The Identification of Genes Associated with the Maintenance of the Tamoxifen Resistant Breast Cancer Cell Phenotype Following Tamoxifen Withdrawal.

A thesis presented for the degree of

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Summary

Despite the benefit tamoxifen has provided for millions of breast cancer patients worldwide, almost all patients with metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen treatment will acquire resistance to the drugs inhibitory effect on breast cancer cell growth. Previous studies in the Tenovus Centre have demonstrated that the development of anti-oestrogen resistance *in vitro* is associated with aberrant growth factor signaling which facilitates a more aggressive cell phenotype. The aim of the present study was to determine whether the undesirable characteristics of tamoxifen resistant cells were maintained following withdrawal from the drug. Interestingly, the accelerated rate at which resistant cells proliferated was sustained following a 6 month withdrawal period despite decreased expression of epidermal growth-factor receptor and reduced sensitivity to gefitinib. Following the assessment of long-term tamoxifen exposure on classically regulated oestrogen gene targets progesterone receptor and trefoil factor 1, it was apparent that the genes were no longer inducible by oestradiol following the acquisition of resistance. In contrast, when cells were co-treated with a demethylation agent in combination with oestradiol, genes were once again responsive to oestrogen stimulation, providing proof of principle that long-term tamoxifen exposure can silence oestrogen regulated gene expression through promoter hyper-methylation. Importantly, this combination treatment was shown to significantly reduce cell growth, inferring that a proportion of the genes that were reactivated by this treatment were associated with a tumour suppressive function. Using microarray technology, methylight analysis and polymerase chain reaction validation, several genes with tumour-suppressive ontology were identified as being silenced by promoter hypermethylation in tamoxifen-withdrawn tamoxifen-resistant cells, including p53 gene target, prostate differentiation factor, and inhibitor of Ras signaling, Ras protein activator-like 1. It is therefore proposed that anti-hormone induced epigenetic modification of tumour-suppressor genes, alongside aberrant growth factor signaling, can promote resistant cell survival and progression.

Publications

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Table of Contents

1. INTRODUCTION

1.1 Cancer

Cancer is a group of diseases characterised by the inappropriate management of the regulatory circuits that govern normal cell proliferation and homeostasis, permitting cells to divide uncontrollably and gain otherwise restricted metastatic function, leading to invasion and erosion of surrounding tissues. This phenomenon can occur within all major components of the human anatomy, with over 100 different forms of cancer identified which are further sub-categorised according to the specific organ affected. It has been estimated that cancer was the cause of 7.6 million deaths worldwide in 2007 [American Cancer Society, 2007], which equates to around 20,000 cancer related deaths per day. In the UK, one in three people are expected to develop cancer [Office for National Statistics, 2007]. In 2005, 239,000 people were diagnosed with the disease which claimed the lives of 126,600 patients in the same year, accounting for 29% of all deaths in males and 24% in females [Office for National Statistics, 2007].

While cancer accounts for an increasing proportion of deaths in the UK due to the decline of other main causes such as heart disease, stroke and infectious disease, cancer-related mortality decreased by 12% for men and 9% for women between 1997-2006, with significant reductions in cervix, stomach, bowel, lung and breast cancer patient mortality [Cancer Research UK, 2008a]. Reduced cancer death is primarily the result of improved primary prevention

of cancer, earlier detection and better treatment. However, the multitude of molecular events governing the genetic changes observed in cancer onset remains a hugely active area of scientific research. Insight into the underlying mechanisms that dictate malignant cell growth, commonly identified as self-sufficient growth signalling, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [Hanahan & Weinberg, 2000], will provide essential therapeutic targets; imperative to relinquishing the grasp cancer has on the health of global society.

1.2 Breast cancer- Incidence and risk factors.

Breast cancer is now considered the most prevalent cancer amongst women, with over 1 million cases diagnosed per year worldwide [Ferlay *et al*, 2005]. Approximately 44,000 women and 300 men are diagnosed with breast cancer every year in the UK, and 12000 female and 100 male breast cancer patients will die from the disease annually [Cancer Research UK, 2008b]. Over the twenty five year period between 1981 and 2005, the incidence rate increased by 57% [Cancer Research UK, 2008c]. This was, in part, due to the introduction of a national screening programme in 1988, leading to a transient additional increase in breast cancer incidence in women aged 50-64, as early undiagnosed cancers were detected [Quinn & Allen, 1995]. Approximately 14,000 cases are annually diagnosed as a result of the NHS breast screening programme, and it is thought to have saved the lives of approximately 1400 women per year [Advisory Committee on Breast Cancer Screening, 2006].

Indeed, since 1980, mortality rates have reduced by a third, and since 1999, breast cancer was superseded by lung cancer as the most common cause of death from cancer in women in the UK [Cancer Research UK, 2008d].

The greatest risk factor in the development of breast cancer is age; with 8 out of 10 newly diagnosed cancers in women over the age of 50 [Office for National Statistics, 2005]. There is considerable evidence that associates the increased breast cancer risk in individuals of this age with long-term exposure to elevated oestrogen levels [Key *et al,* 2001]. A catalogue of events over a women's life time can contribute to this phenomenon; such as early menarche and late menopause [Kelsey *et al*, 1993], late first full-term pregnancy and reduced parity [Layde *et al*, 1989], and the choice not to breast-feed [Lipworth *et al*, 2000]. Oral contraceptives [Collaborative Group on Hormonal Factors in Breast Cancer, 1996] and oestrogen-replacement therapy [Beral, 2003] have also been implicated in breast cancer risk. In addition, other modifiable risk factors include obesity [Van den Brandt, 2000], alcohol consumption [Hamajima, 2002] and lack of exercise [Key *et al,* 2001], although their true involvement in breast cancer remains to be determined.

Although over 85% of women who develop breast cancer have no family history of the disease, evidence suggests that a genetic pre-disposition to breast cancer can be passed on through generations of women. An individual with one affected first degree relative (mother or sister) has approximately double the risk of breast cancer of a woman with no family history of the disease; if two (or more) relatives are affected, her risk increases further [Collaborative Group on Hormonal Factors in Breast Cancer, 2001]. Several

genes have been identified as major contributors to familial breast cancer, most significantly, the breast cancer susceptibility gene, BRCA1, which is aberrantly expressed in 2-5% of all breast cancer cases. Women carrying a BRCA1 mutation have a 50-80% chance of developing the disease [Ford *et al,* 1998].

Breast cancer incidence and mortality varies significantly across the world, with highest rates recorded in more economically developed areas such as the US and affluent European nations, where incidence is five-fold higher than in the less economically developed countries of Africa and Asia [Key *et al,* 2001]. Worryingly, in areas of the world with historically low rates of breast cancer, such as Eastern Europe and the Far East, incidence has begun to rise rapidly as countries adopt western ideals and consequently, the breast-cancer related risk factors associated with this way of life. Interestingly, migrants from low to high risk countries acquire the risk of the host country within two generations [Key *et al,* 2001], highlighting the role of life-style, socio-economic status and environmental factors in the development of breast cancer.

1.2.1 Oestrogen and breast cancer.

The circulating oestrogens, of which 17β-oestradiol (E2) is biologically the most important, are members of a unique family of aromatic steroids that are produced primarily by the aromatase enzyme system in the ovaries, as well as in other tissues such as the brain, liver and body fat, which become the main sites of oestrogen synthesis in women following the menopause [Dowsett *et al*

2005]. Oestrogens serve to maintain the homeostasis of many different tissues, such as bone, blood vessels, urinogenital tissues, and breast [White and Parker, 1998]. Although the actions of oestrogen are primarily beneficial to health of normal female development, experimental and clinical data dating back over 100 years indicate that a significant proportion of breast cancers are dependent on oestrogens for their development. In 1896, George Beatson first demonstrated that removal of the ovaries resulted in regression of the primary tumour in a proportion of women [Beatson, 1896]. Four years later, a study reported by Stanley Boyd established that a third of patients could expect regression of their disease following this surgery [Boyd, 1900]. The mechanism by which this phenomenon occurred was not identified until 1962, when the first oestrogen receptor (ER) was described by Jensen and Jacobson in the uterus of rats [Jensen and Jacobson, 1962]. Following extensive biochemical analysis, Jensen and colleagues translated the basic science into clinical utility by formulating the first ER assay, to determine whether patients would respond to endocrine ablation either by oophorectomy in pre-menopausal women, or by adrenalectomy in post-menopausal women [Jensen *et al,* 1971]. It was observed that women with ER-rich tumours responded to endocrine ablation, whilst women with ER negative breast tumours were unlikely to respond and were faced with a poorer prognosis [McGuire *et al,* 1975]. It is now established that approximately 70% of breast cancers are initially dependent on oestrogen and functional ER for their growth and development [Clark *et al*, 1984].

1.3 The oestrogen receptor- Structure and activation.

There are two main forms of human ER, ERα and ERβ, both of which are members of the nuclear hormone receptor family. ERα was cloned and sequenced from MCF-7 human breast cancer cells in 1986 by Green *et al* [Green *et al,* 1986]. It was 10 years later that the second receptor, ERβ, was identified and cloned from a rat prostate complementary DNA library [Kuiper *et al*, 1996]. The human ER α gene resides on chromosome 6q sub band 25.1 and is transcribed as a single mRNA of 6.5kb that encodes a protein of 595 amino acids, with an approximate molecular mass 66kDa. The ERβ gene is located on chromosome 14q22-24 and encodes a protein of 530 amino acids, with a molecular mass of around 60kDa. Both receptors have been separated into six functional domains which were designated A – F, from N- to C terminus and share substantial homology [Kumar *et al,* 1987] [Fig 1.1].

The A/B domain contains one of the two activation functions (AF1) present in ER, which is responsible for the activation of gene expression by ER in the absence of ligand. AF-1 is constitutively active and often works in synergy with the second, ligand-dependent activation function of ER (AF-2), to further activate gene transcription in a promoter- and cell -specific manner [Tzukerman *et al,* 1994]. However, post-translational modifications to AF-1 such as phosphorylation can increase its activity, permitting independent activation of gene transcription [Chen *et al,* 1999]. The amino terminal A/B domain is the site of greatest variability between ERα and ERβ, sharing a homology of only 18% [Hall and McDonnell, 2005]. A comparison of the AF-1 domain in the two ERs has revealed that AF-1 is very active in ERα on a

variety of oestrogen responsive promoters, but under identical conditions, the activity in ERβ is minimal [Barkhem *et al,* 1998].

The C region contains both the DNA binding domain (DBD) and the dimerisation domain. The DBD consists of two zinc-fingers that fold into two helical structures; one directly binds to the major groove of the DNA helix, while the other serves to support this interaction [Schwabe *et al*, 1993]. ER that lacks the DBD cannot bind DNA *in-vitro* or *in-vivo* which is crucial for genomic, ER-regulated gene activation [Kumar *et al,* 1987]. The dimerisation domain has been discovered equally critical for ER activation, as mutations that prevent or impede dimerisation result in receptors that are insoluble or transcriptionally inactive [Tamrazi *et al,* 2002]. The C-domain shares a homology of 97% between ERα and ERβ, indicative of this regions importance in ER function [Hall and McDonnell, 2005].

The D domain functions as a hinge region between the DBD and the ligand binding domain (LBD), and also contains sequences for dimerisation, nuclear localisation signal (NLS) function and interactions with receptor co-activator and co-repressor proteins [Klinge, 2001].

The C- terminal E domain is the largest of the six regions and comprises of the LBD, which is intrinsically linked to the ligand-dependent activation function of ER, AF2, located in the same region [Parker *et al,* 1993]. The LBD has a compact three layer structure comprising of 12 α-helices, 5 of which (H3, H6, H8, H11, and H12) form a hydrophobic ligand binding pocket [Sommer and Fuqua, 2001]. Binding of endogenous ligand 17-β oestradiol (E2) to the LBD alters its conformation, with helix 12 forming a lid over the pocket that secures

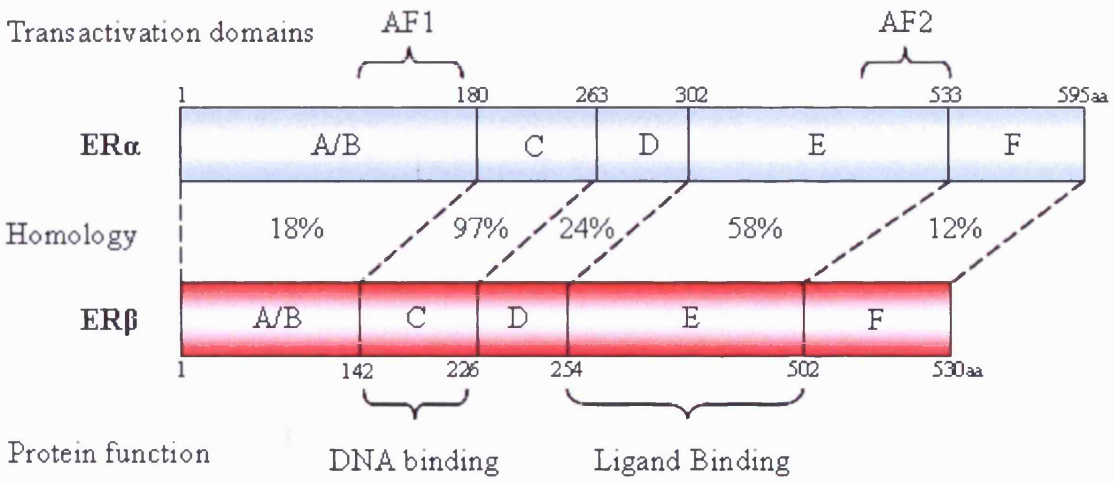
the ligand. The agonist induced positioning of H12, together the with amino-acid residues in H3, H4 and H5, provides an adequate co-activator interaction surface permitting the recruitment of the co-factors necessary for efficient AF-2 mediated, ER transcriptional activity [Brzozowski *et al,* 1997]. The E-domain also contains sequences for heat-shock protein 90 binding, ligand independent nuclear localisation signalling (NLS) function, and a dimerisation domain [Parker *et al,* 1993].

Though less is known about the F domain, it is thought to play a role in distinguishing between ER agonists and antagonists through interactions with cell specific factors [Klinge, 2001].

ERα and ERβ share poor to moderate homology of these C-terminal domains; D - 24%, E - 58%, F - 12%, highlighting the potential for varying patterns of gene expression incurred following endogenous/exogenous ligand-dependent activation of ERα and ERβ [Hall and McDonnell, 2005].

The understanding of the role of ERβ in breast cancer is rapidly expanding, though still in its infancy compared to ERα; thus for the purpose of this thesis, ERα will be the focus of the investigation, and is hereafter referred to as ER.

Fig 1.1 Comparison of the ER-α and ER-β functional domains.



1.3.1 Genomic oestrogen receptor signalling.

In the absence of ligand, ER is sequestered in cell nuclei within a large inactive oligomeric complex containing heat shock proteins HSP90, HSP70, and cyclophilin-40 and p23 [Pratt and Toft, 1997]. E2 diffuses through the cell plasma-membrane, translocates to the nucleus, and binds to ER [Rao, 1981] causing conformational alterations to the LBD and phosphorylation of ER residues, releasing of ER from the inhibitory protein complex and permitting maximal AF-1 activity. Receptors rapidly dimerise and translocate to the oestrogen response element (ERE) in the promoter sequence of an oestrogen responsive gene [Osborne and Schiff, 2005]. The ERE consists of inverted repeats of the sequence GGTCA separated by three variable nucleotides, e.g. 5'- CAGGTCAnnnTGACCTG-3,' however, most oestrogen-regulated genes

contain imperfect, non-palindromic ERE sequences [Driscoll *et al,* 1998]. Once bound to the DNA, the receptor complex can either positively or negatively regulate target gene transcription through the specific recruitment of co-regulatory transcription factors. Co-regulators can influence the recruitment of general transcription machinery as well as other co-factors, and can also modify the chromatin environment surrounding the promoter of the targeted gene to further facilitate gene activation/inactivation (further discussed in section 1.3.1.1) [Klinge, 2001]. This is referred to as the classical model of ER signalling. Ligand bound ER can also regulate gene expression by tethering to other DNA bound transcription factors and acting as a co-activator by strengthening DNA binding and recruiting other co-activators to the transcription-factor complex. For example, by associating with the c-fos and c-jun transcription factors, ER can promote the expression of genes that contain the AP-1 response element in their promoter region, such as cyclin D1, ovalbumin, collagenase, and the growth factor ligand IGF-1 [Kushner *et al,* 2000].

1.3.1.1 Co-regulation of genomic oestrogen receptor activation.

Co-Activators

Co-activators (CoAs) are proteins that interact with steroid receptors and enhance transcription of their target genes by further facilitating recruitment of general transcription machinery and other CoAs, stabilising the transcription complex and importantly, some are capable of post-translational modification

to the chromatin environment within which the target gene is organised, leading to efficient ER-regulated gene transcription.

Three related co-activators, collectively known as the p160 co-activators, contribute to ligand bound ER activity through direct interaction with AF2; nuclear-receptor co-activator 1 (NCoA1; also known as SRC1), NCoA2 (also known as TIF2 or GRIP1) and NCoA3 (also known as P/CIP, ACTR, AIB-1, RAC3 or TRAM1) [McKenna *et al,* 1999]. They contain a conserved motif called the nuclear receptor (NR) box, which comprises of an α-helical LxxLL structure (L represents leucine and x represents any amino acid) in which the leucines create a hydrophobic surface that fits into the major groove of the receptor's AF-2 [Hall and McDonnell, 2005]. Other co activators contain NR boxes, including the SWI/SNF complexes [Sudarsanam & Winston, 2000], p300/CBP-associated factor (PCAF) [Vo & Goodman, 2001], and the TRAP/DRIP/SMCC complex [Ito & Roeder, 2001].

A common feature of the co-activators recruited by ER is that of intrinsic histone-acetylase (HAT) activity [Ogryzko *et al,* 1996; Spencer *et al,* 1997]. The positive charge on un-acetylated (tri-methylated) lysine residues of the histone protein-octamer round which DNA is tightly wrapped is highly attracted to the negatively charged DNA phosphate backbone, producing a compact chromatin state that limits access to general factors of transcription, and thus limits the efficiency of gene activation. HAT initiates local hyper-acetylation of the lysine-rich tails of histones H3 and H4, removing the positive charge and resulting in a less condensed chromatin environment, facilitating gene transcription [Tsukiyama & Wu, 1997]. Histone deacetylase

(HDAC) removes the acetyl groups from the lysine residues, which reverses this process and reduces gene activation [Fig 1.2].

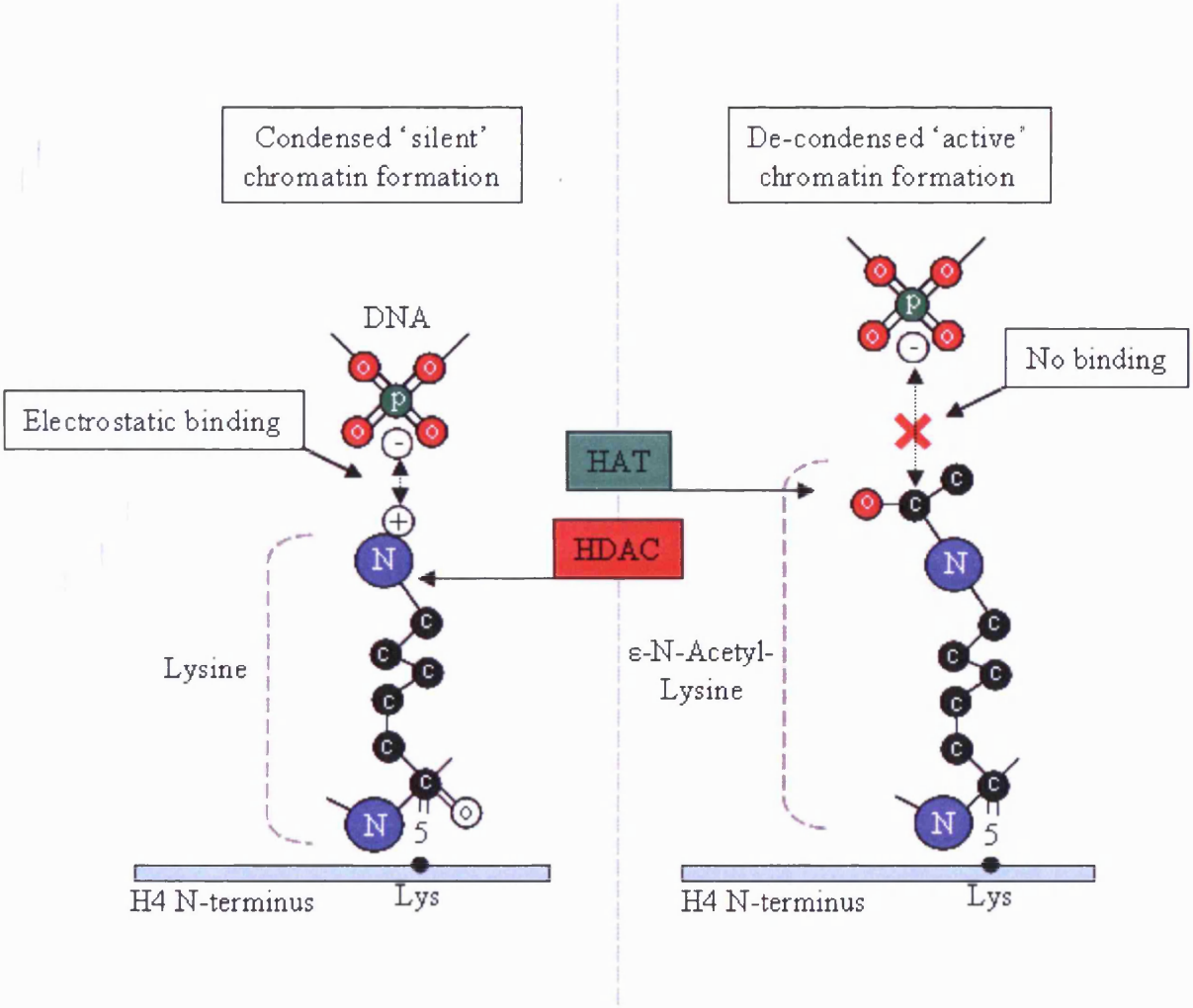
In addition to the CoAs that enhance receptor activity by interacting with the LBD, AF-1-interacting CoAs have also been described, specifically steroid receptor RNA-activator (SRA) and p68RNA helicase [Lanz *et al,* 1999; Endoh *et al,* 1999].

Co-Repressors

Co-repressors (CoRs) interact with nuclear receptors and serve to reduce transcription of the gene to which the receptor/CoR is tethered. This is achieved through a wide variety of mechanisms including chromatin remodelling, binding competition with CoAs, sequestration of ER in the cytoplasm, and interference with DNA binding [Dobrzycka *et al*, 2003]. The first CoRs to be identified were the nuclear receptor co-repressor (NCoR) [Horlein *et al,* 1995] and silencing mediator for retinoid and thyroid hormone receptor (SMRT) [Chen and Evans, 1995]. The interaction between these CoRs and the LBD is mediated by two NR-interacting domains (CoR-NR boxes) similar to those found in the NR boxes of CoAs [Hu and Lazar, 1999]. NCoR and SMRT both serve to suppress transcriptional activation of ER in the absence of ligand by the recruitment of other CoRs, such as mSin3 which associate with HDACs [Hu and Lazar, 2000]. Other recently described CoRs, including repressor of oestrogen receptor activity (REA) [Montano *et al,* 1999], interact with the ER at novel interaction sites, confirming the existence of additional binding motifs, other than CoR-NR boxes.

NCoR and SMRT are functionally similar as they interact with un-liganded receptors, enhance repression, dissociate upon agonist binding, and contain intrinsic silencing domains [Horlein *et al,* 1995; Chen and Evans, 1995]. However, other CoRs of ER, such as nuclear receptor-interacting protein 1 (RIP140), associate with ligand bound ER, and occlude access of CoAs to the AF-2, whilst interacting with HDAC complexes [Smith & O'Malley, 2004]. Thus the existence of CoRs that moderate agonist activities of oestrogens provides an additional mechanism for the fine tuning of the expression of ER target genes and attenuating the physiological output in situations where there are chronically elevated levels of the hormone.

Fig 1.2 N-terminal histone tails contain several lysine residues which have a positively charged amino group in their side chain. This amino group can be acetylated by histone acetyletransferase (HAT), a reaction that can be reversed by histone deacetylase (HDAC). The presence of a positive charge on the lysine amino acids of the histone tails increases electrostatic interactions with the negatively charged phosphate backbone of the DNA, and hence serves to condense the chromatin environment. The removal of this positive charge (by acetylation) opens the chromatin structure, permitting gene transcription. Thus, active genes are usually located in hyperacetylated chromatin, whilst inactive genes are found in hypoacetylated chromatin.

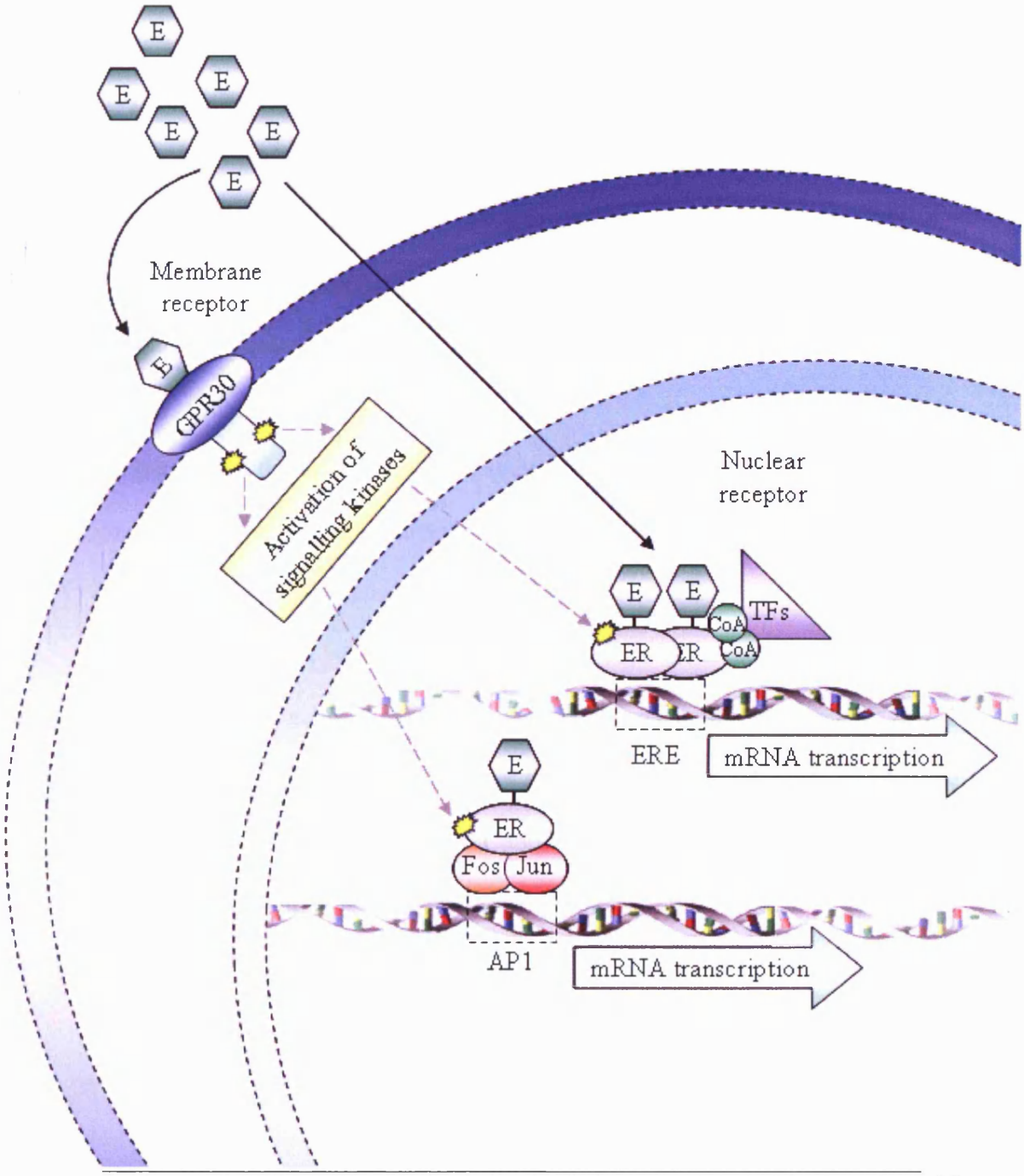


1.3.2 Non-nuclear oestrogen receptor signalling.

It is evident that in some oestrogen responsive tissues, such as bone and the epithelium, non-nuclear, membrane bound-ER is predominant in these cells [Nemere *et al,* 2003]. In breast cancer cells, data indicates that in addition to nuclear ER, cytoplasmic and membrane ER are present [Losel *et al,* 2003]. Significantly, GPR30, the newly described membrane ER, can activate key growth-factor signalling pathways, such as epidermal growth factor receptor (EGFR) [Filardo *et al,* 2000], to initiate a rapid primary cellular response to hormonal signalling [Gee *et al,* 2005; Levin, 2003]. Such interaction leads to the downstream activation of mitogen activated protein kinase (MAPK) and serine/threonine protein kinase (Akt) signalling pathways, which up-regulate the transcriptional activity of E2-bound nuclear ER by phosphorylating Ser118 and Ser167 residues present within the AF1 domain of the receptor [Kato *et al,* 1995; Martin *et al,* 2000]. Thus, non-nuclear ER can deliver pro-transcriptional signals to ER-responsive genes rapidly, and in a fashion complementary to nuclear ER activation through crosstalk with growth factor signalling.

The ER activation pathways are summarised in [Fig 1.3].

Fig 1.3 ER Activation: Upon ligand binding (E), nuclear ER activates transcription either by direct DNA binding to its own response element (ERE) in the targeted gene promoter followed by co-activator (CoA) and general transcription factor (TF) recruitment (classical mode), or by tethering to other transcription factors, such as the Fos/Jun activating protein-1 (AP1) complex (non-classical mode). Ligand-bound membrane ER (GPR30) can interact with different signalling intermediates at the vicinity of the membrane causing rapid induction of key growth-factor-dependent kinases, which phosphorylate nuclear ER at specific residues in the AF-1, promoting receptor activity (non-nuclear action).



1.4 Oestrogen receptor activation of cell growth.

In order to achieve E2 mediated cell proliferation, the E2/ER transcription complex up-regulates the expression of a variety of genes which directly promote breast cancer cell growth, including key components of the insulin-like growth factor (IGF) signalling system, IGF-1R and insulin-receptor substrate-1 (IRS-1) [Ye, 1998]. In addition, promoters of the cell-cycle, like cyclin D1 [Wilcken *et al,* 1997] and cMyc [Dubik *et al,* 1987] (which can also regulate genes associated with proliferation and/or apoptosis – reviewed in Oster *et al*, 2002), genes associated with DNA synthesis, such as replication factor C4 (RFC4) and minichromosome maintenance genes (MCM2, MCM3, MCM6), and genes encoding proteins for anti-apoptotic signals, like survivin, are all up-regulated by E2 in ER-positive breast cancer cells [Frasor *et al*, 2003].

In parallel to the up-regulation of oncogenes, recent studies have shown that the ability of the E2/ER transcription complex to act as a suppressor of gene expression also facilitates oestrogen activation of ER-positive breast cancer cell growth. In a study conducted by Frasor *et al* (2003), E2-induced enhancement of cell proliferation was shown to be significantly reliant on the down-regulation of numerous factors that are known to inhibit the cell cycle such as B-cell translocation gene 1 and 2 (BTG-1, BTG-2), and cyclin G2, a gene known to induce cell cycle arrest and to be up-regulated in cells during apoptosis [Frasor *et al*, 2003]. Other down regulated genes included transcriptional repressors, such as Max dimerization protein-4 (Mad4), which inhibits Myc action by competing for their binding partner Max [Grandori *et*

al, 2000]. Thus, E2 could enhance Myc activity not only by up-regulating c-Myc, but additionally by down-regulating an inhibitor of Myc.

E2 was also shown to down-regulate the expression of growth factors that are known to inhibit cell proliferation, such as members of the transforming growth factor beta (TGFß) family members TGFβ-3 and bone morphogenic protein 4 (BMP4), and pro-apoptotic genes, B-cell lymphoma protein 2 (BCL-2) interacting killer (BIK), BCL-2 antagonist/killer 1 (BAK-1), and caspase-9. These findings highlight the variety of molecular functions that activated ER manipulates to enhance cell growth, and in particular the emerging significance of E2/ER mediated gene suppression. The underlying molecular biology of this phenomenon remains poorly understood. One mechanism proposed is that the E2/ER complex enters into protein/protein interactions with further transcription factors (e.g. NFκB) leading to repression at diverse response elements [Stein and Yang, 1995]. There are also data to suggest that the E2/ER complex may recruit repressors to some gene promoters. For example, the E2/ER complex recruits NCoR and interestingly, HDACs to the promoter of the anti-proliferative gene cyclin G2, in association with release of RNA polymerase II and transcriptional repression of the gene's oestrogen responsive-half-ERE [Stossi *et al,* 2006]. There is emerging data that such repressive events can occur at other ERE-bearing promoters in breast cancer cells [Kaipparettu *et al,* 2008; Ye *et al,* 2008].

1.5 Anti-oestrogen therapy

Having established the extensive involvement of oestrogen in breast cancer, it is hardly surprising that traditional therapies for ER-positive breast cancer patients have included hormone-ablative surgery, such as oophorectomy, adrenalectomy and hypophysectomy. Such procedures have largely been superseded by current endocrine therapies which are based on the targeting of the ER signalling pathway by either partial antagonism of ER function with selective modulators of ER activity (SERMs), down-regulation of ER using pure anti-oestrogens (SERDs) or reducing oestrogen synthesis with aromatase inhibitors (AIs).

1.5.1 Clinical use of tamoxifen.

The most commonly used anti-hormone treatment for patients with ER-positive breast cancer is tamoxifen. Tamoxifen was first approved in the UK in 1973 and by the Food and Drug Administration (FDA) in the USA in 1977 for the treatment of advanced breast cancer. The drug has since been approved as an adjuvant therapy with chemotherapy (1986), as a single adjuvant therapy in postmenopausal patients with metastatic disease (1988), and in pre- and postmenopausal ER-positive non-metastatic breast cancer patients [Park and Jordan, 2002]. Its widespread use over the last 30 years in all stages of ER-positive breast cancer has significantly contributed to the decrease in national breast cancer mortality rates observed during this period [Jaiyesimi *et al,* 1995].

1.5.2 Selective oestrogen receptor modulation by tamoxifen.

The ability of tamoxifen to act either as an agonist or antagonist in an oestrogen-sensitive cell specific context can be explained by the fact the tamoxifen only blocks ER activation through one of its two main activation functions. Contrary to the initial understanding that tamoxifen simply stopped ER activation upon receptor binding, it is now established that tamoxifen-bound ER is able to dissociate from its inhibitory protein complex and dimerise in a manner equivalent to E2-bound ER [Nicholson *et al,* 2002]. The binding of ER to its natural ligand causes a conformational change to the LBD, whereby H12 of the LBD seals the ligand into the receptor, forming an active AF-2 domain by providing the necessary surface required to recruit general components and co-activators of transcription (see section 1.3). However, when tamoxifen binds to the LDB, helix 12 is prevented from sealing the binding pocket due to the bulky alkylaminoethoxyphenyl side chain of tamoxifen interacting with the Asp 351 residue of the LBD [Shiau *et al,* 1998]. Therefore co-facilitators of transcription cannot bind to the LBD of tamoxifen-bound ER and AF-2 activation of gene transcription is prevented. Furthermore, tamoxifen induced conformational changes to the LBD have been shown to facilitate the recruitment of co-repressors to the site of transcription, further repressing AF-2 gene activation [Lavinsky *et al.* 1998]. Tamoxifen however does not inhibit ER's constitutively active, ligand independent activation function AF-1; therefore it has been proposed that for genes where AF-2 is required for ER transcriptional activity, tamoxifen functions as a pure antagonist, and conversely, genes for which AF-1 is

sufficient for ER transcriptional activity, tamoxifen can act as an agonist [Tzukerman *et al,* 1994].

1.5.3 Tamoxifen resistance.

Despite the obvious benefit tamoxifen has provided for millions of breast cancer patients worldwide, almost all patients with metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen will acquire resistance to the drugs inhibitory effect on breast cancer cell growth [Schiff *et al,* 2003]. Unfortunately, patients who experience disease relapse may develop a more aggressive form of the disease, and face a poorer prognosis and premature death [Hiscox *et al,* 2004]. The biological mechanisms underlying acquired resistance to tamoxifen are therefore of considerable clinical significance.

Originally it was thought that the acquisition of anti-oestrogen resistance was caused by a loss or mutation of the ER, as is often the case in patients with intrinsic anti-hormone resistance [Ring, and Dowsett, 2004]. However, it has since been shown that breast cancer cell lines that have lost anti-oestrogen sensitivity can retain an ER positive phenotype with normal ER functionality [Brünner *et al,* 1993]. Indeed tamoxifen-resistant breast cancer remains sensitive to other hormonal therapies that target the ER such as the pure anti-oestrogen fulvestrant (Faslodex®) [Robertson, 2001]; although it too is subject to the development of resistance mechanisms (see section 1.6).

1.5.3.1 Positive growth signals up-regulated in tamoxifen resistant breast cancer.

Many mechanisms have been associated with tamoxifen resistance in ER positive breast cancer (reviewed in Ring and Dowsett, 2004). Significantly, the up-regulation of growth factor receptors, e.g. EGFR and HER2, and their downstream kinases, notably Ras/Raf/MAP kinases and phosphoinositide 3-kinase [PI3K]/Akt signalling, has been extensively associated with both de-novo and acquired tamoxifen resistant breast cancer cell growth [Gee *et al*, 2005; Nicholson *et al*, 2007]. These growth factor signalling pathways can be directly and indirectly activated by tamoxifen binding to membrane-bound ER [Shou *et al,* 2004]. The subsequent activation of the multiple signalling kinases leads to the phosphorylation of nuclear ER at specific sites in the AF-1 domain and promotes ER activity. Significantly, phosphorylation of ER serine 118 by the MAPKs extracellular signal-regulated kinase (ERK) 1 and ERK2, down-stream components of the EGFR/HER2 pathway, and phosphorylation of serine 167 by ribosomal S6 kinase (RSK) (activated by ERK1 and ERK2) enhances sensitivity of the ER to oestrogenic stimulation and have been cumulatively implicated in promoting the agonistic behaviour of tamoxifen in resistance [Nicholson *et al,* 2004b].

Other activated kinases serve to phosphorylate co-regulators of ER, further propagating their ability to activate ER-regulated gene transcription. For example, NCoA3 is phosphorylated by multiple kinases including p42/44MAPK, which can be activated by HER2 [Font de Mora & Brown, 2000]. Therefore, high levels of activated CoAs like NCoA3 could profoundly

limit the efficiency of anti-hormone treatment of tumours that also overexpress tyrosine kinase receptors [Osborne *et al,* 2003]. In contrast, phosphorylation of CoRs can result in their nuclear export, preventing their recruitment to the ER transcriptional complex, and therefore limiting gene suppression [Arpinol *et al,* 2008].

In addition to activation of ER-regulated growth pathways, some kinase activity can independently stimulate proliferation via activation of their associated gene networks. For example, Akt, which is directly activated as a consequence of ER interaction with the p85a regulatory subunit of PI3K, can promote cell growth, survival and motility [Vivanco & Sawyers, 2002].

EGFR and HER2 expression and activity have been found to be considerably increased in several *in vitro* models of tamoxifen-resistance, including the MCF-7 cell based model developed at the TCCR. The contribution to resistant-cell growth has been highlighted in studies using the EGFR specific tyrosine kinase inhibitor, gefitinib (Iressa®), and the HER2 monoclonal antibody trastuzumab (Herceptin®). Tamoxifen resistant cells exhibited significant growth inhibition in response to these agents compared to tamoxifen-responsive MCF-7 cells [Nicholson *et al,* 2001; Nicholson *et al,* 2004a]. The use and success of these drugs in both experimental and clinical representations of anti-hormone resistant cancer are discussed in section 1.6.1.

1.5.3.2 Inactivation of tumour suppressor genes in tamoxifen resistant breast cancer.

In addition to the activation of oncogenes, it has become clear that the inactivation of tumour suppressor genes like p53 is at least as important in the development and progression of breast cancer. In many cases, gene inactivation is the result of genetic events such as of loss of heterozygosity and mutation, however, it also been shown that functional inactivation of tumour suppressor genes can be caused by epigenetic mechanisms [Jones and Baylin, 2002]. Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency, are reversible upon treatment with pharmacological agents and occur at defined regions in a gene [Jones and Laird, 1999]. Epigenetic gene inactivation is a heritable trait that is not based upon a change in primary DNA sequence, but rather alterations that impact on chromatin organisation and gene promoter accessibility, like histone modification and DNA hypermethylation of promoter CpG islands.

Promoter hypermethylation has been revealed as one of the most frequent mechanisms of loss of gene function in human neoplasia, and in the specific example of breast cancer, genes involved in every step of tumour development have been shown to be silenced by this epigenetic mechanism (reviewed by Widschwendter and Jones, 2002).

CpG island methylation is catalysed by a family of DNA methyl-transferases (DNMTs) by use of the universal methyl donor S-adenosyl-methionine, and inhibits transcription by interfering with recruitment and function of basal transcription factors or co-activators [Tate & Bird, 1993]. It has also been

suggested that the hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate recruitment of members of the methyl-CpG binding domain (MBD) protein family, which may mediate silencing of genes through reducing chromatin accessibility to co-activating complexes [Wade *et al,* 2001].

Literature indicates that by suppressing E2-stimulated genes, long-term ER signalling disruption has the capacity to completely silence gene transcription through hypermethylation of CpG islands in their promoters [Leu *et al,* 2004]. Proof of principle studies describe the impact of ER siRNA on the ER-regulated genes progesterone receptor (PR) and trefoil-factor 1 (pS2), where initial repressive chromatin modifications with longer-term progressive accumulation of DNA methylation at the EREs of the promoters were observed. Their expression could be restored by treatment with a methylation inhibitor and E2 treatment. Subsequently, evidence has been generated to suggest that epigenetic modifications to E2-stimulated gene promoters can occur following long-term anti-hormone induced gene ER-signalling disruption [Badia *et al,* 2000]. In addition, a study conducted by Fan *et al* (2006) showed that 75% of genes that were identified as up-regulated in oestradiol treated wt-MCF-7 breast cancer cells (≥2 fold increase in expression), were no longer inducible in the tamoxifen-resistant cell sub-line, highlighting the potential scale of this phenomenon.

In section 1.5.2, it was mentioned briefly that the conformational changes induced in the LBD by ER binding tamoxifen served to provide docking sites for co-repressors of transcriptional activity. Interaction studies showed that

various corepressors including NCoR/SMRT [Lavinsky et al. 1998], REA [Montano *et al,* 1999], repressor of tamoxifen transcriptional activity (RTA) [Norris *et al.* 2002], scaffold attachment factor-B1 (SAFB1) [Oesterreich *et al.* 2000], and Smad4 [Wu *et al,* 2003] which recruit different HDAC protein complexes as part of their co-repressive function, bind more strongly to ERα in the presence of tamoxifen. During tamoxifen-induced suppression of the classically regulated oestrogen responsive gene, pS2, the time course of recruitment of the HDAC complexes precisely coincided with that of deacetylation of histone H3 and H4 tails at the target promoter, providing crucial support for the hypothesis that tamoxifen functions as an antagonist in the breast cells by inducing epigenetic modification [Liu *et al,* 2004]. Interestingly, significant crosstalk between the histone code and gene promoter methylation has been established. DNMT-1 and DNMT3a/DNMT3b have been shown capable of binding HDAC2 and HDAC1 respectively to achieve effective gene silencing [Fuks *et al,* 2000; Rountree *et al,* 2000; Bachman *et al,* 2003]. Although several studies have shown that long-term tamoxifen treatment can induce distinct global gene expression and promoter DNA methylation profiles in breast cancer cells [Fan *et al,* 2006; Badia *et al,* 2000], few studies have whether this event could contribute to anti-hormone resistant cell growth. Interestingly, a study by Treeck *et al* (2004) reported that long-term tamoxifen treatment of MCF-7 cells decreased levels of several pro-apoptotic genes and impaired subsequent apoptotic response to etoposide treatment. Another study conducted by Wu *et al* (2007) used SAGE analysis to show that the expression of the tumour suppressor gene retinoblastoma

binding protein 8 (CtIP) was decreased in acquired tamoxifen resistant models, where its knockdown in MCF-7 cells promoted tamoxifen resistance, and induction restored response. Although promoter methylation of the tumour suppressor genes identified in these studies was not confirmed, data supports the concept of anti-hormonal silencing of potential tumour suppressors.

1.6 Over coming tamoxifen resistance in the clinic – Alterative anti-hormone therapies.

The successful use of tamoxifen over the last 30 years as first-line treatment for ER-positive breast cancer has established ER modulation as an effective therapeutic strategy for this disease. However, as discussed in section 1.5.3, approximately 40% of patients receiving tamoxifen acquire resistance to the drug, and frequently relapse with worsened outlook. Failure to continue a response to tamoxifen therefore, does not mean patients remain at the same prognostic level; they are faced with a more life-threatening disease. Consequently, huge efforts have been made to identify more effective alternative SERMs with better efficacy. First generation SERMs, like toremifene and idoxifene, were closely related to the chemical structure of tamoxifen and were entered into clinical trials in the early 1990s. Phase III trials showed postmenopausal women with advanced breast cancer treated with these agents produced similar objective response rates to tamoxifen [Pyrhonen *et al,* 1997; Arpinol *et al,* 2003]. Second generation SERMs, which featured an altered triphenylethylene ring structure, such as raloxifene and

arzoxifene, were then introduced following preclinical studies that suggested that these agents possessed less agonist activity than tamoxifen [Black *et al,* 1982; Sato *et al,* 1998]. Unfortunately these findings were not supported by Phase II trials with the drugs [Budzar *et al*, 1988; 2001]. Paradoxically however, the agonist effect of raloxifene helped to preserve bone mineral density and the drug is now used in the prevention of osteoporosis [Ettinger *et al,* 1999].

Having established that the new generation SERMs were no more effective than tamoxifen in advanced breast cancer, and that alternative SERMs shared a high level of cross-resistance with tamoxifen, attention was turned to developing other methods of ER signalling attenuation. One such method is the pure antagonism of ER signalling with the steroidal analogue of 17β-oestradiol, fulvestrant (Faslodex®). Fulvestrant binds to the ER with a similar affinity (89%) to oestradiol, but unlike SERMs, it has no agonist potential due to the steric hindrance caused by the alkylsulphinyl side-chain [Howell, 2006]. This causes an abnormal conformation of the ER protein, inhibiting receptor dimerisation and nuclear localisation. In addition, the ER-fulvestrant complex is unstable and leads to the subsequent rapid degradation of ER [Osborne *et al,* 2004]; hence fulvestrant is classified as a selective oestrogen receptor down-regulator (SERD). Fulvestrant was the first pure anti-oestrogen to enter clinical development; disappointingly it failed to demonstrate superiority over tamoxifen or aromatase inhibitors (AIs) in the treatment of advanced breast cancer possibly due to delivery problems [Robertson *et al,* 2002; 2003]. However, fulvestrant is the only drug of its class to be licensed for the

treatment of advanced breast cancer in postmenopausal women with disease recurrence or progression following anti oestrogen therapy, as both patients with acquired tamoxifen resistance and acquired AI-resistance, have been shown to gain clinical benefit from the drug when used as a second-line therapy [Howell *et al,* 2002; Ingle *et al,* 2006].

Aromatase inhibitors have been developed to suppress the activity of aromatase cytochrome P450, thereby reducing oestradiol synthesis [Smith & Dowsett 2003]. Through this mechanism AIs circumvent oestradiol-induced regulation of transcription via nuclear and non-nuclear pathways. They do not, however, directly target any contribution that oestrogen-independent ER activation may play in the development and progression of breast cancer.

Although the first and second generations of aromatase inhibitors failed to show any statistical benefit compared to tamoxifen in clinical trials, the third generation of more potent aromatase inhibitors have now been developed that almost completely inhibit aromatase activity, leading to a substantial reduction in oestrogen production [Geisler *et al,* 2002]. These include the steroidal aromatase inhibitor exemestane (Aromasin®), which binds to the p450 site of the aromatase complex, and the non-steroidal aromatase inhibitors anastrazole (Arimidex®) and letrozole (Femara®), which bind to the enzymes substrate binding pocket [Lønning, 2004]. Data from the phase III ATAC (anastrazole, tamoxifen and combination) clinical trial suggested that adjuvant anastrozole was superior to tamoxifen in terms of disease-free survival, time to recurrence, time to distant recurrence and prevention of contralateral breast cancer in postmenopausal women with early ER-positive breast cancer [Howell *et al,*

2005]. They are therefore set to challenge the status of tamoxifen as current first-choice treatment for these patients, pending further investigation into the effect that long-term inhibition of oestrogen synthesis has on other oestrogenic tissues. Interestingly, the combination treatment arm of tamoxifen and anastrozole showed no clinical benefit compared to tamoxifen alone, and was terminated early. This was thought to be due to the weak oestrogenic properties of tamoxifen activating growth signalling pathways, circumventing the growth inhibitory effect of oestradiol deprivation. AIs have also proven effective as a second-line therapy in post-menopausal patients with acquired tamoxifen resistant ER-positive breast cancer; although both fulvestant and AI acquired resistance have been reported in tamoxifen sensitive and resistant ER-positive breast cancer patients, as well as in numerous experimental models [Nicholson and Johnston, 2005].

1.6.1 Overcoming tamoxifen resistance in the clinic – Growth factor signalling pathway inhibitors.

As mentioned in section 1.5.3.1, the growth of tamoxifen resistant MCF-7 cells can be inhibited following the inactivation of the EGFR signalling pathway with gefitinib (Iressa®), or the inactivation of HER2 pathway using trastuzumab (Herceptin®). Pre-clinical studies in tamoxifen-resistant MCF-7 cells (TAM-R) developed in the TCCR showed that gefitinib treatment subsequently induced a concentration dependent inhibition of TAM-R cell growth, where 1μM gefitinib reduced proliferation by approximately 60% in correlation with a loss of phosphorylated EGFR [Nicholson *et al.* 2002,

2004b, Knowlden *et al.* 2003]. Phosphorylation of its heterodimerisation partner HER2 was also reduced in TAM-R cells together with pMAPK. Cell loss, however, was not achieved following gefitinib challenge, and ultimately resistance developed in association with a further gain in aggressive behaviour [Jones *et al.* 2004]. Treatment with trastuzumab similarly reduced pMAPK activity in TAM-R cells and temporarily inhibited cell growth. In the clinical setting, responses to trastuzumab as a single agent only occur in approximately 30% of HER2+ patients, and again, relapse is inevitable [McKeage and Perry, 2002].

The failure of sequential delivery of anti-hormone therapy followed by growth factor signaling inhibition to sufficiently block tumour progression has raised the possibility of combinational treatments being the most effective form of therapy for ER-positive breast cancer patients. In the specific example of tamoxifen and gefitinib combination treatment, the onset of tamoxifen resistance was significantly delayed in MCF-7 cells treated with both drugs, compared to cells receiving only tamoxifen; however, once again, cell loss was not achieved and resistance eventually developed [Gee *et al,* 2003]. Diverse clinical studies are also exploring the value of blocking downstream signalling components using including MAP kinase inhibitors, farnesyltransferase inhibitors and mammalian target of rapamycin [mTOR] inhibitors, however, to date many of these studies have proved relatively disappointing, with therapeutic resistance again a pervading problem [Johnston *et al*, 2007].

It is therefore apparent that blocking the up-regulated growth pathways featured in tamoxifen resistant cells, either singularly or in combination, may not be sufficient to cause complete tumour regression. In this context it is therefore possible that the cells are able to continue to grow as a result of a concurrent loss of growth 'braking mechanisms', i.e. loss of tumour suppressor/pro-apoptotic gene expression. Although the loss of tumour suppressor genes is a phenomenon identified in all stages of breast cancer, it is the ability of anti-hormone treatment, specifically tamoxifen, to permanently alter the expression of such genes by epigenetic mechanisms, as a contributing factor to tamoxifen-resistant cell growth that provides the focus of investigation for this thesis.

1.7 Aims

The aim of the current study will be to investigate whether the long-term tamoxifen treatment can cause the epigenetic silencing of oestrogen responsive genes associated with tumour suppressor / pro-apoptotic function, enhancing the cells ability to survive and potentially limiting the effect of second-line treatments.

In order to test this hypothesis, the following aims were pursued;

- The establishment of a tamoxifen-resistant cell sub-line, withdrawn from tamoxifen for up to 6 months, to determine the permanency of the aggressive tamoxifen-resistant cell phenotype, as assessed by cell morphology, growth and motility.
- The characterisation of ER, EGFR and IGF-1R mRNA and protein expression levels by real-time PCR, and Western blotting/immunocytochemistry (ICC) respectively, and their contribution to MCF-7, TAM-R and tamoxifen withdrawn TAM-R (TAM-Wd) cell growth using signalling inhibitors.
- The provision of proof of principle data to assess whether long-term tamoxifen treatment permanently silences well characterised, classically regulated ER-gene targets, pS2 and PR, using real-time PCR and ICC.
- The determination of whether genes silenced following long-term tamoxifen exposure could be re-expressed with the de-methylation

agent 5-Azacytidine (5-Aza) +/- oestradiol (E2) using real-time PCR and ICC in correlation with methylight analysis.

- The determination of whether the re-expression/activation of demethylated genes in tamoxifen withdrawn TAM-R cells effects cell growth.
- The use of micro-array technology to identify oestrogen responsive genes whose ontology and array profiles following 5-Aza + E2 co-treatment associate with cell growth (as determined above).
- The confirmation of gene expression profiles of the candidate genes identified in the microarray analysis, using semi-quantitative PCR.

2. MATERIALS AND METHODS

The equipment and disposables used throughout this study are listed below alongside the supplier from which they were sourced. Equipment is only referenced once, but may have been used in more than one experimental procedure.

Tissue Culture	
Equipment	Manufacturer
0.2µm Supor membrane VacuCap 60 filter unit	Gellman Laboratory Pall, Ann Arbour, USA
Aspiration pump	Gardner Denver Alton Ltd, Alton, UK
Cecil CE 2041 Spectrophotometer	CECIL, Cambridge, UK
Cell scrapers	Greiner Bio-One Ltd, Gloucestershire, UK
Class II biological safety cabinet	MDH Intermed Airflow from Bioquell, Andover, UK
Corning Standard Transwell® inserts (6.5mm diameter, 8µm pore size)	Fisher Scientific, Leicestershire, UK
Coulter Counter counting cups and lids	Sarstedt AG and Co., Nümbrecht, Germany
Coulter Multisizer II	Beckman, High Wycombe, UK
Cryo Storage Chamber	Taylor Wharton, Alabama, USA
Denly BA852 Autoclave	Thermoquest Ltd, Basingstoke, UK
Gilson Pipettes (1-10µl, 5-50µl, 20-200µl, 100µl-1000ml and 500µl-5ml)	Gilson, Luton UK
Glass coverslips (thickness no. 2, 22mm2)	BDH Chemicals Ltd, Poole, Dorset, UK
Hamamatsu C4742-96 digital camera	Hamamatsu Photonics UK Ltd, HERTS, UK
Hoffman Condenser	Leica Microsystems Imaging Solutions Ltd, Cambridge, UK
Improvision OpenLab V4.04 software	Improvision, Coventry, UK
Jouan C312 Centrifuge	Thermo Fisher Scientific Inc., MA, USA
Leica DM-IRE2 inverted microscope	Leica Microsystems Imaging Solutions Ltd, Cambridge, UK
Multiskan MCC/340 plate-reader	Titertek, USA

Nikon Eclipse TE200 Phase Contrast Microscope	Nikon, Kingston-upon Thames, UK
PowerMAC G5 computer	Apple Computer Inc., CA, USA
Quartz Cuvettes	Sigma-Aldrich, Poole, Dorset, UK
Sanyo MCO-17AIC incubator	Sanyo E&E Europe BV, Loughborough, UK
Sterile Syringe Needles (BD microbalance™ 3 characteristics: 25 G5/8 (0.5 X 16)	Becton Dickinson (BD) Biosciences Ltd, Oxford, UK
Syringes (5ml and 10ml)	Sherwood Medical Davis and Geck, Gosport, UK
Tissue culture plasticware (24, 96-well plates, filter flasks, 35mm, 60mm and 100mm dishes)	Nunc Int., Roskilde, Denmark
Vacuum flask	Gardner Denver Alton Ltd, Alton, UK

Semi-Quantitative and Real-time PCR	
Equipment	Manufacturer
Alpha Digidoc RT Densitometry Software	Alpha Innotech Corp. California, USA
GeneQuant RNA/DNA Calculator	Biochrom Ltd Cambridge, UK
IBM Personal Computer	IBM, UK
IEC Micromax RF Micro-centrifuge	Thermo Electron Corporation, Hampshire, UK
Labconco Purifier PCR Enclosure	GRI, Rayne, UK
Olympus 8mp Digital Camera	Olympus, Oxford, UK
Opticon 2™ real-time PCR machine	MJ Research Ltd, Massachusetts, USA
OpticonMONITOR™ Version 2.01	MJ Research Ltd, Massachusetts, USA
Powerpac 1000 power pack	Bio-Rad Laboratories Ltd, HERTS, UK
PTC-100 thermocycler	MJ Research Ltd, Massachusetts, USA
Sanyo 950W Microwave	Sanyo Europe, Loughborough, UK
Sub-cell® Agarose Electrophoresis System	Bio-Rad Laboratories Ltd, HERTS, UK
UV Transilluminator	Alpha Innotech Corp. California, USA

SDS-PAGE/Western Blotting	
Equipment	Manufacturer
HyperCassette™ developing cassette	Amersham, Little Chalfont, UK
DRI Block DB2A - Heating block	Techne, New Jersey, USA
IEC Micromax RF micro-centrifuge	Thermo Electron Corporation, Hampshire, UK
Magnetic Stirrer	Fisher Scientific UK Ltd, Loughborough, UK
Mini-Protean ® 3 electrophoresis apparatus	BioRad Laboratories Ltd (Hertfordshire, UK)
MXB Autoradiography Film (Blue Sensitive; 18 X 24 cm)	Genetic Research Instrumentation (GRI), Rayne, UK
Nitrocellulose membrane BA85(0.45μM)	Schleicher and Schuell, Dassell, Germany
Platform Rocker STR6	Stuart Scientific, Bibby Sterilin Ltd. (Stone, UK)
Powerpac Basic™ power pack	BioRad Laboratories Ltd (Hertfordshire, UK)
Roller Platform	Stuart Scientific, Bibby Sterilin Ltd. (Stone, UK)
X-O-graph Compact X2 x-ray developer	X-0-graph Imaging System, Tetbury, UK

Immunocytochemistry	
Equipment	Manufacturer
Olympus BH-2 phase contrast microscope	Olympus, Oxford, UK
Olympus DP-12 digital camera system	Olympus, Oxford, UK

Other Plastics and Disposables	
Equipment	Manufacturer
15-ml Phase Lock Gel (PLG) tubes	Eppendorf, Hamburg, Germany
Bijou tubes (5ml)	Bibby Sterilin Ltd., Stone, UK
Cryotube™ vials (1.8ml, starfoot, round)	Nunc Int., Roskilde, Denmark
Disposable Cuvettes	Fisher Scientific UK Ltd, Loughborough, UK
Eppendorf tubes	Eppendorf, Hamburg, Germany
Filter Paper (No. 4), Filter Paper (grade 3; 460 X 370mm)	Whatman, Maidstone, UK
General laboratory glass- and plasticware	Fisher Scientific UK Ltd, Loughborough, UK
Glass coverslips (thickness no. 2, 22mm2)	BDH Chemicals Ltd, Poole, Dorset, UK
Glass slides	Fisher Scientific UK Ltd, Loughborough, UK
Micro-centrifuge tubes (0.5ml and 1.5ml)	Elkay Laboratory Products, Basingstoke, UK
Pipette Tips	Greiner Bio-One Ltd, Gloucestershire, UK
Pipette tips	Greiner BioOne Ltd, Gloucestershire, UK
Sterile disposable pipettes (5ml, 10ml and 25ml), Falcon tubes (50ml), Coulter Counter lids and cups	Sarstedt AG and Co., Nümbrecht, Germany
Sterile Falcon tubes (15ml and 50ml)	Sarstedt AG and Co., Nümbrecht, Germany
Sterile universal containers (30ml)	Greiner Bio-One Ltd, Gloucestershire, UK
Sterile, disposable serological pipettes (5ml, 10ml and 25ml)	Sarstedt AG and Co., Nümbrecht, Germany
White 96 well qPCR plates and caps	Bio-Rad Laboratories Ltd, HERTS, UK

The chemicals and reagents used throughout this study are listed below alongside the supplier from which they were sourced. Chemicals are only referenced once, but may have been used in more than one experimental procedure.

Tissue Culture	
Reagent	Manufacturer
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich, Poole, Dorset, UK
Activated charcoal	Sigma-Aldrich, Poole, Dorset, UK
Amphotericin B (Fungizone)	Invitrogen, Paisley, UK
Antibiotics (penicillin/streptomycin)	Invitrogen, Paisley, UK
Bovine Trypsin	Lorne Laboratories Ltd, Reading, UK
Cell culture medium: RPMI 1640 and Phenol-red-free RPMI 1640	Invitrogen, Paisley, UK
Crystal violet	Sigma-Aldrich, Poole, Dorset, UK
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, Poole, Dorset, UK
Ethylene diamine tetraacetic acid (EDTA)	Sigma-Aldrich, Poole, Dorset, UK
Fibronectin (from Human Plasma; 1mg/ml in 0.05M TBS; pH 7.5)	Sigma-Aldrich, Poole, Dorset, UK
Foetal calf serum (FCS)	Invitrogen, Paisley, UK
Isoton® II azide-free balanced electrolyte solution (sodium chloride at 7.9g.l-1, disodium hydrogen orthophosphate at 1.9g.l-1, EDTA disodium salt at 0.4g.l-1, sodium dihydrogen orthophosphate at 0.2g.l-1 and sodium fluoride at 0.3g.l-1)	Beckman Coulter Ltd, High Wycombe, UK
L-glutamine	Invitrogen, Paisley, UK
Phenol/Chloroform/Isoamyl (25:24:1)	Sigma-Aldrich, Poole, Dorset, UK
Proteinase-K	Sigma-Aldrich, Poole, Dorset, UK
Sodium Acetate (NaOAc)	Sigma-Aldrich, Poole, Dorset, UK
Solvents (acetone, chloroform, ethanol, formaldehyde, isopropanol and methanol)	Fisher Scientific UK Ltd, Loughborough, UK
Sterile phosphate buffered saline (PBS)	Invitrogen, Paisley, UK
TRI-Reagent	Sigma-Aldrich, Poole, Dorset, UK
Tris HCl	Sigma-Aldrich, Poole, Dorset, UK
Triton X-100	Sigma-Aldrich, Poole, Dorset, UK

Semi-Quantitative and Real-time PCR	
Reagent	Manufacturer
Agarose	Bioline Ltd, London, UK
Di-thiothreitol (DTT)	Sigma-Aldrich, Poole, Dorset, UK
dNTPs (dGTP, dCTP, dATP, dTTP; 100mM)	Amersham, Little Chalfont, UK
DyNAmo™ qPCR kit	Finnzymes Oy, Espoo, Finland
Ethidium bromide (EtBr)	Sigma-Aldrich, Poole, Dorset, UK
Glacial Acetic Acid	Fisher Scientific UK Ltd, Loughborough, UK
Hyperladder™ I and Hyperladder™ IV	Bioline Ltd, London, UK
Magnesium chloride (MgCl2)	Sigma
Molony-murine leukaemia virus (MMLV) reverse transcriptase	Invitrogen, Paisley, UK
QIAquick PCR purification kit	QIAGEN Ltd, Crawley, UK
Random hexamers (RH)	Amersham, Little Chalfont, UK
RNase-free H2O	Sigma-Aldrich, Poole, Dorset, UK
RNasin® ribonuclease inhibitor	Promega, Southampton, UK

SDS-PAGE/Western Blotting	
Reagent	Manufacturer
Acrylamide/bis-acrylamide (30% solution (v/v), 29:1 ratio)	Sigma-Aldrich, Poole, Dorset, UK
Ammonium persulphate (APS)	Sigma-Aldrich, Poole, Dorset, UK
Aprotinin	Sigma-Aldrich, Poole, Dorset, UK
Bio-Rad DC Protein Assay (Reagents A, B and S)	Bio-Rad Laboratories Ltd, HERTS, UK
Bovine serum albumen (BSA)	Sigma-Aldrich, Poole, Dorset, UK
Bromophenol blue (BPB)	BDH Chemicals Ltd, Poole, UK
Di-potassium hydrogen orthophosphate anhydrous (K2HPO4)	Fisher Scientific UK Ltd, Loughborough, UK
Di-thiothreitol (DTT)	Sigma-Aldrich, Poole, Dorset, UK
Glycerol	Fisher Scientific UK Ltd, Loughborough, UK
Glycine	Sigma-Aldrich, Poole, Dorset, UK
Leupeptin	Sigma-Aldrich, Poole, Dorset, UK
Lower buffer for SDS-PAGE Gels (Tris 1.5M, pH 8.8)	Bio-Rad Laboratories Ltd, HERTS, UK
N,N,N',N'-tetramethylene-diamine (TEMED)	Sigma-Aldrich, Poole, Dorset, UK

Perbio Chemiluminescent Supersignal® West Pico, Dura and Femto	Pierce and Warriner Ltd, Cheshire, UK
Phenylarsine oxide	Sigma-Aldrich, Poole, Dorset, UK
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Poole, Dorset, UK
Polyoxyethylene-sorbitan monolaurate (Tween 20)	Sigma-Aldrich, Poole, Dorset, UK
Rainbow protein size markers (10-250 kDa)	Amersham, Little Chalfont, England
Sodium chloride (NaCl)	Sigma-Aldrich, Poole, Dorset, UK
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Poole, Dorset, UK
Sodium fluoride (NaF)	Sigma-Aldrich, Poole, Dorset, UK
Sodium molybdate (Na2MoO4)	Sigma-Aldrich, Poole, Dorset, UK
Sodium orthovanadate (NaVO4)	Sigma-Aldrich, Poole, Dorset, UK
Trizma (Tris) base	Sigma-Aldrich, Poole, Dorset, UK
Upper buffer for SDS-PAGE Gels (Tris 0.5M, pH 6.8)	Bio-Rad Laboratories Ltd, HERTS, UK
Western Blocking Reagent	Roche Diagnostics, Mannheim, Germany

Immunocytochemistry	
Reagent	Manufacturer
Anti-rabbit/Anti-mouse EnVision™+ System, Peroxidase (DAB) kits	DAKO, Cambridgeshire, UK
Di-butylpthalatexylene (DPX)	Raymond A Lamb Ltd, Eastbourne, UK
Liquid DAB+ substrate chromogen system	DAKO, Cambridgeshire, UK
Methyl green	Sigma-Aldrich, Poole, Dorset, UK
Sucrose	Fisher Scientific UK Ltd, Loughborough, UK

2.1 Tissue Culture

2.1.1 Cell Seeding from Cryogenically Stored Cell Reserves.

ER positive, MCF-7 breast cancer cells, kindly given to our laboratory by AstraZeneca Pharmaceuticals (Cheshire, UK), were stored in liquid nitrogen in RPMI-1640 based medium containing 5% (v/v) fetal calf serum (FCS), antibiotics (streptomycin (10μg/ml), penicillin (10IU/ml), fungizone (2.5μg/ml) (RPMI+5%) with the addition of 7.5% DMSO. Upon removal from liquid nitrogen, 1ml aliquots of cell solution, containing approximately 10^6 cells, were rapidly defrosted and vials were sterilised with 75% EtOH before opening. Cells were re-suspended in 9mls of RPMI+10% cell culture medium and were centrifuged at 1340g for 5 minutes at room temperature. The supernatant was aspirated and the cell pellet was re-suspended in 3ml of medium through gentle pipetting with a 10ml pipette until no clumping of cells was visible. The cell suspension was then seeded in a 12.5 cm^2 filter flask and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was refreshed the following day, and subsequently twice a week, until cell confluency was reached. Cells were then passaged at a ratio of 1:2 with RPMI+5% medium until cell reserves were sufficient to permit routine culture (described below).

2.1.2 Routine MCF-7 Cell Passage Procedure

All cells were routinely cultured in 75cm^2 flasks (T75) containing 15mls of culture medium, refreshed twice weekly; upon reaching 70-80% confluency the cells were passaged as follows. The culture medium was aspirated and replaced with 10mls of pre-warmed (37°C) trypsin (0.05%)/EDTA (0.02%) in PBS. The flask was returned to an incubator at 37°C for approximately 5 minutes, until the cells had detached from the flask surface. The trypsinised cell suspension was then mixed with an equal volume of pre-warmed RPMI+5% culture medium and centrifuged at 1340g for 5 minutes at room temperature. The supernatant was aspirated and the cells were resuspended into 10mls of RPMI+5% by gently pipetting through a 10ml pipette, ensuring no clumping of cells was visible. 1ml of cell suspension was then seeded into 14mls of RPMI+5% in T-75 flasks and put in an incubator (5% CO_2 at 37°C).

2.1.3 Experimental Cell Media and Passage procedure

T75 flasks containing MCF-7 cells at 70-80% confluency were trypsinised and centrifuged as described in the routine cell passage procedure. The pellet obtained was then resuspended in 10ml of oestrogen depleted experimental media. Experimental media was necessary to avoid the unwanted oestrogenic properties of both the phenol red in standard RPMI-1640 medium, and the steroidal hormones present in the standard FCS. Therefore, standard RPMI was replaced with a phenol red free equivalent medium (wRPMI), and FCS was charcoal-stripped to produce steroid-depleted FCS (csFCS). The csFCS was prepared by firstly aliquoting the standard FCS (100ml) and adjusting the

pH to 4.2 using 5M HCL. This was then allowed to equilibrate for 30 minutes at 4°C. A charcoal/dextran solution was prepared using distilled water with Norit A (charcoal, 11.1%) and Dextran C (0.06%). This mixture was then stirred vigorously for 1 hour. 5ml of charcoal solution (5% v/v) was added to each 100ml aliquot of FCS and incubated with gentle agitation for 16 hours at 4°C. The charcoal was then removed by centrifugation (12,000g for 40 minutes) and the solution was filtered with filter paper (grade 4) to remove any traces of charcoal. The pH of the solution was then readjusted to pH7.2 and sterilised by filtering with a 0.2μM membrane filter to remove fine impurities and contaminating micro-organisms. The experimental media for all cell-lines used in this study comprised of wRPMI containing 5% (v/v) csFCS, antibiotics (same as routine culture media) and glutamine (4mM) (wRPMI+5%).

To ensure that equivalent cell numbers were obtained for each experiment, the cells were counted and diluted appropriately prior to plating into the selected experimental receptacle. The cell pellet obtained by centrifugation was resuspended into 10mls of wRMPI+5% using a syringe with a 25 G5/8 0.5 X 16 needle, to separate the cells and achieve a single-cell suspension. 100μl of this solution was then added to 10ml of Isoton solution in a counting cup and counted using a Beckman Coulter counter Multisizer II. Cell counts were carried out in duplicate, and an average was calculated. An appropriate volume of RPMI+5% was then added to a known number of cells to obtain a suitable cell density.

2.1.4 Establishment of the tamoxifen resistant cell-line (TAM-R).

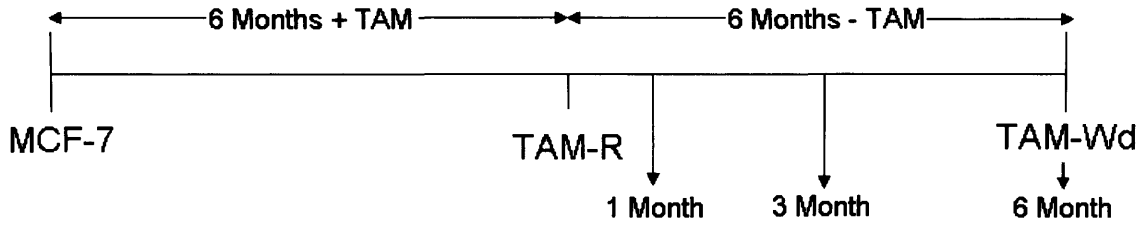
A tamoxifen-resistant MCF7 cell-line was generated by the long-term culture of MCF-7 cells in wRPMI+5% containing 4-OH-tamoxifen (10^{-7}M) (TAM). During the first two months of this culture regime, cell growth was significantly inhibited. However, cell growth rate then began to steadily increase following the emergence of cells capable of overcoming the growth inhibitory effects of TAM. After six months culture, the resultant tamoxifen resistant-MCF-7 derived cell sub-line (TAM-R) was characterised [Knowlden *et al,* 2003; Hutcheson *et al,* 2003; Hiscox *et al,* 2004]. Large stocks were generated and cells were periodically brought up from frozen for experimental analysis. Cells were routinely cultured for up to 25 passages under the same conditions described for the MCF-7 cells (section 2.1.2), with wRPMI+5% +TAM medium.

2.1.5 Establishment of the tamoxifen withdrawn TAM-R cell-line (TAM-Wd).

In order to establish a cell model representative of tamoxifen-resistant breast cancer cells which have subsequently been withdrawn from the drug, TAM-R cells were maintained in wRPMI+5% in the absence of tamoxifen for up to six months. Cells were frozen in liquid nitrogen after a tamoxifen withdrawal period of either 1, 3 or 6 months. Briefly, cells were harvested from T75 flasks and counted as previously described in section 2.1.3. The cell suspension was centrifuged and cells were resuspended in wRMPI+5% + 7.5% DMSO at a cell density of 10^6cells/ml. 1ml aliquots were transferred to freezer vials, labelled and placed into a lag box which was transferred to a -80°c freezer.

Vials were transferred to liquid nitrogen storage after at least 24hours. The resultant tamoxifen-withdrawn TAM-R cell sub-lines, TAM-Wd 1 Month (TAM-Wd 1M), TAM-Wd 3M and TAM-Wd 6M, were simultaneously brought up from frozen (as described in section 2.1.1 with wRMPI+10%), and were routinely cultured (as described for MCF-7 cells in section 2.1.2) with wRPMI+5% medium for up to ten passages before being discarded. See Fig.2.1 for a time-line depicting the establishment of experimental cell-models.

Fig 2.1. Time-line of established experimental cell-lines.



2.2 Cell Behaviour Analysis

2.2.1 Morphology

T75 flasks containing cells at around 50-60% confluency were visualised at 20x magnification using an inverted microscope fitted with a Hoffman condenser. Representative images of live cells were obtained using a digital camera and Improvision® OpenLab software.

2.2.2 Growth Assays

Cells from T75 flasks were passaged as described in section 2.1.3. The cell suspension was diluted to a suitable density ($4x10^4$ cells/ml unless otherwise stated), and cells were seeded in 24-well plates (1ml/well). Cells were incubated for 24 hours prior to treatment, with each condition and/or time-point allocated triplicate wells. Concentration-response assays were run for 7 days, while growth assays were assessed over an 11-14 day period, typically taking cell counts every 2-3 days. In both instances, the medium was refreshed every 3-4 days. To record the number of cells per well, the medium was firstly removed and replaced with 1ml of trypsin solution. The plate was returned to the incubator until the cells could be detached with gentle rocking (usually 3-5 minutes). Using a 5ml syringe with a 25 G5/8 0.5 X 16 needle, the detached cells were pipetted up and down twice to encourage a single-cell suspension for accurate analysis of cell number. 1 ml of Isoton solution was added to the well and the solution was pipetted up and down twice more before being drawn into the syringe. This process was repeated twice to give a total volume

of 4ml. The cell suspension was transferred to a counting pot containing 6ml of Isoton, to give a final volume of 10ml. The number of cells in each pot was then determined in duplicate using the Coulter™ Multisizer II set to count the number of cells in 500µl. Pots were counted a minimum of twice, with gentle agitation between each count to resuspend the cells. Duplicate counts for the three wells/condition were averaged and multiplied by the dilution factor of 20 to give the average number of cells per well. All growth/concentration-response assays were carried out as independent triplicate experiments unless otherwise stated. Treatments are summarised in table 2.1.

2.2.2.1 *MTT Cell Proliferation Assay*

Cells were harvested as previously described in section 2.1.3 and seeded into 96-well plates at a density of $5x10^3$cells/well. Cells were incubated for 24hrs prior to treatment, with each condition allocated 8 wells. Following a further 7 days of culture (with 1 medium change) medium was removed, and cells were washed gently with PBS. Sterile-filtered 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in wRPMI (0.5mg/ml; 150µl) was added to the cells and plates were left to incubate at 37°C for 4 hours. During this time, the MTT compound was metabolised by mitochondrial dehydrogenase enzymes in the cells to produce insoluble purple formazan crystals. The MTT solution was then removed and cells were lysed in 10% (v/v) Trition-X100 in PBS (150µl/well) for 12hrs at 4°C. Cell lysis causes the formazan crystals to dissolve and the absorbance of the resultant solution is proportional to cell number. Plates were warmed to room temperature and

gently tapped before being read on a Multiskan® MCC/340 plate-reader at 540nm.

2.2.3 Wound Healing Assay

Cells were harvested and seeded into 24-well cell-culture plates as described in section 2.2.2. After confluent cell mono-layers were established, cells were wounded by running a pipette tip across the diameter of the base of the well, manually removing cells. The well was rinsed with 1ml sterile PBS, refreshed with new medium, and incubated for a further 48 hours. Cells were then fixed with 3.7% formaldehyde, and stained with crystal violet (0.5% in PBS). Wells were visualized on a phase contrast-microscope and were photographed using a digital camera at ×4 magnification. An allocation of wells that had not been wounded 48hours previous, were wounded on the day of fixing to provide a positive control. Three independent experiments were carried out for this analysis, with images presented representative of the average degree of wound recovery.

2.2.4 Cell Migration

To analyse a cells capability to migrate, 24-well plate inserts 6.5mm in diameter with an 8μm pore membrane, were basally coated with fibronectin (10μg/ml in wRPMI+5%) by submerging an insert into a well containing 200μl of fibronectin solution for 2 hours at 37°C. The inserts were then washed with PBS and allowed to air-dry.

Cells from T75 flasks were passaged as described in section 2.1.3 and the cell suspension was diluted with experimental medium to a density of $2.5x10^5$cells/ml. 200µl of cell suspension was seeded into the upper compartment of the insert. Each cell-line was seeded in duplicate per experiment, and independent experiments were carried out in triplicate. 650µl of media was then added to the lower compartment of each insert and the plate was incubated at 37 °C for 24 hours. The medium in the upper compartment of the inserts was then aspirated. Non-migratory cells attached to the upper-surface of the membrane were manually removed using a cotton swab. Migratory cells, attached to the basal membrane surface were fixed with 3.7% (v/v) formaldehyde in PBS for 10 minutes. Cells were then washed with PBS and stained with crystal violet (0.5% w/v in water) for 30 minutes. PBS was then used to thoroughly remove excess crystal violet stain and the inserts were air-dried at room temperature. Inserts were visualised at 10x magnification using a phase contrast microscope, and the number of migratory cells present in 5 random fields of view was recorded for each insert. Data is presented as mean cell count/field (n=3).

Table 2.1 - Treatments Used in Cell Culture Procedures.

Treatments for Concentration-Response and Growth Assays

Treatment	Classification	Target	Typical Conc. Used	Vehicle	Manufacturer
17β-Oestradiol	Hormone	ER	1nM	EtOH	Sigma-Aldrich
4-hydroxytamoxifen	Anti-hormone	ER	100nM	EtOH	Sigma-Aldrich
Fulvestrant (Faslodex™)	Anti-hormone	ER	100nM	EtOH	AstraZeneca
Gefitinib (Iressa™)	Inhibitor	EGFR	1μM	DMSO	AstraZeneca
ABDP (4-anilino-5-bromo-2-[4-(2-hydroxy-3-(N,N-dimethylamino) propoxy) anilino] pyrimidine)	Inhibitor	IGF-1R	1μM	DMSO	AstraZeneca
5-Azacytidine	De-methylation agent	-	1μM	dH_20	Sigma-Aldrich

Treatments for RNA/DNA Extractions

Treatment	Classification	Target	Typical Conc. Used	Vehicle	Manufacturer
17β-Oestradiol	Hormone	ER	1nM	EtOH	Sigma-Aldrich
4-hydroxytamoxifen	Anti-hormone	ER	100nM	EtOH	Sigma-Aldrich
5-Azacytidine	De-methylation agent	-	1μM	dH_20	Sigma-Aldrich

Treatments for ICC Analysis

Treatment	Classification	Target	Typical Conc. Used	Vehicle	Manufacturer
17β-Oestradiol	Hormone	ER	1nM	EtOH	Sigma-Aldrich
5-Azacytidine	De-methylation agent	-	1μM	dH_20	Sigma-Aldrich

2.3 Gene Expression Analysis

Gene expression in wt-MCF7, TAM-R and TAM-Wd cell-lines was analysed using both semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and real-time (quantitative) PCR (qPCR). For both procedures, RNA and DNA were prepared using RNA/DNase free reagents and autoclaved disposables, and ethanol rinsed gloves were worn at all times.

2.3.1 Cell Culture Procedure for RNA Harvest

Cells were harvested and counted as described in section 2.1.3, and resuspended in fresh experimental medium at cell density of $3x10^6$ cells/ml. 1ml of cell suspension was seeded into a 150mm petri-dish containing 9mls of pre-warmed wRPMI+5%. Cells were incubated at 37°C for 24hr before treatments were added (treatments are summarised in table 2.1). Cells remained in culture for a further six days before RNA harvest, with media typically refreshed every 2-3 days.

2.3.2 RNA Extraction

Media was drained from the petri-dish, and cells were rinsed with x1 PBS three times to ensure removal of all traces of cellular debris and media components. Petri-dishes were drained thoroughly and 1ml of TRI-reagent was distributed across the cell-monolayer. Dishes were gently rocked to ensure total-surface coverage, and then left to incubate for 5 minutes at room temperature. Dishes were gently tapped to dislodge cells, and a cell scraper

was used to ensure all cells had detached. The viscous cell solution was transferred to a 2-ml eppendorf tube and placed on ice (at this point, cell-lysates were stored at -80°C for at least 24 hours before the RNA extraction was carried out). 0.2mls of Chloroform was added to the cell lysate, and the solution was vortexed to ensure complete mixture. Samples were incubated for 10mins at room temperature before centrifugation at 13000g, 4°C for 10 mins. Samples separated into two phases; 0.5ml aliquots of upper (aqueous) phase which contained the RNA were transferred to fresh 1.5ml eppendorf tubes, to which 0.5ml isopropranol was added. Samples were vortexed for 5secs, and incubated for a further 10 mins at room-temperature before a repeat centrifugation step.

The supernatant was carefully removed and the remaining RNA pellet was resuspended in 1ml 75% ethanol (EtOH). Samples were centrifuged at the same speed and temperature as before for 5 mins, after which the EtOH was carefully removed. Pellets were briefly air-dried and re-dissolved into 50µl dH_2O. Samples were stored at -80°c prior to analysis.

2.3.3 RNA Quantification

Total RNA was typically diluted 1: 200 in TE buffer pH 8.0 (10 mM Tris-Cl, 1 mM EDTA) and measured in a quartz cuvette using a spectrophotometer. Optical densities (OD) observed at wavelengths of 260 and 280nm were recorded and subsequently used to produce the A260/A280 ratio as an indicator of nucleic acid purity, with a ratio of 1.8-2.1 representing an acceptable RNA solution. The RNA integrity was checked by running RNA

through a 2% agarose gel. The following calculation was used to obtain the RNA concentration:-

$[RNA] = OD_{260} \times 200 \times 40 = \mu g/ml$ $(RNA - 40\mu g = 1OD_{260})$

2.3.4 Reverse Gene Transcription

To generate complementary DNA (cDNA) from Total RNA obtained, 1µg of total RNA was made up to 7.5µl with RNase-free H_2O. This RNA solution was then added to 11µl of RT master mix comprising 5µl dNTP (0.625mM of dGTP, dCTP, dATP and dTTP), 2µl 10X PCR buffer (containing 25mM $MgCl_2$), 2µl di-thiothreitol (DTT; 0.1M) and 2µl random hexamer oligonucleotides (RH; 100µM). Samples were placed in a thermal cycler and denatured by heating to 95°C for 5 minutes. Samples were then removed and cooled on ice for 5 min. 1µl MMLV (reverse transcriptase enzyme (200U/µl) and 0.5µl RNase inhibitor (40U/µl) were added to each tube to give a final volume of 20µl. Tubes were mixed gently and centrifuged briefly to collect volume and then returned to the thermal cycler and reverse transcribed using the cycle program below. The resultant cDNA was stored at -20°C.

Thermocycling Procedure for RT

		Temp	Duration
1	Annealing	22°C	10 minutes
2	RT Extension	42°C	42 minutes
3	Denaturing	95°C	5 minutes
4	Short-term storage	4°C	No more than 12hrs

2.3.5 Polymerase Chain Reaction

The reaction mixture for the amplification of genes by semi-quantitative PCR consisted of 2.5µl 10x PCR buffer, 2µl dNTP mix (2.5mM), forward primer (20µM), 0.6µl gene reverse primer (20µM), 0.1µl Taq polymerase (5U/µl) and 0.5µl cDNA (equivalent to 25ng of RNA used in RT assuming 100% efficiency of reaction), with the final volume made up to 25µl with sterile H_2O. A negative control in which cDNA was substituted with an equal volume of sterile H_2O was also run for each experiment. All primers used were synthesised at Invitrogen and were micro-column purified. Primer sequences for all genes amplified can be found in table 2.2. The reaction tubes were mixed gently, pulsed in a micro-centrifuge and placed in a thermal cycler. The PCR reaction was then run for the optimal cycle number for gene identification using the temperature cycling conditions stated below. PCR data was normalised using amplification of the β-actin housekeeping gene.

Thermocycling Procedure for PCR:

		Temp	Duration	Cycle Number
1	Denaturing	95°C	5 minutes	x 1
2	Annealing	55-60°C	1 minute	
3	Extension	72°C	1 minute	
4	Denaturing	95°C	1minute	x 24-32
5	Annealing	55-60°C	1minute	
6	Extension	72°C	1minute	
7	Final Extension	72°C	10 minutes	
8	Short-term storage	4°C	No more than 12hrs	

2.3.6 Agarose Gel Electrophoresis and Gel Densitometry

Samples were resolved using a 2% (w/v) agarose gel made up with Tris-Acetate-EDTA (TAE) buffer (Tris-base 2M, Glacial acetic acid 1M, EDTA 0.05M (stock is 50x) -pH 8.3- made up to 1L with distilled H_2O, diluted 1:50 before use) containing ethidium bromide (1µl of a 10mg/ml solution per 100ml gel solution). The agarose was firstly dissolved in the TAE buffer by heating in a microwave at full power for 1 minute, with intermittent mixing. The gel was left to cool to approximately 40°C and the ethidium bromide was added. The gel was cast and a comb was added and the gel was left to set. The solidified gel was then submerged in an electrophoresis tank containing TAE buffer, and 10µl of each PCR product was mixed with 5µl loading buffer and

dispensed into a well of the gel. A 100bp DNA size marker was run in parallel with the samples. The gel was run at 100V constant voltage for approximately 45-60 minutes. Gels were visualised using a UV trans-illuminator and photographed with a digital camera attached to a shroud for densitometric analysis. Using the spot density application within the Digidoc computer software, the light intensity of the UV fluorescence of ethidium-bromide chelated DNA was standardised and quantified relative to β-actin. Densitometry data is representative of 3 independent experiments. Single gels that reflect the observations from three independent experiments are also presented.

2.3.7 Real-Time PCR

Quantitative PCR (qPCR) uses fluorescence to measure the amount of DNA material present in a PCR reaction after each temperature cycle and, thus, follows the amplification of a gene in 'real-time' until the system becomes saturated. Following the reaction, the amount of starting template material in an unknown sample can be quantified by comparing its fluorescence at a suitable cycle number to that of a pre-defined standard curve. The present study used the DyNAmo™ SYBR Green qPCR Kit according to the manufacturer's instructions. SYBR green is an intercalating DNA dye that is only fluorescent when bound to double-stranded DNA. Briefly, each reaction mix contained 12.5μl SYBR Green PCR Mastermix (hot start taq DNA polymerase, 1x SYBR Green PCR buffer, SYBR Green 1, dNTPS, and $MgCl_2$), 0.375μl forward- and reverse specific primers (20μM) (table 2.2), and

11.3µl sterile H_2O, giving a total volume of 24.5µl of master mix which was aliquoted into the wells of a 96 well qPCR plate, to which 0.5µl of cDNA product (equivalent to 0.05 µg RNA starting material) was added. Serial dilutions of specific cDNA-amplicon preparations of known concentration were extracted from freshly purified PCR product using a QIAquick PCR purification kit according to manufacturer's instructions. They were then quantified using a RNA/DNA calculator and used to produce a standard curve from which experimental samples could be quantified. Final standard concentrations ranged from 0.001-10ng/µl. Each standard/sample was analysed in duplicate. The wells of the plate were capped and gently tapped to ensure complete mixture of the reaction components. Plates were lightly pulsed in a centrifuge to collect volume and placed into an Opticon 2™ real-time PCR machine. Throughout the set-up procedure, effort was made to protect the master-mix from light, due to the photo-sensitive nature of its constituents. The thermocycling protocol was as below. Each qPCR reaction was set to run for 50 cycles to ensure product saturation.

Thermocycling Procedure for qPCR:

		Temp	Duration	Cycle Number
1	Initial denaturing phase	95°C	15 minutes	
2	Annealing	55°C	30seconds	x 1
3	Extension	72°C	30seconds	
4	Denaturing	95°C	30seconds	
5	Annealing	55°C	30seconds	x 50
6	Extension	72°C	30seconds	
7	Final Extension	72°C	10 minutes	
8	Cooling period	4°C	-	

Plate fluorescence readings were collected after each 72°C extension time. The concentrations of starting template material in the samples were extrapolated from the standard curve using Opticon 2™ computer software. The quantities of cDNA recorded were then corrected against ß-actin expression to normalise the data. Data presented shows cDNA detected, normalised to actin for 3 independent experiments, run on the same qPCR plate. After each run, melting curves were inspected to ensure valid product specificity.

Table 2.2. Primers used in Semi-Quantitative and Quantitative PCR

Primers Used in Semi-Quantitative PCR					
Gene		Primer Sequence	Amplicon size (bp)	Annealing Temp(°c)	Cycle no.
ADCY9	For	5'- ctgctgcccttatccaacat -3'	487	60	28
	Rev	3'- ctgggaaacactgagcacac -5'			
β-Actin	For	5'- ggagcaatgatcttgatctt -3'	208	55	24
	Rev	3'- ccttcctgggcatggagtcct -5'			
CA12	For	5'- atgggatgcactctcagacc -3'	506	60	25
	Rev	3'- aaagcttggagaagcagcag -5'			
COL6a3	For	5'- tcaatccaaattccctctgg -3'	456	60	28
	Rev	3'- gggcagggaatgaaaaagtt -5'			
CXCL12	For	5'- agagatgaaagggcaaagac -3'	132	56	30
	Rev	3'- cgtatgctataaatgcaggg -5'			
GDF15	For	5'- actgctggcagaatcttcgt -3'	536	60	32
	Rev	3'- cacatggtcacttgcacctc -5'			
HBA2	For	5'- caagacctacttcccgcact -3'	435	60	25
	Rev	3'- aggcagtggcttaggagctt -5'			
KAZRIN	For	5'- ggcagaggatgtggtctgtt -3'	543	60	29
	Rev	3'- acaaacccagccaagacaag -5'			
RASAL1	For	5'- ctgtgtgccttgagtccaga -3'	514	60	28
	Rev	3'- cagctgtatccagcagctca -5'			
RGC32	For	5'- ctgccactgtcactcctcag -3'	486	58	30
	Rev	3'- ttgagtgcacgtctttgtcc -5'			
ST6	For	5'-tgttcaatataggacaccccagctt-3'	275	58	28
	Rev	3'-catcctgttggtgacaaggtggtga-5'			
WISP2	For	5' –ggtctgtctggacgagtatgg -3'	191	60	27
	Rev	3'- ggactgcttgtcccatctcttgcc -5'			

Table 2.2. Continued

Primers Used in Quantitative PCR					
Gene		Primer Sequence	Amplicon size	Annealing Temp(°c)	Cycle no.
β-Actin	For	5'- ggagcaatgatcttgatctt -3'	204	55	50
	Rev	3'- ccttcctgggcatggagtcct -5'			
EGFR	For	5'- caacatctccgaaagcca -3'	636	55	50
	Rev	3'- cggaactttgggcgactat-5'			
ERα	For	5'- ggagacatgagagctgccaac -3'	432	55	50
	Rev	3'- ccagcagcagcatgtcgaagatc -5'			
IGFR	For	5'- actgacctcatgcgcatgtg -3'	285	55	50
	Rev	3'- ctcgttcttgcggcccccgt -5'			
PR	For	5'- ccatgtggcagatcccacaggagtt -3'	320	55	50
	Rev	3'- tggaaattcaacactcagtgcccgg -5'			
pS2	For	5'- catggagaacaaggtgatctg -3'	336	55	50
	Rev	3'- cagaagcgtgtctgaggtgtc -5'			

2.4 Protein Expression Analysis

Protein expression in wt-MCF7, TAM-R and TAM-Wd cell-lines was analysed using both SDS PAGE-Western blotting and Immunocytochemistry (ICC).

2.4.1 Cell Culture Procedure for Protein Harvest

Cells were harvested as described in section 2.1.3, counted and resuspended in fresh experimental media at a cell density of $2x10^6$ cells/ml. 1ml of cell suspension was seeded into a 100mm petri-dish containing 9mls of pre-warmed wRPMI+5%. Cells remained in culture until they had reached approximately 60-70% confluency (typically 6-7 days), with media refreshed every 3-4 days. Media was then aspirated and cells were washed twice with 1xPBS (10mls). Dishes were drained thoroughly and transferred to ice, and cells were lysed using 250µl of ice-cold lysis buffer (50mM Tris-HCL, 5mM EDTA, 150mM NaCl, 1% Triton X-100 (v/v) in distilled water, pH7.5) supplemented with protease and phosphatase inhibitors (2mM Na3VO4, 20mM NaF, 1mM PMSF, 10 µg/ml leupeptin, 20µM phenylarsine oxide, 10µg/ml aprotinin and 10mM sodium molybdate), which was distributed evenly across the cell monolayer. Following 2 minute incubation on ice, cellular material was collected using a cell-scraper and transferred to a 1.5ml micro-centrifuge tube. The solution was homogenised briefly using a pipette and left on ice for 20 minutes, with occasional mixing. Lysates were then centrifuged at 13,000g for 15 minutes (4°C) to clear cell debris, and the supernatants were stored at -20°C until required.

2.4.2 Protein Concentration Assay

The total protein concentration of the cell lysates was determined using the Bio-Rad DC Protein Assay kit. Lysates were diluted 1:4 in lysis buffer to give a final volume of 50µl, prepared in disposable cuvettes. A standard curve was constructed for spectrophotometric quantification using known concentrations of bovine serum albumin (BSA), diluted in lysis buffer to obtain 50µl of solution at protein concentrations of 0, 0.25, 0.5, 0.75, 1 and 1.45mg/ml. 250µl of reagent A (from BioRad kit) was added to each cuvette, supplemented with substrate S (20µl in 1ml of reagent A) followed by 2ml of reagent B. Each cuvette was then briefly vortexed and colour was allowed to develop for a minimum of 15 minutes. The absorbance at 750nm for each BSA sample of known concentration was then read on a spectrophotometer and a calibration curve was plotted (absorbance versus concentration). Each protein sample was then processed and its concentration was determined from the standard curve.

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

Electrophoresis was carried out using Biorad Mini-Protean® 3 electrophoresis apparatus from BioRad Laboratories. Glass plates were thoroughly cleaned with ethanol and assembled to provide the gel cast. Resolving gel and stacking gel were prepared as follows; resolving gel: 7.5% acrylamide/bisacrylamide, 375mM lower buffer (pH8.8), 0.1% (w/v) SDS, 0.1% (w/v) APS and 70µM of TEMED, stacking gel: 5% acrylamide/bisacrylamide, 125mM upper buffer (pH6.8), 0.1% (w/v) SDS, 0.05% (w/v) APS and 116µM TEMED.

The constituents of the resolving gel, with the exception of TEMED, were added to a universal container and mixed thoroughly. TEMED was then added to the gel solution immediately before casting the gel as it catalyses the polymerisation and cross-linking of the acrylamide/bis-acrylamide, causing the gel to set. The gel was dispensed between the glass plates until the level reached approximately 1.5cm below the top of the exterior plate (to allow room for the stacking gel). 0.05% (w/v) SDS in H_2O was carefully dispensed over the exposed gel surface to ensure the formation of a level gel front and the exclusion of any air bubbles. The gel was left for approximately 30 minutes at room temperature to set.

The SDS solution was then removed from the resolving gel surface, and distilled water was used to rinse the gel. Excess water was drained with a strip of filter paper. The stacking gel solution was prepared and poured onto the resolving gel, filling the remaining volume of space between the glass plates. A 10-well comb was inserted into the stacking gel solution and the gel was allowed to set for 45-60 minutes at room temperature.

After the stacking gel had polymerised, the comb was gently removed and the casting apparatus was transferred to an electrophoresis tank, the upper and lower chambers of which the were then filled with running buffer (250mM Trizma base, 2M Glycine, 40mM SDS - pH8.8).

Forty μg of protein from each quantified protein sample was then mixed with 10μl of loading buffer (4% (w/v) SDS, 20% (v/v) glycerol, 120mM upper buffer (pH6.8), 0.1% (W/V) bromophenol blue, plus 100mM DTT). The protein samples, combined with loading buffer, were heated to 100°C for 10

minutes to denature and reduce the proteins in the sample and were allowed to cool before loading onto the gel. Rainbow protein marker (10-250 kDa) was loaded into the first lane of each gel. Electrophoresis was carried out at a constant voltage of 150volts until the dye front had reached the base of the gel (approximately 45 minutes).

2.4.4 Western Blotting

Proteins were transferred from SDS-PAGE gels to a nitrocellulose membrane using the Bio-Rad Mini-Protean® III transfer apparatus according to manufacturer's instructions. Two pieces of grade 3 filter paper and one piece of nitrocellulose membrane (0.45μm pore size) cut to the same size as the gel, along with two Teflon sponge pads, were pre-soaked in transfer buffer (0.2M of glycine, 25mM of Trizma base, 20 %(v/v) of methanol in distilled water, pH 8.3) for 30 minutes.

Gels were removed from the electrophoresis plates and carefully transferred to a tray containing distilled H_2O to wash off any excess SDS. The stacking gel was gently separated from the resolving gel and discarded.

The resolving gel was assembled with the other components of the western blot transfer cassette according to the manufacturer's instructions [Fig 2.2]. Gentle pressure was applied to the complete cassette assembly to ease out any air bubbles that may formed between the layers. The cassette was then placed into the transfer apparatus which was loaded into a tank along with an ice-block to prevent over-heating of the gel during transfer, and a magnetic flea.

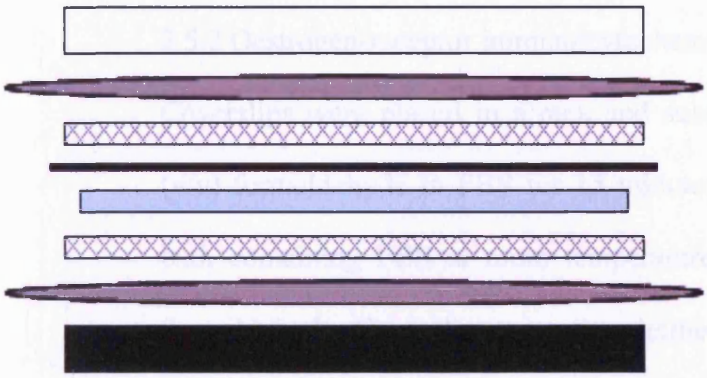
The tank was then filled with transfer buffer, placed on a magnetic stirrer, and the transfer was run at 100V constant voltage for 1 hour.

Afterwards, nitrocellulose membranes were blocked in a solution of 5% (w/v) skimmed milk in TBS-Tween (10mM Tris, 0.1M NaCl, 0.05% (v/v) Tween 20, pH 7.5) for at least 1 hr to prevent non specific binding. Blots were then incubated in the appropriate primary antibodies overnight. A list of the primary antibodies used in this study, along with their corresponding dilutions is shown in table 2.3.

Membranes were then washed three times with TBS-Tween at 5 minute intervals, and incubated for 1 hour with the required secondary antibody labelled with horseradish peroxidase (anti rabbit or anti mouse) diluted 1/10000 in TBS-Tween (1:20mls). Membranes were then washed TBS-Tween (3x10minutes).

Detection was performed by applying a thin film of enhanced chemiluminescence (ECL) reagent (Supersignal™ West Pico, Supersignal™ West Dura) to the membrane, which was then sealed within a plastic sheath in a development cassette. After 5 minutes, cassettes were taken to a dark room, where a sheet of x-ray film was placed on top of the sealed membrane. The cassette was closed, and left for a suitable duration of time until blots could be visualised on the developed film. Blots presented reflect the observations from three independent experiments and were standardised using β-actin detection.

Fig 2.2 Western Blot Transfer Cassette Assembly



2.5 Immunocytochemistry ICC

2.5.1Experimental tissue culture

Cells were harvested as described in section 2.13, counted and resuspended in fresh experimental media at cell density of $1x10^5$ cells/ml. 1ml of cell suspension was seeded onto 22-mm^2, 3-aminopropyltriethoxysilane (APES)-coated glass coverslips (coverslips were submerged in 2% APES in acetone for 60secs followed by 1x10 minute wash and 2x 1 minute washes with water-air-dried and sterilised at 180°C for 2 hours), resting in 35mm culture dishes. Dishes were incubated for 24hrs to allow cells to settle. Treatments were then applied where appropriate (see table 1 for treatments) and cells were routinely cultured for up to seven days. Media was then removed and cells were fixed by a procedure most appropriate to the immunocytochemical assay to be

performed. All ICC antibodies and corresponding fixing methodologies used are shown in table 2.3.

2.5.2 Oestrogen-receptor immunocytochemical assay (ER-ICA) fixation.

Coverslips were placed in a rack and submerged in a bath containing 3.7% (v/v) formaldehyde in PBS for 15 minutes. They were then transferred to a bath containing PBS at room temperature for 5 minutes to remove excess formaldehyde. Coverslips were then immersed in methanol (-10°C to -30°C) for 5 minutes, then acetone (-10°C to -30 °C) for 3 minutes. The coverslips were then washed in PBS at room temperature for at least 5 minutes and stored at -20°C in sucrose storage medium (SSM).

2.5.3 Formal-Saline (F/S) Fixation

Medium was removed from the coverslips and replaced with 1ml formal-saline solution (3.7% formal-saline – 4.5g NaCl, 50ml 37% Formaldehyde solution diluted in 450ml of tap water) for 10 minutes. The cells were then washed with 100% ethanol (5 minutes, followed by a quick ethanol rinse) and PBS (5 minutes, followed by a quick PBS rinse). Coverslips were stored in SSM at -20°C before use.

2.5.4 Phenol Formal-Saline (PFS) Fixation

Medium was removed from the coverslips and replaced with 2ml 2.5% phenol in formal-saline solution for 5 minutes. The cells were then washed with 100% ethanol (5 minutes, followed by a quick ethanol rinse) and PBS (5

minutes, followed by a quick PBS rinse). Coverslips were stored in SSM at -20°C before use.

2.5.4 Immunocytochemical processing of coverslips.

The SSM in which the fixed cells were stored was removed by gently rinsing with PBS. The coverslips were then submerged in PBS containing 0.02% (v/v) Tween-20 (PBS/Tween) for a few seconds to provide a surface that permits the primary antibody solution to spread evenly over the coverslip. 50µl of primary antibody (diluted in PBS) was added to each coverslip and incubated in a humidified atmosphere at 23°C. Antibody dilutions and incubation times varied between proteins (table 2.3).

Following primary antibody incubation, coverslips were washed with PBS/Tween (2 x 5 minutes) and incubated with DAKO EnVision+ system-HRP-labelled polymer, conjugated to goat anti-rabbit or goat anti-mouse immunoglobulins for up to 2hours at 23°C. Coverslips were washed with PBS/Tween (2 x 5 minutes), and staining was visualised using the DAKO Liquid DAB+ substrate and chromogen system. Colour development took 6-10 minutes depending on signal strength. Coverslips were rinsed with distilled water and cells were counter-stained with methyl-green (0.5% (w/v) in H_2O) for 5 minutes. Coverslips were then thoroughly rinsed with distilled water to remove excess methyl-green. Coverslips were dried and mounted onto glass slides using DPX, a xylene-based mounting medium, and left to set over night at room temperature. They were later analysed with an Olympus BH2 phase-

contrast microscope (at 20x magnification unless otherwise stated) and images were produced using a digital camera.

Table 2.3. Antibodies used in Western Blot and ICC procedures

Antibodies Used in Western Blot-Protein Detection System			
Antibody	Source	Dilution	Manufacturer
tEGFR	Rabbit	1/1000	Cell Signaling Technologies
ERα	Rabbit	1/10000	Santa Cruz Biotechnology
tIGFR	Mouse	1/1000	Santa Cruz Biotechnology
β-actin	Mouse	1/20000	Sigma-Aldrich

Antibodies Used in ICC Detection System					
Antibody	Source	Dilution	Incubation Conditions	Fixation Req.	Manufacturer
tEGFR	Mouse	1/140	Overnight at 23°c	Phe/Form/Sal	NeoMarkers
ERα	Mouse	1/75	90minutes at 23°	ER-ICA	Vector
tIGFR	Rabbit	1/125	Overnight at 23°c	Form/Sal	Santa Cruz
PR	Mouse	1/75	60 minutes at 23°	ER-ICA	NovaCastra
pS2	Rabbit	1/500	90minutes at 23°	ER-ICA	NovaCastra

2.6 Methylight Assay

The DNA material was generated in Tenovus as described below, and sent to Dr H. Fiegl of the University of Innsbruck for Methylight analysis.

2.61 Phenol / Chloroform / Isoamyl Alcohol extraction of DNA

Cells were harvested and were seeded on to 100mm^2 culture plates, as previously described in section 2.4.1. They were incubated at 37°C for 24hours before treatments were added (see table 1). Cells were cultured for a further 7 days with media refreshed every 3-4 days. On the day of harvest, media was removed from the dish and the cells were thoroughly washed twice with 10mls PBS. Dishes were drained, and 1.5 ml SDS lysis buffer (0.1 M EDTA, 0.5 % SDS, 100 μg/ml Proteinase K) was added. Dishes were incubated at room temperature for approximately 2 minutes before cells were scraped off with a sterile cell scraper. The viscous solution of cells was then transferred to a 15ml universal tube and incubated in a pre-warmed water bath at 65°C for two hours to allow Proteinase-K digestion.

An equivalent volume of alkaline-calibrated phenol (pH 7.9) was then added to the cell solution, which was then mixed gently. Samples were then centrifuged at 4000g for 10minutes at 4°C. The aqueous phase containing the genomic DNA was transferred to a fresh 15-ml phase lock gel (PLG) tube using a plastic pipette. The organic phase was back extracted by adding 1ml TE buffer pH 8.0 (10 mM Tris-Cl, 1 mM EDTA), mixing gently and re-centrifugation. The aqueous phases were pooled, and an equivalent volume of Phenol/Chloroform/Isoamyl alcohol (25:24:1) solution was added to each

sample, and mixed gently. Samples were then centrifuged for 4000g for 10minutes at 4°C, and supernatants were transferred to a fresh 15ml universal tube. 1/10 of the equivalent volume of NaOAc (2.5M, pH5.2) was added to the supernatant and mixed. Twice the equivalent volume of 100% EtOH was subsequently added. Gentle inversion of the tubes eluted fine DNA strands. Samples were then centrifuged (using the conditions described in the previous step) and the supernatant was discarded. Pellets were air-dried and re-dissolved in 50-200µl DNAse and RNAse-free sterile H_20 at 4°C overnight, then stored at -20°C prior to analysis.

2.6.2 DNA Quantification:

DNA extractions were allowed to thaw thoroughly. 5µl of sample was transferred to a quartz cuvette containing 995µl TE buffer (pH8) (i.e. 1:200). The solution was gently inverted and optical densities (OD) were recorded at wavelengths of 260 and 280nm using a UV spectrophotometer. The A260/A280 ratio was calculated as an indicator of nucleic acid purity, with a ratio of 1.6-2.0 representing an acceptable DNA solution. The following calculation was used to obtain the DNA concentration for each sample:-

[DNA] = OD260 x 200 x 50 = µg/ml (DNA - 50µg = 1OD260)

2.6.3 Methylight Assay – Conducted by Dr H Fiegl at Innsbruck University

Methylight is a sensitive, fluorescence-based real-time PCR technique that is capable of quantifying DNA methylation at a particular locus using DNA oligonucleotides that anneal differentially to bisulfite-converted DNA.

Briefly, DNA samples were firstly treated with sodium bisulphate which converts the unmethylated cytosine bases to uracil whilst methylated cytosines remain unaffected, using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Methylight analysis was carried out as previously described by Eads *et al* (2000) using two sets of primers and probes, designed specifically for bisulfite-converted DNA; a set for the gene of interest and a set for collagen, type II, alpha 1 (COL2A1), an internal reference gene, that would be amplified regardless of the methylation status of the DNA to normalise the differences in the amount of genomic template present in each reaction. Specificity of the reactions for methylated DNA was confirmed separately using CpG methylating enzyme SssI-methylase (M.SssI) (New England Biolabs) treated human white blood cell DNA, which is heavily methylated. The percentage of fully methylated molecules at a specific locus was then calculated by dividing the GENE:COL2A1 ratio of a sample by the GENE:COL2A1 ratio of M.SssI-treated human white blood cell DNA (in which the gene of interest is fully methylated) and multiplying by 100.

Primer and probe sets for TFF1, PGR, COL2A1, SAT2, and ALU (markers of global DNA methylation) have been described recently [Widschwendter *et al,*

2004; Weisenberger *et al,* 2005]. Primer and probe sets for ADCY9, CA12, CXCL12, GDF15, HBA2, Kazrin, RGC32 and ST-6 are listed in Table 4.

Table 4. Primers and Probes used in Methylight Reactions

Primers Used in Methylight Analysis				
Gene	Primer Sequence		Primer Location	Probe Oligo Sequence
ADCY9	For	5'- cctcgacgtcccaaaaacc -3'	Promoter	6F AM-ccgcccgaaatcccgaccct -BH Q1
	Rev	3'- tttttacggtaggcgtttttaggt -5'		
CA12	For	5'- ctctccaactacacaccgaaacc -3'	Exon-1	6F AM-cgtacgcgcaacctaaataccg -BH Q1
	Rev	3'- aagggcggacgtattcgtt -5'		
CXCL12	For	5'-actcgccacctacccgactt-3'	Promoter	6F AM-cgacgcaaccgccgacaaaact-BH Q1
	Rev	3'-gttacgttgattgtaaagacgggttt-5'		
GDF15	For	5'- cgactcgcctcgaccaaa -3'	Exon-1	6F AM-ccatacgacaaccacgaaaacaccaacaa-BH Q1
	Rev	3'- gtaagaatttaggacggtgaatggttt -5'		
HBA2	For	5'- ccgccccgacctaacac -3'	Promoter	6F AM-cgctaaacgcgcatcgactccaa-BH Q1
	Rev	3'- gaagtttttcggttcgtattcgtt -5'		
KAZRIN	For	5'- cgaacgaacgccgaaaact -3'	Promoter	6F AM-cgcgcgccaccaaacactctt-BH Q1
	Rev	3'- cggcgaatggtaggttttattt -5'		
RGC32	For	5'- taaatcctacgaaataacaaccgaaa -3'	Promoter/Exon-1	6F AM-aacttactatcccgcacacttcaaccctacca –BH Q1
	Rev	3'-tttaggaattcgagtcggtggta-5'		
ST6	For	5'-ctccccgcgccctaat-3'	Exon-1	6F AM-cctacgccctcccgctctacgct-BH Q1
	Rev	3'-tttgtttacggttgtttgttcgg-5'		

2.7 Affymetrix gene analysis – Conducted at Central Biotechnology Services, Cardiff University, Heath Park.

RNA extractions were prepared and quantified as described in sections 2.3.2 and 2.3.3. 5μg of total RNA per sample was sent in triplicate to Central Biotechnology Services, who performed RNA integrity analysis, reverse-transcription and labelling and hybridisation of cDNA fragments to the HG-U133A.2 Affymetrix® gene chip. The chip was washed and scanned and signal intensities were determined using Mas.5 Affymetrix software. Data was exported in a variety of file formats enabling subsequent uploading and profile analysis using Genesifter® on-line software.

2.7 Statistical analysis

Where data allowed, the statistical significance of the results obtained when comparing between cell-lines or when comparing treated cells versus controls was analysed using independent, two-tailed Student's t-test. Where multiple data points were present, data were analysed using ANOVA with post-hoc tests. These analyses were conducted using the statistical analysis program, SPSS v12.0.2 (SPSS Inc.).

3. RESULTS

The anti-hormone tamoxifen has been the gold standard therapy for the treatment of ER-positive breast cancer in post-menopausal women for almost thirty years. Although an estimated 50% of patients benefit from tamoxifen treatment, resistance to the anti-hormonal properties of this drug develops in a high proportion of initially responsive patients, leading to disease recurrence. The failure of tamoxifen to prevent tumour progression can be also be accompanied by the disease spreading to life threatening sites and hence a poorer patient prognosis; thus representing a significant obstacle in the treatment of breast cancer in the clinic. In order to investigate the changes that occur within breast cancer cells following the acquisition of tamoxifen resistance, the Tenovus Centre for Cancer Research has developed an *in-vitro* model of acquired tamoxifen resistance using the ER-positive MCF7 breast cancer cell-line.

MCF-7 cells, first derived by the Michigan Cancer Foundation in 1973 from a pleural effusion obtained from an invasive ductal carcinoma, are one of the most commonly used ER-positive breast cancer cell lines [Soule *et al,* 1973]. They display many characteristics of mammary epithelium, including the expression of oestrogen and progesterone receptors and the ability to synthesize and process oestradiol for growth, making them an ideal cell-model for the study of ER-positive breast cancer *in-vitro*. The tamoxifen resistant MCF7 cell-subline (TAM-R) was developed following the long-term culture

of MCF7 cells in the presence of 4-hydroxytamoxifen as described in materials and methods (section 2.1.4). Previous 'in-house' characterisation of the TAM-R cell sub-line revealed fundamental changes in response to hormonal manipulation when compared to the parental MCF-7 cells. Although the TAM-R cells were found to be ER-positive, they were shown to be largely unresponsive to either the growth-stimulatory effects of E2 or to the growth-inhibitory effects of tamoxifen previously seen in the parental cell-line. However, TAM-R cells retained a partial role for ER in the regulation of cell growth, as evidenced by their sensitivity to receptor attenuation achieved through challenge with the pure anti-oestrogen, fulvestrant (Faslodex®) [Hutcheson *et al,* 2003].

Previous observations have also highlighted an essential role for growth-factor receptor signalling in TAM-R cells; with both EGFR and erbB2 being over-expressed and demonstrating increased activity compared to the parental MCF cells [Knowlden *et al,* 2003]. Data suggests that TAM-R cells are significantly dependent on the EGFR/HER-2 signalling pathway to drive proliferation in contrast to the parental MCF-7 cells, as evidenced by considerable growth inhibition following challenge with EGFR-selective tyrosine kinase inhibitor, gefitinib (Iressa®), and HER-2 antibody, trastuzumab (Herceptin®). This contribution to cell growth may be amplified by cross-talk mechanisms that exist between growth-factor and ER signalling pathways and also, between different growth-factor signalling pathways, noticeably the IGF-1R pathway interplaying with EGFR via c-SRc [Knowlden *et al,* 2005].

In parallel with the emergence of growth-factor signalling pathways in the MCF-7 cells following the acquisition of tamoxifen resistance, cells attain a more aggressive phenotype, demonstrating enhanced growth and motile capabilities [Hiscox *et al,* 2004]. In this chapter, we aim to determine whether the presence of tamoxifen is necessary to sustain these undesirable features of TAM-R cells, or whether permanent alterations to the cells phenotype have also occurred. In order to achieve this, TAM-R cells were withdrawn from tamoxifen for up to 6 months (as described in section 2.1.5). During this period, any agonistic contribution of the drug to TAM-R cell morphology, growth or motility would be depleted, whilst the effects of permanent heritable cell re-programming caused by the previous long-term tamoxifen treatment would remain. These tamoxifen-withdrawn (6-month) TAM-R cells (TAM-Wd), were then used to investigate the impact withdrawal had on the expression of components associated with the growth signalling pathways manifest in TAM-R cells, as well as analysing the effect that chronic tamoxifen exposure had on classically activated down-stream gene targets of the ER.

3.1 Assessment of TAM-R cell behaviour following tamoxifen withdrawal.

3.1.1 Cell morphology.

The morphology of MCF7, TAM-R and TAM-R cells withdrawn from tamoxifen for a period of 1, 3 and 6 months was assessed by phase contrast microscopy using a Leica DM-IRE2 inverted microscope fitted with a Hoffman condenser, permitting image capture of live cells. In contrast to the MCF-7 cells, the TAM-R cells appeared more angular, featuring enhanced lamellipodia and filopodia formation; indicative of their highly motile and invasive phenotype [Fig 3.1]. In addition, MCF7 cells grew in tightly packed cell colonies, in contrast to the TAM-R cells, which tended to favour growing independently before being forced to combine to form loosely packed cell colonies when approaching confluency. Following the withdrawal of tamoxifen of up to 6 months, cells appeared to retain the morphological characteristics associated with the acquisition of tamoxifen resistance, demonstrating increased lamellipodia and filopodia formation, though cells appeared less angular than TAM-R cells, and grew in loosely packed colonies rather than independently.

3.1.2 Cell growth rate.

The growth rates of MCF7, TAM-R and TAM-Wd (1, 3 and 6month) cell-lines were measured using anchorage dependent cell counting experiments as described in section 2.2.2. Growth curve analysis showed that compared to the parental MCF-7 cells, the TAM-R cell-line demonstrated a significantly

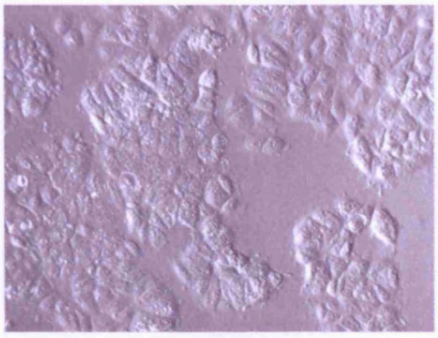
elevated rate of growth from the fourth day of culture ($p<0.001$), which continued until day 11, at which point the cells had reached confluency. This elevated growth rate was sustained in all TAM-Wd cell sub-types over the 14 day culture period [Fig 3.2].

3.1.3 Cell Motility

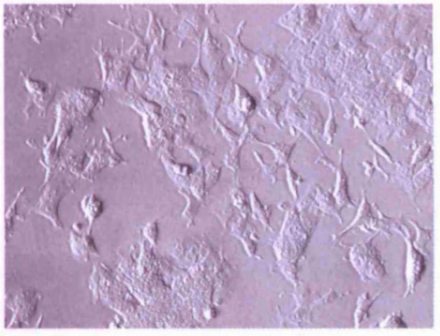
Cell motility was assessed by the ability of MCF-7, TAM-R and TAM-Wd (6-month) cells to migrate through fibronectin coated, polycarbonate membranes, as a simulation of their affinity for matrix-components. Migratory cells were stained with crystal violet and quantified by counting the number of stained cells present in five random fields of view at 10x magnification for each insert. Fig 3.3 shows the number of migratory TAM-R cells observed exceeded that of the MCF-7 cells, with an approximate 4-fold increase in the number of stained cells counted over three independent experiments ($p<0.001$). Following the tamoxifen withdrawal period of 6 months, the number of migratory cells fell ($p<0.001$), and cell counts were reduced to those recorded for the MCF-7 cells. Wound-healing assay analysis (see section 2.2.3) showed that TAM-R cells were significantly more able to achieve wound closure compared to both MCF-7 and tamoxifen withdrawn TAM-R cells, confirming the loss of TAM-R cell motility in the absence of tamoxifen [Fig 3.4] whilst cell proliferation was maintained [Fig 3.2]; emphasising the differential regulation of these events in breast cancer cells.

Fig 3.1 Cell Morphology: Capture of live-cell images using a Leica DM-IRE2 inverted microscope (20x magnification) fitted with a Hoffman condenser, as an assessment of morphological status in MCF-7, Tam-R, and Tam-Wd cells (1, 3 and 6 months).

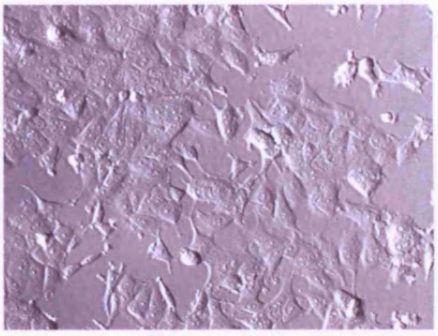
MCF-7



TAM-R



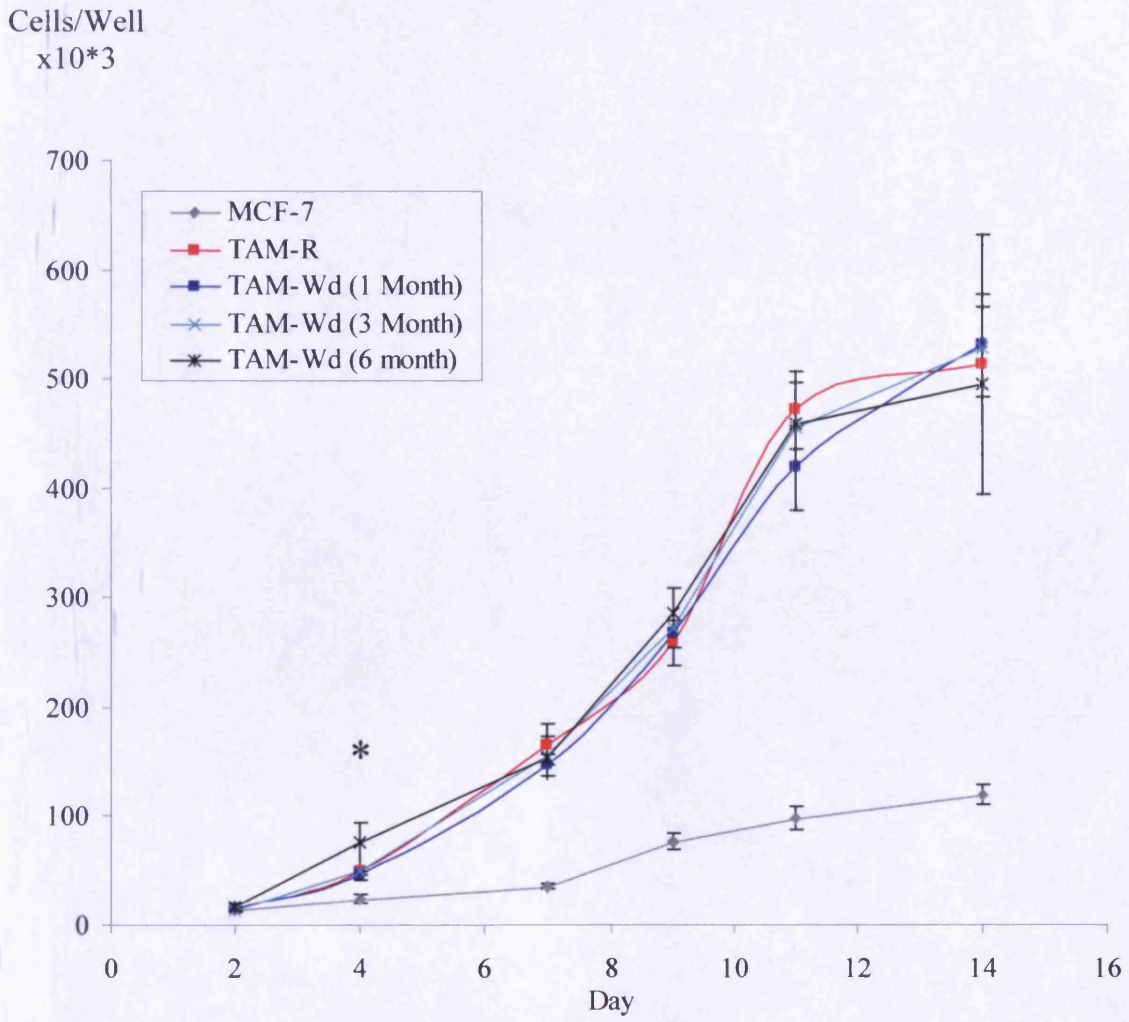
TAM-Wd 1 Month



TAM-Wd 3 Month

TAM-Wd 6 Month

Fig 3.2 Anchorage dependent growth assay: MCF-7, TAM-R, and TAM-Wd (1,3 6 months) cells were seeded at a similar density ($1x10^4$ cells/ well) on 24-Well Corning Co-star plates. Cells were trypsinised and counted using Coulter Counter apparatus on days 2, 4, 7, 11 and 14. The data shown represents actual cell number/well recorded over 3 independent experiments. Counts recorded for all resistant cell-lines were greater than MCF-7 cell counts from day 4 of culture (*p<0.001).



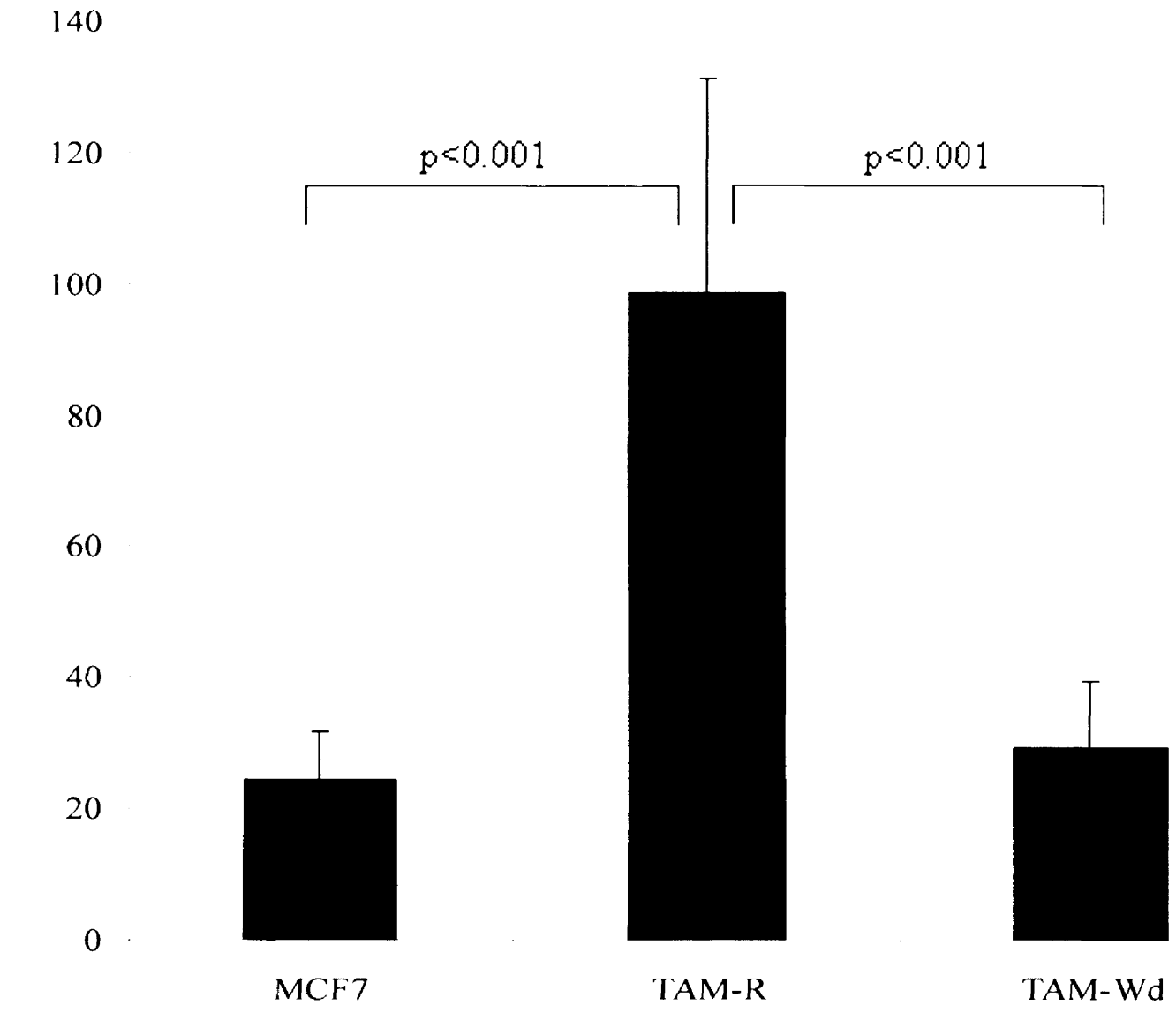
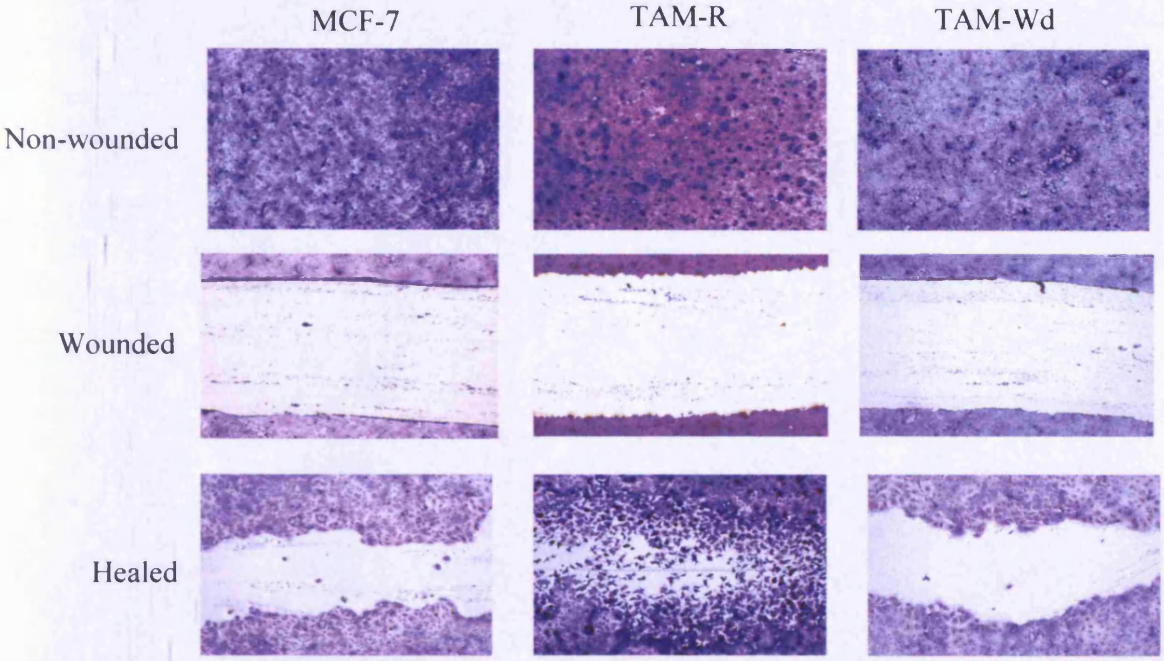

Average cell count per frame
140
120
100
80
60
40
20
0
p<0.001
p<0.001
MCF7
TAM-R
TAM-Wd

Fig 3.4 Wound Healing Assay: Monolayer cultures of MCF-7, TAM-R and TAM-Wd (6 month) cells were wounded by manual scratching with a pipette tip, washed with PBS and fresh medium was added. Some wells were fixed with 3.7% formaldehyde and stained with crystal violet directly after wounding to provide a timed control. After 48 hours, the remaining cells were fixed and stained and photographed to monitor wound closure at ×4 magnification.



3.2 Growth Pathway Analysis in Tam-R cells following Tamoxifen withdrawal

Having shown that despite the long-term withdrawal of tamoxifen, Tam-Wd cells proliferated at the same rate as the TAM-R cells, we sought to determine whether the expression of key growth regulatory elements previously identified in TAM-R cells, namely ER, EGFR and IGF-1R, had been retained following tamoxifen withdrawal. We also investigated how these signalling pathways contributed to cell growth using target specific inhibitors.

3.2.1 Oestrogen Receptor α:

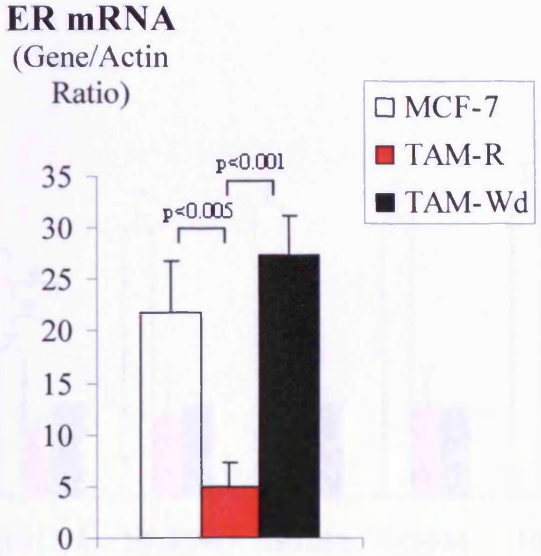
Levels of ER mRNA present in MCF-7, TAM-R and TAM-Wd resistant cell sub-lines were compared by means of Real-Time PCR analysis. It was observed that in TAM-R cells, the quantity of transcribed ER was significantly reduced from that expressed in the parental MCF-7 cells ($p<0.005$). Importantly, tamoxifen withdrawal restored TAM-R cell ER mRNA expression ($p<0.001$) to the level detected in the MCF-7 cells [Fig 3.5a]. Western-blot and ICC analysis confirmed that this phenomenon was also observed at the protein level [Fig 3.5b].

Having shown that ER mRNA and protein were restored to wt-expression levels in the TAM-Wd cell line, concentration response analyses were conducted using oestradiol (E2) and tamoxifen (TAM) in order to determine whether cells had also regained sensitivity to E2 growth stimulation, or TAM induced growth inhibition. E2 was shown to be highly mitogenic to the MCF-

7 cells since growth was significantly promoted over a concentration range of 10^{-12} to10^{-7}M ($p<0.001$). In contrast, E2 was not shown to be significantly mitogenic to TAM-R (in the absence of tamoxifen) or TAM-Wd cells [Fig 3.6a]. MCF-7 cells were shown to be sensitive to the growth inhibitory effect of TAM treatment in a dose-dependant manner. Unsurprising, the TAM-R cells were less sensitive to tamoxifen compared to MCF-7 cells, even at a concentration as high as 10^{-6}M ($p<0.001$). Interestingly, despite having been withdrawn from tamoxifen for 6 months, re-challenge of the TAM-Wd cells with the anti-hormone had no significant effect on cell growth [Fig 3.6b]. However, following the removal of functional ER protein, using the pure-ER antagonist fulvestrant (10^{-7}M), cell growth was shown to be inhibited in MCF-7, TAM-R and TAM-Wd cell lines as assessed by a series of three independent growth assays [Fig 3.7]. Importantly however, the extent to which fulvestrant inhibited growth varied between cell lines. TAM-Wd cells were shown to be the least sensitive (TAM-Wd vs TAM-R $p<0.001$, vs MCF-7 $p<0.001$) (30% growth inhibition), in comparison to TAM-R (60%) and wt (80%) cells (TAM-R vs MCF-7 $p<0.001$) following 11 days of culture with the anti-oestrogen. The data implies that the significance of ER signalling as a contributor to breast cancer cell growth decreases following the acquisition of tamoxifen resistance (presumable due to the emergence of alternative growth pathways like EGFR/IGFR), and further decreases following the withdrawal of the drug.

Fig. 3.5 ERα expression A: Real-Time PCR evaluation of ER mRNA concentration in MCF-7, TAM-R and TAM-Wd cells. Data shown was normalised to actin, hence was represented by an arbitrary unit (n=3). B: ICC staining and western blot for total ER in MCF-7, TAM-R and TAM-Wd cells.

A



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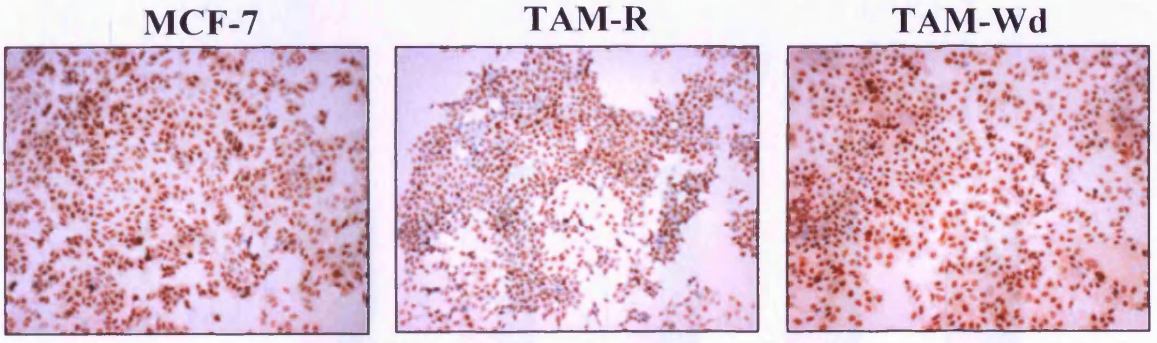


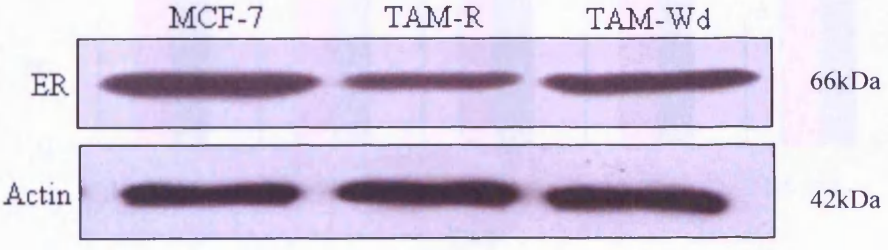
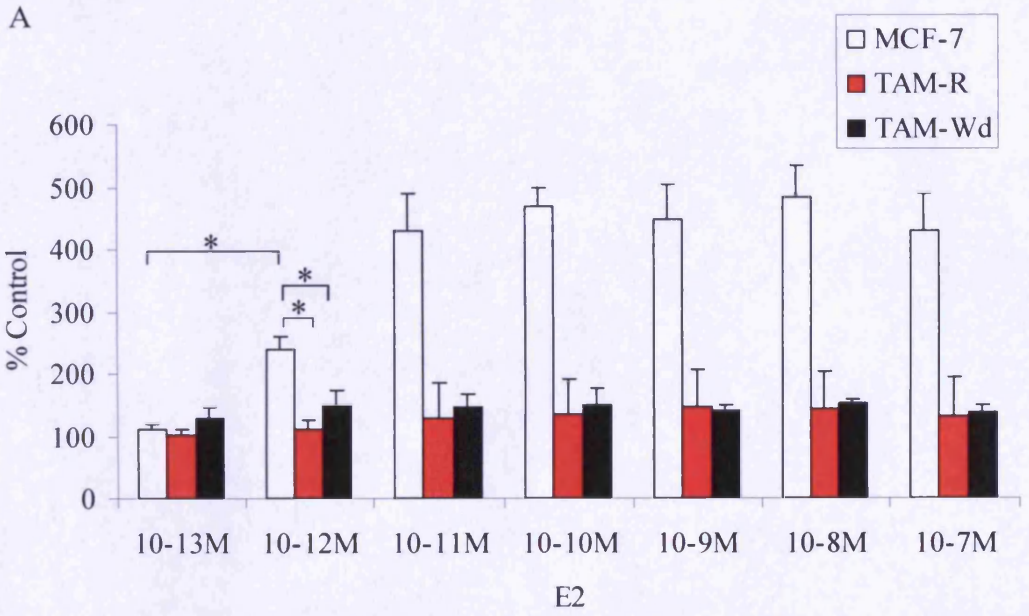


Fig 3.6 MCF-7, TAM-R and TAM-Wd cell concentration responses to E2 (A) and TAM (B). Cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated, and cultured for a further 7days. Cell number was assessed using Coulter Counter analysis. Data is shown as cell number as a percentage of the counts recorded for non-treated cells (n=3). (*p<0.001)



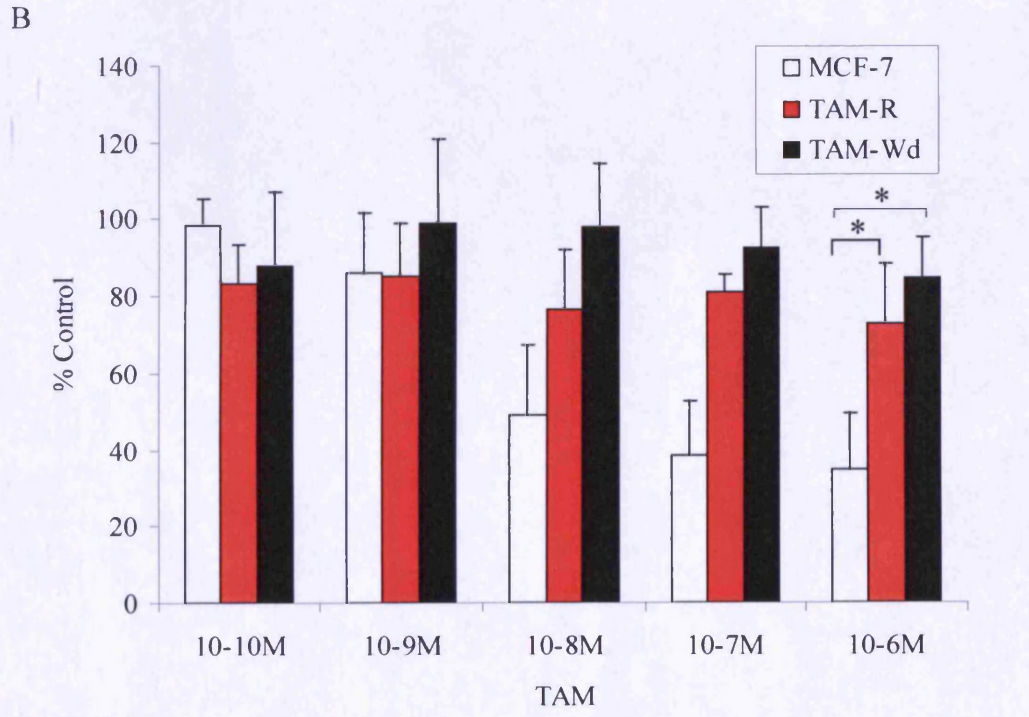
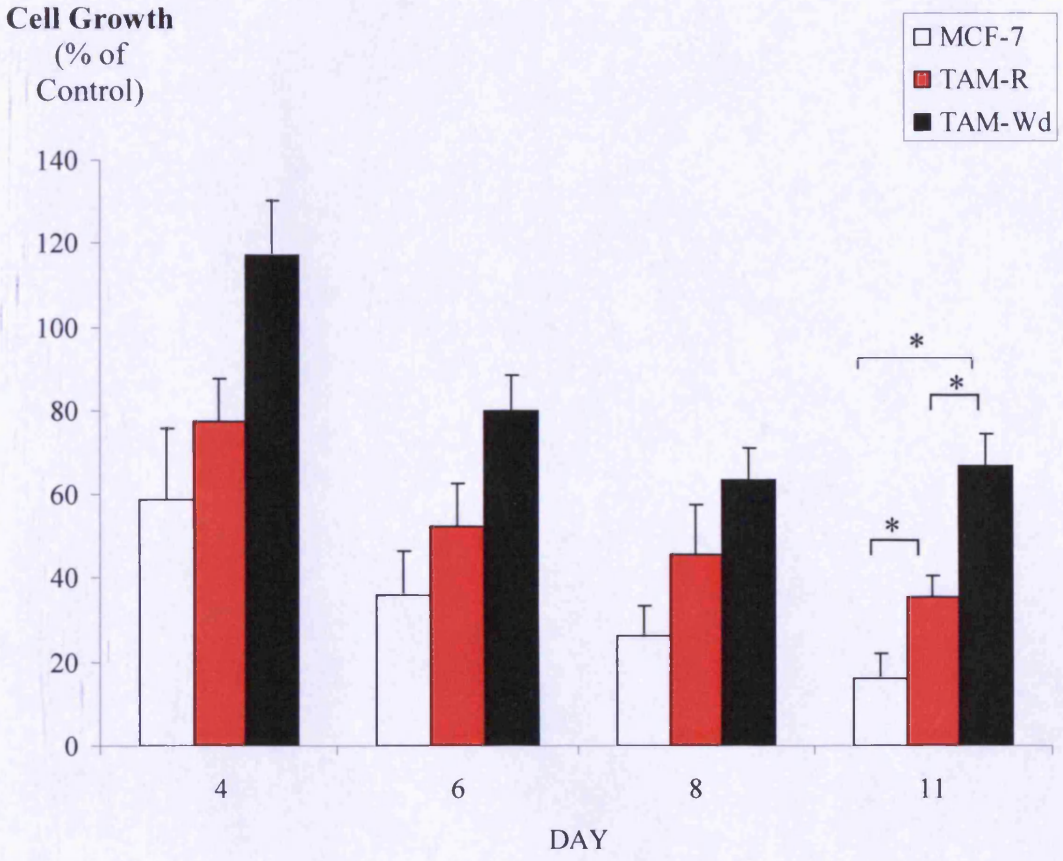


Fig 3.7 Cell growth inhibition in response to fulvestrant: MCF-7, TAM-R, and TAM-Wd (6 months) cells were seeded on 24-Well Corning Co-star plates. Following 24hrs incubation in control media, cells were treated with 0.1μM fulvestrant. Cell media was changed every 4 days. Subsequent counts were taken on days 4, 6, 8 and 11 using Coulter counter apparatus and the data shown represents the percentage cell number relative to non-treated control cells, as a measurement of growth inhibition (*p<0.001).



3.2.2 EGF-R Signalling Pathway

EGF-R expression has been previously shown to be elevated in TAM-R cells and represents a major determinant of their growth. In order to examine whether this signalling pathway contributes to cell growth in tamoxifen withdrawn TAM-R cells, levels of EGFR mRNA were assessed by Real-Time PCR in the TAM-Wd cell sub-line, relative to MCF-7 and TAM-R cells [Fig 3.8a]. Analysis confirmed that in the TAM-R cells, the quantity of EGFR mRNA was significantly increased from that expressed in the parental MCF-7 cells ($p=0.012$) and this phenomenon was also observed at the protein level as assessed by ICC and western blotting [Fig 3.8b]. Interestingly, it was observed that in TAM-Wd cells, EGFR expression at both the mRNA and protein level was reduced compared to the TAM-R cells, although values remained slightly higher than those seen in the MCF-7 parental cell line.

Having shown that EGFR expression was greatly reduced in tamoxifen withdrawn TAM-R cells, dose response and anchorage dependent growth studies were performed to determine the extent to which EGFR-signalling contributed to MCF-7, TAM-R and TAM-Wd cell growth, using the EGFR-specific tyrosine kinase inhibitor, gefitinib.

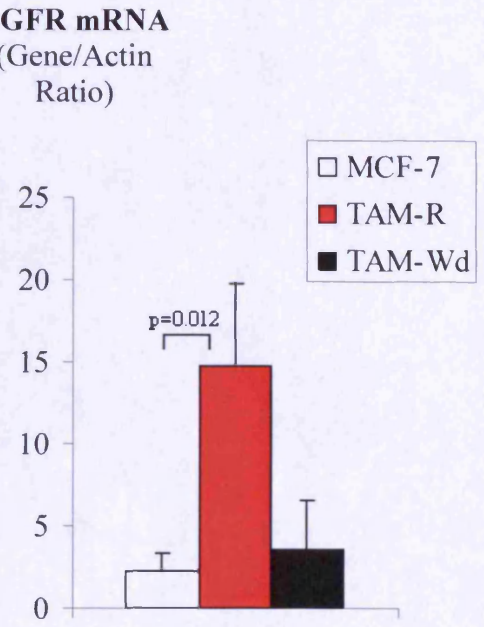
Significantly, while the highly EGFR-positive tamoxifen resistant cells showed a substantial (albeit incomplete) inhibitory response to gefitinib with a 50% growth inhibition being achieved at 1μM ($p<0.001$), tamoxifen withdrawal was associated with a reduced sensitivity to TKI. Indeed, the growth inhibition achieved in the tamoxifen withdrawn TAM-R cells and MCF-7 cells was significantly lower than in the TAM-R cells ($p<0.001$) [Fig

3.9]. This observation was confirmed in growth assays in which all cell-lines were cultured with gefitinib at 1μM for up to 11days, taking cell-counts at days 4, 6, 8 and 11 [Fig 3.10]. TAM-R cells clearly demonstrated the greatest sensitivity to the drug, showing a 5-fold decrease in cell number compared to non-treated control cells by day 8 ($p<0.001$). MCF-7 and TAM-Wd cells again demonstrated their reduced sensitivity to gefitinib compared to the TAM-R cells following 11 days of culture ($p<0.001$).

Therefore, the withdrawal of tamoxifen from TAM-R cells caused a reduction of the EGF-R mRNA/protein expression detectable in these cells, which directly correlated with their loss of sensitivity to gefitinib, however, it is clear that their cell growth rate was not impeded [Fig 3.2]. Interestingly, the withdrawal of tamoxifen was also shown to reduce the motile capacity of the TAM-R cells [Figs 3.3 and 3.4], though the association of this event with reduced EGFR expression/signalling was not investigated.

Fig. 3.8 EGFR expression A: Real-Time PCR evaluation of EGF-R mRNA concentration in MCF-7, TAM-R and TAM-Wd cells. Data shown was normalised to actin, hence was represented by an arbitrary unit (n=3). B: ICC staining/western blot analysis for total EGF-R in MCF-7, TAM-R and TAM-Wd cells.

A



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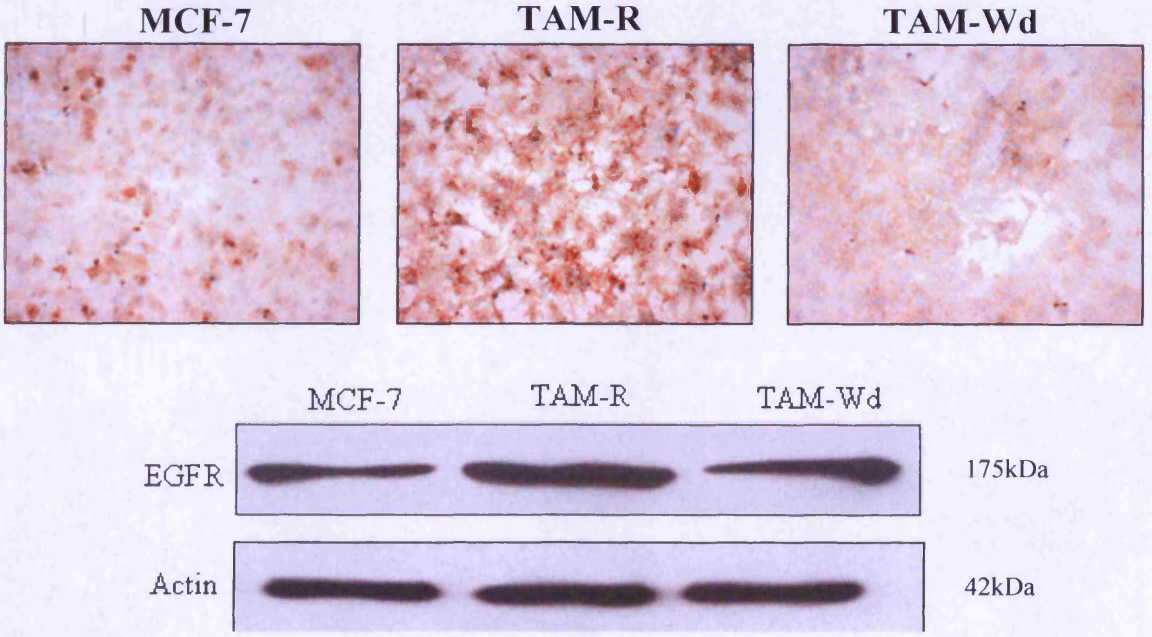


Fig 3.9 Gefitinib concentration response: MCF-7, TAM-R and TAM-Wd cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated, and cultured for a further 7days. Cell number was assessed using Coulter counter analysis. Data is shown as cell number as a percentage of the counts recorded for non-treated cells (n=3) ($p<0.001$).

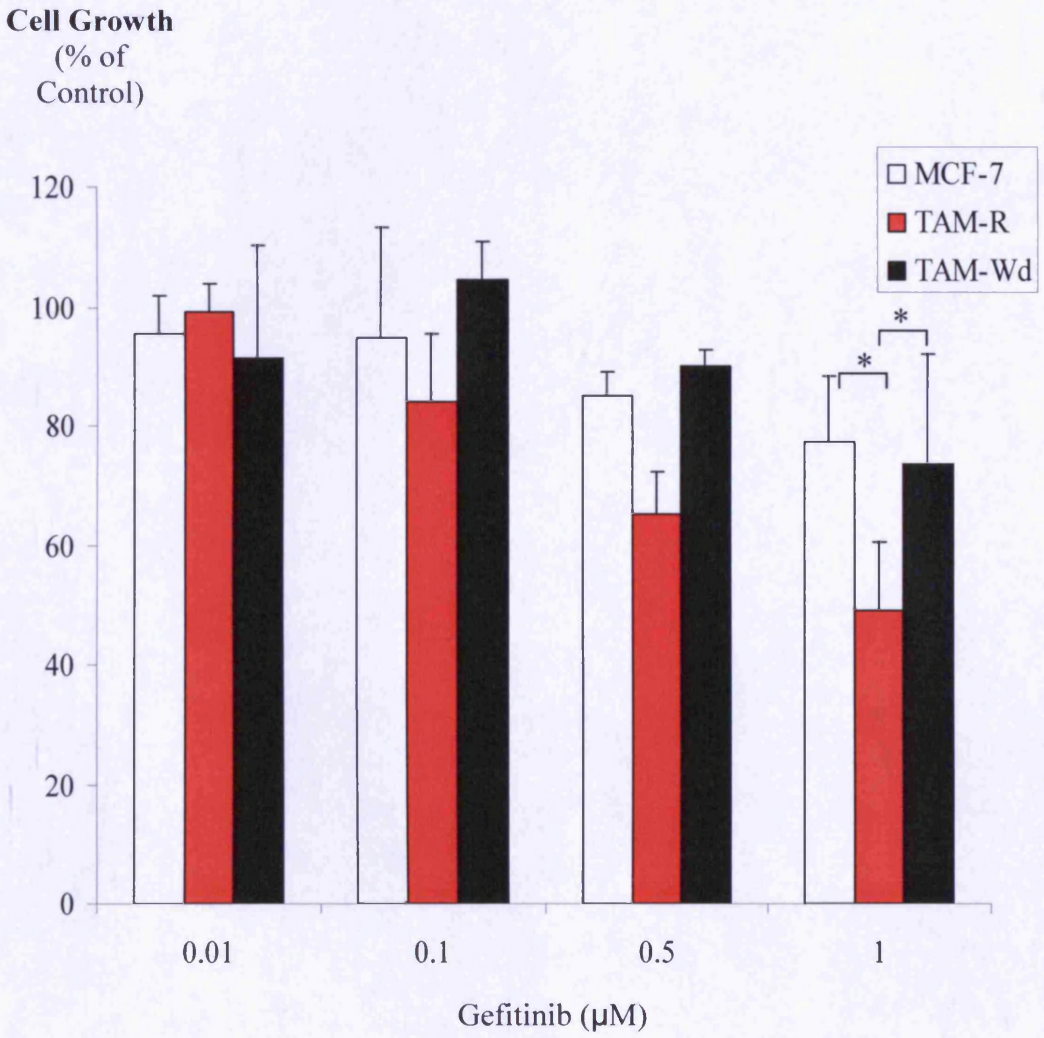
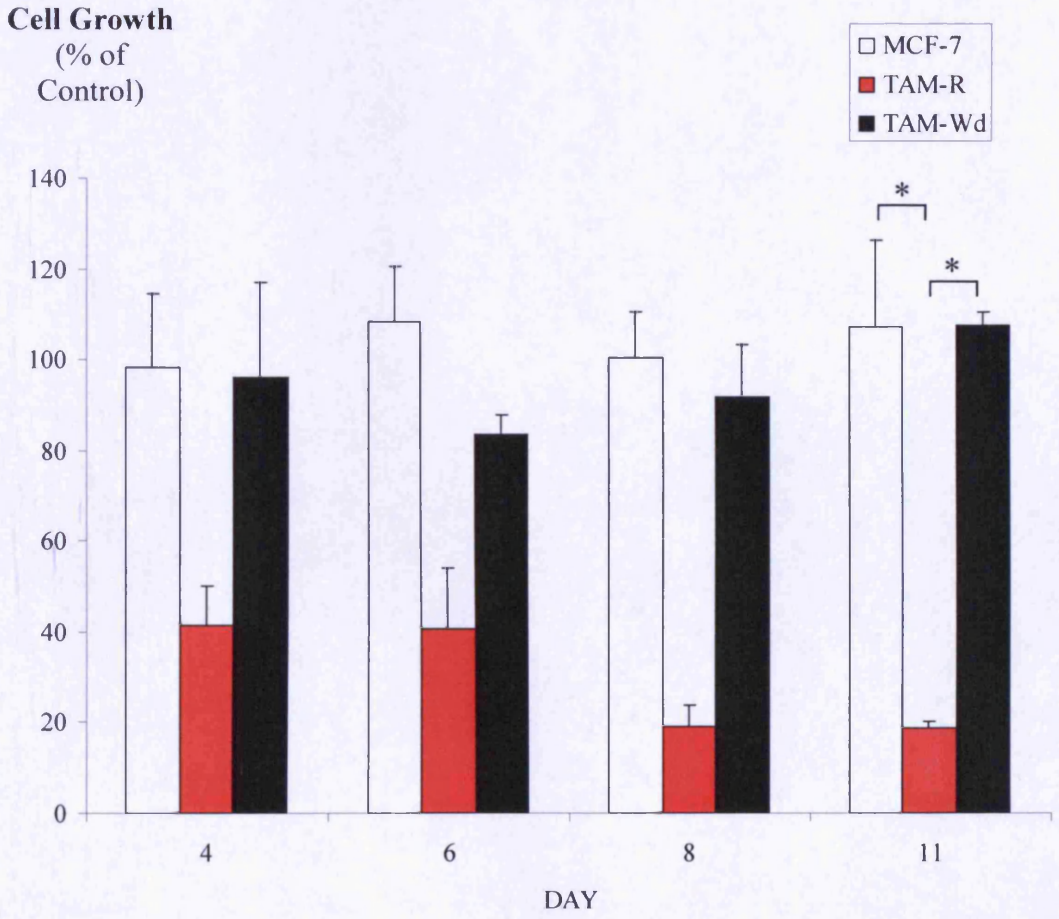


Fig 3.10 Cell growth inhibition in response to gefitinib: MCF-7, TAM-R, and TAM-Wd (6 months) cells were seeded on 24-Well Corning Co-star plates. Following 24hrs incubation in control media, cells were treated with 1μM Gefitinib. Cell media was changed every 4 days. Subsequent counts were taken on days 4, 6, 8 and 11 using Coulter counter apparatus and the data shown represents the percentage cell number relative to non-treated control cells, as a measurement of growth inhibition.



3.3.3 IGF-1R Signalling Pathway

Having established that EGFR expression was reduced in tamoxifen withdrawn TAM-R cells in parallel with the loss of their sensitivity to gefitinib growth inhibition, the role of IGF-1R signalling was next examined, since over-activation of this pathway has previously been associated with anti-hormone resistant cell growth [Knowlden *et al,* 2005]. The expression levels of IGF-1R mRNA present in MCF-7, TAM-R and TAM-Wd cell sub-lines were compared by means of Real-Time PCR analysis. It was observed that IGF-1R mRNA was significantly reduced in TAM-R cells compared to wt-MCF-7 cells ($p=0.013$), though levels still remained detectable. Following tamoxifen withdrawal, IGF-1R expression increased in the TAM-R cells ($p=0.005$) (in concurrence with the decrease in EGFR expression [Fig 3.8a]) to a level similar of that detected in the wt-cells [Fig 3.11a]. Western-blot and ICC analysis showed that this phenomenon was also seen at the protein level [Fig 3.11b].

Significantly, whilst tamoxifen withdrawal served to reverse the down-regulation of IGF-1R expression in TAM-R cells, this did not translate to an increased dependence on this signalling pathway for the growth of these cells. Concentration response analysis using IGF-1R inhibitor ABDP showed that compared to MCF-7 cells (which have higher IGF-1R expression than TAM-R cells), the TAM-R cells were less sensitive to the drugs growth inhibitory effect at a concentration of 0.25 μM, though were similarly effected at a dose of 0.5μM. Despite the restoration of IGF-1R expression following tamoxifen withdrawal, TAM-Wd cells were less sensitive to ABDP than wt-cells at both

concentrations ($p=0.002$ for 0.5 μM), inferring tamoxifen withdrawn TAM-R cells maybe less sensitive to IGF-1R blockade [Fig 3.12].

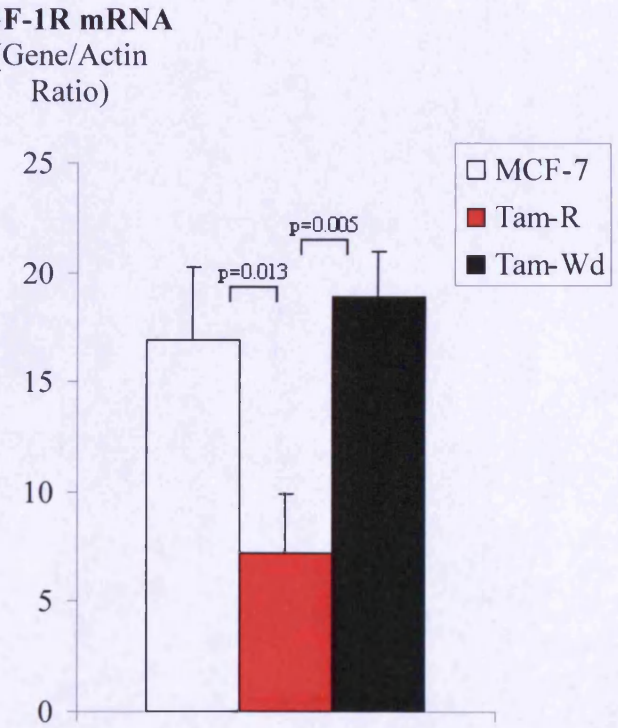
3.4 Summary

In total, we have observed that tamoxifen withdrawal renders TAM-R cells less sensitive to growth inhibition in response to ER, EGFR and IGF-1R inhibitors, yet the elevated rate of TAM-R cell growth remains. This leads us to investigate other possible mechanisms by which long-term tamoxifen exposure could permanently alter rate of cell proliferation. In addition to tamoxifen up-regulating components associated with cell growth, such as EGFR, tamoxifen could also permanently suppress previously oestrogen-responsive pro-apoptotic or tumour suppressive genes, as part of the mechanistic events governing the acquisition of tamoxifen resistance.

In light of this, the focus of the remaining chapters was to determine whether tamoxifen was capable of permanently silencing the expression of genes previously up-regulated by oestrogen, and whether such changes in gene expression could contribute to cell growth in addition to alternate growth factor signalling.

Fig. 3.11 IGF-1R Expression A: Real-Time PCR evaluation of IGF-1R mRNA concentration in MCF-7, TAM-R and TAM-Wd cells. Data shown was normalised to actin, hence was represented by an arbitrary unit (n=3). B: ICC staining/western blot analysis for total IGF-1R in MCF-7, TAM-R and TAM-Wd cells.

A



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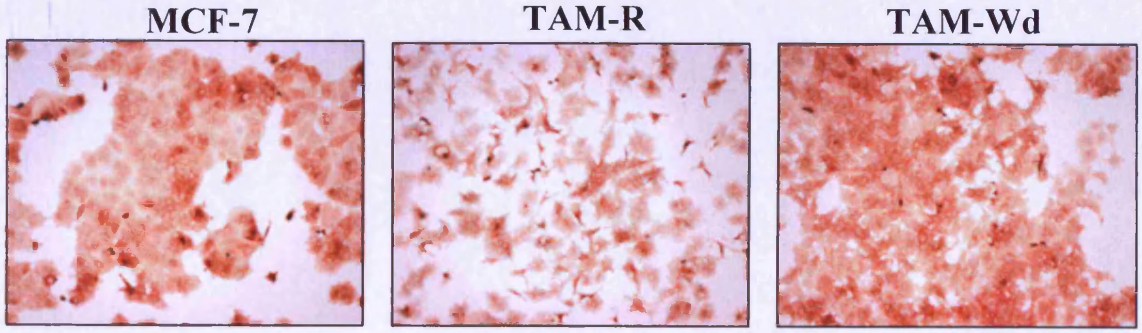


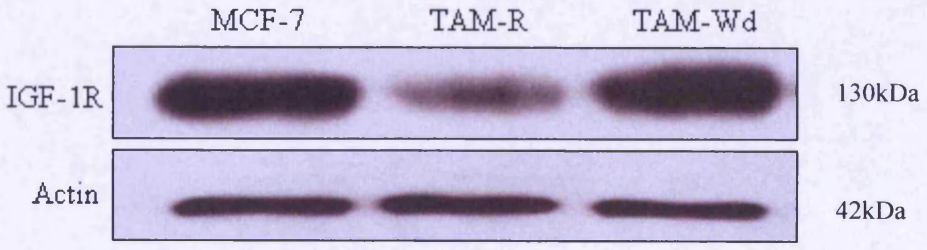
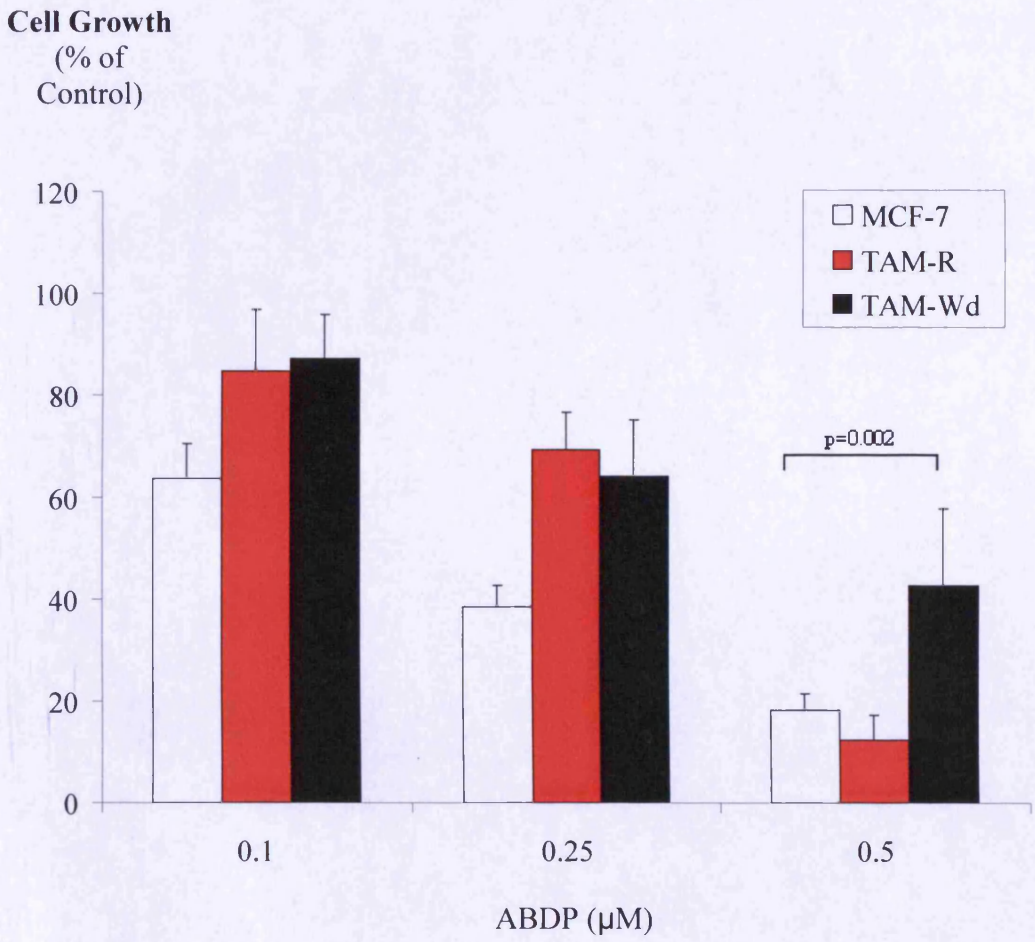


Fig 3.12 ABDP Concentration Response: MCF-7, TAM-R and TAM-Wd cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated, and cultured for a further 7days. Cell number was assessed using Coulter counter apparatus. Data is shown as cell number as a percentage of the counts recorded for non-treated cells (n=3).



4. RESULTS

The inactivation of tumour suppressor genes is central to the development of all common forms of cancer. This inactivation often results from epigenetic silencing associated with promoter hypermethylation rather than intrinsic mutations [Widschwendter and Jones *et al,* 2002]. Hypermethylation of gene promoters associated with tumour suppressor factors, such as p53, has been shown to directly contribute to carcinogenesis in a wide range of tumours and as such, inhibitors of DNA methylation, such as 5-Azacytidine, are being investigated as a potential therapeutic avenue. In human cells, the mechanisms underlying locus-specific or global methylation patterns remains unclear, however, in the present study, current data has led us to investigate the possibility that chronic tamoxifen exposure may serve to permanently silence classically regulated ER gene targets, previously associated with pro-apoptotic or tumour suppressive function, as a contributing factor to tamoxifen-resistant cell survival.

4.1 Oestrogen receptor gene targets can be silenced following chronic tamoxifen exposure in MCF-7 cells.

To provide proof of principle that downstream oestrogen-receptor gene targets could be permanently silenced following tamoxifen exposure, real-time PCR and ICC analyses were conducted to assess pS2 and progesterone receptor

(PR) expression in MCF-7, TAM-R, and TAM-Wd cells, pre-and post-oestradiol challenge. Interestingly, although all three cell lines shared a similarly low basal mRNA expression, pS2 and PR were up-regulated in the MCF-7 cells by 4 ($p=0.049$) and 40-fold ($p=0.032$) respectively following oestradiol challenge; in contrast to the TAM-R and TAM-Wd cells, in which no significant increase in pS2 or PR mRNA was detected (Figs 4.1a and 4.2a). Concurrently, ICC analysis showed that pS2 and PR protein staining was homogenous in all cell-lines, and that expression was only elevated in MCF-7 cells following oestradiol challenge [Fig 4.1b and 4.2b]. This infers that the suppression of ER activation of these genes is maintained in the absence of tamoxifen following long-term exposure to the drug.

To investigate promoter methylation as the mechanism of this apparent gene inactivation, MCF-7 and TAM-Wd cells were exposed to the de-methylating agent, 5-Azacytidine (5-Aza) at a concentration of 1μM, which has previously been used by other groups to provoke an effective DNA de-methylation response with minimal cell cytotoxicity [Van Agthoven *et al,* 1994; Sadikovic *et al,* 2004], for five days prior to E2 challenge (48hrs). Real-time PCR analyses showed that in TAM-Wd cells treated with 5-Aza, pS2 and PR mRNA expression increased 4 ($p=0.001$) and 16 fold ($p<0.001$) respectively following oestradiol challenge, in contrast to non-5-Aza treated cells, for which little up-regulation of these genes were observed with E2. The expression of both genes was up-regulated to a similar degree in E2-treated MCF-7 cells (compared to non-E2 treated cells) both in the presence and absence of 5-Aza [Fig 4.3a and 4.4a].

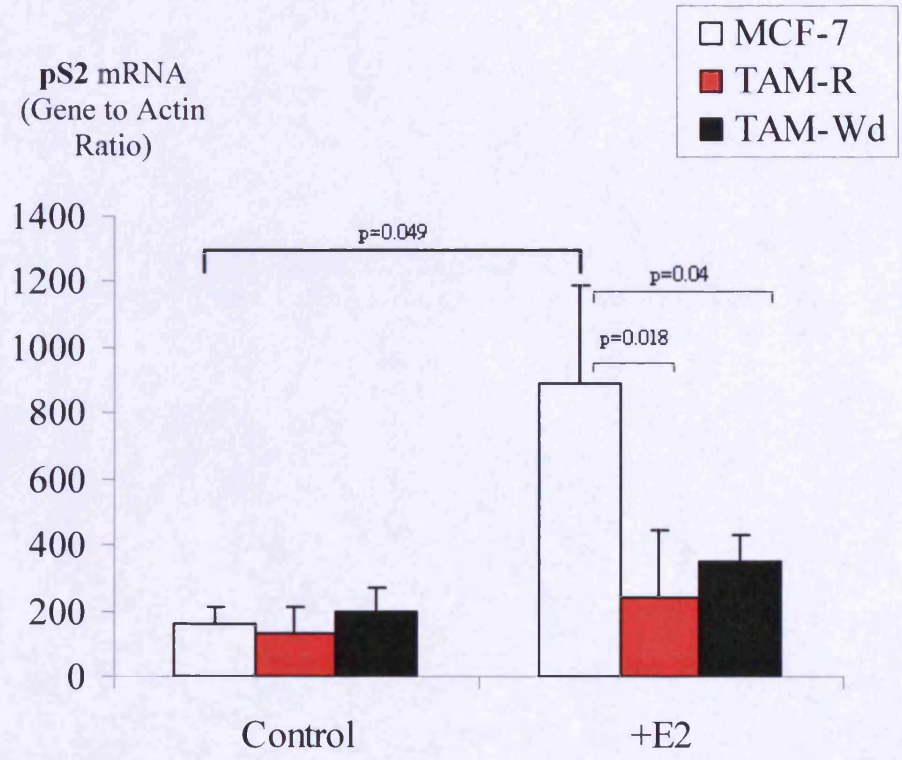
5-Aza treatment alone appeared to have little effect on basal expression of pS2 or PR, though it had restored oestrogen responsiveness at these gene promoter sites in TAM-Wd cells, permitting functional protein transcription [Fig. 4.3b and 4.4b], in contrast to E2-alone treated TAM-Wd cells [Fig 4.1b and 4.2b].

As part of an international collaboration with Dr Heidi Fiegl of the Medical University of Innsbruck, Austria, it was confirmed that 5-Aza was achieving the desired effect of gene promoter de-methylation using DNA samples harvested from similarly treated TAM-Wd cells. The methylation status of ALU and SAT-2 gene repeats, whose methylated status are highly correlative with global DNA methylation measurements, was assessed in TAM-Wd cells using a quantitative Taq-man based real-time PCR system. The expression of methylated ALU and SAT-2 ($p=0.004$) detected in TAM-Wd cells cultured with 5-Aza was reduced compared to non-treated cells (calculated as the percentage of methylated reference (PMR)) [Fig 4.5]. The presence of E2-alone in both treatment groups had no influence on gene methylation status.

Methylight reactions to pS2 and PR were conducted in parallel to the assessment of global methylation markers to confirm their methylated status in TAM-Wd cells. Methylated pS2 gene promoter was readily detectable in non-treated TAM-Wd cells, in contrast to the 5-Aza (+/- E2) treated cells, in which expression was reduced by approximately 4 fold ($p<0.001$) [Fig 4.6]. PR proved undetectable in the Methylight system since the quantity of PR expressed in these cells was too low.

Fig. 4.1 Expression of pS2: A. Real-Time PCR evaluation of pS2 mRNA concentration in MCF-7, TAM-R and TAM-Wd cells ± E2 at 10^{-9}M for 48hrs. Data shown represents cDNA detected normalised to actin, hence was represented by an arbitrary unit (n=3). B. ICC analysis of pS2 in MCF-7, TAM-R and TAM-Wd cells ± E2.

A



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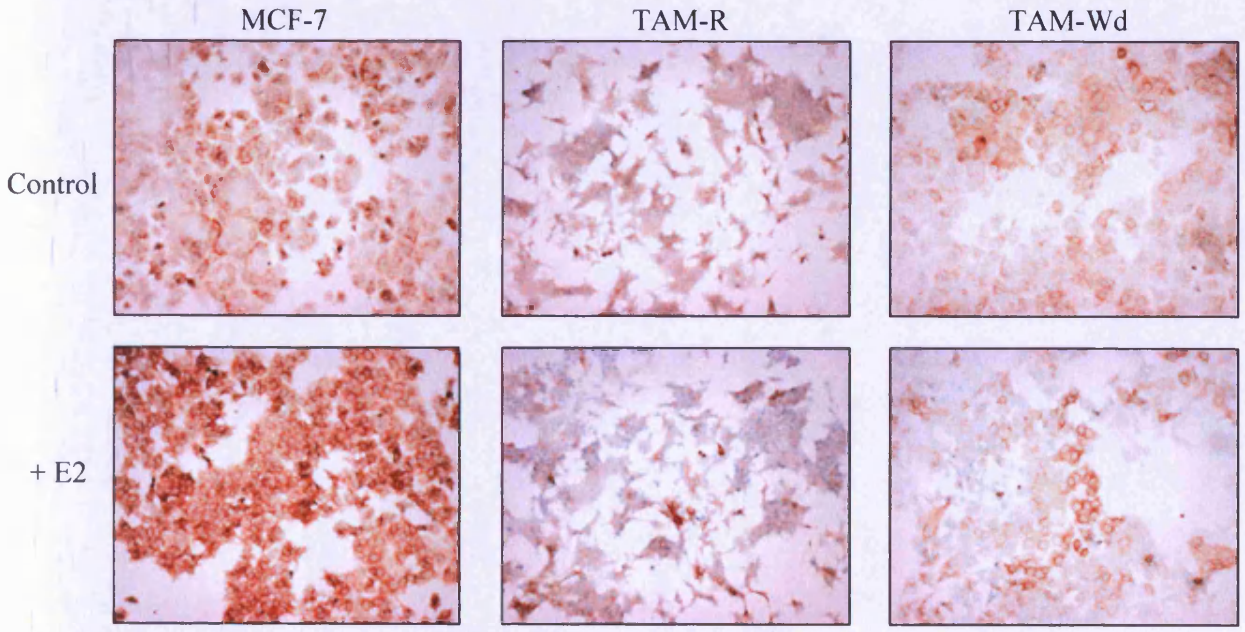


Fig. 4.2 Expression of PR: A. Real-Time PCR evaluation of PR mRNA concentration in MCF-7, TAM-R and TAM-Wd cells ± E2 at 10^{-9}M for 48hrs. Data shown represents cDNA detected normalised to actin, hence was represented by an arbitrary unit (n=3). B. ICC analysis of PR in MCF-7, TAM-R and TAM-Wd cells ± E2.

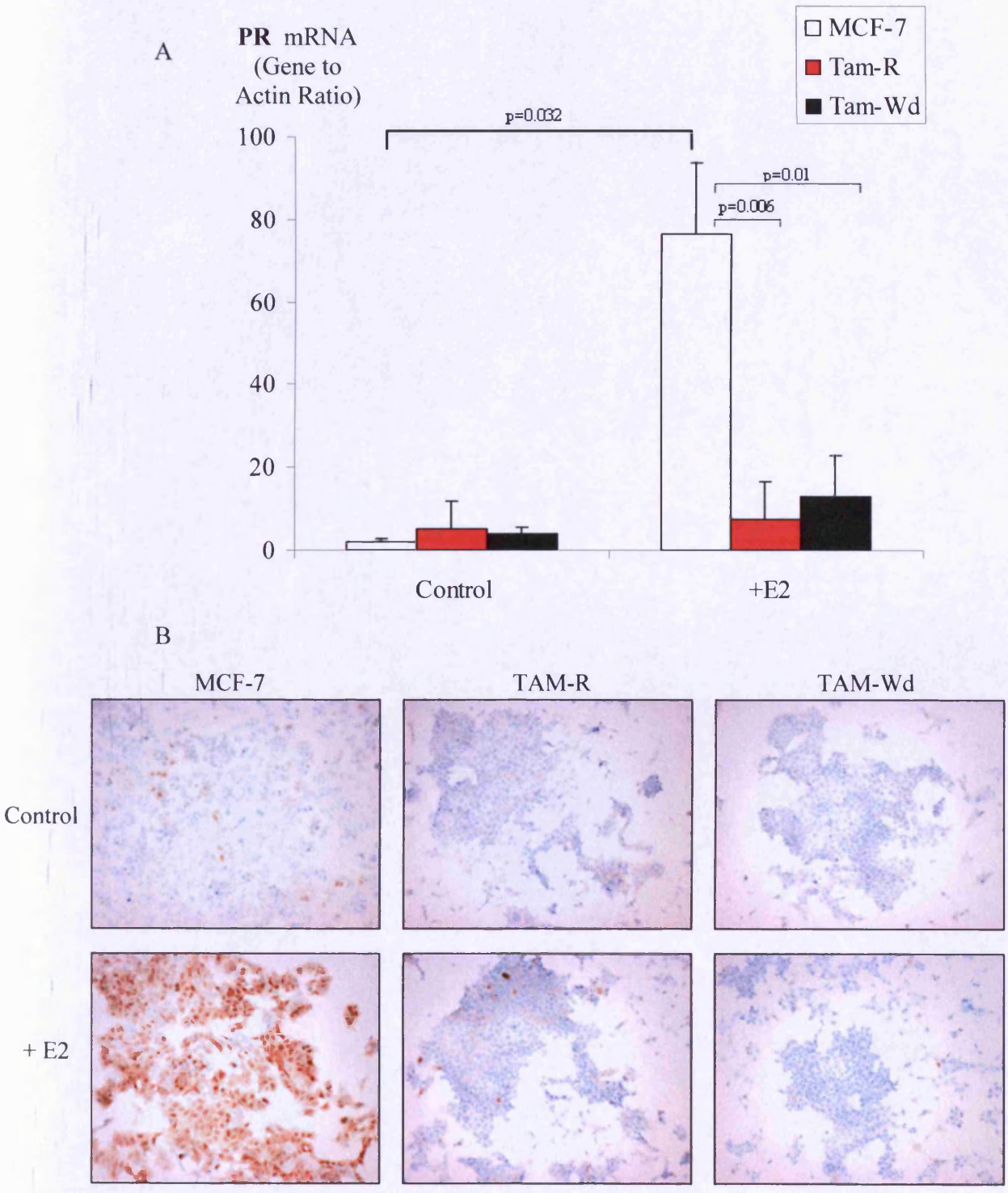
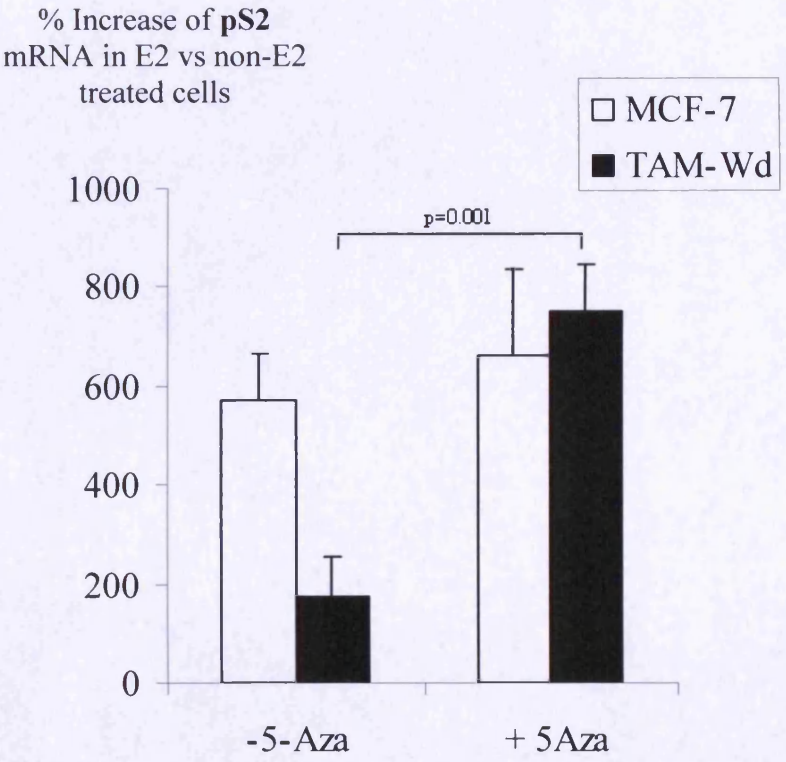


Fig. 4.3 Expression of pS2: A. Real-Time PCR evaluation of pS2 mRNA concentration in MCF-7 and TAM-Wd cells ± 5-Aza for 5 days ± E2 at 10^{-9}M for 48hrs before cell harvest. Data shown represents percentage increase in pS2 cDNA detected in cells ± 5-Aza, following E2 challenge. B. ICC analysis of pS2 protein expression in TAM-Wd cells treated with 5-Aza ± E2.

A



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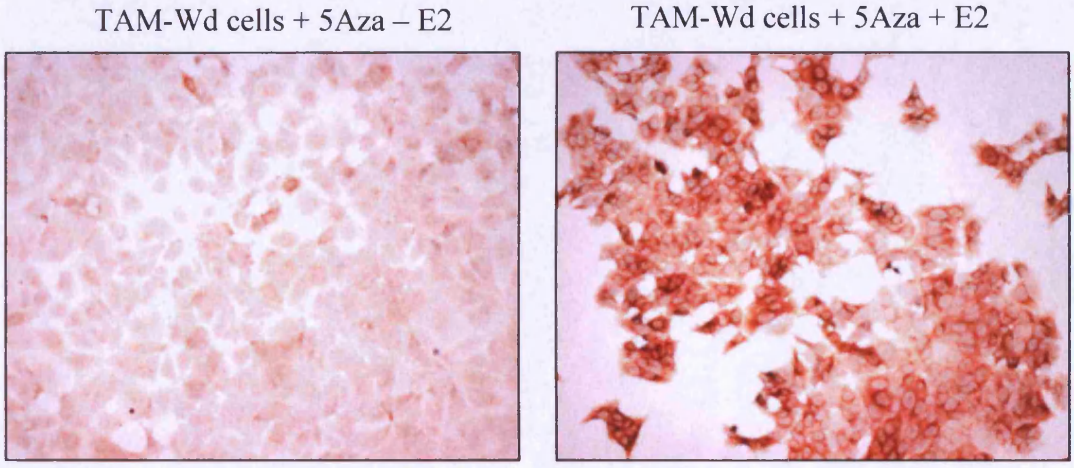
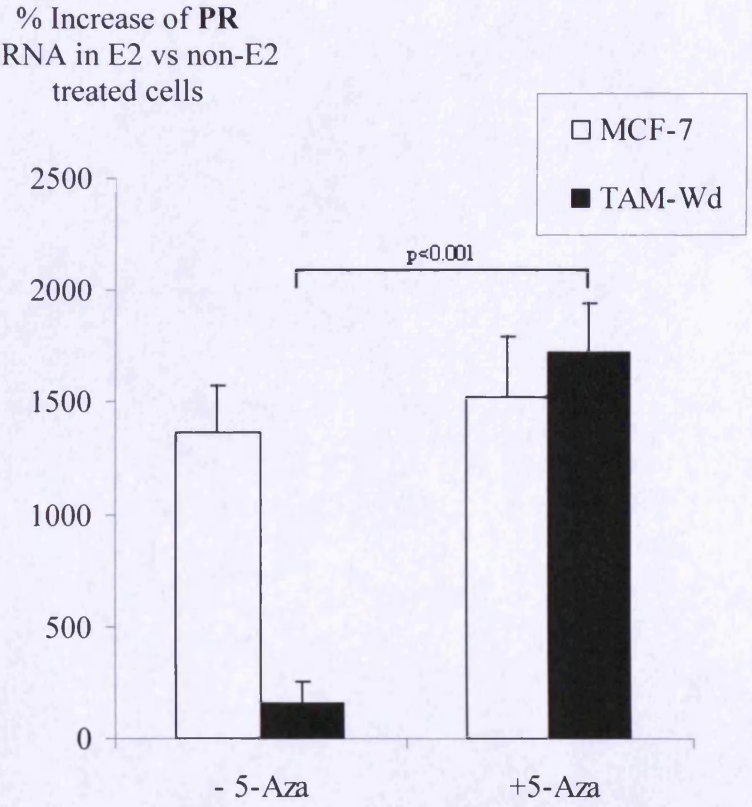


Fig. 4.4 Expression of PR: A. Real-Time PCR evaluation of PR mRNA concentration in MCF-7 and TAM-Wd cells ± 5-Aza for 5 days ± E2 at 10^{-9}M for 48hrs before cell harvest. Data shown represents percentage increase in PR cDNA detected in cells ± 5-Aza, following E2 challenge. B. ICC analysis of PR protein expression in TAM-Wd cells treated with 5-Aza ± E2.

A



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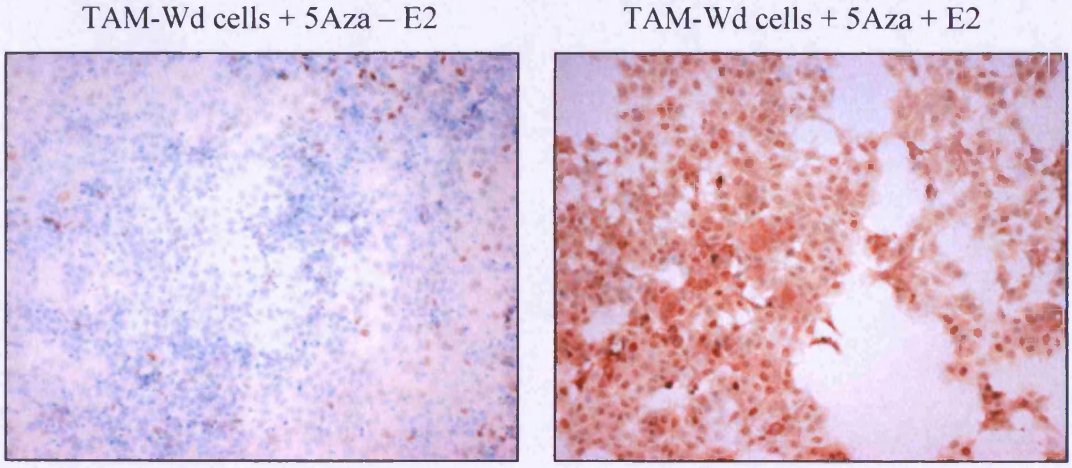
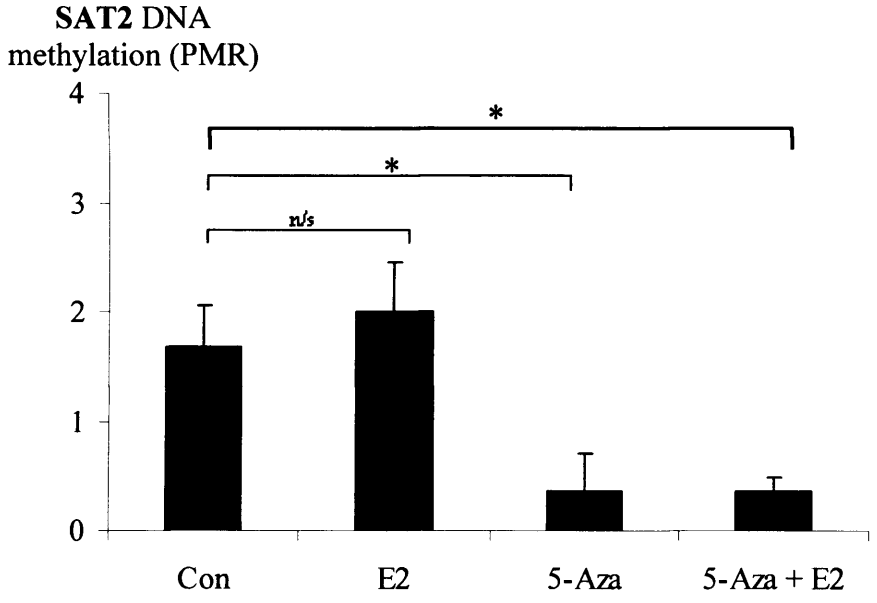


Fig. 4.5 Promoter methylation profile for global methylation marker genes, SAT2 and ALU, in TAM-Wd cells: MethylLight data specific for methylated SAT2 (A) and ALU (B), detected in cells cultured in the presence or absence of 5-Aza for 5 days, ± E2 for 48hrs before cell harvest (data expressed as percentage of methylated reference (PMR)) (*p=0.004).

A



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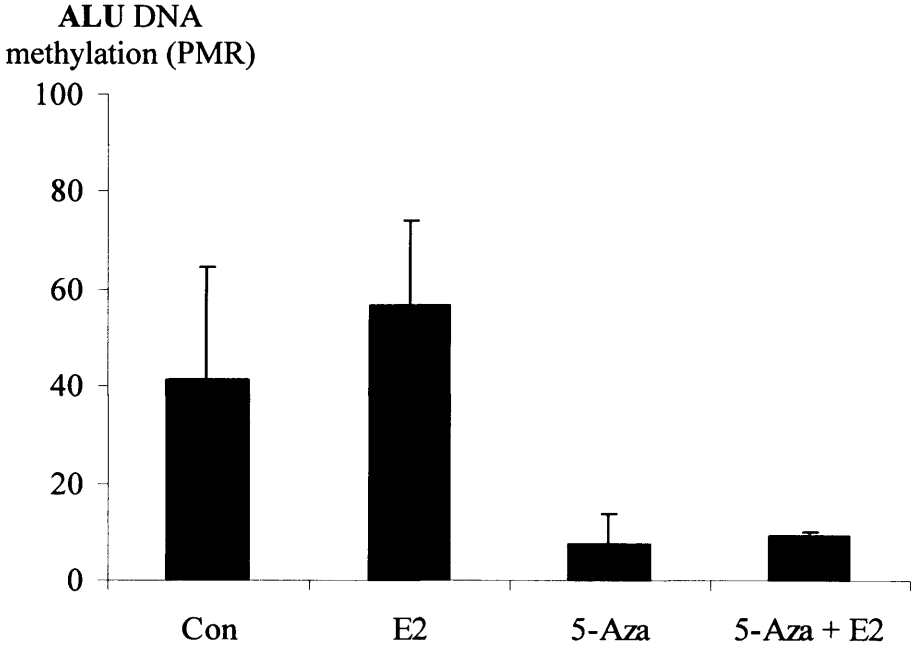
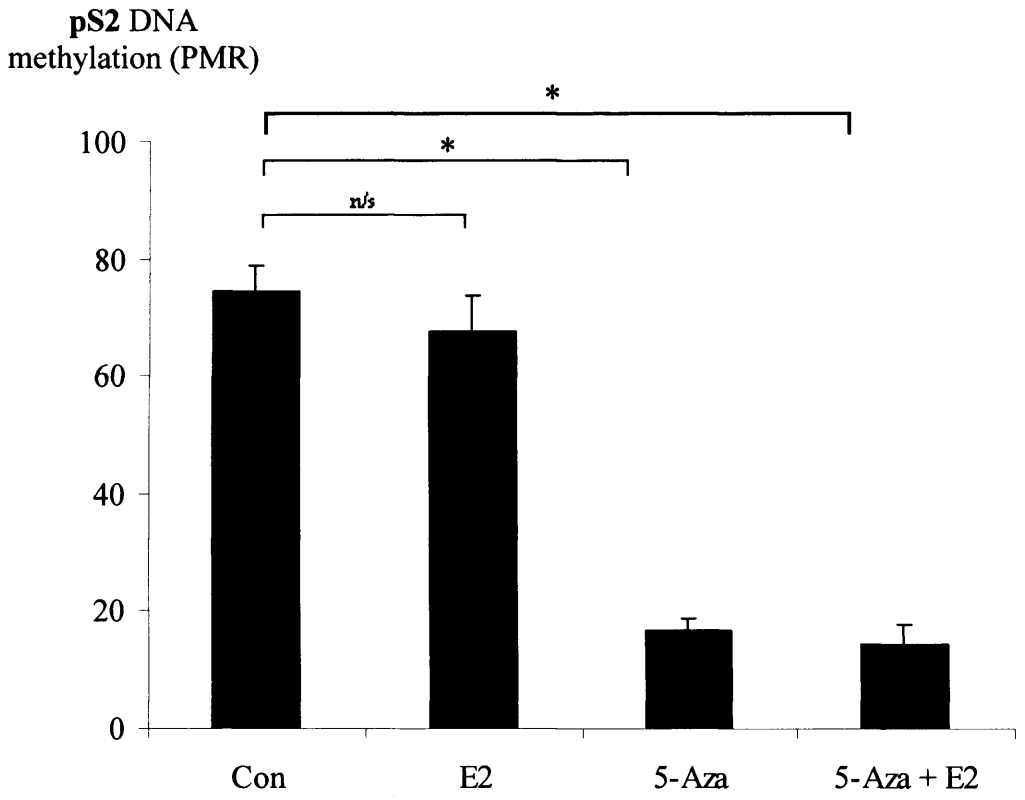


Fig. 4.6 Promoter methylation profile for pS2 in TAM-Wd cells: Methyl Light data specific for methylated pS2 detected in cells cultured in the presence or absence of 5-Aza for 5 days, ± E2 for 48hrs before cell harvest (data expressed as PMR) (*p<0.001).



4.2 The methylation of genes following chronic tamoxifen exposure contributes to TAM-Wd cell proliferation.

Having demonstrated that chronic tamoxifen exposure can be associated with diminished oestrogen-regulated gene expression through promoter methylation, a potential role for this phenomenon to contribute to the increased capacity of the TAM-Wd cells to proliferate was assessed. To determine whether E2-activation of gene promoters that were demethylated in TAM-Wd cells in response to 5-Aza treatment could effect cell growth, it was first necessary to assess the effect that 5-Aza (1μM) had on cell growth when used as a single agent, due to its associated cytotoxic functions. Growth curve analysis over a 7 day period (taking counts at days 2 and 7) showed that 5-Aza did not effect TAM-Wd cell growth [Fig 4.7]. Concentration response experiments were then carried out using TAM-Wd cells cultured in the absence or presence of 5-Aza at 1μM for 7 days, to determine whether the presence of the demethylation agent had any effect on E2 response over a concentration range of 10^{-12}M to 10^{-7}M. Surprisingly 5-Aza facilitated the role reversal of E2 as a mild mitogen, to an inhibitor of TAM-Wd cell growth. The maximal dose of E2 was shown to cause a 50% reduction in the number 5-Aza treated cells detected following 7 days culture compared to non-E2 (5-Aza) treated cells ($p<0.001$). In contrast, cells cultured in the absence of 5-Aza showed a 40% increase in cell number with E2 at 10^{-7}M (compared to non-E2 non-5-Aza treated control cells) ($p<0.001$) [Fig 4.8]. A concentration response to 5-Aza (0, 0.5 and 1μM) showed that the magnitude of the growth inhibitory response to E2 in TAM-Wd cells (10^{-7}M) increased in correlation with 5-Aza

concentration [Fig 4.9]. Further experiments showed that the addition of tamoxifen (10^{-7}M) to TAM-Wd cells cultured with 5-Aza (1μM) and E2, reversed growth suppression [Fig 4.10].

Interestingly, growth analysis of MCF-7 cells treated +/- 5-Aza and E2 over the same concentration range indicated that E2 was unable to inhibit the growth of these cells despite the presence of the demethylation agent, as assessed by MTT cell detection assays [Fig 4.11].

TAM-Wd cells cultured with 5-Aza, 5-Aza and either E2 or tamoxifen, or both, were grown for two weeks, taking cell counts at days 4, 7, 10 and 14 to determine the extent of the growth inhibition achieved by activating de-methylated oestrogen responsive genes. Following the two week culture period, the number of cells treated with 5-Aza and E2 had fallen to approximately 60% of the cell seeding density ($4x10^4$). Although cell counts appear to drop for 5-Aza and 5-Aza/E2/Tam treated cells after day 10 of culture (perhaps due to the initiation of cytotoxic events), cell populations remain significantly greater than the numbers recorded for 5-Aza + E2 treated cells at day 14 [Fig 4.12].

In total, these data provide evidence to suggest that a proportion of the genes silenced by promoter methylation as a consequence of pro-longed tamoxifen exposure are associated with a tumour-suppressive and/or pro-apoptotic function, and that demethylation and activation of such genes (using 5-Aza and E2 respectively) can induce tamoxifen-resistant cell growth inhibition.

Fig 4.7 Anchorage dependent growth assay: TAM-Wd cells were seeded at a density of $1x10^4$ cells/ well in 24-well plates. After 24hrs, cells were treated +/- 5-Aza (day 0), and cultured for a further 7days. Cell number was assessed using Coulter counter analysis. The data shown represents actual cell number/well recorded over 3 independent experiments.

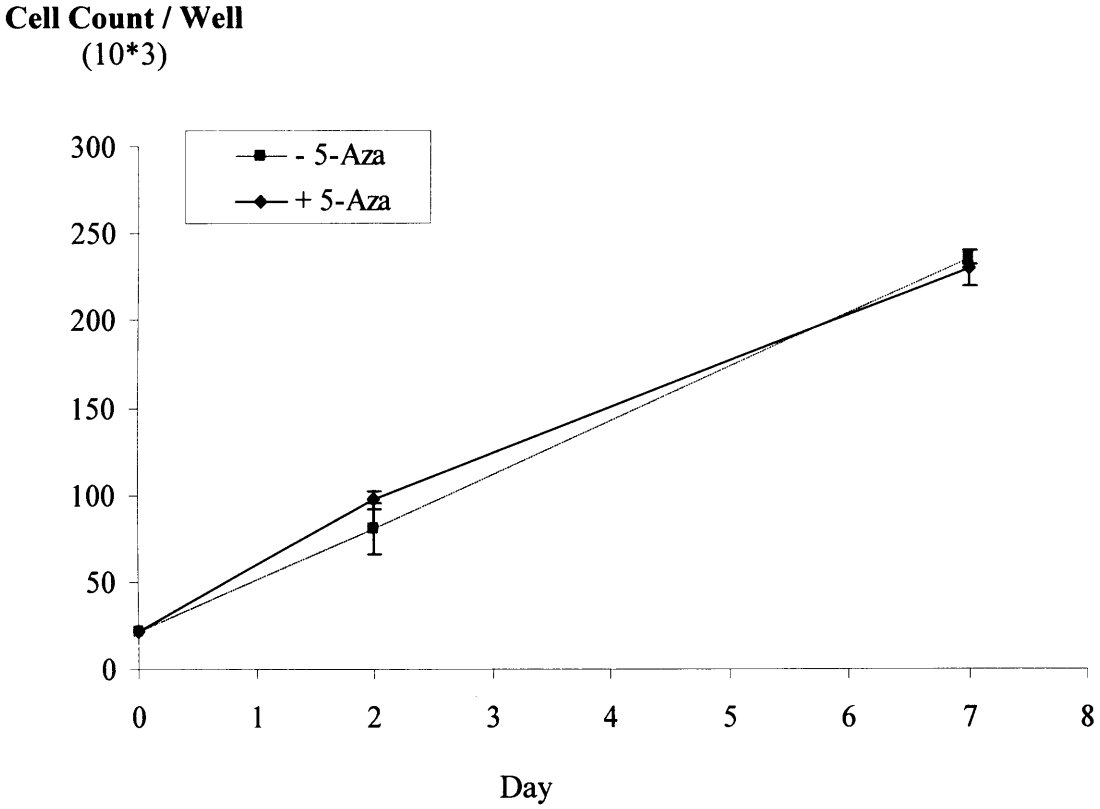


Fig. 4.8 Concentration response to oestradiol: TAM-Wd cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated ± 5-Aza and E2 at a concentration ranging from 10^{-12}M to 10^{-7}M. Cell number was assessed on day 7 of culture using Coulter counter analysis. Data shown represents E2-treated cell counts as a percentage of count recorded for non-E2 treated control cells ± 5-Aza (Significantly different to control (100%); *p<0.001, +p=0.002, °p=0.017).

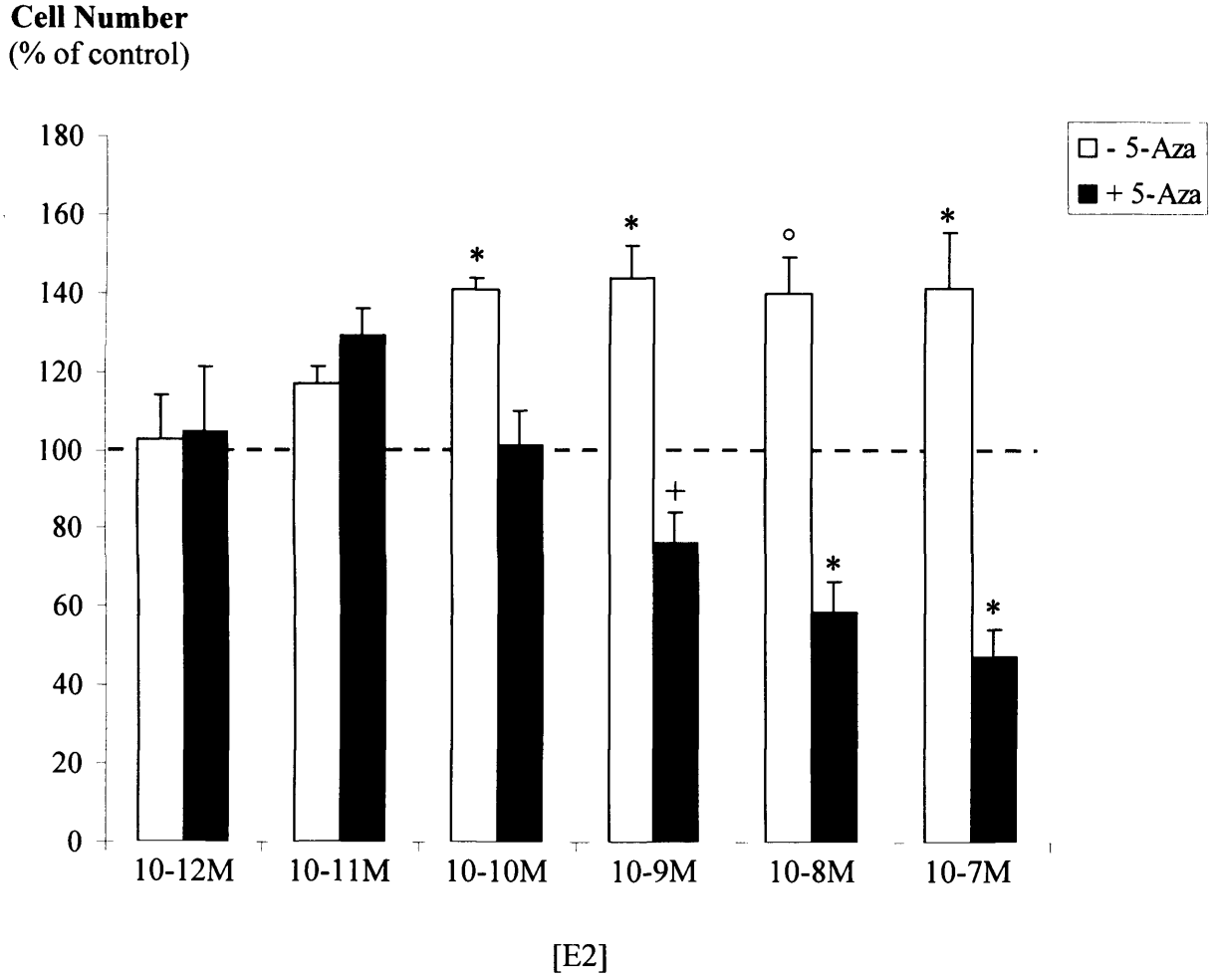


Fig. 4.9 Concentration response to 5-Aza: Tam-Wd cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated ± E2 (10^{-7}M) and 5-Aza at a concentration of 0, 0.5 or 1μM. Cell number was assessed on day 7 of cell culture using Coulter counter analysis. The data shown represents actual cell number/well recorded over 3 independent experiments.

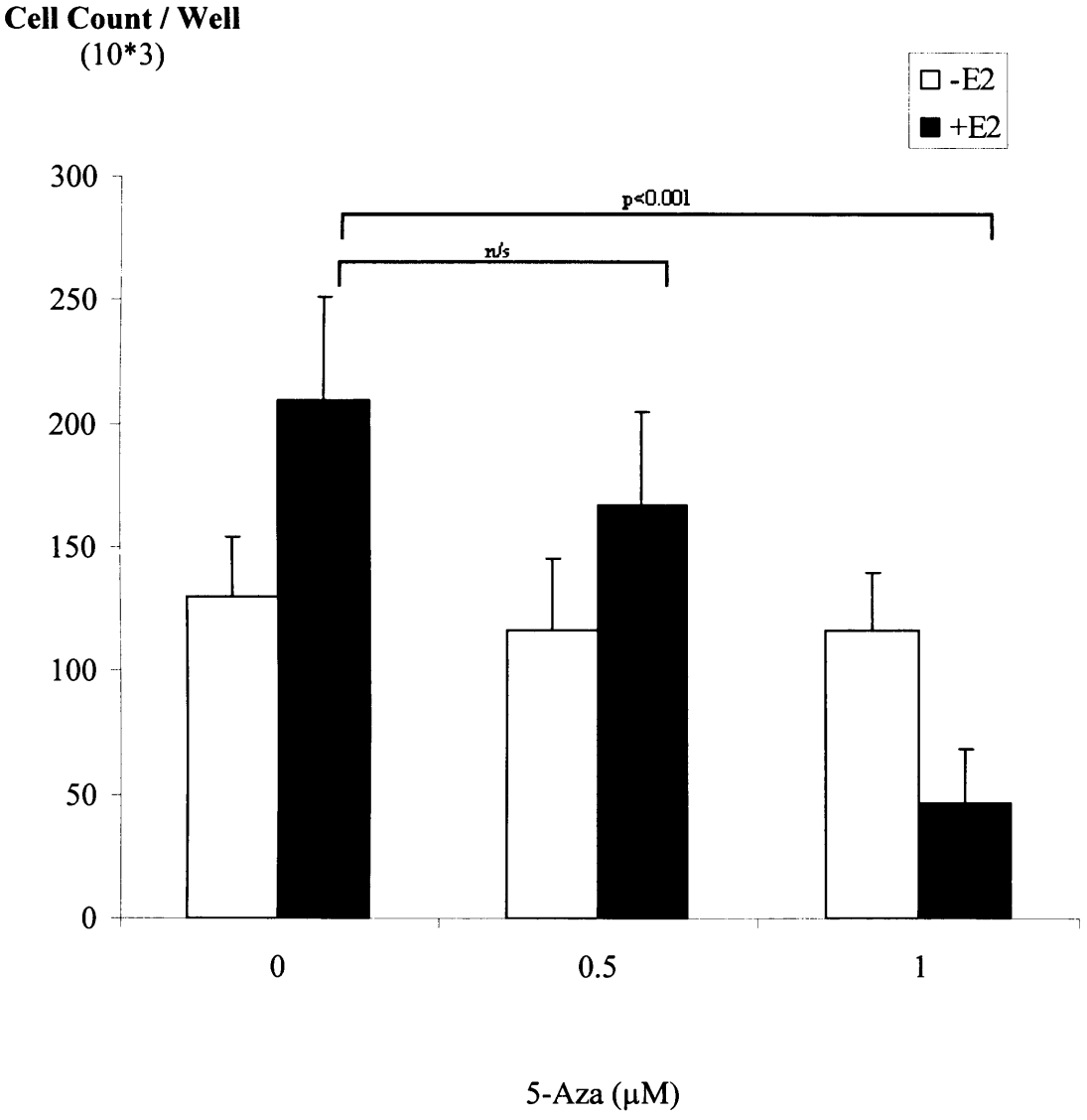


Fig. 4.10 Concentration response to oestradiol in 5-Aza treated TAM-Wd cells in the presence and absence of tamoxifen: TAM-Wd cells were seeded in 24-well plates at an initial density of $4x10^4$cells/well. After 24hrs, cells were treated with 5-Aza +/- tamoxifen and E2 at a concentration ranging from 10^{-12}M to 10^{-7}M. Cell number was assessed on day 7 of culture using Coulter counter analysis. Data shown represents E2-treated cell counts as a percentage of counts recorded for non-E2 treated control cells. (Significantly different to control (100%); *p<0.001, +p=0.002, °p=0.026).

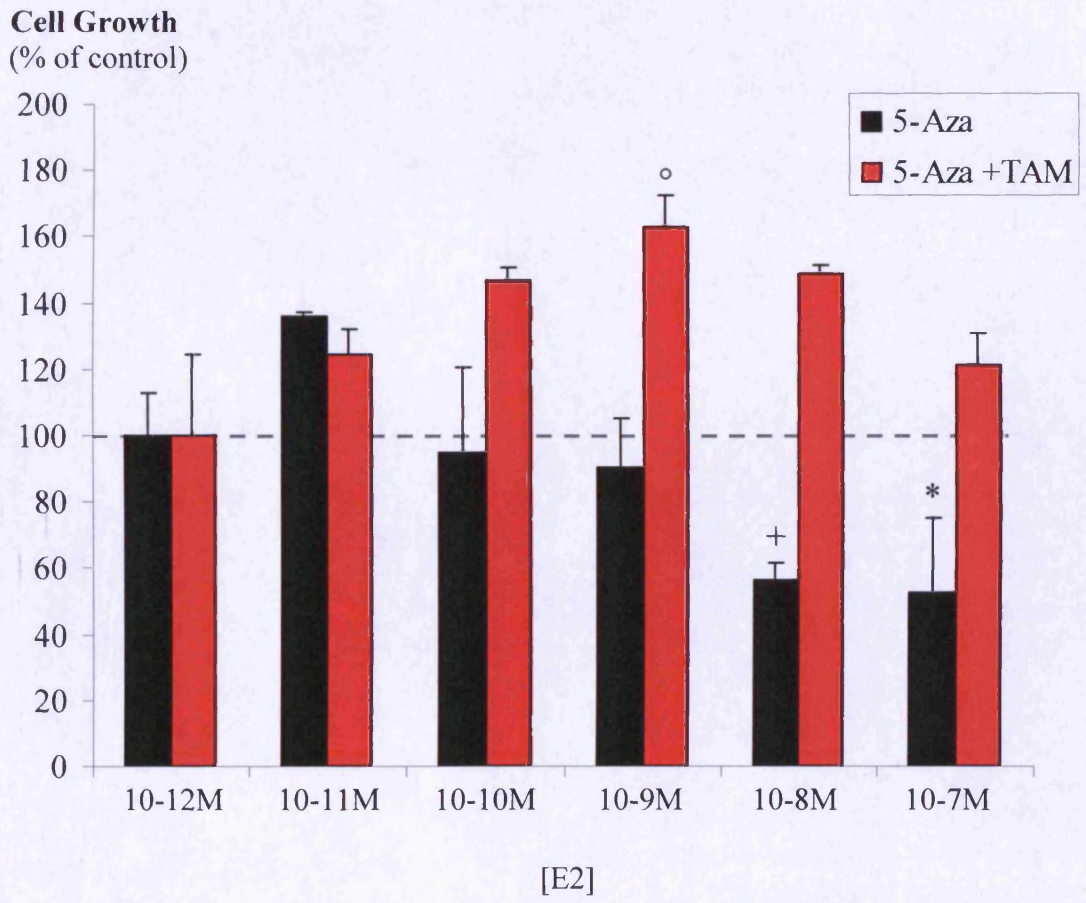


Fig 4.11 MCF-7 cell concentration response to oestradiol: MCF-7 cells were treated ± 5-Aza and E2 at a concentration ranging from 10^{-12}M to 10^{-7}M. Cell number was assessed on day 7 by MTT cell detection assay (n=3). Data shown represents E2-treated cell counts as a percentage of counts recorded for non-E2 treated control cells.

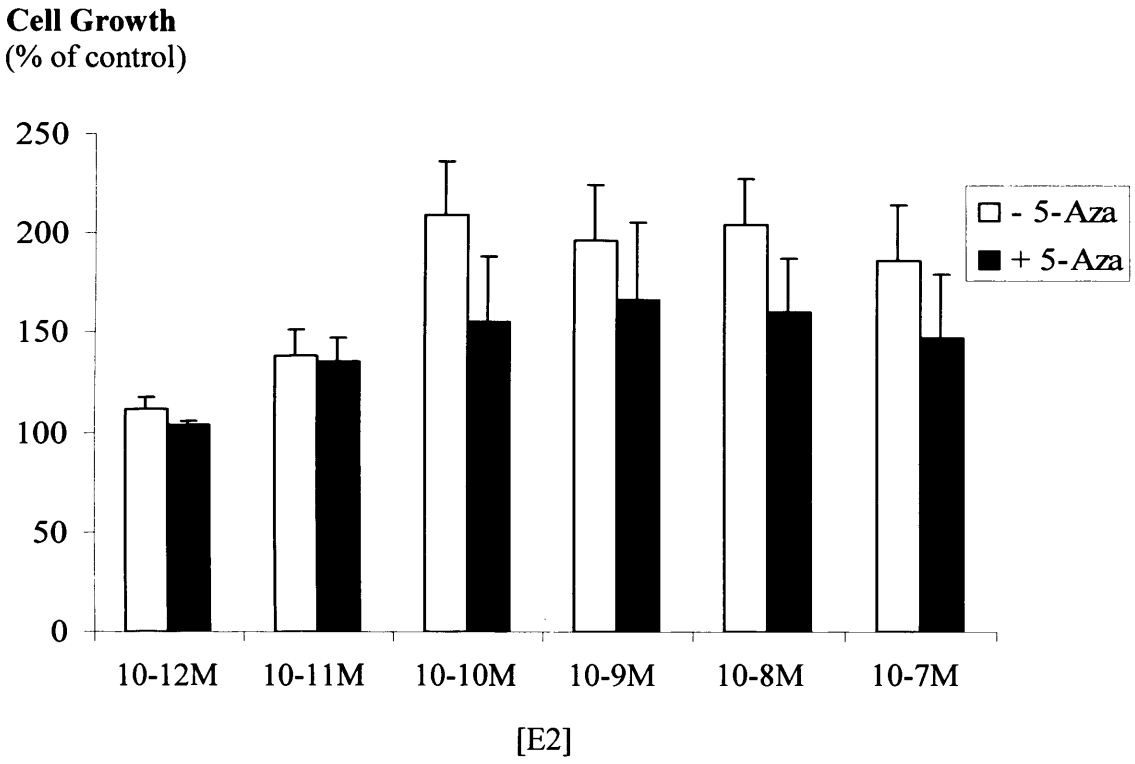
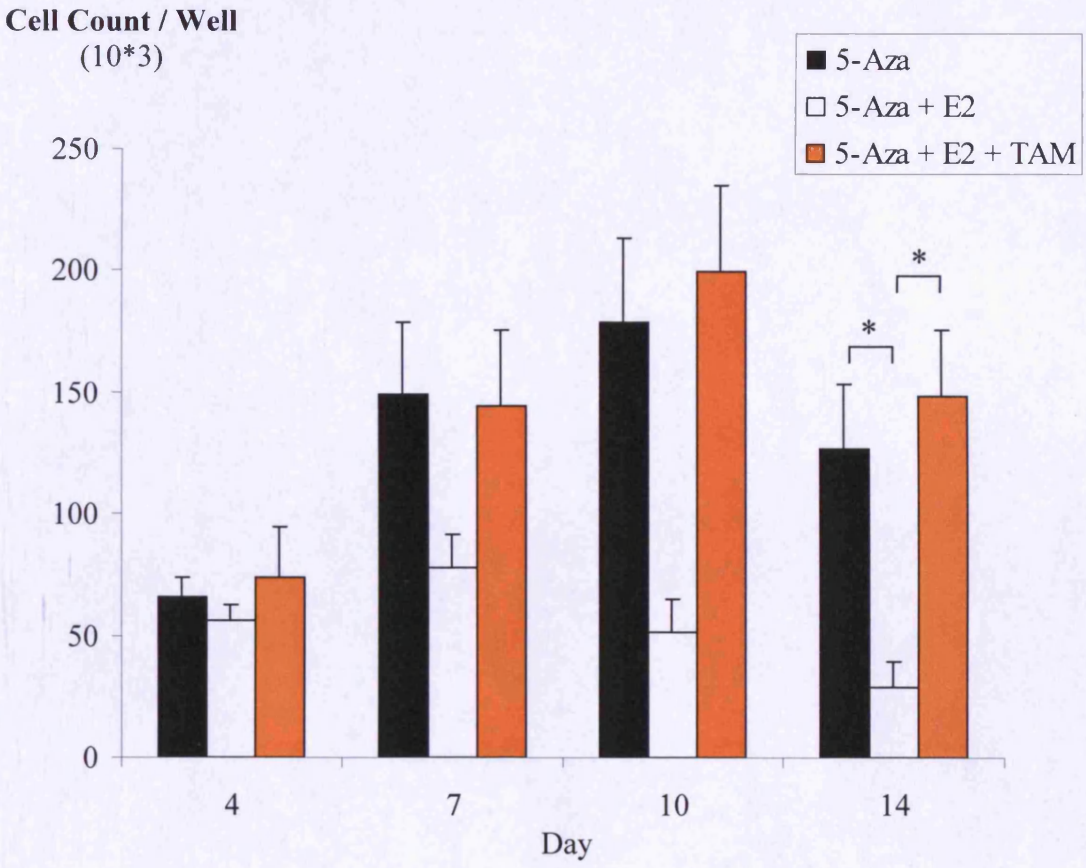


Fig. 4.12 TAM-Wd cell growth response to E2 +/- tamoxifen in the presence of 5-Aza: TAM-Wd cells were seeded on 24-well plates at a density of $4x10^4$cells/well. Following 24hrs incubation in control media, cells were treated with 5-Aza, 5-Aza + E2 or 5-Aza + E2 + TAM. Cell media was changed every 4 days. Subsequent cell counts were taken on days 4, 7, 10 and 14 using Coulter counter apparatus. The data shown represents actual cell number/well recorded over 3 independent experiments (*p<0.001).



5. RESULTS

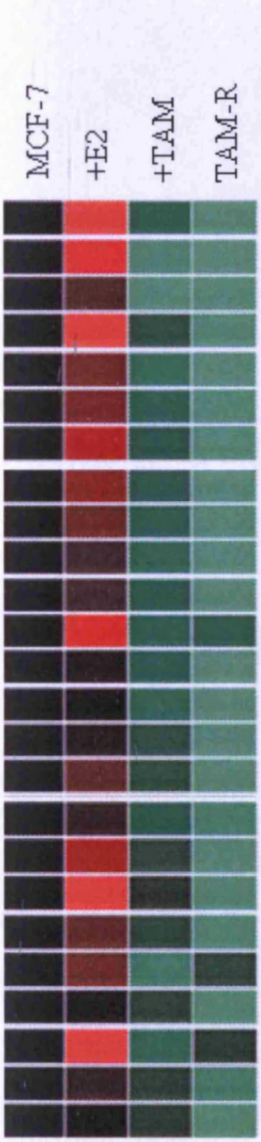
5.1 Identification of potential tumour-suppressor genes silenced in tamoxifen withdrawn TAM-R cells using an Affymetrix gene array approach.

Having demonstrated that the combination of 5-Aza and E2 causes TAM-Wd cells to proliferate at a slower rate than 5-Aza-treated (or E2 treated) controls and furthermore cause cell loss over a 14 day period, we sought to identify genes that were being up-regulated specifically by E2 challenge in the presence of the demethylation agent which could be associated with this growth suppressive phenomenon. According to the hypothesis, these genes would have to be readily up-regulated by E2 in the parental MCF-7 cells, not up-regulated or expressed below basal level following initial (10 day) TAM challenge, and have a comparable or ideally reduced basal expression in the TAM-R cell sub-type, in a similar fashion for that shown for pS2 and PR. Genes would also have to be shown to be free from EGFR-regulation, i.e. expression should not be reversible with EGFR inhibition, to confirm that gene suppression was independent from EGFR signalling input.

Using an existing Affymetrix database (HG-U133A platform) available to Tenovus, previously created from triplicate samples of MCF-7 cells treated to reflect the transition from anti-hormone responsive to cells with acquired

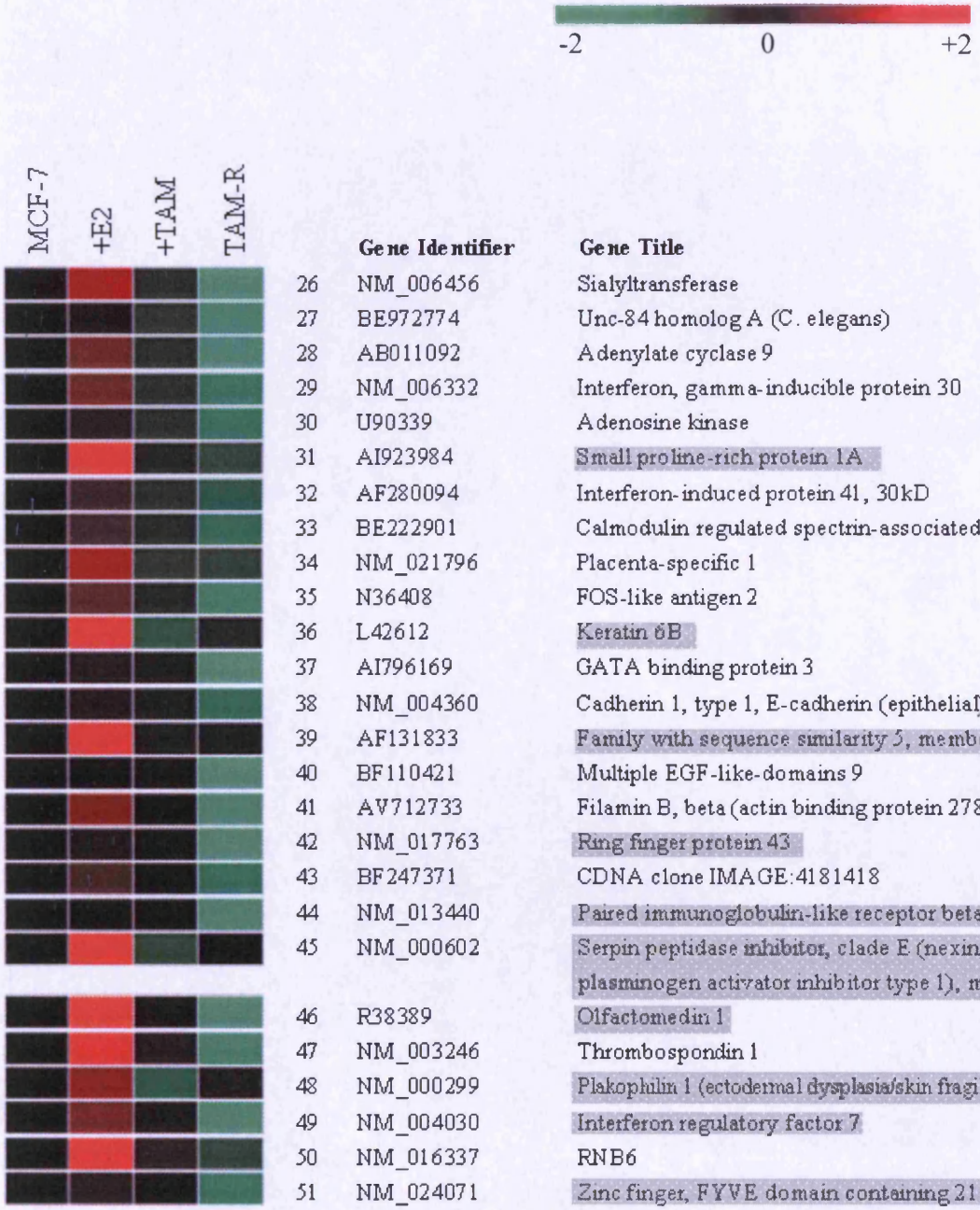
resistance (in this instance tamoxifen) we were able to create a gene shortlist. A pattern navigation analysis was conducted using online Genesifter™ software to reveal genes that were up-regulated by E2 and suppressed by TAM in wt-MCF-7 cells, and also down-regulated in TAM-R cells relative to wt-MCF-7 (ie, wt=1, wt + E2 >2, wt + TAM ≤1 and TAM-R ≤1 with regards to fold-change relative to wt-cell gene expression), at a statistical cut off of $p<0.001$ using ANOVA. This analysis produced a list of 51 gene probes, although probes called 'absent' in the wt-MCF-7 cells were subsequently withdrawn from the list, leaving 33 potential gene probe candidates [Fig 5.1].

Fig 5.1 Genesifter™ pattern navigation analysis: Identification of probes that were up-regulated by E2 and suppressed by TAM in wt-MCF-7 cells, and down-regulated in TAM-R cells relative to wt-MCF-7 (wt=1, wt + E2 >2, wt + TAM ≤1 and TAM-R ≤1 ($p<0.001$). This analysis produced a list of 51 probes, 33 of which were called 'present' in the wt-MCF-7 cells. 'Absent' called were discarded (gene names highlighted in grey). Data is represented by profile heatmaps, where green indicates gene suppression (-2) and red indicates up-regulation (+2) relative to wt-expression (black).



	Gene Identifier	Gene Title
1	AF061812	Keratin 16
2	NM_005978	S100 calcium-binding protein A2
3	NM_003881	WNT1 inducible signaling pathway protein 2
4	AI935123	AHNAK nucleoprotein 2
5	AB017493	Core promoter element binding protein
6	AF133207	Protein kinase H11
7	BE675435	Kruppel-like factor 6
8	NM_006334	Olfactomedin 1
9	AF207990	Fer-1 (C.elegans)-like 3 (myoferlin)
10	U90304	Iroquois homeobox protein 5
11	NM_030941	Exonuclease NEF-sp
12	AI812030	Thrombospondin 1
13	BC003629	RNA, U2 small nuclear
14	AF064771	Diacylglycerol kinase, alpha (80kD)
15	U26744	Dystrobrevin, alpha
16	NM_017459	Microfibrillar-associated protein 2
17	NM_015675	Growth arrest and DNA-damage-inducible, beta
18	NM_013451	Fer-1 (C.elegans)-like 3 (myoferlin)
19	U19495	Stromal cell-derived factor 1
20	NM_020672	S100-type calcium binding protein A14
21	NM_014400	GPI-anchored metastasis-associated protein homolog
22	AI826799	EGF-containing fibulin-like extracellular matrix protein 1
23	NM_012101	Tripartite motif-containing 29
24	AK000300	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
25	AB002301	Microtubule associated serine/threonine kinase family member 4

Fig 5.1 (Continued)

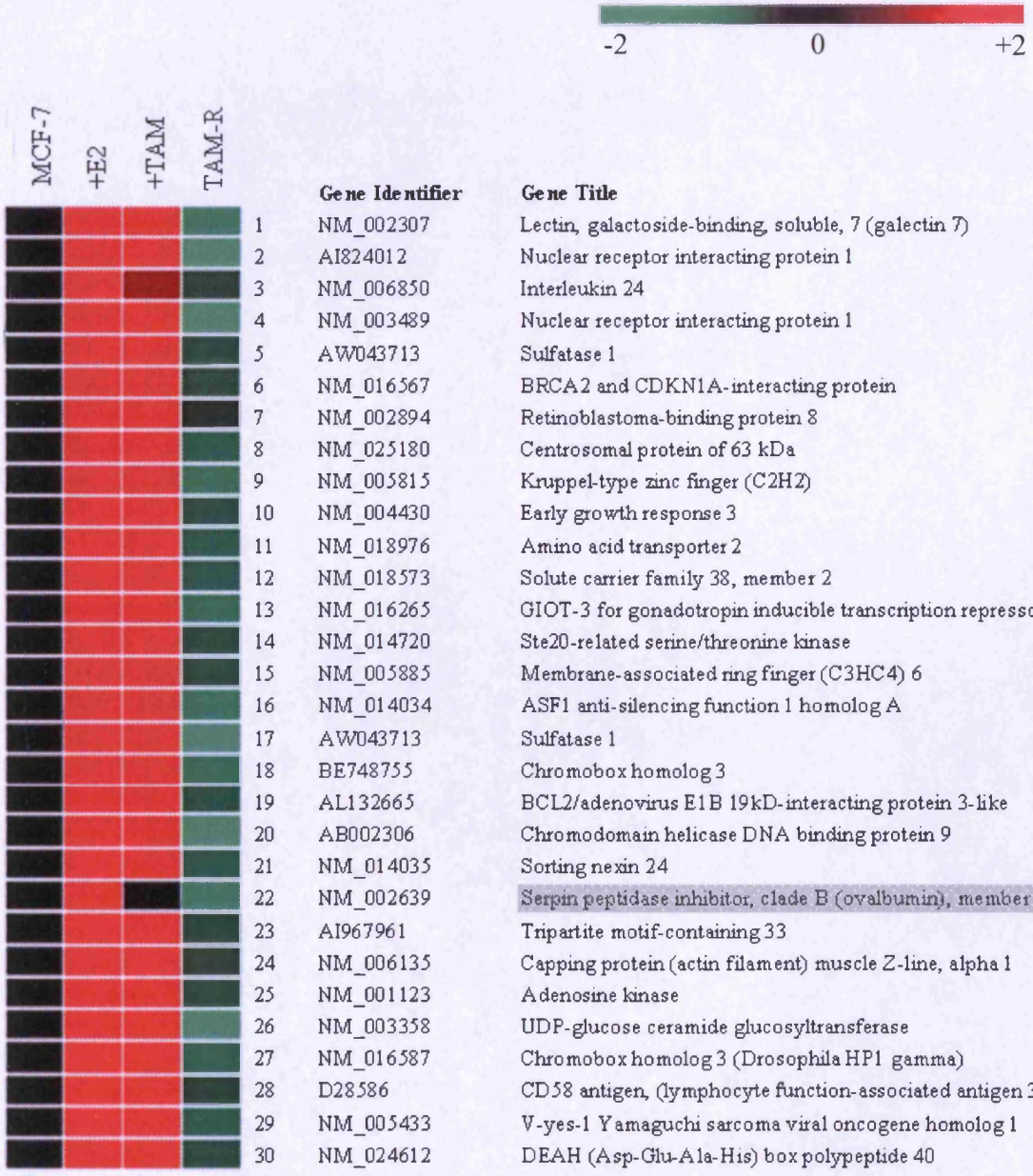


	Gene Identifier	Gene Title
26	NM_006456	Sialyltransferase
27	BE972774	Unc-84 homolog A (C. elegans)
28	AB011092	Adenylate cyclase 9
29	NM_006332	Interferon, gamma-inducible protein 30
30	U90339	Adenosine kinase
31	AI923984	Small proline-rich protein 1A
32	AF280094	Interferon-induced protein 41, 30kD
33	BE222901	Calmodulin regulated spectrin-associated protein 1
34	NM_021796	Placenta-specific 1
35	N36408	FOS-like antigen 2
36	L42612	Keratin 6B
37	AI796169	GATA binding protein 3
38	NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)
39	AF131833	Family with sequence similarity 5, member B
40	BF110421	Multiple EGF-like-domains 9
41	AV712733	Filamin B, beta (actin binding protein 278)
42	NM_017763	Ring finger protein 43
43	BF247371	CDNA clone IMAGE:4181418
44	NM_013440	Paired immunoglobulin-like receptor beta
45	NM_000602	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1
46	R38389	Olfactomedin 1
47	NM_003246	Thrombospondin 1
48	NM_000299	Plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)
49	NM_004030	Interferon regulatory factor 7
50	NM_016337	RNB6
51	NM_024071	Zinc finger, FYVE domain containing 21

In addition, a global sweep of the Affymetrix database was carried out in order to identify any potential targets which had been overlooked due to the statistical stringency of the pattern navigation analysis. To do this, genes whose expression were significantly altered with 10-day E2 challenge versus wt-MCF-7 ($p<0.05$) were first selected using a T-test (n= 4376 probes). Only E2 induced genes were then selected from the list using the same selection process (n= 1691 probes). Heatmaps for these probes were then manually inspected and genes whose expression were not further induced or fell in TAM-R versus wt-MCF-7 cells were selected (n=202 probes). These probes were further filtered to select genes with a reported association with growth/proliferation, cell survival/cell death/apoptosis or transcriptional regulation using the "ontology" function available in Genesifter. A more detailed ontological selection was then carried out for probes specifically with any reported evidence of negative ontology (e.g. pro-apoptosis/cell cycle inhibitor, transcriptional silencing, co-repressor) or reported to be subject to hyper-methylation in any disease state using online Medline software (n=75 probes). This list of 75 probes was fed into the previously-described pattern navigation with no statistical parameters applied, and heatmaps were then manually selected for those probes not showing any up-regulation in TAM treated-MCF-7 versus wt-MCF-7 cells (but E2 regulation and also minimal expression in TAM-R cells). This left 18 probes, 8 of which were called present in MCF-7s [Fig 5.2]. Seven of these 8 probes had not been identified using the pattern navigation analysis.

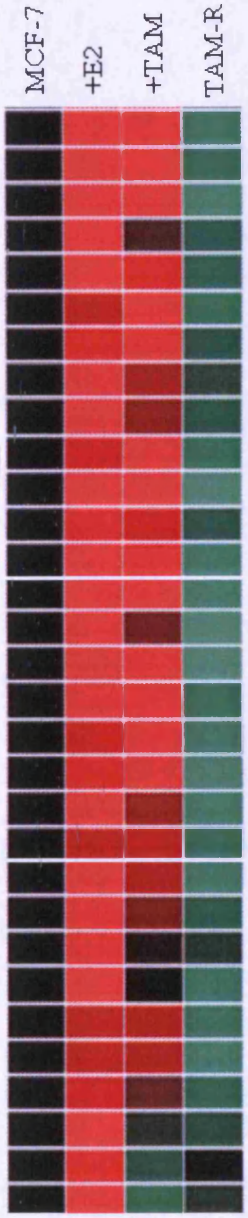
Heat-maps were then generated for the list of 33 probes obtained by pattern navigation and the 7 additional probes from the broad sweep (40 probes in total) to analyse how their expression changed following gefitinib challenge in TAM-R cells. Genes showing any indication of recovery of expression in TAM-R + gefitinib versus TAM-R cells were discarded leaving 25 probes (24 genes) [Fig 5.3].

Fig 5.2 Heatmap expression profile for the 75 gene probes selected from the broad Affymetrix database analysis, in wt, wt + E2, wt + TAM and TAM-R cells (with no statistical stringency). Probe profiles were manually selected to identify probes that showed no up-regulation in response to TAM in wt cells, and that were called 'present' in wt-cells (high-lighted in green n=8 probes). Probes that were not up-regulated by TAM, but were called absent in wt-cells are high-lighted in grey (n=10).



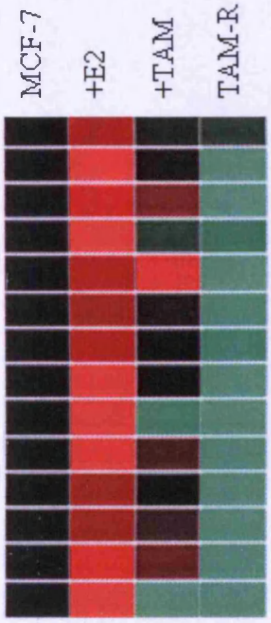
	Gene Identifier	Gene Title
1	NM_002307	Lectin, galactoside-binding, soluble, 7 (galectin 7)
2	AI824012	Nuclear receptor interacting protein 1
3	NM_006850	Interleukin 24
4	NM_003489	Nuclear receptor interacting protein 1
5	AW043713	Sulfatase 1
6	NM_016567	BRCA2 and CDKN1A-interacting protein
7	NM_002894	Retinoblastoma-binding protein 8
8	NM_025180	Centrosomal protein of 63 kDa
9	NM_005815	Kruppel-type zinc finger (C2H2)
10	NM_004430	Early growth response 3
11	NM_018976	Amino acid transporter 2
12	NM_018573	Solute carrier family 38, member 2
13	NM_016265	GIOT-3 for gonadotropin inducible transcription repressor-3
14	NM_014720	Ste20-related serine/threonine kinase
15	NM_005885	Membrane-associated ring finger (C3HC4) 6
16	NM_014034	ASF1 anti-silencing function 1 homolog A
17	AW043713	Sulfatase 1
18	BE748755	Chromobox homolog 3
19	AL132665	BCL2/adenovirus E1B 19kD-interacting protein 3-like
20	AB002306	Chromodomain helicase DNA binding protein 9
21	NM_014035	Sorting nexin 24
22	NM_002639	Serpin peptidase inhibitor, clade B (ovalbumin), member 5
23	AI967961	Tripartite motif-containing 33
24	NM_006135	Capping protein (actin filament) muscle Z-line, alpha 1
25	NM_001123	Adenosine kinase
26	NM_003358	UDP-glucose ceramide glucosyltransferase
27	NM_016587	Chromobox homolog 3 (Drosophila HP1 gamma)
28	D28586	CD58 antigen, (lymphocyte function-associated antigen 3)
29	NM_005433	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1
30	NM_024612	DEAH (Asp-Glu-Ala-His) box polypeptide 40

Fig. 5.2 (Continued)



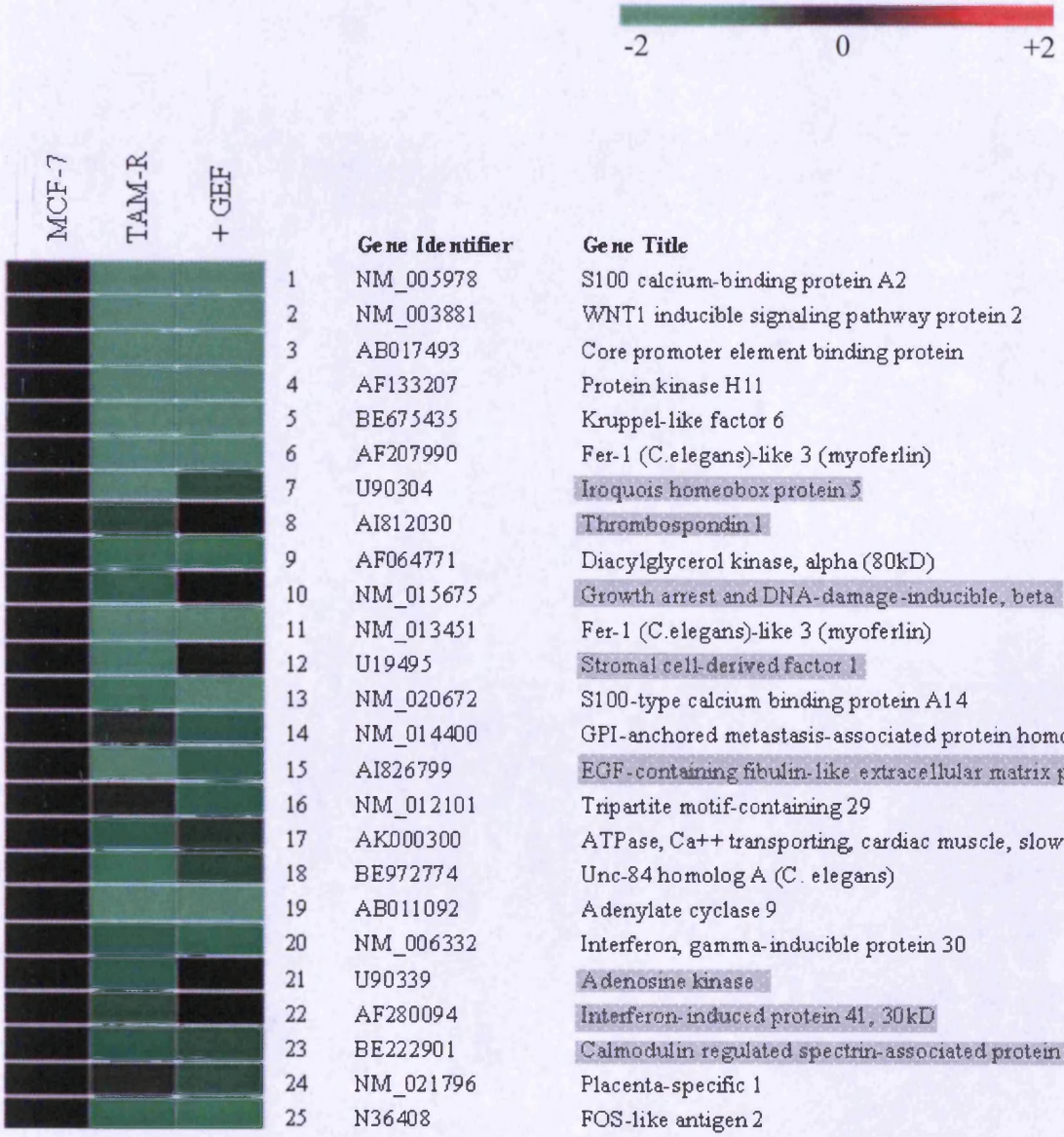
	Gene Identifier	Gene Title
31	NM_017927	Mitofusin 1
32	NM_014415	Zinc finger protein
33	AW612574	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member E
34	NM_004972	Janus kinase 2 (a protein tyrosine kinase)
35	NM_005802	Tumor protein p53-binding protein
36	NM_014899	Rho-related BTB domain containing 3
37	NM_006472	Thioredoxin interacting protein
38	AF208012	Tumor protein D52-like 1
39	AU160695	BUB3 budding uninhibited by benzimidazoles 3 homolog
40	AL080111	NIMA (never in mitosis gene a)-related kinase 7
41	NM_004052	BCL2/adenovirus E1B 19kD-interacting protein 3
42	NM_022133	Sorting nexin 16
43	Y09703	Pinin, desmosome associated protein, some similarity to MDR protein
44	NM_012311	Antigenic determinant of recA protein (mouse) homolog
45	NM_015515	Type I intermediate filament cytokeratin
46	NM_000314	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
47	NM_002956	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein
48	BG253119	Dystonin
49	NM_000321	Retinoblastoma 1 (including osteosarcoma)
50	BC000278	Carbonic anhydrase XII
51	NM_024948	Chromosome 10 open reading frame 97
52	AI913365	Methyl-CpG binding domain protein 4
53	U83410	Cullin 2
54	AF003934	Prostate differentiation factor
55	NM_000700	Annexin A1
56	NM_016626	Mex-3 homolog C (C. elegans)
57	AF051851	Supervillin
58	NM_004415	Desmoplakin (DPI, DPII)
59	NM_001909	Cathepsin D (lysosomal aspartyl protease)
60	AI084226	Fas apoptotic inhibitory molecule 3
61	NM_012101	Tripartite motif-containing 29

Fig 5.2 (Continued)



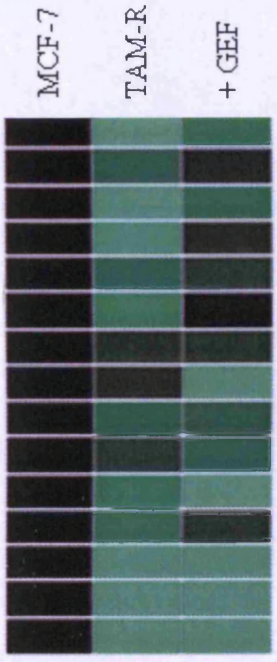
	Gene Identifier	Gene Title
62	AW170602	Regulator of G-protein signaling 12
63	NM_002031	Fyn-related kinase (or RAK)
64	NM_004848	Basement membrane-induced gene
65	NM_022131	Calsyntenin-2
66	AB050468	Ortholog of mouse integral membrane glycoprotein LIG-1
67	BF514079	Kruppel-like factor 4 (gut)
68	NM_014890	Downregulated in ovarian cancer 1
69	NM_000165	Gap junction protein, alpha 1, 43kD (connexin 43)
70	NM_001562	Interleukin 18 (interferon-gamma-inducing factor)
71	AW043713	Sulfatase 1
72	NM_006456	Sialyltransferase
73	NM_001218	Carbonic anhydrase XII
74	BC001012	Carbonic anhydrase XII
75	AI378979	Plakophilin 1

Fig 5.3 Heatmap expression profiles for the 33 probes obtained by pattern navigation and the 7 additional probes from the broad sweep in wt, TAM-R and TAM-R cells + gefitinib (with no statistical stringency). Probe profiles were manually selected to identify probes that showed no up-regulation of expression in TAM-R cells treated with gefitinib (vs non-treated cells). Probes that were up-regulated by gefitinib were discarded (high-lighted in grey n=15).



	Gene Identifier	Gene Title
1	NM_005978	S100 calcium-binding protein A2
2	NM_003881	WNT1 inducible signaling pathway protein 2
3	AB017493	Core promoter element binding protein
4	AF133207	Protein kinase H11
5	BE675435	Kruppel-like factor 6
6	AF207990	Fer-1 (C.elegans)-like 3 (myoferlin)
7	U90304	Iroquois homeobox protein 5
8	AI812030	Thrombospondin 1
9	AF064771	Diacylglycerol kinase, alpha (80kD)
10	NM_015675	Growth arrest and DNA-damage-inducible, beta
11	NM_013451	Fer-1 (C.elegans)-like 3 (myoferlin)
12	U19495	Stromal cell-derived factor 1
13	NM_020672	S100-type calcium binding protein A14
14	NM_014400	GPI-anchored metastasis-associated protein homolog
15	AI826799	EGF-containing fibulin-like extracellular matrix protein 1
16	NM_012101	Tripartite motif-containing 29
17	AK000300	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2
18	BE972774	Unc-84 homolog A (C. elegans)
19	AB011092	Adenylate cyclase 9
20	NM_006332	Interferon, gamma-inducible protein 30
21	U90339	Adenosine kinase
22	AF280094	Interferon-induced protein 41, 30kD
23	BE222901	Calmodulin regulated spectrin-associated protein 1
24	NM_021796	Placenta-specific 1
25	N36408	FOS-like antigen 2

Fig 5.3 (Continued)



	Gene Identifier	Gene Title
26	AI796169	GATA binding protein 3
27	NM_004360	Cadherin 1, type 1, E-cadherin (epithelial)
28	BF110421	Multiple EGF-like-domains 9
29	AV712733	Filamin B, beta (actin binding protein 278)
30	BF247371	CDNA clone IMAGE:4181418
31	NM_003246	Thrombospondin 1
32	NM_016337	RNB6
33	AF003934	Prostate differentiation factor
34	NM_000700	Annexin A1
35	NM_001909	Cathepsin D (lysosomal aspartyl protease)
36	BF514079	Kruppel-like factor 4 (gut)
37	NM_014890	Downregulated in ovarian cancer 1
38	NM_000165	Gap junction protein, alpha 1, 43kD (connexin 43)
39	NM_006456	Sialyltransferase
40	NM_001218	Carbonic anhydrase XII

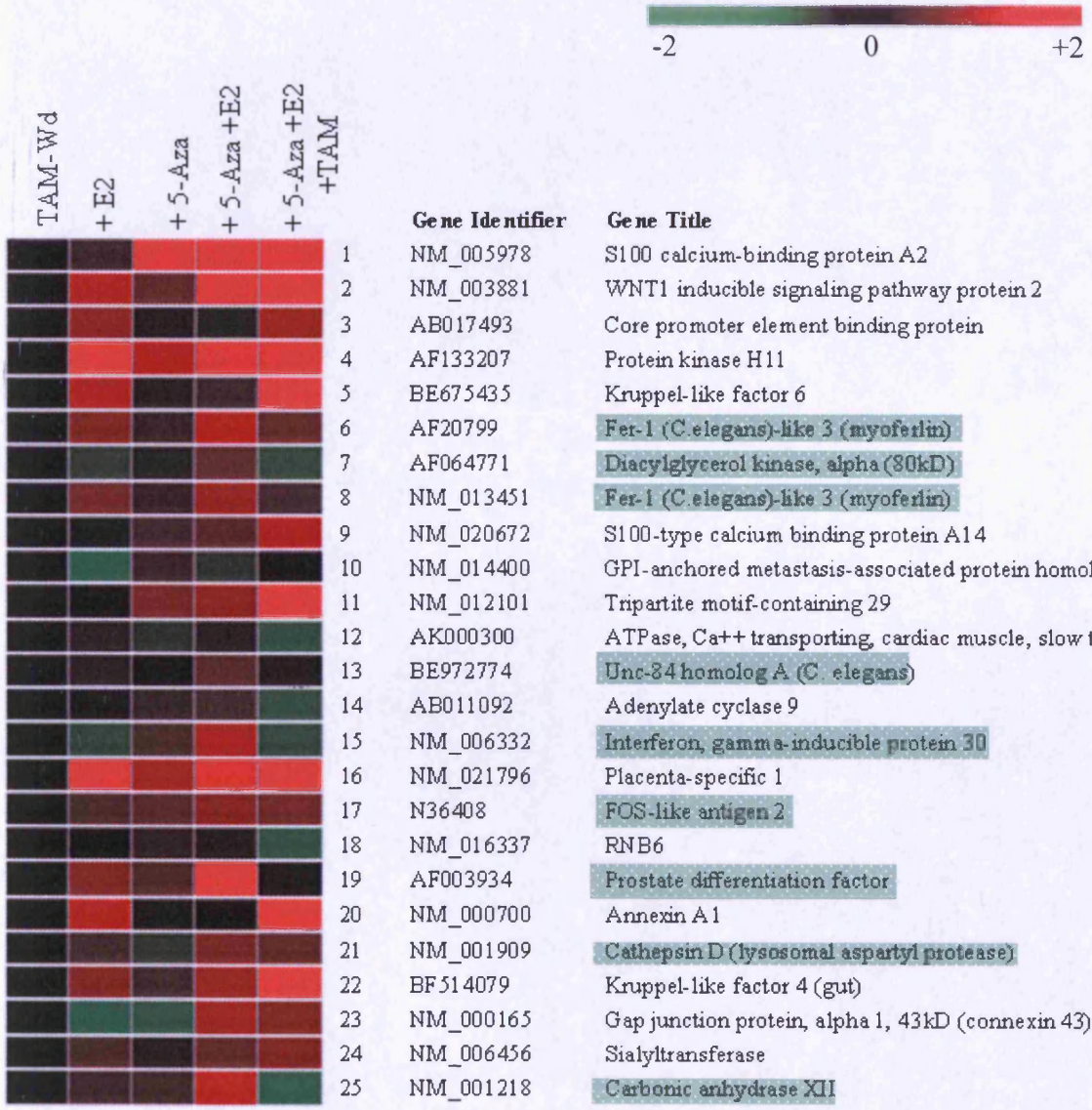
In parallel to this study, another Affymetrix database was created later in this project using triplicate TAM-Wd cell mRNA samples, reverse transcribed and hybridised to a HG-U133A2 platform. TAM-Wd cells were treated with 5-Aza, E2, 5-Aza + E2 or 5-Aza + E2 in combination with TAM (versus non-treated cells) for six days. The 25 probes (24 genes) identified from the first Affymetrix database were profiled within the new data set [Fig 5.4]. Out of the 24 genes, 8 were shown to follow the expected expression profile in relation to the tumour-suppressor hypothesis, i.e. highest expression in 5-Aza + E2 treated TAM-Wd cells, reversible with the co-addition of TAM. All of these genes (namely myoferlin, diacylglycerol kinase-α, unc-84 homolog A, interferon gamma-inducible protein 30, FOS-like-antigen 2, prostate differentiation factor, cathepsin-D and carbonic anhydrase XII) were taken forward as high-priority candidates.

In addition, a broad sweep of the second array database was then conducted in order to identify genes matching the desired profile, which could then be subsequently analysed to determine their status in MCF-7 cells treated with E2 and TAM and TAM-R cells, ensuring all genes with a strong trend toward the required profile were taken forward for consideration for PCR analysis. The project was filtered for all gene probes significantly induced in TAM-Wd cells challenged with 5-Aza + E2 versus non-treated TAM-Wd cells (n=744 probes, $p<0.05$). Genes were further filtered by selecting probes whose expression were further induced with E2 + 5-Aza versus 5-Aza alone (n=240 probes), and subsequently, those genes whose re-expression could be reversed with TAM challenge (n=159) by manually selecting heatmaps. This 159 probe set was

visualised in the first Affymetrix data set to identify the genes that showed a degree of suppression in TAM-R cells versus wt-MCF-7 (n=108 probes). Of these 108 probes, 43 probes (34 genes) were found to be regulated by E2 in MCF-7 cells [Fig 5.5]. On inspection it was observed that there was a degree of overlap between the 8 genes selected from the first method of analysis, and the 34 identified from the second, with 5 genes being present in both lists. Using a multi-layered points system produced in collaboration with Dr Julia Gee, the 37 genes were scored and filtered according to the classifications summarised in table 5.1. Total scores at this stage ranged from 2.5-12, out of a possible 13. Genes scoring 8 or higher, of which there were 13, were taken forward to a second scoring system based on information from screening the genes in the Oncomine online database. Genes were awarded scores for evidence of down-regulation in normal vs cancer (+1), relation to clinical profile and good prognosis (+1), evidence of E2 induction *in-vitro* and anti-hormone suppression (+1) – i.e. on a 0-3 scale. Total overall scores for the 13 selected genes ranged from 9-13 (table 5.2).

The 8 highest scoring genes were selected for their confirmation of expression profile by semi-quantitative PCR and promoter methylation status by methylight assay. The final list of 8 genes consisted of 2 identified in both rounds gene of selection, prostate differentiation factor (GDF15) and carbonic anhydrase XII (CA12), and 6 from the second round of gene selection adenylate cyclase-9 (ADCY9), Kazrin (KIAA1026), haemoglobin alpha-2 (HBA2), RAS-protein activator-like-1 (RASAL-1), collagen type VI alpha3 (Col6a3) and response gene to complement-32 protein (RGC32).

Fig 5.4 Heatmap expression profiles for the 25 probes identified from the first Affymetrix database in the TAM-Wd cells, + E2, +5-Aza, +5-Aza +E2 and + 5-Aza +E2 + TAM (with no statistical stringency). Probe profiles were manually selected to identify probes that were most highly up-regulated by 5-Aza + E2 and down-regulated with TAM co-treatment (high-lighted in green n=9).



	Gene Identifier	Gene Title
1	NM_005978	S100 calcium-binding protein A2
2	NM_003881	WNT1 inducible signaling pathway protein 2
3	AB017493	Core promoter element binding protein
4	AF133207	Protein kinase H11
5	BE675435	Kruppel-like factor 6
6	AF20799	Fer-1 (C.elegans)-like 3 (myoferlin)
7	AF064771	Diacylglycerol kinase, alpha (80kD)
8	NM_013451	Fer-1 (C.elegans)-like 3 (myoferlin)
9	NM_020672	S100-type calcium binding protein A14
10	NM_014400	GPI-anchored metastasis-associated protein homolog
11	NM_012101	Tripartite motif-containing 29
12	AK000300	ATPase, Ca++ transporting, cardiac muscle, slow twitch2
13	BE972774	Unc-84 homolog A (C. elegans)
14	AB011092	Adenylate cyclase 9
15	NM_006332	Interferon, gamma-inducible protein 30
16	NM_021796	Placenta-specific 1
17	N36408	FOS-like antigen 2
18	NM_016337	RNB6
19	AF003934	Prostate differentiation factor
20	NM_000700	Annexin A1
21	NM_001909	Cathepsin D (lysosomal aspartyl protease)
22	BF514079	Kruppel-like factor 4 (gut)
23	NM_000165	Gap junction protein, alpha 1, 43kD (connexin 43)
24	NM_006456	Sialyltransferase
25	NM_001218	Carbonic anhydrase XII

Fig 5.5 Heatmap expression profiles for the 43 probes identified as being most highly expressed in the 5-Aza + E2 treated TAM-Wd cells, and down-regulated with TAM co-treatment from a broad sweep of the second Affymetrix database (shown on left of gene list). Probes were also profiled in MCF-7 cell +/- E2, and TAM-R cells (heatmaps on right of gene list) to provide evidence of E2 regulation in wt-cells. Genes that were also identified from previous rounds of analysis are highlighted in yellow.

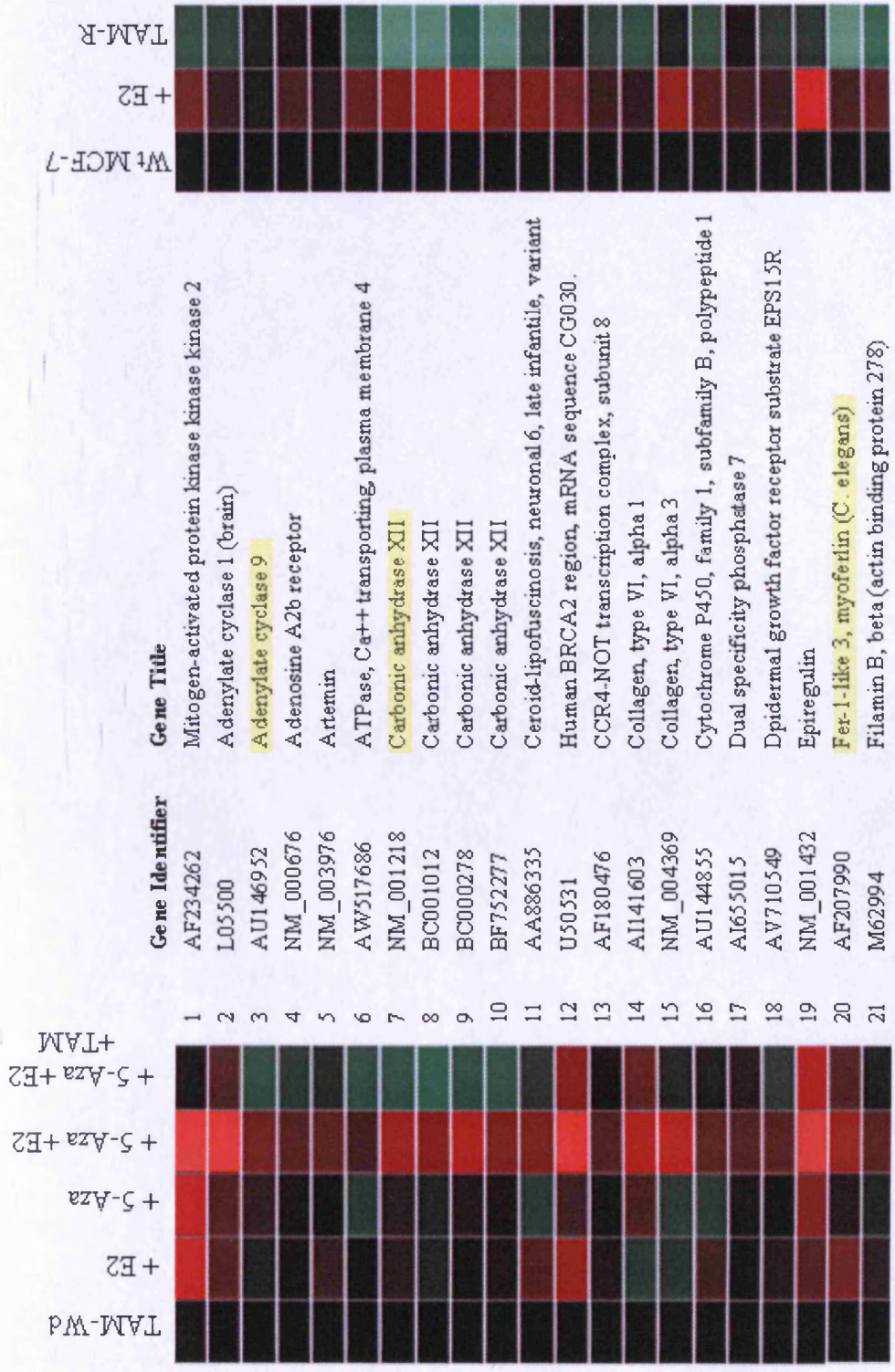


Fig 5.5 Continued

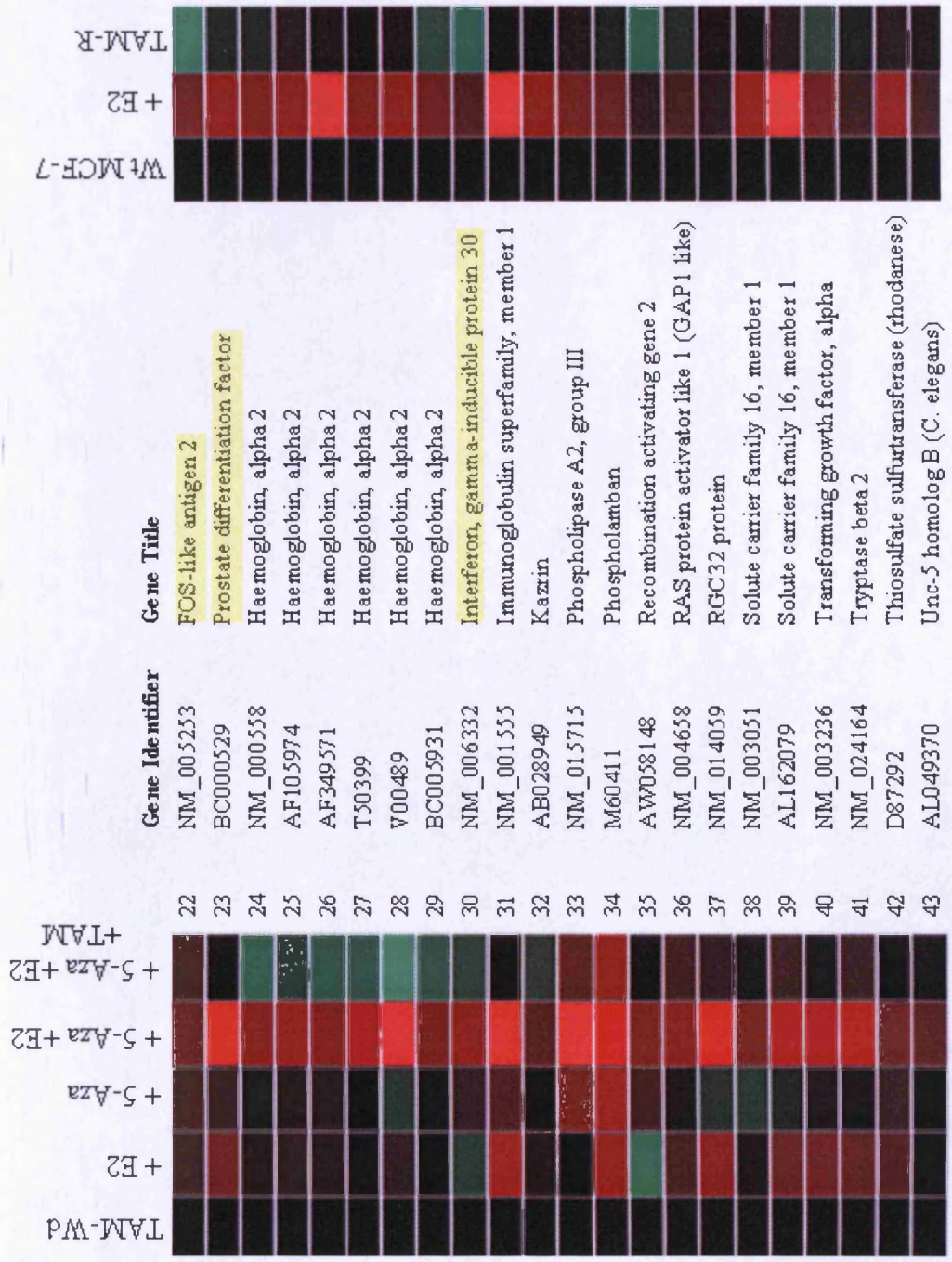


Table 5.1. Classifications for gene scoring system.

Classification	Scoring
E2 induced in MCF-7 and E2/AZA induced in TAM-Wd cells	+1 Yes, 0 No
Anti-hormone suppressed in MCF-7 cells	+ 1 Yes + 0.5 Conflicting probes 0 No
Scored according to Literature Ontology (i.e. tumour suppressive/growth inhibitory ontology).	No, possibly, yes, strongly yes - score 0-3
Replicate probe profile	-1 if conflicting, +1 if profile adequate, 2 if profile good
TKI regulation	-1 Up-regulated, +1 Unaffected or down-regulated
TAM-R heatmap colour vs MCF-7 for all probes	+2 Green, +1 Black, 0 mixed including Red
Present (p), marginal (m) or absent (a) call over MCF-7 E2, TAMR, Tam-Wd, Tam-Wd+E2+5aza groups.	+1 pppp or aaaa or mmmm or aapp or aamm or mmpp (poor call profile); +2 papp or mapp or pamm or pmpp (adequate call profile); +3 mamp or paap or aaap or aaam or maap (good call profile)

Table 5.2 Total scores awarded to the list of 37 genes identified by Affymetrix analysis, following first and second round classifications. Genes scoring 8 or more points in the first round were carried over to the second round and are high-lighted in the table below (n=13) (green: highest 8 scoring genes carried forward for PCR analysis (n=8), blue: remaining 7 genes scoring 8 or over that were not selected for PCR.)

Gene Title	1st Round Score	2nd Round Score	Total
Mitogen-activated protein kinase kinase kinase 2	4	-	
Adenylate cyclase 1 (brain)	3.5	-	
Adenylate cyclase 9	9	3	12
Adenosine A2b receptor	7	-	
Artemin	7	-	
ATPase, Ca++ transporting, plasma membrane 4	7	-	
Carbonic anhydrase XII	10	3	13
Cathepsin D (lysosomal aspartyl protease)	7	-	
Ceroid-lipofuscinosis, neuronal 6, late infantile, variant	5.5	-	
Human BRCA2 region, mRNA sequence CG030.	7	-	
CCR4-NOT transcription complex, subunit 8	2.5	-	
Collagen, type VI, alpha 1	4	-	
Collagen, type VI, alpha 3	8	3	11
Cytochrome P450, family 1, subfamily B, polypeptide 1	7	-	
Diacylglycerol kinase, alpha (80kD)	6	-	
Dual specificity phosphatase 7	4	-	
Epidermal growth factor receptor substrate EPS15R	7	-	
Epiregulin	8	1	9
Fer-1-like 3, myoferlin (C. elegans)	6	-	
Filamin B, beta (actin binding protein 278)	3	-	
FOS-like antigen 2	7	-	
Prostate differentiation factor	10	1	11
Haemoglobin, alpha 2	10	3	13
Interferon, gamma-inducible protein 30	9	0	9
Immunoglobulin superfamily, member 1	9	1	10
Kazrin	8	3	11
Phospholipase A2, group III	7	-	
Phospholamban	2.5	-	
Recombination activating gene 2	9	1	10
RAS protein activator like 1 (GAP1 like)	12	0	12
RGC32 protein	8	3	11
Solute carrier family 16, member 1	7	-	
Transforming growth factor, alpha	5	-	
Tryptase beta 2	3	-	
Thiosulfate sulfurtransferase (rhodanese)	7	-	
Unc-5 homolog B (C. elegans)	8	1	9
Unc-84 homolog A (C. elegans)	7	-	

5.2 Semi-Quantitative PCR for 8 candidate genes.

Triplicate samples of mRNA obtained from TAM-Wd cells treated with E2, 5-Aza, E2 and 5-Aza and E2 5-Aza and TAM were reversed transcribed and amplified using primers designed to the candidate gene fragment expressed on the HG-U133A Affymetrix array platform in a semi-quantitative PCR. Corresponding DNA extractions were sent to Dr Heidi Fiegl of the University of Innsbruck for Methylight analysis.

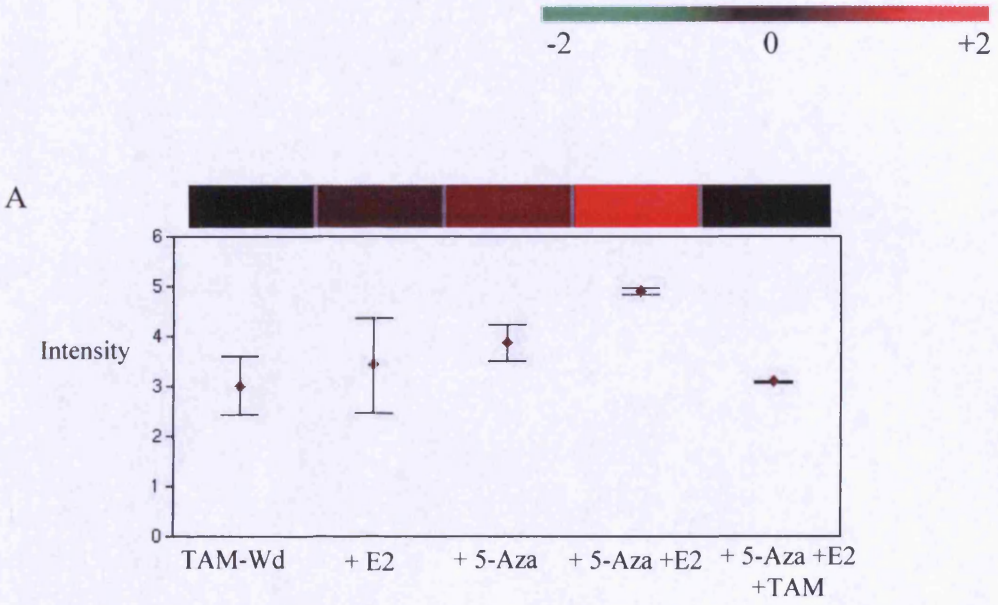
Of the 8 genes, the expression profile of 4 was shown to correlate with the corresponding array profile, as assessed by semi-quantitative PCR and densitometry. GDF-15, RGC-32, HBA2 and CA12 were all most highly expressed in TAM-Wd cells treated with E2 and 5-Aza, and were down-regulated with the co-addition of TAM [Fig 5.6b to 5.9b]. Methylight reactions performed for GDF-15 and HBA-2 showed a significant reduction in methylated gene promoter detected in TAM-Wd cells cultured with 5-Aza (± E2) compared to non-treated and E2 treated cells. Furthermore, the addition of TAM to 5-Aza + E2 cultured cells appeared to partially restore the methylated status of GDF-15, and to a less significant extent, HBA2 [Fig 5.6c and 5.8c]. Methylight assays showed that no methylated CA12 promoter was detected in TAM-Wd cell DNA, and signals for RGC-32 were too weak for accurate detection.

The PCR profile recorded for RASAL-1 and Col6a3 exhibited little resemblance to the array profile, both showing highest expression in cells co-treated with E2, 5-Aza and TAM [Fig 5.10b and 5.11b]. The PCR profile for

ADCY9 and Kazrin also did not match the corresponding array profile, as both genes were partially up-regulated by E2 (+/- 5-Aza) compared with non-treated cells. Contrary to the array data, the highest expression of both genes was again observed in the E2, 5-Aza and TAM co-treated cells [Figs 5.12b and 5.13b].

Methylight reactions for ADCY9 showed no promoter methylation was detected in the DNA samples and signals for Kazrin were too weak for accurate readings. Reactions were not carried out for RASAL-1 and Col6a3.

Fig 5.6 Prostate Differentiation Factor – GDF-15: A Heatmap profile and gene probe box-plot from the Affymetrix database, to assess GDF-15 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).



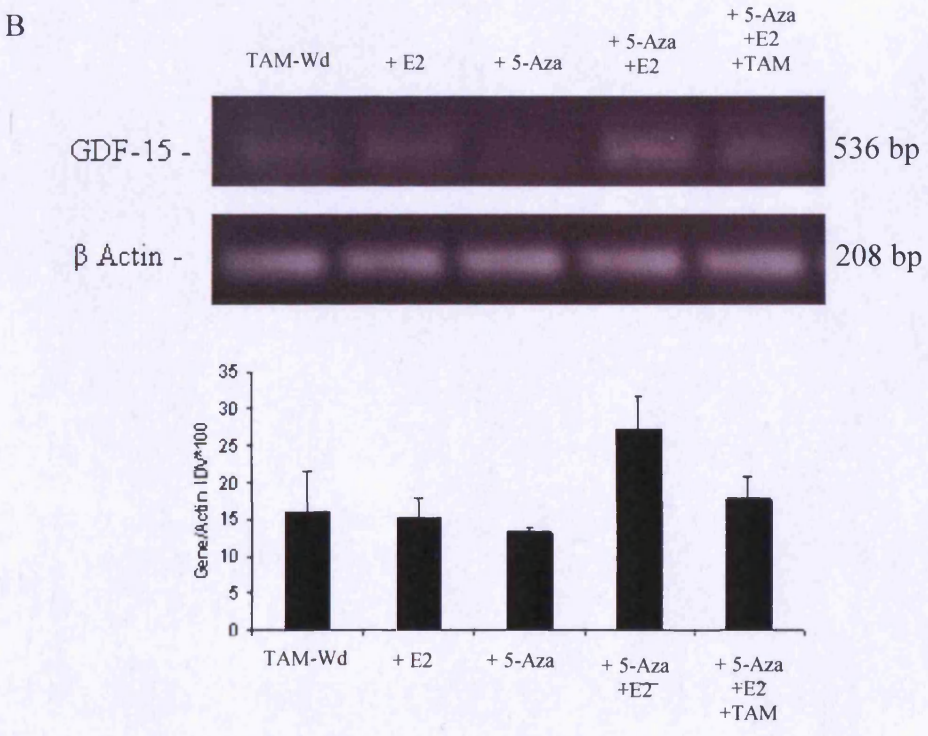


Fig 5.6 C: Promoter methylation profile for GDF-15 in TAM-Wd cells: Methylight data specific for methylated GDF-15 promoter detected in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM 5-Aza (data expressed as PMR).

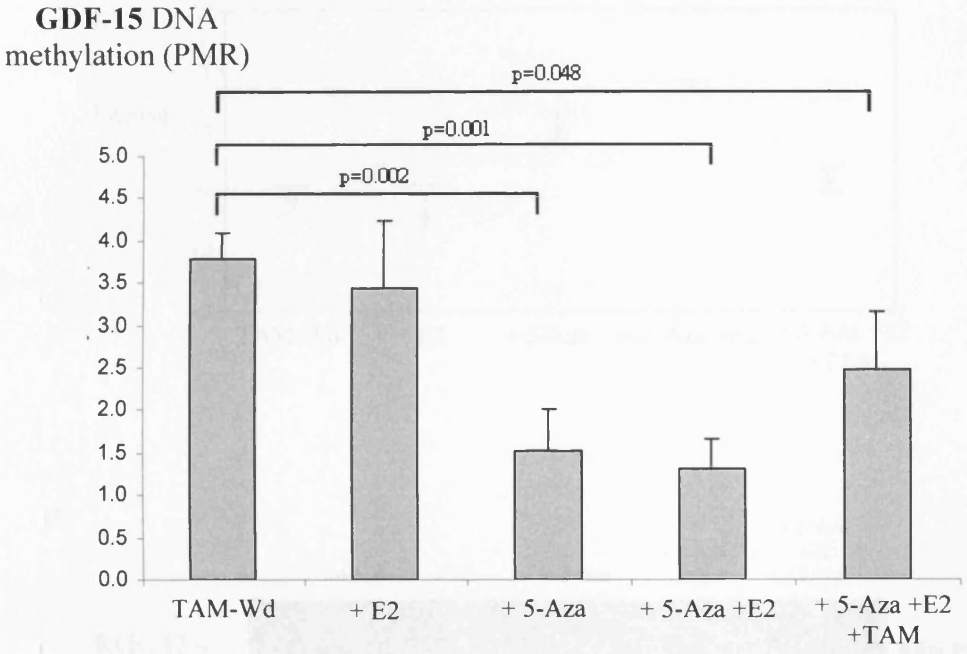


Fig 5.7 Response gene to complement 32 protein - RGC32. A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess RGC-32 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

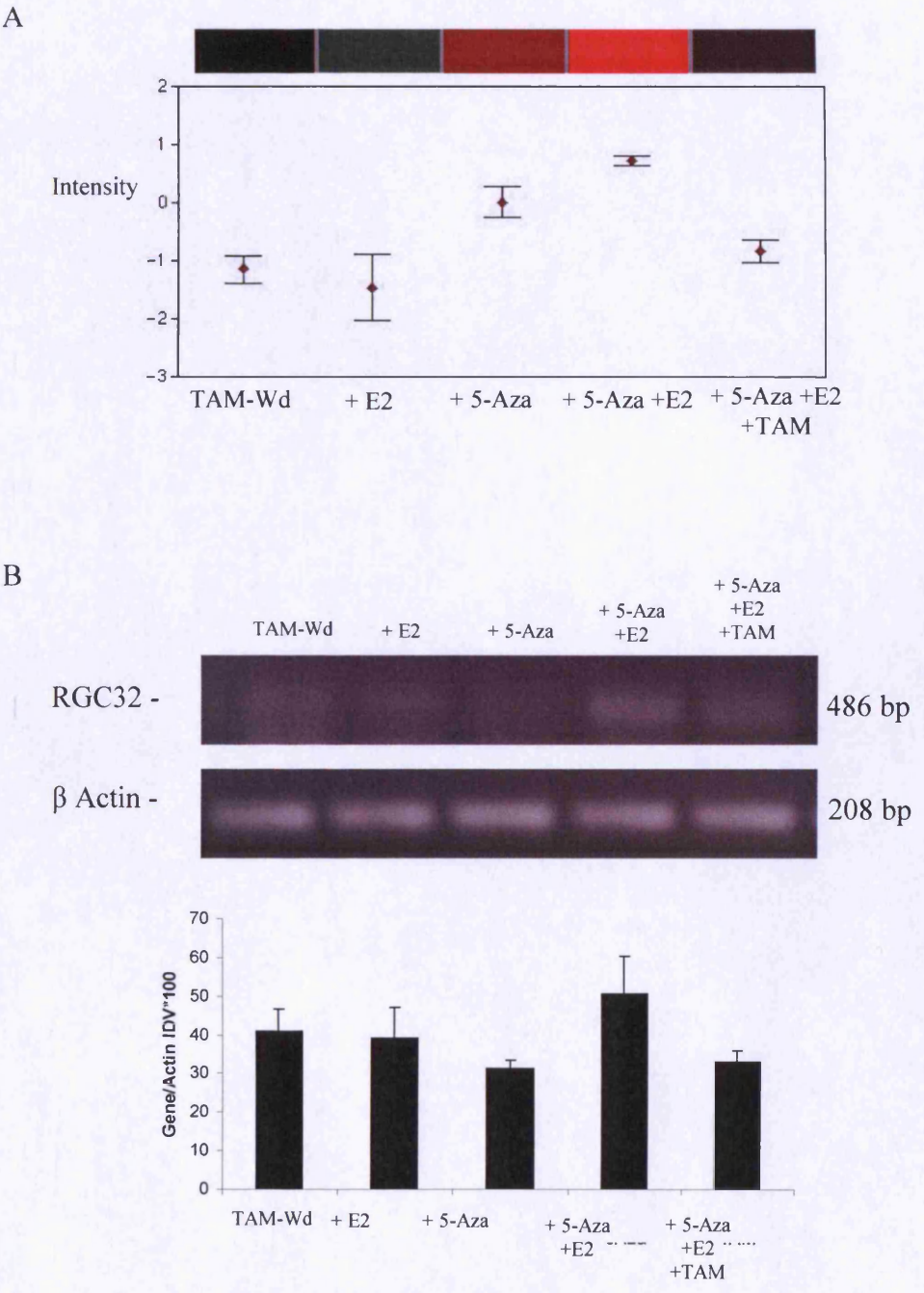


Fig 5.8 Haemoglobin alpha 2 - HBA2 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess HBA2 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

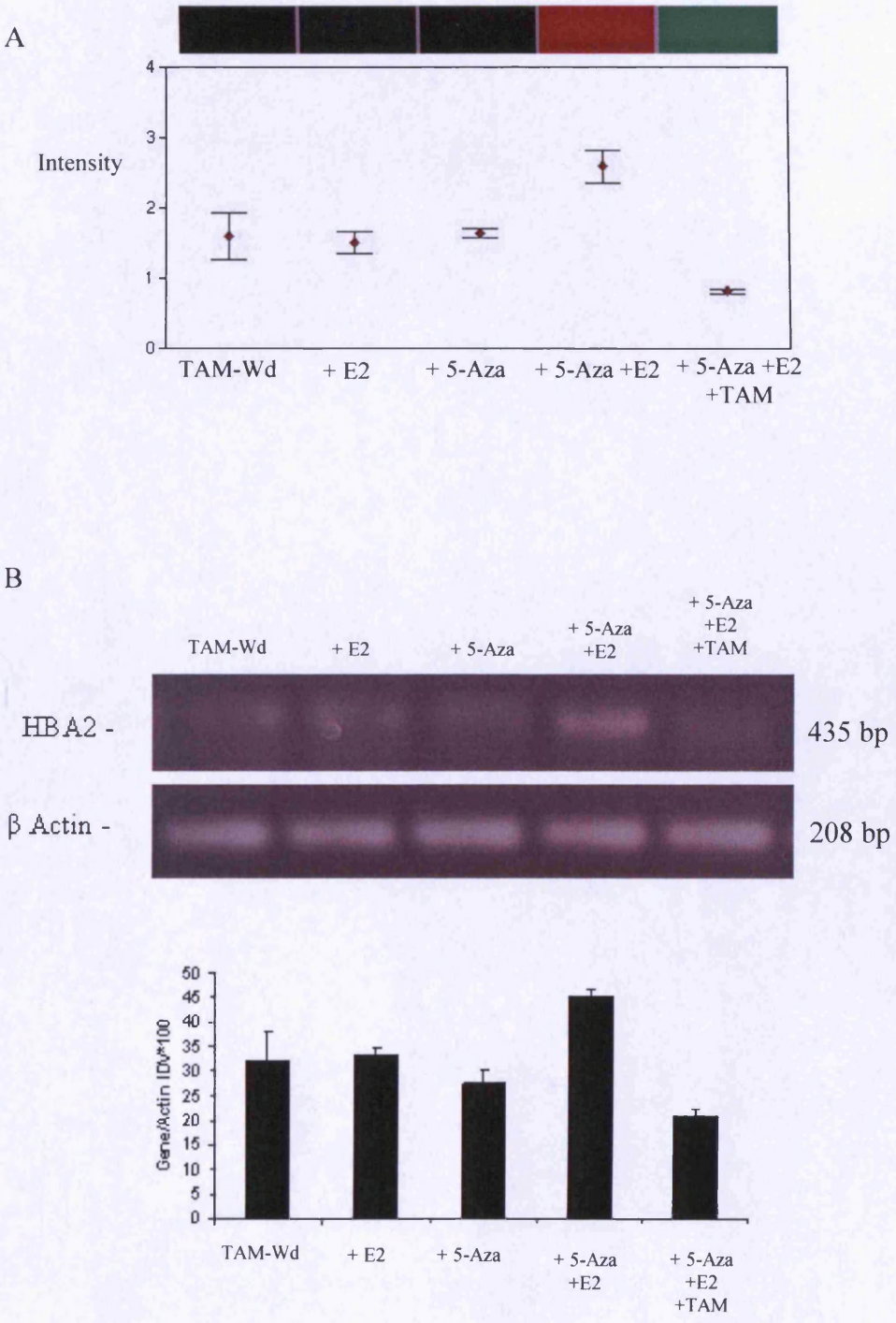


Fig 5.8 C: Promoter methylation profile for HBA2 in TAM-Wd cells: Methylight data specific for methylated HBA2 promoter detected in TAM-Wd cells, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM 5-Aza (data expressed as PMR (*p<0.001).

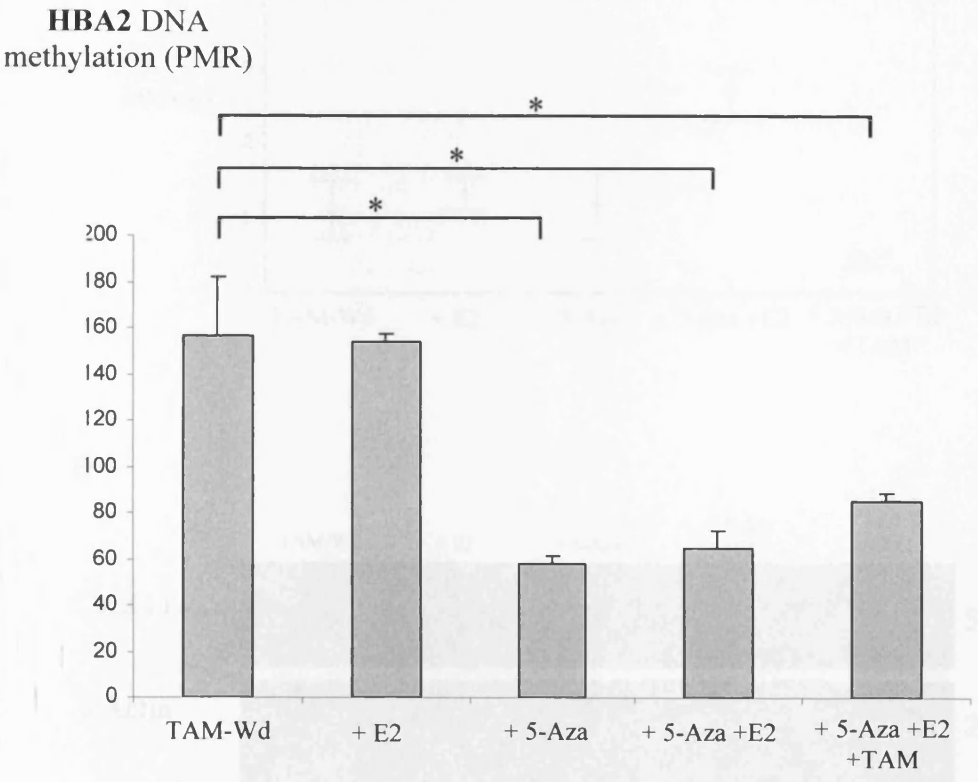


Fig 5.9 Carbonic Anhydrase XII – CA12 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess CA12 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

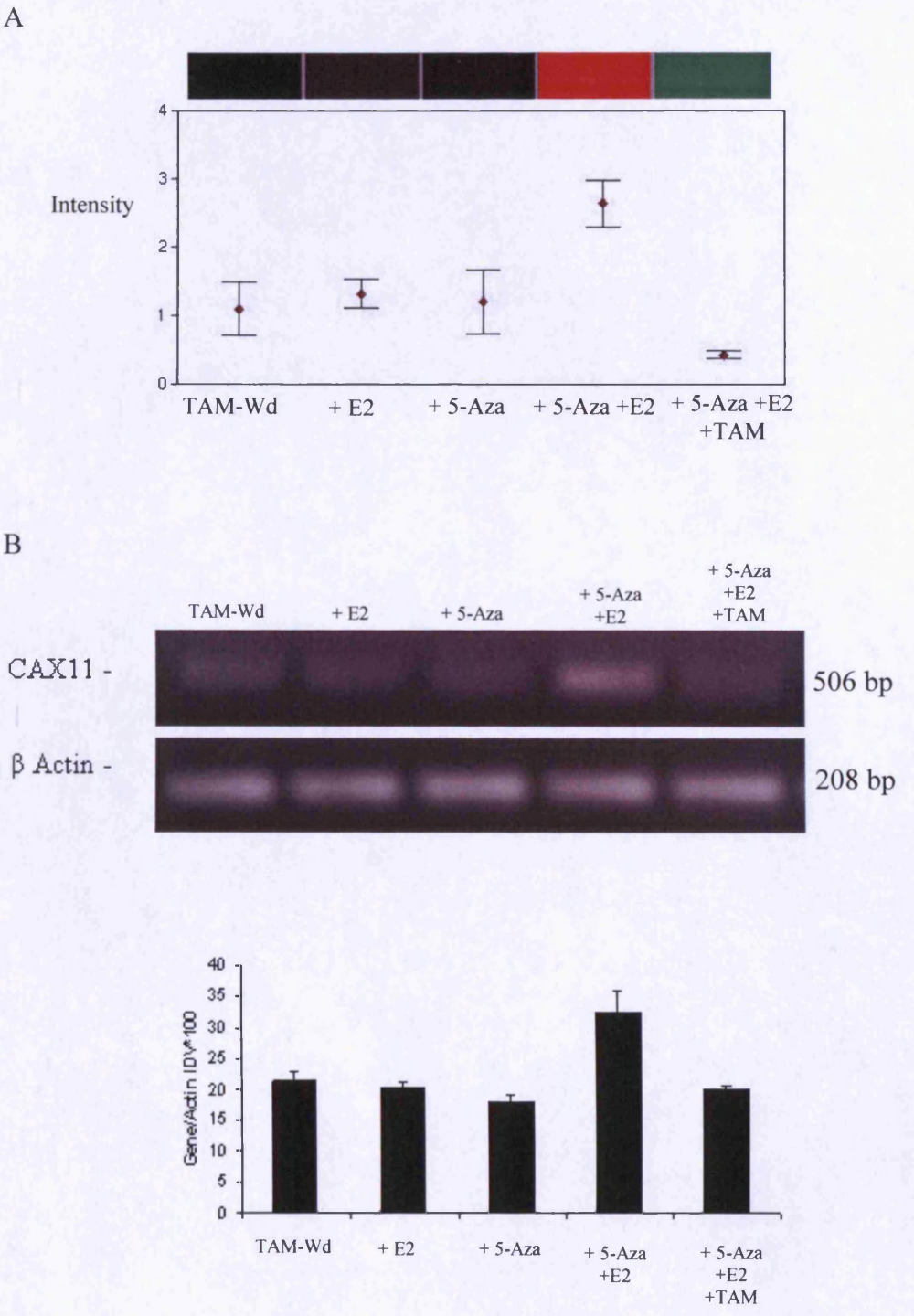


Fig 5.10 RAS-protein Activator Like 1- RASAL-1 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess RASAL-1 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

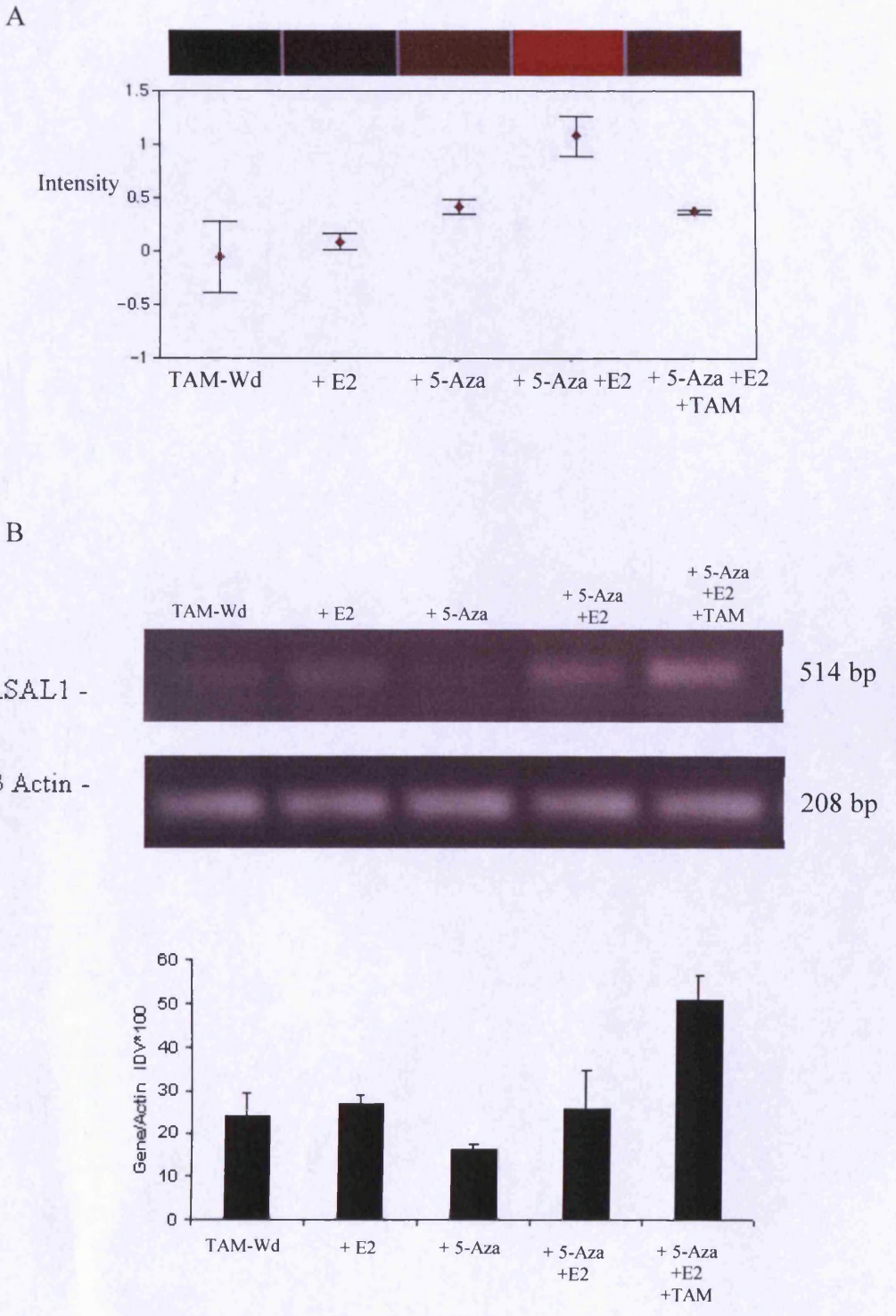


Fig 5.11 Collagen type VI alpha 3 - COL6a3 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess Col6a3 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

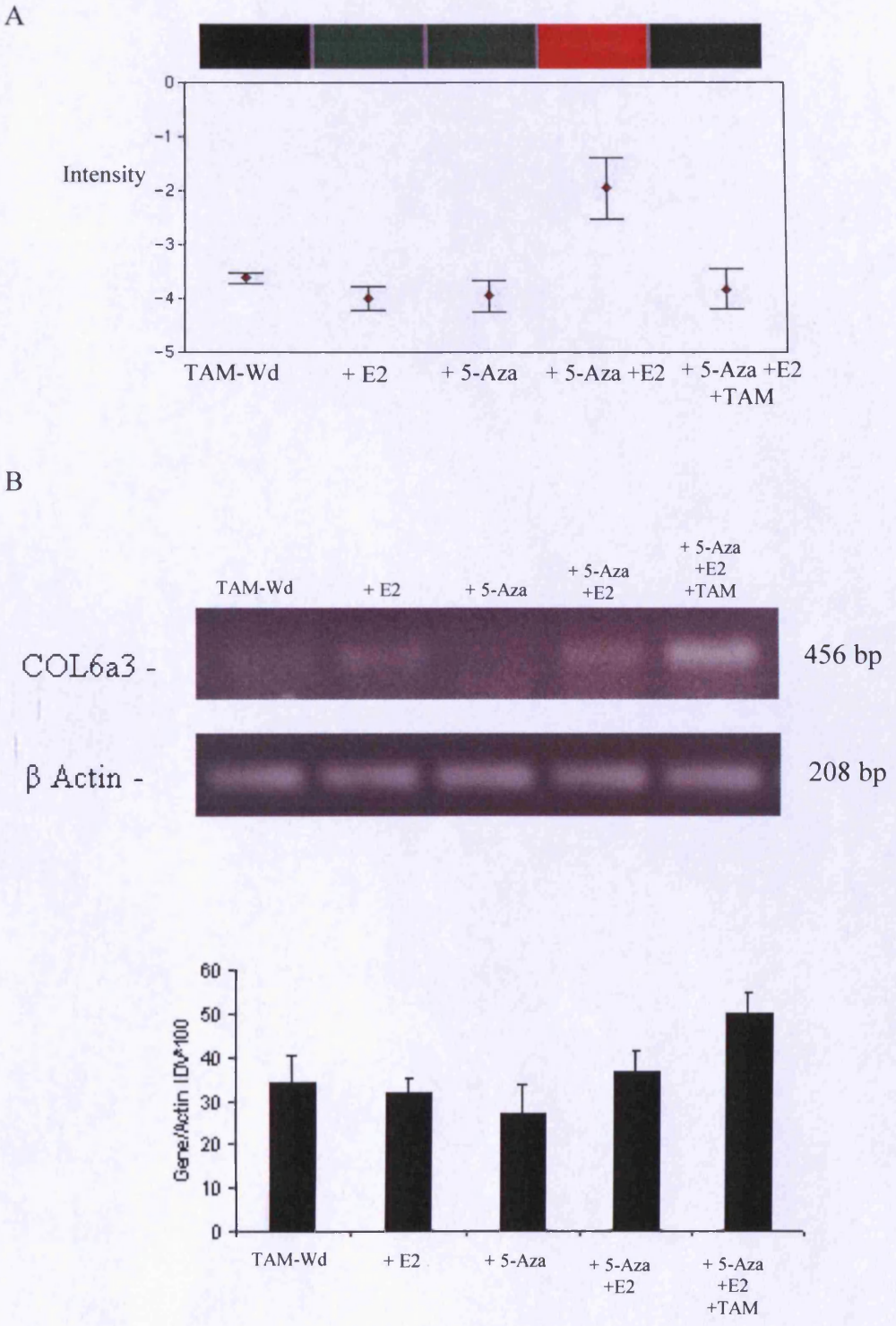


Fig 5.12 Adenylate Cyclase 9 - ADCY 9 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess ADCY9 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

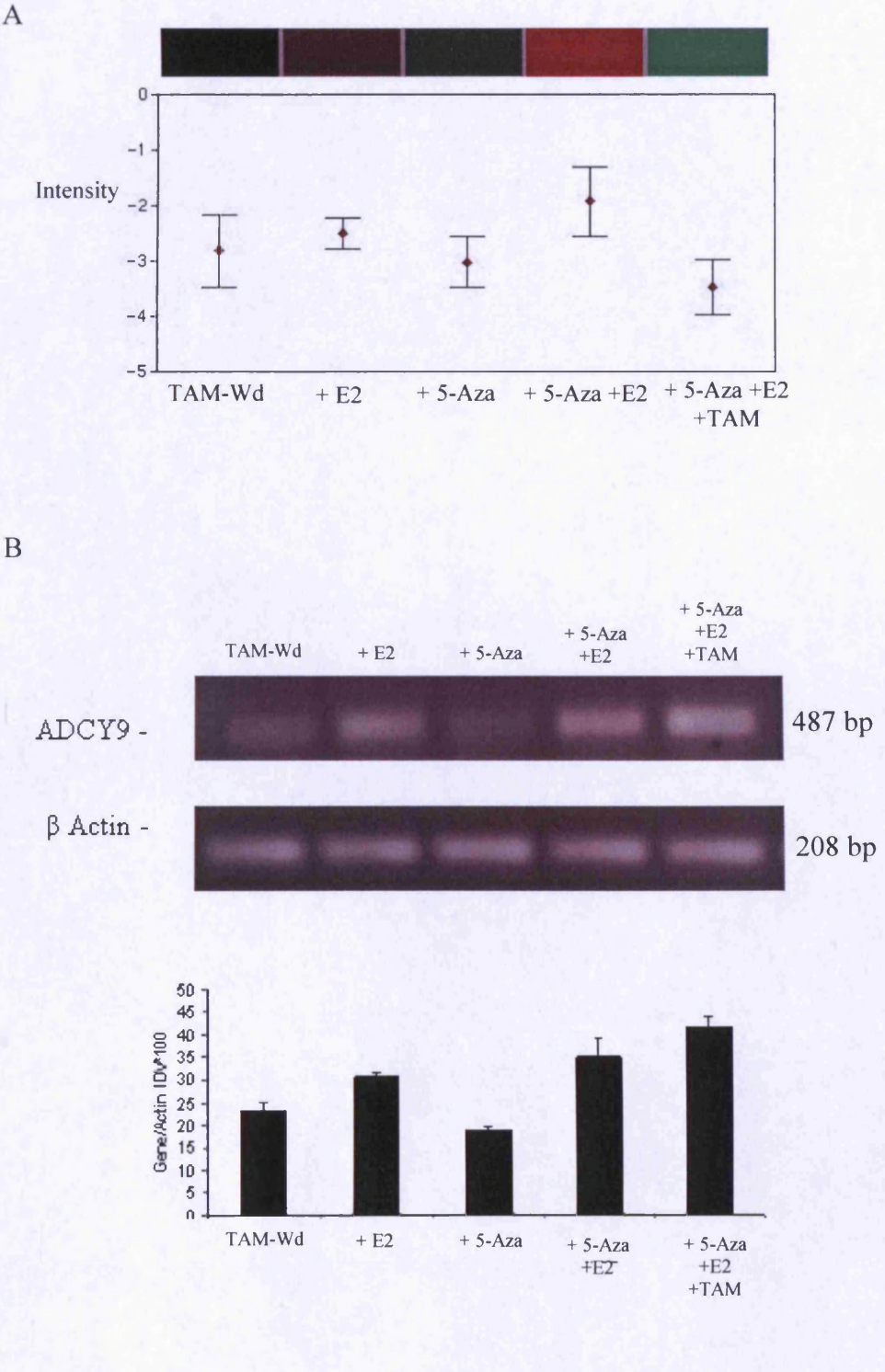
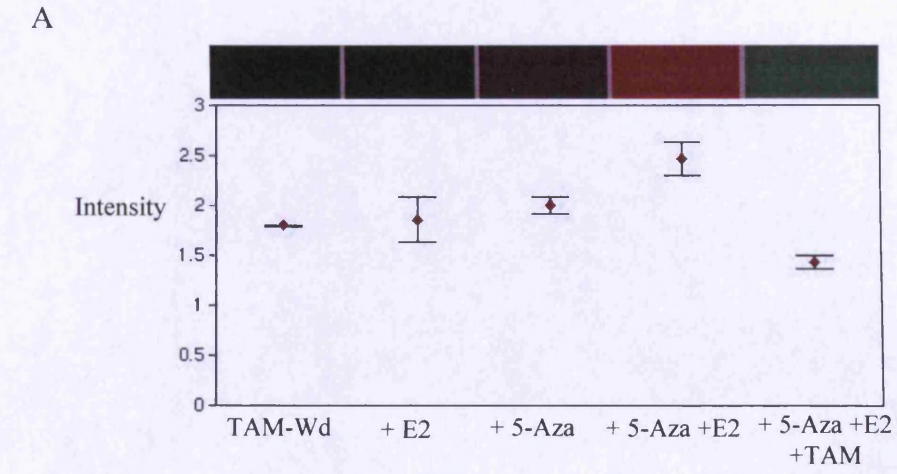
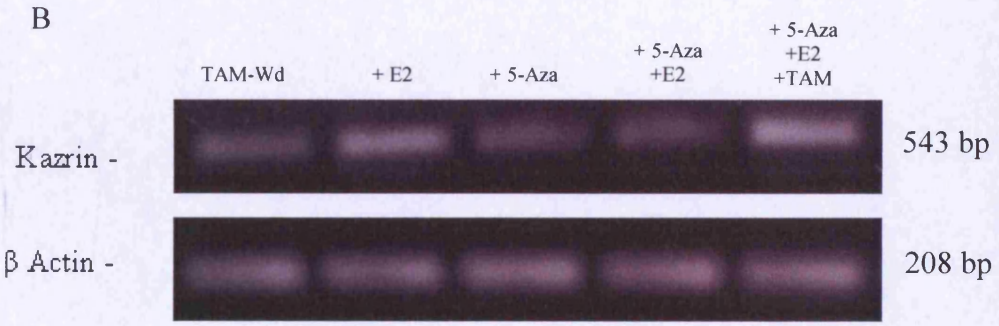
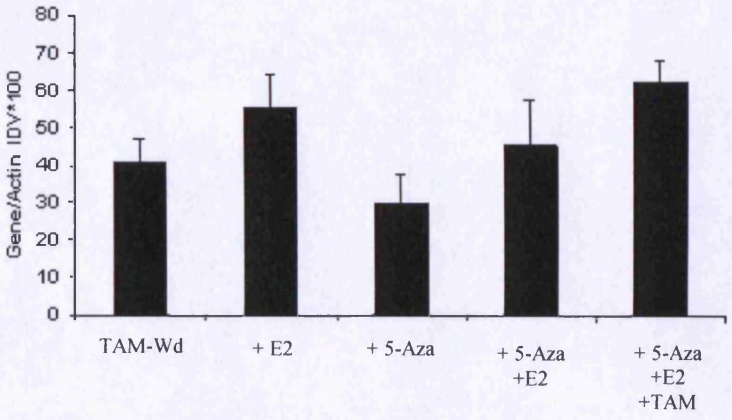


Fig 5.13 KIAA1026 - Kazrin A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess Kazrin expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).







5.3 De-methylation of genes associated with tumour progression.

Although the cell growth analysis from the previous chapter showed that the cumulative effect of 5-Aza and E2 co-treatment in TAM-Wd cells was reduced cell growth [Fig's 4.8-4.10 and 4.12], it should be noted that due to the nature of 5-Aza as a non-targeted de-methylation agent, it is inevitable that not all genes affected will be associated with the reduction of cell growth observed. Interestingly, amongst the gene set selected following the pattern navigation of the Affymetrix database [Fig 5.1], i.e. genes up-regulated by E2, suppressed by TAM in MCF-7 cells and suppressed in TAM-R cells, there were a number of genes whose up-regulation have been associated with tumour progression, eg WNT1 inducible signalling pathway protein-2 (WISP-2) [Saxena *et al,* 2001; Banerjee *et al,* 2003; Davies *et al,* 2007], stromal-cell derived factor-1 (CXCL12) [Luker & Luker, 2006, Kang *et al,* 2005a; 2005b], sialyltransferase (ST6) [Schneider *et al,* 2001; Lloyd *et al,* 1996], diacylglycerol kinase (DAGK) [Filigheddu *et al,* 2007], LY6/PLAUR domain containing 3 (LYPD3) [Fletcher *et al,* 2003; Paret *et al,* 2007; Hansen *et al,* 2008] and S100 calcium binding protein A14 (S100A14) [Yao *et al,*2007]. Indeed, it has been documented that anti-hormones can suppress genes that may advance tumour-progression as part of their protective effect [Frasor *et al,* 2003, Fan *et al,* 2006], and therefore there is a possibility that such genes could be silenced by promoter methylation following long-term treatment. Thus, the expression profiles of three of these genes (CXCL-12, WISP-2 and ST-6) in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 +

TAM treated cells was assessed using the Affymetrix data and PCR verification, and methylight reactions were carried out to determine promoter methylation status.

CXCL-12 was shown to be up-regulated in TAM-Wd cells cultured with E2 as assessed by both PCR and array analysis. Although the array data shows that 5-Aza and E2 treated cells show a higher expression of CXCl-12 than non-treated cells, the up-regulation is lower than that induced by E2 alone [Fig 5.14a]. This observation differs from the PCR data, where up-regulation is similar for both treatments, and reversed when E2 and 5-Aza are used in combination with TAM [Fig 5.14b]. Interestingly, methylight analysis showed a significant reduction of CXCL-12 promoter methylation in all 5-Aza treated cells compared to non-5-Aza treated cells ($p<0.001$) [Fig 5.14c].

WISP-2 was partially induced in TAM-Wd cells challenged with E2 in both PCR and array data sets. The addition of 5-Aza and E2 further increased expression over E2-only treated cells however; the addition of TAM caused no reversal of expression in the array data, and further increased expression in the PCR data [Fig 5.15].

In a similar fashion, ST6 was partially induced in TAM-Wd cells challenged with E2 in both PCR and array data sets. The addition of 5-Aza and E2 marginally further increased expression compared to E2-only treated cells in the array profile, though not in the PCR profile. The addition of TAM caused a further increase in ST6 expression in both data sets [Fig 5.16]. Methylight assays showed that no methylated ST6 promoter was detected in TAM-Wd cell DNA, and primers for accurate WISP-2 detection could not be designed.

Interestingly, two EGFR ligands were present in the list of 37 genes identified as being most highly up-regulated in TAM-Wd cells treated with 5-Aza + E2 (compared to non-treated, E2, 5-Aza + E2 + TAM treated cells) [Fig 5.5], namely epiregulin and transforming growth factor alpha (TGFα). This prompted further investigation of the impact 5-Aza + E2 co-treatment has on EGFR expression, and expression of other EGFR ligands. Heatmap profiles were selected for all 6 EGFR probes, 3 TGFα probes, and the single amphiregulin and epiregulin probes present on the HG-U133A Affymetrix chip. 2 of the 6 EGFR probes [Fig 5.17], 2 of the 3 TGFα probes [Fig 5.18] and the epiregulin probe [Fig 5.19] were most highly expressed in 5-Aza + E2 treated cells compared to non-treated cells, E2, 5-Aza and 5-Aza + E2 + TAM treated cells. Amphiregulin was up-regulated by E2 +/- 5-Aza [Fig 5.20].

The up-regulation of EGFR expression in TAM-Wd cells in response to 5-Aza + E2 was confirmed by real-time PCR (n=2). In the presence of 5-Aza, expression was up-regulated by 150% with E2-co-treatment, in contrast to E2-only treated cells, in which EGFR expression was down-regulated by approximately 50% (n=2) [Fig 5.21]. Parallel analysis of the MCF-7 cells showed that EGFR expression remained suppressed by E2 (approximately 50%) despite the presence of the de-methylation agent.

Fig 5.14 Stromal Cell Derived Factor 1 – CXCL-12 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess CXCL12 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

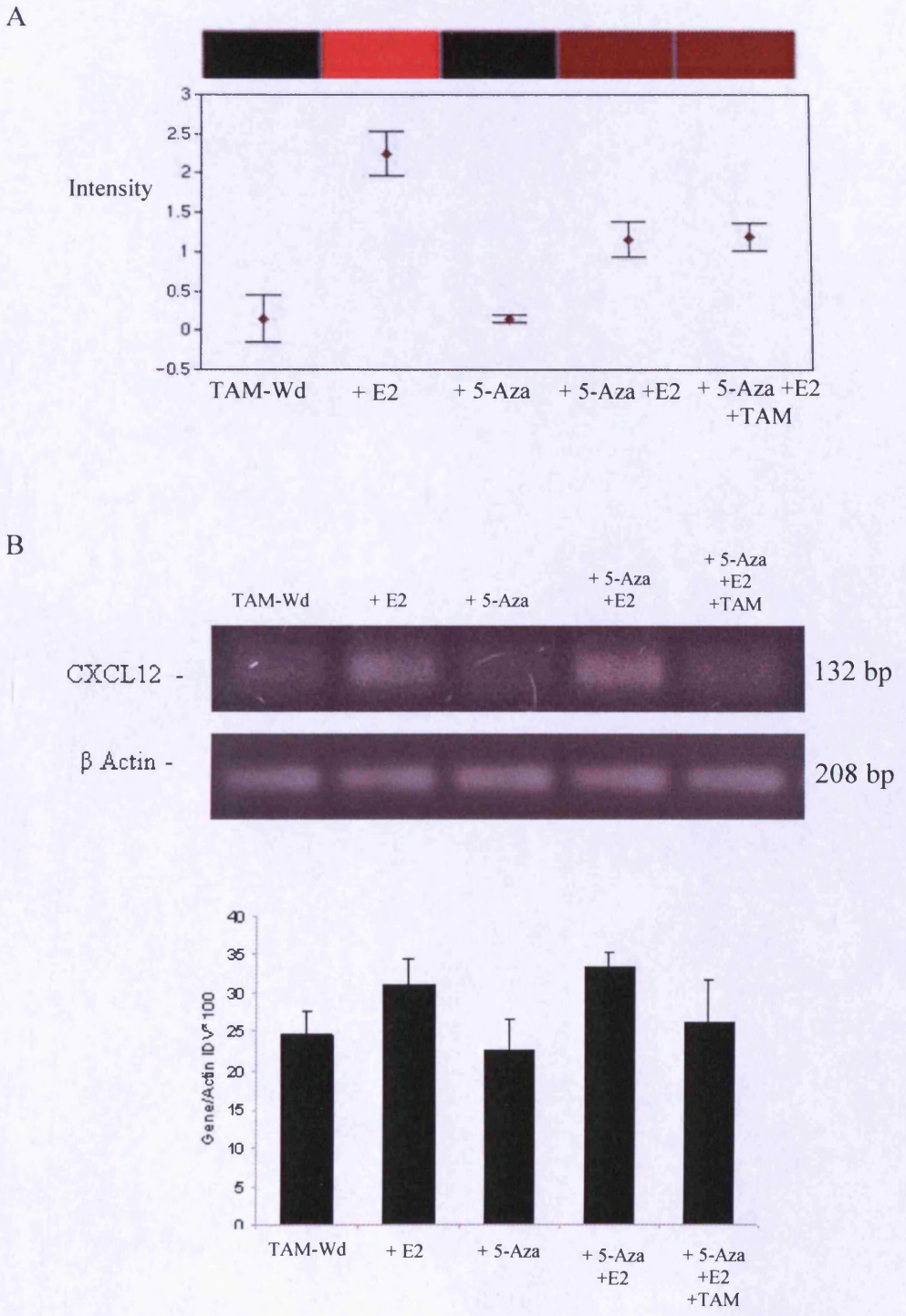


Fig 5.14 C: Promoter methylation profile for CXCL-12 in TAM-Wd cells: Methylight data specific for methylated CXCL-12 promoter detected in TAM-Wd cells, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM 5-Aza (data expressed as PMR) (*p<0.001).

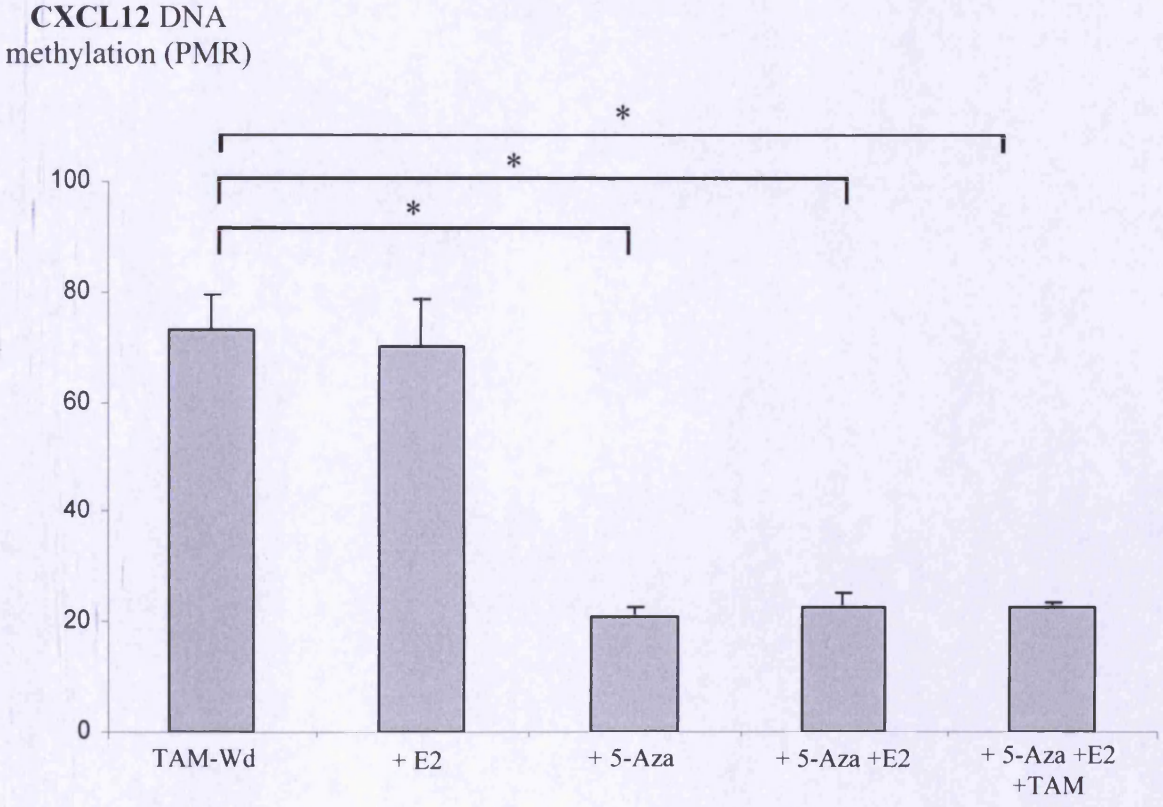


Fig 5.15 WNT1 Inducible Signalling Pathway protein-2 – WISP2 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess WISP2 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).

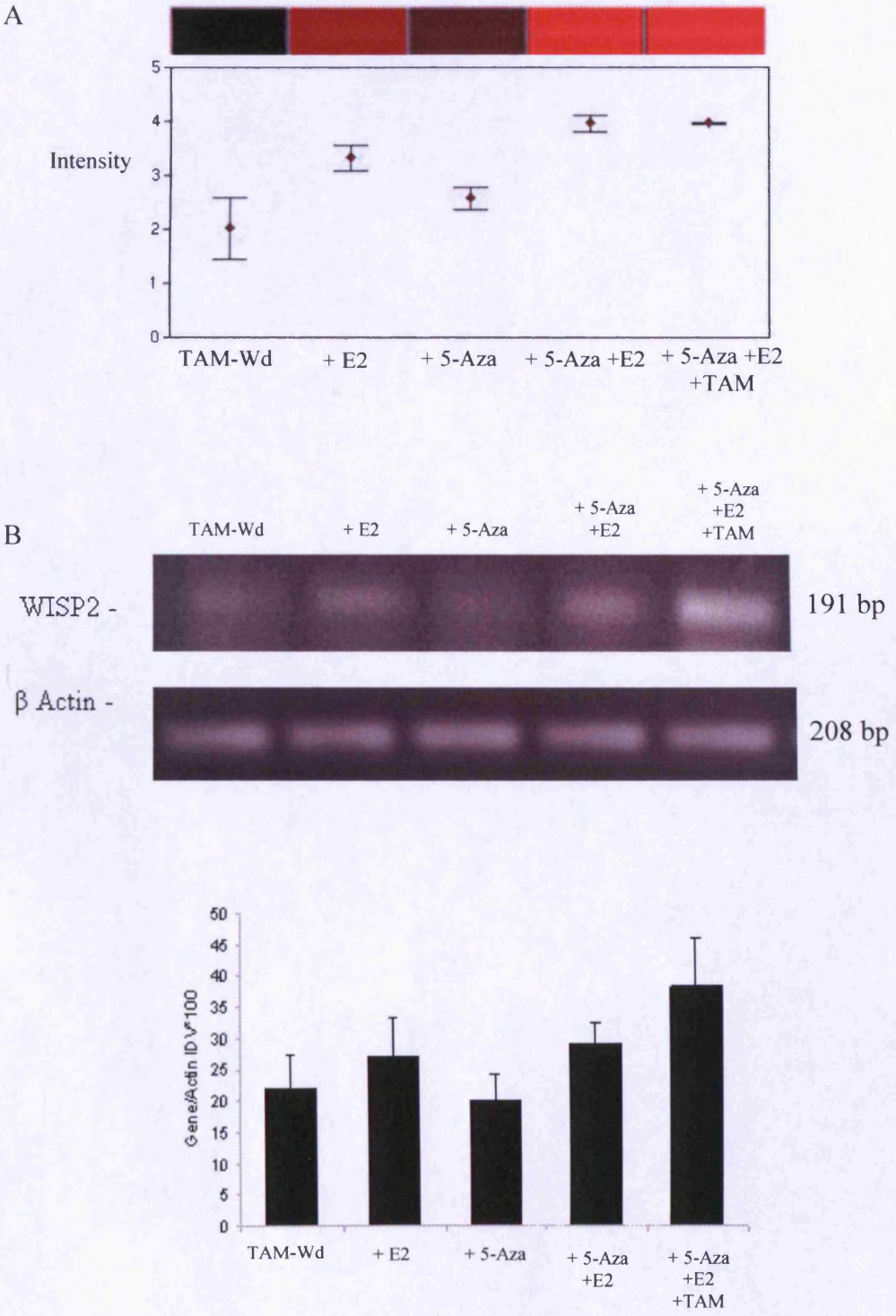
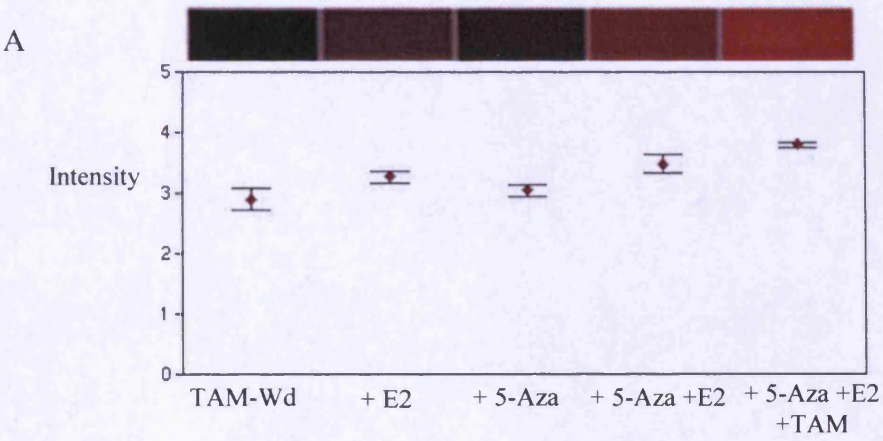
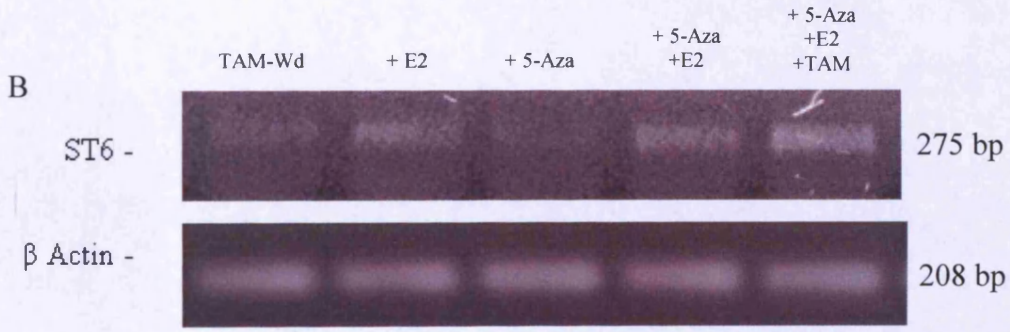


Fig 5.16 Sialytransferase – ST6 A: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess ST6 expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells. B: PCR validation of array profile and corresponding densitometry data (n=3).





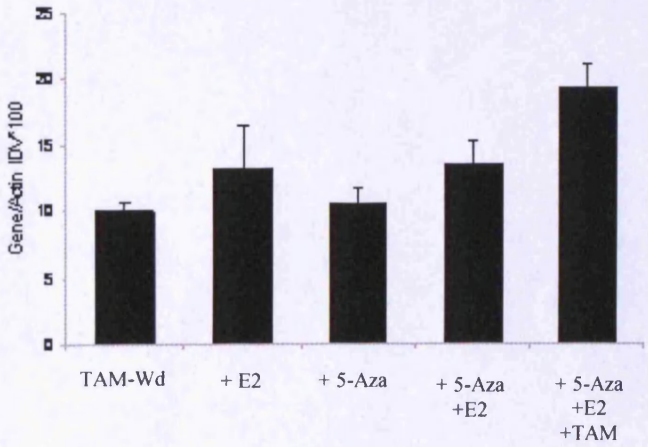
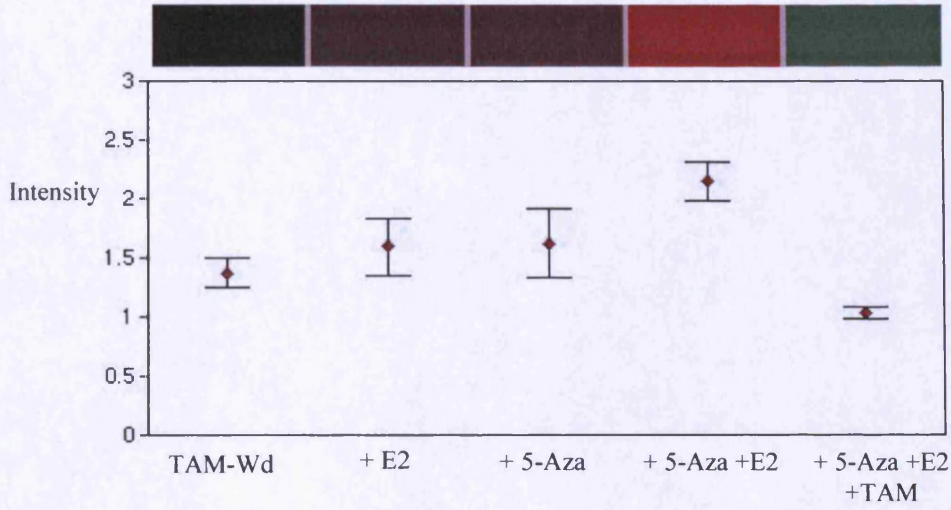
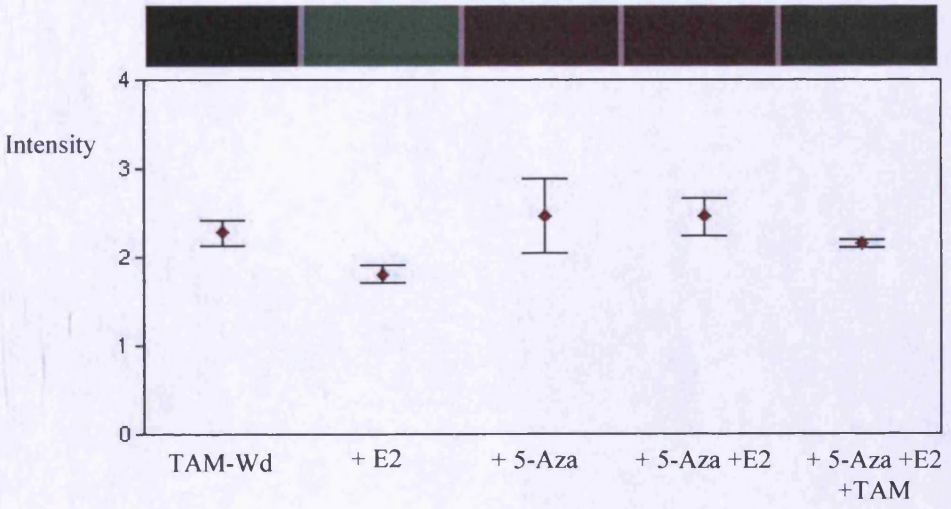


Fig 5.17 Epidermal Growth Factor Receptor – EGFR: Heatmap profiles and gene probe box-plots from the Affymetrix database, to assess EGFR expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells (n=6).





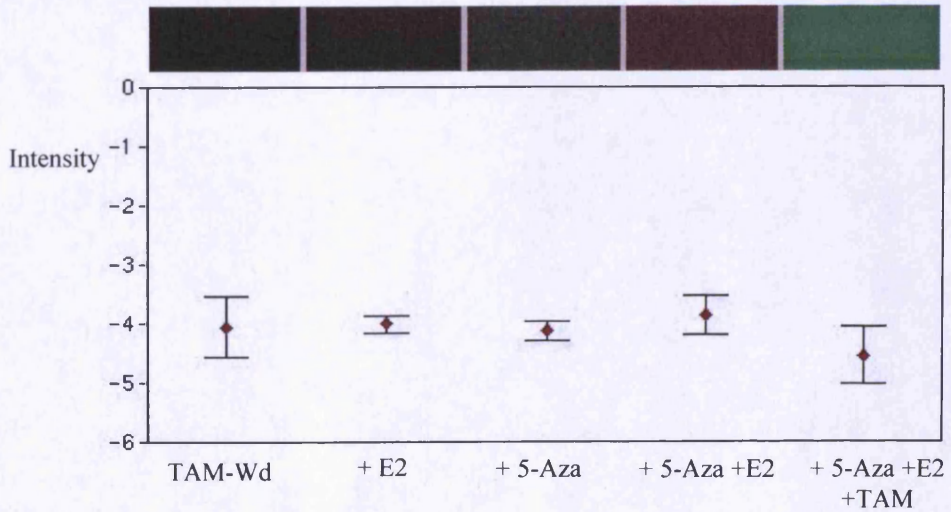
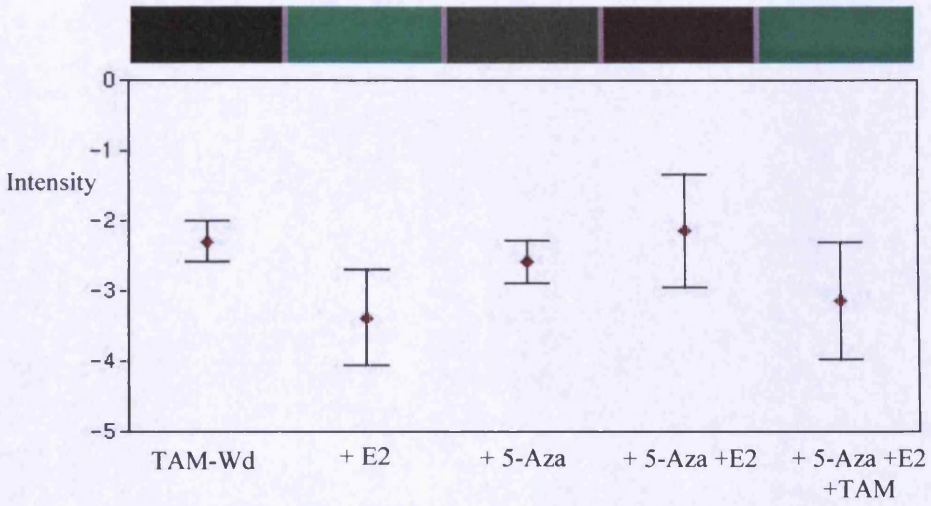
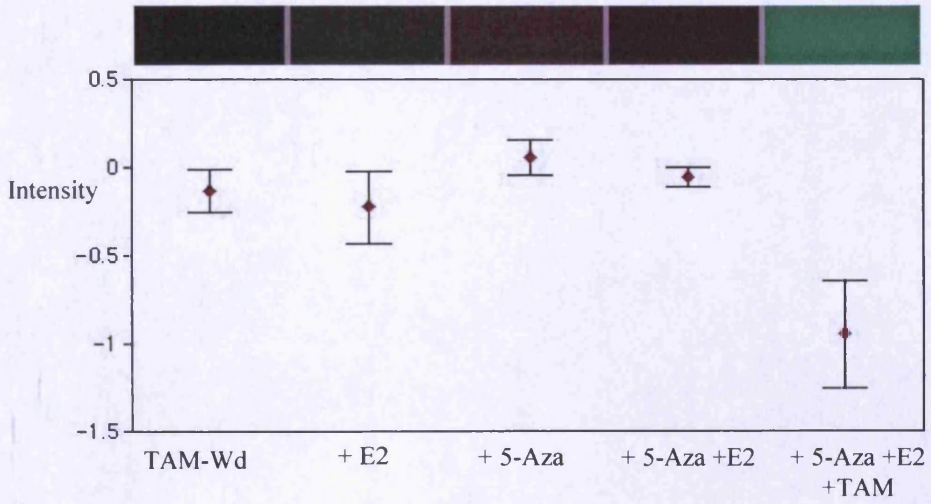


Fig 5.17 EGFR continued





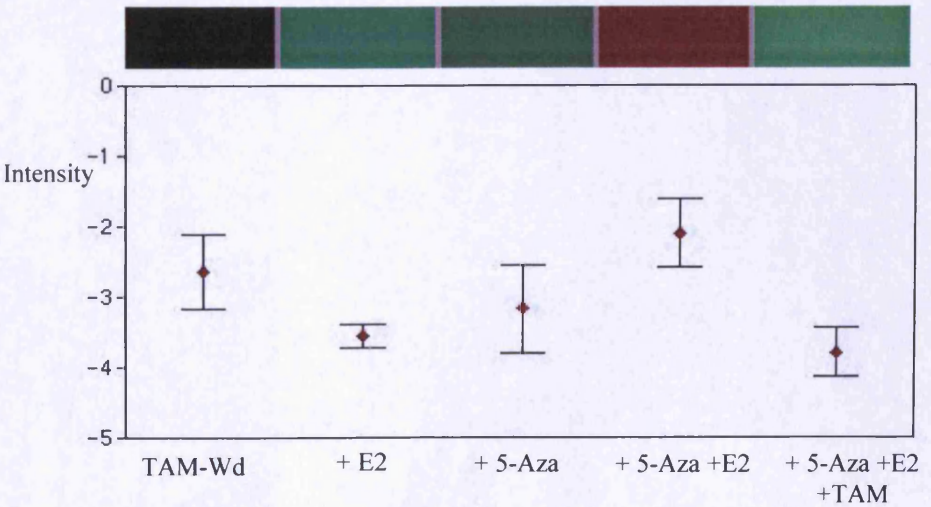


Fig 5.18 Transforming Growth Factor alpha - TGFα: Heatmap profiles and gene probe box-plots from the Affymetrix database, to assess TGFα expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells (n=3).

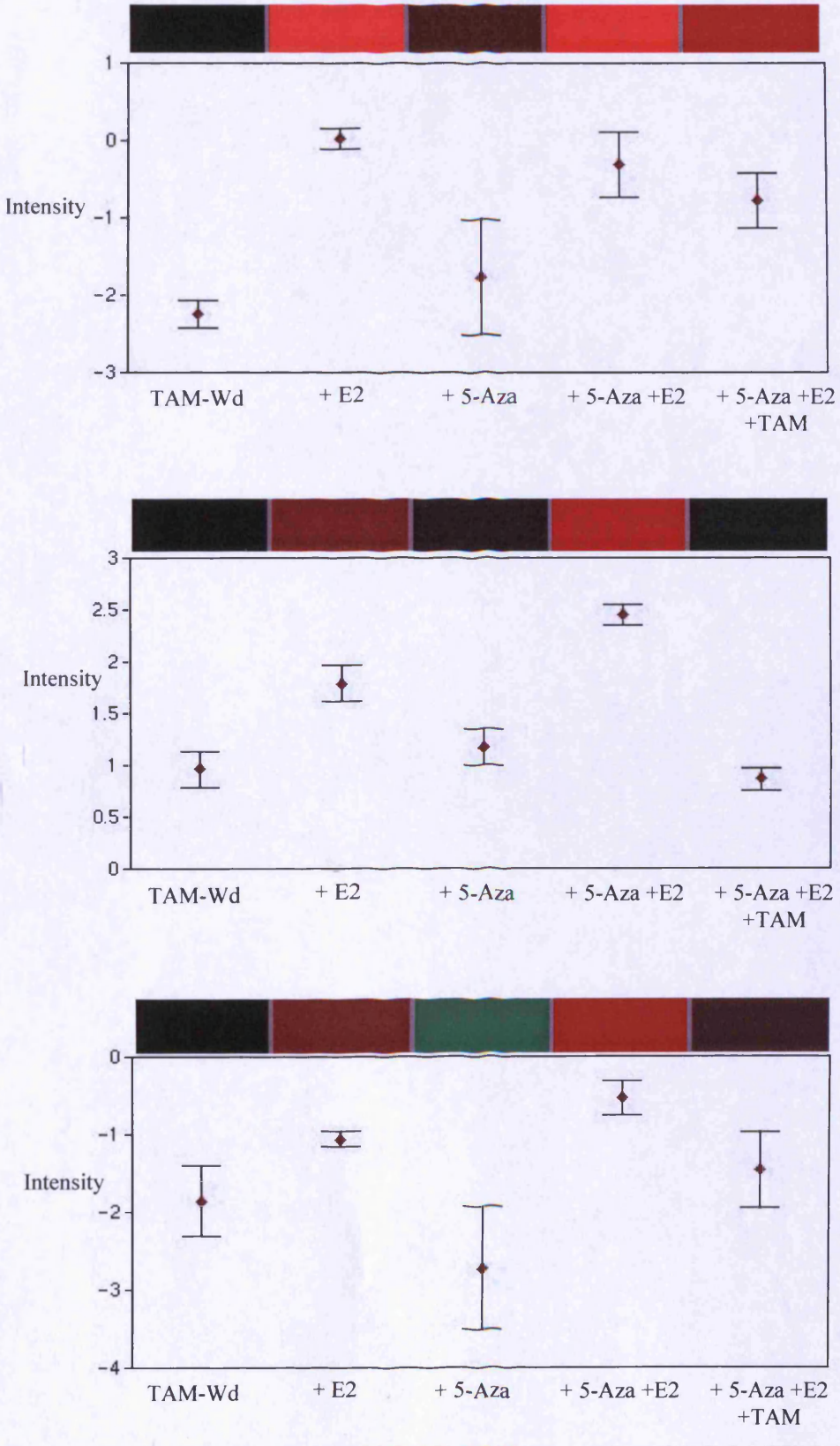


Fig 5.19 Epiregulin: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess epiregulin expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells (n=1).

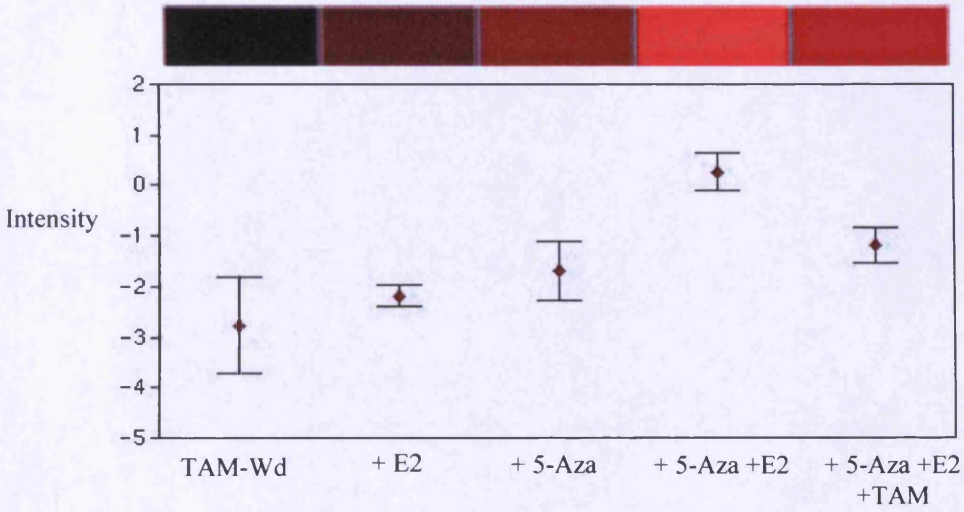


Fig 5.20 Amphiregulin: Heatmap profile and gene probe box-plot from the Affymetrix database, to assess amphiregulin expression in TAM-Wd, +E2, + 5-Aza, + 5-Aza, +5-Aza + E2 and +5-Aza + E2 + TAM treated cells (n=1).

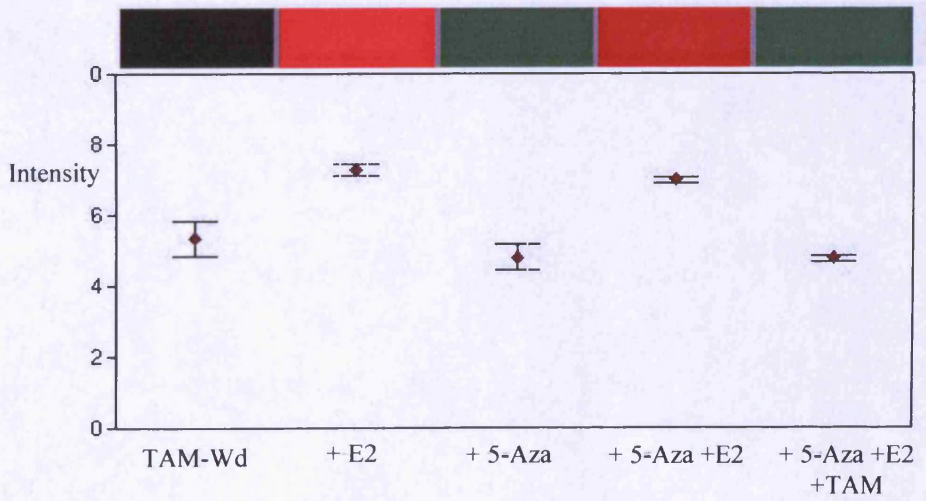
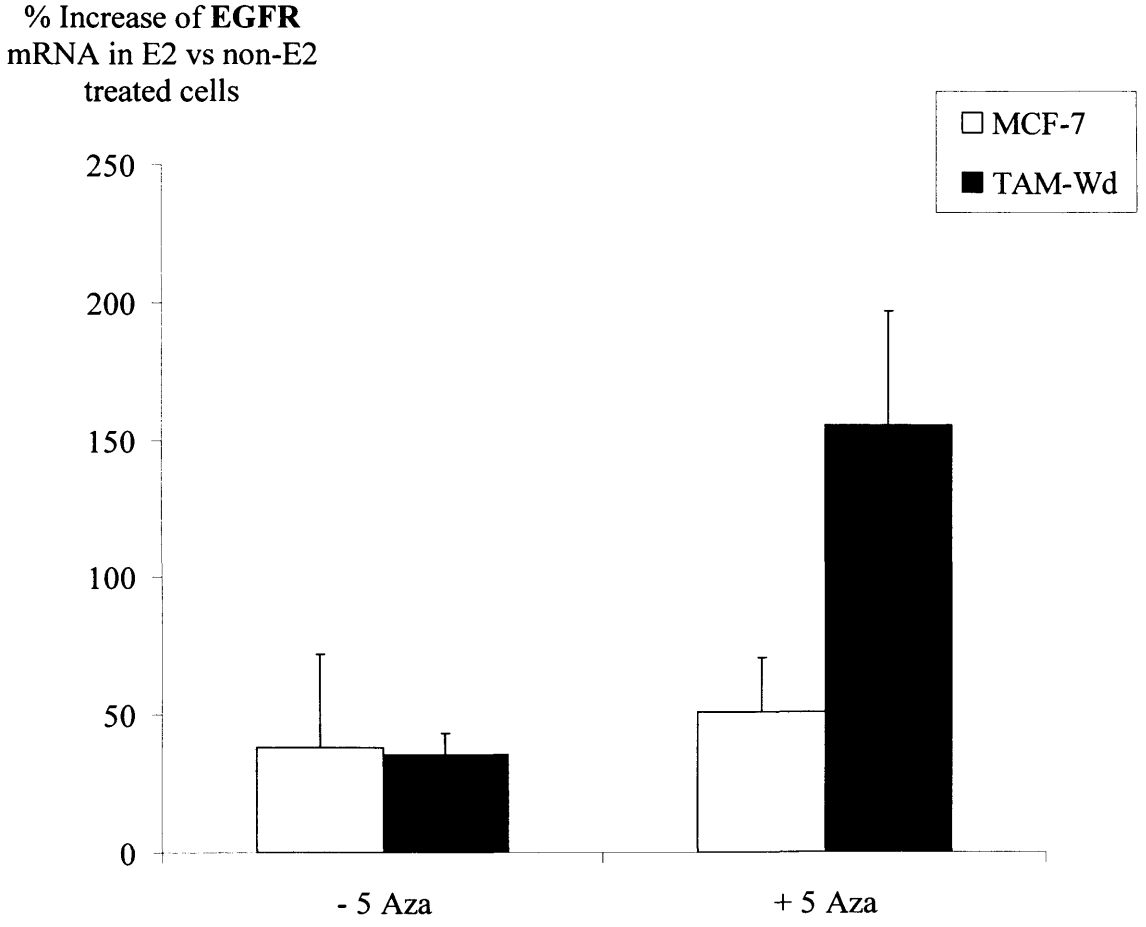


Fig 5.21 Expression of EGFR: Real-Time PCR evaluation of EGFR mRNA concentration in MCF-7 and TAM-Wd cells ± 5-Aza ± E2 at 10^{-9}M for 6 days. Data shown represents percentage increase of EGFR detected in cells ± 5-Aza, following E2 challenge (n=2).



6. DISCUSSION

It is now widely accepted that the acquisition of anti-hormone resistance is associated with a variety of molecular mechanisms which serve to not only to compensate for the growth inhibition caused by decreasing oestrogen receptor activity, but also further enhance the aggressiveness of the breast cancer phenotype [Hiscox *et al,* 2003; 2004]. Preliminary analysis of our cellular model of ER-positive tamoxifen-resistant breast cancer has shown that resistant MCF-7 cells possess enhanced proliferative and invasive capacities compared to wt-MCF-7 cells. The increased activation of a growth-factor receptor signalling network featuring EGFR, HER-2 and IGF-1R [Nicholson, *et al,* 2005] has been shown to contribute to both the accelerated rate of growth, and to a lesser extent, the motile and invasive potential of these cells. Interestingly however, although inhibition of these growth factor receptors, either alone or in combination, can provoke some growth inhibition, the effects on cell proliferation are incomplete and rarely promote cell kill [Nicholson *et al,* 2001; Knowlden *et al,* 2003; 2005]. Consequently, breast cancer cells survive drug treatment and resistant cell growth resumes within several months. The apparent shortfall of such inhibitors to meet their expected effectiveness in the experimental setting is indicative of additional mechanisms mediating the tamoxifen-resistant breast cancer cell phenotype. In light of this it is noteworthy that tamoxifen, through its genomic mechanism of action, classically acts to reduce expression of ER-regulated genes, where

conformational changes induced by tamoxifen binding to ER provides docking sites for corepressors such as NCoR/SMRT [Lavinsky et al. 1998], REA [Montano *et al,* 1999], RTA [Norris *et al.* 2002], SAFB1 [Oesterreich *et al.* 2000], and Smad4 [Wu *et al,* 2003]. This in turn can facilitate the recruitment of HDACs to ERE-bearing genes causing the condensation of the surrounding chromatin environment and gene suppression [Hu & Lazar, 2000]. Since gene promoters that are silenced by histone modifications are considered vulnerable to permanent silencing through hypermethylation of CpG islands in their promoter sequences [Cheng *et al,* 2008], it is possible that epigenetic alterations (such as histone code modification and promoter hyper-methylation) to oestrogen regulated tumour suppressor gene promoters may contribute to the development of tamoxifen resistance. Although at first sight this may appear incongruent since oestrogens are considered mitogenic to many breast cancer cell-lines, the proliferative actions of oestrogens are not limitless and exponential cell growth in culture rapidly ceases, possibly due to the induction of growth suppressive pathways [Stender *et al,* 2007; Safe, 2001]. Oestrogen has been shown to induce apoptosis in other breast cancer cell models including oestrogen deprived cells [Song *et al,* 2001; Lewis *et al,* 2005] and ER-negative cells stably transfected with ER [Levenson *et al,* 1994; Licznar *et al,* 2003]. Furthermore, high doses of synthetic oestrogens, such as diethylstilbestrol (DES), have been used effectively to treat postmenopausal women with ER-positive breast cancer, and cause tumour-regression [Peethambaram *et al,* 1999]. Cumulatively, these reports indicate that under certain circumstances oestrogen can positively influence tumour suppressor/

pro-apoptotic gene networks, and infer that the loss of function of these estrogen responsive genes may facilitate tumour progression.

The focus of the present study, therefore, was to determine whether oestrogen responsive genes associated with tumour-suppressor function, were silenced by promoter methylation in TAM-R cells as a direct consequence of long-term tamoxifen treatment, and whether re-activation of such genes could provide a potential therapeutic avenue for the management of tamoxifen-resistant breast cancer.

6.1 Long-term tamoxifen exposure is associated with a permanent alteration to breast cancer cell phenotype.

In order to assess the extent to which the presence of tamoxifen contributed to the TAM-R cell phenotype, cells were withdrawn from tamoxifen for up to 6 months. It was reasoned that during this period, any agonistic contribution of the drug to TAM-R cell morphology, growth or motility would be depleted, whilst the effects of permanent heritable cell re-programming caused by the previous long-term tamoxifen treatment would remain.

6.1.1 Growth and Invasion

Significantly, following a tamoxifen withdrawal period of 1, 3 and 6 months, it was noted that the enhanced growth rate of the TAM-R cells compared to the parental MCF-7 cells was sustained in the absence of tamoxifen, as were features of their morphological appearance; with Tam-Wd cells demonstrating

increased lamellipodia and filopodia formation. In contrast to these observations, the motile capacity of the TAM-R cells was significantly reduced following tamoxifen withdrawal. The divergence between the effects of tamoxifen withdrawal on proliferation and motility emphasises the differential regulation of these events in breast cancer cells and suggests that although the suppression of anti-invasive genes has been described in breast cancer [Yang *et al,* 2007], in this instance, it is unlikely that they have been permanently down-regulated in TAM-R cells by long-term tamoxifen treatment.

6.1.2 Assessment of the effects of tamoxifen withdrawal on ER and growth factor signalling.

In light of the continued proliferation of the tamoxifen withdrawn tamoxifen-resistant cells, their expression and use of ER, EGFR and IGF-1R signalling pathways was reassessed. In general terms, tamoxifen withdrawn cells were less sensitive to inhibitors of these pathways.

6.1.2.1 Oestrogen Receptor

Numerous reports suggest that the oestrogen receptor may be silenced through promoter methylation in a variety of cancers, including lung [Issa *et al,* 1996], colon [Issa *et al,* 1996], prostate [Sasaki *et al,* 2002], ovarian [O'Doherty *et al,* 2002] and breast [Ottaviano *et al,* 1994]. Recent studies have shown that this phenomenon can contribute to the ER-negative status of both *de-novo* ER-negative cells, and cells that have acquired ER-negative status as a result of

long-term anti-hormone exposure, and can facilitate an anti-hormone resistant phenotype. The use of 5-Aza resulted in demethylation of the ER promoter and re-expression of ER mRNA and functional protein in both MBA-231 ER-negative cells, and ER-depleted, fulvestrant-resistant-MCF-7 cells [Ferguson *et al,* 1995; Yang *et al,* 2001]. In the specific example of tamoxifen resistance, ER expression is rarely lost, though long-term tamoxifen treatment of MCF-7 cells has been shown to irreversibly inhibit the expression of oestrogenic genes through chromatin remodelling [Badia *et al*, 2000]. In the present study, ER mRNA expression was reduced in the TAM-R cell-line compared to the anti-hormone sensitive parental cell-line; however, withdrawal of tamoxifen restored ER expression, suggesting no permanent gene suppression had occurred. Significantly, the loss of ER expression in TAM-R cells has been observed in cells cultured in the anti-hormone for longer periods of time. This occurs through a reduction in mRNA transcripts from the A, B and C ER promoters (Abdel Bensmail – personal communication). This effect is reversible on tamoxifen withdrawal and may account for the raised ER expression in the TAM-Wd cells used in the present study.

Despite their increased ER expression levels, TAM-Wd cells showed reduced sensitivity to Faslodex (40% growth inhibition), in comparison to TAM-R (60%) and wt (75%) cells. They do not respond to tamoxifen re-exposure and are relatively insensitive to the growth promoting actions of oestradiol. Importantly, hyper-sensitivity to E2 stimulation was not observed in either TAM-R or TAM-Wd cells, contrary to findings from other groups using models of anti-hormone resistance, in which oestrogen can be apoptotic [Yao

et al, 2000; Jordan *et al,* 2007]. In total, these data imply a diminishing significance for ER signalling in the tamoxifen withdrawn model.

6.1.2.2 EGFR and IGF-1R

An increased reliance of breast cancer cells on EGFR and IGF-1R has been linked to the development of anti-hormone resistance [Nicholson *et al,* 2001] and gefitinib resistance [Jones *et al,* 2004] respectively and can occur by ER dependent or independent mechanisms. It was deemed feasible therefore, that changes in the usage of these growth factor receptors in the tamoxifen withdrawn tamoxifen resistant cells might underpin their continued growth rate. This, however, was not the case since the withdrawn cells showed reduced sensitivity to gefitinib and ABDP, selective inhibitors of these pathways. Interestingly, the reduced sensitivity to gefitinib in the tamoxifen-withdrawn cells was associated with a parallel reduction in EGFR expression, indicating tamoxifen was maintaining EGFR levels; while the reverse was true for the expression of IGF-1R, which increased on tamoxifen withdrawal. Although the precise molecular mechanisms associated with this differential response remain to be established in the tamoxifen withdrawn cells, they are consistent with the cellular actions of tamoxifen in MCF-7 cells, where it rapidly promotes EGFR expression through the repression of oestrogen induced negative regulatory elements in the first intron of the EGFR gene [Wilson & Chrysogelos, 2002], whilst repressing the positive regulation of IGF-1R expression by oestradiol [Guvakova *et al,* 1997].

6.2 Proof of principal that epigenetic silencing of ER regulated genes occurs in tamoxifen withdrawn tamoxifen resistant cells.

In concordance with observations of other research groups [Badia *et al,* 2000; Fan *et al,* 2006], the TAM-R cells used in the present study show significantly reduced E2-induction of the classically regulated, oestrogen responsive genes, PR and pS2, compared to the MCF-7 cells. Critically however, their suppression was maintained in the tamoxifen withdrawn cells, suggesting that the effect may be epigenetic in nature. The loss of oestrogen-regulation of these genes in TAM-R and TAM-Wd cells was further illustrated by their lack of response to oestradiol at a dose which up-regulated PR and pS2 by 40 and 4 fold in MCF-7 cells.

Confirmation of the epigenetic nature of the suppression of PR and pS2 was shown by the reversal of the oestrogen induction of their expression by the DNA de-methylating agent 5-Aza, which enabled oestradiol to increase their levels 18 and 7 fold in the tamoxifen withdrawn TAM-R cells. Methylight assays, performed through collaboration with Dr. H. Fiegl (Medical University of Innsbruck), showed that the pS2 promoter was methylated in the tamoxifen-withdrawn TAM-R cells, and that the quantity of methylated promoter detected was reduced 4-fold by 5-Aza treatment. The quantity of PR expressed in these cells, which was observed at a much lower level than pS2 under basal conditions, proved undetectable in TAM-Wd cells using the Methylight system. In accordance with studies carried out using ER siRNA to induce signalling disruption [Leu et al, 2004], these data indicate that epigenetic

silencing of classically E2-regulated genes can arise as a result of ER-signalling disruption caused by long-term tamoxifen challenge, and that this is apparent in the emerging resistant state.

6.3 Relationship between epigenetic silencing and tumour cell growth.

Although the studies on PR and pS2 clearly demonstrate that epigenetic silencing of ER regulated genes occurs in TAM-R cells, these genes are not considered to be significant regulators of cell growth. Experiments were conducted to determine whether other genes specifically activated in TAM-Wd cells by 5-Aza + E2 co-treatment in TAM-Wd cells could be associated with growth regulation. Care was taken to select a dose of 5-Aza which was not growth inhibitory since high doses of this drug can be severely cytotoxic. A dose of 1μM 5-Aza was chosen since it had little growth inhibitory effect, yet, as discussed above, it reversed promoter methylation of the pS2 gene. Importantly, subsequent studies revealed that 5-Aza treatment of TAM-Wd cells switched oestradiol from being mildly mitogenic into a growth inhibitory agent. Thus, in contrast to oestradiol promoting an approximate 40% increase in cell numbers when used as a single agent, in combination with 5-Aza, cell number was reduced by 50% at 7 days, and had fallen below the initial seeding density after 14 days culture. This previously unidentified phenomenon appears to be ER related, since it was reversible by tamoxifen co-addition and was not evident in MCF-7 cells, implying it was associated with long-term tamoxifen treatment. Evidently, long-term tamoxifen treatment

of breast cancer cells is able to suppress the expression of oestrogen regulated genes that inhibit cell growth and/or cell death. Significantly, two previous studies are supportive of the concept of anti-hormonal silencing of potential tumour suppressors. SAGE studies revealed that expression of CtIP (or retinoblastoma binding protein 8) was decreased in acquired tamoxifen resistant models, where its knockdown in MCF-7 promoted tamoxifen resistance, while its induction restored response [Wu *et al,* 2007]. Secondly, a study by Treeck *et al,* (2004) has reported that long-term tamoxifen treatment of MCF-7 cells decreased levels of several pro-apoptotic genes and impaired subsequent apoptotic response to etoposide treatment. To our knowledge, only two other groups have reported oestrogens to be growth inhibitory to anti-hormone resistant breast cancer cells [Liu *et al* 2003; Yao *et al,* 2000], and these particular instances occurs, the growth inhibitory effect observed was thought to be due to cells becoming 'addicted' to the increased growth factor signalling that oestrogens subsequently suppress. Critically, our novel observation directly links anti-hormone induced hypermethylation of oestrogen regulated tumour suppressor gene promoters to anti-hormone resistant cell proliferation. Importantly, data suggested that this process is reversible with co-treatment of 5-Aza and E2, and thus the identification of genes associated with phenomenon could provide valuable insight into the mechanisms that govern the tamoxifen-resistant cell phenotype.

6.4 Identification of oestrogen regulated genes potentially associated with 5-Aza induced inhibition of cell growth.

Having demonstrated that the combination of 5-Aza and E2 caused the TAM-Wd cells to proliferate at a slower rate than 5-Aza-treated controls and furthermore cause cell loss over a 14 day period, we sought to identify genes that were being up-regulated specifically by E2 challenge in the presence of the demethylation agent which could be associated with this phenomenon. This was achieved by Affymetrix profiling of our cells, examining for potential tumour suppressor/pro-apoptotic genes that had the following profile:

- Oestradiol induced and anti-hormone suppressed in wt-MCF-7 cells.
- Suppressed in TAM-R and tamoxifen withdrawn TAM-R cells.
- Not induced by Gefitinib in TAM-R cells.
- Induced by E2 + 5Aza co-treatment (but not by single agent treatment) and reversed by tamoxifen in tamoxifen-withdrawn TAM-R cells.

Candidate genes carried forward from our microarray study for PCR verification and promoter methylation analysis included GDF-15, ADCY9, CAX11, KIAA1026, HBA2, RASAL1, COL6a3 and RGC32, and are distinct from the pro-apoptotic genes identified as suppressed by Treek *et al* (2004). Two of the candidate genes, namely GDF-15 and RGC32, have been shown to be down-stream targets of the putative tumour-suppressor, p53 [Tan *et al,* 2000; Saigusa *et al,* 2006]. P53 functions to eliminate and inhibit the

proliferation of abnormal cells, thereby preventing neoplastic development [Gasco *et al,* 2002]. It remains the most commonly mutated gene in many human cancers, with mutations (principally, but not exclusively, mis-sense) estimated to occur in 50% of all cancers. Interestingly, although the frequency of p53 mutation is lower in breast cancer than in other solid tumours; various regulators of p53 activity and some downstream transcriptional targets of p53 have been reported to be silenced by both genetic and epigenetic mechanisms, leading to the mismanagement of this pathway in breast cancer cells, and hence promoting cell survival [Gasco *et al,* 2002]. The identification of p53 gene targets that have been silenced by promoter hypermethylation as a result of long-term tamoxifen treatment is a novel observation which could have value in the diagnosis, prognostic assessment and, ultimately, in the treatment of tamoxifen resistant breast cancer.

6.4.1 Growth Differentiation Factor-15

Growth Differentiation Factor-15 (GDF-15), which is also known by other names including prostate-derived factor, macrophage inhibitory cytokine 1, placental bone morphogenetic protein, placental transforming growth factor ß, and nonsteroidal anti-inflammatory drug-activated protein 1, is a member of the type β transforming growth factor (TGF-ß) superfamily and was first isolated from a subtracted cDNA library enriched for genes associated with macrophage activation [Bootcov *et al,* 1997]. It has since been reported to inhibit prostate epithelial proliferation, induce colon and mammary epithelial cancer cell apoptosis *in vitro* and inhibit colon and glioblastoma tumor growth

in vivo [Tan *et al,* 2000; Baek *et al,* 2001; Li *et al*, 2000; Graichen *et al,* 2002; Albertoni *et al,* 2002]. Tan *et al* (2000) identified GDF-15 as a p53 target gene that inhibits tumour cell growth via the TGFβ signalling pathway. Studies revealed that p53 activation is mediated through two p53 binding sites in the promoter of GDF-15 in a p53 dose- as well as p53 binding site-dependent manner by wild-type p53, but not by several p53 mutants. The p53 binding and transactivation of the GDF-15 promoter was enhanced by etoposide, a p53 activator, and was largely blocked by a dominant negative p53 mutant. GDF-15 was found to be a secretory protein, associated with the inhibition of tumour cell growth via p53-induced G1 arrest in both the cells secreting it (autocrine), and their neighbouring cells (paracrine). Indeed, conditioned medium collected from GDF-15-over expressing cells, but not from the control cells, suppressed tumour cell growth. Growth suppression was not, however, seen in cells that lack functional TGF-β receptors or Smad4, confirming that GDF-15 acts through the TGF-β signalling pathway. It is therefore apparent that this gene is an important link between p53 activation and TGFβ-mediated growth suppression, and that the loss of this gene could contribute to tumour cell proliferation. Interestingly, Ibanez de Caceres *et al* (2006) identified GDF-15 gene promoters as being silenced by hyper-methylation in renal cancer cells compared to normal cells, and demonstrated that this gene could be de-methylated following 5-Aza treatment.

The microarray data obtained from the present study shows that GDF-15 is an oestrogen regulated gene in wt-MCF-7 cells, which is suppressed following the acquisition of tamoxifen resistance. Using Methylight technology, we have

shown that GDF-15 gene promoter was significantly methylated in the tamoxifen withdrawn TAM-R cells, and that 5-Aza could be used to reverse the promoter's methylated status. Indeed, microarray and PCR verification showed that GDF-15 was most highly expressed in tamoxifen withdrawn TAM-R cells treated with 5-Aza and E2 compared to non-treated, 5-Aza or E2 treated cells. Intriguingly, the addition of tamoxifen caused a reduction in GDF-15 expression, in parallel with an increase in methylated promoter detected; indicating that silencing of GDF-15 by tamoxifen induced promoter methylation may indeed contribute to tamoxifen-resistant breast cancer cell growth.

6.4.2 Response gene to complement 32 protein.

The response gene to complement (RGC)-32 gene was first cloned from rat oligodendrocytes by differential display during a search conducted by Badea *et al* (1998) for genes that were differentially expressed in response to complement activation. Since then, several studies have reported changes in RGC-32 expression in a wide range of cell lines and tissues in response to a variety of stimuli including steroid hormones and growth factors [Vlaicu *et al,* 2008; Chen *et al,* 2005]. It has also been reported that RGC-32 transcription is directly regulated by p53 in glioblastoma/astrocytoma, osteoblastic, and colon cancer cell lines [Saigusa *et al,* 2007]. The expression of RGC-32 mRNA was dramatically increased by exogenous p53 in p53-mutant glioma cells, and also by endogenous p53 in response to DNA damage in colon-cancer cells over-expressing p53, but not in p53-deleted cells. Transiently and stably

overexpressed RGC-32 suppressed the growth of glioma cells through the suspected formation of a protein complex with polo-like kinase-1 (Plk-1), thus inhibiting its activity. Plk-1 is a key regulator of mitosis [Barr *et al*, 2004], but is also associated with oncogenesis since expression and kinase activity of Plk-1 is elevated in many kinds of cancers [Takai *et al,* 2005]. Inactivation of Plk-1 by a variety of methods has caused cell-cycle arrest at mitosis and/or apoptosis in cancer cells of several types [Elez *et al,* 2000; Spankuch-Schmitt *et al,* 2002], indicating that inhibition of Plk1 function might be a promising approach to cancer therapy [Gumireddy *et al,* 2005]. As RGC-32 binds to and is phosphorylated by Plk1 *in vitro*, it is possible that RGC-32 may inhibit G2/M progression through interaction with Plk1 in tumour cells. RGC-32 expression is down-regulated in invasive prostate cancer, multiple myeloma, and drug-resistant glioblastoma [Vlaicu *et al,* 2008]. Furthermore, Takai *et al* (2005) identified RGC-32 as suppressed in 5 out of 6 endometrial cancer cell lines as part of a study conducted to reveal novel tumour-suppressor genes that are epigenetically silenced in this disease. However, other studies have shown that RCG-32 expression is up-regulated in cutaneous T cell lymphoma and colon, ovarian, and breast cancer tissue, and can act as a substrate and regulator of cell division cycle 2 (CDC2) kinase activity, and thus induce S-phase entry and mitosis in tumour cells [Vlaicu *et al,* 2008]. Although there is no evidence to explain the discrepancies between the reported findings, it is possible that RGC-32 may function differently among different types of cells depending on which activation targets were available. Further examination will be needed to clarify the significance of RGC-32 in anti-hormone resistant

breast cancer, however, preliminary investigations using our microarray database show that Plk-1 expression is significantly up-regulated in tamoxifen resistant MCF-7 cells versus wild-type cells, whilst CDC2 expression remains consistently low in both cell-lines (data shown at the end of chapter - Fig 6.1), highlighting the possibility that RGC-32 could contribute to the inhibition of proliferation by deactivating Plk-1 in tamoxifen-resistant cells. Microarray data from the present study shows that RGC-32 expression is up-regulated in E2 challenged MCF-7 cells, and suppressed following the acquisition of tamoxifen resistance. Microarray and PCR verification showed that RGC-32 was most highly expressed in tamoxifen withdrawn TAM-R cells treated with 5-Aza and E2 compared to non-treated, 5-Aza or E2 treated cells. The addition of tamoxifen caused a reduction in RGC-32 mRNA expression in cells co-treated with 5-Aza and E2; suggesting the up-regulation of Plk-1, combined with the epigenetic silencing of RGC32, may facilitate TAM-R cell growth and the re-activation of RGC-32 could serve to reverse this phenomenon.

6.4.3 Ras protein activator type 1

Another oestrogen regulated gene identified from the present study as potentially associated with 5-Aza induced inhibition of cell growth, is Ras protein activator type 1 (RASAL-1). Unlike GDF-15 and RGC-32, RASAL-1 is not activated by p53; instead its activity is promoted in response to agonist-induced, intracellular Ca2+ oscillations [Walker *et al,* 2004]. RASAL-1 is a GTPase-activating protein (GAP) which is reported to normally terminate Ras activation, a GTP-ase oncogene which is constitutively active in

approximately 30% of all human tumours [Downward, 2003]. As with other small GTPases, Ras switches between two distinct conformations, an inactive GDP-bound state and active GTP-bound complex. Once in the GTP-bound conformation, Ras engages a number of effectors that couple this GTPase to the regulation of multiple signalling cascades important for cellular proliferation, differentiation, and survival [Mitin *et al,* 2005]. RASAL-1 enhances the intrinsic GTPase activity of Ras, thereby leading to its inactivation through the conversion of GTP into GDP. In a study conducted by Jin *et al* (2007), RASAL-1 was identified as being suppressed in several breast, lung, nasopharyngeal, hepatocellular and oesophageal tumour cells compared to normal-cells, and was subsequently found to be silenced by promoter hyper-methylation in a large proportion of the tumour cells, and corresponding tissue samples. They showed that ectopic expression of catalytically active RASAL-1 led to growth inhibition of these tumour cells by Ras inactivation, and hence provided evidence that epigenetically silencing of this gene represents a new mechanism of aberrant Ras activation in certain cancers.

In our models of anti-hormone resistant breast cancer, it has been established that Ras is an important transactivation target of EGFR, and it's activation of a down-stream signalling cascade involving Raf, MEK and ERK, can contribute to TAM-R cell proliferation and survival. The loss of Ras regulators could therefore potentiate this event and hence, it is reasonable to assume that re-introduction of RASAL-1 in TAM-R cells could compromise Ras down-stream signal transduction, and diminish the associated growth activation.

Microarray data from the present study shows that RASAL-1 expression is up-regulated in MCF-7 cells in response to E2, down-regulated by tamoxifen treatment and potentially suppressed following the acquisition of tamoxifen resistance. Microarray data also showed that for all 3 gene probes, RASAL-1 was most highly expressed in tamoxifen withdrawn TAM-R cells treated with 5-Aza and E2 compared to non-treated, 5-Aza or E2 treated cells. The addition of tamoxifen caused a reduction in RASAL-1 mRNA detected in cells co-treated with 5-Aza and E2; suggesting that long-term tamoxifen exposure may indeed cause epigenetic silencing of this gene, a mechanism by which cell growth could be facilitated. PCR verification of this observation was not achieved; however, the microarray gene probe profiles in question are so convincing, work has started on alternative RASAL-1 primer design due to the significant implications this observation would have on the understanding of the mechanisms that govern the growth of anti-hormone resistant breast cancer cells.

6.5 Potential induction of adverse genes by 5-Aza.

Although the cell growth analysis showed that the cumulative effect of 5-Aza and E2 challenge in TAM-Wd cells was reduced cell growth, it is noteworthy that due to the nature of 5-Aza as a non-targeted de-methylation agent, it is likely that not all induced genes affected will be associated with the reduction of cell growth observed. Indeed, EGFR, which was down-regulated following tamoxifen withdrawal from TAM-R cells, was greatly up-regulated by 5-Aza

and E2 co-treatment versus E2 alone. Importantly, this was observed for 2 of the 6 EGFR probes on the micro-array chip, and was complemented by increased expression of its ligands TGFα (2 of 3 probes) and epiregulin (1 of 1 probe). The data, subsequently confirmed by real-time PCR, contrasts with both the suppressive effects of E2 on EGFR levels in wt-MCF-7 and tamoxifen withdrawn TAM-R cells in the absence of 5-Aza, and the inability of E2 to induce EGFR in wt-MCF-7 cells in the presence of 5-Aza. Clearly the acquisition of anti-hormone resistance followed by tamoxifen withdrawal has facilitated a (5-Aza sensitive) reduction in the expression of the EGFR; presumably at a promoter site which oestrogens positively regulate the expression of this gene. In this context, it is noteworthy that early reports on the effects of oestrogens on EGFR expression suggested it was biphasic, with oestrogen first rapidly stimulating EGFR expression, and then inhibiting it [Yarden *et al,* 1996]. Whatever the mechanism, it is conceivable that the induction of EGFR (together with its ligands) in 5-Aza and E2 treated tamoxifen withdrawn cells may facilitate limited growth and survival signalling to counteract the induction of tumour suppressor/pro-apoptotic genes and that much greater inhibitory responses might be achieved by the concurrent targeting of the EGFR.

It is noteworthy that of the 24 genes selected from the first round of microarray analysis (i.e. up-regulated in MCF-7 cells in response to E2, down-regulated or suppressed in tamoxifen treated and tamoxifen resistant cells, no EGFR regulation), 6 adverse genes were potentially down-regulated (to the point of epigenetic silencing) by tamoxifen treatment, possibly as part of the

anti-hormone's protective effect. Genes included WISP-2 [Saxena *et al,* 2001; Banerjee *et al,* 2003; Davies *et al,* 2007], CXCL12 [Luker & Luker, 2006, Kang *et al,* 2005a; 2005b], ST6 [Schneider *et al,* 2001; Lloyd *et al,* 1996], DAGK [Filigheddu *et al,* 2007], LYPD3 [Fletcher *et al,* 2003; Paret *et al,* 2007; Hansen *et al,* 2008] and S100A14 [Yao *et al,*2007]. If these genes were indeed methylated in TAM-Wd cells, restoration of their expression could also limit the effectiveness of 5-Aza and E2 co-treatment. However, no evidence was produced to suggest that the expression any of the 3 genes selected for further analysis, namely CXCL-12, WISP-2 and ST-6, was significantly up-regulated by 5-Aza +E2 co-treatment (compared to E2 treated cells) in tamoxifen withdrawn TAM-R cells, suggesting they were free from epigenetic regulation. Interestingly however, CXCL-12 gene promoter was found to be significantly methylated in non-5-Aza compared to 5-Aza treated tamoxifen-withdrawn TAM-R cells as assessed by Methylight assay, despite the lack of any significant increase in mRNA expression in 5-Aza +E2 co-treated cells as assessed by microarray and PCR analysis. It may well be the case that 5-Aza has caused the demethylation of a promoter sequence that is free from oestrogenic regulation, and hence its reactivation in this instance is inconsequential.

In total, although the adverse gene candidates selected for PCR verification did not demonstrate sensitivity to 5-Aza + E2 co-treatment, it is likely that this event will occur (as is the case with EGFR). Interestingly, chromatin remodelling drugs (e.g. HDAC and methylation inhibitors) are of clinical interest in cancer although many researchers remain cautious regarding the

broader genomic impact of such agents [Miyamoto & Ushijima, 2005], and thus potential restoration of tumour suppressors on an individual basis may prove more desirable if this can ultimately be achieved through alternative strategies.

6.6 Oestrogen repression of tumour suppressors: a potential role in breast cancer and development and progression.

Classically, E2 acts to induce expression of genes whose target promoters bear oestrogen response elements (EREs). However, it is transcriptional repression of genes that has been reported to comprise the bulk (70%) of expression changes associated with E2 challenge of ER-positive breast cancer models. In a study conducted by Frasor *et al*, 2003, a significant proportion of the genes identified as being down-regulated by E2 in breast cancer cells were associated with anti-apoptotic/growth-regulatory function, and therefore are presumably down-regulated as part of the E2-mediated activation of MCF-7 cell growth. However, there is emerging literature to indicate that the gene-suppressive effects of E2 at tumour suppressors may, in some instances (e.g. RUNX3, Jiang *et al.* 2008), culminate in their epigenetic silencing and possibly advance breast cancer progression. Studies of several E2-repressed genes, including the tumour suppressor gene cyclin G1, indicate that alongside corepressors such as N-CoR, the E2/ER complex can recruit HDACs to gene promoters, creating a repressive chromatin conformation that inhibits gene expression [Stossi *et al,* 2006] and renders the promoter sequence vulnerable

to CpG-hyper-methylation. Therefore it is reasonable to assume that de-methylation agents such as 5-Aza could be used in these cells to provoke re-expression of E2-silenced tumour-suppressor genes, and consequentially induce growth inhibition. However, when we treated MCF-7 cells with 5-Aza at a concentration of 1μM, no growth inhibition was observed and in contrast to the tamoxifen-withdrawn TAM-R cells, E2 remained mitogenic despite the presence of the de-methylation agent. It could be suggested that the gene promoters that are aberrantly methylated by long-term E2 exposure in MCF-7 cells will again be suppressed by E2 despite their de-methylated status, rendering them unable to make an impact on the regulation of cell growth. In contrast, in the case of the tamoxifen-withdrawn TAM-R cells we have determined that tumour suppressor genes that were previously activated by E2 in MCF-7 cells can be epigenetically silenced by long-term tamoxifen exposure, and that following the de-methylation of gene promoters (using 5-Aza), gene expression can once again be up-regulated by E2 to provoke cell-growth inhibition. These data therefore indicate that if the genes that were de-methylated by 5-Aza in MCF-7 cells were activated (by an anti-hormone) rather than suppressed by E2, an inhibitory growth response may be achieved. However, evidence from studies of Slug, an E2-repressed gene with pro invasive ontology indicates that adverse signalling genes can equally be subject to E2-driven silencing [Ye *et al,* 2008] and interference with such events could prove undesirable in the context of cancer development and progression.

6.7 Might the development of tamoxifen resistance be prevented by 5-Aza and tamoxifen co-treatment?

In the present study, it has been shown that oestrogen-regulated tumour-suppressor genes have been epigenetically silenced in our MCF-7 breast cancer cells following long-term tamoxifen treatment; and that this event may contribute to anti-hormone resistant cell growth. It has previously been suggested that co-treatment of MCF-7 cells with 5-Aza + tamoxifen (as opposed to tamoxifen alone), may extend the time-taken to achieve anti-hormone resistant cell growth, as the presence of the de-methylation agent will prevent such genes from becoming silenced. However, consistently throughout the study, we have shown that 5-Aza alone has little effect on gene expression itself, and merely acts to provide access for transcriptional regulation of the de-methylated promoter. Thus, if 5-Aza was used in combination with tamoxifen to prevent the onset of resistance, the de-methylation agent would presumably prevent epigenetic silencing of genes down-regulated by tamoxifen. As we have shown for GDF-15 and RGC-32 however, in our 5-Aza +E2 treated tamoxifen withdrawn TAM-R cells, due to the affinity of the anti-hormone for ER compared to physiological concentrations of oestrogen, the presence of tamoxifen would still cause gene suppression. Therefore, the presence of 5-Aza would be inconsequential and have little effect on the time taken to develop anti-hormone resistance. Indeed, in a study conducted by Van Agthoven *et al* (1994), the application of 5-Aza was able to accelerate the emergence of an EGFR-positive tamoxifen resistant cell-phenotype in ZR75-1

breast cancer cells. Although data from the present suggests a possible explanation as to why 5-Aza did not prevent the emergence of an EGFR-positive tamoxifen resistant phenotype, it does not offer any explanation as to why 5-Aza accelerated this process. One possible explanation is that long-term 5-Aza exposure could ultimately induce hypo-methylation at promoters of genes associated with tumour development in anti-hormone sensitive cells, including potential growth inducing elements. Hypo-methylation has been closely associated with genomic instability and the development of a multitude of tumours [Lund & Van Lohuizen, 2004].

6.8 Conclusion

Alongside anti-hormone-induced compensatory signalling, the data presented in this thesis indicates that the silencing of various E2-regulated tumour suppressor genes could play a significant role in limiting the growth inhibitory effects achieved by long-term tamoxifen treatment, and promote resistance. It has been shown that the growth of tamoxifen withdrawn TAM-R cells is inhibited following co-treatment with 5-Aza and a physiological concentration of oestradiol, and that this occurs in parallel with the de-methylation and activation of p53-activated tumour-suppressor genes GDF-15 and RGC-32, as well as other genes such as RASAL-1, that could serve to suppress anti-hormone resistant cell growth and/or promote apoptosis. To date the concept that anti-hormones silence growth inhibitory genes has not been examined in

clinical breast cancer, although the reversal of epigenetic events is being examined [Miyamoto & Ushijima, 2005].

Importantly, however, the data presented in the thesis also shows that due to the un-targeted mechanism of action of this combination treatment, it may also serve to up-regulate genes which may limit the growth inhibition achieved, in particular EGFR. Therapeutically, a combination of 5-Aza + E2 and gefitinib might be used to overcome this problem, and could even improve the growth inhibition realised by 5-Aza + E2 in tamoxifen-resistant breast cancer cells. This however is only one example of an adverse gene that was re-activated by 5-Aza + E2 co-treatment and it is inevitable that there will be others. How this phenomenon might affect other aspects of the breast cancer cell phenotype long-term remains to be determined.

The data from this study add evidence to the theory that anti-hormones are not passive bystanders during the acquisition of resistance; they directly promote adverse compensatory mechanisms within tumour cells. This 'dark side of anti-hormonal action' has been recently reviewed by Gee *et al* (2008), and adds weight to the argument that perhaps after 30 years service, we need to take a new look at the way we use anti-hormonal drugs and how we treat anti-hormone resistance, possibly moving away from single agent therapy to intelligent combinations of drugs targeting various aspects of the complex anti-hormone-induced resistance mechanism.

6.9 Future Work

In addition to the PCR-verification of RASAL-1 as an oestrogen regulated gene potentially associated with 5-Aza induced inhibition of tamoxifen resistant cell growth, and the assessment of the effect that gefitinib has on 5-Aza + E2 mediated growth inhibition in these cells (which have already been discussed); the following lines of investigation will be pursued to further assess the significance of the novel observations identified in the present study:

Examination of expression and promoter methylation status of GDF-15, RGC-32 and RASAl-1 in further models of endocrine resistant and insensitive breast cancer.

The tissue culture unit of the Tenovus Centre routinely maintains multiple models of anti-hormone resistance/insensitivity, and it is our intention to screen those cell lines for the expression of the candidate genes. Where the genes are suppressed, we will once again assess the methylation status of their promoters in the presence and absence of 5-Aza, and determine the effects of 5-Aza and oestradiol on cell growth.

Examination of the functional significance of the candidate genes in anti-hormone resistant/insensitive cells.

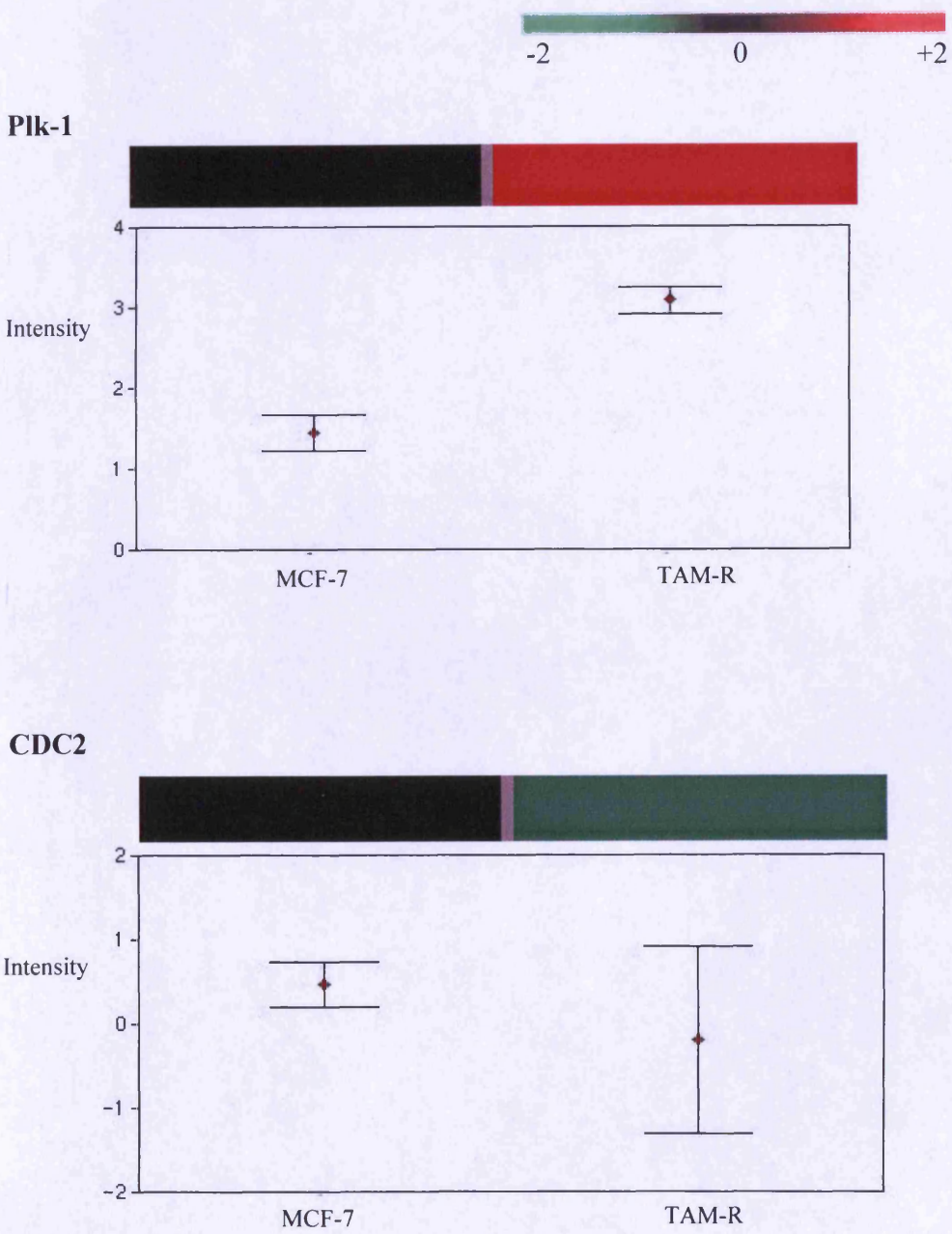
It is our intention to reduce cellular levels of candidate gene mRNA using SiRNA knockdown. This will be primarily undertaken in our tamoxifen-withdrawn TAM-R cells treated with 5-Aza and oestradiol, using reversal of the growth inhibitory response associated with this co-treatment as the initial marker of biological reference of this gene. More detailed studies on the mechanism of reversal will subsequently be undertaken using FACS analysis, to determine the proliferative and apoptotic events associated with the use of the siRNAs, and by signalling studies, which will be driven by what is currently known about the genes and the availability of reagents.

Assessment of the importance of the candidate suppressor genes in clinical breast cancer.

The Tenovus Centre for Cancer Research and the University of Nottingham have enjoyed a longstanding collaboration associated with the translation of experimental observations into clinical studies and trials. Significantly this joint interest has mostly focused around the use of various endocrine therapies and as such, multiple breast cancer specimens relevant to our current work have been accumulated. These include frozen and paraffin embedded samples from anti-hormone treated patients, removed before, during and at relapse. Such material will be examined for the expression of the candidate genes

using quantitative PCR and immunohistochemical procedures to determine clinical relevance.

Fig 6.1 Polo-like kinase (Plk-1) and cell division cycle 2 protein (CDC2) Affymetrix gene probe expression profiles (boxplots and heat-maps shown) in MCF-7 and TAM-R cells.



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