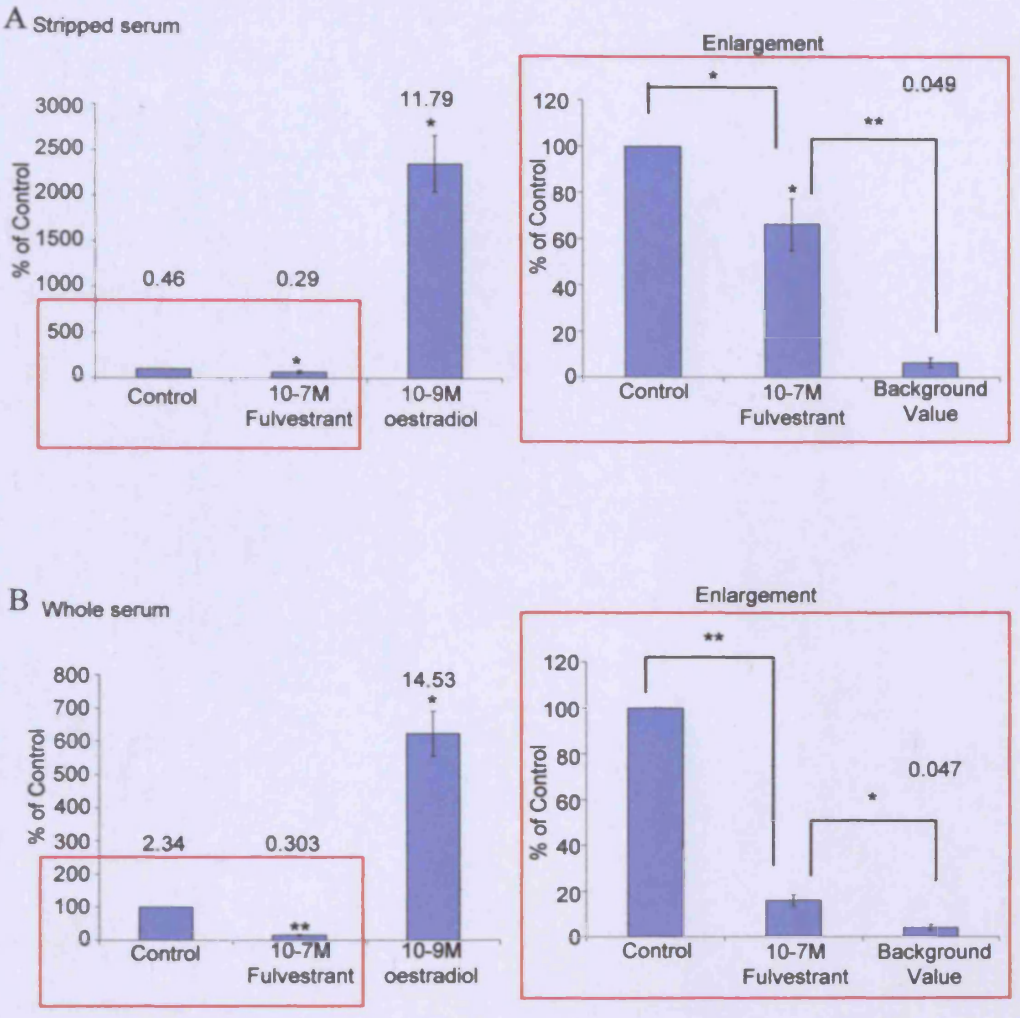
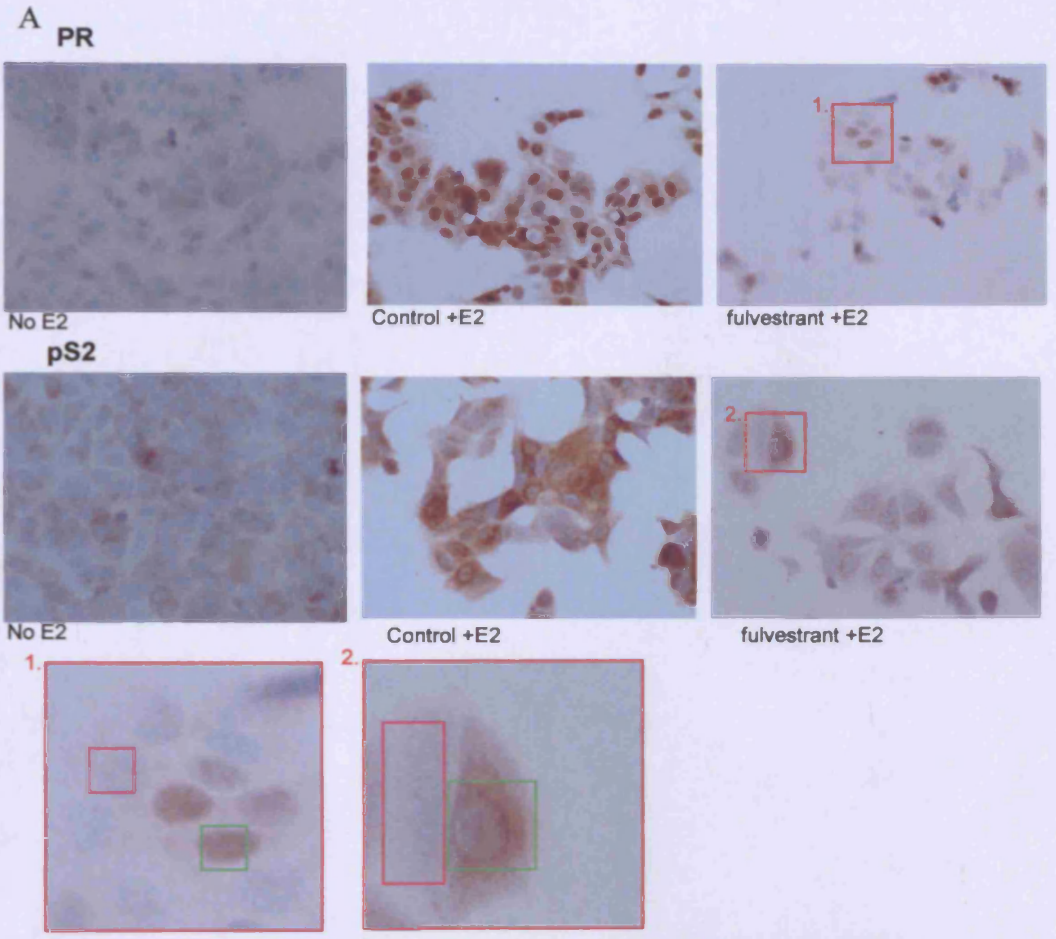


*Figure 3.7.(A) Reporter gene assay showing average ERE expression (n=3) in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS, treated with either 10-7M fulvestrant, 10-9M oestradiol or vehicle control on day 4 from initial treatment; with enlargement of scales to show background fluorescence. (B) Reporter gene assay showing average ERE expression (n=3) in MCF-7 cells cultured in phenol-red free RPMI containing 5% FCS, treated with either 10-7M fulvestrant, 10-9M oestradiol or vehicle control on day 4 from initial treatment; with enlargement of scales to show background fluorescence. N.B. Basal normalised fluorescence values shown above bars *= p≤0.05 **= p≤0.01 vs Untreated Control or where indicated by lines.*

PR and pS2 protein expression in MCF-7 cells cultured in stripped serum supplemented with oestradiol and treated with fulvestrant.



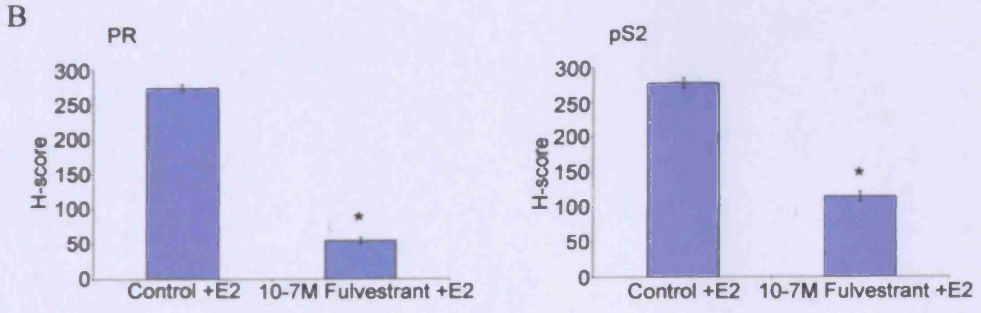


*Figure 3.8.(A). Immunocytochemical staining of PR and pS2 (enlargements, indicating residual staining and negative cells in green and pink respectively) magnification x20 (B) and average nuclear PR and cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS and 10-9M oestradiol and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control +E2.*

IGFR signalling in MCF-7 cells cultured in stripped or whole serum and treated with fulvestrant.

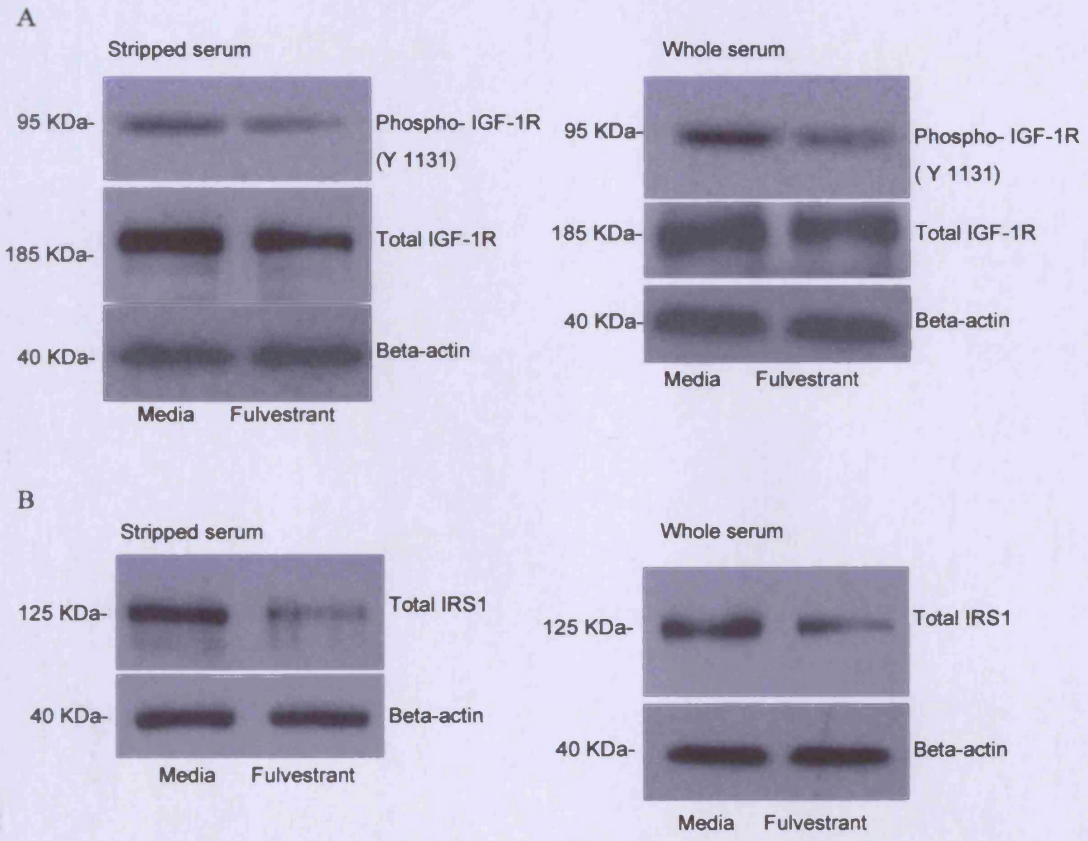
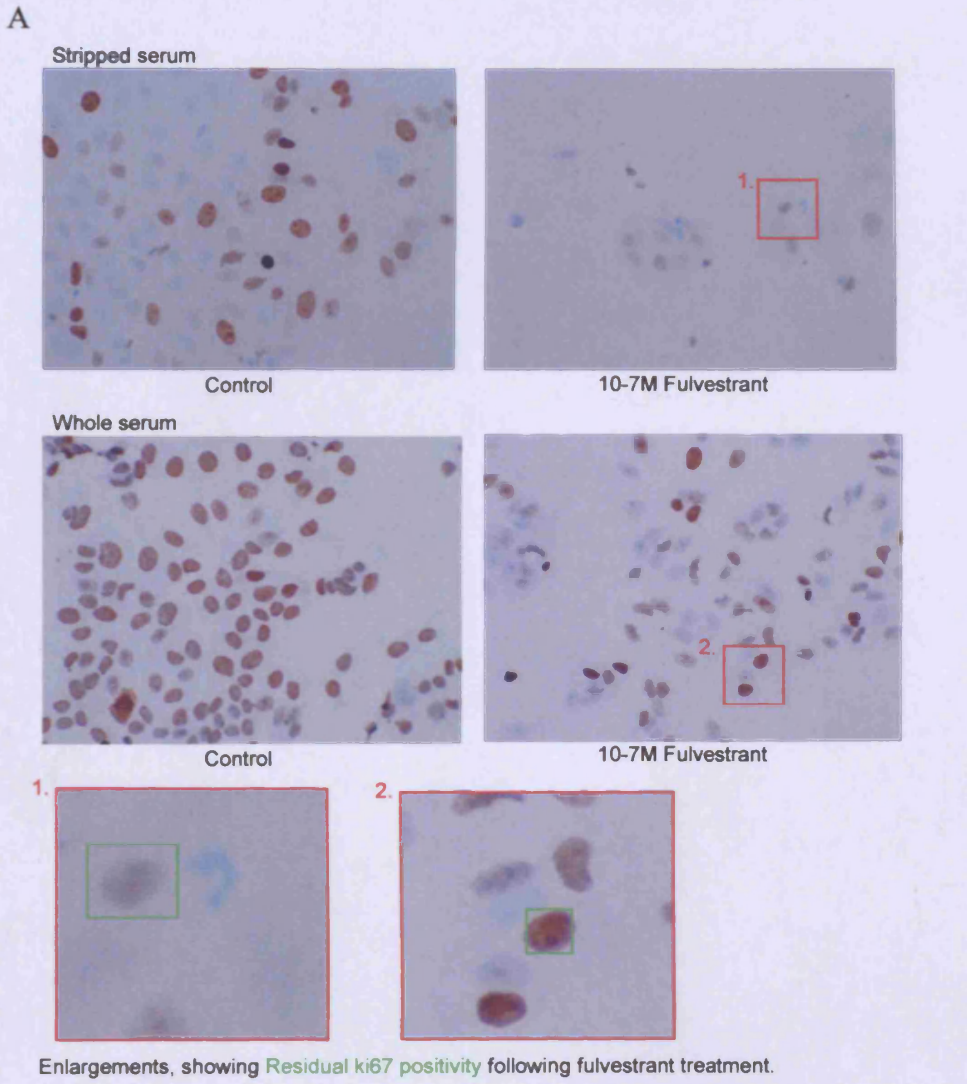


Figure 3.9.(A) Western blotting analysis of Total IGFR, Phospho-IGFR and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. (B) Western blotting analysis of Total IRS1 and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment.

Ki67 expression of MCF-7 cells culture in stripped or whole serum, treated with fulvestrant.



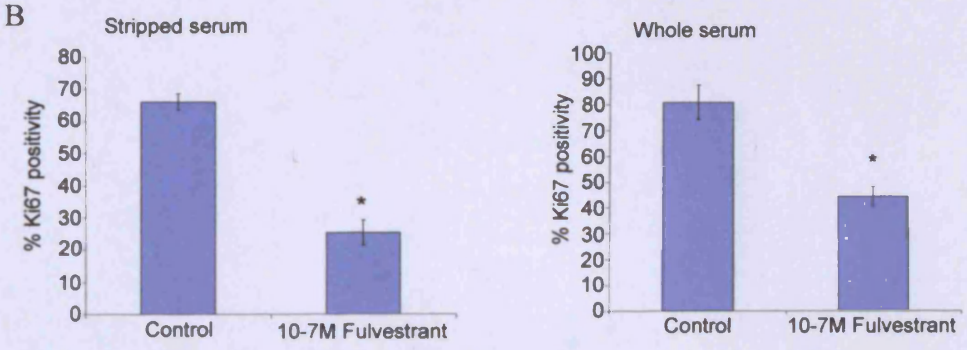


*Figure 3.10.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average total Ki67 positivity (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with 10-7M fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control.*

The effect of fulvestrant on growth of Hormone-responsive cells cultured in stripped or whole serum.

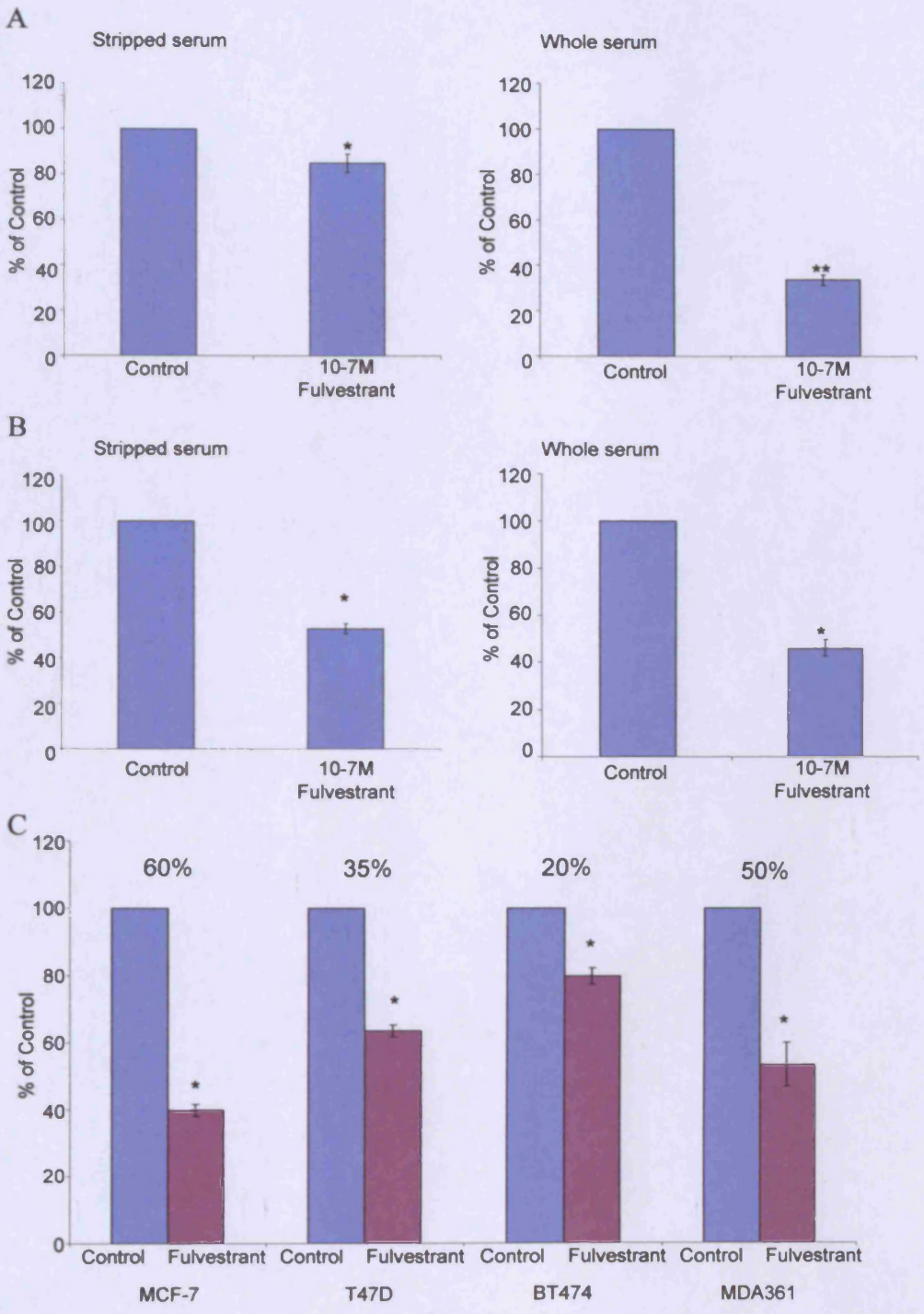


Figure 3.11.(A) Effect of 10-7M fulvestrant on basal MCF-7 cell growth, cultured in phenol-red free RPMI containing 5% SFCS or FCS on day 8 from initial treatment and represented as percentage of vehicle control after MTT assessment (n=3) or (B) Coulter counting (n=3). (C) Effect of 10-7M fulvestrant on basal MCF-7, T-47D, BT-474 and MDA-MB-361 cell growth, cultured in phenol-red free RPMI containing 5% SFCS or FCS on day 8 from initial treatment and represented as percentage of vehicle control after Coulter counter analysis % growth inhibition indicated above bars (n=3) . N.B. . = p≤0.05, ** = p≤0.01 vs Control.*

Complete ER H-score analysis of MCF-7 cells following fulvestrant exposure.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum).	238.3(+/-5.1)	0
Fulvestrant (stripped serum)	41.7(+/-5.1)	0
Control (whole serum)	249.1(+/-11.1)	0
Fulvestrant (whole serum).	49.1(+/-3.7)	0

Table 3.1. ER H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days fulvestrant exposure (n=3).

Complete PR H-score analysis of MCF-7 cells following fulvestrant exposure.

	Nuclear H-score	Cytoplasmic H-score	Total H-score
Control (stripped serum)	102.5(+/-6.8)	48.6(+/-5.3)	122.5(+/-6.6)
Fulvestrant (stripped serum)	53.3(+/-6.0)	20.4(+/-2.1)	75.6(+/-4.4)
Control (stripped serum +E2)	273.3(+/-5.1)	185.7(+/-13.5)	284.8(+/-9.4)
Fulvestrant (stripped serum +E2)	55(+/-4.5)	32(+/-3.1)	81.8(+/-7.3)
Control (whole serum)	244.1(+/-7.4)	177(+/-11.6)	263.6(+/-8.8)
Fulvestrant (whole serum)	58.3(+/-6.1)	30.3(+/-2.4)	84.7(+/-7.2)

Table 3.2. PR H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days fulvestrant exposure (n=3).

Complete pS2 H-score analysis of MCF-7 cells following fulvestrant exposure.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum)	0	155.8(+/-8.0)
Fulvestrant (stripped serum)	0	50(+/-7.7)
Control (stripped serum +E2)	0	277.5(+/-7.6)
Fulvestrant (stripped serum +E2)	0	115.0(+/-7.1)
Control (whole serum)	0	263.3(+/-5.2)
Fulvestrant (whole serum)	0	123.3(+/-6.8)

Table 3.3. pS2 H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days fulvestrant exposure (n=3).

3.2. The effect of the Dharmafect transfection lipid, and optimisation of ER siRNA knockdown in MCF-7 cells.

3.2.1. Optimisation of transfection lipid and knockdown efficiency.

After observing residual ER expression and the incomplete inhibition of ER activity and growth inhibition following fulvestrant treatment, an alternative mechanism of ER ablation was investigated using the alternative mechanism of protein 'knock-down' by utilising siRNA technology. This procedure targets specific mRNA expression as opposed to direct protein interaction as with fulvestrant. However, during initial siRNA transfection studies in the MCF-7 cell line, as per the manufacturer's methodology, a toxic effect was observed for the siRNA and lipid control conditions. This was typified by a decrease in cell number following initial application, with increased toxicity following re-transfection of the siRNA after 4 days, hindering taking the experiment to an 8 day conclusion. The Dharmafect#1 transfection lipid being used was eventually determined to be the cause, and an optimised method was developed.

MCF-7 cells cultured in the absence of lipid for 8 days showed a healthy culture of cells, grown almost to 100% confluency. These cells appeared packed tightly together as a complete monolayer, with the cells attached securely to the bottom of the culture apparatus (figure 3.12.A.). In

contrast, MCF-7 cells cultured for 8 days exposed to the recommended concentration (10nM) of transfection lipid demonstrated a substantial decrease in cell number, with a greater number of dead cells observed floating in the media (enlargement 2.). Some of the remaining lipid exposed cells also showed classic symptoms of cellular distress, being more rounded in appearance with vacuoles and pyknotic nuclei (an indication of apoptosis) being observed, and cells also demonstrated reduced adhesion to the bottom of the culture dish (enlargement 1.).

After observing the lipid effect on the MCF-7 cells an experiment was performed to assess the effect of this lipid concentration on cell growth, and to determine an optimal lipid concentration for future studies that would maximise siRNA transfection but show a less toxic profile. Studies showed that at 10nM transfection lipid there was a significant, 70%, decrease in MCF-7 cell number compared to untreated cells following an 8 day exposure (figure 3.12.B.). MCF-7 cells exposed to lower lipid concentrations of 8, 6 and 4nM transfection lipid also showed significant growth inhibition compared to untreated cells. The lowest concentration of transfection lipid assessed (2nM), showed no significant growth inhibition compared to control. An 8 day exposure to this reduced lipid concentration showed a healthier MCF-7 cell culture that was far closer in appearance to the untreated MCF-7 cells than the cells exposed to 10nM of lipid (figure 3.12.C.). The cell number was far greater, the cells were less rounded in appearance and more securely attached to the surface of the culture flask and were arranged in a tighter monolayer.

Though the reduced concentration of the transfection lipid showed significantly reduced toxicity, if this concentration was unable to form viable lipid-siRNA complexes and facilitate siRNA passage, in biologically active quantities into cells then the reduced toxicity profile would be irrelevant. To assess the siRNA uptake efficiency of the reduced lipid concentration siTOX siRNA was used. When siTOX siRNA is successfully transfected into a cell, the cell becomes arrested, and can not progress through its cell cycle and subsequently dies. Using Coulter counter assessment, at the reduced lipid level of 2nM, siTOX successfully reduced cell number by 60% and 90% after 4 and 8 days respectively (figure 3.13.A.) showing a conservative transfection efficiency of at least 60% after 4 days, though due to the time taken for siTOX to kill transfected cells the initial transfection efficiency is likely to be higher.

ER siRNA Knockdown.

Western blot analysis was used to assess the effect of a 4 day oestrogen receptor siRNA transfection, using the optimised Dharmafect#1 lipid concentration, on the expression of total ER protein (figure 3.13.B.). There was no observable difference in ER expression between the untransfected MCF-7 cells, the lipid treated control and the scrambled siRNA-treated cells. SiGenome ER siRNA showed a significant down regulation in ER protein expression in the MCF-7 cells transfected with siGenome ER siRNA combined with the reduced lipid concentration. It further showed that the reduced lipid concentration was able to facilitate siRNA transfection and

allowed a good knockdown of the gene of interest and as such could be used for all future experiments. Though not directly compared the ER siRNA knockdown of ER following transfection with either 2nM or 10nM Dharmafect was equivalent and potentially more efficient at the lower optimised level, due to cells not needing to survive both the effect of ER protein inhibition and severe lipid toxicity. This experiment also showed that the controls selected for use in future siRNA experiments do not effect ER expression.

3.2.2. Efficiency of knockdown using an individual siRNA or a pool.

The siGenome ER siRNA purchased from Dharmacon was a pool comprising of 4 different individual siRNA molecules provided in equal parts. It is provided in this manner as a method to reduce the severity of possible 'off-target' effects that can occur when using siRNA. However use of a pool of siRNA can also increase the frequency of possible 'off-target' effects. In a single cell line one of the components of any siRNA pool may be more effective at down regulating the target gene than the other components of the pool. To ensure in the MCF-7 cell model being used that the ER siRNA was being utilised in the most optimal manner possible, the pool and its individual components were assessed for protein knockdown efficiency and growth inhibition.

ER protein knock-down following ER siRNA transfection with a pool or individual siRNA.

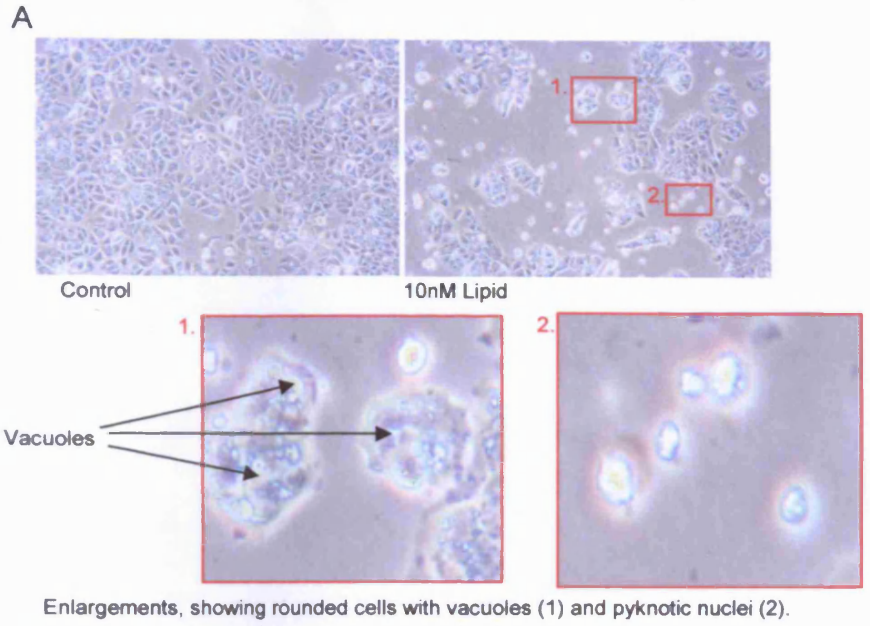
A Western blot, probed for total ER expression of MCF-7 cells after a 4 day transfection with either the complete ER siRNA pool or each of the 4 components showed that the untransfected MCF-7 cells, the lipid-treated and the scramble siRNA-treated cells again showed no differential ER expression (figure 3.13.C.). The cells transfected with any of the four individual ER siRNA constructs or the pool all showed clear down regulation of ER protein expression. There was only slight variations observed in effectiveness of protein down regulation between the individual siRNA components of the ER siRNA pool; with construct 1 promoting greatest ER down regulation and the least efficient construct being number 3. However the ER siRNA pool of all four constructs was shown to be the most effective at down regulating ER protein expression.

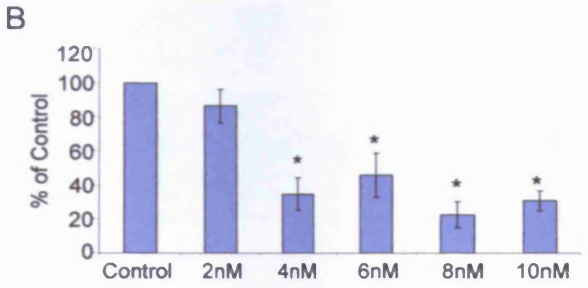
Effect of ER siRNA pool and components on cell proliferation.

An experiment to determine whether the complete siRNA pool or one of its components showed the greatest growth inhibition was also performed (figure 3.13.C.). MCF-7 growth after an 8 day transfection with either the total ER siRNA pool, or one of its four components showed that the scrambled siRNA treatment and the lipid treatment caused no significant growth inhibition compared to the untreated MCF-7 cells. There was an equal and significant

60% decrease in growth observed after transfection with all individual ER siRNA constructs. The total siGenome ER siRNA pool however showed the greatest growth inhibition compared to the individual components, though the difference was not significant. Based on these findings the complete ER siRNA pool was selected for all future experiments.

Effect of the transfection lipid Dharmafect #1 on MCF-7 cells.





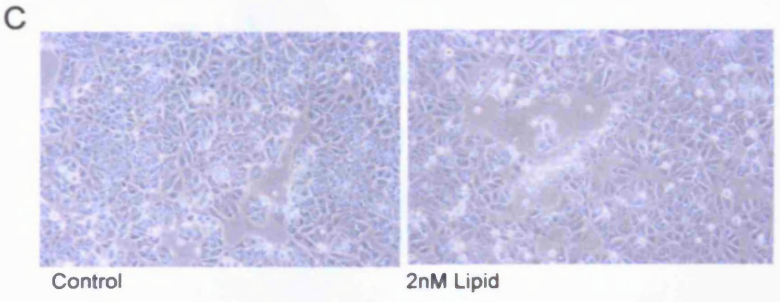


Figure 3.12.(A) MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS, treated with 10nM Dharmafect or no lipid (control) on day 4 from initial treatment (x20 magnification). (B) Effect of increasing concentrations of Dharmafect (2nM-10nM) on basal MCF-7 cell growth cultured in phenol-red free RPMI containing 5% SFCS on day 8 from initial treatment and represented as percentage of untreated control after Coulter counting (n=3). (C) MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS, treated with 2nM Dharmafect or no lipid (control) on day 4 from initial treatment (x20 magnification). N.B.= P≤0.05 vs Control.*

SiRNA transfection efficiency, and protein knockdown effects in MFC-7 cells using ER siRNA pool or individual ER siRNA with reduced transfection lipid.

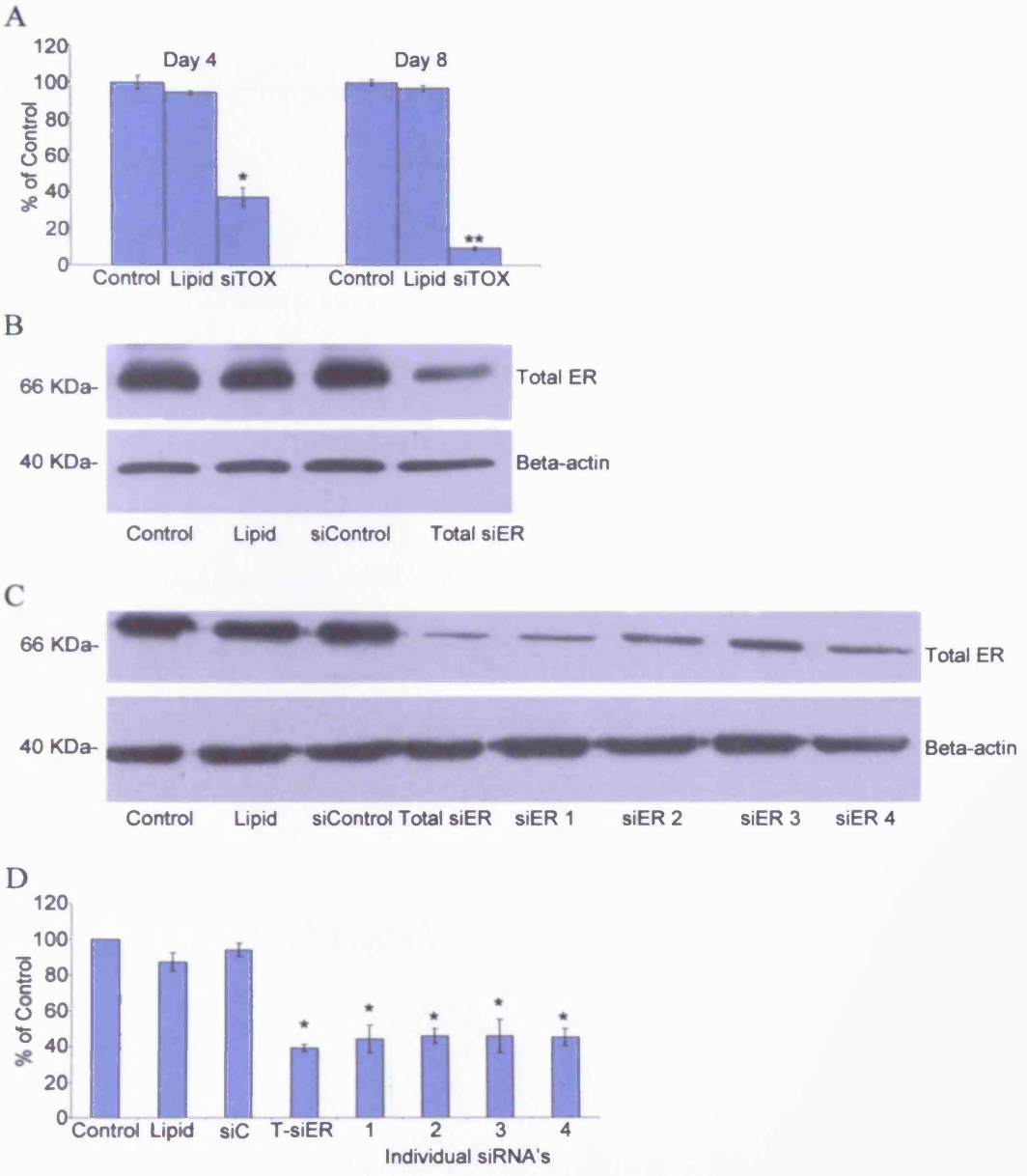


Figure 3.13.(A) Effect of siTOX, and 2nM lipid on basal MCF-7 cell growth cultured in phenol-red free RPMI containing 5% SFCS on day 4 from initial treatment and represented as percentage of untreated control after Coulter counting (n=3). (B) Western analysis of ERα and β-actin expression in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS and treated with either siGENOME ER siRNA pool or (C) individual siGENOME ER siRNA's, on day 4 from initial treatment. (D) Effect of siGENOME ER siRNA pool and components on basal MCF-7 cell growth cultured in phenol-red free RPMI containing 5% SFCS on day 4 from initial treatment and represented as percentage of untreated control after Coulter counting (n=3). N.B.= P≤0.05. .**= P≤0.01 vs Control for lipid and siTOX and siControl for siER's respectively.*

3.3. The Effect of specific ER siRNA on MCF-7 cells.

Down-regulation of ER protein expression following fulvestrant exposure in MCF-7 cells was previously shown to be incomplete (section 3.1.). In this section experiments were conducted to see how effective ER siRNA was at reducing oestrogen receptor levels, ER signalling activity and growth, and thus whether targeting ER mRNA was a more effective strategy for reducing the residual ER levels than targeting the ER protein levels with fulvestrant. During the course of the project, Dharmacon released a second class of siRNA products, called On-target siRNA in addition to their siGenome siRNA range. This new class of siRNA's had been optimised and selected for greater knockdown and less off-target effects. The new On-target siRNA construct to the oestrogen receptor was also assessed in this project.

3.3.1. ER siRNA response on oestrogen receptor levels in MCF-7 cells.

A 4 day transfection with either siGenome or On-target ER siRNA pools revealed a clear down-regulation of oestrogen receptor mRNA expression in MCF-7 cells cultured in both stripped and whole serum when compared to lipid-treated, scrambled siRNA-treated, and untransfected MCF-7 control cells (figure 3.14.A.). The fulvestrant treated arm again showed no effect on ER

mRNA expression. The down regulation with either ER siRNA constructs was substantial; however residual ER mRNA was still present.

Western blotting analysis also revealed an impressive down regulation of total ER protein expression following a 4 day transfection with either ER siRNA pool in MCF-7 cells cultured in stripped or whole serum (figure 3.14.B.). MCF-7 cells transfected with either ER siRNA constructs again showed the presence of residual ER protein expression at largely equivalent levels to that seen following fulvestrant treatment in both whole and stripped serum conditions.

Incomplete down regulation of ER protein expression following siRNA knockdown was also apparent following immunocyto-chemical assessment and subsequent nuclear ER H-score analysis (figure 3.15.A. and B.). A 4 day exposure of MCF-7 cells to scrambled siRNA, transfection lipid and culture medium showed no effect on ER protein expression, with high staining intensity in 95% of the cells nuclei and moderate staining in the remaining nuclei being observed. Subsequent nuclear H-score analysis (figure 3.15.B. and table 3.4.) for both ER siRNA constructs utilised in this study showed a clear decrease of ER protein expression, with only 40% of cells showing any nuclear staining. This residual nuclear ER staining was of low intensity but still clearly apparent. MCF-7 cells grown in stripped or whole serum treated with ER siRNA also showed a few highly stained nuclei, possibly an indication of untransfected cells within the population, as it occurred regardless of siRNA construct used (see enlargements). ER down regulation observed following ER siRNA transfection, and fulvestrant

exposure was statistically significant ($p \leq 0.05$, n=3) under both serum conditions compared to the controls, but there was no significant difference in ER protein expression levels observed between the two ER siRNA constructs and fulvestrant exposed cells in either serum condition.

3.3.2. The Effect of ER siRNA transfection on oestrogen signalling.

Transfection of ER siRNA in MCF-7 cells produced a significant but incomplete down regulation of ER protein expression, comparable to fulvestrant treatment. Further studies were subsequently performed to investigate whether the residual ER remaining following ER siRNA transfection possessed similar activity by assessment of ER transcriptional activity.

The effect of ER siRNA transfection on PR and pS2 mRNA and protein expression.

The transcriptional activity of the residual ER following ER siRNA transfection was initially investigated by measurement of PR and pS2 mRNA and protein expression.

PR mRNA expression.

A down regulation of PR mRNA expression was observed when MCF-7 cells, incubated in both stripped and whole serum, were transfected with either ER siRNA construct, and was comparable to levels observed following fulvestrant exposure. Once again the down regulation of PR at the mRNA level was incomplete, with residual levels observed following ER siRNA transfection (Figures 3.16.A.).

pS2 mRNA expression.

In stripped and whole serum culture conditions, analysis of pS2 mRNA expression also showed that levels were reduced following transfection with either ER siRNA, and the pS2 mRNA expression was comparable to the levels observed following fulvestrant treatment (figures 3.16.B.). As with PR gene expression, the down regulation of pS2 mRNA expression was incomplete in both serum conditions again suggesting the presence of residual ER transcriptional activity.

PR protein expression.

Untransfected MCF-7 cells cultured in stripped serum showed low cytoplasmic (40% of cells) and nuclear (60% of cells) PR protein expression with the remainder of the cells being PR negative (figure 3.17.A.). The ER

siRNA transfected cells revealed a reduction in PR protein expression, with detectable staining in the nucleus and cytoplasm of only 10% of the cells with the remaining cells being PR negative. Confirmation of these observations by subsequent nuclear H-score analysis showed a significant ($p \leq 0.05$, n=3) down regulation of PR expression in cells transfected with either ER siRNA construct, however the presence of residual nuclear PR was also detected in a small population (see enlargements 1. and 2.). The reduction in expression observed following transfection with both ER siRNA constructs was similar to that observed following fulvestrant exposure (figure 3.17.B. and table 3.5.).

Both cytoplasmic and nuclear PR protein expression was observed in MCF-7 cells cultured in whole serum under all control conditions (figure 3.17.A.), with medium to high intensity staining being observed in the nucleus of 70% of the lipid-treated, scrambled siRNA-treated and untransfected MCF-7 cells. These cells also displayed very high expression of cytoplasmic PR in 60% of these control cells with moderate staining in the remaining cells. ER siRNA transfection with either construct significantly reduced nuclear PR protein expression but low level of residual nuclear PR staining was still observed in 40% the cells (with some high intensity staining) with no expression observed in the remainder (enlargements 3. and 4.) which was similar to fulvestrant treatment. Cytoplasmic staining was also decreased following transfection with ER siRNA but low level staining was again observed in 40% of cells and no PR expression in the cytoplasm of the remaining cells. Corresponding nuclear H-score assessment (figure 3.17.B. and table 3.5.) also demonstrated both a statistically significant ($p \leq 0.05$, n=3)

reduction of PR protein expression after transfection, and residual PR expression levels equivalent to expression following fulvestrant exposure.

pS2 protein expression.

Overall pS2 protein expression levels in MCF-7 cells cultured in stripped serum (figure 3.18.A.) was exclusively within the cytoplasm. Lipid-treated, scrambled siRNA-treated and untransfected cells showed some moderate intensity staining in the cytoplasm of 40% of the population, with lower pS2 expression in the cytoplasm of the remaining cells. Transfection with either ER siRNA construct caused a loss of all high intensity cytoplasmic staining, but residual pS2 expression was observed in the cytoplasm of 10% of the population. The corresponding cytoplasmic H-scores showed a significant ($p \leq 0.05$, $n=3$) down regulation in the presence of either ER siRNA and also demonstrated the presence of residual pS2 protein expression, with levels comparable to that observed in fulvestrant exposed cells (figure 3.18.B. and table 3.6.).

In whole serum culture conditions, all MCF-7 cells again showed only cytoplasmic pS2 protein expression (figure 3.18.A.). Lipid-treated, scramble siRNA-treated and untransfected MCF-7 cells showed moderate cytoplasmic staining across 40% of the population with low staining in 20%, and the remaining cells showed no cytoplasmic staining. There was a clear reduction in pS2 protein levels across all the population following transfection with either ER siRNA construct, no nuclear staining was observed but 15% of the

cells demonstrated moderate to low residual cytoplasmic staining and the remainder were pS2 negative. Corresponding cytoplasmic H-score analysis (figure 3.18.B. and table 3.6.) reflected these observations, showing a statistically significant ($p \leq 0.05$, n=3) down regulation in the ER siRNA transfected cells but still clear evidence of residual pS2 protein expression identical to that seen in the fulvestrant exposed cells.

ERE activity.

An ERE driven dual *luciferase* assay was again utilised to quantitatively measure the level of ER signalling following ER siRNA transfection of MCF-7 cells. These studies showed an equivalent and statistically significant ($p \leq 0.05$, n=3) fall in ERE activity in both ER siRNA transfected and fulvestrant-treated cells, cultured in media containing stripped serum (figure 3.19.A.). However residual ERE transcriptional activity was still apparent after exposure to either ER siRNA construct or the pure anti-oestrogen with all treatments reducing ERE activity to a similar degree.

In the MCF-7 cells cultured in whole serum (Figure 3.19.B.) a statistically significant ($p \leq 0.01$, n=3) 70-80% reduction in ERE transcriptional activity was observed following ER siRNA transfection (with either construct) or fulvestrant treatment, with fulvestrant treatment showing a significantly greater reduction than either ER siRNA alone. Though ER siRNA transfection or fulvestrant treatment decreased ERE transcriptional activity significantly, there was still a residual activity equivalent to 20% of control, which was

statistically significantly greater than the background fluorescence produced from cells not transfected with ERE constructs.

ER siRNA effect on oestradiol stimulation.

To determine whether ER siRNA transfection, like fulvestrant treatment, was able to overcome enhanced ER signalling following oestradiol challenge. The ability of ER siRNA to reduce oestradiol-stimulated ER regulated PR and pS2 protein expression was assessed.

When MCF-7 cells cultured in stripped serum were stimulated with the addition of 10^{-9}M oestradiol to the media the basal expression levels of both PR and pS2 protein expression were increased though only slightly for PR expression (figure 3.20.) when compared to unstimulated cells previously shown (in figure 3.17.). The oestradiol stimulated cells treated with either scrambled siRNA, transfection lipid or culture media showed PR expression in the nucleus of all of the cells, and high expression of PR in the cytoplasm was also observed in 40% of the cells, with less intense staining also observed in the remainder of the cells (figure 3.20.A.). When stimulated with oestradiol, MCF-7 cells cultured with stripped serum also showed increased cytoplasmic levels of pS2 expression, compared to unstimulated cells. All control cells showed intense cytoplasmic staining. A significant ($p \leq 0.05$, $n=3$) down regulation of oestradiol-induced PR protein expression was apparent, following ER siRNA transfection, with 30% of cells showing reduced intensity staining in the nucleus with occasional strong staining (see

enlargements) and some residual PR expression present in the cytoplasm, with 30% of cells being PR negative. Oestradiol-treated, ER siRNA transfected cells, showed only low levels cytoplasmic pS2 staining, with 15% of cells showing no expression of pS2 at all (figure 3.20.A.). Subsequent H-score analysis of nuclear PR and cytoplasmic pS2 staining supported these observations and showed a statistically significant ($p \leq 0.05$, $n=3$) down regulation of both PR and pS2 expression following ER siRNA transfection with either construct (figure 3.20.B. and tables 3.5. and 3.6.). However both the pictures and the H-score analysis showed presence of residual PR and pS2 after ER siRNA transfection and these levels of reduction and residual expression was comparable to the MCF-7 cells treated with fulvestrant.

3.3.3. The Effect of ER siRNA transfection on MCF-7 cell proliferation.

These previous findings clearly show that ER siRNA transfection resulted in an incomplete down regulation of ER expression and ER transcriptional activity in a manner comparable to fulvestrant treatment. As fulvestrant treatment subsequently produced an incomplete blockade of cell proliferation the influence of ER siRNA transfection on cell proliferation was examined to determine whether a similar pattern was evident with this therapeutic strategy. Again effects on proliferation were assessed in multiple ways, through monitoring effects on IGF-1R signalling component expression, nuclear Ki67

expression analysis and through the use of cell viability and cell counting assays.

IGF-1R signalling component analysis.

Measurement of IGF-1R signalling component expression, assessed by Western blotting following a 4 day transfection with either ER siRNA construct, showed a down regulation of total and phosphorylated IGF-1R levels, as well as total IRS1 expression, in ER siRNA transfected cells compared to scrambled siRNA-treated, lipid-treated and untransfected MCF-7 cells cultured in either stripped or whole serum. This reduction was comparable to the down regulation observed following fulvestrant treatment (figures 3.21.A. and B.) and again the inhibition observed was incomplete for both ER siRNA constructs regardless of serum conditions.

Ki67 analysis.

In MCF-7 cells treated with scrambled siRNA, transfection lipid and untransfected cells, cultured in stripped serum, high levels of nuclear staining for the proliferation marker Ki67 was observed in 40% of the population and less intense staining recorded in a further 20% of the population with the remaining cells showing no expression of the antigen (figure 3.22.A.) The total Ki67 positivity score was roughly 65% for all these conditions (figure 3.22.B.). Similarly scrambled siRNA-treated, lipid-treated and untransfected

MCF-7 cells cultured in whole serum all showed a high percentage (80%) of cells expressing the Ki67 antigen in the nucleus (figures 3.22.A. and B.). ER siRNA transfection with either construct decreased staining significantly compared to the controls, with staining observed in 30% and 40% of cells cultured with stripped and whole serum respectively. The reductions were again comparable to levels observed following exposure to fulvestrant. Once more despite a significant ($p \leq 0.05$, $n=3$) reduction in nuclear Ki67 positive cells in both serum conditions, some cells still showed presence of the marker in the ER siRNA transfected cell populations (see enlargements). Transfection with either ER siRNA was also shown to reduce Ki67 expression to a similar degree in both stripped and whole serum.

Proliferation assays.

MTT assays revealed incomplete growth inhibition in response to ER siRNA transfection with either construct. An 8 day transfection with On-target ER siRNA resulted in a small but significant growth inhibition of 20% in MCF-7 cells cultured with stripped serum, an inhibition comparable following fulvestrant exposure (figure 3.23.A.). However a greater growth inhibition of 60% was observed following siGenome ER siRNA transfection under these culture conditions. In whole serum culture conditions, a significant growth inhibition of 50% was observed following transfection with On-target ER siRNA (figure 3.23.A.), transfection with siGenome ER siRNA caused a 70% growth inhibition, which was comparable with fulvestrant treatment.

Coulter counter analysis (figure 3.23.B.) of MCF-7 cells transfected with siGenome ER siRNA showed a significant decrease of 70% and 40% in total cell number when cultured with stripped and whole serum respectively. A significant growth inhibition of 40% was observed following On-target ER siRNA transfection under both serum conditions. Fulvestrant exposure caused a greater growth inhibition than On-target ER siRNA transfection under both culture conditions, and a greater growth inhibition when compared to siGenome ER siRNA transfection with whole serum, but not under stripped serum conditions. Residual growth was again observed following transfection with either ER siRNA construct under both culture conditions.

3.3.4. Possible Off target effect of siGenome ER siRNA.

During the course of the previous growth experiments, transfection with siGenome ER siRNA seemed to produce a more substantial impact than the reputedly more specific On-target ER siRNA construct in MCF-7 cells. Further studies revealed that use of SiGenome ER siRNA could cause an off-target effect, leading to, or directly causing a down-regulation of total ERK protein expression, an important growth regulatory protein in MCF-7 cells. This down regulation was observed under both culture conditions (figures 3.24.A. and B.), all other treatments, including the On-target ER siRNA transfected cells and the fulvestrant exposed cells, showed no effect on ERK protein expression. Evidence of an off-target effect acting either directly or indirectly on ERK regulation made the siGenome class of ER siRNA

unreliable for future experiments due to the importance of ERK on MCF-7 cell proliferation and survival.

ER mRNA and protein expression in MCF-7 cells cultured in stripped or whole serum, treated with ER siRNA.

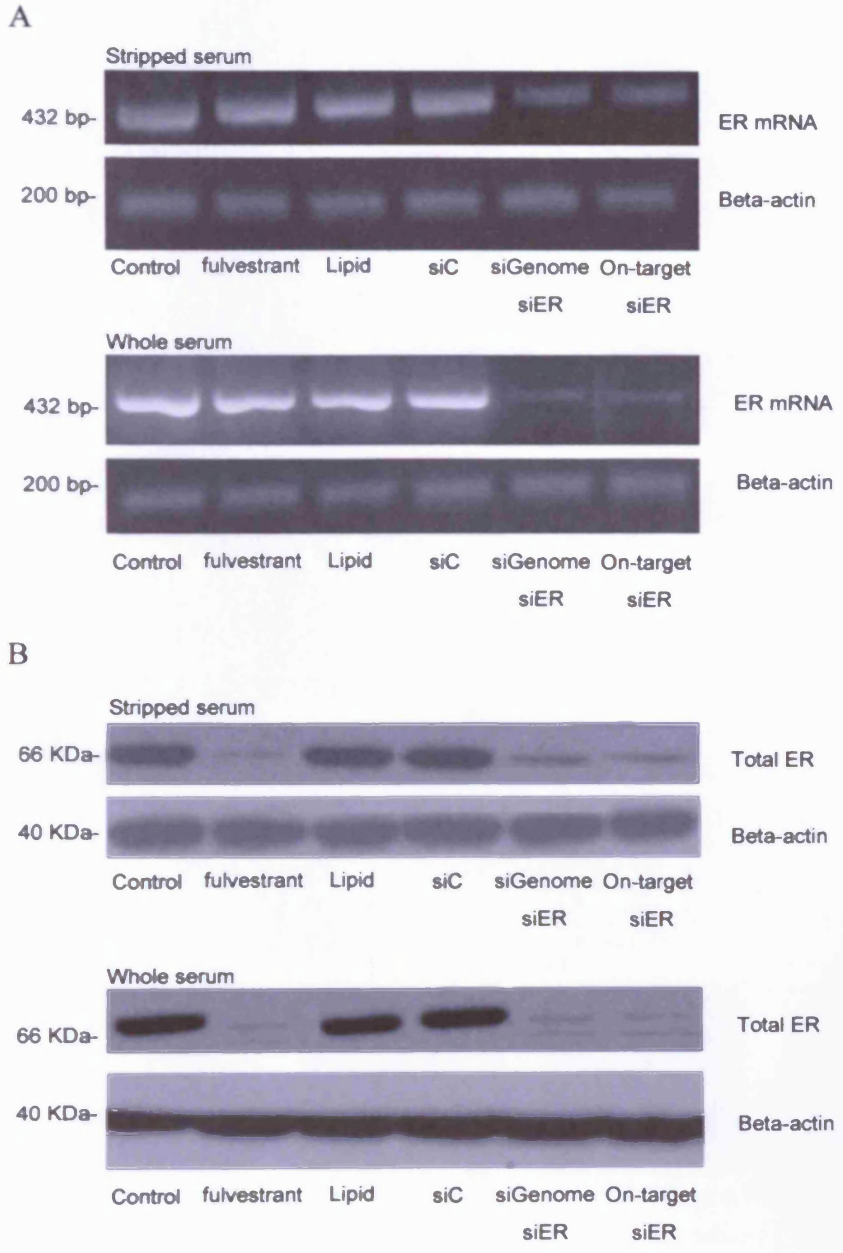


Figure 3.14.(A) Expression of ERα and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment. (B) Western analysis of ERα and β-actin expression in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment.

ER protein expression in MCF-7 cells cultured in stripped or whole serum, treated with ER siRNA.

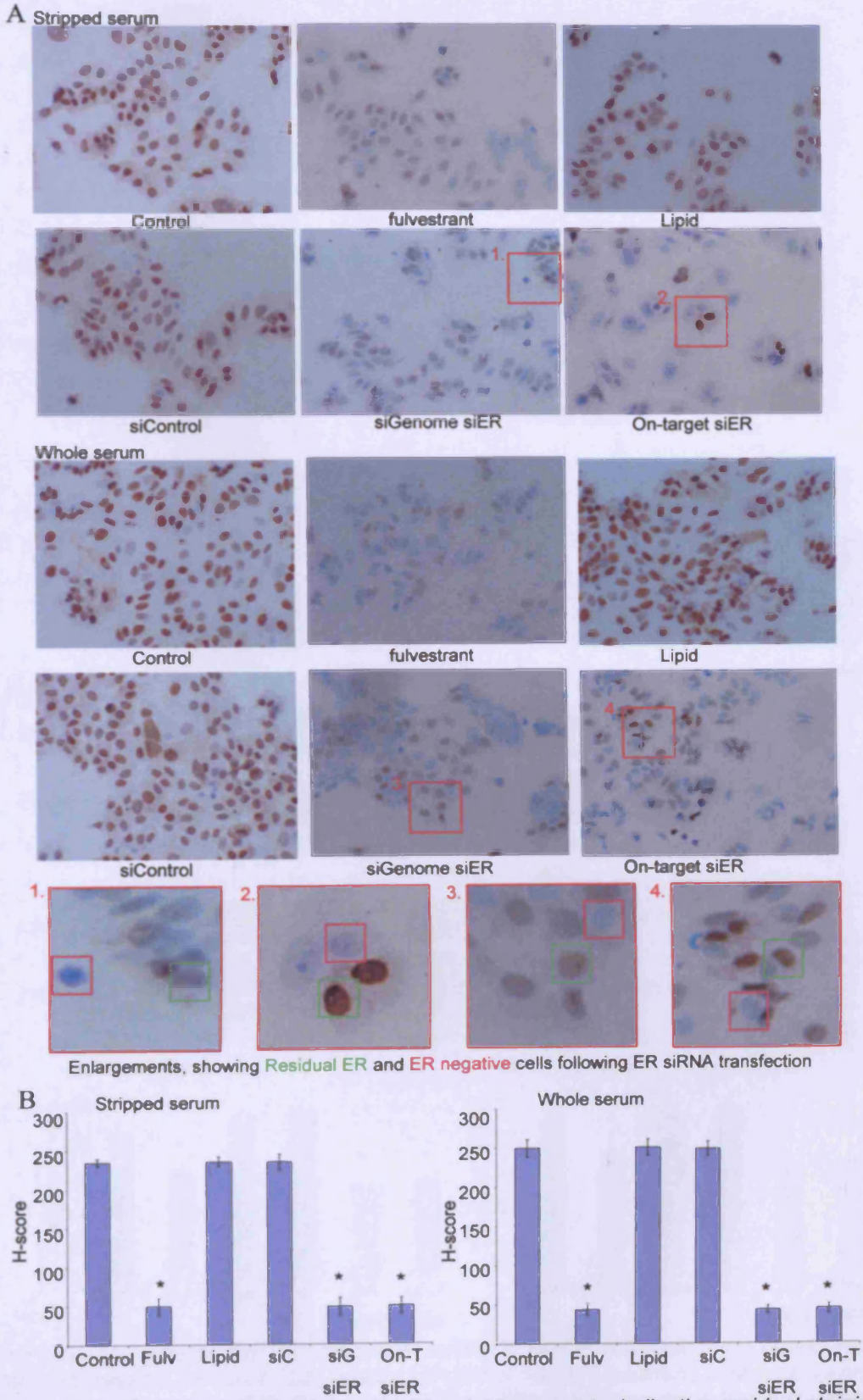


*Figure 3.15. (A) Immunocytochemical staining (enlargements, indicating residual staining and negative cells in green and pink respectively) magnification x20 and (B) average nuclear ER H scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control or siC for Fulv and siER's respectively.*

PR and pS2 mRNA expression in MCF-7 cells cultured in stripped and whole serum, treated with ER siRNA.

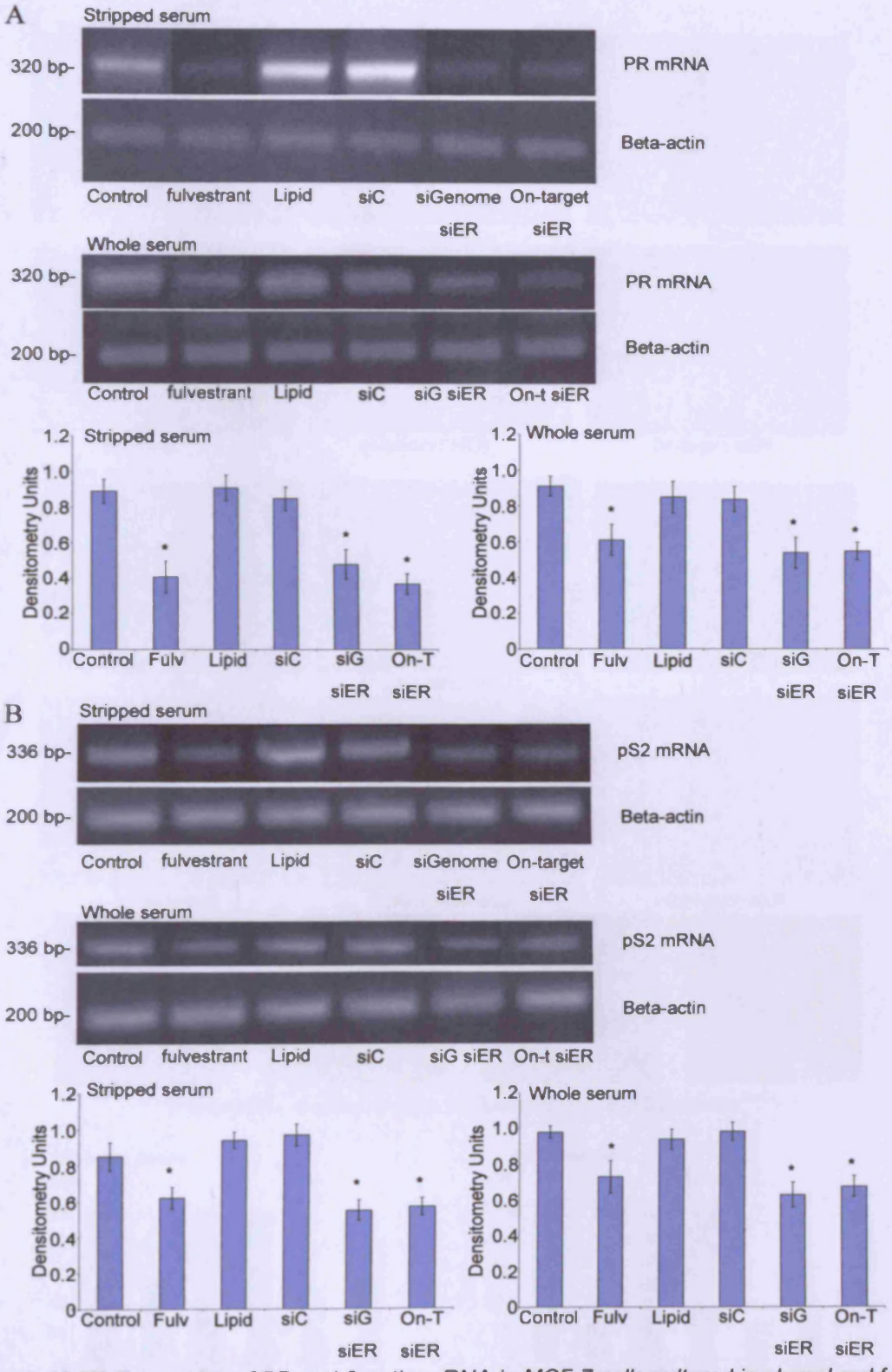
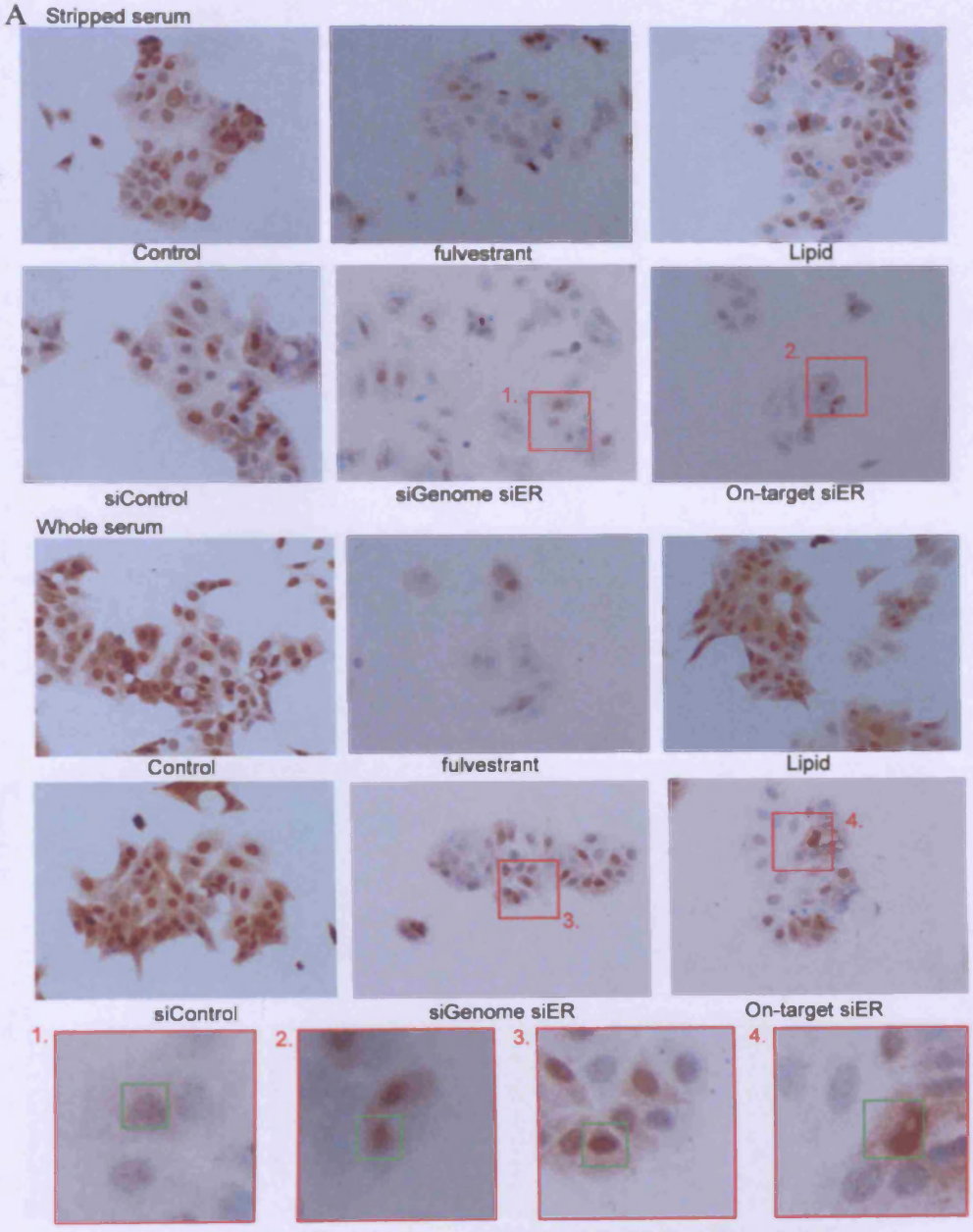


*Figure 3.16.(A) Expression of PR and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment and actin normalised densitometry values (B) Expression of pS2 and β-actin expression in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment and actin normalised densitometry values. N.B. *= P≤0.05 vs Control or siC for Fulv and siER's respectively.*

PR protein expression levels in MCF-7 cells cultured in stripped or whole serum, and treated with ER siRNA.



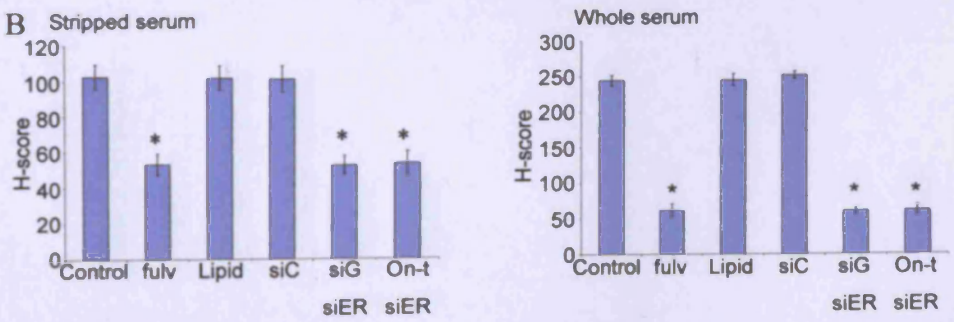


Figure 3.17.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 *and (B) average nuclear PR H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control or siC for fulv and siER's respectively.*

pS2 protein expression levels in MCF-7 cells cultured in stripped or whole serum, and treated with ER siRNA.

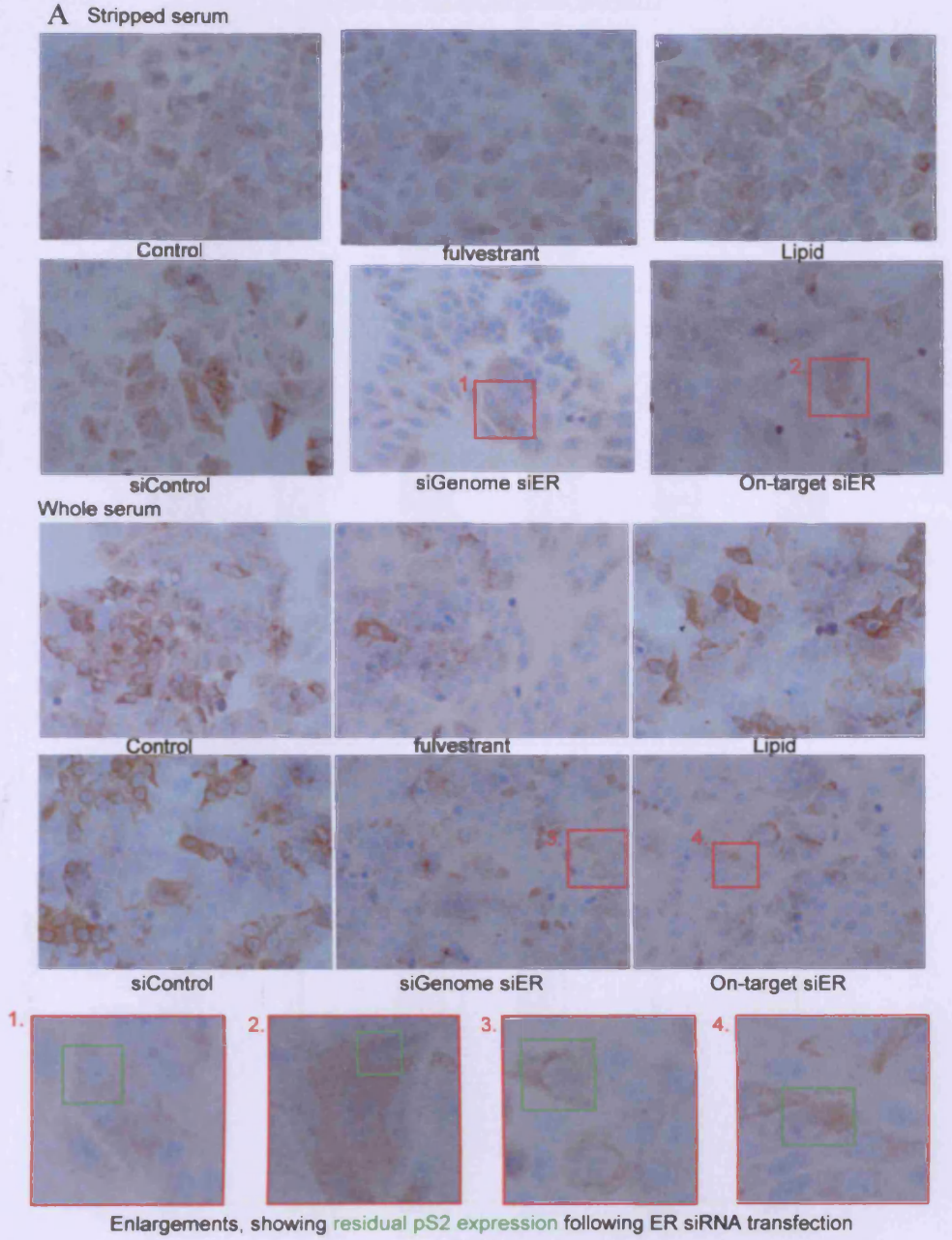


*Figure 3.18.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control or siC for fulv and siER's respectively.*

Level of ERE signalling in MCF-7 cells treated with ER siRNA, cultured in stripped or whole serum.

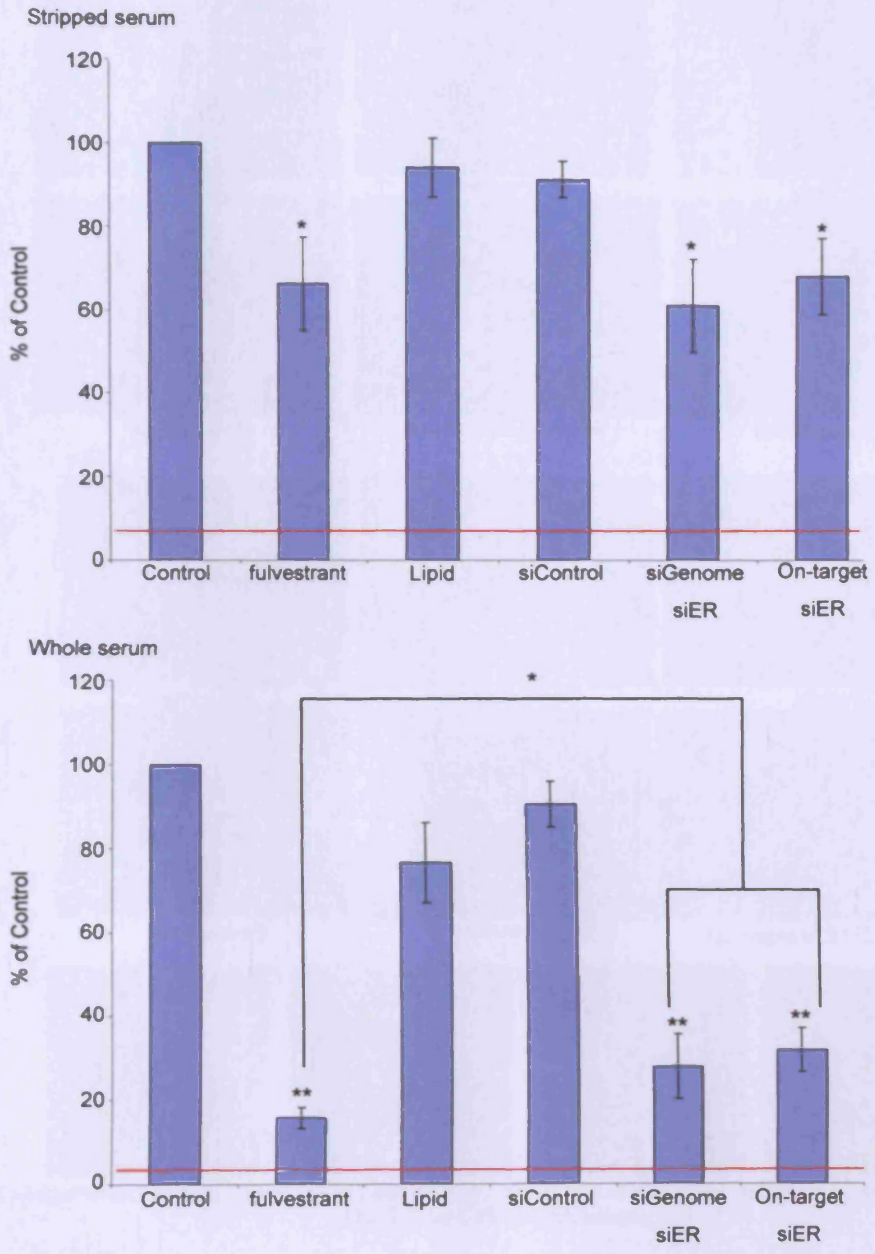
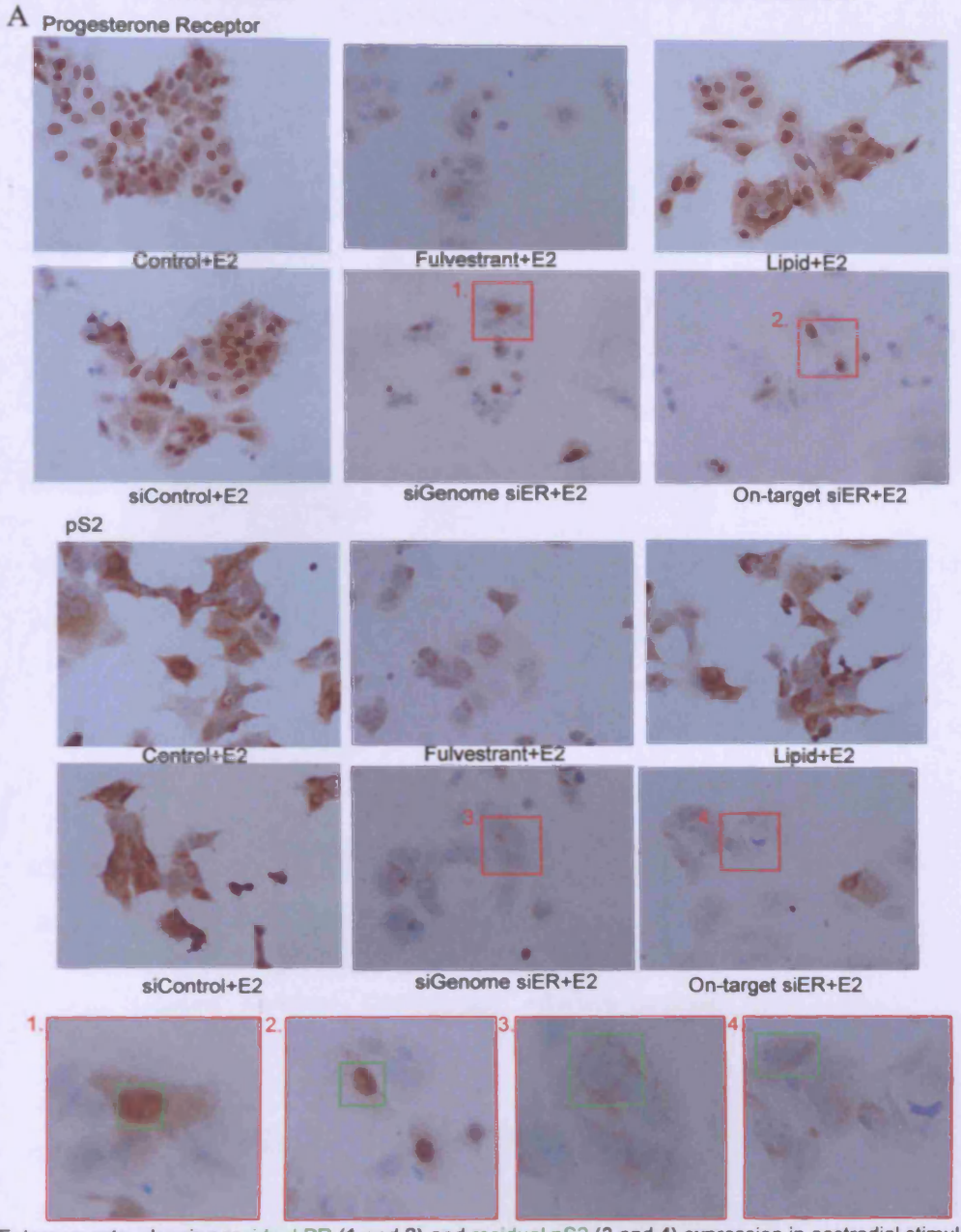


*Figure 3.19. Reporter gene assay showing average ERE expression (n=3) in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER, or vehicle control on day 4 from initial treatment; with background fluorescence levels indicated by red line (n=3). N.B. *= p≤0.05 **= p≤0.01 vs control or siControl for fulv and siER's respectively or where indicated by bars.*

PR and pS2 protein expression in MCF-7 cells cultured in stripped serum supplemented with oestradiol and treated with ER siRNA.



Enlargements, showing residual PR (1 and 2) and residual pS2 (3 and 4) expression in oestradiol stimulated cells, following ER siRNA transfection.

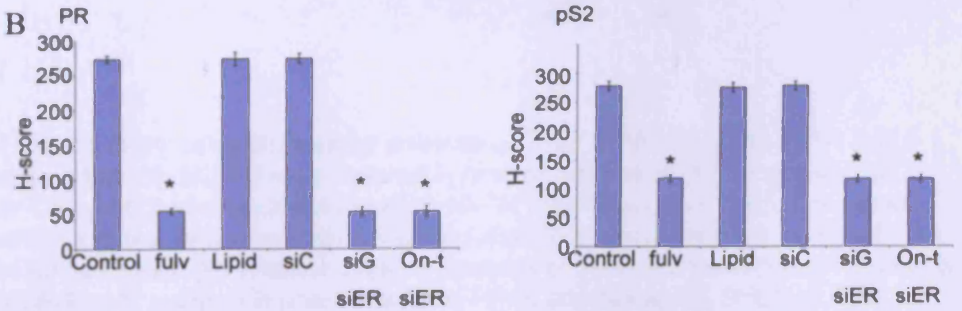


*Figure 3.20.(A) Immunocytochemical staining of PR and pS2 (enlargements, indicating residual staining in green) magnification x20 and (B) average nuclear PR and cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS and 10-9M oestradiol and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control or siC for fulv and siER's respectively.*

The effect of ER siRNA on IGFR signalling in MCF-7 cells cultured in stripped or whole serum.

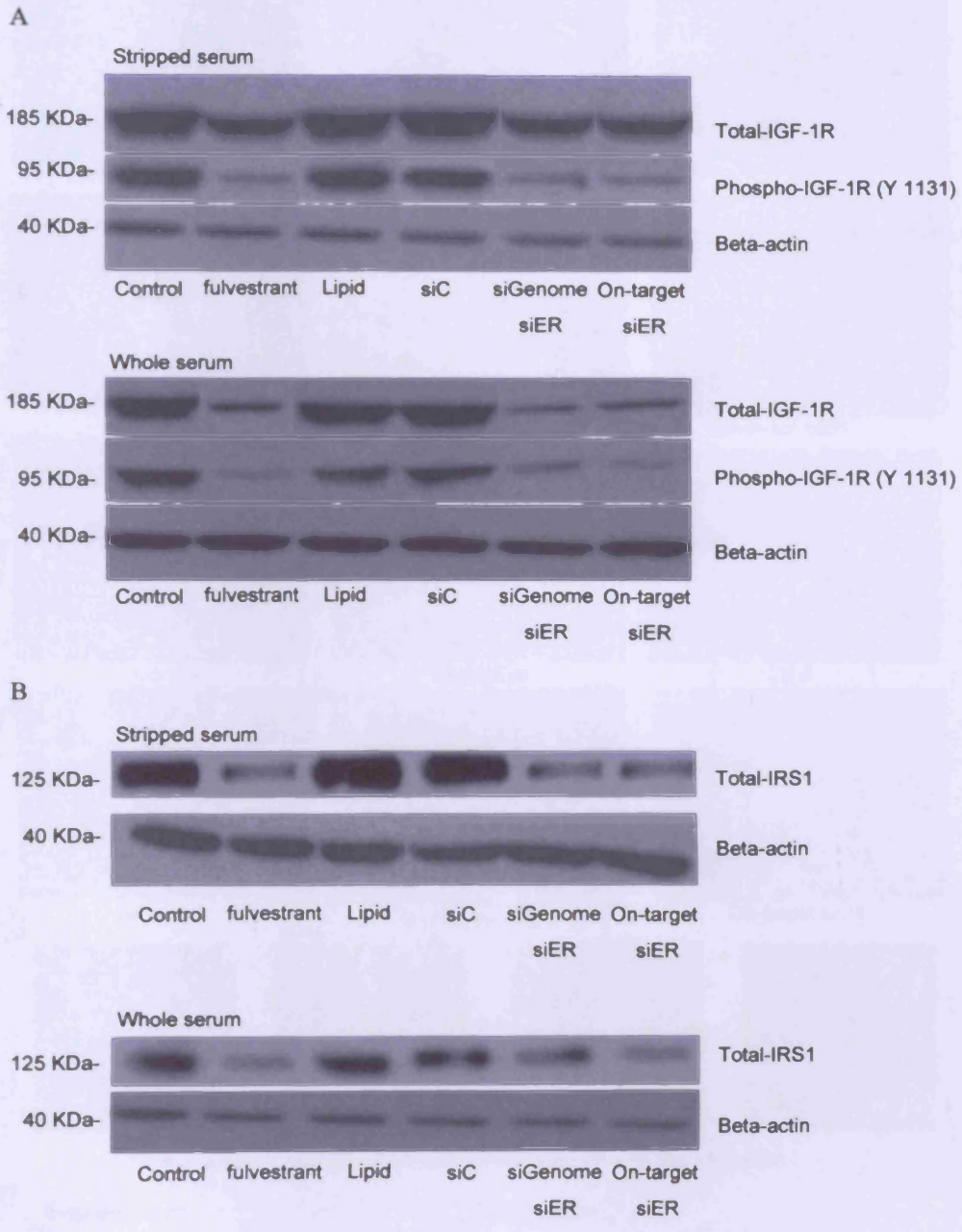


Figure 3.21.(A) Western blotting analysis of Total IGFR, Phospho-IGFR and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. (B) Western blotting analysis of Total IRS1 and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment.

Ki67 expression in MCF-7 cells cultured in stripped or whole serum, treated with ER siRNA.

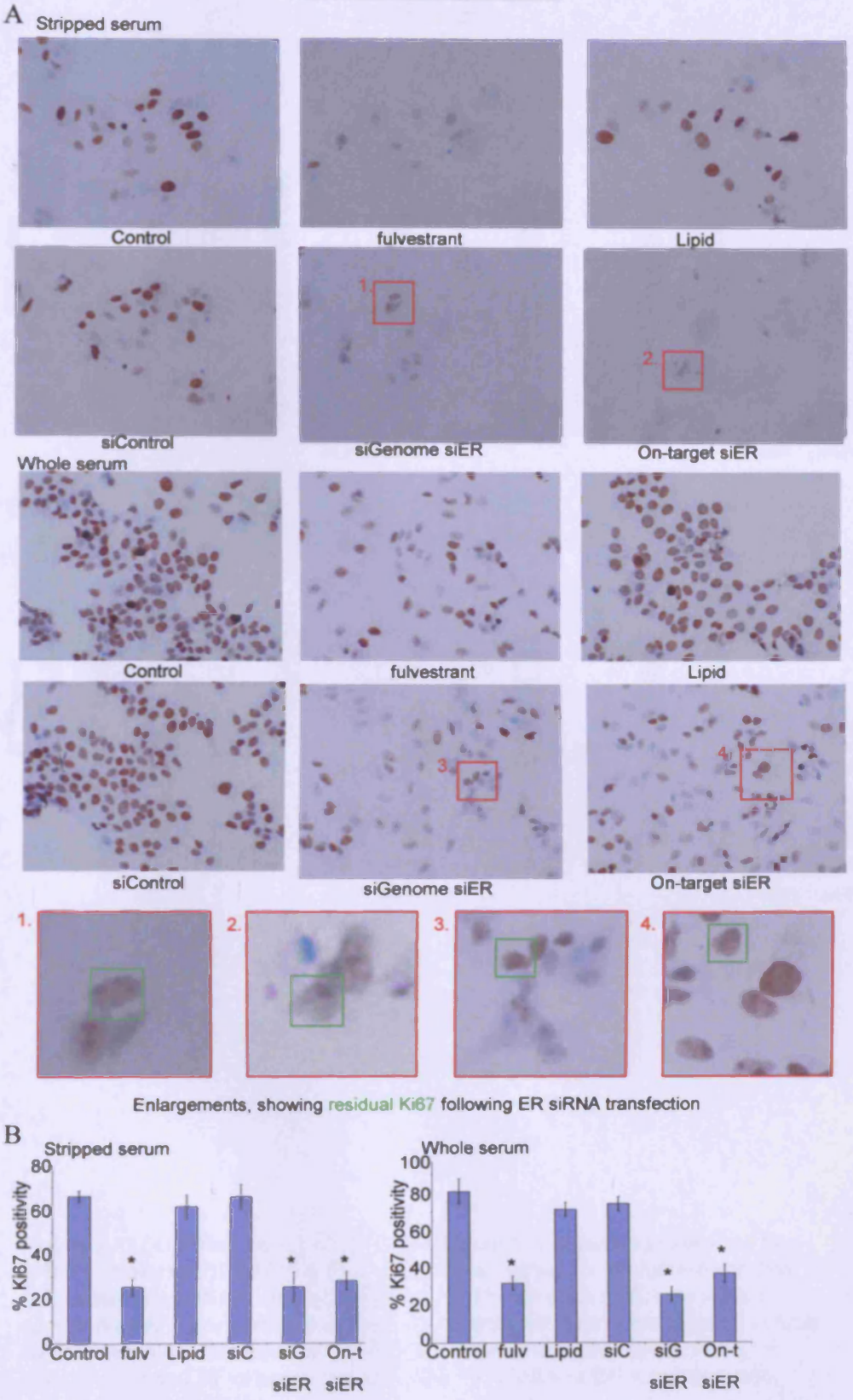


*Figure 3.22.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average total Ki67 positivity (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs control or siC for fulv and siER's respectively.*

The effect of ER siRNA on growth of MCF-7 cells cultured in stripped or whole serum.

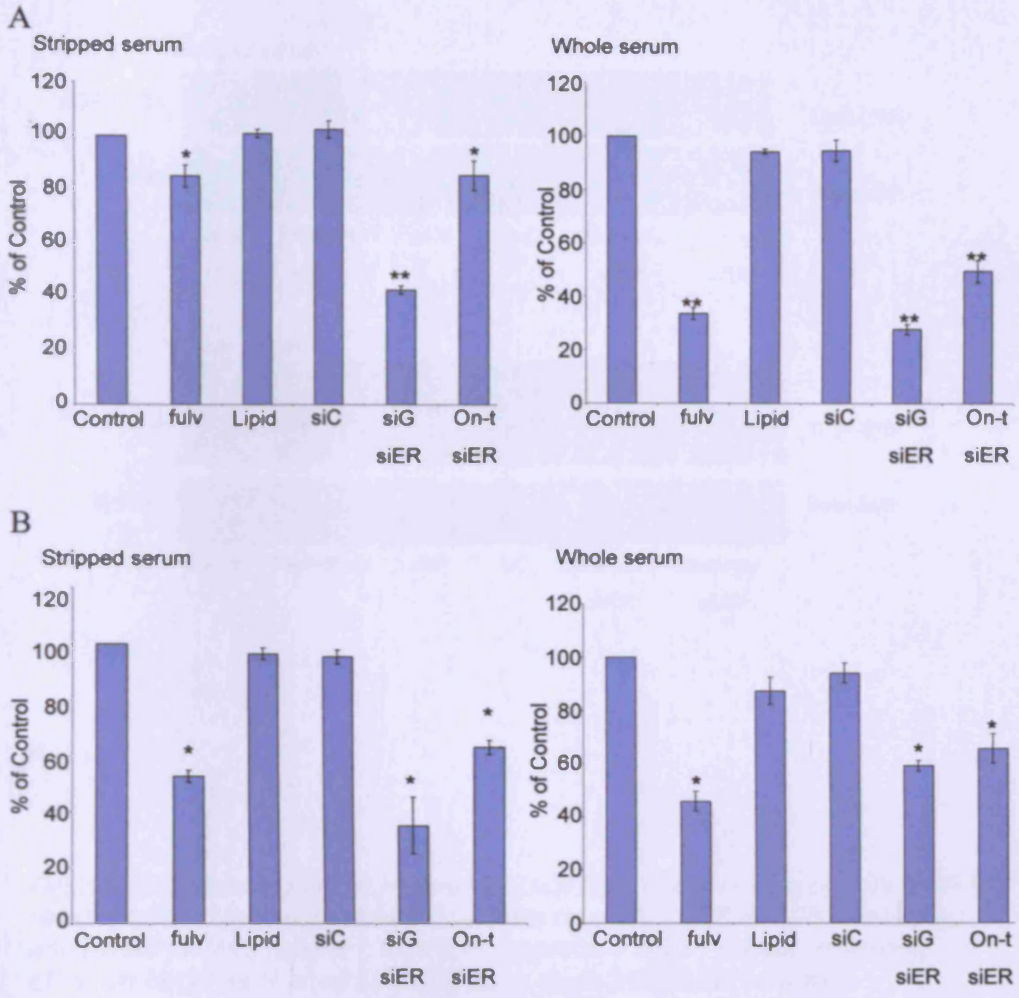


Figure 3.23.(A) Effect on basal MCF-7 cell growth, cultured in phenol-red free RPMI containing 5% SFCS or FCS treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER, or vehicle control on day 8 from initial treatment and represented as percentage of vehicle control after MTT assessment (n=3) (B) or Coulter counting (n=3). N.B. . = p≤0.05, ** = p≤0.01 vs control or siControl for fulv and siER's respectively.*

The effect of ER siRNA on ERK signalling in MCF-7 cells cultured in either striped or whole serum.

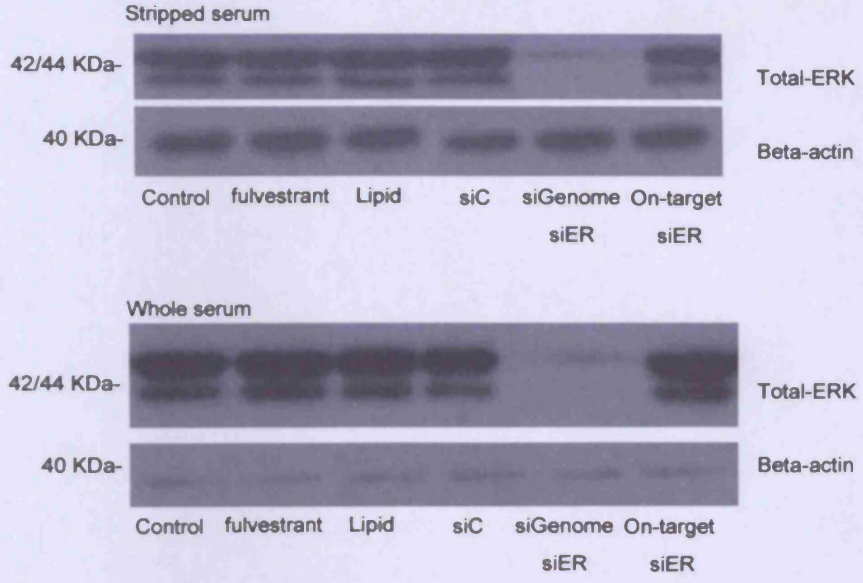


Figure 3.24. Western blotting analysis of Total ERK and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control, on day 4 from initial treatment.

Complete ER H-score analysis of MCF-7 cells following ER siRNA transfection.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum)	235(+/-4.5)	0
Fulvestrant (stripped serum)	49.2(+/-11.1)	0
Lipid (stripped serum)	235.8(+/-5.8)	0
siRNA control (stripped serum)	233.3(+/-6.8)	0
siGenome ER siRNA (stripped serum)	48.3(+/-11.7)	0
On-target ER siRNA (stripped serum)	49.2(+/-10.7)	0
Control (whole serum)	249.2(+/-11.1)	0
Fulvestrant (whole serum)	43.3(+/-7.5)	0
Lipid (whole serum)	250(+/-10.5)	0
siRNA control (whole serum)	248.3(+/-9.3)	0
siGenome ER siRNA	42.5(+/-5.2)	0
On-target ER siRNA	44.2(+/-6.6)	0

Table 3.4. ER H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA transfection or fulvestrant exposure (n=3).

PR H-score analysis of MCF-7 cells following ER siRNA transfection.

	Nuclear H-score	Cytoplasmic H-score	Total H-score
Control (stripped serum)	102.5(+/-6.9)	48.6(+/-5.3)	122.5(+/-6.6)
Fulvestrant (stripped serum)	53.3(+/-6.1)	20.4(+/-2.1)	65.6(+/-4.4)
Lipid (stripped serum)	101.7(+/-6.8)	45.7(+/-3.3)	121.8(+/-7.2)
siRNA control (stripped serum)	100.8(+/-7.4)	45.2(+/-7.2)	123.4(+/-6.2)
siGenome ER siRNA (stripped serum)	52.5(+/-5.2)	21.3(+/-5.3)	66.2(+/-6.5)
On-target ER siRNA (stripped serum)	53.3(+/-6.8)	21.7(+/-3.8)	65.9(+/-7.2)
Control (stripped serum +E2)	273.5(+/-5.2)	205.7(+/-13.5)	274.8(+/-9.4)
Fulvestrant (stripped serum +E2)	55(+/-4.5)	52(+/-3.1)	90(+/-7.3)
Lipid (stripped serum +E2)	274.2(+/-9.7)	201.1(+/-12.1)	272.5(+/-8.2)
siRNA control (stripped serum+E2)	275(+/-7.1)	207.7(+/-14.6)	273.2(+/-8.9)
siGenome ER siRNA (stripped serum +E2)	54.2(+/-6.6)	56.2(+/-7.2)	85.2(+/-7.1)
On-target ER siRNA (stripped serum+E2)	54.2(+/-8.6)	52.2(+/-4.5)	87.6(+/-8.1)
Control (whole serum)	244.4(+/-7.3)	177(+/-11.6)	253.6(+/-8.8)
Fulvestrant (whole serum)	61.7(+/-8.8)	40.3(+/-2.4)	89.7(+/-7.2)
Lipid (whole serum)	245(+/-8.4)	178.2(+/-10.3)	255.7(+/-8.1)
siRNA control (whole serum)	251.6(+/-5.2)	176.6.(+/-9.9)	257.3(+/-10.2)
siGenome ER siRNA	60.8(+/-4.9)	38.6(+/-3.9)	91.2(+/-8.6)
On-target ER siRNA	61.6(+/-6.8)	41.6(+/-4.3)	89.4(+/-6.8)

Table 3.5. PR H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA or fulvestrant exposure (n=3).

pS2 H-score analysis of MCF-7 cells following ER siRNA transfection.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum)	0	155.8(+/-8)
Fulvestrant (stripped serum)	0	58.3(+/-5.2)
Lipid (stripped serum)	0	154.2(+/-4.9)
siRNA control (stripped serum)	0	153.3(+/-5.2)
siGenome ER siRNA (stripped serum)	0	59.2(+/-5.8)
On-target ER siRNA (stripped serum)	0	60.8(+/-3.8)
Control (stripped serum +E2)	0	278.3(+/-8.2)
Fulvestrant (stripped serum +E2)	0	118.3(+/-6.1)
Lipid (stripped serum +E2)	0	275.8(+/-8)
siRNA control (stripped serum+E2)	0	279.2(+/-7.4)
siGenome ER siRNA (stripped serum +E2)	0	117.5(+/-6.9)
On-target ER siRNA (stripped serum+E2)	0	118.3(+/-6.1)
Control (whole serum)	0	258.3(+/-6.8)
Fulvestrant (whole serum)	0	118.3(+/-5.2)
Lipid (whole serum)	0	255.8(+/-5.8)
siRNA control (whole serum)	0	255(+/-4.5)
siGenome ER siRNA	0	117.8(+/-5.2)
On-target ER siRNA	0	116.7(+/-6.1)

Table 3.6. pS2 H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA or fulvestrant exposure (n=3).

3.4. The Effect of the combination of 'pure' anti-oestrogen fulvestrant and ER siRNA treatment on MCF-7 cells.

The previous work in this project showed that fulvestrant exposure and On-target ER siRNA transfection both reduced ER protein expression to an equivalent degree, fulvestrant by facilitating degradation of the oestrogen receptor protein, and ER siRNA by initiating down regulation of ER mRNA, reducing translation of ER protein. These treatments also reduced oestrogen driven signalling and growth in MCF-7 cells, however the level of ER protein down regulation and the subsequent effects on down-stream ER signalling and growth inhibition were incomplete when each agent was used alone. Residual ER was present in both serum conditions and in the presence of exogenous E2 and exerted residual signalling and potentially growth. The studies in this next section were designed to assess whether the combination of ER siRNA and fulvestrant, targeting both ER mRNA and protein simultaneously would be a more effective anti-oestrogen strategy and eliminate expression of residual ER.

3.4.1. The effect of the combination of fulvestrant and ER siRNA on ER levels in MCF-7 cells.

The combination treatment of fulvestrant and on-target ER siRNA transfection for 4 days showed no greater down regulation of ER mRNA expression in

MCF-7 cells cultured, in either stripped or whole serum, when compared to ER siRNA transfection alone (figure 3.25.A.). MCF-7 cells treated with either scrambled siRNA, or fulvestrant and scrambled siRNA showed no reduction in ER mRNA expression.

Assessment of ER protein expression by Western blotting in MCF-7 cells cultured with either stripped or whole serum revealed a greater down regulation of total ER protein levels after a 4 day combination treatment of fulvestrant and ER siRNA (figure 3.25.B.), when compared to either fulvestrant or ER siRNA treatment alone. Residual ER protein levels were barely detectable in the combination treatment arm in both serum conditions. Extremely low levels of residual ER protein expression were detected following the 4 day fulvestrant and ER siRNA combination treatment of MCF-7 cells by immunocyto-chemical assessment under both stripped and whole serum conditions (figure 3.26.A.). The untransfected cells and the scrambled siRNA-treated MCF-7 cells cultured in either stripped of whole serum showed high levels of ER expression, with high staining intensity observed in 90% of the cell nuclei and moderate staining in the remaining nuclei (figures 3.26.B. and in table 3.7.). Regardless of culture conditions the combination treatment of fulvestrant and ER siRNA resulted in extremely low levels of nuclear ER staining with only 5-10% of cells showing any ER protein expression (see enlargements). This decrease in ER expression as determined by H-score assessment was statistically significant ($p \leq 0.05$, $n=3$) under both experimental conditions compared to untransfected MCF-7 cells. In both serum types however the combination treatment also showed a greater

ER protein down regulation compared to either fulvestrant or ER siRNA alone. The corresponding nuclear ER H-score analysis showing a significant ($p \leq 0.05$, n=3) 70% decrease in ER expression in the combination condition compared to either fulvestrant or ER siRNA treatment alone.

3.4.2. The Effect of fulvestrant and ER siRNA combination treatment on oestrogen receptor signalling.

As the combination treatment of fulvestrant and ER siRNA showed greater, (and almost complete) down regulation of ER protein expression compared to either agent alone, further studies were performed to assess whether this greater impact on nuclear ER expression had a superior inhibitory effect on ER transcriptional activity. Any transcriptional activity following combination treatment was initially investigated by measurement PR and pS2 mRNA and protein expression.

Effect of the Combination treatment on ER-regulated genes.

PR and pS2 mRNA expression.

As previously observed with each agent alone, a down regulation of both PR and pS2 mRNA expression was observed in MCF-7 cells following a 4 day combination treatment incubated with both stripped and whole serum. The

down regulation of PR at the mRNA level was not complete in the combination condition, with residual PR expression being observed (figure 3.27.A.). As with PR gene expression, down regulation of pS2 mRNA expression was also incomplete in both culture conditions indicating the possibility of residual ER transcriptional activity (figure 3.27.B.). Importantly, the combination treatment showed no greater PR or pS2 mRNA down-regulation compared to fulvestrant or ER siRNA treatment alone, despite the greater loss of residual ER previously observed.

PR protein expression.

Under both stripped and whole serum culture conditions, both cytoplasmic and nuclear PR protein expression was observed in scrambled siRNA-treated and untransfected MCF-7 cells (figure 3.28.A.), with 20% and 30% of cells showing cytoplasmic and nuclear expression, respectively, under stripped serum conditions, with the remainder of the cells being PR negative in both treatment arms. Following fulvestrant and ER siRNA combination treatment MCF-7 cells showed a reduction of PR expression, with detectable staining only observed in the cytoplasm and nucleus of 10% of the cells. Under whole serum conditions scrambled siRNA-treated and untreated MCF-7 cells showed high expression in the nucleus of 40% of the cells and moderate to low expression observed in a further 40% of cells, with no expression observed in the remaining cells. There was also high to moderate cytoplasmic PR expression in 60% of the cells with no further expression observed across the

population. The combination of fulvestrant and ER siRNA treatment significantly ($p \leq 0.05$, n=3) reduced PR protein expression, under whole serum conditions, though very low level nuclear staining was still observed in 10% the cells (figures 3.28.A. and B. and table 3.8.). Cytoplasmic staining was also completely undetectable after combination treatment (figure 3.28.A.). Corresponding nuclear PR H-score analysis showed a significant ($p \leq 0.05$, n=3) down regulation of PR expression following combination treatment, compared to untreated MCF-7 cells under stripped serum culture conditions. Under whole serum conditions however, nuclear H-score assessment revealed that there was a slightly reduced nuclear PR protein expression level when the effect of the combination treatment was compared to ER siRNA transfection alone; though this was not determined to be statistically significant. The presence of low levels of residual nuclear PR was observed under both culture conditions. There was also no significant difference in the level of nuclear PR protein expression when the effect of the combination treatment was compared to either fulvestrant expression or ER siRNA transfection alone, under both culture conditions (figure 3.28.B. and table 3.8.).

pS2 protein expression.

pS2 protein expression in MCF-7 cells cultured with stripped serum (figure 3.29.A.), was exclusively observed within the cytoplasm. Scrambled siRNA-treated and untreated MCF-7 cells showed moderate intensity staining in the cytoplasm of 20% of the population, with lower pS2 expression in the

cytoplasm of 60% of cells, the remaining cells showed no pS2 expression. The combination of fulvestrant and ER siRNA treatment caused a significant loss of cytoplasmic staining, with only low level expression present in 20% of the population. However pS2 expression levels were similar in cells treated with the combination of fulvestrant and ER siRNA and cells treated with either fulvestrant or ER siRNA alone. MCF-7 cells cultured with whole serum all showed no nuclear staining for pS2 protein (figure 3.29.A.). Untreated and scrambled siRNA transfected MCF-7 cells cultured in whole serum all showed moderate cytoplasmic staining across 50% of the population with 10% showing low staining and 40% no cytoplasmic staining. There was again a clear reduction of pS2 protein expression across all the cells following combination treatment, with only 20% of the cells demonstrating moderate to low staining with the remainder of the cells pS2 negative. pS2 expression following combination treatment was comparable to the expression levels observed after fulvestrant and ER siRNA treatment alone. Corresponding H-scores of cytoplasmic staining under both culture conditions revealed the down regulation of cytoplasmic pS2 to be significant ($p \leq 0.05$, $n=3$) following the combination treatment and again demonstrated that residual pS2 protein expression was present and that the combination was no more effective than either fulvestrant or ER siRNA treatment alone (figure 3.29.B. and table 3.9.).

ERE studies.

ERE reporter gene studies showed a statistically significant ($p \leq 0.05$, $n=3$) drop of 35% following combination treatment compared to the untransfected and scrambled siRNA-treated MCF-7 cells. Though the combination treatment showed a slightly greater down-regulation of ERE activity compared to the ER siRNA transfected cells alone, this series of experiments showed no greater inhibition of ERE activity compared to fulvestrant treatment alone in MCF-7 cells cultured with stripped serum (figure 3.30.A.), and residual ERE activity was still clearly apparent.

In the MCF-7 cells cultured with whole serum (figure 3.30.B) a similar profile was observed, the combination treatment causing a statistically significant ($p \leq 0.01$, $n=3$) reduction of 85% compared to untreated and scrambled siRNA transfected controls. Combination treatment again also demonstrated a significantly greater down-regulation of ERE activity when compared to ER siRNA transfection alone ($p \leq 0.05$, $n=3$). However, again there was no significant difference in ERE activity following the combination treatment when compared to fulvestrant treatment alone ($p > 0.05$, $n=3$), with both culture conditions showing significant residual ERE activity after combination treatment.

Oestradiol Challenge.

When MCF-7 cells grown in stripped serum were stimulated with the addition of 10^{-9}M oestradiol to the media the basal expression levels of both PR and pS2 were increased. The oestradiol-stimulated scrambled siRNA-treated and untransfected MCF-7 cells showed intense staining of PR in the nucleus of all of the cells, and high expression of PR in the cytoplasm was also observed in 70% of the cells, with less intense staining also being seen in the remainder of the cells (figure 3.31.A.). A significant ($p \leq 0.05$, n=3) down regulation of oestradiol-induced PR expression was apparent, following exposure to the combination treatment of ER siRNA and fulvestrant with only 30% of cells showing low intensity staining in both the nucleus and the cytoplasm, and 30% of cells observed being PR negative. When stimulated with oestradiol, cells cultured in stripped serum showed increased cytoplasmic levels of pS2 expression, when compared to unstimulated MCF-7 cells (previously shown in figure 3.29.). Scrambled siRNA-treated and untransfected MCF-7 cells showed intense to moderate cytoplasmic staining in 80% of cells, with detectable pS2 expression in the remainder. Oestrogen stimulated cells exposed to fulvestrant and ER siRNA in combination showed less intense cytoplasmic staining, with 10% showing no expression of pS2 at all (figure 3.31.A.). However the combination treated cells showed no greater down regulation of PR or pS2 expression than fulvestrant or ER siRNA treatment alone. Corresponding H-score analysis of nuclear PR and cytoplasmic pS2 expression both clearly showed a statistically significant ($p \leq 0.05$, n=3) down

regulation of expression following combination treatment (figure 3.31.B. and tables 3.8. and 3.9.). However both the immuno-histochemical images and the subsequent H-score analysis showed presence of residual PR and pS2 after exposure to ER siRNA and fulvestrant in combination when MCF-7 cells were oestradiol stimulated.

3.4.3. The Effect of the combination of fulvestrant and ER siRNA treatment on MCF-7 cell growth.

Although there was no greater effect on classical ER signalling following the combination treatment, the greater loss of residual ER may have a greater impact on growth and proliferation than the use of either agent alone by another mechanism. The effect of the combination treatment on IGF-1R signalling component expression, nuclear Ki67 expression, cell viability and cell number were therefore assessed in the following studies.

IGFR signalling.

Following the fulvestrant and ER siRNA combination treatment a down regulation of total and phosphorylated IGF-1R was observed when compared to scrambled siRNA-treated and untreated MCF-7 cells under both culture conditions but no greater down regulation when compared to either fulvestrant or ER siRNA treatment alone (figures 3.32.A. and B.). This inhibition was

again incomplete following combination treatment in both serum conditions. Furthermore, an incomplete inhibition of IRS1 was also observed following a 4 day combination treatment in MCF-7 cells cultured with either serum type, again there was no observable difference between the combination treatment and either fulvestrant treatment or ER siRNA transfection alone (figures 3.33.A. and B.).

Ki67 analysis.

When the effect of the combination treatment on nuclear expression levels of the proliferation marker Ki67 was examined, scrambled siRNA-treated and untreated MCF-7 cells showed a total Ki67 positivity score of 65% and 80% for the stripped and whole serum culture conditions, respectively (figures 3.33.A. and B.). ER siRNA and fulvestrant exposure in combination caused a decrease in nuclear Ki67 positivity under both culture conditions. The observed staining was less intense across the population compared to the controls, with only 25% and 30% of cells showing any presence of the antigen in the nucleus in stripped and whole serum, respectively. There was no difference in the combination treatment arm when compared with either ER siRNA or fulvestrant treatment alone. Once more, despite a clear reduction in Ki67 positive cells, some positively stained cells were still evident in the combination treated cell populations.

Growth and proliferation assays.

MTT assays revealed incomplete growth inhibition in response to the combination treatment, an 8 day exposure showed a small but significant drop in growth of 20% in stripped serum conditions, similar to that seen with fulvestrant alone and equal to siRNA transfection alone (figure 3.34.A.). There was a significant ($p \leq 0.05$) but incomplete growth inhibition of 70% in combination treated cells cultured in whole serum; this was again equivalent to fulvestrant exposure alone but significantly greater than that seen by siRNA transfection alone (figure 3.34.A.). The incomplete decrease in growth was also reflected in total cell number observed following analysis by Coulter counter. Under stripped serum culture conditions a significant ($p \leq 0.05$) decrease of 50% in total cell number was observed following exposure to the combination treatment (figure 3.34.B.). Under whole serum culture conditions studies showed a statistically significant ($p \leq 0.01$) decrease in total cell number of 60% in response to combination treatment, significantly greater than ER siRNA alone (figure 3.34.B.). Both culture conditions showed residual growth after combined fulvestrant and ER siRNA exposure with the combination treatment providing no greater growth inhibition compared to fulvestrant exposure alone. However the combination treatment showed greater growth inhibition when compared to ER siRNA transfection alone under both culture conditions.

3.4.4. Combination treatment of ER siRNA and fulvestrant effect on the EGFR signalling in MCF-7 cells.

The apparent lack of further growth inhibition in the combination condition could also possibly be explained by an increase in compensatory signalling through the epidermal growth factor receptor pathway (EGFR) on ER blockade, so the effect of the combination condition on components of EGFR signalling pathway were assessed by Western blotting.

Basal levels of EGFR and HER2 expression in MCF-7 cells were low and difficult to detect, however a combination treatment of ER siRNA and fulvestrant treatment showed no increase in phosphorylated EGFR expression in either serum condition (figure 3.35.A.). Interestingly however, there was an observable increase in total EGFR at this early time point, however this was very slight and a similar increase was also observed following fulvestrant treatment as well under stripped serum conditions. This was also the case under whole serum conditions though ER siRNA transfection alone was also observed to induce increased Total EGFR expression, though again this was slight. Furthermore, there was also evidence of slight induction of Total HER2 expression in the combination condition, above fulvestrant treatment alone, though this was only observed in the stripped serum cultured MCF-7 cells, but together indicates the possibility of induction of growth factor signalling in response to severe ER down regulation, albeit not in general exceeding that observed following fulvestrant treatment alone in this study.

ER level expression in MCF-7 cells cultured in stripped and whole serum, treated with a combination of fulvestrant and ER siRNA.

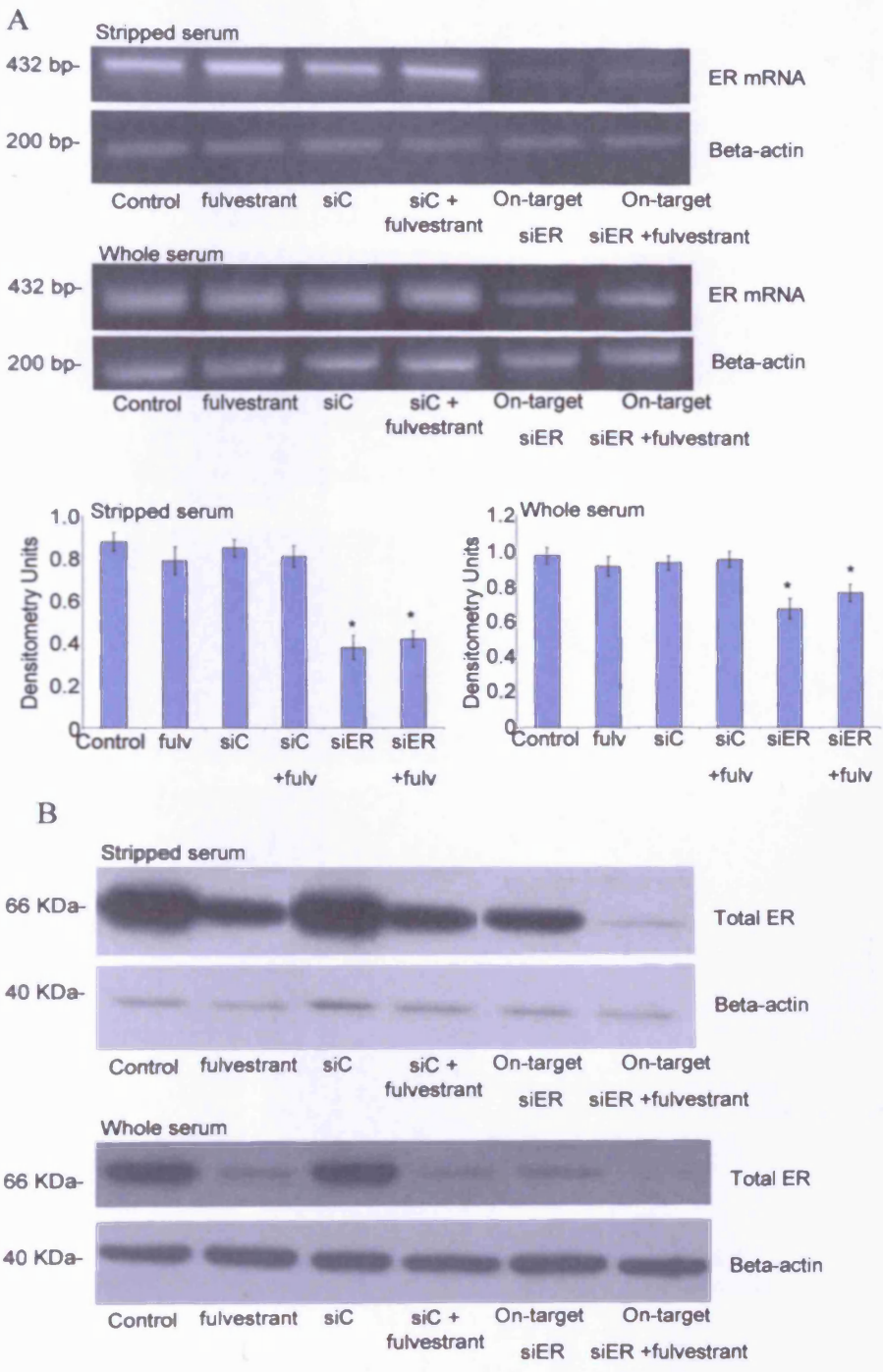


Figure 3.25.(A) Expression of ERα and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant or vehicle control. on day 4 from initial treatment and actin normalised densitometry. (B) Western analysis of ERα and β-actin expression in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant or vehicle control, on day 4 from initial treatment. N.B. . = p≤0.05 vs siControl or siControl +fulv for siER and siER +fulv respectively.*

ER protein expression in MCF-7 cells cultured in stripped or whole serum, treated with a combination of fulvestrant and ER siRNA.

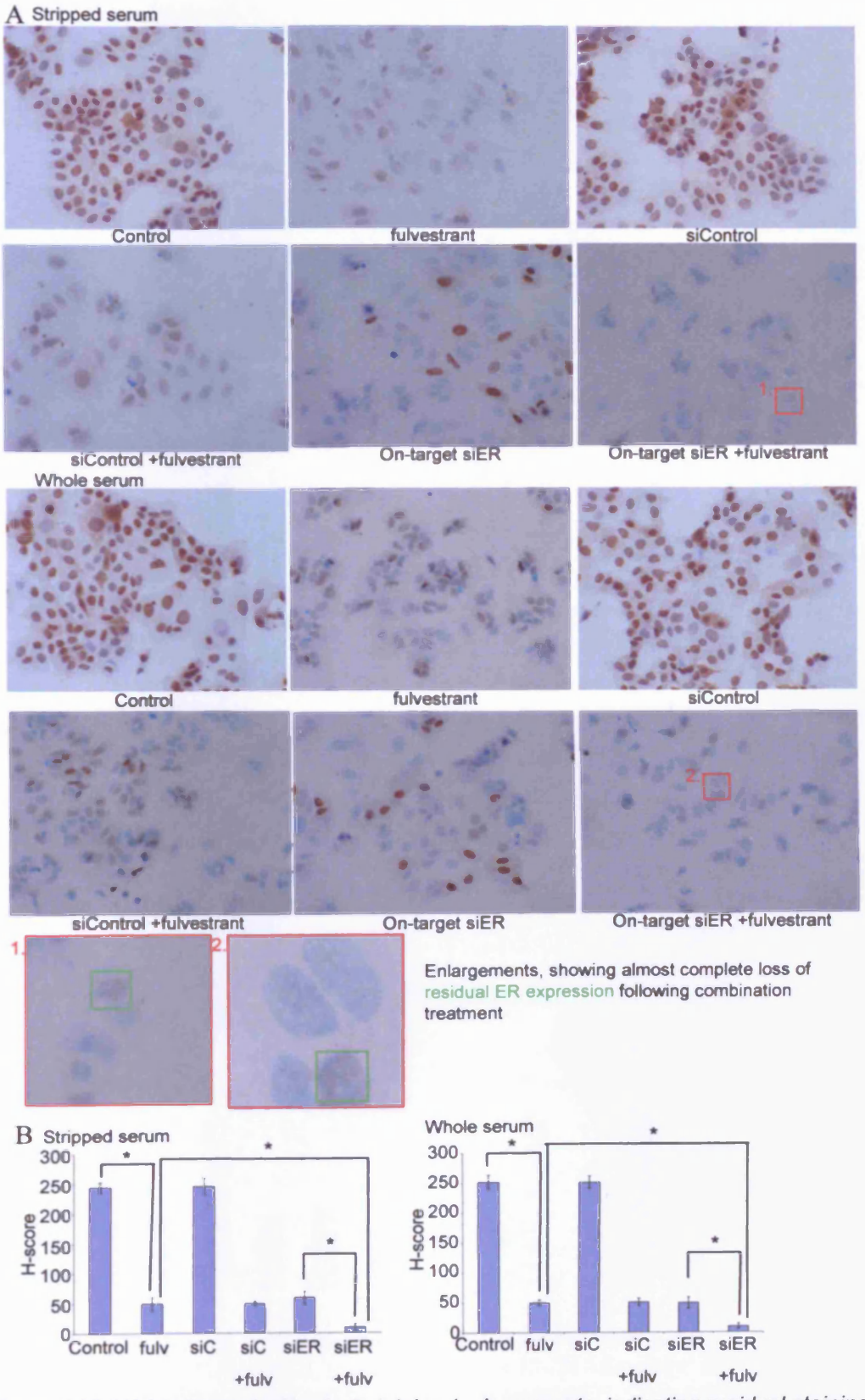


*Figure 3.26.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average nuclear ER H scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 indicated by lines.*

PR and pS2 mRNA expression in MCF-7 cells cultured in either stripped or whole serum, and treated with a combination of fulvestrant and ER siRNA.

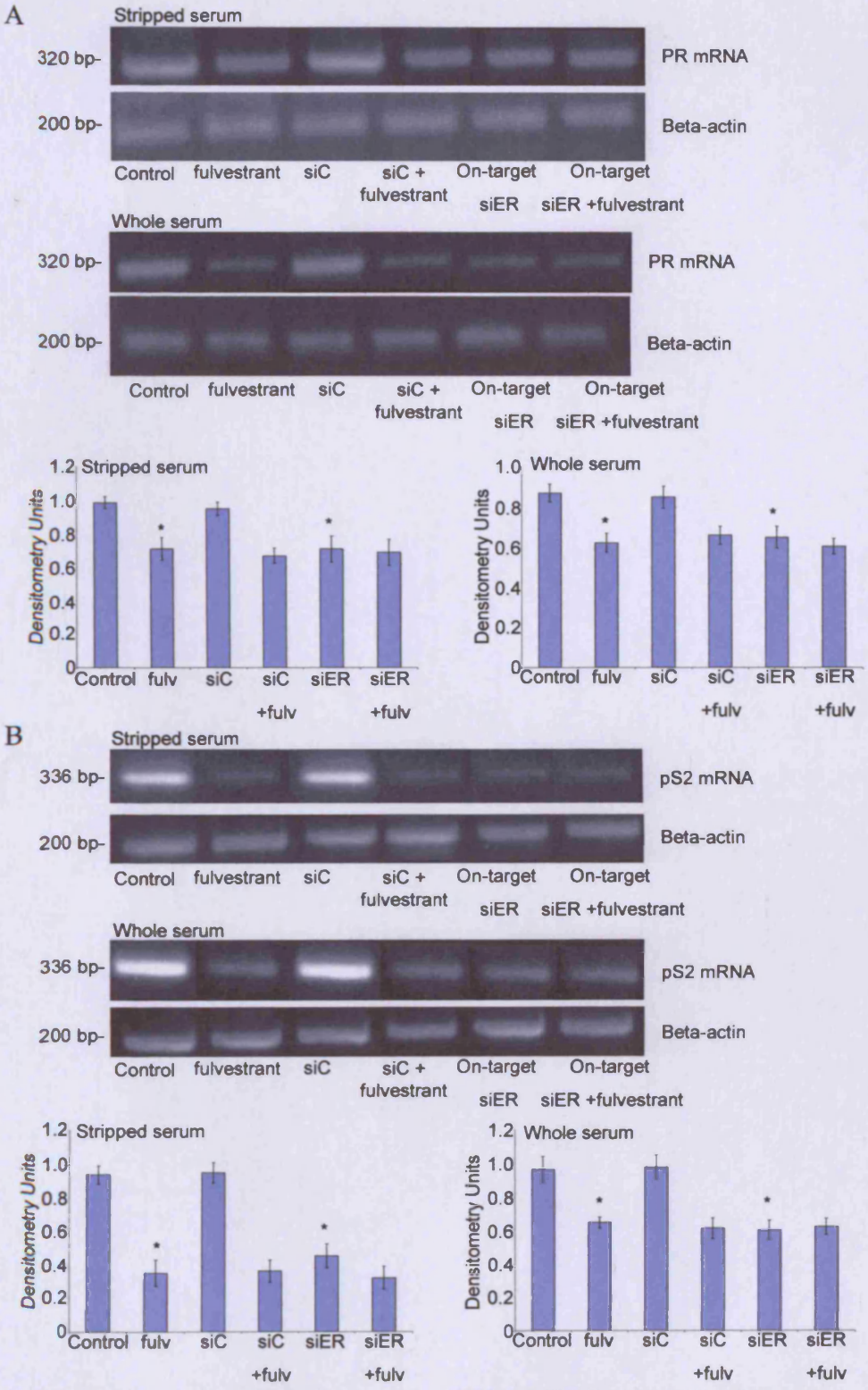


Figure 3.27.(A) Expression of PR and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant or vehicle control on day 4 from initial treatment and actin normalised densitometry. (B) Expression of pS2 and β-actin mRNA in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, 2nM lipid, scrambled siRNA control, siGenome siER, On-target siER or vehicle control on day 4 from initial treatment and actin normalised densitometry. N.B. . = p≤0.05 vs control or siControl or siControl +fulv for fulv, siER and siER +fulv respectively.*

PR protein expression in MCF-7 cells cultured in stripped serum or whole serum, and treated with a combination of fulvestrant and ER siRNA.

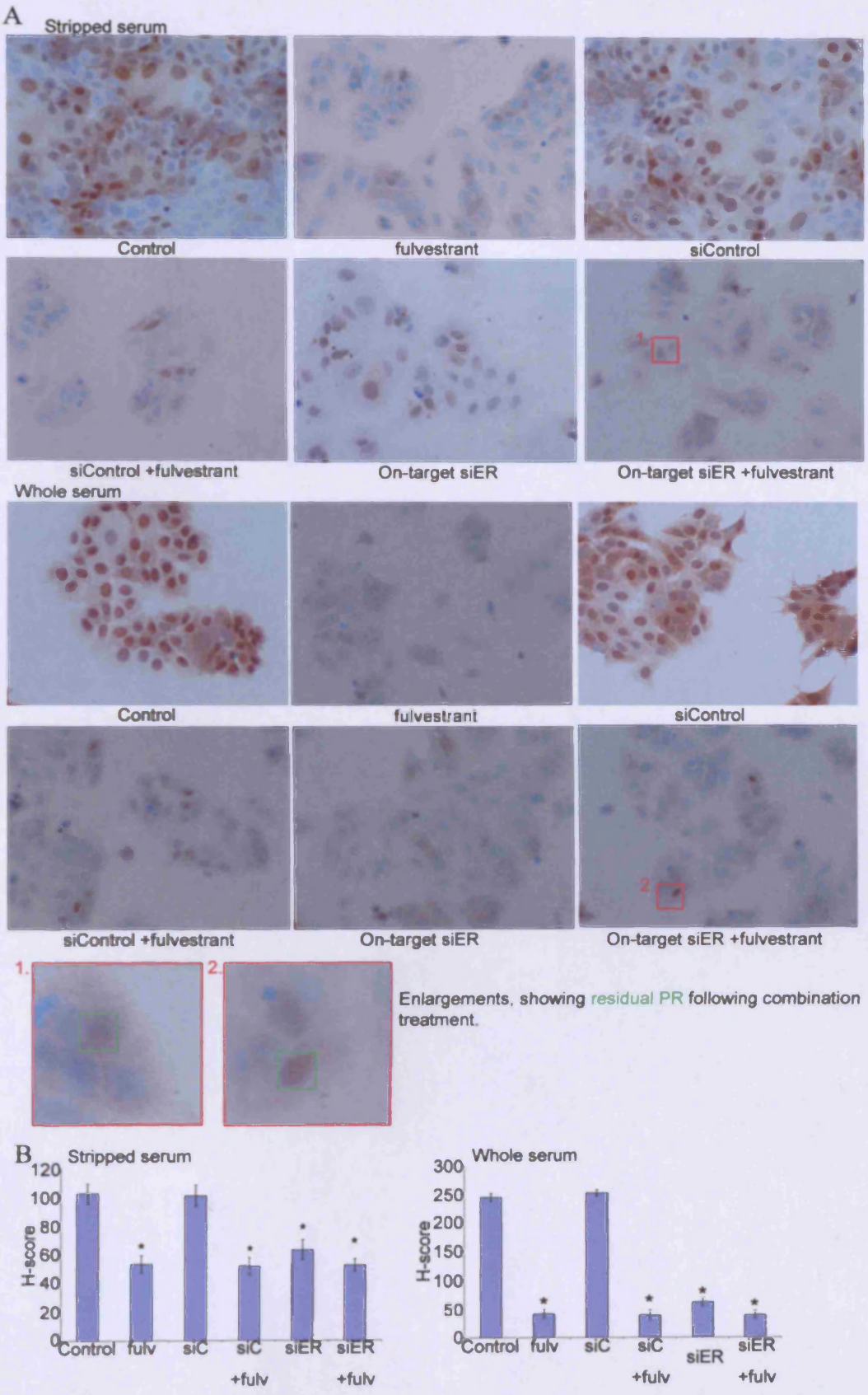


Figure 3.28.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average nuclear PR H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs control or siC for fulv and siER respectively.*

pS2 protein expression in MCF-7 cells cultured in stripped serum or whole serum, and treated with a combination of fulvestrant and ER siRNA.

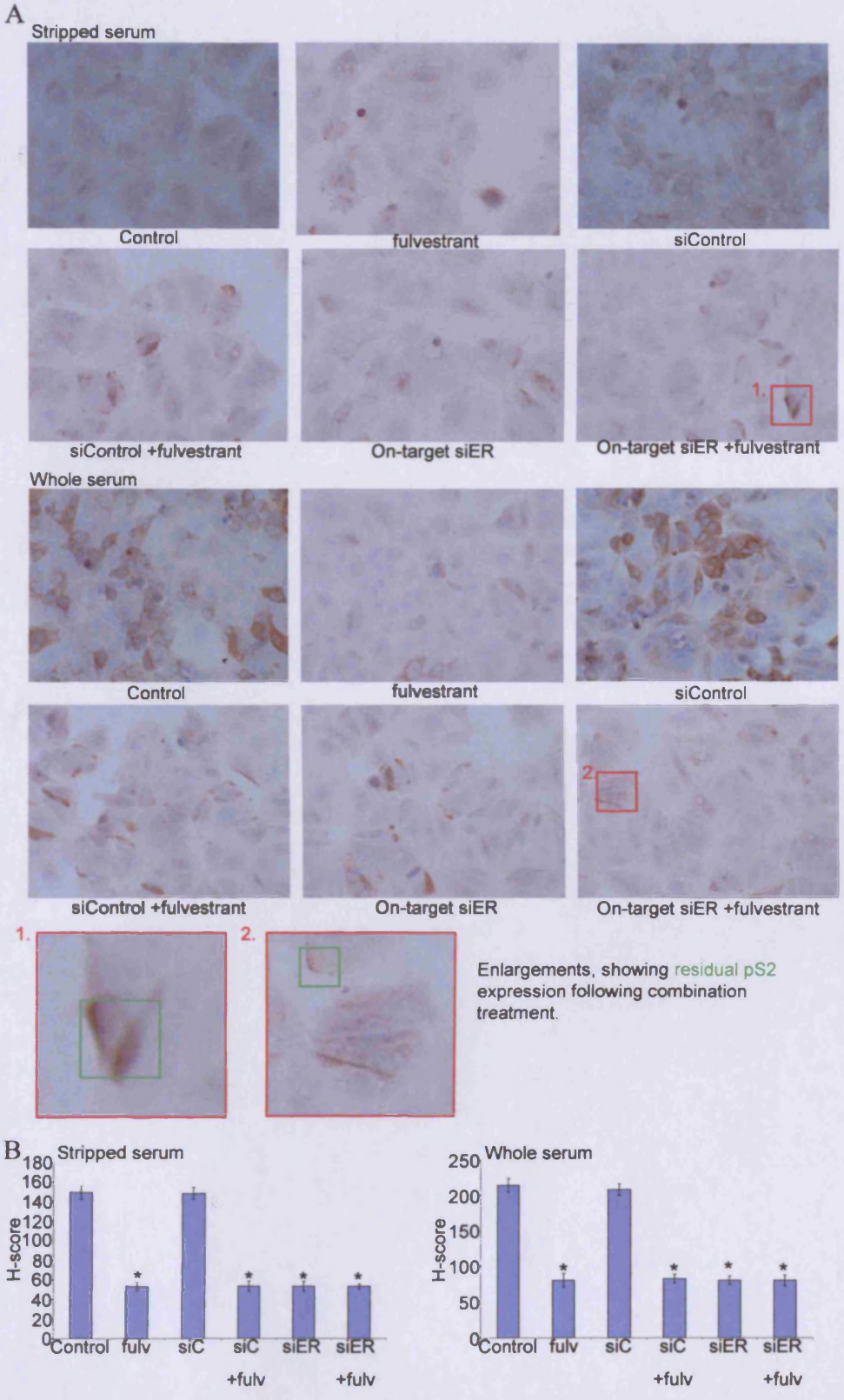


Figure 3.29.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 and (B) average cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs control or siC for fulv and siER respectively.*

Level of ERE signalling in MCF-7 cells cultured in stripped or whole serum, treated with a combination of fulvestrant and ER siRNA.

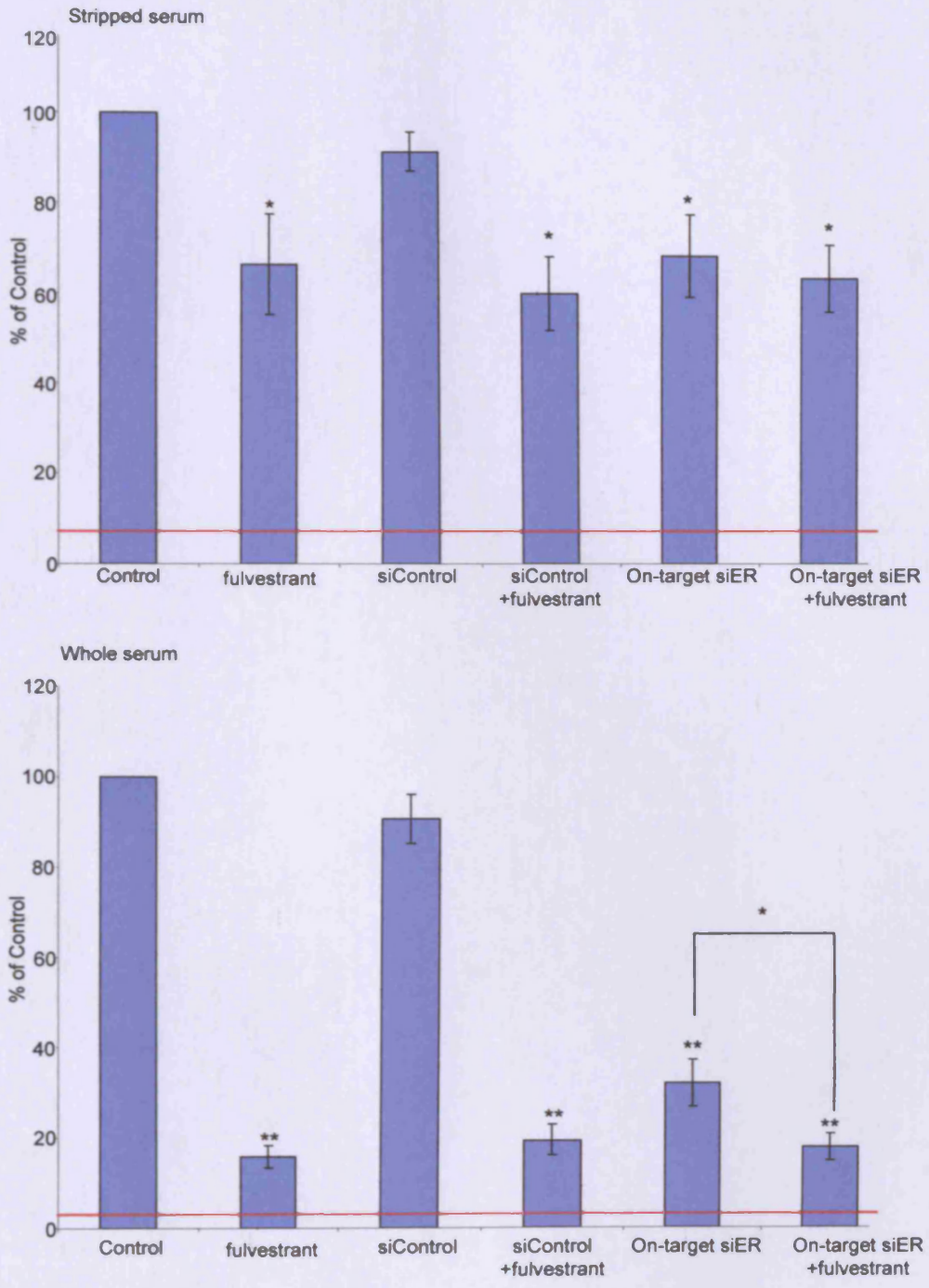


Figure 3.30. Reporter gene assay showing average ERE expression (n=3) in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment; with background fluorescence indicated by red line (n=3). N.B. *= p≤0.05 **= p≤0.01 vs control or siControl for fulv and siER respectively or indicated by lines.*

PR and pS2 expression of MCF-7 cells cultured in stripped serum supplemented with oestradiol and treated with a combination of fulvestrant and ER siRNA.

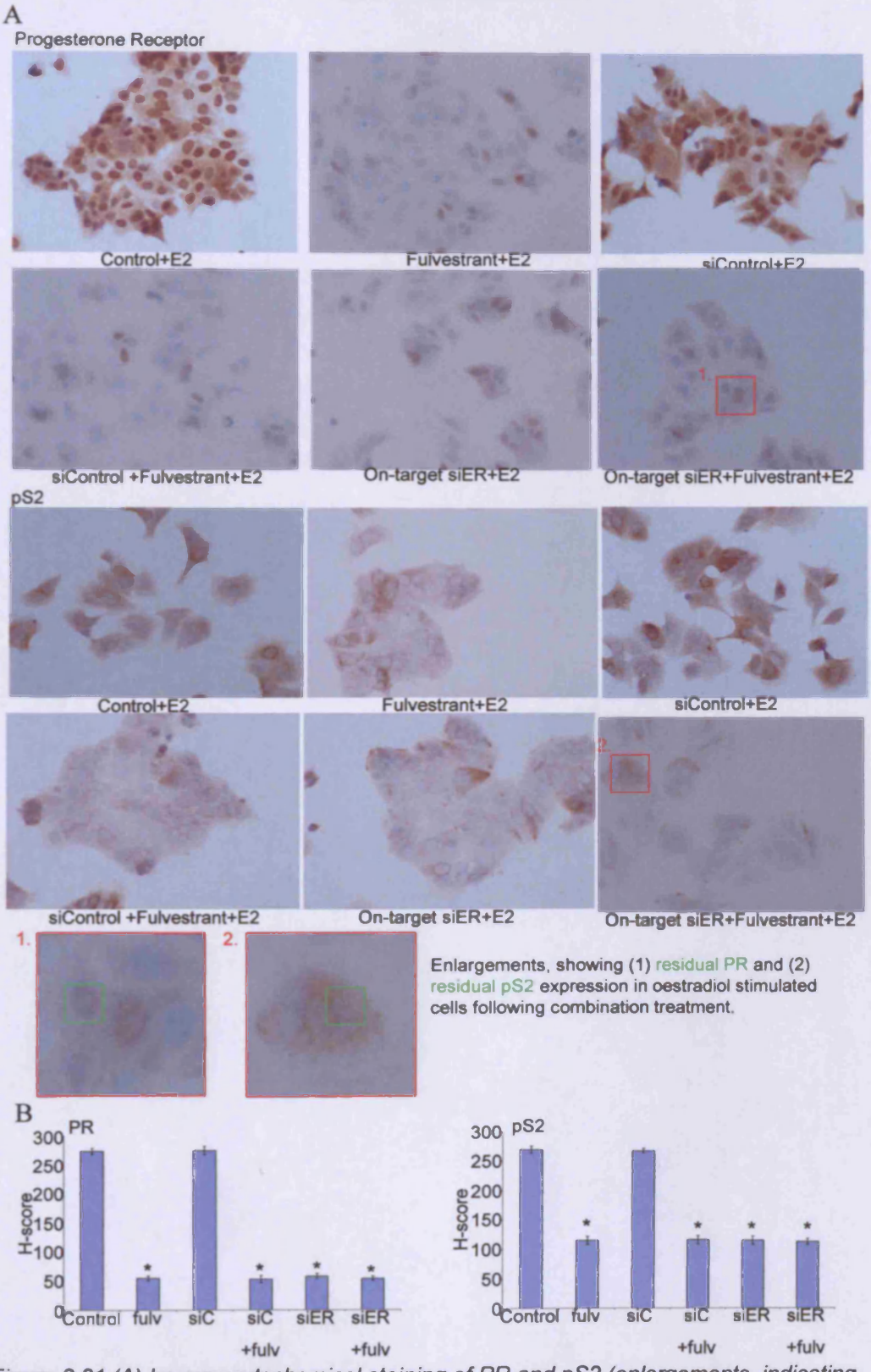


Figure 3.31.(A) Immunocytochemical staining of PR and pS2 (enlargements, indicating residual staining in green) magnification x20 and (B) average nuclear PR and cytoplasmic pS2 H-scores (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS and 10-9M oestradiol and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs Control +E2 or siC +E2 for fulv+E2 or siER+E2 respectively.*

The effect of the combination of fulvestrant and ER siRNA on IGFR signalling in MCF-7 cells, cultured in stripped or whole serum.

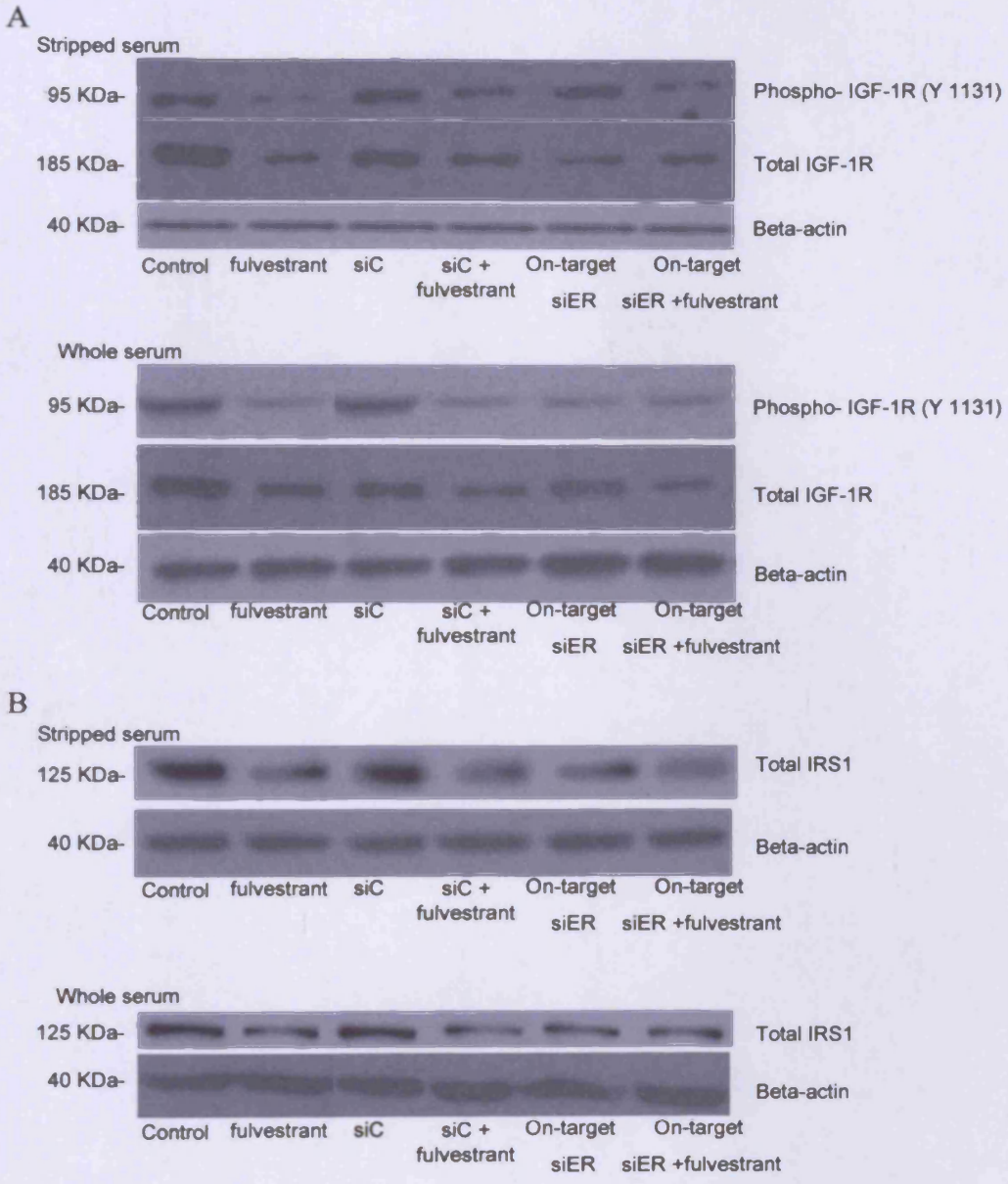


Figure 3.32.(A) Western blotting analysis of Total IGFR, Phospho-IGFR and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment. (B) Western blotting analysis of Total IRS1 and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER* and fulvestrant *or vehicle control on day 4 from initial treatment.*

Ki67 expression of MCF-7 cells cultured in stripped or whole serum, treated with a combination of fulvestrant and ER siRNA.

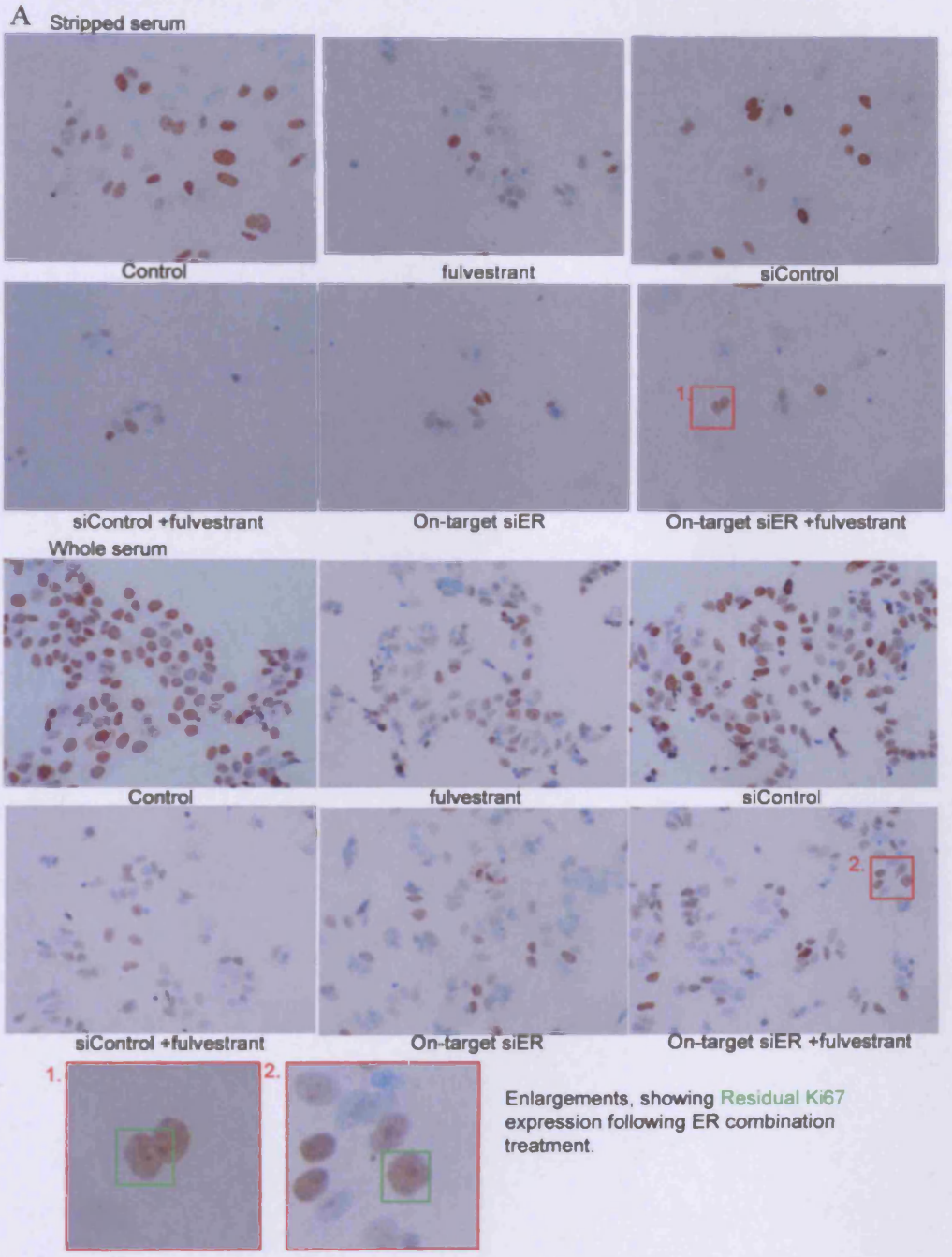


Figure 3.33.(A) Immunocytochemical staining (enlargements, indicating residual staining in green) magnification x20 *and (B) average total Ki67 positivity (n=3) of MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER* and fulvestrant *or vehicle control, on day 4 from initial treatment. N.B. *= P≤0.05 vs control or siC for fulv and siER respectively.*

The effect of the combination of fulvestrant and ER siRNA on growth of MCF-7 cells cultured in stripped or whole serum.

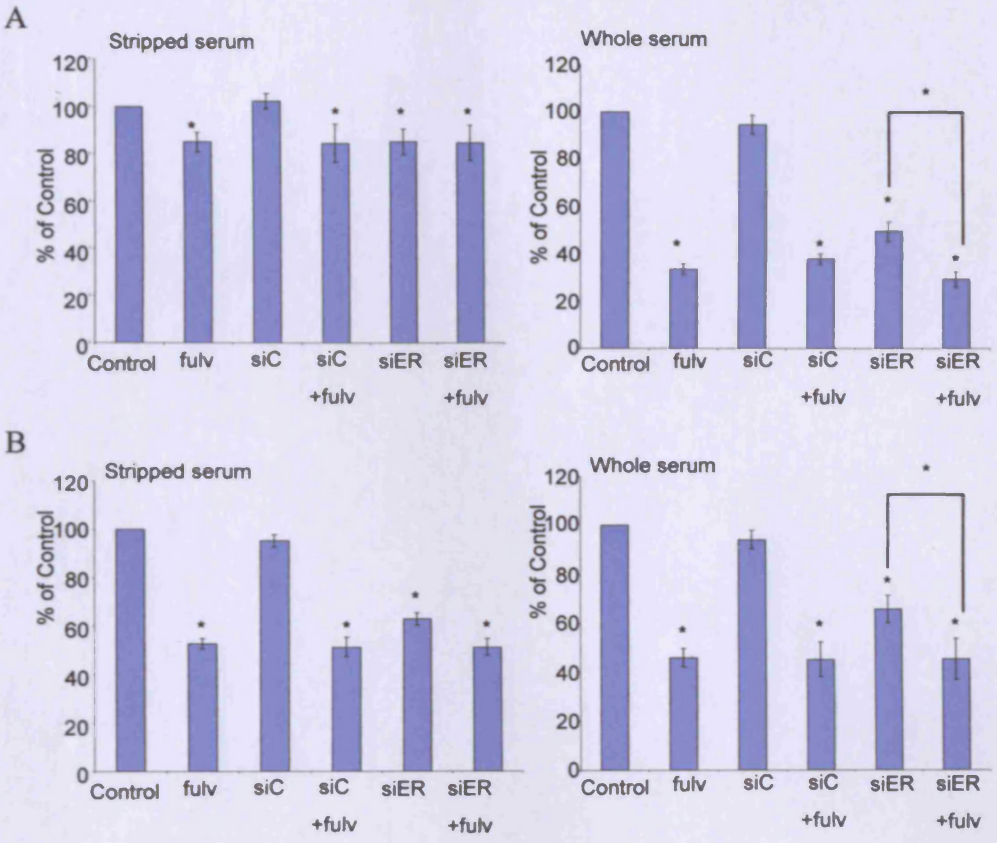


Figure 3.34.(A) Effect on basal MCF-7 cell growth, cultured in phenol-red free RPMI containing 5% SFCS or FCS *treated with either 10-7M fulvestrant, scrambled siRNA control, scrambled siRNA control and fulvestrant, On-target siER, On-target siER* and fulvestrant *or vehicle control on day 8 from initial treatment and represented as percentage of vehicle control after MTT assessment (n=3) (B) or Coulter counting (n=3). N.B. .* = p≤0.05, ** = p≤0.01 vs control or siC for fulv and siER respectively or indicated by lines.*

The effect of the combination of fulvestrant and ER siRNA on EGFR signalling in MCF-7 cells, cultured in stripped and whole serum.

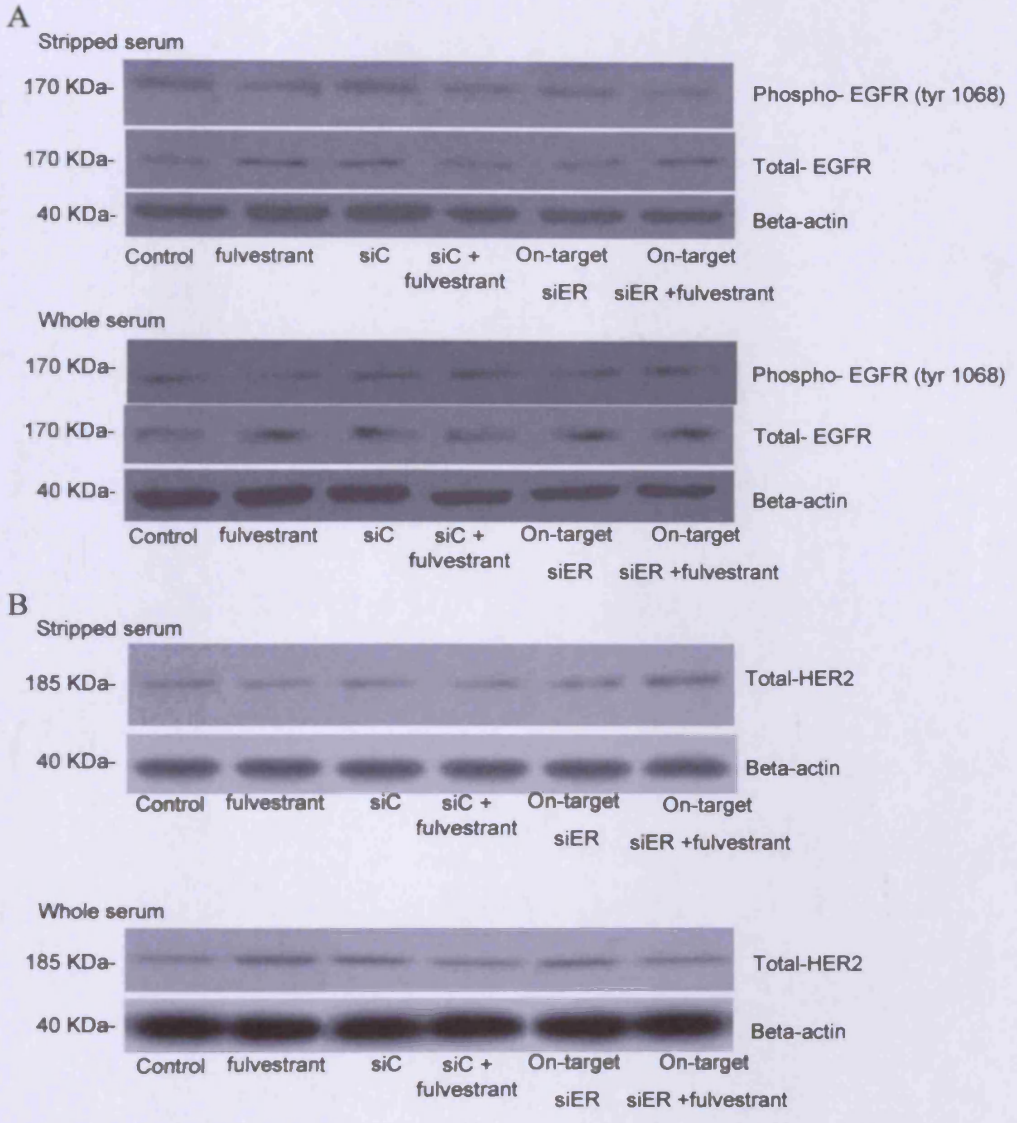


Figure 3.35.(A) Western blotting analysis of Total EGFR, Phospho-EGFR and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS and treated with either treated with either 10-7M fulvestrant, Scramble siRNA control, Scramble siRNA control and fulvestrant, On-target siER, On-target siER and fulvestrant *or vehicle control, on day 4 from initial treatment. (B) Western blotting analysis of Total HER2 and β-actin protein in MCF-7 cells cultured in phenol-red free RPMI containing 5% SFCS or FCS, treated with either 10-7M fulvestrant, Scramble siRNA control, Scramble siRNA control and fulvestrant, On-target siER, On-target siER* and fulvestrant *or vehicle control on day 4 from initial treatment.*

ER H-score analysis of MCF-7 cells following combination treatment.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum)	244.2(+/-8.6)	0
Fulvestrant (stripped serum)	49.2(+/-11.1)	0
siRNA control (stripped serum)	245.8(+/-13.9)	0
siRNA control+fulv (stripped)	49.2(+/-3.8)	0
On-target ER siRNA (stripped serum)	59.2(+/-10.7)	0
Combination (stripped serum)	9.2(+/-5.8)	0
Control (whole serum)	251.7(+/-11.7)	0
Fulvestrant (whole serum)	49.2(+/-4.9)	0
siRNA control (whole serum)	250.8(+/-10.2)	0
siRNA control+fulv (whole serum)	50.8(+/-5.8)	0
On-target ER siRNA	49.2(+/-9.7)	0
Combination (whole serum)	10(+/-4.5)	0

Table 3.7. ER H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA transfection and fulvestrant exposure (n=3).

PR H-score analysis of MCF-7 cells following combination treatment.

	Nuclear H-score	Cytoplasmic H-score	Total H-score
Control (stripped serum)	102.5(+/-6.9)	28.6(+/-5.3)	112.5(+/-6.6)
Fulvestrant (stripped serum)	53.3(+/-6.1)	10.4(+/-2.1)	55.6(+/-4.4)
siRNA control (stripped serum)	100.8(+/-7.4)	25.2(+/-7.2)	113.4(+/-6.2)
siRNA control+fulv(stripped serum)	51.7(+/-6.1)	10.3(+/-5.2)	54.2(+/-6.9)
On-target ER siRNA (stripped serum)	53.3(+/-6.8)	11.7(+/-3.8)	55.9(+/-7.2)
Combination (stripped serum)	52.5(+/-4.2)	10.9(+/-5.8)	56.1(+/-2.1)
Control (stripped serum +E2)	273.3(+/-5.2)	50.7(+/-13.5)	274.8(+/-9.4)
Fulvestrant (stripped serum +E2)	55(+/-4.5)	22(+/-3.1)	66(+/-7.3)
siRNA control (stripped serum+E2)	275(+/-7.1)	57.7(+/-14.6)	273.2(+/-8.9)
siRNA control +Fulv(stripped serum+E2)	53.3(+/-6.1)	21.9(+/-4.2)	68.4(+/-6.2)
On-target ER siRNA (stripped serum+E2)	58.3(+/-4.1)	22.2(+/-4.5)	67.6(+/-8.1)
Combination (stripped serum+E2)	54.2(+/-3.8)	23.2(+/-5.7)	68.2(+/-7.2)
Control (whole serum)	244.2(+/-6.6)	157(+/-11.6)	253.6(+/-8.8)
Fulvestrant (whole serum)	41.7(+/-8.6)	10.3(+/-2.4)	39.7(+/-7.2)
siRNA control (whole serum)	251.7(+/-5.2)	156.6.(+/-9.9)	257.3(+/-10.2)
siRNA control +fulv (whole serum)	39.2(+/-8.6)	12.2(+/-4.2)	40.8(+/-8.6)
On-target ER siRNA (whole serum)	61.7(+/-6.8)	11.6(+/-4.3)	39.4(+/-6.8)
Combination (whole serum)	39.2(+/-6.6)	10.8(+/-5.6)	41.5(+/-9.1)

Table 3.8. PR H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA and fulvestrant exposure (n=3).

pS2 H-score analysis of MCF-7 cells following combination treatment.

	Nuclear H-score	Cytoplasmic H-score
Control (stripped serum)	0	149.2(+/-6.6)
Fulvestrant (stripped serum)	0	53.3(+/-4.1)
siRNA control (stripped serum)	0	148.3(+/-6.1)
siRNA control +fulv (stripped serum)	0	53.3(+/-2.2)
On-target ER siRNA (stripped serum)	0	53.2(+/-5.2)
Combination (stripped serum)	0	53.3(+/-2.6)
Control (stripped serum +E2)	0	269.2(+/-5.8)
Fulvestrant (stripped serum +E2)	0	115(+/-7.1)
siRNA control (stripped serum+E2)	0	266.7(+/-4.1)
siRNA control+fulv (stripped serum+E2)	0	115.8(+/-7.4)
On-target ER siRNA (stripped serum+E2)	0	115(+/-7.1)
Combination(stripped serum+E2)	0	112.5(+/-5.2)
Control (whole serum)	0	214.2(+/-9.7)
Fulvestrant (whole serum)	0	80.8(+/-9.7)
siRNA control (whole serum)	0	208.3(+/-8.2)
siRNA control +fulv (whole serum)	0	83.3(+/-6.1)
On-target ER siRNA (whole serum)	0	80.8(+/-5.8)
Combination (whole serum)	0	80.8(+/-7.4)

Table 3.9. pS2 H-score analysis of MCF-7 cells grown in stripped or whole serum after 4 days ER siRNA and fulvestrant exposure (n=3).

Chapter 4. Conclusions and Discussion Section.

The majority of breast cancers and breast cancer cell models typically express tens of thousands of ER proteins per cell at any one time; these act to coordinate the growth and survival signalling of these cells, which are mediated by oestrogen (Bundred 2001 and Veronesi *et al* 2005). This involves actions both at the cell membrane/cytoplasm and the nucleus, where they enhance growth factor signalling and gene transcription respectively (Osborne *et al* 2005). However, while much detail is understood about the molecular actions of E2/ER complexes (Howell 2006), many fundamental issues involved in their signalling remain poorly understood; critically within this thesis, how many of the thousands of ER present within the breast cancer cells are required to mediate the full anti-tumour actions of anti-hormone drugs and to what degree does it matter if the ER is not completely ablated by the treatment. Indeed, it is currently unknown whether complete loss of all cellular ER could lead to more effective therapies and a delay or even prevention of the development of an acquired resistance phenotype. Currently, only one anti-hormonal drug, fulvestrant, is registered for use as an ER down-regulator and clinically it achieves only an approximate 50% ER protein knockdown, with numbers of tumour remissions approximately equal to those achieved by other anti-hormone therapies. With respect to this, the current project set out to illuminate the relationship between the capacity of fulvestrant to promote growth inhibition in MCF-7 cells (an ER positive breast cancer cell line, commonly used to investigate hormone and anti-hormone actions in oestrogen responsive cancers) and its ability to reduce ER levels and subsequent signalling activity. These studies were then compared with the more modern

method of RNAi to promote ER down-regulation, and finally the two methods were employed in combination to investigate whether residual ER remaining in the breast cancer cells following each treatment was at biologically active levels and therefore potentially able to contribute to subsequent acquired resistance mechanisms. As the combination treatment effectively reduced the ER to undetectable levels, this study effectively answers the issue of whether a complete ER down-regulation is a desirable clinical goal.

4.1. The effect of the pure anti-oestrogen fulvestrant on ER expression and activity in MCF-7 cells.

4.1.1. Acute fulvestrant exposure promotes ER down regulation but not complete ER loss.

Initial studies presented in this thesis focused on the ability of fulvestrant to impact on ER expression levels and activity in ER-positive MCF-7 cells under differing culture conditions. In broad terms, at a concentration of 10^{-7}M fulvestrant this was a highly effective ER down-regulator. This concentration reduced ER levels by upwards of 80%, a value comparable to that achieved in other studies using this pure anti-oestrogen (Lykkesfeldt *et al* 1994, Nicholson *et al* 1996, McClelland *et al* 1996, McClelland *et al* 2001, Pink and Jordan 1996 and Staka *et al* 2005). The residual ER staining remaining with 10-7M fulvestrant following ICC and Western blotting techniques used in this project

was believed to be residual ERα and not non-specific binding, an artefact of the detection assays used, or indeed ERβ due to residual ERα present following fulvestrant exposure in the literature (McClelland *et al* 2001) and also that further reduction of this residual staining was achieved following a combination of specific ERα inhibition using the ER siRNA. The fulvestrant effect was achieved at 96 hours (day 4), a time point which, although shorter than usually employed by others, enabled a direct comparison to be made with the siRNA studies (later discussed in part 4.2.). Importantly, the capacity of fulvestrant to significantly down-regulate ER levels by day 4 is not surprising since it has been calculated that fulvestrant is able to reduce the half-life of the ER protein from 5 hours to less than an hour (Long and Nephew 2006). Theoretically, therefore, a 95% loss of ER could occur within the first 5 hours of fulvestrant treatment, providing no further ER was produced by the cell. The observation of unchanged ER mRNA levels following 4 days fulvestrant exposure, however, suggests that ER mRNA transcription and translation continues during early treatment presumably to, in part, counteract the capacity of fulvestrant to fully eliminate all cellular ER. It is of note that the capacity of fulvestrant to reduce ER protein within cells:

1. Does not increase if higher concentrations are used, as no further ER loss was observed at 10^{-6}M fulvestrant, suggesting that maximum rates of ER degradation have been achieved.
2. Does not significantly alter by day 8, or as cells become resistant to fulvestrant (FasMCF) (McClelland *et al* 2001), and the levels of residual ER remain at approximately 20% of control levels.

3. Occurs in other ER-positive breast cancer cell lines, giving a 65-85% down-regulation in ER indicating that ER down-regulation by fulvestrant is not cell line specific.
4. Is independent of serum or E2 levels *in vitro*, when fulvestrant is used at the 10^{-7}M concentration.
5. Parallels in part the effect achieved by E2, which reduces ER half-life to roughly 3 hours by facilitating ER ubiquitination and degradation via the ubiquitin-proteasome pathway, although the effects of fulvestrant are more pronounced than the 50% ER down-regulation usually seen following E2 exposure (Laïos *et al* 2005).

In total, the above data suggests that although fulvestrant is a more efficient ER down-regulator than E2, it is unable as a single agent to completely reduce ERα levels within breast cancer cells *in vitro*. The inability of fulvestrant to completely down-regulate all ER protein may be due that maximal activity of the ubiquitination proteosome pathway responsible for degradation of ER protein has been reached, another potential explanation could be that some ER is bound in multi-protein complexed 'signalsomes' preventing ubiquitinisation.

4.1.2. The effect of fulvestrant on E2-regulated transcription and residual ER activity.

The ability of fulvestrant to act as an ER down-regulator within breast cancer cells further implies that it would correspondingly reduce subsequent ER signalling, leading to anti-tumour actions. Indeed, as fulvestrant is defined as a pure anti-oestrogen, lacking oestrogen-like activity, it is suggested that it has a further property by producing biologically inactive ER complexes and out competing E2 binding to the ER (Howell *et al* 2000). Further corroborating this data, fulvestrant exposure in the present study was shown to reduce the expression of two well established E2 regulated genes, PR and pS2 and ER regulated growth relevant genes IGF-1R and IRS1, at both the mRNA and protein level as well as causing a significant reduction in the activity of an ERE construct transiently transfected into fulvestrant treated MCF-7 cells. Importantly however, while the effects of fulvestrant on these endpoints were considerable, and were consistent with the established literature both *in vitro* (Howell *et al* 2000, McClelland *et al* 1996) and *in vivo* investigations (Osborne *et al* 1994, 1995), as well as the data generated in limited clinical studies (Robertson *et al* 2001), it is noteworthy that, in all instances, the knockdown of E2 regulated events by fulvestrant was incomplete. Thus following fulvestrant treatment of MCF-7 cells, at a concentration which produces maximum ER down-regulation, limited PR and pS2 expression was still evident at approximately 30 and 25% of their initial values respectively.

Unfortunately, explanations for these observations are numerous and could, for example, result from the activation of the many growth factor driven response elements which are known to be present in the promoters of various E2-regulated genes, indeed, residual production of PR following ER degradation and inactivation could potentially result from growth factor sensitive SP1 sites present in its promoter sequence (Björnstorm and Sjöberg 2005). Continuing residual IGF-1R expression (another E2-regulated gene), may result from non-classical genomic activation by ER tethering to TPA and NF-κB response elements in the presence of the anti-oestrogen (Nicholson *et al* 2002, Osborne *et al* 2005). Alternatively non-genomic ER signalling in the presence of anti-hormones has been described (Levin 2005, Song and Santen 2006) and has shown this can lead to residual IGF-1R expression and activity at the cell membrane, also involving residual IRS-1 expression (Lee *et al* 1999, Stewart *et al* 1990).

Importantly, studies from the Tenovus Centre for Cancer Research have also identified a further mechanism which may contribute to growth factor driven residual ER signalling which directly results from the capacity of oestrogens to suppress the expression of a number of growth factor signalling elements, most notably members of the erbB receptor family (Hutcheson *et al* 2003, Britton *et al* 2006). Several studies have described the reversal of the suppression of these genes following anti-hormone exposure, thereby enabling alternative compensatory signalling to occur during the anti-hormone responsive phase to allow initial promotion of cell survival and eventually resistant growth (Nicholson *et al* 2004). Interestingly, these events are able to

promote cell growth and survival in either an ER dependant or independent fashion depending on the strength of the growth factor signalling (Nicholson *et al* 2004). Thus, at moderate levels of growth factor receptor activation (primarily EGFR and HER2), such as is present in cells with acquired resistance to tamoxifen, ER can be activated by growth factor signalling elements, including AKT and ERK (Staka *et al* 2005, Nicholson *et al* 2004, Knowlden *et al* 2003) through the phosphorylation of the ER and its co-activators (Nicholson and Johnston 2005). However, at a higher level of receptor activation the growth factor driven responses are so strong that they proceed in an ER independent manner (Sonne-Hansen *et al* 2010). Within the context of this thesis, therefore, the incapacity of fulvestrant to apparently block all ER signalling may result from growth factor driven ER activation or growth factor promoted expression of ER regulated genes through alternative signalling elements and pathways. Interestingly, this project showed some evidence of induction of growth factor signalling pathways when EGFR and HER2 were assessed following fulvestrant exposure.

Importantly, although a further explanation for the above could be in an inability of fulvestrant to saturate, and thus occupy all the ER and fully prevent all ER/E2 binding, however this explanation is unlikely as the concentration of fulvestrant used throughout this thesis was shown to fully block cellular actions of exogenous E2 in MCF-7 cells, following E2 stimulation studies.

4.1.3. Comparison of the effects of fulvestrant on tumour growth and ER down-regulation.

As expected with MCF-7 cell growth being primarily driven by oestrogens, the significant loss of ER protein expression levels and subsequent signalling following fulvestrant treatment was associated with a potent growth inhibitory effect of the anti-hormone as determined by MTT and Coulter counter assays, and ICC assessment of Ki67 expression levels. Critically however, once again the effect of fulvestrant was incomplete, and limited cells growth was observed at 10^{-7}M fulvestrant. These data, once again are consistent with the literature and lead to the development of a fully fulvestrant resistant phenotype within 10-12 weeks (McClelland *et al* 2001).

Importantly, the incomplete growth inhibitory capacity of fulvestrant in MCF-7 cells was mirrored in the other ER positive breast cancer cell lines investigated in this study and is entirely consistent with the reported effects of this anti-hormone in the literature in several other *in vitro* breast cancer cell lines (Wakeling and Bowler 1987), *in vivo* cell models (Wakeling *et al* 1991, Osborne *et al* 1995), and in clinical material when fulvestrant was used either as a first-line therapy (Howell 2006, Robertson 2007) or following relapse from previous endocrine therapy (Howell *et al* 2002, Ingle *et al* 2006). The similar ER protein down-regulation across the ER positive cell-lines against the markedly different growth inhibition effects following fulvestrant treatment shows the quantity of mechanistic response (ER protein down-

regulation) does not always equate to quality of growth inhibitory response, something further proven by the combination of siRNA and fulvestrant, which completely removed ER protein but still showed residual growth equal to that following fulvestrant treatment alone. This suggests factors other than ER inhibition may determine the effectiveness of antihormone response. This may potentially be a consequence of induction of compensatory growth signalling pathways (de-repression of oestrogen suppressed genes such as EGFR or HER2) or as a consequence of the inherent genotype of the cell lines conferring partial *de novo* resistance. For example, the BT-474 cell line has higher expression of HER2 and may be more adapted to grow via growth factor signalling than the MCF-7 cell line, potentially explaining the differing fulvestrant induced anti-tumour effect shown in this project.

4.2. The use of ER siRNA to reduce ER levels and its value as an anti-tumour therapeutic strategy.

Though siRNA is now routinely used in many laboratories, optimisation for its successful application is required in most cases. After initial issues with the toxicity profile caused by the concentrations of transfection lipid used, the system was optimised for MCF-7 cells with the use of a substantially reduced concentration of Dharmafect #1 transfection lipid (2nM) and a pooled ER siRNA. The ER siRNA pool provided the greatest ER protein knockdown and growth inhibition when compared to any of the individual constructs alone.

Greater knockdown with use of a pool/mix of ER siRNA in MCF-7 cells has also been shown by Bouclier (Bouclier *et al* 2008), who arrived at the use of a mixture of two siRNA sequences to halve the off-target effects occurring by random sequence complementation between unrelated mRNA and the siRNA used.

A few recently published studies have also utilised ER siRNA within breast cancer cell lines. However, reported ER siRNA use has been in the main utilised as a mechanism of ER inhibition to elucidate the relationship between the ER and another molecules/mechanisms of interest, rather than the effect of ER inhibition alone. For example, a study by Araizi showed an 85% ER protein knockdown following ER siRNA transfection by Western blotting, though, this was only used as a comparison to show that the GPR30 siRNA being utilised was specific, and did not have an effect on ERα levels (Araizi *et al* 2010).

4.2.1. ER siRNA is able to reduce ER mRNA levels and consequently protein levels in MCF-7 cells.

To confirm the potential of targeting ER mRNA to ablate ER protein expression and activity, the use of ER siRNA was assessed and directly compared to the post-translational ER inhibition observed following fulvestrant exposure. The data presented within this study initially showed that the ER siRNA constructs assessed were both working via the previously

understood mechanism of RNAi action (De Fougerolles 2007). The ER siRNA utilised were able to reduce ER mRNA levels, leading to subsequent loss of protein expression, whereas fulvestrant was shown to have no effect on ER mRNA as expected. This result was consistent with other studies which have shown ER mRNA down-regulation following the use of a transient ER siRNA transfection system both in cell models (Araizi *et al* 2010, Bourdeau *et al* 2008), and *in vivo* studies (Bouclier *et al* 2008). Furthermore, the use of stable short-hairpin ER siRNA transfection into MCF-7 cells has also demonstrated an ER mRNA reduction of 50-80% in three generated clones when assessed by real-time PCR (Luqmani *et al* 2009).

Importantly, transient ER siRNA transfection was also shown to lead to significant but incomplete loss of ER protein expression, comparable to the previous findings for fulvestrant. An approximate 80-90% ER loss was observed when assessed by ICC and subsequent H-score regardless of the culture conditions used. Previous optimisation data using siTOX siRNA indicated that, using ER siRNA by the employed methodology, uptake would be incomplete and not all cells within the population would be transfected. Thus, a small number of cells within the ER siRNA treated population showed high nuclear ER protein expression probably indicating that these cells were untransfected. This was not observed within the fulvestrant treated arm as each cell was exposed equally to the anti-hormone. The untransfected cells introduce the complication of false-positive readings on the true effect of the ER siRNA within the cell population. However, even though many ER-negative cells were observed post siRNA treatment, a population of low ER-

expressing cells were still apparent suggesting that even in cells likely to have been transfected, ER mRNA and protein down-regulation was still incomplete. Interestingly, in the cells most likely to have been transfected with ER siRNA loss of ER expression appeared to be greater than in cells treated with fulvestrant. This could indicate the possibility that post-transcriptional ER targeting could be more effective than use of fulvestrant to down-regulate ER expression in this model.

Slight differences in levels of ER protein down-regulation were observed between ER siRNA transfection using pooled ER siRNA and individual constructs, which has also been shown by other groups. For example, while Bouclier's initial transfection experiments in MCF-7 cells showed only a 60% loss of ER protein expression after 5 days when using either of two individual ER siRNA sequences, when using a 50% pooled mix of both siRNA constructs an 85% loss of ER protein was achieved, by densitometry assessment of western blots (Bouclier *et al* 2008). As previously mentioned an 85% ER protein knockdown following ER siRNA transfection was also reported by Araizi (Araizi *et al* 2010), while similar ER levels were reported in this thesis by ICC methods. While a loss of ER expression has also been reported using the pre-cursor technology of anti-sense nucleic acids (Taylor *et al* 2001), the efficiency of the transient siRNA system employed within this thesis was far more effective and thus further comparisons with this technology were unlikely to be meaningful. The higher oestrogenic environment within the whole serum culture condition employed within this study had no apparent effect on ER protein expression following ER siRNA

transfection. This observation is corroborated by Bourdeau who also reported a significant (but incomplete) reduction of ER protein expression following transient ER siRNA transfection in MCF-7 cells cultured with stripped serum, regardless of E2 stimulation (Bourdeau *et al* 2008).

The significant but incomplete ER protein knockdown effect following ER siRNA transfection reported in this study has also been shown when using stable ER siRNA transfection, with Luqmani reporting residual ER after 15 and 35 passages, in a stably ER siRNA transfected MCF-7 cell model designated pII (Luqmani *et al* 2009). Interestingly, due to use of a selection media employed by Luqmani to identify 'pure' clones containing the ER siRNA construct and thus generate a homogeneous model, there were no untransfected cells present (and hence false-positive readings). This further indicates the possibility of a greater ER protein down-regulation due to the transient transfection system employed within this thesis.

The ER knockdown effect observed within this study support the previous premise of the value of continued siRNA research, and to employing this technique as a potential therapy with increased clinical benefit. Indeed further evidence for this hypothesis has been reported in a pre-clinical study using MCF-7 xenograft models (Bouclier *et al* 2008). The use of an ER siRNA mix incorporated into a PEG-PCL/MA (PEG- -caprolactone-malic acid) nanocapsule was reported to produce the same ER protein down-regulation as when it was in a complex with a simple transfection lipid in MCF-7 cells; conditions similar to the transfection lipid employed within this thesis. However the former preparation had the added advantage of being a

viable *in vivo* formulation for siRNA delivery. Subsequent subcutaneous injection of this preparation into a xenograft model, resulted in a significant reduction of ER protein expression, though only a modest 40-50% decrease was observed (Bouclier *et al* 2008), rather than the 85% observed *in vitro* in this thesis. Cumulatively the data presented within this thesis, and the corroborative evidence cited above support the potential of ER siRNA activity as a possible therapeutic option and further highlights the importance of efficient delivery of agents *in vivo* to gain greatest knockdown effect.

Furthermore, with fulvestrant not yet recommended for use in the clinic for pre-menopausal women, this project indicates that the use of an ER siRNA that does not have to compete with E2 to achieve successful ER down-regulation could be a potential therapeutic option in this clinical setting since this thesis showed an ER down-regulation and growth inhibition using ER siRNA under all serum or oestrogen stimulated conditions. However, a significant hurdle that would need to be overcome in such a clinical setting would be effective delivery of the ER siRNA to its target.

4.2.2. Treatment with ER siRNA inhibits ER signalling activity in a comparable manner to fulvestrant.

The data presented in this study showed a clear reduction in ER transcriptional activity following ER siRNA transfection when PR and pS2 mRNA and protein levels were assessed. However, these reductions in gene

and protein expression were again incomplete, comparable to the findings with fulvestrant exposure. While some of the residual ER transcriptional activity may potentially be due to the unaltered ER activity present in the untransfected cells within the ER siRNA treated population, residual protein expression of the investigated genes was shown to be present in many cells (when assessed by ICC). This would indicate potential residual activity of remaining ER within the transfected cell population. Indeed, in a stably ER siRNA transfected MCF-7 cell line where all cells contained ER siRNA, a significant but incomplete loss of approximately 90% of both PR and pS2 mRNA expression was reported following assessment by real-time PCR. This was after several weeks of continuous culture when compared to parental cells (Luqmani *et al* 2009). These findings support the evidence presented here of continued transcription of PR and pS2 possibly by residual ER transcriptional activity, rather than detection of PR and pS2 protein levels which would continue to reduce over time following natural turnover/half-life. Furthermore, another transient ER siRNA study showed incomplete inhibition of cyclin D1 mRNA expression, another E2 regulated gene (Bordeau *et al* 2008) and further indication of continued transcription possibly by residual ER.

Further evidence of potential residual ER transcriptional activity could be deduced from this thesis following the observation of residual ERE transcription present following ER siRNA transfection. The residual ERE expression observed following either ER siRNA of fulvestrant treatment was approximately five fold higher than the controls used to obtain a background reading. Cells were read in the absence of the Stop and Glo reagent, cells not

transfected with the ERE construct were read, as well as readings taken from a sterile saline solution to obtain a background fluorescence value for the assay and the spectrophotometer. This indicates the residual ERE activity observed is a measure of continued transcription rather than an artefact of the assay.

Interestingly, the residual ERE transcriptional activity in the whole serum culture conditions (an environment with higher levels of exogenous oestrogens) was greater following ER siRNA transfection than observed following fulvestrant exposure. This was despite ER protein expression being equal between the ER siRNA and fulvestrant treatment arms. These data shows the potential importance of receptor occupancy by fulvestrant causing inhibition of transcription, rather than its ability to down-regulate ER protein expression. This also reveals the possibility of a potential synergistic effect being achieved if fulvestrant and ER siRNA were used in combination.

Incomplete loss of ERE signalling following ER siRNA treatment has also been shown in other transient ER siRNA *in vitro* transfections in MCF-7 cells (Bouclier *et al* 2009), further supporting data presented in this study. However, Bouclier also showed complete loss of PR mRNA expression at 4 and 6 hours following transfection, though observed basal levels of control cells were also very low and clear residual ERE signalling was detected (Bouclier *et al* 2009).

There has also been some reported *in vivo* data supporting the observation within this study of potential continued ER transcriptional activity. ER siRNA treated mouse xenografts showed residual protein expression of the oestrogen regulated gene CD34 (Bouclier *et al* 2009)

following assessment of biopsies. This is unsurprising however as the levels of residual ER were much higher following siRNA transfection in xenograft models due to limitations in efficacy of delivery.

The implications of residual signalling potentially via remaining ER following siRNA transfection are identical to that following fulvestrant exposure; that residual signalling could confer some growth stimulation in these cells, possibly allowing for future adaptation and survival of initial ER insult and future acquisition of resistance.

4.2.3. ER siRNA transfection showed comparable growth inhibition and anti-proliferative effects to fulvestrant.

With ER siRNA treatment producing similar effects on ER expression and activity to that seen with fulvestrant, a significant but incomplete growth inhibition and reduction of proliferative capacity in these cells was also expected following ER siRNA transfection. This proved to be the case with incomplete inhibition of growth and proliferation following ER siRNA transfection being observed regardless of culture conditions. The growth inhibition following ER siRNA transfection was comparable to fulvestrant treatment indicating some continued growth promoting signal potentially due to the presence of the residual ER signalling previously observed or the induction of compensatory growth factor signalling elements as discussed previously. The effect of transient ER siRNA transfection on MCF-7 cell

growth has recently been reported, with groups showing inhibition of proliferation (Bourdeau *et al* 2008, Bouclier *et al* 2009). Both papers report an approximate growth inhibition of 50% in MCF-7 cells following transfection, complementary to the data presented in this thesis, again growth inhibition was shown to be incomplete, and additionally irrespective of E2 stimulation (Bouclier *et al* 2009). Also interestingly, recent pre-clinical evidence of the anti-tumour activity of ER siRNA *in vivo* has emerged, supporting the data within this thesis. MCF-7 xenograft tumours injected with ER siRNA, showed only a doubling in size over a four week period (when measured with callipers), with control tumours more than trebling in size in the same period (Bouclier *et al* 2009). Furthermore when biopsied it was also reported that these tumours contained fewer cells, large banks of fibrotic tissue and extensive areas of necrotic and apoptotic damage.

The reason for the incomplete inhibition of growth and proliferation could potentially be due to a change in ER level equilibrium, similar to that described for maximal fulvestrant exposure previously. While ER mRNA is rapidly degraded via use of an siRNA, following the use of RNAi there is no mechanism in place to stop continued transcription of further ER mRNA within the cells. Thus, potentially some of the ER mRNA will escape degradation, and be translated which could account for the residual ER protein expression observed within this thesis. Its potential continued activity therein, could be providing a continued growth promoting effect on these cells, though at the markedly reduced rate observed. However there is also a noted incomplete inhibition of IGFR and IRS1 which can also promote growth. No

subsequent loss of ERK was observed following On-target ER siRNA transfection, however, the siGenome ER siRNA which had some off target effects, lead to a loss of ERK and showed a greater growth inhibition, showing the vital importance of ERK signalling within these cells. Under the conditions of residual ER the observed induction of HER2 and EGFR following siRNA transfection is indicative of subsequent compensatory signalling which could contribute to continued growth and survival and ERK lies downstream of such induced signalling (Gee *et al* 2003).

In conclusion, the use of ER siRNA within this thesis has illuminated two important points; firstly that ER siRNA does have the potential to be a therapeutic strategy in the management of breast cancer if an effective delivery method can be designed. Its use showed significant if incomplete anti-tumour activity in the cell model studied in this thesis, corroborated by some early promising recent pre-clinical evidence. Secondly, the incomplete loss of ER expression and residual signalling could be contributing to continued growth within these cells. Together these data support the original rational for combination of post-transcriptional and post-translational methods of ER interference to further increase loss of remaining ER and, thus, potentially improve on the efficacy of the anti-ER treatment. Furthermore, ER siRNA transfection while shown to have a potentially greater capacity for ER down-regulation than fulvestrant treatment showed no subsequent improvement in inhibition of signalling or growth inhibition. This could potentially again indicate the importance of fulvestrant occupancy of the receptor causing transcriptional repression, and indicate a possible synergy

between the two mechanisms of action as the residual ER protein is not further antagonised following ER siRNA transfection.

4.3. The effect of the combination treatment of fulvestrant and ER siRNA on MCF-7 cells.

The reported residual ER present in this study, following either fulvestrant exposure or ER siRNA transfection, and the subsequent residual ER signalling observed left open the possibility that the two agents could act synergistically to promote greater ER down-regulation and reduction in ER activity, and might thereby effect a more substantial tumour cell growth inhibition. However, while greater loss of residual ER could potentially improve the anti-tumour response, it could also induce potentially undesirable compensatory signalling, which could override the potential benefit.

4.3.1. The combination of ER siRNA and fulvestrant gives greater ER down regulation than either agent alone.

Prior to the commencement of this thesis, the combination of ER siRNA and fulvestrant had not been reported in the literature and complete ER negativity had only been reported following the generation of endocrine resistant cell models via a prolonged period of exposure to anti-hormones (Nicholson *et al* 2007, Hiscox *et al* 2006, Liu *et al* 2006). Notably, the stably

ER negative fulvestrant resistant MCF-7 cell line created by the Tenovus Centre for Cancer Research which took over two years of continuous culture to achieve a completely irreversible ER negative phenotype (Nicholson *et al* 2007, Hiscox *et al* 2006). The data presented in this thesis showed a rapid and almost complete loss of ER protein expression, following only 96 hours of combination treatment, significantly greater than the use of either agent alone. This appears to be achieved as a consequence of the targeting of both ER mRNA and protein levels. Significantly, however, complete ER negativity was not achieved, with approximately 5% of cells showing some (although weak) immunostaining and most likely reflects cells that were untransfected by the ER siRNA, and hence have low levels of residual ER due to the action of fulvestrant alone. However, in the majority of cells, complete ER negativity was achieved, something previously unreported within the literature following short-term exposure to such treatment. Post-transcriptional and post-translational methods may thus potentially work synergistically to ablate ER protein expression.

While no evidence of the synergistic effect of ER siRNA and SERD's on ER loss had been published prior to the work in this study, a very recent study supports the evidence presented herein (Bouclier *et al* 2009). Bouclier and colleagues reported a greater loss of ER protein expression following combination treatment in MCF-7 cells, though the delivery method of the siRNA, the sequence of siRNA itself (as previously shown to be important within this project, for siGenome versus On-target ER siRNA), the SERD (RU556) used, and its delivery method were all different from those employed

in the present study. Significantly, Bouclier assessed ER level by Western blot analysis, and a long exposure time with the x-ray film was required to obtain any residual ER signal in the combination condition by this method when employed in this thesis. This might indicate an over-estimation of the level of ER down-regulation following combination treatment by Bouclier and colleagues. Importantly, they also reported a similar effect *in vivo* when ER levels were assessed by ICC in MCF-7 xenografts (Bouclier *et al* 2009). The levels of residual ER, however, were much higher following treatment *in vivo* with 30-40% of tumour cells still expressing observable, significant residual ER following the combination treatment. This indicates that treatment with either agent (and hence the combination treatment) is still not optimal within this setting, when compared to maximal ER protein down-regulation observed in MCF-7 cells by fulvestrant (and similar levels achieved by ER siRNA transfections) shown in this thesis and by Bouclier's *in vitro* studies (Bouclier *et al* 2009).

4.3.2. The combination treatment of ER siRNA and fulvestrant showed greater ER protein down-regulation, but no further inhibition of ER activity.

With the greater ER loss observed in this study following combination treatment, a greater loss of ER activity was also expected when compared to the single agents alone. Interestingly however this thesis reports that the levels

of PR and pS2 mRNA expression, while reduced compared to parental cells were equivalent following either the combination treatment or either agent alone when assessed by RT-PCR, however, this is not a quantitative measure of expression and, thus slight differences in mRNA levels are difficult to detect. These data contrast with those of Bouclier (Bouclier *et al* 2009) who showed a complete PR mRNA loss following SERD and ER siRNA combination treatment under E2 stimulated conditions. Importantly, they also observed a complete loss of PR following ER siRNA transfection alone making an additive effect impossible to determine. Unsurprisingly, in this thesis as both PR and pS2 mRNA expression following combination treatment or fulvestrant exposure were equal, protein expression levels of PR and pS2 also showed no difference in levels following combination treatment or fulvestrant under all conditions examined.

When ER transcription was examined quantitatively using ERE reporter gene assays the combination treatment did not improve on the response shown with fulvestrant alone. It is likely, therefore, that although the effect was greater than that achieved with the ER siRNA alone, the effect of fulvestrant was dominant, as in the study using a SERD in MELN cells by Bouclier (Bouclier *et al* 2009), with the combination treatment and SERD utilised both showing equal down-regulation of ERE activity, and ER siRNA alone showing slightly higher residual activity. As with ER expression levels, following combination treatment *in vivo* there was still marked residual protein expression of the E2 regulated genes GLUT-1 and CD34 though this was lower than treatment with either agent alone (Bouclier *et al* 2009) but this

not surprising due to the limited effectiveness of ER ablation following combination treatment within this setting shown by Bouclier. While the presence of residual PR and pS2 following combination treatment could be simply ascribed to the fact that their transcription can be driven by factors other than ER, the presence of residual transcriptional activity (significantly higher than background levels) at ERE sites is more complicated to explain. However, there are several possible reasons for this continued ERE activity following the combination treatment. One explanation is that the ER is not completely saturated by fulvestrant and thus unrepressed residual ER is able to drive transcription at these sites. It is also possible that the assays used to assess ER expression levels underestimated the residual ER and more ER is present than believed. It is also possible that compartmentalised ER (such as membrane associated ER) was able to avoid degradation by the combination treatment and able to drive transcription in this assay. Some evidence also suggests that the ER can be phosphorylated in the presence of fulvestrant, as seen in the LTED model MCF-7X (Staka *et al* 2005) and this could drive ERE transcription potentially driven by growth factor signalling kinases. Finally, it is possible that the residual ERE expression is due to the action of ERβ. While ERβ expression has not been directly assessed within this thesis, Luqmani and colleagues reported increased ERβ within their stable transfected ER siRNA cell model (Luqmani *et al* 2009). It has also been established that siRNA can be specific for mRNA containing a single base pair change (Elbashir *et al* 2001, 2001a) and thus the siRNA used was unlikely to effect ERβ expression. The role of ERβ in breast cancer still remains controversial and while it shows

preferential hetero-dimerisation with ERα, in the absence of ERα its function is not fully understood. Some studies report that increased ERβ expression correlates with an ERα negative phenotype and prevents ERE signalling (Ogawa *et al* 1998), and that increased ERβ relates to tamoxifen resistance (Speirs *et al* 1999). Other studies have shown lowered ERβ expression can be indicative of tamoxifen resistance (Elledge *et al* 2000). While it has been suggested that ERβ is not just a surrogate for ERα and it may contribute to growth and proliferation through an alternative set of downstream target genes (Bates *et al* 2008), another recent study showed an overlap of ERα and ERβ target genes in MCF-7 cells, and when both ERα and ERβ were present ERα was shown to be dominant over ERβ and to displace it to alternative binding sites. Importantly however, if ERα was absent then ERβ would preferentially bind to these former sites to drive transcription, though less efficiently (Charn *et al* 2010). This may offer an explanation of the continued transcription at ERE sites, and potentially the residual PR and pS2 observed following combination treatment, and it would be of possible future benefit to assess whether there is a change in ERβ expression or its activity within the combination treated cells presented in this thesis. ERβ expression and activity may potentially be able to promote growth following the combination treatment.

Taken as a whole these data illuminates several interesting biological points. The first is that transcription at ERE elements (and by extension other oestrogen regulated genes), in the system assessed may not be solely dependent on the presence of ERα as it is clear that the ERE reporter gene

studies reveal ER transcriptional activity despite almost complete absence of ERα expression following the combination treatment. Another point to note is the similarity in activity between fulvestrant exposure alone and the combination condition. If the observed increased loss of ER following combination treatment is accurate, this result could indicate that transcriptional activity of the residual ER following fulvestrant exposure is blocked by occupancy by fulvestrant. This again raises the important clinical point that further reduction of residual ERα following fulvestrant treatment may give no further clinical benefit as the receptor is fully occupied and inhibited by fulvestrant.

4.3.3. The combination of ER siRNA and fulvestrant showed no greater growth-inhibitory or anti-proliferative affects *in vitro.*

With the effect on signalling shown to be equal between the combination treatment condition and fulvestrant exposure alone, this study also reports that while a significant reduction in proliferation and growth was observed following the combination treatment this was also equal to fulvestrant treatment alone. This was despite the greater ER loss achieved following combination treatment within 4 days. When viability and proliferation of combination treated MCF-7 cells was assessed by MTT assay and Ki67 positivity respectively, a similar pattern to ERE signalling was

observed. The greatest reduction in proliferation was observed in the combination and fulvestrant alone arms and was equivalent in each arm; with ER siRNA transfection alone shown not to be as effective. The slight differences in proliferation measured by MTT compared to the larger difference in Ki67 positivity observed most likely results from the relatively low sensitivity of the MTT assay technique when at low cell number under stripped serum conditions. This may be due to use of smaller wells, and thus smaller sample size, plus possible disruption of the more delicate monolayer when washing cells of media and application of MTT solution. These data are comparable to the study by Bouclier (Bouclier *et al* 2009), which used a colourmetric Cell Titer 96 Aqueous One Solution Cell Proliferation Assay from Roche. In the absence of E2, the same conditions under which growth was assessed in this thesis, reduction of proliferation in the combination condition was equal to RU556 exposure alone at both 10^{-8}M RU556 (35% inhibition) and at 10^{-6}M RU556 (60% inhibition) and there was no significant difference between this and the combination (Bouclier *et al* 2009). This data was also mirrored by the Coulter counter assessment of total cell growth employed in this project.

In conclusion, the above data suggests that not all growth of ER positive breast cancer cells is dependant on the activity and presence of ERα, since the combination treatment was unable to further growth arrest these cells beyond fulvestrant alone, despite a greater loss of ER. In biological terms, the complete dependence on a single factor is not an optimum survival strategy and many alternative signalling pathways are available to breast cancer cells

since they are surrounded by a rich milieu of hormone, steroids and various growth factors (Nicholson *et al* 2004). Indeed, many paracrine options are available to the cells in addition to autocrine and endocrine factors. Finally, as stated previously, it is conceivable that since anti-hormones are known to induce compensatory growth and survival signalling, that a more complete loss of ER promotes such elements more efficiently to counteract the effectiveness of the combination treatment. The induction of genes, such as EGFR and HER2 observed in this thesis for example, could potentially contribute to the growth response observed within this study following fulvestrant or the combination treatment. Indeed, these genes have been identified as mediators of anti-hormone resistance both in the clinic and preclinical *in vivo* and *in vitro* cell models. When EGFR and HER2 were assessed in the present thesis, there was an increase in total EGFR and HER2 following the combination treatment and fulvestrant treatment potentially leading to subsequent ERK and AKT signalling and an induction to a hormone dependant phenotype. This rapid induction of potential compensatory signalling has potential clinical implications as there is a noted correlation between activation of growth signalling pathways and endocrine insensitivity and resistance to anti-hormone therapy (Nicholson *et al* 2004), and consequently more aggressive disease and poor prognosis (Gee *et al* 2001). These data could indicate that maximal ER inhibition could potentially induce this unfavourably phenotype at an earlier stage, and thus targeting ER alone may not be an optimal strategy. There has been further evidence for an induction of an unfavourable phenotype in Luqmani's ER siRNA stable

transfected cell model pII (Luqmani *et al* 2009), these cells that had markedly reduced ER expression showed increases in vimentin (VIM), CD68, carbonic anhydrase IX (CA9), loss of keratin (KRT) and over-expression of CD68 and stathmin (STMN1) which have been associated with ER loss and increased aggression and migration (Thompson *et al* 1992, Sommers *et al* 1989, Cobleigh *et al* 2005, Curmi *et al* 2000), however Luqmani also showed a reduction in EGFR and HER2 rather than the induction observed in this thesis, showing the treatment and cellular context is important in adaptation to anti-hormone treatment. While only some of the EGFR family members were assessed within this thesis and while no obvious inherent behavioural changes were observed, future work looking for the appearance of these markers could indicate that the combination treatment is pushing the cells towards a more aggressive phenotype, with induction of EGFR family members and downstream signalling kinases seen in some pre-clinical models of anti-hormone resistance (Gee *et al* 2011). Whatever the explanation, the data suggest that maximum ER blockade in the clinic may not necessarily promote further clinical benefit.

4.4. Conclusions and future prospects.

This thesis answers the fundamental questions that residual ER following fulvestrant treatment can be further reduced and ER can be almost completely removed in the *in vitro* setting within this project, but that this has no greater

influence on growth inhibition than fulvestrant treatment alone. This does however open up new areas of work and questions, such as

- Clarifying any differences in induction of compensatory signalling following ER siRNA and fulvestrant combination compared with fulvestrant alone.
- Whether the combination treatment is more effective in a co-targeting setting (ER and HER2/EGFR) than fulvestrant alone.
- Is the combination treatment improved in Tamoxifen resistant disease above fulvestrant alone? (As fulvestrant is the current clinical treatment following Tamoxifen relapse).
- Whether there is a greater duration of response associated with the combination treatment, potentially answerable by use of a stably transfected ER siRNA cell line treated with fulvestrant.
- The phenotype of the cells with residual ER and determining the occupancy of residual ER following fulvestrant treatment.

While historically increased efficacy of ER ablation has been desirable and clinically beneficial, this thesis has taken it to its logical conclusion by employing a strategy of combining a SERD with an siRNA and has shown that it is not a 'cure' in its own right. However it must be noted that this study used short-term treatment and looked at initial response and not duration of response, whereas increasing dosage in clinical trials has shown greater time to progression (Robertson *et al* 2007). An example of this is the FIRST study, comparing 500mg monthly fulvestrant to 1mg daily Anastrozole. Both

treatment regimens showed similar clinical benefit rate and objective response, however the time to progression was significantly higher in the fulvestrant treated arm and at data cut-off only 29.4% of fulvestrant treated patients had progressed in comparison to 41.7% receiving anastrazole (Robertson *et al* 2009). Importantly, however, while many explanations maybe proposed to explain the incomplete growth inhibition observed following the combination therapy, the induction of compensatory signalling seen in this thesis and in models of endocrine resistance suggests that combination therapies attacking such induced elements may be beneficial alongside fulvestrant therapy. If this is so, the direction of breast cancer treatment must therefore progress from targeting ER as a single target in isolation to an individual patient tailored therapy targeting ER and the genes responsible for resistance mechanism(s). Significantly, there has been some work on this to date, exposure to both fulvestrant and the EGFR specific tyrosine kinase inhibitor Iressa prevented development of resistance to either agent in MCF-7 cells. Indeed there was a high level of cell loss associated with the co-treatment with any remaining cells being growth arrested for over six months (McClelland *et al* 2001). Similar results have been obtained by combining the pan-ErbB inhibitor CI-1033 with fulvestrant (Sonne-Hansen *et al* 2010). Critically, there is tentative evidence that the combination of an ER antagonist with inhibition of growth factor pathways may also increase clinical benefit. Thus, a recent phase II neo-adjuvant study showed the efficacy of the AI letrozole was significantly enhanced when co-treated with an mTOR inhibitor, a downstream ErbB target (Baselga *et al* 2009). Further clinical trials combining

signal transduction inhibitors and traditional ER antagonists have been proposed and the work presented in this thesis indicates that this maybe the way forward in the future successful management of breast cancer. However, there is a need to screen for compensatory signalling within clinical disease during treatment to ensure patients receive the most beneficial therapy for their disease. As evidence that the screening for compensatory signalling in patients to find the correct targets for a combination treatment is absolutely paramount, a phase III clinical trial by Johnston *et al.* combining the AI Letrozole and a dual tyrosine kinase inhibitor against EGFR and HER2 (lapatinib) showed only limited value in HER2 negative patients compared to AI alone (although the combination was superior in hormone receptor positive, HER2 positive patients (Johnston *et al* 2009)).

Chapter 5. References Section.

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Chapter 6. Appendices Section.

Appendix 1. Charcoal stripping of foetal calf serum.

To strip 100ml of foetal calf serum (FCS), the following solution is made

- 2g Activated charcoal
- 0.01g Dextran T70
- 18ml distilled H_2O

This solution is stirred vigorously for one hour. FCS is pH adjusted to 4.2 with 5M Hydrochloric acid (HCL) and allowed to equilibrate for 30 minutes at 4°C. 5ml charcoal solution is added to FCS and stirred for 16 hours at 4°C. Charcoal is removed by centrifugation at 12,000g for 40 minutes. Pass supernatant through Whatman filter paper No.4 (repeat filtration 2-3 times). Adjust serum to pH to 7.2 with NaOH (5M) and sterilise by passing through 0.2µm bottle-top filter. Stripped serum can be aliqouted and stored at -20°C.

Appendix 2. 0.01M Phosphate buffered saline (PBS) for Immunocytochemistry.

42.5g NaCl (Sodium chloride) and 7.15g di-potassium hydrogen orthophosphate anhydrous (K_2HPO_4) is made up to 5L with distilled H_2O.

Appendix 3. Sucrose storage medium for Immunocytochemistry.

42.8g Sucrose

0.33g $MgCl_2$ (Magnesium Chloride)

Dissolved into 250ml 0.01M PBS (appendix 2.).

Added to 250ml glycerol and stored at -20°C prior to use.

Appendix 4. 0.02% PBS Tween.

50µl Tween added to 250ml 0.01M PBS (see appendix 2.)

Appendix 5. Reverse-transcription master mix for PCR.

11µl of Reverse Transcriptase master mix solution is comprised of:-

5µl dNTPs (2.5mM stock comprised of 0.625mM each of dGTP, dCTP, dATP and dTTP).

2µl PCR buffer (comprised of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$ 0.001% w/v gelatine)

2µl dithiothreitol (DTT) (0.1M stock)

2µl Random hexamer oligonucleotides (RH) (100µM stock).

This is sufficient for one 7.5µl sample containing 1µg of RNA, and should be scaled up accordingly

Appendix 6. PCR master mix.

2.5µl 10x PCR buffer (comprised of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$ 0.001% w/v gelatine).

2µl dNTPs (2.5mM stock, comprised of 0.625mM each of dGTP, dCTP, dATP and dTTP)

0.2µl Taq polymerase (5 u/µl)

And 0.625µl each of desired forward and reverse Primers (20µM stock)

Made up to 24µl with sterile H_2O

N.B. if β-actin primers are being used, 0.3µl of forward and reverse primers were used, with an additional 0.65µl DNase free H_2O added due to abundance of gene present.

Appendix 7. 50x Tris-Acetate-EDTA (TAE) buffer.

Component	Amount (for 1L)	Final concentration
Tris base	242g	2M
Glacial acetic acid	57.1ml	1M
EDTA (0.5M, pH8)	100ml	0.05M

Make up to 1L with distilled H_2O and adjust pH to 8.3, dissolve 1 in 50 with distilled H_2O prior to use.

Appendix 8. RNA loading buffer.

Component	Amount (for 10ml)	Final concentration
Sucrose	6g	60% (w/v)
Bromophenol blue	0.025g	0.25% (w/v)

Add to 10ml RNase-free H_2O, and pass through 0.2μm syringe filter to remove undissovled bromophenol blue.

Appendix 9. Complete protein lysis buffer.

Lysis buffer stock comprised of:-

Component	Amount (for 100ml)	Final Concentration
Tris base	0.6g	50mM
EGTA	0.19g	5mM
NaCL	0.87g	150mM
Triton X-100	1ml	1% (v/v)
Distilled H_2O	100ml	-

Adjust pH to 7.5 with HCL (5M), and store at 4°C.

Add following phosphatase/protease inhibitors immediately prior to use (See overleaf);

Inhibitor	Stock concentration	Solvent	Volume to add to 5ml Lysis buffer	Final concentration
Sodium orthovandate	100mM	H_2O	100µl	2mM
PMSF	100mM	Isopropanol	50µl	1mM
Sodium fluoride	2.5M	H_2O	50µl	25mM
Sodium molybdate	1M	H_2O	50µl	10mM
Phenylarsine oxide	20mM	Chloroform	5µl	20µM
Leupeptin	5mg/ml	H_2O	10µl	10µg/ml
Aprotinin	2mg/ml	H_2O	25µl	10µg/ml

Appendix 10. 12% Resolving Gel for SDS-PAGE.

Component.	To make 15ml (12% w/v)	Final concentration.
Lower buffer (1.5M Tris-HCl buffer pH 8.8)	3.75ml	12% (w/v)
Acrylamide/Bis-acrylamide (30% solution).	4.95ml	375mM
Distilled H_2O	6ml	-
SDS (10% solution in H_2O)	150µl	0.1% (w/v)
APS (10% solution in H_2O)	150µl	0.1% (w/v)
TEMED	15µl	0.1% (v/v)

Acrylamide solution had an acrylamide:bis-acrylamide ratio of 29:1, and TEMED was added last to commence polymerisation.

Appendix 11. 4.5% Stacking gel for SDS-PAGE.

Component	To make 10ml (4.5% w/v)	Final concentration.
Upper buffer (0.5M Tris-HCL pH 6.8)	2.5ml	125mM
Accrylamide/Bis-acrylamide (30% solution).	1.3ml	4.5% (w/v)
Distilled H_2O	6.1ml	-
SDS (10% solution in H_2O)	100µl	0.1% (w/v)
APS (10% solution in H_2O)	50µl	0.05% (w/v)
TEMED	10µl	0.1% (v/v)

Acrylamide solution had an acrylamide:bis-acrylamide ratio of 29:1, and TEMED was added last to commence polymerisation.

Appendix 12. 10x Running buffer for SDS-PAGE.

Component	Amount (to make 1L)	Final concentration
Tris base	30g	2.5M
glycine	144g	19.2M
SDS	10g	1% (w/v)
Distilled H_2O	1L	-

Adjust to pH 8.3 with 5M hydrochloric acid (HCL), and diluted to 1 in 10 with distilled H_2O prior to use.

Appendix 13. 2x Laemli sample loading buffer.

Component	To make 10ml (2x stock)	Final concentration (when diluted with cell lysate)
SDS (10% solution in H_2O)	4ml	2% (w/v)
Glycerol	2ml	10% (v/v)
Tris Base (1M stock pH 6.8)	1.2ml	60mM
Distilled H_2O	Make up to 10ml	-
Bromophenol blue	0.002g	0.01% (w/v)

Prior to use 8µg DTT is added per 0.5ml buffer to be used.

Appendix 14. Transfer buffer for SDS-PAGE.

Component	Amount (to make 1L)	Final concentration
Tris base	3.03g	0.25M
Glycine	14.4g	1.92M
Methanol	200ml	20% v/v
Distilled H_2O	800ml	-

Appendix 15. Tris-buffered saline (TBS) and TBS-tween.

Component	Amount (to make 1L)	Final concentration
Tris base	1.21g	10mM
Sodium chloride (NaCl)	5.8g	100mM
Distilled H_2O	1L	-

Adjust to pH 7.6 with 5M hydrochloric acid (HCL).

TBS-tween.

TBS is made as above, but with addition of 50µl Tween 20, to give a final concentration of 0.05% (v/v).

Can current Oestrogen Receptor Therapies be further improved, and is this desirable in the clinic?

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Breast cancer is the most common cancer in females, affecting 1 in 9 women worldwide. This number is increasing every year. Approximately three-quarters of these cancers are termed hormone-responsive. This means that the growth of these tumours is driven by the female hormone oestrogen, via its interaction with the Oestrogen Receptor (ER). The most common treatment of breast cancer is by use of anti-oestrogens, the first and most widely used agent in this class being Tamoxifen. Although effective in many cases Tamoxifen only partially impairs ER function and can also be oestrogenic, another issue is that long-term use can promote Tamoxifen resistance, leading to more aggressive tumour growth and poor prognosis. Because of this newer anti-oestrogens, more able to ablate ER function were developed, the leading molecule from this research being Faslodex. Faslodex has no oestrogenic activity and gives a more complete ER blockade; it is also able to degrade the ER at the protein level. Although Faslodex is well tolerated in the clinic, its inhibition of ER function is not complete and long-term Faslodex treatment can lead to resistance. The presence of Residual ER is believed to be responsible for development of resistance in both cases, and consequently this project to assess whether a more complete ER inhibition can be achieved, by utilisation of siRNA to the ER in combination with Faslodex, and if a more complete ER inhibition can be achieved to see whether this is of benefit within the clinic and in what setting.

Ribonucleic acid interference (RNAi) is a relatively new technology that by addition of a specific small interfering RNA molecule (siRNA), is able to utilise a natural cellular process to down regulate a specific gene at the mRNA level, and subsequently at the protein level as well. However these siRNAs are relatively large and charged molecules and do not readily pass across the cell membrane without the aid of a transfection agent. Transfection lipids allow siRNA uptake but disrupt the cell membranes integrity resulting in cell death. This lipid toxicity is a major limitation of the use of siRNAs, and an optimal balance must be found, this can then be used in combination with Faslodex as a treatment *in vitro*.

Initially the toxic effect of the recommended concentration transfection lipid was optimised, while minimising toxicity the optimised concentration was still able to deliver siRNA effectively, down-regulating the ER at both mRNA and protein levels. Most siRNA are purchased and used as pools of four different siRNA to the same gene, to give a good knockdown in most cell lines while minimising off-target effects. The four components of the ER siRNA pool were assessed individually and against the pool to see whether the were differential levels of ER knockdown in the cell lines used, as each component of the pool gave comparable knockdown and the pool gave the greatest knockdown, the pool was used throughout. The combination treatment of ER siRNA and Faslodex was compared against Faslodex treatment alone and the combination showed a greater knockdown of ER at mRNA and protein levels. The combination treatment was also shown to have a greater effect on 'classical' oestrogen regulated genes, showing greater down-regulation on estrogen induced genes such as progesterone receptor (PR) and pS2 than Faslodex treatment alone. The combination treatment showed greater down regulation of oestrogen signalling than Faslodex alone when components of the Insulin-like growth factor pathway were observed. While there was some indication that the combination condition had a greater reduction of proliferation than Faslodex there did not seem to be any significant difference on cell growth. This pattern of results from the hormone-sensitive cell line (MCF-7) seems to be true for the Tamoxifen resistant line derived from MCF-7's (TAM-R), the combination of ER siRNA and Faslodex give a more complete ER blockade than Faslodex alone, however the growth effects still need to be assessed.

Work will now focus on investigating whether the effects shown in MCF-7 cells are comparable in other hormone dependant breast cancer lines and whether this combination treatment is effective in anti-hormone resistant, ER positive breast cancer. With siRNA technology constantly developing, a newer construct to ER is available and will be assessed for consistency.

Using new technology to assess the importance of the Residual Oestrogen Receptor protein in the growth of breast cancer cells.

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Breast cancer affecting 1 in 9 women worldwide, is the most common female cancer. Seventy-five percent of these cancers are termed hormone-responsive; this means the growth of the cancer is driven by the female hormone Oestrogen via its interaction with the Oestrogen receptor protein (ER). As such the most common way to treat these tumours is by use of an anti-Oestrogen compound. The most common drug of this class is Tamoxifen, a drug which partially impairs normal ER function, and as such its use has been shown to inhibit breast cancer growth.

Although impeding ER function has an effect on growth, Some ER remains and resistance develops. The role this Residual Oestrogen Receptor plays in formation of resistance in a range of different breast cancer cell is not yet fully understood.

Recently a new technology (RNAi) which has the ability to completely eliminate the Oestrogen Receptor is being used to shed further light on this area of research.

Optimisation of RNAi technology for use in Oestrogen Receptor Knockdown studies to understand growth in Breast cancer cells.

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Breast cancer is the most common cancer in females, affecting 1 in 9 women worldwide[1]. This number is increasing every year. Approximately three-quarters of these cancers are termed hormone-responsive. This means that the growth of these tumours is driven by the female hormone oestrogen, via its interaction with the Oestrogen Receptor (ER). This is why the most common treatment of breast cancer is by use of anti-oestrogens, the most widely used agent in this class being the anti-oestrogen Tamoxifen. Tamoxifen partially impairs ER function, and as such its use has been shown to slow or stall the progress of hormone-responsive breast cancer. This compound acts as an antagonist in breast tissue but as an agonist in other tissues and is therefore termed a selective oestrogen receptor modulator or SERM. A further class of molecules called 'pure' anti-oestrogens are coming into wider use, the most common of these being fulvestrant (Faslodex). Faslodex is also able to slow or stall breast cancer growth through down regulation of the ER protein. Though it is known that impairing the ER function has an effect on growth, the role of the residual ER present in anti-oestrogen treatment and its function in the continued growth of a range of different breast cancer cells is not yet fully understood. The aim of this project is to assess and to fully understand the role of the residual Oestrogen Receptor on growth of breast cancer cells.

Ribonucleic acid interference (RNAi) is a relatively new technology that by addition of a specific small interfering RNA molecule (siRNA), is able to utilise a natural cellular process to down regulate a specific gene at the mRNA level, and subsequently at the protein level as well. This technology is able to almost completely eliminate a desired protein from a cell. However these siRNAs are relatively large and charged molecules and do not readily pass across the cell membrane without the aid of a lipid based transfection agent. Transfection lipids allow siRNA uptake but disrupt the cell membranes integrity resulting in cell death. This lipid toxicity is a major limitation of the use of siRNAs, and an optimal balance must be found.

The RNAi technology was used in a range of breast cancer cells and a series of *in vitro* cell growth experiments were conducted to see whether a less toxic concentration of lipid could be found. An optimum concentration displaying minimum detrimental affects on cell number over a prolonged time period was found. A second series of experiments were run to determine whether this optimum lipid concentration still facilitated siRNA uptake. The optimised siRNA protocol gave significant ER knockdown at the protein level. Also the difference in ER knockdown between the pool of four siRNAs most commonly used and the individual siRNAs was investigated. The individual siRNAs all gave a significant protein knockdown compared to all the control conditions, but the siRNA pool gave a more efficient knockdown though this was not significantly different when compared to the individual siRNAs.

The optimised siRNA protocol is able to consistently and effectively knockdown the ER. We are now investigating this effect of removing the ER on the growth of the Cancer cells. The next step will be to look at changes in downstream signalling targets of the ER, and to detect this using certain classical markers. This use of optimised RNAi for breast cancer should prove a powerful tool in elucidating the full function of the Oestrogen Receptor and its importance in the progression of breast cancer.

1. Office for National Statistics. *Registrations of cancer diagnosed in 1993-1996, England and Wales.* Health Statistics Quarterly 1999; 04:59-70.