Characterisation of Mammalian Cell Lines for use in Proliferative and Estrogenic Response Assays

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by D. J. Inglis

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Department of Medical Biotechnology

Faculty of Health Sciences

Flinders University

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Summary

Characterisation of Mammalian Cell Lines for use in Proliferative and Estrogenic Response Assays

A significant number of endocrine disrupting chemicals (EDCs), which have the ability to act as estrogens or disrupt the estrogen signalling pathway in humans and other species, have been reported in environmental waters,. Bioassays using human cell lines, which contain endogenous or inserted estrogen receptors, are useful to rapidly assess this (xeno)estrogen content of water.

This thesis presents a comparative assessment of the suitability of MCF7, ZR-75-1, H23, RL95-2, Ovcar and T-47D cell lines for (xeno)estrogen testing in a 96 well plate format. Cell lines were assessed for attachment time, proliferation rate, optimal time for producing a standard curve relating absorbance to cell number and the expression of ERα and ERβ mRNA and protein. Six hours was the optimum time for attachment and gave the smallest 95% confidence interval (CI) for a standard curve for MCF7, Ovcar, RL95-2 and T-47D cells. ZR-75-1 and H23 cells were best after 4 hours of attachment. Incubation for 24 hours gave the highest 95% CI and produced the least accurate standard curve. The reporting of a single doubling time for each cell line may not be accurate as cell lines had a doubling time that was density related. ERα expression was greatest in MCF7, followed by T-47D and ZR-75-1. ERβ was not detected in any cell line.

MCF7 cells are characterised as proliferating in response to (xeno)estrogens primarily via the ERα. The E-screen was originally developed using MCF7 cells, however, many modifications have lead to only semi-standardisation of the methodology. T-47D and ZR-75-1 cells have also been shown to proliferate in response to estrogen and were compared to MCF7 cells in an E-screen assay. The responses of cells using a 4hr or 6hr or an overnight attachment period in estrogen-free pre-treatment before exposure to estrogen for 3, 5 or 7 days were compared. The pre-treatment/attachment period did not affect EC_{50} values, however the proliferation of MCF7 cells almost doubled and the 95% CI was greatly reduced by reducing the attachment/ pre-treatment time to 6 hours. MCF7 cells are used in the E-screen

within a given passage range. The changes in sensitivity and response to estrogen of the three cell lines after extended time in culture (>18 passages) were also assessed. Although each cell line maintained similar EC_{50} values, the proliferative response decreased and variability increased.

The regulation of individual genes by estrogen has been widely studied. VEGF, IGFBP-4, pS2, MaoA and EGR1 were selected from microarray and *in vivo* data based on the magnitude and speed of response to estrogen exposure. The promoter regions, inclusive of their estrogen responsive elements, were identified. pS2 and MaoA promoter regions were successfully amplified and inserted into the pGL4.21(luc2P/Puro) reporter vector. Stable integration into T-47D cells resulted in non-estrogen inducible expression of the luciferase gene. Transient transfection of the vector into MCF7, ZR-75-1 and T-47D cells also resulted in expression of luciferase that could not be induced by estrogen.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in and university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed

Daniel Inglis

Acknowledgements

List of Abbreviations

Amp	Ampicillin
ANOVA	Analysis of variance
AR	Androgen receptor
ATCC	American type culture collection
AU	Absorbance unit
bp	Base pairs
BPA	Bisphenol A
CHX	Cycloheximide
CI	Confidence interval
CPS	Counts per second
Cq	Quantification cycle
DBD	Deoxyribonucleic acid binding domain
DC-FCS	Dextran-charcoal treated fetal calf serum
DMEM	Dulbecco's modified Earles' medium
DNA	Deoxyribonucleic acid
E_2	17β-estradiol
EC_{50}	Effective concentration for half-maximal effect
EDC	Endocrine disrupting chemical
eGFP	Enhanced green fluorescent protein
ER	Estrogen receptor
ERE	Estrogen response element
EtBr	Ethidium bromide
EtOH	Ethanol
FCS	Fetal calf serum
GCMS	Gas chromatography / mass spectrometry
GWRC	Global water research coalition
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulphonic acid
Hsp	Heat shock protein
kDa	kilodalton
LB	Luria broth
LBD	Ligand binding domain
LOD	Limit of detection

Luc	Luciferase
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OD	Optical density
PCR	Polymerase chain reaction
PE	Proliferative effect
PR	Phenol red
RIA	Radioimmunoassay
RPE	Relative proliferative effect
RPP	Relative proliferative potency
RT	Reverse transcription
RT-PCR	Reverse transcription real-time polymerase chain reaction
SD	Standard deviation
SEM	Standard error of mean
SOC	Super optimal broth with catabolite repression
SPE	Solid phase extraction
SRB	Sulforhodamine B
STP	Sewage treatment plant
TSS	Transcription start site
UTR	Untranslated region
UV	Ultraviolet
YES	Yeast estrogen screen

Publications and Conference Proceedings

Publications

- Characterisation of Mammalian Cell Lines for use in Proliferative and Estrogenic Response assays. Daniel J Inglis, Andrew R Humpage, Suzanne M Froscio, George C Mayne, Fiona M Young. (*Under Review*)

Conference Proceedings

- Flinders Research Centre for Coastal and Catchment Environments and Flinders University Bioknowledge Research Group Postgraduate Research Conference.. 18th-19th July 2006. Adelaide, Australia. *Oral Presentation "Development of a bioassay for measuring xenoestrogens in recycled water"*

- Society For Reproductive Biology 38th Annual Conference. 2nd-5th September 2007. Christchurch, New Zealand. *Oral Presentation "Development of an improved E-screen"*

- The Endocrine Society of Australia 51st Annual Scientific Meeting.. 25th – 28th August 2008. Melbourne, Australia. *Poster Presentation " A comparative Assessment of three cell lines for use in the E-screen"*

- Society of Environmental Toxicology and Chemistry World Congress. 3rd-7th August 2008. Sydney, Australia. *Oral Presentation "Improving the E-Screen: Effect of FCS Concentration on ZR75-1 Cell Proliferation and Estrogen Receptor Expression"*

- 2nd Australian Symposium on Ecological Risk Assessment and Management of Endocrine Disrupting Chemicals (EDCs), Pharmaceuticals and Personal Care Products (PPCPs) in the Australasian Environment. November 21st-22nd 2007. Canberra, Australia. *Poster Presentation " Development of an Improved E-Screen"*

- 13th Australasian Society for Ecotoxicology Conference: Toxicants in a changing environment. 20-23 September 2009. Adelaide, Australia. *Oral Presentation "Passage effect on MCF-7 and T-47D cells for use in the E-screen"*

CHAPTER 1

Introduction and Literature Review

1 Introduction

1.1 Endocrine disrupting chemicals (EDCs)

Rachel Carson's book of 1962, Silent Spring, warned that certain synthetic chemicals can be detected in the environment (Carson, 1962). Widespread use of synthetic chemicals for domestic, industrial, agricultural and medical use has resulted in these chemicals, and their degradation products, being a part of our environment (Sumpter and Jobling, 1995).

Endocrine disruptors are chemicals that can alter the normal function of the endocrine system and act by mimicking or blocking endogenous hormones at a receptor or non-receptor level (Manning, 2005). The existence of these endocrine disrupting chemicals in the environment, surface water, recycled water and food are of concern(Falconer, 2006). Effects by exogenous chemicals mainly occur in the androgen, thyroid and estrogen hormone systems (Mueller, 2004).

Xenoestrogens are a class of endocrine disruptors that are natural or synthetic compounds that can mimic estrogen or interfere with the estrogen receptor and signalling pathway *in vivo* and come from a large variety of sources such as pharmaceuticals, plastics and detergents (Routledge and Sumpter, 1996; Moggs, 2005). Endocrine disruption caused by exposure to these anthropogenic chemicals occurring in our environment is of concern. Multiple xenoestrogens, combined at below individual no-observed-effect concentrations, have also been shown to have biological effect (Rajapakse et al., 2002).

Surface water primarily receives the bulk of these chemicals from treated and untreated wastewater. Reports of feminisation and the occurrence of intersex amongst aquatic fauna caused by endocrine disrupting chemicals and xenoestrogens (Guilette et al., 1994; Sumpter, 1995; Jobling et al., 1998) has lead to a large international assessment of the character and relevance of endocrine disrupting chemicals in the environment to humans and wildlife (Falconer, 2006).

The development of technologies that are able to assess low concentrations of xenoestrogens has also driven interest in the topic. Current chemical approaches are

able to give information about specific contaminants, and bioassays can measure activation of the estrogen receptor.

1.2 Objectives

This project aims to develop a bioassay using human cell lines that can give physiologically relevant data about xenoestrogens present in water samples. This will be done by characterising a set of selected cell lines, refining and developing the existing "E-screen" proliferation assay, and developing a novel high throughput assay using estrogen responsive genes.

- **To evaluate cell lines for use in estrogenic response assays**

  Five cell lines will be assessed for their expression of estrogen receptors alpha and beta at the mRNA and protein level. Cells will also be assessed for attachment time, proliferation, and the reproducibility and the accuracy of these measures.

- **To evaluate and develop the E-screen assay**

  Multiple cell lines will be assessed in order to develop the E-screen. Parameters that will be optimised will include the time taken to complete the assay, the effect of a reduced attachment / pre-treatment period and media conditions.

- **To develop a bioassay to detect estrogenic exposure**

  Genes that are estrogen-regulated within a short time frame will be identified using existing microarray data. The promoter and regulatory regions of the genes will be used to drive the expression of a reporter gene in response to estrogenic stimuli.

1.3 Hypotheses

- **Cell lines express the estrogen receptors alpha and beta to different levels.** Certain cell lines will express one or both of the receptors to greater levels than other cell lines. Identification of a cell line(s) with characteristics facilitating estrogen receptor activation and use in a 96-well plate format will lead to the development and optimisation of new and existing xenoestrogen bioassays.

- **Cell lines have different growth characteristics such as attachment time, and doubling time, and biological variability derives from both of these.** We can assess cell lines and select those that generate least variability in *in vitro* assays and compare growth characteristics, then use this knowledge to inform assay development.

- **The E-screen assay is currently performed with MCF7 and T-47D cells, one of which (or another cell line), may be better suited to the assay.** There may be a cell line, currently used or not, that performs best in the E-screen. The time taken to perform the E-screen, through some modifications, can also be reduced.

- **Exposure to Estrogen in mammalian cell culture rapidly induces endogenous genes.** The rapid induction of endogenous genes, identified from microarray data, can be used to develop a novel bioassay in which the promoter and regulatory region of an identified gene is coupled to a reporter gene.

1.4 Steroid receptors of the endocrine system

The steroid receptor super-family is the largest known group of transcription factors in eukaryotes and are traditionally characterised as being nuclear, ligand-binding transcription factors regulating the expression of responsive genes (Tsai and O'Malley, 1994). When these receptors are bound by the corresponding ligand, the receptor-ligand complex binds to another receptor-ligand complex forming a dimer. The dimer can then bind to the specific steroid response element on DNA, recruit cofactors and begin transcription (Willemsen et al., 2004). Alternative mechanisms of action are also

possible where the receptor dimer will interact with another signalling pathway giving rise to a rapid response without binding to its own DNA response elements (Reid et al., 2002).

1.4.1 Estrogen Receptor alpha and beta

The estrogen receptor is part of the steroid receptor super-family and is highly conserved in many species from chordates to mammals. The natural ligand for the receptor is 17β- estradiol (E$_2$). The estrogen receptor was discovered by Elwood Jenson in 1958 and cloned from a uterus cDNA library in 1986. Initially only one estrogen receptor was thought to exist until Kuiper et al. (1996) identified another estrogen receptor in a rat prostate cDNA library which was termed ERβ. The original receptor therefore became known as ERα. The human homolog of ERβ was cloned later the same year by Mosselman (1996).

The two receptors are highly separated on the chromosomes; ERα is encoded by chromosome 6 at 6q25.1 and ERβ encoded by chromosome 14 between 14q11.1 and 14q11.2 (Reid et al., 2002). Tissue distribution of ERα and ERβ is quite different and indicative of their different roles. The kidney, heart, pituitary, vagina and uterus have the most abundant amount of ERα whereas ovary, lungs, prostate, brain and central nervous system have the most ERβ (Mosselman et al., 1996; Enmark and Gustafsson, 1999; Dechering et al., 2000; Foltin et al., 2005). Xenoestrogens with different binding affinities for ERα and ERβ can therefore be expected to have variable effects across tissue types, further mediated by differences in uptake, absorbance and access to different body compartment and tissue types.

The estrogen receptor resides both in the nucleus and the cytoplasm and has been reported also to be bound to the membrane in cholesterol rich pockets (Zivadinovic and Watson, 2005). Transient transfection of ER into Chinese hamster ovary cells show that the same ER gene product localises to both the nucleus and to the membrane (Razandi et al., 1999). The full length ERα is 66 whereas the ERβ is slightly smaller at 61 kDa (Dechering et al., 2000).

1.4.2 Genomic organisation of ER

1.4.2.1 ER α

The transcript for ERα itself contains seven identified promoters and eight exons (Pinzone et al., 2004) . This leads to variation in the 5'UTR only as there is a common splice site directly upstream of the initiation codon of the ERα itself. The promoters either have no or low consensus TATA, CCAAT or GC box sequences making them relatively weak promoters (Kos et al., 2001). There are three identified variant forms of ERα with molecular weights of 66, 46 and 39 kDa. All three have been identified in osteoblast cells and two (66 and 46 kDa) identified in MCF7 cells (Flouriot et al., 2000). The promoters, labelled A-F, can be tissue specific. The liver expresses almost solely promoter E which is not expressed in other tissue (Reid et al., 2002). In terms of development and differentiation of cells, it is possible that the large separation between promoters is required for definitive control of activation and suppression of different forms of the receptor (Reid et al., 2002). It has also been shown that the presence of estradiol will either up-regulate (promoters C and F) or down-regulate estrogen receptor expression (Chicken promoter A) (Reid et al., 2002). The implications of multiple promoter control is that tissue specific regulation of the receptor is possible (Kos et al., 2001).

1.4.2.2 ER β

ERβ is reported to have multiple isoforms caused by alternative splicing of codon 8 resulting in proteins of 59, 56, 54 and 53kDa in size (Leung et al., 2006). This also is thought to enable tissue specific regulation of the receptor (Pettersson and Gustafsson, 2001).

1.4.2.3 Promoter methylation

Methylation of the estrogen receptor promoter is also fundamental in the transcriptional regulation of the receptor. Extensive methylation of CpG islands in the promoter of a gene can prevent transcription and expression of the gene product. This is seen to occur to ER in some tumour and breast cancer cell lines (Pinzone et al., 2004).

1.4.2.4 ER stability

The 3' UTR of ERα is twice as long as the coding region and has a potential role in the post transcriptional control of the receptor. High amounts of AU sequences exist in the 3'UTR which suggest a definitive role in mRNA destabilisation. A transcript prone to

destabilisation facilitates a fast turn-over rate and rapid response. The half life of reporter mRNA was only 3 hours when the ERα 3'UTR was present compared to over 24 hours without it (Kenealy et al., 2000). The ubiquitin-proteosome pathway is used in the removal of the estrogen receptor and is activated when a receptor is bound by ligand and has a half life of 3hr compared to an unbound receptor which has a half life of 5 days (Nirmala and Thampan, 1995).

1.4.3 Estrogen receptor structure

ERα and ERβ have partial homology and bind to identical or very similar ERE (Pettersson et al., 1997). This is due to 96% homology between the DNA binding domains (Dechering et al., 2000). The ligand binding domain however has only a 60% homology. The similarities should not be overstated though as they not more similar than some receptor pairs with different ligands (Enmark and Gustafsson, 1999).

The estrogen receptor contains the following major features as shown in Figure 3:

- N terminal domain containing ligand-independent activating function 1 (AF1)
- Zinc finger DNA-binding domain
- Hinge region
- C-terminal ligand-binding domain (LBD) containing ligand-dependent activating function 2 (AF2)
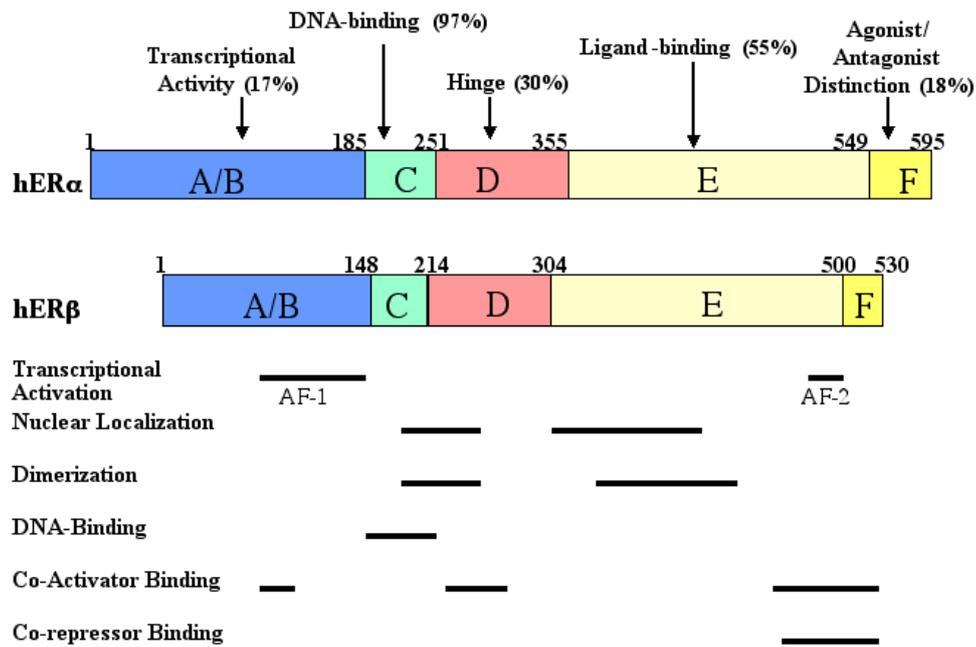
Figure 1.1: The human ERα and ERβ coding regions and a comparison of their homology (%). The lines indicate the position of the coding region responsible for the described characteristics. Taken from Klinge (2000).

ERα and ERβ, in their inactive form, are bound to either heat shock protein 90 or 70 (hsp90 or hsp70). When a ligand, such as estradiol, binds to the receptor, hsp90 or hsp70 is released and the receptor-ligand complex dimerises with other estrogen receptor-ligand complexes. The ER will dimerise with the same receptor type although it is also possible that ERα can dimerise with ERβ. Upon binding a ligand, the receptor-ligand protein folds into a final configuration which differs according to the bound ligand. If the bound ligand, such as an antagonist, does not fold the receptor correctly, cofactors such as AF2 cannot be activated and transcription will not proceed.

The estrogen receptor dimer binds to conserved estrogen response elements (ERE) or to non classical transcription elements such as Activator Protein 1 (AP1) or SP1 (Lin et al., 2004).
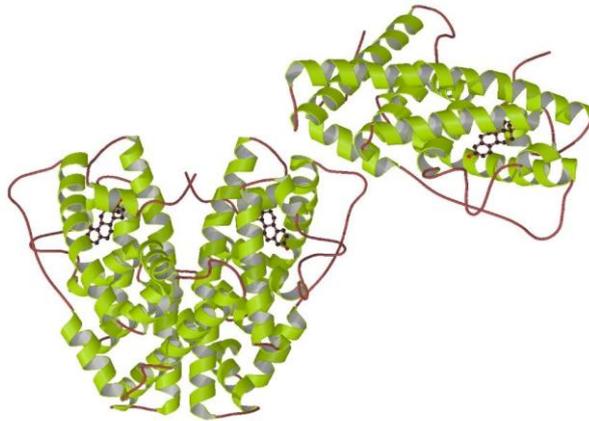
Figure 1.2: Protein Structure of the Estrogen Receptor

A dimerised and a single estrogen receptor (grey) with bound estradiol (black)

Taken from (Institute) http://www.ebi.ac.uk/msd-srv/msdtarget/gallery.html

1.4.4 Receptor elements

1.4.4.1 AF1 and AF2

AF1 and AF2 are important cofactors on the estrogen receptor and can modulate the binding and transcription initiating ability of the receptor (Zacharewski, 1997). These two domains function independently but in some cases are both required for full activation of transcription (Cavailles et al., 1995).

AF1 acts as a ligand-independent activator of the receptor. AF-1 activity in ERβ is minimal when compared to ERα (Barkhem et al., 1998). AF2 is ligand dependent and can modulate transcription by binding cofactors that influence transcription (Reid et al., 2002). AF2 is not active in the presence of an antagonist as the receptor does not fold into suitable conformation (Dechering et al., 2000).

1.4.4.2 Ligand binding domain of the ER

The ER has a ligand binding domain (LBD) that renders it a promiscuous receptor (Brzozowski et al., 1997). The actual parts of the ERα LBD that are responsible for binding are very similar to ERβ. There are, however, small differences in the affinities for various compounds (Pettersson and Gustafsson, 2001). The ligand binding domain of the ER has been studied using x-ray crystallography and it was determined that the LBD comprises of a three layered cluster of α-helices. An outer layer of two helices

sandwiches a central layer of 3 helices forming a wedge shape with a sizeable cavity for the binding ligand (Brzozowski et al., 1997). The LBD is almost twice the volume of the estradiol molecule and with the essential requirement of an effective ligand to contain one aromatic ring, the mostly hydrophobic remaining regions can accept a large number of different molecules containing hydrophobic groups (Brzozowski et al., 1997).

1.4.5 Estrogen receptor action

The estrogen receptor has some important features that regulate its action (Dechering et al., 2000):

- The same ligand can exert agonist or antagonist activity dependent on tissue type
- The ligand-receptor complex recruits a number of co-activators which can modulate the response
- Estrogen receptors can be activated through ligand-independent mechanisms
- The estrogen receptors can function independently from their ERE through cross-signalling pathways.

1.4.6 Estrogen response elements

The variation between the two receptors does not extend to the DNA binding domain (DBD). The P box motif which is critical for receptor-DNA recognition is identical between the receptors. This allows the receptors to bind the EREs with similar specificity and affinity (Matthews and Gustafsson, 2003).

A palindromic ERE consensus sequence in the promoter of estrogen responsive genes has been found in *Xenopus laevis* (5' CAGGTCAnnnTGACCTG 3'), in humans, however, most EREs are non palindromic (Anolik et al., 1995). The flanking nucleotide at position -7 in the 5' region allows the DBD to compensate for a one nucleotide mutation in the consensus sequence and also increases the rate of transcription (Gruber et al., 2004).

It has been shown that a 46 kDa ERα dimer shows a higher preferential binding ability to the ERE than a 66 kDa ERα dimer. Interestingly, the two isoforms can form dimers together which show a medium affinity for the ERE. In an environment where AF1 activation and the 66 kDa ERα is required for transcription, the 46 kDa ERα can

actually competitively inhibit activation of the complex. Physiologically this translates to the estrogen receptor being used for activation or repression under the control of estradiol in a site specific manner dependant on which form of the ER is being produced.

1.4.7 Non-ERE transcriptional pathways

Non-ERE activation pathways are possible whereby the ER binds to other DNA-bound transcription factors such as activating protein 1 (AP1) and specificity protein 1 (Sp-1). AP1 transcriptional ability is enhanced by binding the estrogen receptor, glucocorticoid receptor or CREB- binding protein. Again, either an agonist or antagonist effect on the AP-1 element is possible dependent on which ligand is bound to the estrogen receptor. AP1 activates transcription at a transcription site that is similar to an ERE which requires the presence of the transcription factors Fos and Jun (Paech et al., 1997). Similar to an ERE, ERα and ERβ activate transcription at the AP1 site differently depending on the bound ligand. AP-1 can act in a very different manner to an ERE with transcriptional assays showing that the classic estrogenic antagonist, tamoxifen, can actually act as an agonist at AP-1 sites (Webb et al., 1995).

1.4.8 Non transcriptional pathways

The phosphorylation of the receptor causes the receptor to integrate with other signalling pathways and can also modulate the activity of AF1 and AF2. Phosphorylation at multiple sites is usually enhanced by the presence of growth factors and cytokine signalling pathways. EGF is an example where cross signalling between steroid and growth factor pathways occur (Reid et al., 2002).

1.5 Endocrinology of estrogen

1.5.1 The endocrine system

Human physiological processes are regulated primarily by the endocrine and nervous systems. The nervous system acts by point to point electrical communication whereas the endocrine system acts through the release of hormones into the blood stream which are then received by specific cell and tissue types (Foltin et al., 2005). Although the two systems are responsible for a different network of actions and responses, they function in a complimentary manner. The endocrine system specifically controls the internal secretion of hormones, for communication and effect throughout the body. The

endocrine system is relevant to control of metabolism, sexual function, reproduction, growth, development, tissue function and behaviour (Greenspan and Forsham, 1997).

The endocrine system is based on the synthesis of hormones and secretion of hormones into the blood stream. The hormones are transported with the assistance of binding proteins which not only act as carriers but also regulate the amount of unbound hormone available for delivery to target cells. In the case of the estradiol circulating in the blood, albumin binds 60% of the hormone and gonadal steroid-binding globulin binds 38% of the hormone allowing 2% to be available for binding to receptors (Foltin et al., 2005). All cell and tissues types are exposed to the numerous hormones in the blood stream meaning that specific receptors are required for action on the target cells. The specific receptors are either membrane bound or nuclear (Razandi et al., 1999). Nuclear receptors sometimes are located in the cytosol and traffic to the nucleus when they are bound to their ligand. Traditionally, endocrine hormones are actioned at distant sites to the secreting gland but there are also two other types of hormones. Paracrine hormones act locally within a tissue or organ. Autocrine hormones are ones that act on the same cell that secreted them.

More than one specific hormone type can bind to receptors and cause a physiological outcome. This allosteric nature of receptors is an important yet vulnerable feature of receptors as it allows mimics of natural hormones to bind to receptors and cause an array of outcomes. An agonist is a molecule that binds the receptor and causes a biological effect, and an antagonist is a molecule that binds the receptor blocking it from receiving any further signals.

1.5.2 Production of estrogen

Estrogen is commonly known as the primary female sex hormone and is an integral part of the endocrine system. Estrogens, and the male equivalent androgen, are classified as steroids. There are more than 25 types of estrogen identified in the human body most of which are synthesised via enzymatic modifications of cholesterol (Foltin et al., 2005). Three forms of estrogen; 17β-estradiol (E_2), estrone and estriol, are the most common types found in the human body. The enzymatic action of aromatase (CYP19) converts testosterone to estradiol and androstenedione to estrone (Foltin et al., 2005) (Figure 2). E_2 is the most physiologically potent of the estrogens. Estrone has a lower relative

potency and estriol is predominantly an excretion product with very little estrogenic ability (Bidlingmaier and Knorr, 1978).

1.5.3 Estrogen and pre-puberty

Sex steroids during pre-puberty are at a low concentrations and are not active in general pre-pubertal growth (Greenspan and Forsham, 1997). It has, however, been shown that the pre-pubertal gonads in males are able to respond to stimulants such as human chorionic gonadotropin (hCG) and produce testosterone. Similarly in females, stimulation of ovaries with LH and FSH produces estrogen (Bidlingmaier and Knorr, 1978).

E_2 levels in females less than 8 years old are below 7pg/mL and in males less than 10pg/mL (Greenspan and Forsham, 1997). The developmental requirements for the steroids estrogen and androgen are not significant until the onset of puberty.

1.5.4 Estrogen at puberty

Estrogen has major effects on females at puberty where it essentially prepares the female body for reproduction. From the ages of 8 to 16 the levels of estradiol range from 8 – 68pg/ml (Greenspan and Forsham, 1997).

Figure 1.3: Synthesis of Estrogens in the human body.

Adapted from Foltin et al. (2005)

Cholestrol → Pregnenolone → 17^α-Hydroxypregnenolone

Progesterone → 17^α-Hydroxyprogesterone

Dehydroepiandrosterone

Androstenedione

CYP19

Estrone

Testosterone

16-

CYP19

16α-Hydroxyestrone

Estriol

17β -Estradiol
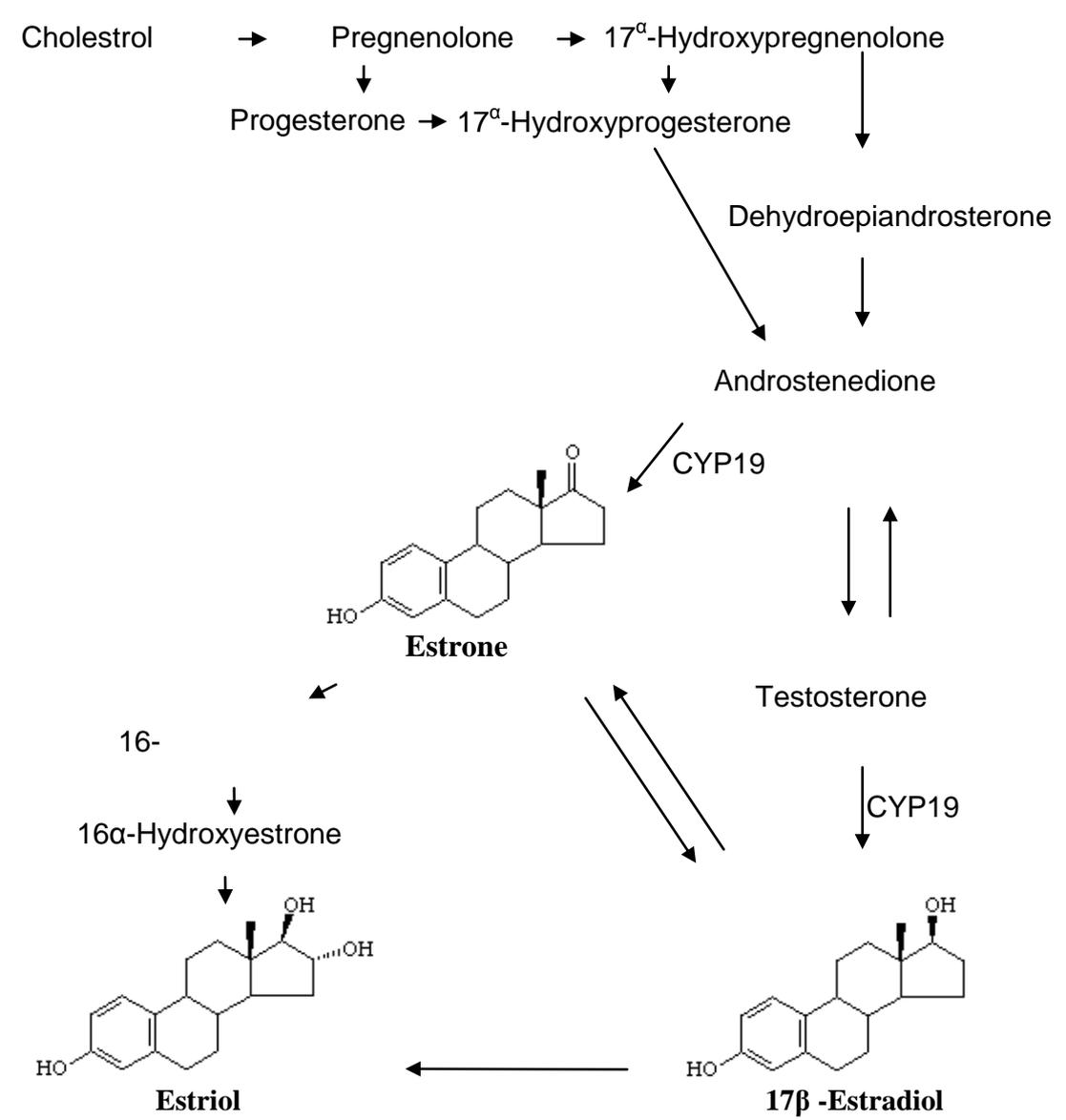
1.5.5 Estrogen in males and spermatogenesis

In the male, estrogen is formed in relatively low amounts. Estrogen production in the testes accounts for 10-25% of estrogen in circulation with the remainder being produced by adipose cells (Akingbemi, 2005). The testes primarily produce the hormones testosterone and inhibin. Testosterone is produced by enzymatic conversion of androstenedione to testosterone. The production of estrogen in men is limited to the testes with most estradiol production occurring by aromatisation of the testosterone and androstenedione synthesised by Leydig cells (Carreau et al., 2003). The actual concentration of estrogen, however, is much higher in the reproductive tract than in plasma suggesting a definitive action (Carreau et al., 2003). There are large amounts of ERα and ERβ found in the region of the rete testis where spermatozoa are concentrated and at early stages of development throughout the testes indicating a distinct but yet not yet fully understood function (Carreau et al., 2003; Akingbemi, 2005; Foltin et al., 2005). Carrueau et al. (2006) showed that sperm ejaculate has ERα, ERβ and a membrane bound truncated form of ERα suggesting that estrogen plays a definitive role in gamete maturation. In case studies it has been shown that an aromatase deficiency caused by a mutation in the CYP19 gene leads to sterility as does a mutation in the ERα (Carreau et al., 2003). Despite a relatively low circulating estrogen level in adult males (20-50pg/ml) (Greenspan and Forsham, 1997) local bone production of aromatase is important to the normal bone formation and resorption (Meier et al., 2005).

An over-production or administration of estrogen in males will lead to the onset of female characteristics (Levy et al., 2003). A typical dosage to effect these changes is 50ug E_2 per day. An increase in breast size will occur within 2-3 months, fertility will decrease, a reduction in libido is experienced and erectile function is disrupted. Adipose tissue will accumulate around the thighs and muscle mass will decrease (Levy et al., 2003).

1.6 Xenoestrogens and endocrine disruption

1.6.1 Endocrine disruption

The European Union commission defines an endocrine disruptor as "an exogenous substance or mixture that alters the function(s) of the endocrine system and

consequently causes adverse health effects in an intact organism or its progeny or (sub) populations" (http://ec.europa.eu/research/endocrine/background_disruption_en.html)

Xenoestrogens are a class of endocrine disruptors that are natural or synthetic chemicals that mimic or disrupt the effects of estrogen in mammals by interactions with (but not limited to) the estrogen receptor (Moggs, 2005).

1.6.2 Receptor based disruption

Studies of the 3D chemical structure of the estrogen receptor has shown it to be unique because it can accept a wide variety of non-steroidal compounds (Brzozowski et al., 1997). The ligand binding domain is relatively large compared to the native ligand E_2. It is able to accept a potential ligand providing it contains an aromatic ring, and the remainder of the receptor domain can accept many different hydrophobic groups (Katzenellenbogen et al., 1995) . Therefore, many chemicals that have similar shape or structure to E_2 are able to bind to the ER (Brzozowski et al., 1997).

In cellular systems it has been widely documented that synthetic chemicals will interact directly with ER and alter the normal function of the cells by modulating activities (Nikov et al., 2000; Mueller, 2004) and also cause subtle effects through incorrect intra-cellular communication (Manning, 2005). The folding of the receptor, interaction with AF-1 and AF-2 and recruitment of co-activators and transcriptional elements means that the receptor will act very differently depending on the bound ligand. Ligands that bind poorly may still activate the receptor (Manning, 2005).

Receptor based disruption also occurs when a ligand is bound to the ER with no activation, preventing endogenous hormones from binding to the receptor, effectively stopping estrogen signalling within the cell (Manning, 2005).

It is possible that ERα and ERβ can respond differently with the same ligand, and this is commonly taken advantage of with the therapeutic drug tamoxifen (4-hydroxytamoxifen). In breast tissue, tamoxifen blocks the ERβ from activation acting as an antagonist, in uterus cells however the tamoxifen acts as an agonist on the ERα receptor (Dechering et al., 2000). These agents are called selective estrogen receptor modulators (SERMs) (Glidewell-Kenney et al., 2005).

A group of receptors called orphan receptors also exist, which when activated can cause ER based disruption. The aromatic hydrocarbon receptor can stop the function of the ER by binding DNA, changing its shape, and preventing an ER complex from attaching to the ERE (Manning, 2005) or by interacting with the ER directly (Ohtake et al., 2003).

1.6.3 Non-receptor binding based disruption
There are many more ways through which non-receptor based disruption can occur. These processes disrupt the cellular communication before ER activation occurs.

1.6.3.1 Receptor numbers
The number of receptors present will determine the response to an agonist. It is possible that some chemicals may prevent the production of receptors, or interrupt cell surface receptors and thereby limit cellular response to estrogen (Hollande et al., 1998).

1.6.3.2 Limiting enzymes for cholesterol – estrogen production
Production of natural estrogen is a complex pathway beginning with the absorption of cholesterol from the digestive tract and requiring many enzymes including aromatase. The process can easily be disrupted when high levels of enzymes are recruited or produced for removing unwanted chemicals from the body. This limits the availability of enzymes for normal hormone production (Figure 1.3) (Danzo, 1997; Crisp et al., 1998)

1.6.3.3 Transport proteins
Transport proteins and sex hormone binding globulin (SHBG) are required to move estrogens around the body to target tissues. If these transport proteins are disrupted or another chemical is present in the blood stream that can bind SHBG or displace the ligand then delivery to the cell is disrupted (Quigley et al., 1995).

1.7 Xenoestrogens
Some major sources and types of xenoestrogens include;

1.7.1 17β-estradiol and estrone
Occurring in both male and female, these hormones are constantly excreted in urinary and faecal waste and constitute the majority of EDCs from humans and animals. Estrogens are removed from the body in a conjugated highly soluble form, usually as estrone. The oxidation of estrogens with glucuronides and sulphates makes them not only soluble but also largely inactive. It has been found that the water treatment process

can reverse the conjugation and "reactivate" the estrogens (Shang et al., 1999). This means that "active" estrogen is constantly released in effluent from sewage treatment plants.

1.7.2 Pharmaceuticals

Pharmaceuticals are designed to be lipophilic with the ability to cross cell membranes and have a targeted biological effect. Pharmaceuticals are often designed for oral administration which makes them extremely persistent in harsh or degrading environments. After ingestion the substance will be excreted through urine or faeces in a mixture of unchanged, metabolised or conjugated compounds. The metabolites are often more hydrophobic than the parent compounds, allowing them to bio-accumulate.

The contraceptive pill is a highly prescribed pharmaceutical in most developed nations. In 1997 over 3.9 million prescriptions for the contraceptive pill were written in Australia (Wilks, 1997). The pill is a dosage of 17α-ethinylestradiol, a synthetic estrogen, delivered to the body to modulate the reproductive cycle. This form of estrogen is also used for hormone replacement therapy. Designed to be taken orally a far more stable form than 17β-estradiol was developed to withstand the human digestive system and still be delivered to the body in an active form. This persistence means that 17α- ethinylestradiol is not only far more stable in the gastrointestinal tract, it is also far more stable in the environment and resilient to degradation in waterways and water treatment plants. Given the highly specific nature of 17α-ethinylestradiol only very low concentrations are required to elicit an effect. For example, in 1995 the population of Denmark (5.2 million) used in total 49 kilograms of pure 17α-ethinylestradiol. Given the extremely low concentration required for a physiological effect this represents an enormous load over a relatively small area (Halling-Sorenson et al., 1998).

1.7.3 Pesticides

DDT and other organochlorine pesticides have been used since the 1940s extensively to control a variety of pests, most commonly mosquitoes. Although DDT has been banned in most developed countries it has a high persistence level. These chemicals and their metabolites/degradation products (such as the degradation product of DDT, DDE) have been shown to interact with the estrogen receptor. DDT most commonly interacts with the androgen receptor as an antagonist.

Herbicides such as atrazine are used extensively to control weeds in crops. These chemicals are used in very large quantities and are currently under investigation for their endocrine disrupting ability.

1.7.4 Surfactants

Non-ionic surfactants such as alkyl phenol ethoxylates (APEO) are a class of compounds used in industrial and domestic applications such as cleaners, paints, herbicides and pesticides as well as the pulp, paper and textile industries. The metabolites of APEO such as nonylphenol ethoxylate and nonylphenol mono-ethoxylate can be even more toxic than the primary compound and have endocrine disrupting ability, primarily through binding to the estrogen receptor (Manning, 2005). The metabolites usually associate with sediment and can further be broken down by microbes, however an extensive half life reported as being 60 years in the aquatic environment is possible for the metabolites, which stay in the effluent or sewage sludge (Shang et al., 1999).

1.7.5 Bisphenol A

Bisphenol A (BPA) is the monomer used in the production of polycarbonate plastics, epoxy resins and polycarbonate resins (vom Saal and Hughes, 2005). The most common application of BPA is in polycarbonate products, such as single use and reusable food containers, automobile parts, electronic components and laminates. BPA is one of the most produced chemicals worldwide; approximately 2.2 million metric tonnes were produced globally in 2003 with increasing production rates each year (vom Saal and Hughes, 2005). Bisphenol A shares structural characteristics with diethylstilbestrol (DES), the first synthetic estrogen, and also binds directly to the estrogen receptor (Ben-Jonathan and Steinmetz, 1998). Based on a study by Krishnan et al (1993) it was found that BPA bound to the ER, stimulated proliferation in MCF7 cells, induces progesterone receptors and its estrogenic action was reversed by tamoxifen. It is perhaps the hydroxylated metabolic products of BPA that are the most potent estrogen mimics, similar to the metabolism of diethylstilbestrol (DES) (Ben-Jonathan and Steinmetz, 1998).

The estrogenic properties of BPA were discovered accidentally when Feldman et al. (1984) reported that a yeast strain was producing 17β-estradiol, later confirmed by RIA. It was in 1993 that Krishnan et al. reported the effect was actually caused by BPA

leaching into water autoclaved in polycarbonate flasks that was used for media preparation. 2-3ug per litre of BPA was detected (Krishnan et al., 1993).

In vivo studies have shown that injections of high doses of BPA can induce uterus and vagina cell proliferation and induce c-fos expression in F344 rats (Steinmetz et al., 1998). Low dose injections of BPA (0.3mg per kg) for 3 days resulted in hypertrophy, hyperplasia and mucus secretion in the uterus. Lobular maturation and altered cell cycle kinetics of mammary epithelial cells has also been demonstrated with BPA (Colerangle and Roy, 1997).

1.8 Exposure risks

1.8.1 Wildlife exposure risk

It has been shown both in Australia and overseas that feminisation and in some cases the occurrence of intersex in fish stocks is occurring in rivers and waterways downstream from sewage treatment plants (Sumpter, 1995; Jobling et al., 1998; Batty and Lim, 1999; Jobling et al., 2003). Effects have been widely reported in UK waterways, to a lesser extent in northern America and just recently in Australia. An important factor in these findings is the intensity of land use compared with the dilution of treated water available. That is, the fast flowing rivers of the US tend to give a higher dilution of treated water compared to the relatively low volume slow moving waters of the UK. The coastal location of Australia's major cities and associated deep sea outlets allows for effluent dilution.

In the UK it has been shown that some waterways are comprised of more than 50% water from STPs especially in periods of low rainfall (Sumpter and Jobling, 1995).
A study by Batty and Lim (1999) showed that male Mosquitofish (*Gambusia affinis holbrooki*) are being adversely effected by EDCs in Hawkesbury-Nepean River system in New South Wales, Australia. The EDCs are from a STP outlet in the river system and also one area affected by agricultural run-off. The male mosquitofish had a modified anal fin or gonopodium (GP) for sperm transfer. The formation of the GP is caused by hormonal responsive elements during growth and development. Control mosquitofish were taken from the same river system where it emerged from a national park. The STP outlet and testing sites were downstream from this. It was found that fish at sites downstream from the STP showed a reduced GP length. It is proposed that the estrogens

or the anti-androgen effects of the estrogen were responsible for the reduction in length of the GP.

1.8.2 Human exposure risk

The endocrine disrupting ability of xenoestrogens on fauna is well documented, showing conclusively that low doses of EDCs have real measurable effects on animals. In humans, however, the ability to gather endocrine disrupting data is very difficult. The subject is open to controversial debate in the literature as to whether EDCs at low doses are even capable of adverse endocrine disruption in humans.

There are no established procedures for measuring the effect of EDCs in humans and so researchers have looked to occupational or pharmaceutical exposure as a way of understanding the roles of EDCs and their measurable effects. The use of diethylstilbestrol (DES), a synthetic estrogen, widely administered from 1947 until 1971 is one such example. Human exposure to DES was either through ingestion, via beef consumption from supplemented cattle feed, or was prescribed during pregnancy to prevent miscarriage and has conclusively been shown to cause significant adverse effects on the children exposed *in utero*. The so called DES- "sons and daughters" display symptoms that range from rare forms of clear cell adenocarcinoma (Lin et al., 2007) to testicular cancer and malformation. Studies looking at decrease in sperm quality and count, increase in testicular cancer, breast cancer and endometriosis have all failed to provide conclusive evidence that environmental EDCs can have the same effect (Safe, 2000). Other researchers such as Hood (2005) have acknowledged this, but also argue that the highly sensitive pathways in a developing fetus are responsive to minute amounts of hormone especially in very early development.

In the cases of occupational and accidental EDCs exposure we can study high dosage and/or long term effect. The fact that exposure to current low concentrations of EDCs from environmental sources will increase with the reuse of water, suggests that persistent EDCs will reach higher concentrations and humans will be constantly exposed to them. The current findings of 'conclusive no effect' from low dose EDCs have not prevented water providers from being aware of and examining ways to measure and regulate the amounts of EDCs being delivered in drinking water.

1.9 EDCs in surface water and STP effluent

During 1999-2000, the first US nationwide reconnaissance by the US Geological Survey determined the occurrence of pharmaceuticals, hormones and organic wastewater contaminants in surface water resources including 139 streams in 30 states (Kolpin et al., 2002). Concentrations of EDCs were determined, using five different chromatography/ mass spectrometry methods suited to the type of compound being detected. Of the streams sampled, 80% contained contaminants with coprostanol, cholesterol and N,N-diethyltoluamide occurring at highest frequency. Xenoestrogens were also found, reported here as frequency, median level and maximum level: Bisphenol A (41%, med.0.14µg/L, max. 12µg/L); 4-nonylphenol (50.6%, med. 0.8µg/L, max. 40µg/L); 17α-Ethinyl estradiol (15.7%, med. 0.073µg/L, max. 0.831µg/L), 17β-estradiol (E_2) (10.6%, med. 0.16µg/L, max.0.2µg/L) and estriol (21.4%, med. 0.019µg/L, max 0.051µg/L).

A large number of other studies have determined the levels of these and other estrogenic chemicals in surface water, with particular focus on STP effluent (Halling-Sorenson et al., 1998; Jobling et al., 1998; Korner et al., 1999; Kolpin et al., 2002; Atkinson et al., 2003; Jobling et al., 2003; Beck et al., 2005; Tan et al., 2007; Williams et al., 2007; Jackson and Sutton, 2008). In Australia, where major population centres are next to the sea, the majority of our STP effluent is released directly to the ocean. Nonetheless a study by Williams et al (2007) examined the effluent from 11 STPs which had median concentrations of estrone, E_2 and 17α ethinyl estradiol of 23.9, 3.8 and 0.45 ng/L. The concentration of such EDCs can of course vary with the level of treatment applied to the wastewater. A separate study by Leusch et al (2005) found that advanced sewage treatment reduced the estrogenicity of the effluent to below detection levels.

1.10 Xenoestrogens and the Water Industry

The World Health Organisation (WHO), United States (of America) Environmental Protection Agency (US EPA), US Food and Drug Administration, European Commission's Registration, Evaluation, Authorisation and Restriction of Chemical substances (REACH) program, and the National Health and Medical Research Council (NHMRC) all provide regulatory guidelines for the safe provision of drinking water. These guidelines contain the regulatory framework in which water providers, from the

relevant regions, must operate, and give indications of the concentrations of contaminants that are acceptable for human exposure and consumption.

There are however currently no consistent guideline values for (xeno)estrogens such as 17β-estradiol, 17α ethinyl estradiol, estrone and estriol (www.epa.gov). The US EPA has classified these contaminants on their drinking water contaminant candidate list 3 (CCL3), a document identifying contaminants that are known to or are anticipated to occur, and may require regulation.

In the 'Australian guidelines for water recycling: managing health and environmental risks (phase 2) augmentation of water supplies (May 2008)', estrogenic compounds were included in the recommended drinking water guideline for human pharmaceuticals (and pharmaceuticals with agricultural and veterinary applications). The concentrations of the following estrogenic chemicals were recommended: 17β-estradiol, 0.175µg/L; 17α ethinyl estradiol, 0.0015µg/L; estrone, 0.03µg/L. and estriol 0.05µg/L.

Australian water suppliers use various techniques during the treatment process to remove contaminants from drinking water. These processes can include addition of coagulants, flocculation, sludge collection, filtration, chlorination (or chloramination - addition of chlorine and ammonia for long distance distribution of water) and fluoridation (www.sawater.com.au). At all stages of collection and distribution the water is tested using various chemical and microbiological techniques consistent with the ADWG.

The use of STP effluent as a non-potable water resource is now common practice, with diverse applications including irrigation, industry and household amenities. The commissioning and use of desalination plants for the delivery of potable water to Australian cities has been realised during the period of this study, reducing the requirement to use recycled water as a potable option. Certainly, however, in parts of Australia and many cities of the world, the use of desalinisation is not always feasible and recycled water is a potentially viable option to augment water supplies.

The case mounts for xenoestrogens to be included in the battery of tests applied to water quality. This now not only incorporates 'single use' water, that is water used from traditional water sources, but also extends to recycled water applications.

As described, EDCs with a xenoestrogen action encompass a large number and combination of chemicals. *In vitro* bioassays are best suited for detection due to their ability to determine the total estrogenic content of a sample. This can be applied as an initial screening tool prior to further *in vivo* or chemical identification methods (Leusch et al., 2010).

The standardisation of bioassays with consistent operating protocols makes integration of bioassays into risk assessments and tiered routing monitoring practical (Leusch et al., 2010).

1.11 Assays for detecting estrogens

Extensive progress has been made in the development and refinement of methods for the detection of estrogens and xenoestrogens in environmental samples. The use of bioassays in chemical or pharmaceutical industries is well established, however, the correlation between chemical assays and bioassays for environmental/water industry testing has been reported only recently (Tan et al., 2007; Leusch et al., 2010). The reliability of bioassays has been scrutinised due to high variability when dealing with complex or highly polluted samples (Leusch et al., 2010). This is partly due to poor standardisation of methodology between laboratories (Andersen et al., 1999). The study by Leusch et al (2010) compared five bioassays; ER-CALUX, E-screen, MELN, T47D-KBluc and YES assays. The first two compared most favourably with chemical methods, although there was good consensus between all assays. Validation of existing methods and development of new assays is critical to a tiered screening approach to water quality

1.11.1 Sample Collection

Typically water samples are collected and the micro-pollutants concentrated by means of solid phase extraction (SPE) and elution in a solvent. Filtered water samples are either passed through SPE columns, or polar discs are left immersed at testing sites for passive sampling (Leusch et al., 2006). Solvents such as methanol or methanol:isopropanol mixtures are used to release the chemicals from the column once salts and all other unbound material is removed.

1.11.2 Yeast assays

The robust nature of yeast and ease of growth provides a relatively cost-effective high-throughput approach to testing compounds for their estrogenicity. Yeast are generally more resilient to salts (Bovee et al., 2004a) and cytotoxins such as the solvents used to extract micro-pollutants from water samples, and provide a relatively simple assay for high-throughput applications. The major disadvantage of the system is the non-physiological condition of the yeast. They are not regulated by the same co-activators and repressors found in mammalian cells and are simply measuring the response of ligand binding to a single, over-expressed receptor. This in turn can be advantageous as it avoids the combined response of other receptors such as androgen and progesterone receptors, which in cells such as MCF7 can antagonise the E_2-induced cell proliferative response (Widmaier et al., 2004) . Steroid free media also makes the use of yeast highly desirable, as there is no background interference to the assay arising from the yeast's own growth requirements.

There is good comparison of results between mammalian systems and yeast, however yeast assays were found to be less sensitive by an order of magnitude leading to a lot of non-detects (Leusch et al., 2010). In some cases, results from yeast assays conflict with other assays i.e. DES is an agonist in mammalian systems but an antagonist in yeast (Klein et al., 1994).

The yeast strain *Saccharomyces cerevisiae,* which lacks endogenous steroid hormone receptors, is most commonly used. The yeast is stably transfected with the human ER which allows for standardisation and negates interference from other steroid pathways. Some studies have used ER from alternate species such as rainbow trout estrogen receptor (Petit et al., 1997).

1.11.2.1 The Yeast Estrogen Screen (YES)

Routledge and Sumpter (1996) developed an estrogen screen in yeast to test the estrogenic properties of surfactants and their metabolites. A recombinant yeast strain was produced that was stably transfected with the human ERα and a plasmid containing an ERE within a strong promoter designed to drive the expression of lac-Z (Figure 1.4). The samples are incubated with the yeast in 96 well plates for 3 days. In the presence of estrogen (or any chemical acting like estrogen with a binding ability to the receptor) the

receptor is activated and shows a high affinity for the ERE and drives the expression of the lac-Z reporter gene. Lac-Z encodes for the beta galactosidase enzyme which is secreted into the media. A colourimetric endpoint can then measure the production of beta-galactosidase which cleaves red-beta-D-galactopyranoside (CPRG) into chlorophenol red. CPRG is added for the duration of the assay then measured in a spectrophotometer at 540nm. The samples are examined simultaneously with a standard curve to calculate the estrogenic potential of the sample. An increase in β-galactosidase activity was detected in response to 2 ng/L E_2 (7.3pM) .

The YES assay can also be used for the detection of antagonistic chemicals. The yeast are incubated with a concentration of E_2 known to elicit a sub-maximal response. When the test sample is co-incubated with this concentration of E_2, a reduction in the expected response can be measured (Sohoni and Sumpter, 1998). YES has often been applied to environmental water monitoring (Gaido, 1997; Beck et al., 2005; Vermeirssen et al., 2006).

The YES assay was optimised by Schultis and Metzger (2004) to significantly reduce the time required to complete the assay from to 72 hours to 7 hours by including a lysis step of the yeast cells. This releases the β-galactosidase from the cells which normally is a time limiting step.

1.11.2.2 Variations of YES

A study by Yoo et al (2002) used an improved YES to successfully detect estrogens. They argued that they improved the YES by using the *Saccaromyces pombe* strain, which divides evenly by binary fission, and is evolutionarily much closer to a mammalian system than the traditional yeast strain *S. cerevisiae,* which divides through budding. Also, by limiting the copy number of the lac-Z gene by genomic integration they propose that the system is much closer to a mammalian system. The assay was tested and showed sensitivity to E_2 levels of 0.1nM.
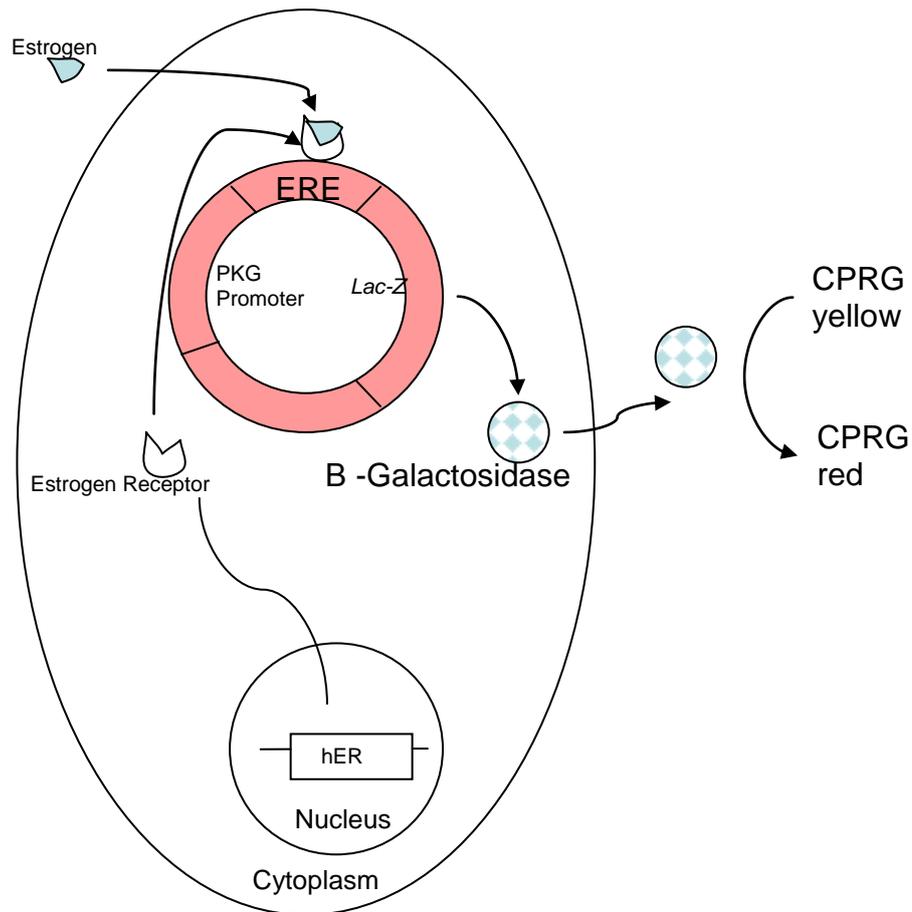
Figure 1.4 : The Yeast Estrogen Screen (YES). Estrogen(-like substances) activate the human estrogen receptor (hER) and bind to the estrogen response element (ERE). This allows for activation of the PKG promoter which drives the expression of the lac-Z gene producing β-galactosidase. Chlorophenol red-ß-D-galactopyranoside is cleaved to chlorophenol red which can be measured by absorbance at 562nm.
Adapted from Routledge and Sumpter (1996).

1.11.3 Yeast eGFP reporter gene assay

Bovee et al (2004) developed a set of vectors containing two EREs placed within a truncated cytochrome c oxidase promoter (CYC-1) driving the expression of reporter gene. Three reporter genes were tested to determine which reporter gene had the best response time and sensitivity to E_2. Luciferase (luc), yeast Enhanced Green fluorescent

protein (yEGFP) or β galactosidase (β-gal) reporter genes were each tested. Homologous recombination of the vectors and the human ERα, isolated from T-47D cells, into yeast were used to determine the best reporter construct.

The yEGFP assay was chosen in preference as it could be performed in a 96 well plate format without the need for substrates or cell disruption and also had lower variability than the other two more laborious assays. When exposed to E_2 the yEGFP reporter gave an EC_{50} of 0.4nM after 4 hours respectively. The two other reporter genes required much longer exposure periods to generate significant differences to the control.

Bovee et al (2004b) further expanded the study by producing a recombinant yeast strain that expressed ERβ, which had an E_2 EC_{50} about 5 times lower than when the ERα was present, but gave only 40% the activity of the ERα model(Bovee et al., 2004b). A range of common EDCs were tested in the study showing different potencies through each receptor i.e. ERα; E_2 **>** 8-prenylnaringenin > coumestrol > zearalenone**>** genistein **>** genistin > naringenin. When the ERβ model was used the results were; E_2 > coumestrol > genistein > zearalenone > 8-prenylnaringen >daidzein > naringenin > genistin > daidzin.

1.11.4 Yeast Two Hybrid Assay

The yeast two hybrid assay is designed around the Gal4 DNA binding domain (Gal4DBD) as well as the Gal4 activation domain (Gal4AD) linked to a co-activator such as TIF2. The assay is advantageous compared to the YES as it can be performed in a reduced time-frame of 4 hours (Nakano et al., 2002). The estrogen ligand binding site is linked to the Gal4DBD. When a co-activator binds to a ligand-hormone complex, β-galactosidase is expressed. Nankano (2002) expressed concerns that proliferation of the yeast was highly variable dependant on the compound being tested, which lead to varying results.
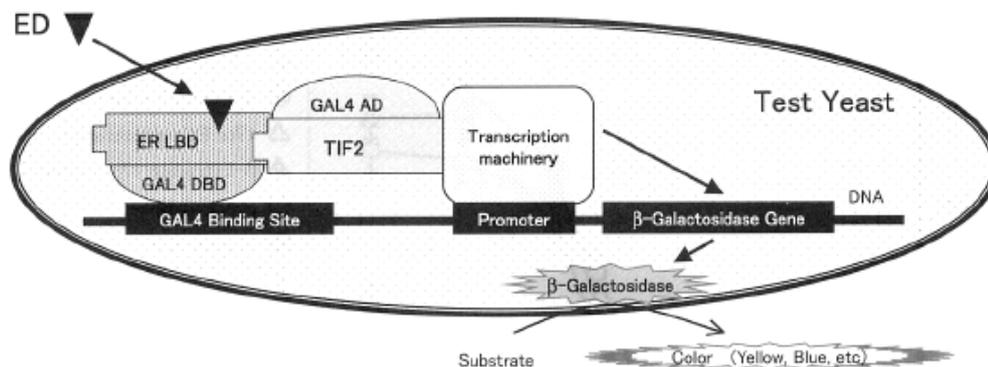
Figure 1.5: The yeast two hybrid assay. An estrogenic endocrine disruptor (ED) binds to the estrogen receptor ligand binding domain (ERLBD) activating the ligand dependant interaction between the estrogen receptor (ER) and co-activator (TIF2). The expression of β-galactosidase can be measured by the cleavage of CPRG, as described. *Taken from Nishihara and Nishikawa (2001).*

1.11.5 Chemical analysis

High Pressure Liquid Chromatography (HPLC), Gas chromatography (GC) and Mass Spectrometry (MS) are all highly sensitive methods for the identification and quantification of known compounds from mixtures. These methods can be used when pure samples of identified target compounds can be used to calibrate the assay and be used as standards. When applied to water samples, as performed by Liu et al. (2004) water samples are passed through SPE columns which bind the compounds of interest. Columns are then washed and the compounds eluted from the column using solvents. The eluted compounds pass through single or multiple analytical tools such as gas chromatography for size separation (based on vaporisation) and then into a mass spectrometer for mass determination. This type of assay is very appropriate if particular compounds are being analysed such as different natural steroidal estrogens (Hanselman et al., 2006) or used in conjunction with a bioassay or to provide valuable information about extraction and recovery of compounds. Although this type of assay can measure the concentrations of selected xenoestrogens and EDCs very accurately it fails to measure estrogenic bioactivity, that is, identify estrogenic action from unknown compounds and synergistic effects from multiple low level compounds.

1.11.6 Estrogen receptor competitive binding assay

An ER competitive binding assay measures the ability of an environmental or test chemical to bind to the estrogen receptor compared to a radio-labelled estradiol standard. The more test chemical that binds the receptor in preference to the labelled estradiol, the lower the scintillation count. This method does not discriminate between agonist and antagonist chemicals and simply measures binding ability. Rat uterine cytosolic ER was used by Laws et al (2006) to compare 50 high production volume chemicals listed in the United States Environmental Protection Agency's toxic substances control inventory. Of the 50 tested, 17 showed affinity for the receptor.

A competitive receptor binding assay cannot distinguish between agonist and antagonistic responses nor provide any measure of bioactivity and can only measure the binding response of a sample compared to an estrogen standard curve. Steric hindrance can also interfere with the accuracy of the assay whereby proximity of the chemical to the receptor prevents the natural estradiol from binding (Laws et al., 2006).

1.11.7 Fluorometric Polarisation Estrogen Receptor Binding Assay

The Fluorometric Polarisation assay works on the same principle as the ER competitive binding assay where a fluorescein-labelled estradiol is substituted for the radio-labelled estradiol. Estrogen-like compounds compete for binding to the ER and displace the fluoromone (Parker et al., 2000). A fluorescent ligand bound to the Estrogen receptor has a high polarisation value. A competing ligand will displace the fluorescent ligand and result in low polarisation value. The assay does not require the use of radioactive components and eliminates the need for a separation of the unbound labelled ligand (www.invitrogen.com). Generally a much shorter incubation time of 2-6 hours is also used compared to 18-24 hours for the ER competitive binding assay.

1.11.8 Mammalian cell systems

Mammalian cell lines are a useful tool for measuring environmental estrogens in a way that may have more physiological relevance. The induction of a reporter gene is a very attractive and reliable system for measuring transcription of a marker gene or response element linked to a known promoter. The concentration of compounds in water samples however continues to be problematic with cytotoxic effects commonly seen (Seifert et al., 1999). While transient transfection of cell lines can be invaluable for fast assessment of response elements, the inherent variability, and requirement for internal

controls such as co-transfected reporters makes transient systems less desirable than stably transfected systems for long term monitoring tools.

1.11.8.1 ER-CALUX

Legler et al. (1999b) developed an estrogen receptor mediated, chemical-activated luciferase reporter gene-expression (ER-CALUX) assay. A plasmid was constructed from pGL3-basic (Promega). Three repeats of the ERE (5'-GAGCTTAGGTCACTGTGACCT-3') were inserted upstream of minimal human E_1B TATA promoter sequence linked to an enhanced luciferase reporter gene. The plasmid was titled pEREtata-luc (Figure 5).

The plasmid was transfected into MCF7, T-47D and ECC-1 cells and clonal populations of cells expressing the plasmid were selected. MCF7 and ECC-1 cells failed to expand or produce stable estrogen responsive clones as measure by luciferase activity. The T-47D cell line did however, and a clone was selected that had maximal response to E_2 of 102 fold induction of the luciferase compared to the control. The assay has an EC_{50} of 5.5 pM E_2 and a detection limit of 0.5pM.

The assays are performed in steroid free medium and can be completed in 24 hours. This was the first bioassay of its kind after the development of the E-screen (section 1.7.11) that could reliably produce highly sensitive results. The ER-CALUX assay is now a commercial enterprise with a licensing fee required per use .

Kpn I
Sac I
Mlu I
Nhe I
ERE ERE ERE
Sph I
Pst I
AccI
Sal I
XbaI
E1B TATA
Bam HI
Bgl II
Hind III

MCS
poly(A)
f1 ori

luc+

amp   **pEREtata-LUC**
       (5200 bps)

SV40
poly (A)

ori
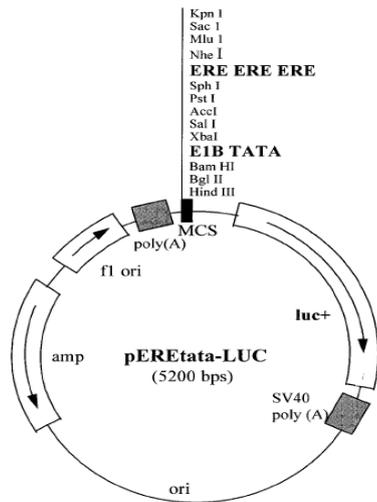
Figure 1.6: The pEREtata-luc vector. The 5200 bp vector includes three estrogen response elements (ERE) and an E1B TATA minimal promoter placed in the multiple cloning site (MCS) of the reporter vector. Binding and activation of the triplet ERE and minimal promoter drives the expression of the luciferase (luc$^+$). A poly (A) region before the promoter region and after the luciferase gene ensure efficient blockage of transcription. The vector contains the selective gene for Ampicillin (Amp) resistance. *Taken from Legler et al. (1999b)*
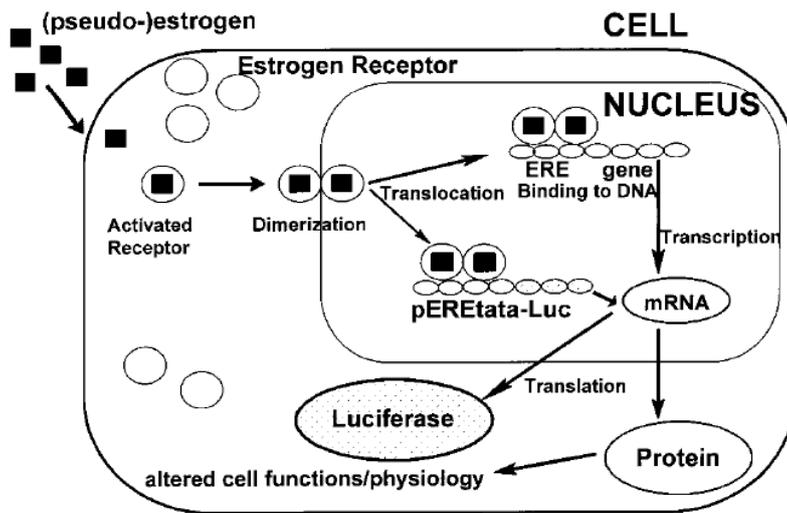
Figure 1.7: Induction of luciferase expression in ER-CALUX. When a (pseudo-)estrogen enters the cell, it binds to and causes dimerisation of the estrogen receptor. The activated receptor translocates to the cell nucleus and binds to estrogen response elements (ERE) either of endogenous promoter regions or to that of the transfected pEREtata-Luc. Activation of pEREtata-Luc leads to luciferase expression which can be quantified. *Taken from Legler et al. (1999b)*

1.11.8.2 T47D-KBluc

Wilson et al. (2004) developed a highly similar cell line called T47D-KBluc, freely available from ATCC, which is a clone of T-47D also transfected with a plasmid carrying triple ERE up-stream from a TATA minimal promoter. The EC_{50} of E_2 was 10 nM compared to 5.5 pM for the ER-CALUX system.

1.11.8.3 GFP response MCF7-ERE

Miller et al (2000) developed a highly responsive clone of MCF7 stably transfected with a plasmid encoding eGFP linked to two repeat EREs within a human phosphoglycerate kinase promoter. Cells were sorted using FACS according to their level of GFP expression. The assay, as reported, relies on microscopy and image analysis with results expressed as fluorescent units per 1000 cells which may not translate well to high throughout systems.

1.11.8.4 Variations of ERE linked luciferase reporter systems

Many variations of the Luciferase assay have been performed with the mammalian cell lines commonly being plated, allowed to attach overnight and then changed to medium devoid of any estrogen or steroids. Cells are then exposed to the samples or an E_2 standard curve for 24-48 hours, luciferin added and luminescence measured in a luminometer.

Pons et al (1990) and Demirpence et al (1993) developed and characterised a MCF7 cell line stably transfected with an ERE from the *Xenopus* vitellogenin A2 gene linked to the luciferase reporter gene. The cell line was called MVLN, and responded to E_2 in a dose dependant manner. Briefly , the cells were attached to a 6-well plate and pre-treated for 1-2 weeks in phenol red free media supplemented with 3% DC-FCS to free the cells of intracellular estradiol and reduce the background signal (Demirpence et al., 1993). Cells were then exposed to E_2 or the test samples for 24 hours before luciferase expression was measured. E_2 generated luciferase induction in the pM range, other xenoestrogens generated an induction at very low concentrations also; zearalenone (0.1nM), hexestarol (10pM) and DES (1pM).

Balaguer et al. (1999) developed 4 cell lines to assess the sensitivity and response of the cells to xenoestrogens from the effluent of sewage treatment works. MCF7 cells

were transfected with a reporter plasmid containing an ERE within the β globulin promoter linked to a luciferase gene (MELN). Also, HeLa cells, which do not express ER, were transfected with the reported plasmid and human ERα (HELN(α)) or ERβ (HELN(β)). This method allows for the elucidation of chemicals reacting with each type of receptor. The HeLa cell line was also transfected using a Gal4 chimeric system (HEGLN). A bound ER ligand site activated the Gal4-DNA binding domain which binds to the response element driving luciferase expression. Although all the cell lines were able to detect estrogenicity of sewage water treatment plant influent and to a lesser degree effluent, the MELN and HELN(α) were found to be the most sensitive. These two cell lines both had an EC_{50} to E_2 of 5pM, followed by the lower affinity ERβ cell line HELN(β) (EC_{50} of 10pM) and the chimeric HEGLN cell lines (EC_{50} of 40pM).

1.11.8.5 Flow Cytometric determination of cell cycle kinetics

Flow cytometry has been applied to the assessment of xenoestrogens (Blom, 1998; Diel et al., 2002; Vanparys, 2006). The assessment of cell cycle dynamics and entry into the cell cycle is a rapid method for assessing proliferation without the requirement for cell number quantification. Flow cytometry allows for the specific physiological state of individual cells to be assessed (Vanparys, 2006). MCF7 cells are typically growth arrested in G_0/G_1 phase using steroid-free medium for 72 hours. Exposure to the test samples which are estrogenic initiates the cell cycle and causes a significantly higher number of cells to enter S phase than the control cells. Stains such as propidium iodide, a DNA-stain, can detect cells with greater DNA content due to increased synthesis with entry to the duplication process. This method gives results which are consistent in sensitivity and response with E-screen data (section 1.7.11) (Vanparys, 2006).

1.11.9 Gene expression assays

1.11.9.1 Vitellogenin induction

It is possible to measure effectively the level of estrogenic exposure in fish by the concentration of vitellogenin (VTG) present in the blood of fish. VTG is a serum phospholipid glycoprotein precursor to egg yolk protein in vertebrates that lay eggs that hatch outside their bodies (Heppel et al., 1995). The presence of VTG is undetectable in immature animals and males and is highly induced in female fish when eggs are being produced. The induction of VTG is a direct response to (xeno)estrogens. Normally vitellogenin is produced in the liver and stored in oocytes as yolk to serve as the main

nutrient source for growing embryos. It is transported in blood plasma and therefore can be easily measured by taking blood samples. The process is under multi-hormonal control but is mainly affected by the presence of 17β-estradiol.

Sumpter and Jobling (1995) reported a 500 to 50,000 fold increase of the amount of vitellogenin in male trout caged in the effluent of 28 different STWs in England and Wales.

1.11.9.2 Specific Gene Regulation

Many studies have been carried out whereby the estrogenicity of water samples or single compound analysis has involved the monitoring of the up- or down-regulation of specific genes (further discussed in Chapter 5). The regulation of specific genes is the primary occurrence for all other measured endpoints, such as proliferation, and is therefore a comparable technology to the other bioassays presented here (Jorgensen et al., 2000).

1.11.10 Xenoestrogen assays performed *in vivo*

Numerous rodent assays are available for testing estrogenic compounds including; vaginal cornification and keratinisation response, and uterine tissue weight increase after exposure to the test compound (Kanno et al., 2001).

The Rodent 3 day Uterotrophic assay measures an increase in uterine tissue mass in ovariectomized or immature rodents after 3 days of exposure to test chemicals as described by Lan and Katznellenbogen (1976).

In vivo assays, despite their accuracy are time and labour intensive and not suited to a high-throughput testing scenario.

1.11.11 Proliferation Assays

The original E-screen was developed to measure estrogenicity of environmental pollutants (Soto et al., 1995) and was earlier described as the MCF7 cell proliferation bioassay (Soto, 1991). The assay was based on the observation that estrogenic stimuli exert a proliferative effect on MCF-7 cells. The end point of the assay is the cell number of the test sample relative to the hormone free/solvent control. Increase in the proliferation of the human breast cancer cell line, MCF7, is thought to be equivalent to

the induction of mitotic action on the rodent endometrium which is describe as the 'hallmark' action of natural estrogen (Soto, 1995).

The mechanism of action of the E-screen, when dextran-charcoal treated human serum was used in the culture of the cells during the test period, was initially based on the hypothesis that human serum contains an unidentified molecule that that inhibits the proliferation of human estrogen-sensitive cells and when estrogen is present cell proliferation is induced by negating the inhibitory effect (Soto, 1985). Non-estrogenic steroids and growth factors do not abolish this inhibitory effect (Soto, 1985). Since the E screen is commonly performed now in dextran-charcoal treated fetal calf serum (DC-FCS) the assay *suggests* that a test chemical is estrogenic (Zacharewski, 1997). Papers to date have not elucidated the specific mechanism of action where a proliferative effect is caused by the presence of estrogens other than to describe estrogen responsive cells as being dependent on estrogen solely for their proliferation.

Korner (1998) validated and optimised the assay by repeating the assay and comparing the estrogenic potencies of nine xenoestrogens. Interestingly the assay used DC-FCS with no reduction in sensitivity of the assay. Korner (1999) also used the E screen in the application of testing water samples from municipal sewage treatment plants. The optimised version of the E-screen was miniaturised to 96 well plate format, used FCS and a simple colorimetric assay to assess total cell number (SRB total protein or MTT assay).

Rasmussen and Nielsen (2002) further expanded on the critical parameters in the E-screen to reduce the labour-intensiveness of the assay, and the amount of test sample required without a reduction in sensitivity. The highly responsive MCF7 BOS cell line was also used.

1.12 Bioassays in perspective

The detection of contaminants in the environment that have the ability to interact with the endocrine system of humans and wildlife has led to a concerted effort to determine the nature of the chemicals, their source, how widely they are distributed in the environment and waterways, and at what concentrations (Safe, 1994; Crisp et al., 1998).

The vast majority of these chemicals are of anthropogenic origin and have been released to the environment in growing quantities since industrialisation (Crisp et al., 1998; Soto et al., 2006). Plasticisers, detergents, agricultural and pharmaceutical products and their degradation products/metabolites are responsible for the bulk of these endocrine disrupting chemicals, which have a unique ability to interact with the estrogen receptor (Jobling et al., 1998; Falconer, 2006).

The two major estrogen receptors, α and β, have been shown to accept a large number of compounds in the ligand binding domain, primarily compounds whose structure includes a phenol ring with hydrophobic regions (Brzozowski et al., 1997). The outcome of this interaction can vary depending on the bound ligand, be it activation or blocking of the ER or disrupting normal activity of the receptor. Other chemicals which do not bind the ER can also interact with and disrupt the related signalling pathways (Manning, 2005). The actual effect of xenoestrogens in highly regulated physiological systems, such as humans, is still being determined (Leusch et al., 2010). The most concerning implications to date, have arisen from the occurrence of intersex fish, in waterways downstream of STP outlets (Sumpter, 1995). Whilst we may be comforted that the bulk of Australia's STP effluents are primarily discharged to open ocean, Australia is not immune, as a reduction in gonopodium length of mosquitofish has been reported in Sydney waterways (Batty and Lim, 1999).

It has been shown that fish displaying the occurrence of intersex are being exposed to high levels of endocrine disruptors, primarily human E_2 and the pharmaceutical estrogen, 17α-ethinylestradiol. The levels of these two estrogens (or the estradiol equivalent in the case of 17α-ethinylestradiol) have been shown to exist in waterways at up to 0.2μg/L, which can be higher than the physiological concentrations of estrogens in the human body (Kolpin et al., 2002). The conceptual model that has arisen, since the chronic effects seen on fish, is that responses on the molecular level, lead to responses on an organism-level effect which then leads to a population-level effect (Zacharewski, 1997). In humans, we are faced with an extremely complex exposure scenario, making it very difficult to assess and determine the exact effects of xenoestrogens on humans.

Whilst focus can be on the potentially estrogenic effects of EDCs from the environment and quality of water we consume, it is easy to overlook the amount of estrogenic equivalents and EDCs that we ingest everyday through diet. An interesting study by Leusch et al (2009) examined the daily estrogenic intake from a typical diet and showed that many items we consume have an estrogenic effect. Coffee, beer, milk, meat, soy products and wine all have estrogenic content. It was estimated that in one day we ingest 1400-fold (*in vitro* estimate) to 620-fold (*in vivo* estimate) more xenoestrogens from our diets than from drinking 2L of recycled water.

Nonetheless, the actual concentration of EDCs and xenoestrogens present in a commodity that is supplied to entire populations should not be ignored. The concept of endocrine disruption from estrogenic compounds has gained momentum in terms of generating guidelines within the last 10 years. Regulatory bodies such as the WHO, NHMRC, US FDA & EDSTAC the EU REACH program have developed guidelines which provide a risk assessment based directive on acceptable levels of identified contaminants within water. The levels recommended are often based on concentrations of chemicals that have no observed effect, or on a fraction of defined therapeutic dose. 'The Australian guidelines for water recycling: managing health and environmental risks (Phase 2) for augmentation of drinking water supplies', released in 2008, have the major estrogenic chemicals estrone, 17β-estradiol, 17α-ethinylestradiol and other estrogenic chemicals or pharmaceuticals included in guidelines. Other xenoestrogens have not yet been regulated as there currently is insufficient toxicological data.

The implementation of regulatory standards drives research to detect estrogenic (and other) EDCs. While regulatory bodies can pinpoint specific chemicals and give guidelines values for each, there are also demonstrated *in vitro* effects from combinations of chemicals at individual no-effect dosage which combine to drive an estrogenic effect.

1.13 Bioassays within the scope of this project

The field of development and application of bioassays for the measurement of total estrogenic load has progressed significantly throughout the duration of this project. The

most important factor towards implementing the assays into risk management assessment and tiered screening is not only the consistency and specificity with which these assays are applied, but also the determination of whether *in vitro* effects actually translate to human responses (Leusch et al., 2010). As the limit of detection of estrogenic bioassays consistently reaches into the pM range, the determination of actual contaminant concentrations below which there are no health risks need to be determined.

Nonetheless, the continual development of assays that can rapidly and reproducibly detect xenoestrogens would be highly valuable to the timely delivery of water.

CHAPTER 2

General Materials and Methods

2.1 Materials

All chemicals and reagents used in this study were of analytical or tissue culture tested grade and were obtained from Sigma-Aldrich (Castle Hill, Australia) unless otherwise noted within each chapter's material and methods sections.

MCF7, ZR-75-1, H23, T-47D, NIH:OVCAR-3 (Ovcar) , RL95-2 and CCD-1064Sk (1064Sk) were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA).

Cell Line (ATCC Number)	Origin and cell type	Age of Donor	Species	Gender
MCF7 (HTB-22)	Breast epithelial adenocarcinoma	69	Human	F
ZR-75-1 (CRL-1500)	Breast epithelial ductal carcinoma (from ascites)	63	Human	F
RL95-2 (CRL1671)	Uterus epithelial endometrial carcinoma	65	Human	F
T-47D (HTB-133)	Breast epithelial ductal carcinoma	54	Human	F
H23 (C-5800)	Lung adenocarcinoma	51	Human	M
CCD-1064Sk (CRL-2076)	Foreskin fibroblast	newborn	Human	M
Ovcar (HTB-161)	Ovarian epithelial adenocarcinoma	60	Human	F

Table 2.1: Summary of cell lines used.

17β-estradiol, 17α Ethinylestradiol and Fulvestrant, all in powder form, were obtained from Sigma-Aldrich and resuspended in analytical grade filtered Ethanol, and stored at -20°C for up to one year in 1mL aliquots. Final concentrations of Ethanol in cell culture medium did not exceed 0.5%.

2.2. Methods

As the majority of methods used in this study are specific to individual chapters they have been described in full within the chapters, and have not been repeated here. Methods described here are common to all chapters.

2.2.1 Cell culture media and preparation

ZR-75-1, H23 and T-47D were cultured in 10.4 g/L RPMI-1640, supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco), 1% (v/v) penicillin/streptomycin (Thermo Scientific) and 0.11g/L sodium pyruvate, 2.5g/L d-Glucose, 2.38g/L HEPES and 1.5 g/L sodium bicarbonate. Ovcar cells were grown in similar conditions supplemented with 20% heat inactivated FBS (v/v).

MCF7 were maintained in EMEM Earles (Thermo Scientific) supplemented with 1.5 g/L sodium bicarbonate, 0.11gL sodium pyruvate, 0.01mg/ml bovine insulin and 10% heat inactivated FBS (v/v).

RL95-2 were grown in Minimum Essential Medium Eagle supplemented with 0.01mg/ml bovine insulin and 10% FBS.

CCD-1064Sk were grown in Iscoves Modified Dulbecco's Medium supplemented with 10% FBS (v/v).

All media were initially prepared in 5 litre batches (without FBS or insulin if required), combined with mechanical stirring, adjusted to pH 7.4, and filter sterilised before storage at -20°C. Medium was prepared for use in 200mL batches with the addition of FBS and insulin (if required) and filtration, then stored at 4°C. pH and temperature of medium were equilibrated for 30 minutes in the incubator immediately prior to use.

2.2.1.1 Subculture of cells

Cultured cells were allowed to grow to near confluence in a humidified 37^0C, 5% CO_2 incubator in T75cm^2 flasks (Nunc, Denmark) before washing with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4$_)$ and detachment with 1 mL 0.25% trypsin-EDTA (Thermo Scientific). Fresh growth media was added and cells were split 1:5 into new T75 cm2 flasks containing 12mL of growth media. All cell manipulations were done using washed and autoclaved glass pipettes.

A record was kept of the number of times a cell line had been subcultured or passaged. Cells lines received from ATCC, were designated as being passage 1.

2.2.1.2 Fetal bovine serum (FBS)

FBS was heat inactivated at 56^0C for 30 minutes in a waterbath with occasional shaking. Heat inactivated FBS was stored in 10mL aliquots at -20°C.

2.2.1.3 Cell line storage

Cell lines were stored in liquid nitrogen in their respective media + 5% DMSO + 20% (final v/v) FCS. MCF7 cells were stored at -80^0C due to poor viability and recovery after liquid nitrogen storage.

2.2.2 Trypan Blue exclusion

A solution of Trypan blue (0.2%) and sodium chloride (0.9%) was filtered through Whatman grade 1 filter paper. Cells that had been resuspended in their respective media after trypsinisation were added to a trypan blue solution at a 1:1 ratio (typically 10μl each) before loading to a haemocytometer for counting. Cells that stained blue were not included in the cell count as they were non-viable(Freshney, 2005).

2.2.3 MTT Assay

The MTT (3-(4,5-dimehtylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was an adapted from Mossman(1983). MTT was dissolved in sterile PBS (5 mg/ml), and 1ml aliquots stored at -20^0C.

Media from wells of a 96 well test plate was aspirated and replaced with 100μl per well media supplemented with 0.5 mg/ml MTT and returned to incubator for 1 hour. 80μl of acidified 20% SDS (1mM HCl) per well was the added to lyse cells and solubilise the formazan in darkness for 1 hour. Absorbance of the homogenised lysate was read at 570nm with a reference wavelength of 640nm on a μQuant automatic spectrophotometer (Bio-TEK instruments, NSW, Australia) using KC Junior software (Bio-Tek, VT, USA).

2.2.4 Crystal Violet Assay

This method was adapted from Bonnekoh et al (1989) and Kueng et al (1989). Crystal violet (Hexamethylpararosaniline chloride) 0.5% w/v in 50% methanol solution was made and stored at room temperature. Wells of a 96 well plate were carefully aspirated. 50 μl crystal violet stain was added to each well with cells including empty wells to adjust for sorbance of the stain to the plastic growth surface. Plates were left at RT for 10 minutes to allow the cells to stain and be fixed by the methanol. Plates were gently rinsed with RO water. Excess water was removed from the wells and plates were left to

dry either on the bench or in a fume cupboard. 50 µl 30% acetic acid was added to dry wells and plate gently tapped to help solubilise and homogenise the stain. Absorbance of the crystal violet was read at 570nm with a reference wavelength of 640nm on a µQuant automatic spectrophotometer (Bio-TEK instruments, NSW, Australia) using KC Junior software (Bio-Tek, VT, USA).

3.2.2 Cell quantification standard curves

Viable cell suspensions were serially diluted in a 96 well plate (Nunc, Denmark) using a multi-channel pipette (80,000 - 1250 cells per well in six replicates) in 100µl of their respective media. Cell numbers were measured using the MTT or crystal violet assay as described after a designated attachment time (as described relevant to each cell line). The mean absorbance of the six replicate wells was related to the initial cell number in each well using a linear regression function in Excel. The equation describing the relationship between absorbance and cell number, was then used to convert absorbance to cell number after application of the MTT or crystal violet assay to the remaining plates within the assay.

CHAPTER 3

Characterisation of mammalian cell lines for use in proliferative and estrogenic response assays

3.1 Introduction

The activating feature of the majority of human cell-based xenoestrogen bioassays such as the E-screen (Soto, 1995) or ER-CALUX (Legler et al., 1999b) is the binding of an (ant)agonist to the estrogen receptor. This causes receptor dimerisation and activation of the receptor (Dechering et al., 2000). The cascade of cellular events that follow such as binding to the Estrogen Response Element (ERE), or the induction of proliferation, can be measured.

The majority of these assays utilise the MCF7 and T-47D cell lines (Zacharewski, 1997) which express ERα and/or ERβ (Dechering et al., 2000; Chen et al., 2004). The MCF7 cell line, a pleural effusion derived from a breast carcinoma by Soule et al (1973), has been characterised as expressing both estrogen receptors, although levels of mRNA for ERβ were much lower than ERα (Dechering et al., 2000; Chen et al., 2004; Lin et al., 2004) or even undetectable (Jorgensen et al., 2000). MCF7 cells have been used widely in xenoestrogen testing, in such assays as the E-Screen (Soto, 1995) because of their recognized estrogen sensitivity (Soto, 1995).

T-47D, a breast ductal carcinoma cell line (Keydar et al., 1979) which expresses mRNA for ERα with ERβ at a lower level (Dechering et al., 2000), have been used also in the E-screen (Soto et al., 1999; Matsuoka et al., 2005) but also with stably transfected luciferase reporter elements such as in the ER-CALUX assay (Legler et al., 1999b).

Other human cell lines express the estrogen receptors and may provide an alternative for xenoestrogen testing. Another breast ductal carcinoma cell line, ZR-75-1, has been shown to proliferate in direct response to the presence of estrogen (Engel et al., 1978) and has been shown to express mRNA for ERα with ERβ at a lower level (Grandien et al., 1993; Dechering et al., 2000). Alternative ER α promoter splicing has been shown between MCF7 and ZR-75-1 cells (Grandien et al., 1993).

RL95-2, an endometrial carcinoma cell line, expresses ERα and low levels of ERβ mRNA (Way et al., 1983; Dechering et al., 2000). Ovcar, an ovarian carcinoma cell line (Hamilton et al., 1983) and H23 (Stabile et al., 2002; Pietras et al., 2005) a lung cell

line, were shown from previous studies to contain the ERα or ERβ at the mRNA or protein level.

As breast carcinoma cell lines are most commonly used for *in vitro* testing, these additional cell lines originating from different organs of the body were chosen, in order to exploit potentially different physiological responses to estrogens. Aims of this study were to do a basic assessment on these cell lines for their attachment times (optimum culture time for the production of a standard curve), growth rate in a 96 well plate format and expression of the ERα and ERβ.

An overnight cellular attachment time is commonly used in assays such as the E-screen (Soto, 1995) or ER-CALUX (Legler et al., 1999b) before exposure to samples. Standard curves are also commonly derived from measurements taken after an overnight incubation of the cells (~16-24 hours). The determination of the optimum time point to perform a standard curve is an important factor in the accurate quantification of cell numbers where absolute cell number is derived from a single time point. However, due to entry of the cells to the growth cycle during this period of time, attachment times of 4 and 6 hours after dispensing a serial dilution of 80000 to 1250 cells per well to the growth surface of a 96 well plate were also investigated.

This would determine the least possible time required to get maximum attachment to the growth surface whilst minimising artefacts caused by cell proliferation. The standard curves were analysed using linear regression and determination of the 95% CI of each standard curve. Using this knowledge assay times could be also reduced with earlier media manipulations and subsequent exposure to samples possible.

Growth characteristics are important for reproducibility of assays in which the cells will proliferate, such as the E-screen. Page et al (1983) showed that MCF7 cells have a density dependent response to estradiol and have a doubling time of greater than 36 hours(Chhipa et al., 2007). T-47D and ZR-75-1 have a range of reported doubling times; 32 hours (ATCC) to 48 hours (Mathivadhani et al., 2007) for T-47D cells and 72 hours (Daly and Darbre, 1990) to 80 hours (ATCC) for ZR-75-1 cells.

It is desirable to achieve the lowest possible inter-assay variation for standardised testing in order to maintain reproducibility and accuracy. The variation between assays

can be described by calculating the coefficient of variance (CV), a normalised measure of dispersion, which is the ratio of the standard deviation to the arithmetic mean(Hendricks and Robey, 1936). The E-screen as performed by deCastro and Neuberg (2007) was shown using MCF7 cells to have a relatively high CV of 29.6 %. Variation can arise from the removal of cells during aspiration and replacement of media during experimental manipulations and differences in batch and preparation techniques of media. In proliferation assays variability can also arise from relatively small differences in the initial numbers of cells per well, with the variation increasing considerably during exponential growth (deCastro and Neuberg, 2007).

In other assays where a short time frame is involved, such as the monitoring of gene regulation by the presence of (xeno)estrogenic substance, growth rates are not important. The cell plating density is important so that cells are in a responsive state to (xeno)estrogens that may alter gene expression. Primarily a cell line selected for (xeno)estrogen screening requires the presence of the ERα and/or ERβ protein for an ant/agonist binding/interaction and the beginning of the cascade of estrogen signalling events. Each cell line was therefore assessed in a comparable and optimum growth conditions for expression of ERα and ERβ mRNA by RT-qPCR and western blotting.

In this study, the suitability of six human cell lines, MCF7, T-47D, ZR-75-1, RL95-2, Ovcar and H23, was evaluated for use in xenoestrogen screening assays. The expression of estrogen receptor alpha and beta mRNA and protein, attachment times, growth characteristics and optimal times for the production of standard curves in the 96 well plate format were analysed to determine which cell lines gave the lowest intra- and inter-assay variation.

3.2. Material and Methods

3.2.1 Cell adherence time

5000 viable MCF7, T-47D, ZR-75-1, RL95-2, Ovcar or H23 cells, in 100μl of their respective media, were dispensed into six replicates wells of a 96 well plate (Nunc, Denmark). Cell metabolism was measured using the MTT assay (section 2.2.3) after 4, 6 and 24 hours incubation. One 96 well plate per cell type was used for each time point and the experiment was repeated on three separate occasions (n=3) for each cell line.

3.2.2 Standard curves and cell proliferation

Viable MCF7, T-47D, ZR-75-1, RL95-2, Ovcar or H23 cell suspensions were serially diluted in a 96 well plate (Nunc, Denmark) using a multi-channel pipette (80,000 - 1250 cells per well in six replicates) in 100μl of their respective media (section 2.2.1). Cell metabolism was measured using the MTT assay after 4, 6, 24, 48 and 72 hours incubation. One 96 well plate per cell type was used for each time point and the experiment was repeated on three separate occasions (n=3) for each cell line.

3.2.3 Statistical analysis

Cell proliferation analysis was performed on the means of six replicate wells performed on three separate occasions (n=3). Data from the MTT assay was entered into Excel and the mean and standard deviation of the six replicate wells plotted for each separate occasion. GraphPad Prism was used to analyse 95% confidence intervals. The slope obtained from linear regression of data from the 4 or 6 hour time point was used as the standard curve to convert time points thereafter to actual cell number. The results were used to calculate intra- and inter-assay coefficient of variance (CV) using the formula CV (%) = (standard deviation / mean) x 100 (Hendricks and Robey, 1936). The intra-assay CV was a measure of the variance within each replicate and the inter-assay CV was a measure of the variation between the three separate occasions (n=3) on which the assay was performed.

3.2.4 RNA isolation and gene expression analysis using reverse transcriptase PCR and real time quantitative PCR.

3.2.4.1 TRIzol® extraction

RNA was isolated using 2.5ml TRIzol® (Invitrogen Life Technologies, New York, USA) from near confluent cells in a T25cm^2 flask according to the manufacturer's instructions. Briefly, monolayers were rinsed with PBS which was then aspirated from the flask. TRIzol® was dispensed onto the monolayer and cells homogenised in the solution by repeat pipetting. After the addition of chloroform and centrifugation, an aqueous and organic phase separated. RNA remains exclusively in the upper aqueous phase and can be purified with subsequent precipitation and washing in isopropyl alcohol and ethanol. Total RNA was dried to a pellet in a microfuge tube (Sarstedt, Numbrecht, Germany) and resuspended in 100ul water for irrigation (Baxter, Australia). Cell lysate in TRIzol® was stored at -80^{0}C for several days if required.

In triplicate, 1 μl was diluted 1/80 in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.4) for quantification of total RNA with Genequant Pro (Amersham Biosciences, Uppsala Sweden). Purified samples were stored at -80°C until required.

3.2.4.2 Electrophoretic verification of RNA integrity

Integrity of each sample or the presence of genomic DNA contamination was assessed by agarose gel electrophoresis. 500 ng of each sample was diluted in 6 x RNA loading dye and resolved for 30 minutes at 100 volts on a 1.5% agarose gel in 0.5x TBE buffer (45mM Tris-Cl, 45mM boric acid, 1mM EDTA, pH 8.0), containing 10 μg/ml^{-1} ethidium bromide. Quality of the preparation was determined by visualization of distinct 28S and 18S rRNA species.

3.2.4.3 Removal of DNA contamination

Total RNA was purified in a 50μl reaction using TURBO DNA-free (Ambion,TX USA) according to the manufacturer's instructions. Briefly 10 μg RNA, 5μl 10x TURBO DNase buffer and 1 μl TURBO DNase were incubated at 37°C for 30 minutes. 5μl DNAse Inactivation Reagent was added, mixed and left at RT for 2 minutes, followed by centrifugation to remove the inactivation complex.

Again, in triplicate, 1 μl was diluted 1/80 in TE buffer for quantification using a Genequant Pro.

3.2.4.4 Reverse transcription of total RNA to cDNA

cDNA was synthesised using 2μg purified total RNA at 37^0C for 60 minutes in a reaction with Omniscript Reverse Transcriptase (Qiagen), 15-17mer oligo dT (Invitrogen), 10 Units RNase Out Recombinant Ribonuclease Inhibitor (Invitrogen) and 0.5mM of each dNTP (Roche, Basel, Switzerland). Template was diluted 4x in water for irrigation (Baxter Healthcare, Old Toongabbie, Australia) for use in all reactions.

3.2.5 ERα , ERβ and Cyclophilin A primers

Gene sequences were obtained using the UCSC Genome Browser(Kent et al., 2002). Primer3 (Rozen and Skaletsky, 2000) and Oligo Calc (Kibbe, 2007) were used in the generation of primers and to calculate annealing temperatures. ERα specific primers were designed to amplify a 90 bp region in the 3' UTR region of the 1080bp cDNA of ERα (cDNA NM_ 000125). ERβ specific primers amplified a 144bp region in the 3'UTR of the 2169 bp cDNA (AB209620). Primers were synthesised by Geneworks (Thebarton, Australia). The use of primers in the 3'UTR enabled detection without interference from splice variants. A 139 bp amplicon of Cyclophilin A was validated and used as a reference gene to normalise data instead. Cyclophilin A was reported previously as being non responsive to estradiol(Arbogast and Hyde, 2000; Uray et al., 2004). Primer sequences are shown below in Table 3.1.

Gene	Forward Primer	Reverse Primer
ERα	5' GGGAAAAGGCTCAAATGC 3'	5' GACAAAACCGAGTCACATC 3'
ERβ	5' GGGCAGGGTGAAAAAGAAAC 3'	5' CACCGTGTATCCAAAACTGG 3'
Cyclophilin A	5' GGTTGGATGGCAAGCATGTG 3'	5' TGCTGGTCTTGCCATTCCTG 3'

Table 3.1: Primer sequences for PCR

3.2.6 Reverse transcription PCR

A gradient annealing temperature of 52- 66^0C was used in PCR reactions to determine the optimum primer annealing temperature. Reactions used 10μl 2x Quantitect SYBR

Green PCR master mix (Qiagen, Venlo, Netherlands), 1μl each of 5μM forward and reverse primers (Geneworks, Adelaide, Australia), 1 μl of template and 7 μl water (Baxter Healthcare, Old Toongabbie, Australia). PCR product was resolved and visualised on EtBR-stained 1.8% agarose gel using a LAS-4000 imager (FujiFilm Global, Tokyo, Japan). A 100 bp molecular weight marker (Qiagen) was used to determine amplicon sizes. Annealing temperatures were chosen by inspection of the gel for the most abundant amplicon without the formation of primer dimers.

3.2.9 Primer set amplification efficiency

Primers to be used in real time quantitative polymerase chain reaction (RT-qPCR) were initially qualified for their specificity and amplification efficiency (AE). MCF7 and T-47D cDNAs were pooled and serially diluted 1:10 eight times. Reactions were performed in triplicate on three separate occasions (n=3) with 10μl 2x Quantitect SYBR Green Master Mix (Qiagen), 1μl each of 5μM forward and reverse primers (Geneworks, Adelaide, Australia), 0.25 μl cDNA and 7.75 μl water (Baxter Healthcare, Old Toongabbie, Australia). Amplification was carried out on the Rotorgene 6000 cycler (Corbett, Australia) using Rotorgene 6 software (Corbett Research, Sydney, Australia) and the following protocol: enzyme activation (15 minutes, 95°C), then 40 cycles of denaturation (15 seconds, 94°C), primer annealing (20 seconds, ER- 52°C, Cyclophilin A – 60°C), and extension/fluorescence acquisition (20 seconds, 72°C). Data were transferred to an Excel macro designed by George Mayne and repeated on three separate occasions. Serial template dilution resulted in standard curves that had a linear regression value to be $R^2 > 0.98$. Amplification Efficiency (AE) was accepted if in the range of 1.9 - 2.1.

Melt curve analysis was performed using incremental increases in temperature from 72°C to 95°C, held for 5 seconds per degree.

3.2.10 ERα and ERβ RT-qPCR

Reactions were performed with 10μl 2x Quantitect SYBR Green Master Mix (Qiagen), 1μl each of 5μM forward and reverse primers (Geneworks, Adelaide, Australia), 0.25 μl cDNA and 7.75 μl water (Baxter Healthcare, Old Toongabbie, Australia). Reactions were performed in triplicate from three separate total RNA extractions. The gene of interest expression data was normalised by dividing the average quantification cycle (Cq) of each gene by that of the reference gene, Cyclophilin A.

3.2.10 ERα and ER β protein detection

3.2.10.1 Protein lysates

Total protein was extracted from a 80-90% confluent T25cm^2 flask of each cell line using modified RIPA (mRIPA) buffer (Appendix 2). Cells were rinsed with cold PBS (Appendix 1) and 500μl mRIPA was added to each flask on ice for 15 minutes with occasional rocking. The cell lysate was centrifuged at 16000xg for 15 minutes at 4^{0}C and the supernatant retained.

3.2.10.2 Protein quantification

Protein assays were performed using bovine serum albumin (BSA) standards, diluted in mRIPA from 0 to 500 μg/ml. It was found that mRIPA interfered with the Bradford Assay resulting in non-linear standard curves.

The Bicinchoninic Acid (BCA) Protein Determination Kit (Pierce, Illinois, USA) is a detergent- compatible assay based on the alkaline reduction of Cu^{2+} to Cu^{1+} by proteins, and the formation of a bicinchoninic acid:Cu^{1+} complex(Smith et al., 1985). The complex has an absorbance maximum at 562 nm. Each sample was quantified according to the manufacturer's instructions in duplicate using a 96 well plate and an automated microplate reader.

3.2.10.3 Protein electrophoresis

10μg protein from each cell type was boiled for 5 minutes with reducing 3x reducing sample buffer and loaded into a handcast 4-12% resolving SDS polyacrylamide gel (Appendix 3). 1μl MagicMark XP (Invitrogen) was used as a protein size marker. BioRad mini PROTEAN 3 cells were used to resolve the protein at 200V for approximately 40 minutes. MagicMark XP^{TM} protein molecular weight standard was resolved alongside samples.

3.2.10.4 Detection of ERα and ERβ by western analysis

Proteins separated on SDS-PAGE were transferred onto PVDF membrane in Transfer Buffer (Appendix 2) using an ice-cooled mini-Protean II cell (BioRad) for 1.5 hours at 100V on a magnetic stirrer. The transfer was checked using Ponceau S stain (0.2% w/v) in acetic acid (10% v/v).

Membranes were left at 4° C overnight in Blocking Buffer (Appendix 2). Membranes were rinsed 3 three times for 10 minutes each time in Tris-Tween (Appendix 2).

Membranes were incubated with a 1:500 dilution of rabbit anti-ERα polyclonal antibody (HC-20) (Santa Cruz Biotechnology, Inc., U.S.A.) or 1:200 dilution of rabbit anti-ERβ polyclonal antibody (H-150) or goat anti-ERβ polyclonal antibody (L-20) in Blocking Buffer for 1 hour at 37°C with gentle rocking. This was followed by 3 x 10 minute rinses in Wash Buffer (Appendix 2).

The goat anti-Rabbit HRP secondary antibody (Sigma) was diluted 1:10000 with Blocking Buffer and incubated with the membrane for 1 hour at 37°C with gentle rocking.

After washing in Tris-Tween (Appendix 2), ECF substrate (GE Healthcare) was added to each membrane after Tris-Tween had been drained from the membrane. ECF was added at approximately 1mL per 10cm ^{2} membrane. The membrane was left in darkness to develop for 5 minutes. Fluorescence of the HRP bound ECF was visualised on a Typhoon imager (Amersham Biosciences)

The blot was repeated on three separate occasions (n=3) and quantified by pixel count on image J software (Abramoff et al., 2004)

3.2.11 T-47D-Beta recombinant cell line

Since ERβ was not detected in any cell line by either qPCR or western blotting, a control T-47D recombinant ERβ positive cell line was constructed. T-47D cells were plated to near confluence in a 6 well plate and allowed to attach. The following day media was replaced with OptiMEM (+ 10% FCS) and cells were transfected with 4μg ERβ-FLAG plasmid (Hartman et al., 2009) using 10μl LipofectAmine2000 (Invitrogen) as instructed by manufacturer. Transfected cells were seeded at low density and selected using 1.5 mg/ml G418 for 7 days. Cells were challenged periodically with 1.5mg/ml G418 to maintain expression.

3.3 Results:

3.3.1 Cell adherence time

Cells (5000/well) were left to adhere to the growth surface of a 96 well plate for 4, 6 and 24 hours. The number of viable cells was determined using the MTT assay after each of these times.

For MCF7, T-47D and H23 cell lines mean absorbance increased after 6 hours compared to 4 hours (Figure 3.1). The mean absorbance of Ovcar, RL95-2 and ZR-75-1 cell lines did not change after 6 hours compared to after 4 hours.

In all cell lines the absorbance was highest after 24 hours.
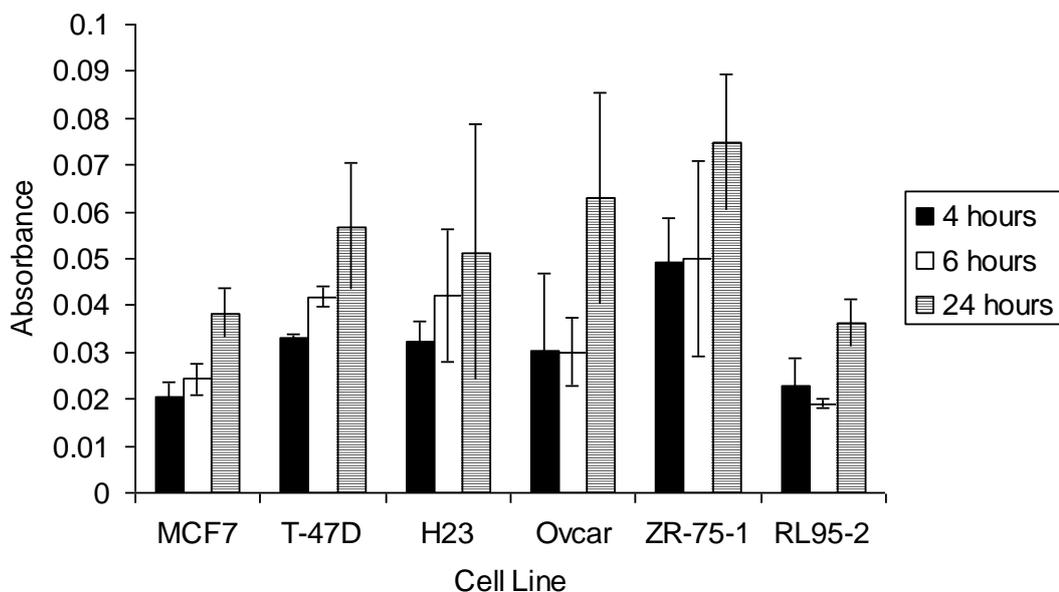
Figure 3.1. A comparison of attachment of each cell line to the growth surface after 4, 6 and 24 hours.

5000 cells per well of MCF7, T-47D, H23, Ovcar, ZR-75-1 and RL95-2 were cultured in a 96 well plate for 4 ■ ,6 □ and 24 ⊟ hours in six replicate wells. Media were aspirated and replaced with 0.5 mg/ml MTT supplemented media for 1 hour. Acidified SDS was added to each well for 1 hour and absorbance read at 570nm (reference 640nm). The experiment was repeated on three separate occasions (n=3) and the means and standard deviations are shown (n=3).

3.3.2 Optimisation of standard curves

Serial dilutions of cells from 1250 to 80000 cells/well were allowed to attach to the growth surface of 96 well plates and the MTT assay was applied after 4, 6 or 24 hours. A linear relationship between the number of cells per well and formazan production after 4 and 6 hours attachment for all cell lines was observed ($R^2 \geq 0.95$). After 24 hours ZR-75-1 and Ovcar lower density cells had proliferated sufficiently to reduce linearity of the standard curve. Data from three separate experiments were used to determine 95% confidence intervals (CI) of standard curves, represented in Figure 3.2, where linear regression indicates the 95% CI of the data. The 95% CI in MCF7, T-47D and Ovcar cell lines was lowest after 6 hours attachment compared to after 4 or 24 hours. This represents the time for the most accurate and reproducible standard curve.

The 95% CI of ZR-75-1 was smaller after 4 hours than after 6 or 24 hours. The slope of the ZR-75-1 cell line standard curve decreased as time increased, whereas the slope of the other cell line standard curves were relatively similar at each time point.

Figure 3.2. A comparison of standard curves of MCF7, T-47D, ZR-75-1 and Ovcar cell lines after 4, 6 or 24 hours.

In a 96 well plate, 1250 to 80 000 cells per well were left to attach to the growth surface for 4, 6 or 24 hours, after which media were aspirated from wells and replaced with 0.5mg/ml MTT supplemented media. Cells were incubated for 1 hour before the addition of acidified SDS. Absorbance was measured at 570nm (reference 640nm) and data exported to GraphPad Prism. The mean (shown by solid line ——) of 3 separate experiments was used to calculate a 95% confidence interval (shown by dotted line ----) for each cell line at 4, 6 or 24 hours using linear regression.

3.3.3 Cell proliferation

The equations that described the relationship between cells/well and absorbance after 4 or 6 hours culture were used to convert absorbance after 24, 48 and 72 hours to actual cell number. Cell number per well was plotted against incubation time for each cell line. After 72 hours, growth was limited in the 40000 and 80000 cells per well plates due to cells reaching confluence in the well. The absorbance and cell densities of 1250, 2500, 5000, 10000 and 20000 cells per well consistently increased for the period of the assay (Figure 3.3).

3.3.4 Cell doubling time

Using the cell number after 72 hours, doubling times (hours) were calculated. The values in Table 3.2 are the rate to be expected from cells grown under optimal conditions. Maximum achievable growth rates are dependent on the initial cell density in the wells, but are often reported for only one initial cell density. In our cell culture conditions when initial cell densities were 5000 cells/well, RL95-2 (29.8 hours) and H23 (32.0 hours) had the fastest doubling time and T-47D the slowest (41.6 hours). At the highest initial cell density possible without reaching confluence in the wells after 72 hours, 20000 cells/well, H23 (34.4 hours) was the fastest and ZR-75-1 was the slowest (80.6). The change in doubling times is evident as the ZR-75-1 cells had a more than two fold increase in doubling time between 5000 and 20000 cells/well whereas T-47D, RL95-2 and H23 only slightly increased. All cells increased their doubling time as the initial cell density increased, except for T-47D which decreased at 10000 cells/well.

Figure 3.3: Proliferation of MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 for 72 hours from initial densities of 5000, 10000 and 20000 cells/well.

In a 96 well plate, 5000, 10000 and 20000 cells per well were left to attach to the growth surface in normal media. After 4 (ZR-75-1 cell line standard curve), 6 (remaining cell line standard curves), 24, 48 or 72 hours, media were aspirated from wells and replaced with 0.5mg/ml MTT supplemented media. Cells were incubated for 1 hour before the addition of acidified SDS. Absorbance was measured at 570nm (reference 640nm). The equations that described the relationship between cells/well values and absorbance after 4 or 6 hours culture was used to convert absorbance derived after 72 hours to actual cell number. The experiment was repeated on three separate occasions (n=3) and the mean ± standard deviations are represented.
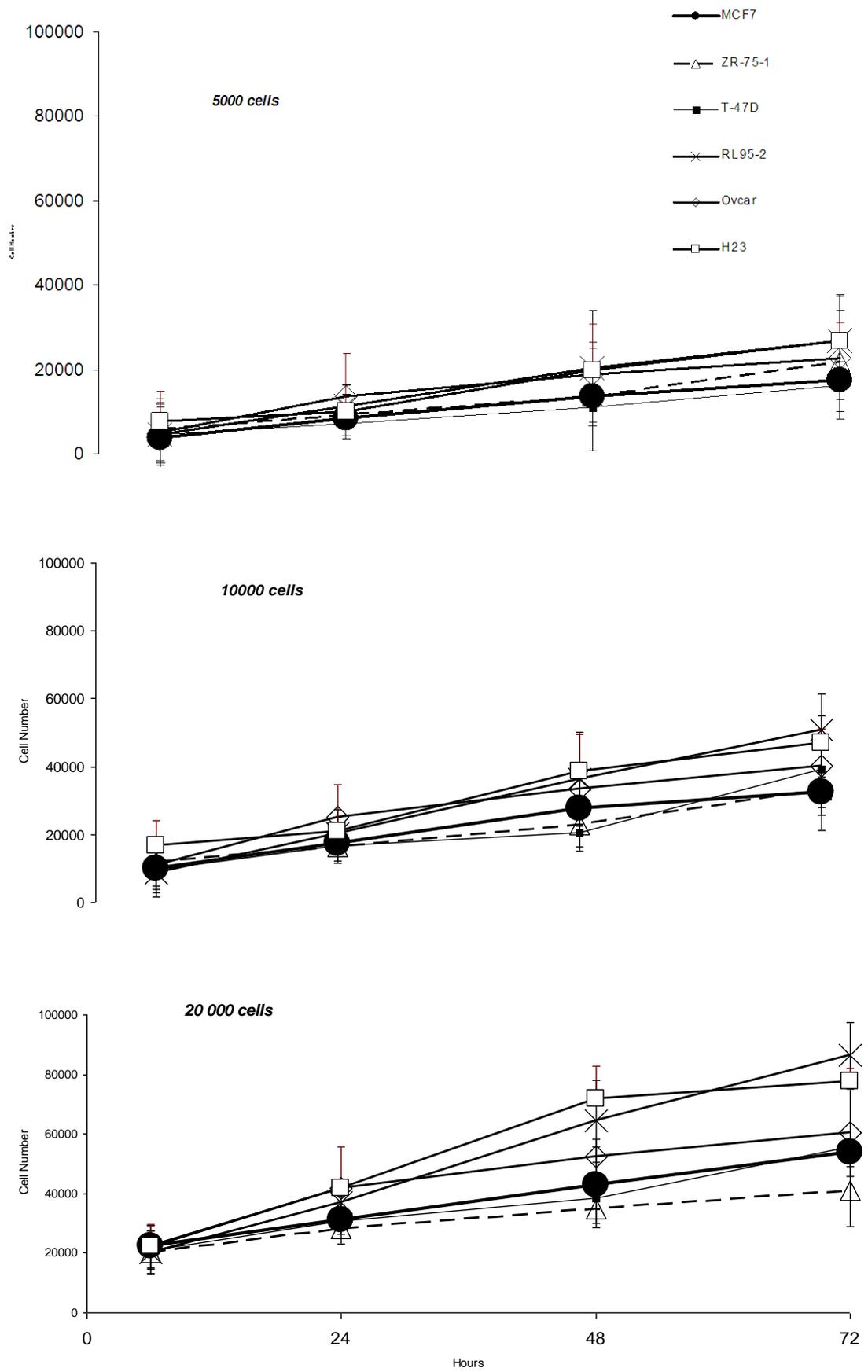
Cell Line	Doubling Time (hours)		
	5000	10000	20000
MCF7	41.6 ± 8.9	42.3±1.8	50.9 ± 4.6
ZR-75-1	36.9 ± 11.9	46.2±16.0	80.6 ± 32.2
T-47D	36.0 ± 5.1	31.0±2.7	40.6 ± 2.8
Ovcar	32.9 ± 0.7	36.0±2.2	48.4 ±13.9
H23	32.0 ± 7.7	33.3±5.1	36.8 ±1.5
RL95-2	29.8 ± 1.8	30.9±2.1	34.4 ± 2.8

Table 3.2: The doubling time (hours) of each cell line at three initial densities of 5000, 10000 and 20000 cells/well.

The slope obtained from the linear regression of the 4hr (ZR-75-1 cell line) or 6hr (remaining cell lines) attachment plate was used to calculate the final cell number per well of each cell line from absorbance at 72 hours. An initial density of 5000 cells per well was compared to the cell density at 72 hours on three separate occasions (n=3) to calculate a doubling time, except T47D which was repeated on two separate occasions.

3.3.5 Intra and inter coefficient of variation

The CV was derived from cell number after 72 hours of culture from three separate repeated experiments (Figure 3.4). The intra-assay CV measures the variation between replicate wells of a single experiment. ZR-75-1 had the lowest intra-assay CV at 5000 (6.4%), 10000 (3.9%) and 20000 cells/well (3.0%)

At the initial cell density of 5000 cells/well the intra-assay CV was highest in the RL95-2 cell line (11.8%). At 10000 and 20000 cells/well MCF7 had the highest CV (15.5% and 12.9% respectively).

The inter-assay CV was used to assess variation between repeat experiments (Figure 3.4). The lowest variation between separate experiments after 72 hours when the initial cell density was 5000 cells/well was generated by Ovcar cells (3.5%). At higher cell densities of 10000 and 20000 cells/well the lowest inter-assay CV was generated using MCF7 cells (4.8%) and H23 cells (5.5%). At each initial cell density T-47D gave the highest inter-assay CV (>40%).

Figure 3.4: Intra- and inter-assay coefficients of variation (CV) after 72 hours of culture of MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 cells with initial densities of 5000, 10000 and 20000 cells/ well.

In a 96 well plate, 5000, 10000 and 20000 cells per well were left to attach to the growth surface in normal media. After 4, 6 or 72 hours, media were aspirated from wells and replaced with 0.5mg/ml MTT supplemented media. Cells were incubated for 1 hour before the addition of acidified SDS. Absorbance was measured at 570nm (reference 640nm). The equations that described the relationship between cells/well values and absorbance after 4 or 6 hours culture was used to convert absorbance derived after 72 hours to actual cell number. The cell numbers were used to calculate intra- and inter-assay Coefficients of Variation (CV). The mean intra (CV) ± standard deviation is represented and the mean inter (CV) is represented for the assay performed on three separate occasions.
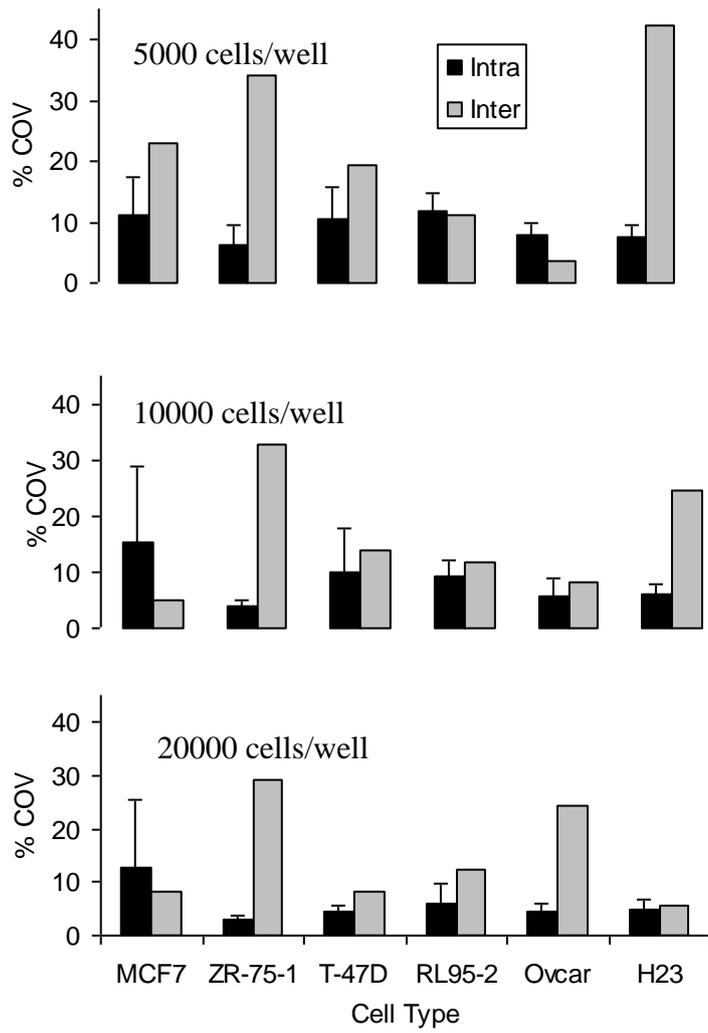
3.3.6 Amplification efficiency and qualification of primers

Serial dilutions of cDNA template in RT-qPCR reactions allowed the assessment of the amplification characteristics of primers sets (Figure 3.5). A linear regression of quantification cycle (Cq) values against a log scale of arbitrary copy number i.e. template dilutions had R^2 values greater than 0.95 for Cyclophilin A and ERα. Primers specific to ERβ did not produce a standard curve with an $R^2 > 0.95$ despite optimisation of annealing temperature. Amplification Efficiency (AE) for Cyclophilin A primers was 2.1 and ER α primers was 2.0.
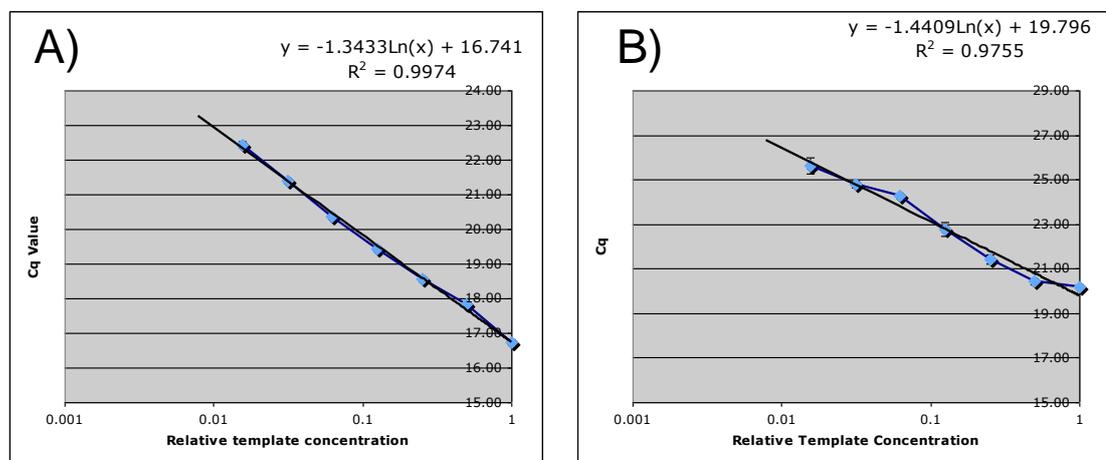
Figure 3.5: Amplification efficiency of Cyclophilin A and ERα primer set.
Combined MCF7 and T-47D cDNA was serially diluted ten fold. RT-qPCR was performed on the diluted template and the Cq plotted against a log scale of arbitrary copy number (Relative template Concentration). The primer sets specific to Cyclophilin A (A) and ERα (B) were tested for their amplification efficiency. As separate dilution series were made for each replicate, initial Cq changed, therefore the mean of duplicates performed on one representative reaction are shown here.

3.3.7 Amplification of target gene within each cell line

3.3.7.1 Cyclophilin A

The amplicon specific to Cyclophilin A was amplified in each cell line. The melt curve analysis (Figure 3.6) shows that a single product was amplified in the reaction only.
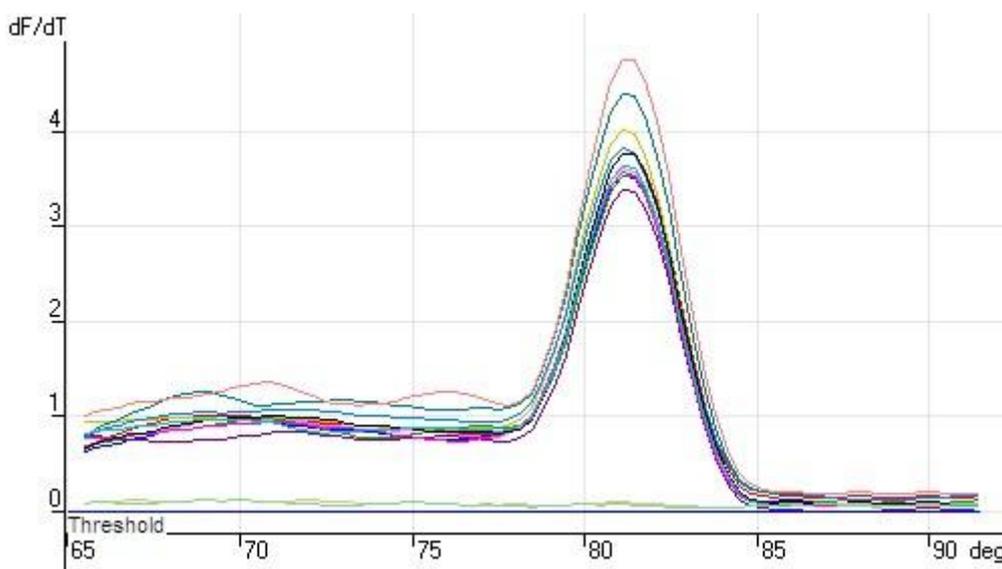
Figure 3.6: Melt Curve Analysis of Cyclophilin A amplicon from MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 cell lines.

cDNA from each cell line was used as template in a RT-qPCR reaction with primers specific to Cyclophilin A. Each line represents one of a duplicate reaction for each cell line. All cell lines had the same amplicon. Negative control reactions produced no amplicon or primer dimerisation.

3.3.7.2 ERα

The amplicon specific to ERα was amplified in each cell line except H23 and RL95-2. RL95-2 had no amplification product whereas H23 showed two separate products not consistent with the ERα amplicon. The melt curve analysis (Figure 3.7) shows each cell line and negative control in which no amplicon was present at the end of the reaction.
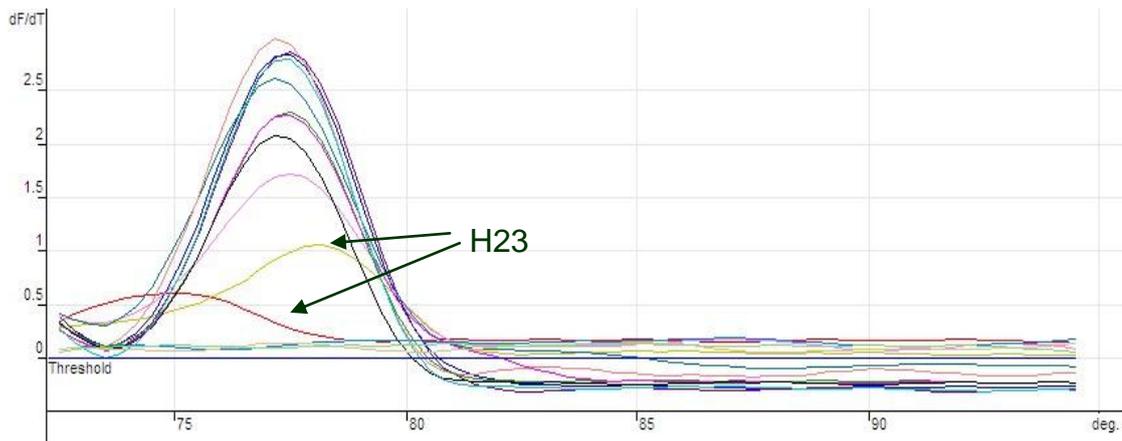
Figure 3.7: Melt Curve Analysis of ERα amplicon from MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 cell lines. cDNA from each cell line was used as template in a RT-qPCR reaction with primers specific to ERα. Each line represents one of a duplicate reaction for each cell line. All cell lines had the same amplicon except H23. Negative control reactions produced no amplicon or primer dimerisation.

3.3.7.3 ERα – Verification of RT-qPCR amplicon

Reaction mixtures from the above RT-qPCR were resolved on a 1.8% agarose gel (Figure 3.8). The single amplicon can be see in MCF7, T-47D, ZR-75-1 and Ovcar. .
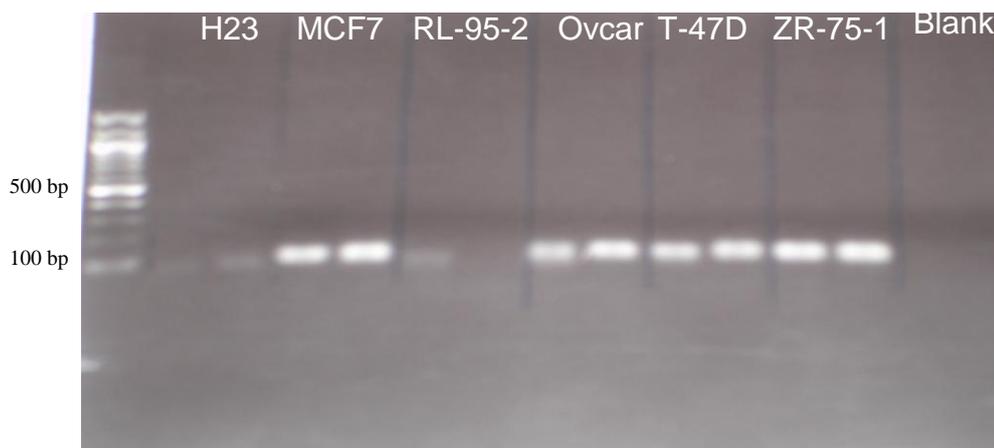
Figure 3.8: RT-qPCR using ERα primers resolved on 1.8% agarose gel
The 90bp amplicons from the reaction using the ERα primers were resolved on a 1.8% agarose gel stained with EtBr.

3.3.7.2 Relative expression of Estrogen Receptor Alpha mRNA

Total RNA was extracted and reverse transcribed on three separate occasions. cDNA from each cell line was used in a RT-qPCR reaction with primers specific to ERα. ERα expression data was normalised by dividing the corresponding levels of Cyclophilin A for each sample (Figure 3.9).

MCF7 had the highest relative expression level of ERα mRNA (100%) followed by T-47D (97.7%), ZR-75-1 (85%) and Ovcar (67%). ERα mRNA was not detected in RL95-2 or H23 cells.

Comparative Expression of ER alpha mRNA
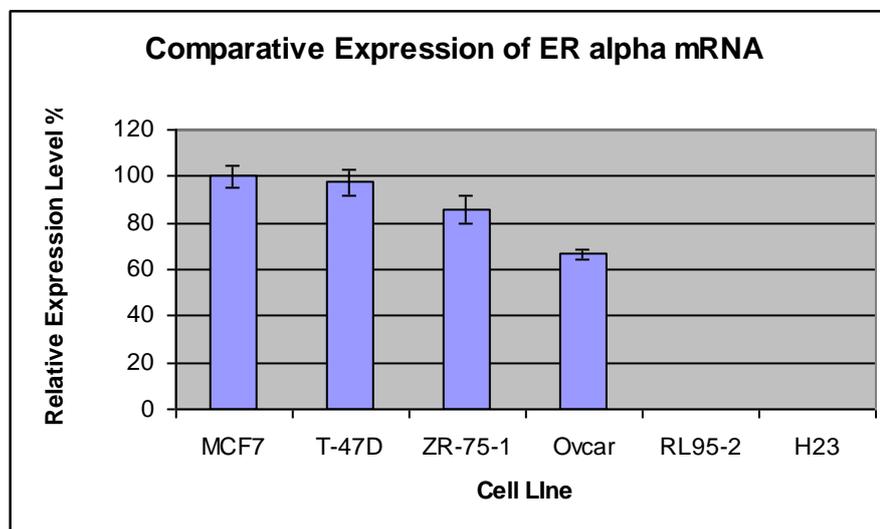
Figure 3.9: Relative abundance of ERα mRNA compared with the reference gene Cyclophilin A in MCF7, T-47D, ZR-75-1, RL95-2, Ovcar and H23.

RT-qPCR reactions using primers specific to a 126bp amplicon in the 3'UTR of ERα were performed using reverse transcribed RNA from each cell line as a template. Values are means ± standard deviations from at least three separate total RNA extractions and reverse transcriptions normalised by dividing by the corresponding levels of Cyclophilin A expression for each sample.

3.3.8 Estrogen Receptor Beta mRNA

Reverse Transcriptase PCR showed that the 144bp amplicon in the 3'UTR of ERβ was not identifiable in any cell line except the transfected control cell line T-47D-ERβ (Figure 3.10).
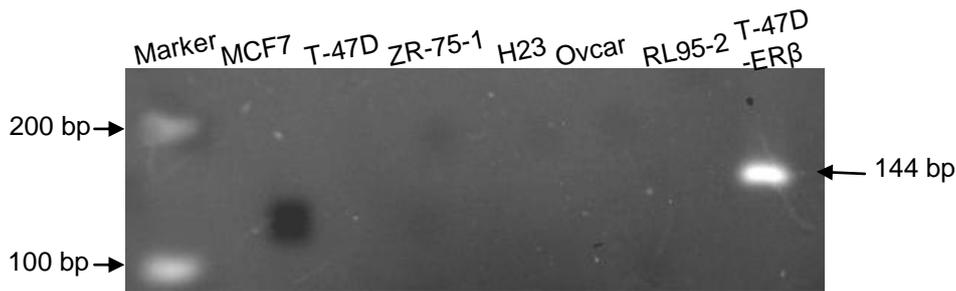
Figure 3.10: Reverse Transcription PCR of ERβ.

Total RNA was extracted from MCF7, T-47D, ZR-75-1, H23, Ovcar, RL95-2 and T-47D-ERβ and reverse transcribed on three separate occasions. A PCR reaction using primers specific to a 144bp amplicon of ERβ was performed using reverse transcribed RNA from each cell line as template. The PCR reaction was resolved on a 1.8% agarose gel. The 144bp ERβ amplicon is only present in the control cell line T-47D ER beta.

3.3.9 Estrogen Receptor Alpha protein expression

Western blot analysis of protein lysates from each cell line identified the 66kDa ERα protein in MCF7, T-47D and ZR-75-1 cells (Figure 3.11). H23, RL95-2 and Ovcar did not show expression of the 66kDa full length protein. A band at ~90KDa was also present in all cell lines including CCD-1064Sk the negative control. Densitometry of blots performed on 3 separate occasions showed that expression of ERα in other cells relative to that in MCF7 was MCF7 (1) > T47D (0.74±0.18) > ZR-75-1 (0.36±0.12).

Interestingly ERα mRNA in the Ovcar cell line did not coincide with expression of the ERα protein. Western blot analysis of nuclear extracts produced very similar results (data not shown).
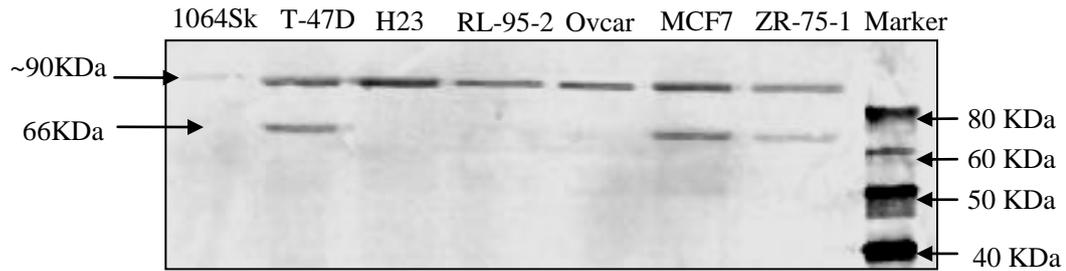
Figure 3.11: Western blot of MCF7, ZR-75-1, T-47D, H23, Ovcar, RL95-2 and 1064Sk probed with anti-ERα (HC-20).

Total protein was extracted from near confluent monolayers of MCF7, ZR-75-1, T-47D, H23, Ovcar, RL95-2 and 1064Sk in T25cm^2 flasks. Protein concentrations were quantified using a BCA Protein Determination Kit and BSA standards. 2ug of protein per cell line was resolved using SDS/PAGE. Protein was transferred to a PVDF membrane. A rabbit polyclonal antibody to ERα (HC-20) identified the 66KDa protein in MCF7, ZR-75-1 and T-47D protein lysate only. CCD-1064Sk was used as a negative control in which the 90KDa also appeared. A ~90 KDa band was also present in all cell lines, albeit weakly in CCD-1064Sk protein isolate.

3.4 Discussion:

This study assessed the human cell lines MCF7, T-47D, ZR-75-1, RL95-2, Ovcar and H23 for their application in estrogenic response assays. Although many studies use one or more of these cell lines, this study directly compared the estrogen receptor α and β mRNA and protein expression and also the growth characteristics of the cell lines in a 96 well plate format.

Cell attachment was assessed by dispensing 5000 cells/well into a 96 well plate. Media were removed from the wells after 4, 6 and 24 hours at which time cells that were not attached to the growth surface were also removed. Media were replaced with media supplemented with MTT, which cells metabolise to formazan, the absorbance of which is proportional to the number of viable cells (Mosmann, 1983). In MCF7, T-47D and H23 cell lines, absorbance increased at 6 hours compared to 4 hours. An increase in absorbance due to proliferation between 4 and 6 hours would be expected to be minimal

and therefore would be a result of a greater number of cells attaching to the growth surface and metabolising the MTT.

The absorbance of ZR-75-1, RL95-2 and Ovcar was similar after 6 hours compared to 4 hours indicating no increase in attachment. One replicate of Ovcar after 4 hours showed much higher absorbance than the other two replicates at this time point, generating a higher mean which may have been due to pipetting variability or 'clumping' of cell. Cells that remain joined together in small groups after trypsinisation and resuspending can result in counting errors and a higher than calculated number of cells being dispensed to each well.

Absorbance after 24 hours was highest in all cell lines. Although all viable cells would have attached after this time, it would also be approximately half of a doubling cycle. The absorbance data at this point would therefore also be inclusive of an expanded population of cells.

After 6 hours, all cells were presumably closer to their normal metabolic rate and less involved with attachment functions, which may need to be considered when deriving cell numbers using a metabolic indicator such as the MTT assay. It has been shown that the metabolism of MTT in conditions challenging to the cell can alter the metabolism of MTT (Mothersill and Austin, 2003). Similarly, cells may have an altered metabolic rate as they attach to the growth surface leading to inaccurate cell numbers being derived from absorbance. MTT should be used with caution when cells are at high confluence or exposed to mixtures which may inhibit cell metabolism resulting in under reporting of cell numbers (Marshall et al., 1995; Mothersill and Austin, 2003). The use of cell quantification techniques such as nuclear counts or cell/protein staining methods such as crystal violet (Gillies et al., 1986) or SRB (Skehan et al., 1990) may in this case be able to elucidate the process.

A linear relationship between initial cell number from 1250 to 80000 cells and absorbance was established after 4 and 6 hours attachment time ($R^2 > 0.95$). The use of a standard curve must not only capture the correct number of cells but also be reproducible. MCF7, T-47D and Ovcar gave a smaller 95% CI after 6 hours attachment than 4 hours. Greater cell attachment was shown after 6 hours in MCF7 and T-47D and

similar amounts in Ovcar. 6 hours was the best time after which to perform a standard curve in these cell lines. ZR-75-1 cells however, did not increase attachment after 6 hours compared to 4 hours and had the smallest 95% CI after 4 hours. The differences in attachment in ZR-75-1 between 4 and 6 hours are minimal and therefore this cell line would be best represented by a standard curve after 4 hours. Interestingly, the slope of the ZR-75-1 standard curve reduced after 6 and 24 hours compared to after 4 hours. This was possibly due to how ZR-75-1 cell metabolism was affected during the attachment process. ZR-75-1 cells after 4 or 6 hours attachment had at least twice the absorbance than MCF7 cells. Given the identical species and tissue origin of the cells, we would have to assume either ZR-75-1 cells were twice as active at metabolising MTT to formazan or twice as many cells were attaching to the growth surface than MCF7 cells, neither of which is known.

In this study a side-by-side comparison of the growth rates achievable in optimal media conditions was made. The doubling times shown in this study for each cell line show the differences that initial cell density can make to a reported doubling time. ZR-75-1 had a doubling time of 36.9 ± 11.9 hours when an initial density of 5000 cells/well was used compared to 80.6 ± 32.2 hours when initially 20000 cells/well were used. This potentially was the result of inter-cell signalling and availability of media components. MCF7 and T-47D doubling times were similar to those reported previously when 5000 cells/well was the initial cell density, however, individual sub-lines, growth conditions, media preparation techniques and normal batch variation could all effect the actual growth rate of cells (Osborne et al., 1987; Villalobos et al., 1995). Confluence within wells was not a factor in these calculations as determined by visual inspection of the wells. Initial cell numbers exceeding 40000 cells per well led to non-linear responses to MTT as cells reached confluence and reduced their metabolism of MTT in line with their general metabolism being reduced as the cells entered stationary phase.

ERα mRNA was shown to be present at the highest levels in MCF7 followed by T-47D, ZR-75-1 and Ovcar. Although H23 and RL95-2 have both previously been shown to express ERα (Way et al., 1983; Pietras et al., 2005) it could not be detected in this study.

Western blotting confirmed that the 66 KDa ERα protein was present in MCF7, ZR-75-1 and T-47D cells. Interestingly, Ovcar showed ERα mRNA expression which was not translated into protein. This was shown using multiple extractions and total and nuclear protein extraction methods. A block to translation may be present in the line of Ovcar cells we have used.

The band present in all samples and very weakly in the negative control at ~90 KDa, has been investigated in other studies (Pavao and Traish, 2001; Stirone et al., 2003; Nonaka et al., 2009) and occurs with use of the COOH-terminal (HC-20) antibody to ERα. In fact, bands at ~100, 93, 82 and 50 KDa have all been described with use of this antibody in western blotting (Stirone et al., 2003). Despite thorough blocking, titration of primary and secondary antibodies and the use of PVDF and nitrocellulose membranes the band remained present. Although this band has been reported in literature as non-specific binding (Nonaka et al., 2009) it has also been shown that immunoreactivity with this band is due to specific ERα antibody interactions (Stirone et al., 2003; Nonaka et al., 2009). Given that it is a higher mass than the 66 KDa protein, degradation is not a cause. Post translational modifications of ERα such as phosphorylation of Serine[118] would result in an 83KDa band (Stirone et al., 2003). Primers used in RT-qPCR specific to ERα were designed in the 3' UTR and able to detect all splice variants meaning that the band detected was not a splice variant. The major splice variant of ERα that is transcribed at 45 KDa was not seen in the western blot in this study. Whilst the presence of the 45 KDa ERα protein may have consequences for cellular signalling after activation it does not impact on proliferative responses (Barraille et al., 1999). The higher molecular weight band needs further characterisation as to its identity and what, if any, implications it has in estrogen signalling.

Although ERα has been the focus of the majority of assays it would also be useful to characterise cell lines that express ERβ. It was expected that ERβ could be found in some of the cell lines chosen but unfortunately all of the cell lines in this study did not express ERβ at the mRNA level. Conflicting information about the expression of ERβ has been reported for MCF7 cells previously (Jorgensen et al., 2000) .

The characteristics of attachment time, proliferation, inter-assay variability and expression levels of the ERα and β in a cell line are useful tools in the selection and application of a cell line to be used for the development of (xeno)estrogen testing assays. In this study, of the cell lines examined (ZR-75-1, H23 and T-47D, Ovcar, MCF7 and RL-95-2), it was found that MCF-7 > T-47D > ZR-75-1 cell lines alone expressed the ERα protein critical for estrogenic response assays. Whilst ZR-75-1 had the fastest attachment time of 4 hours, compared to 6 hours for MCF7 and T-47D, it also had the highest inter-assay variability of the three. It was assessed therefore that MCF7 and T-47D cells were the best candidates for further characterisation, although for completeness of the study we also included the ZR-75-1 cell line.

CHAPTER 4

Assessment of MCF7, T-47D and ZR-75-1 cell lines for use in the E-screen

4.1 Introduction:

The E-screen is based on the observation that estrogenic stimuli exert a proliferative effect on MCF7 cells, and was developed to measure estrogenicity of compounds and environmental pollutants (Soto, 1995). MCF7 cells are placed in replicate wells of a plate and left to attach for 24 hours or longer in steroid-free medium. The medium is then changed to fresh steroid-free medium with the E_2 standards or the sample. After 5 to 7 days exposure, the number of cells is determined. The proliferative effect of the sample can then be compared to that of the E_2 standards. The E-screen has since been developed by different research groups, with different methodologies and components of the E-screen assay such as plate format, media components, pre-exposure period and the length of exposure and cell quantification techniques reported (Villalobos et al., 1995; Korner et al., 1999; Rasmussen and Nielsen, 2002). These differences in protocol are summarised in Table 4.1.

Two primary characteristics impacting the efficacy of the E-screen are expression of estrogen receptor (ER) and the ability of the cell to respond to estrogenic stimuli with increased proliferation. These two characteristics are regulated by the physiological status of the cell and by the *in vitro* culture conditions.

The mechanism underlying the proliferation of cells in response to an estrogenic stimulant are not completely understood (Bronzert et al., 1987; Aakvaag et al., 1990; Nilson et al., 2001), however, the stimulation of growth factor expression by estrogen has been described. Factors such as Transforming Growth Factor α (TGFα) and Insulin-like Growth Factor 1 (IGF-1) (Bronzert et al., 1987)may act in a paracrine and autocrine manner. Another mode of action possible is where estrogen neutralises factors inhibitory to proliferation which are present in serum, a common supplement to cell culture media (Soto and Sonnenschein, 1987).

Author/s	Cell Line	Culture Media	Attachment Conditions and Pretreatment	Exposure Conditions	Quantification
Ruedl et al., (1990)	MCF7 T-47D ZR-75-1	PR – DMEM/F12 + 5% FCS	24 well plate, 24hrs, 40000 – 100000 cells/well	PR- DMEM/F12 + 5% CD-FCS, 7 days	% DNA content
Soto et al., (1995)	MCF7	DMEM + 5% FCS	12 well plate, 24hrs, 20000 cells/well	PR- DMEM + 5% CD-HuS, 5 days	Nuclei counted with Coulter counter
Villalobos et al., (1995)	MCF7 BOS MCF7 (ATCC) MCF7 (BB) MCF7 (BB104)	DMEM + 5% FCS (MCF7 (BB104) : PR- DMEM + 10% DC-FCS)	24 well plate, 24 hrs, 10000 cells/well	PR- DMEM + 10% DC-FCS, 6 days	SRB total protein assay
Korner et al., (1999)	MCF7	DMEM + 5% FCS	24 well plate, 24 hrs, 10,000 cells/well	PR- DMEM + 5% DC-FCS, 6 days	SRB total protein assay and MTT
Rasmussen and Nielson (2002)	MCF7 BOS	DMEM + 10% FCS	96 well plate, 24hrs, 4500 cells/well	PR- DMEM + 5% CD-FCS, 7 days	SRB total protein assay
Rajapakse et al., (2004)	MCF7 BOS	DMEM + 5% FCS, 1% MEM-NEAA	12 well plate, 24 hrs, 20000 cells	PR-DMEM + 10% DC-FCS, 5 days	SRB total protein assay

Matsuoka et al., (2005)	T-47D	DMEM + 5% FBS	24 well plate, 24 hrs, 40000 - 60000 cells/well	PR- DMEM + 5% CD-FCS, 6 days	SRB total protein assay
vanLipzig et al., (2005)	T-47D	DMEM/F12 + 7.5% FCS	48 well plate, 24 hrs 12500 cells/well, 48 hrs pre treat in test medium	PR- DMEM/F12 + 5% DC-FCS, 5 days	SRB total protein assay
Leusch et al., (2006)	MCF7	PR- DMEM + 10% FCS	96 well plate, 24 hrs 20000 cells/well	PR- DMEM+ 10% CD-FCS, 5 days	Fluorescamine (protein content)
Vanparys et al., (2006)	MCF7 (ECACC)	DMEM + 5% FCS	96 well plate, 24 hrs, 2000 cells/well, 72 hrs pre treat in test medium	PR-DMEM + 5% DC-FCS, 6 days	CyQUANT assay (MolecularProbes, Belgium)
Oh et al., (2006)	MCF7 BOS	DMEM + 5% FCS	48 well plate, 48hrs, 5000 cells/well,	PR- DMEM + 10% DC-FCS, 6 days	SRB total protein assay
Tan et al., (2007)	MCF7	DMEM+ 10% FCS	96 well plate, 24hrs, 2000 cells/well	PR- DMEM+ 10% CD-FCS, 5 days	CellTiter 96 AQueous one Solution

deCastro and Neuberg (2007)	MCF7	DMEM + 5% FCS	12 well plate, 24 hrs, 30000-40000 cells/well	PR- DMEM + 5% DC-FCS, 5 days	Nuclei counted with coulter counter

Table 4.1: Summary of E-screen protocols.

E-screen assays were summarised. Cell lines were maintained in Culture Media as described. Certain number of cells/well were placed in various dimension plates and left to attach for 24 hrs in the test medium. In some cases, cells were attached in culture media for 24 hrs then pretreated for the described period of time in test medium (van Lipzig et al., 2005; Oh et al., 2006; Vanparys, 2006). Cells were exposed to E_2 or the test samples for the described period of time before quantification of final cell number or equivalent assay.

Abbreviations. ATCC: American Type Culture Collection, ECACC: European Collection of Cell Cultures, DC-FCS: Charcoal-dextran treated fetal calf serum, FCS: Fetal Calf Serum, CD-HuS: Charcoal-dextran treated Human Serum,
PR- : Phenol Red free, SRB- sulforhodamine B, MEM-NEAA – Modified Essential Medium non essential amino acids.

4.1.1 Fetal Calf Serum

Human serum was initially used as a supplement to media used in the E-screen, but fetal calf serum (FCS) is now most commonly used (due to availability) and has been shown to have no impact on sensitivity or response of cells used in the E-screen (Rasmussen and Nielsen, 2002). Fetal Calf Serum, as used in this study, is produced from blood collected by cardiac puncture. The whole blood is clotted, centrifuged and the supernatant filtered (Invitrogen). FCS contains a large number of components including growth factors, hormones, adhesion factors, minerals, lipids and transport/binding proteins that cells use to maintain viability and proliferation *in vitro* (Freshney, 2005).

Platelet- derived growth factor (PDGF) is released during the natural clotting of blood (Freshney, 2005). This mitogenic polypeptide is one of the major growth factors in FCS and can stimulate endothelial cell proliferation, migration and angiogenesis (Battegay EJ et al., 1994; Freshney, 2005). Other major growth factors include Fibroblast Growth Factors (FGFs), Epidermal Growth Factor (EGF) , Insulin like Growth Factor-I and -II, Fibroblast Growth Factor , Interleukin -1 and -6 (Aakvaag et al., 1990; Freshney, 2005). The presence of active receptors and signalling pathways on the cell determines what effect the growth factors will have.

The major hormones in FCS are hydrocortisone, insulin, triiodothyonine and thyroxine (Freshney, 2005). Another hormone in FCS of interest to this study is 17β-estradiol (E_2). The physiological concentration of E_2 in fetal calves is 50-130 pM (Peterson et al., 1975). E_2 has been measured using a commercial radioimmunoassay in preparations of FCS at 75 pg/ml (275 pM) (Fortunati et al., 1999), however, batch to batch variations would be expected due to variation in animals, climate and pasture conditions (Freshney, 2005).

Heat inactivation (56°C for 30 minutes) is often used in the routine preparation of FCS for cell culture use. Although the process itself is based on a standard procedure rather than a specific outcome (Giard, 1987) , it is thought to inhibit the activity of factors such as TGF-β or cytotoxic components (from bacteria) introduced during the production of FCS that are inhibitory to proliferation (Freshney, 2005).

4.1.2 Charcoal-dextran stripping of serum

FCS can be treated with a charcoal-dextran slurry as a means of binding and removing non-polar lipophilic material without affecting the salt, glucose or amino acids content (Invitrogen). Charcoal-dextran treatment reduces the concentration of many hormones, growth factors and cytokines such as estradiol, cortisol, corticosterone, the B vitamins, T3, T4 and prostaglandins found in FCS (Aakvaag et al., 1990; Dang and Lowik, 2005).

Charcoal-dextran treatment of FCS (DC-FCS) has been shown to reduce E_2 content by 97%, from 275pM (75 pg/ml) to 8pM (2.2 pg/ml)(Fortunati et al., 1999). We would therefore expect that cells that are estrogen-dependent for growth would significantly reduce their proliferation when media supplemented with FCS is instead supplemented with DC-FCS.

Although it was thought that heating during the charcoal-dextran treatment may release steroids from binding proteins and allow them to bind to the charcoal, it was found that charcoal-dextran treatment of serum without heating or heat inactivation, removed estrogen to a higher efficiency (Heringa et al., 2004).

4.1.3 Phenol Red

Phenol red (PR) has been shown to be weakly estrogenic. Although PR has an affinity to the ER that is 0.001% that of estradiol, a normal PR concentration in media (30μM) has been shown to increase the number of MCF7 cells over 8 days by 200% (Berthois et al., 1986). It is for this reason that medium is prepared without PR for use in estrogen-response assays.

4.1.4 ERα up-regulation in response to reduced estrogen concentration

The expression of the ERα, vital for a proliferative response to E_2, has been shown to be regulated by components of the medium in which the cells are grown, notably the estrogen content of the medium (Saceda et al., 1998).

A reduction in the estrogen content of the medium is correlated with an increase in the expression of ERα. Extended culture of MCF7 cells, obtained from the Michigan Cancer Foundation, in PR-free media supplemented with 10% DC-FCS increased ERα

expression 3-fold (Katzenellenbogen et al., 1995). Welshons et al (1987) showed by whole cell uptake of [^{3}H]estradiol, that MCF7 cells grown in PR-free media supplemented with 5% DC-FCS increased ER concentration from 2.9 pmol/mg DNA to 5.5 pmol/mg DNA after 3 days.

The up-regulation of ERα, after extended culture of MCF7 cells in PR-free medium supplemented with DC-FCS, also resulted in a loss of proliferative response to 10nM E_2 compared to MCF7 cells grown in normal complete medium. Another indicator of estrogenic exposure is the up-regulation of the progesterone receptor, a response that was maintained in these conditions (Katzenellenbogen et al., 1995).

A low E_2 environment is consistently shown in the literature to up-regulate the expression of ER in MCF7, T-47D and ZR-75-1 cells (Welshons, 1987; Daly and Darbre, 1990; Katzenellenbogen et al., 1995; Villalobos et al., 1995; Pink and Jordan, 1996). The relationship however, between ER expression and proliferative response to E_2 requires critical assessment. Katzenellenbogen et al (1995) showed using MCF7 cells that after one month of growth in estrogen-free medium, during which time growth was initially greatly reduced, the cells adapted and proliferated at a very rapid rate. This rate could not be further increased by E_2 (Daly and Darbre, 1990). The effect is either caused by altered regulation of growth factor production, or an altered sensitivity to paracrine factors or exogenous growth factors in the medium (Katzenellenbogen et al., 1995).

The 46kDa isoform of ERα has been shown to inhibit the proliferative effect mediated by the 66kDa ERα (Penot et al., 2005). This 46kDa isoform is not regulated by E_2 and has been shown in MCF7 cells to remain stable and have relatively low expression regardless of E_2 exposure.

It was shown that Cyclin E expression is decreased when T-47D cells are placed in estrogen-free media (Strom et al., 2004). Cyclin E is required for the progression of the cell cycle and it may be the down-regulation of Cyclin E and other cell cycle components, which result in decreased proliferation. It may in fact be the down regulation of Cyclin E, whose activity is required for cell cycle G1/S transition, in the absence of E_2 stimulation that decreases the proliferative effect (Strom et al., 2004)

4.1.5 Response of MCF7 (and MCF7 sub-lines), T-47D and ZR-75-1 to E_2-stimulated proliferation

'Wild type' MCF7 (ATCC) cells display a range of estrogen sensitivities. Clonal selection of populations that are highly responsive to estradiol has generated sub-lines of MCF7 cells that display increased estrogen responsiveness over a wide range of passages (Rasmussen et al., 2003).

Villalobos et al (1995) compared four established MCF7 sub-lines and found that BOS cells (Tufts University, Boston (as described in Soto el al (1995)) were most responsive to a range of E_2 concentrations with a maximal 6 fold increase in stimulated cell number compared to unstimulated cells after 144 hours. BB (Institute Jules Bordet, Brussels, Belgium) and BB104 (derived from BB cells maintained in estrogen free medium for more than 24 months, as described by Osborne et al (1987)) cells showed a 2 fold increase. ATCC cells showed a non-significant 1.5 fold increase.

The highest expression of total ER did not correspond to the cell line with highest fold proliferation in response to 17β-estradiol. The BOS sub-line has the lowest ER expression of ~183 fmol/mg protein (with a 6 fold maximal increase in cell number in response to E_2) and BB-104 cell line had the highest expression of ~400fmol/mg protein (with a only a 2 fold maximal increase in cell number in response to E_2) . The ATCC sub-line had ~260 fmol/mg (1.5 fold increase) and BB sub-line had 220 fmol/mg protein of ER (2 fold increase).

Villalobos et al ((1995) showed that after 72 hours of exposure to 1nM E_2, the ER was down-regulated in all cell lines. The ATCC sub-line and the highly responsive BOS cell lines both showed 40% decrease in ER expression. BB and BB104 both showed ~70% decrease in ER expression.

There appears to be no direct correlation between ERα expression and the fold-proliferation caused by E_2. There also appears to be no correlation between the extent to which ER is down-regulated by E_2 or the relationship between ER and proliferative response to E_2.

Interestingly, the BOS cells were shown by flow cytometry to only divide twice before ceasing proliferation when placed in PR-free medium supplemented with 10% DC-FCS. In the same medium, BB and BB-104 slowed their proliferation rate, whereas ATCC cells did not change their proliferation rate. This would greatly affect the reporting of a fold proliferation compared to control (Rasmussen and Nielsen, 2002).

T-47D cells have been compared previously to MCF7 cells (we assume the BOS sub-line) by Soto et al (1999). Sensitivity of T47D cells to E_2 (EC_{50}= 30pM ±9) was similar to MCF7 cells (EC_{50} = 7.3pM ±1.2). van Lipzig et al (2005) determined T-47D cells to have an EC_{50} of 11pM ± 4.

ZR-75-1 have been reported as being stimulated by estrogen (Marth and Daxenbichler, 1985; Harnagea-Theophilus and Miller, 1998) or used in an E-Screen style assay (Ruedl et al., 1990) however, EC_{50} values were not reported in these studies.

A study comparing the cell lines showed that MCF7 (ATCC) expressed 393 fmol ER/mg protein (higher than previously determined by Villalobos et al (1995), potentially due to variation in cell culture condition), T-47D expressed 75 fmol/mg protein and ZR-75-1 expressed 14 fmol/mg protein (Schafer et al., 1999). Despite the discrepancy between the two studies in the reported expression level of ER in MCF7 cells, the relative order of expression is the same as we determined.

4.1.6 Effect of DC-FCS concentration during the 17β-estradiol exposure period

The concentration of DC-FCS during exposure to E_2 affects the fold proliferation of the cells. Villalobos et al (1995) exposed MCF7 cells to E_2 in the presence of PR-free medium supplemented with 5% to 50% DC-FCS and found that maximal proliferation over control was achieved when 10% DC-FCS was used.

It has been hypothesised that the presence of unknown factors in DC-FCS mediate this effect (Ruedl et al., 1990). Sex-hormone binding globulin (SHBG) in DC-FCS, is a specific carrier of estrogen and androgen and is active in mediating estradiol action (Fortunati et al., 1999). Cyclic adenosine monophosphate (cAMP) is a secondary messenger, used for intracellular signal transduction of E_2. It was found that E_2 alone in

serum devoid of SHBG was unable to elicit a cAMP response regardless of SHBG- free serum concentration (Fortunati et al., 1999).

4.1.7 ERβ in the E-Screen

The expression of transfected ERβ at similar mRNA concentration to ERα has been shown to inhibit E_2 stimulated proliferation in T-47D and MCF7 cells. The reduction in proliferation mediated by activated ERβ occurs through an associated decrease in components of the cell cycle associated with proliferation (Strom et al., 2004; Hartman et al., 2006), such as Cyclin E. Lower ERE binding activity was also identified by an ERE-reporter gene (luciferase) construct in MCF7 cells transfected with ERβ compared to 'wild-type' MCF7 cells (Omoto et al., 2003).

4.1.8 Effect of cell passaging on E_2 stimulated proliferation

MCF7 cells have been used in the E-screen within certain passage ranges. Korner et al (1999) used MCF7 cells for up to 7 months and found no loss of estrogen sensitivity whereas other groups constrain their use to much shorter periods. Rasmussen et al (2002) used MCF7 cells to a maximum of 5 passages, Tan et al (2007) reports using MCF7 cells from 6th to 20th passage. Vanparys et al (2006) reports using MCF7 cells up to 25 passages as beyond this range, cells lost their sensitivity to estradiol. It has not been reported what effect passaging may have on other cell lines used in the E-screen or what effect passaging the cells has on the expression level of ER.

4.1.9 Pre-treatment of cells prior to an E-screen

E-Screen protocols can include a pre-treatment period of 24 to 72 hours in PR-free medium supplemented with DC-FCS. The purpose of this estrogen-free period may be to increase sensitivity of the cells to estrogenic stimulus by reducing the basal level of proliferation, synchronising cells to a stationary phase and reducing E_2 related cellular activity. Rasmussen *et al.* (2002), however, found that pre-treatment of the MCF7 BOS cells reduced the fold proliferative response of the cells to E_2 compared to the control cells. Although the cells would have had time to adjust to an E_2 free environment, and were closer to a cessation of proliferation, the re-stimulation of cells by 10nM E_2 led to a lower fold proliferation. The lowest concentration of E_2 causing a significant increase in cells, compared to control, was not affected.

Conversely, Jones et al (1997) found that pre-treatment of MCF7 cells, sourced from ECACC, doubled their fold proliferation (4-fold) when pre-treated for 48 hours in PR-free medium supplemented with 10% DC-FCS, compared to cells that had no pre-treatment period.

4.1.10 E-screen incubation period

Cells used in the E-screen proliferate for 5-7 days before cell numbers are determined. Table 4.1 summarises the different proliferation times used by research groups. Laboratories may validate this incubation based on individual observations, for example Soto et al (1999) stated that T-47D cells were incubated for 6 days instead of 5 days used for MCF7 BOS cells, as T-47D cells have a longer doubling time.

4.1.11 Cell Quantification

Other conditions affecting the E-screen include cell density and associated dimensions of the cell culture plates used (Rasmussen *et al.,* 2002), and quantification methods for total cell numbers, which have varied from metabolic indicators such as MTT (Korner et al., 1999) or MTS (Tan et al., 2007) to cellular or protein stains such as SRB (Rasmussen and Nielsen, 2002; Rajapakse et al., 2004) (Table 1). The use of the MTT assay may be affected by estrogenic treatments modulating metabolic rate (Korner et al., 1999; Berridge et al., 2005). The staining and quantification of cellular components such as of cellular protein using the SRB assay is commonly used in the E-screen as an endpoint (Table 1). Similarly, crystal violet stain binds to acid groups within the cell and can be used to accurately determine cell number (Gillies et al., 1986).

4.1.12 Modelling of the E-screen (The Dose-Response Relationship)

The dose-response (DR) relationship is fundamental to classic toxicology. The proliferative estrogenic response of a cell line can be represented using a Verhulst equation and a log-normal plot (Leusch et al., 2006). The Verhulst equation is shown below where bottom and top are the minimum and maximum x values respectively.

Verhulst equation: $y = \dfrac{bottom + (top - bottom)}{1 + 10 \wedge [(logEC_{50} - logx) \times slope]}$

The assumption in modelling the E-screen is that the response being measured is a direct result of the administered chemical (in this case estrogen) (Vanden Heuvel, 2002). Soto et al (1995) showed that factors such as IGF-1, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) did not stimulate MCF7 cell proliferation in an E-screen assay, however, it is known that other non-genomic estrogenic effects via cell surface receptors or receptor isomer-specific responses are still possible (Korner, 1998; Leusch et al., 2010). Contrary to Soto (1995), Bronzert et al (1987) suggests that the proliferative response of the cells to estradiol is a complex non-direct response requiring accumulation of paracrine and autocrine factors such as IGF-1 and TGFα. It is for this reason that we propose a best-fit slope be applied in the modelling of cell lines. To assume a slope of 1 would imply that a direct relationship exists between estrogen receptor binding and proliferation, whereas evidence suggests that the relationship is not that straightforward. E-screen data must be interpreted with this knowledge.

EC_{50} values are most commonly used in a dose-response relationship to compare the E_2 standards with the samples being tested (Vanden Heuvel, 2002)

When reporting the response of a cell line to E_2, or the potency and specificity of an ER agonist, there are two features that are often compared. These are defined by Soto et al (1995; 1999) and Korner (1999) as Relative Potency (RP), the ratio of the EC_{50} value of E_2 to the EC_{50} value of the test chemical and Relative Proliferative Efficiency (RPE) which is the ratio of the maximal proliferative response of the cells to a test chemical and that of E_2 (Figure 4.1).

A chemical is defined, in this context, as being a full agonist if the RPE is above 70% and a partial agonist if in the range of 25-70% (Soto, 1995; Korner et al., 1999)

In Figure 4.1, an equal number of cells are exposed separately to three different agonists in an E-screen for a given period. If curve A is the response of cells to an E_2 standard, and curve B and C are the responses of cells to two different (xeno)estrogens, it could be concluded that the estrogenic bioactivities of the samples were less than the standards because sample B and C have an EC_{50} of 100nM compared to an EC_{50} of

10pM for the E_2 standards. The estrogenic potency of sample B and C is 0.01% that of E_2.

The RPE of sample B is 100% that of E_2 suggesting that sample B is a full agonist, such as ethinyl-estradiol (Rajapakse et al., 2004) whereas sample C is only a partial agonist such as *t*-Butylhydroxyanisole (Soto, 1995).
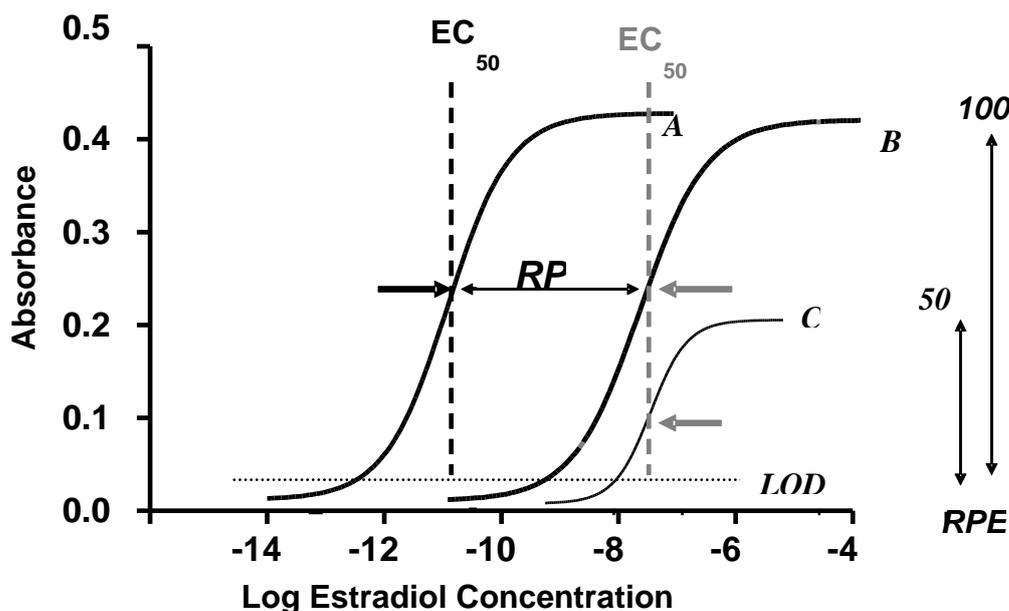
Figure 4.1: Modelling of the E-screen using non-linear regression

Representative log-normal non-linear regression of a dose-response curve to E_2 (A) and a full agonist (B) and a partial agonist (C). The dotted lines indicate the EC_{50} concentration, that is, the concentration of E_2 or sample at which half-maximal proliferation is achieved. Relative Proliferative Effect (RPE) is the comparison of the maximal cell proliferation caused by the E_2 standard divided by the maximal proliferation caused by the sample. This measure determines the extent to which the test chemical is an agonist compared to E_2. Relative Potency (RP) is a comparison between concentrations at which the EC_{50} caused by the test chemical divided by the EC_{50} caused by the E_2 control, expressed as a percentage. This is a measure of the estrogenic potency of the chemical. Note that it is possible that B and C can have the same RP (or EC_{50} values) but C is regarded as only a partial agonist(Korner et al., 1999). Adapted from Soto et al(1999). The Limit of Detection (LOD) is defined as the lowest concentration of E_2 that causes a significant difference (p<0.05) to the negative control.

4.1.13 Data Normalisation

Rajapakse et al (2004) found large inter-assay variability of approximately 60% between identical repeats of the E-screen assay (5 days exposure of MCF7 BOS cells to a range of E_2 concentrations quantified using an sulforhodamine B (SRB) assay). Data normalisation was proposed as a technique to control for the high intra- and inter-experiment variability in final cell numbers(Rajapakse et al., 2004). Data normalisation usually used defines the negative control and maximal proliferation in the positive control as 0 and 100% respectively. The negative control is the cells exposed to the EtOH (solvent) control only and the positive control is the maximum O.D that usually occurs at the upper plateau of OD in the higher concentrations of E_2 tested.

All data points within the range are then determined using the equation:

Normalised proliferation is expressed as a percentage equal to:

$$\frac{\text{O.D (test)} - \text{Average O.D. (negative control)}}{\text{Average O.D.(positive control)} - \text{Average O.D.(negative control)}} \times 100\%$$

4.1.14 *Purpose of this study*

A reduction or cessation of growth in estrogen-free media is critical when reporting an increase in proliferation stimulated by estrogen, as this is the end point of E-screen assays. MCF7 (ATCC) cells have been shown previously to not decrease proliferation when placed in estrogen-free medium (Villalobos et al., 1995), however, it is apparent that a cell line best suited to the E-screen is one that significantly decreases or stops proliferating when placed in estrogen-free medium. All cell lines were therefore assessed for their growth rate in serum–free or estrogen-free (PR-free medium supplemented with 1% DC-FCS) medium for up to 72 hours, the longest reported pre-treatment period (Vanparys, 2006).

The cell lines that were determined to express ERα; MCF7, T-47D and ZR-75-1, were assessed for an increase in ERα protein when incubated in PR-free medium supplemented with 1% DC-FCS for 48 hours, similar to the pre-treatment conditions used in many E-screen protocols (Table 1). It has been determined that pre-treatment in estrogen–free medium causes an upregulation of ERα in MCF7 cells (Welshons, 1987; Katzenellenbogen et al., 1995) which is not necessarily beneficial to a better

proliferative outcome (Villalobos et al., 1995; Rasmussen and Nielsen, 2002). A comparison was therefore done with the other two cell lines, T-47D and ZR-75-1, to determine if, in the absence of estrogen from the media, the ERα of the other cell lines was affected in the same way or was maintained at the same level.

In some reports (Wang et al., 2004; Vega-López et al., 2007), the E-screen is performed with media replacement during the exposure period. The complex response of proliferation by estrogen involves recruitment and generation of paracrine and autocrine factors and other mechanisms (Bronzert et al., 1987; Aakvaag et al., 1990). Cells were pre-treated in estrogen free medium for 48 hours followed by a 72 hours exposure period to E2 with daily media changes to assess if a change in proliferation rate occurred.

The three cell lines; MCF7, T-47D and ZR-75-1, were compared directly in the E-screen assay for their proliferative response to a range of E_2 concentrations. It has been reported that 24 hours attachment in estrogen-free medium without further pre-treatment gave the best response in an E-screen (Rasmussen and Nielsen, 2002) . In this study, 6 hours, determined previously as the optimal time of complete attachment, was also used to see if an increase in proliferative response and sensitivity (while reducing assay time) was possible. A variation in the reported exposure time of 5-7 days is summarised in Table 1. Soto (1995) exposed cells in E-screen to E_2 and samples for 6 days, however, found significant differences after 4 days. Proliferation of cells was determined after 3, 5 and 7 days to determine the proliferative responses after these times.

A difference in cell morphology by visual examination of MCF7 cells could be seen after approximately 18 passages. Many studies limit the passage range of the MCF7 cells used. The cell lines characterised in the E-screen, were tested before and after this passage number was reached to determine if T-47D or ZR-75-1 retained E_2 responsiveness for longer.

Long term adaptation and culture of MCF7 cells in estrogen-free medium resulted in a sub-line of MCF7 cells that had a higher ER content than other MCF7 sub-lines, and a slightly better proliferative response in the E-screen than MCF7 (ATCC) cells(Villalobos et al., 1995). A similar study showing the effect of culture conditions

on the ERα expression of ZR-75-1 cells was performed whereby they were adapted to continuous culture in 5 and 2.5% FCS supplemented medium. The subsequent ERα expression and proliferative response of the cells in the E-screen was determined.

4.2 Methods:

4.2.1 Dextran charcoal stripping of Serum

4.2.1.1 Dextran charcoal stripping of Serum –Method A:

FCS was stripped of hormones and growth factors using a protocol described by Sigma-Aldrich (http://www.sigmaaldrich.com). Activated charcoal (cell culture tested) (0.25% w/v) and Dextran T-70 (0.0025% w/v) were mixed and left overnight at 4°C in a solution containing 0.25M sucrose, 1.5 mM $MgCl_2$, 10mM HEPES, pH 7.4. 500mL of the solution were then centrifuged at 500 x g for 10 minutes in 50mL centrifuge tubes (Falcon) using a bench top centrifuge (Beckmann Coulter). Supernatant was removed gently without dislodging the pellet and the same volume of heat inactivated FCS placed into each tube. Tubes were vortex thoroughly to mix the charcoal with the serum and incubated for 12 hours at 4°C. Tubes were again centrifuged for 10 minutes at 500x g and the serum gently poured off. Serum was filtered using a 0.45µm then 0.22µm sterile vacuum filtration unit. DC-FCS was stored at -20°C in 10 ml aliquots.

4.2.1.2 Dextran charcoal stripping of serum – Method B:

A second approach for stripping serum as used by Dept Clinical Pharmacology, Flinders Medical Centre (supplied by Anne Rogers) was used and replaced the previous method. It was found that greater stimulation from E_2 was possible when this method was used.

A stripping buffer (1.211g Tris (0.01M), 0.558g EDTA (1.5mM), 2.42g sodium molybdite (0.01M) and 10% glycerol) was made to 1 litre in RO water and the pH adjusted to 7.4. Separately, 5g charcoal (0.5%), 0.5g Dextran (55mM) and 100mL glycerol were placed in a beaker and made to 1 Litre with the stripping buffer. The solution was left on a magnetic stirrer overnight at RT.

Twenty 50 mL centrifuge tubes (Falcon) of the solution were spun at 500 x g for 30 mins using a bench top centrifuge (Beckmann Coulter) and the supernatant aspirated. Ten of the tubes were each filled with 50mL FCS (not heat inactivated) and left on a rotating platform at RT for 2 hours. Tubes were then spun at 500 x g for 30 mins at 4°C. The serum supernatant was then poured into the remaining ten tubes and placed on a rotating platform for another 2 hours at RT, followed by another 500 x g centrifugation for 30 mins at 4°C. Serum was pre-filtered using multiple 0.45 µm

vacuum filters, followed by a final sterile 0.22 µm vacuum filtration. DC-FCS was stored at -20°C in 10mL aliquots.

4.2.2 The effect of 0% FCS, 1% DC-FCS or 10% FCS, and presence of PR, on cell proliferation over 72 hours.

Cells are best suited to the E-screen if they cease or significantly reduce proliferation in E_2 free medium. Each cell line was therefore tested to determine if the absence of FCS and phenol red would effect their proliferation.

4.2.2.1 Standard Curve

Viable MCF7, T-47D, ZR-75-1, RL95-2, Ovcar or H23 cell suspensions were serially diluted in a 96 well plate (Nunc, Denmark) using a multi-channel pipette (80,000 - 1250 cells per well in six replicates) in 100µl of their respective media. Cell metabolism was measured using the MTT assay as described after 6 hours attachment time. Data was exported to Excel and a line of best fit plotted. The equation relating the absorbance to the cell number was applied to all further absorbance readings.

4.2.2.2 Cell Treatments

20,000 viable MCF7, T-47D, ZR-75-1, RL95-2, Ovcar or H23 cells, in 100µl of their respective media, were dispensed into six replicate wells of a 96 well plate (Nunc, Denmark). After 6 hours attachment time, media was changed to 0, 1% DC-FCS or 10% FCS with or without PR. Cell numbers were assessed after 24, 48 and 72 hours using the MTT assay as described. O.D data were converted to cell number using the equation generated from the standard curve. The experiment was repeated on three separate occasions (n=3) for each cell line.

4.2.2.3 Statistical Analysis

Data was entered to SPSS version 17 and significance tested using one-way ANOVA with a Tukey-Kramer post-hoc test.

4.2.2 ERα protein expression in PR-free medium supplemented with 1% DC-FCS

A pre-treatment period in E_2 free medium is sometimes used in the E-screen assay, theoretically to acclimatise cells and slow proliferation to a basal level before the test samples are applied. In this experiment, we aimed to show what effect if any a 48 hour

pre-treatment in E_2 free medium had on the ERα protein expression of MCF7, T-47D and ZR-75-1 cells.

In a T25cm^2 flask, 1 x 10^6 MCF7, T-47D, ZR-75-1 and H23 cells were left to attach for 6 hours in their respective media, after which monolayers were thoroughly washed twice with PBS and aspirated. Respective media were replaced with PR-free media supplemented with 1 % DC-FCS. Cells were incubated for 48 hours before protein was isolated from the cells using mRIPA buffer as described.

10μg protein of each cell line, including a negative control Sk1064 cell protein isolate (described previously), was electrophoresed using SDS/PAGE, transferred to a PVDF membrane and probed with anti-ERα (HC-20) antibody and HRP tagged secondary antibody as described. Blots were visualised with ECF (GE Healthcare) using a Typhoon imager (Amersham Biosciences) as described. The extraction and blot was performed on three separate occasions (n=3) and pixel density of the 66kDa band quantified using Fujifilm Multi Gauge software.

4.2.4 Determination of E2 concentration using SouthPath Pty Ltd clinical ELISA

E_2 stock made to 4×10^{-5}M in EtOH (Refer to Chapter 2) was provided to SouthPath Pty Ltd (Flinders Medical Centre) to check the concentration in a NATA accredited ELISA used for clinical testing.

The stock solution was determined to be an actual concentration of 1.39×10^{-5} M. As the initial E-screens were performed prior to this testing, the results were adjusted to the actual concentrations.

4.2.5 E-Screen Methodologies:

4.2.5.1 Method 1: 6 hour attachment, 48 hour pre-treatment, 72 hour E2 exposure, media changed every 24 hours

It has been shown that a pre-treatment of MCF7cells in estrogen-free medium is beneficial to increasing the fold-proliferative response of cells to E_2 (Jones et al., 1997). Also, estrogen related proliferation is supported by an accumulation of autocrine and paracrine factors (Aakvaag et al., 1990). In this experiment, we wanted to demonstrate

if daily media/treatment changes would allow proliferation to occur in not only MCF7 but also T-47D, ZR-75-1 and H23 cells.

5000 viable MCF7, T-47D, ZR-75-1 and H23 cells, in 100µl of their respective media supplemented with 10% FCS, were dispensed into six replicate wells of a 96 well plate (Nunc, Denmark). A standard curve was also prepared as described. Cell numbers in the standard curve plate were measured using the crystal violet assay as described after 6 hours incubation. At this time, media was aspirated from all remaining wells and PBS gently applied to rinse cells of the full serum media. Media was replaced with PR-free media supplemented with 1% DC-FCS (Method A) and incubated for 48 hrs.

After 48 hrs media was gently aspirated from wells and replaced with media containing E_2 at 10^{-6}, 10^{-8}, 10^{-10}, 10^{-12} M or the same E_2 concentrations with 10^{-7}M Fulvestrant. Media including treatments were changed every 24 hours. After 72 hours, cell numbers were determined using the crystal violet assay as described.

One 96 well plate per cell type was used for each time point and the experiment was repeated on three separate occasions (n=3) for each cell line.

4.2.5.2 Method 2: 6 or 16-24 hour attachment combined with pre-treatment, 3, 5 or 7 day E_2 exposure (Passage <18)

The three cell lines were compared directly in an E-screen to test the estrogenic response of T-47D and ZR-75-1 to that of MCF7 cells. A variation in the time that cells are exposed to E2 has been reported. Here we wanted to demonstrate what, if any, differences a 3, 5 or 7 day exposure to E_2 existed.

The MCF-7 cell proliferation assay (E-Screen) protocol was provided by Dr Fred Leusch with modifications as described. Adapted from SOP: GWRC-TDE-04A - Rev 4 (24 Jan 2008).

Viable MCF7, T-47D or ZR-75-1 cells (passage <18) were aspirated and rinsed twice in PBS. Cells were trypsinised as described and resuspended in the cell's respective PR-free medium supplemented with 10% DC-FCS and counted using trypan blue exclusion as previously described. A multichannel pipette was used to dispense 5,000 cells per well into the 60 central wells of a 96 well plate in 100 µl final volume. Plates were incubated for either 6 hours or for those that were G_o synchronised, were left overnight (16-24 hours).

After 6 or 24 hours, 100 μL 2x final concentration of E_2 were added to each well. The 2 x final concentration samples were a serial dilution of E_2 ranging in concentration from 2x 10^{-6}M to 2x 10^{-14}M plus an EtOH control (0.5%). This represented final concentration of E_2 in wells of 10^{-6}M to 10^{-14}M . 200 μL of PBS or sterile water was pipetted to the outside wells to avoid any edge effects that may occur.

After an exposure time of 3, 5 or 7 days, a single plate was used for each time point and the crystal violet assay applied. The experiment was repeated on three separate occasions (n=3) for each cell line.

4.2.5.3 Method 2B: 6 or 16-24 hour attachment combined with pre-treatment, 3, 5 or 7 day E_2 exposure. (Passage >18)

The exact procedure was repeated using cells that were grown in normal conditions and passaged more than 18 times. The experiment was repeated on three separate occasions (n=3) for each cell line.

4.2.5.4 The E-screen using the ZR-75-1 cell line adapted to growth in PR-free media supplemented with 5% or 2.5% DC-FCS

Briefly, ZR-75-1 cells, cultured in $75cm^2$ flasks were sequentially reduced from the standard 10% FCS to 5%, 2.5% and 1% FCS. Cells were allowed to grow to confluence at least twice before resuspending and placing in a new flask with a reduced FCS concentration. The cells were then kept in continuous culture at the designated FCS supplementation level. This work was performed by Mr Rhys Hamon.

Two of the resulting cell lines were used in the E-screen as described using Method 2. Each cell line was attached and exposed to E_2 in the E-screen at either the equivalent concentration (2.5 or 5%) DC-FCS or at 10% DC-FCS..

4.2.5.5 Statistical analysis of E-screen data

Data generated from the automated spectrophotometer were adjusted on a per plate basis. An average absorbance was calculated from the replicate control wells (cells exposed to EtOH control only) which was subtracted from individual absorbance values of each of the test wells i.e

Absorbance = O.D. (test well) − O.D. (average of the control wells)

Data was then imported to GraphPad Prism Version 5.03, data sets were analysed using a non-linear regression (variable slope) function with a "Bottom value" constrained to constant = 0.0.

For comparative purposes, data was also transformed within GraphPad Prism by a normalisation function. The lowest and highest values within the data set were defined 0 and 100% respectively. The data was then again analysed using non-linear regression (variable slope).

A best fit non-linear curve was generated from the data sets by GraphPad Prism which also reported on the degree of fit and a best fit EC_{50} value, the concentration of E_2 yielding half-maximal effect. In cases where GraphPad Prism could not generate a best-fit curve, the data was labelled as non-converged. 95% CI were reported for each best-fit EC_{50} value, however, in cases where the 95% CI exceeded GraphPad Prism's constraints, it was reported as 'very wide'. A one-way ANOVA with Dunnet's Multiple Comparison test was used to determine the lowest E2 concentration that caused proliferation significantly different to that of the control ($p < 0.05$).

Fold increase in cell numbers was determined by dividing the average maximal O.D. by the average negative control O.D. on a per plate basis. This value was calculated from E-screens performed on three separate occasions.

A limit of detection value (LOD) was determined using a one way ANOVA with Dunnett's multiple comparison test in GraphPad Prism. The LOD represents the lowest concentration of E_2 which causes O.D.values/cell numbers to be significantly different ($p < 0.05$) to the negative control.

4.3 Results:

4.3.1 The effect of 0% FCS, 1% DC-FCS or 10% FCS, and presence of PR, on cell proliferation over 72 hours

Cells that significantly reduce or cease proliferation in estrogen-free media are desirable for use in the E-screen assay.

The cell lines grown in medium supplemented with 0%FCS, 1% DC-FCS or 10% FCS with or without PR proliferated or remained the same after 24, 48 and 72 hours depending on treatment. The only exception to this was T-47D cells grown in medium which was not supplemented with FCS (0% FCS), cell numbers decreased below the initial cell number (20000) after 48 or 72 hours. This apparent decrease in number may be due to either a detachment of cells which could not be sustained in the absence of media or a reduction in the metabolism of cells. The reduction in the metabolism of the cells would report lower cell numbers than are actually present, due to a lower metabolism/conversion of MTT during exposure in the MTT assay.

4.3.1.1 Effect of Serum Concentration

10% FCS supplemented medium significantly supported ($p<0.05$) more cell proliferation than 1% DC-FCS or 0% FCS supplemented medium in the cell lines shown previously to express ERα (mRNA): MCF7, T-47D, ZR-75-1 and Ovcar. After 72 hours in medium supplemented with 10% FCS, there were approximately 40000 cells per well more than cells grown in 1% DC-FCS or 0 % FCS supplemented medium (Figure 4.2).

The supplementation of medium with serum had minimal effect on the number of cells after 72 hours in the two cell lines, RL-95-2 and H23, which were shown not to express ERα.

T-47D, ZR-75-1 and Ovcar cells did not proliferate in the absence of serum (0% FCS) whereas the MCF-7, H23 and RL-95-2 cells did (Figure 4.2).

Although non-significant, T-47D, ZR-75-1 and Ovcar cells grown in medium supplemented with 1 % DC-FCS had ~20000 cells/well more after 72 hours than cells

grown in medium supplemented with 0% FCS. MCF7, RL95-2 and H23 had a much smaller difference in cell numbers between cells grown in 0% FCS or 1% DC-FCS supplementation amounts.

4.3.1.2 Effect of Phenol Red

The absence of phenol red in the medium supplemented with 10% FCS slowed the growth of MCF7, T-47D and ZR-75-1 cells after 72 hours, however the difference in cell number was not significant. (Figure 4.3).

There was no significant difference in growth in the presence or absence of PR in media supplemented with 0 and 1% serum.

Figure 4.2: Proliferation of 20000 cells/well MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 for 72 hours in 10% FCS, 1% DC-FCS or 0% FCS with or without PR.

In a 96 well plate, 20000 cells per well were left to attach to the growth surface in the cell's respective medium supplemented with 10% FCS. To produce a standard curve, the MTT assay was applied after 4 or 6 hours. At the same time the media in the remaining plates was aspirated and replaced with media supplemented with 10% FCS, 1% DC-FCS or 0% FCS with or without PR. The equations that described the relationship between cells/well values and absorbance were used to convert absorbance derived after 24, 48 or 72 hours to actual cell number. The experiment was repeated on three separate occasions (n=3) and the means ± standard deviations are represented for 24, 48 and 72 hours.
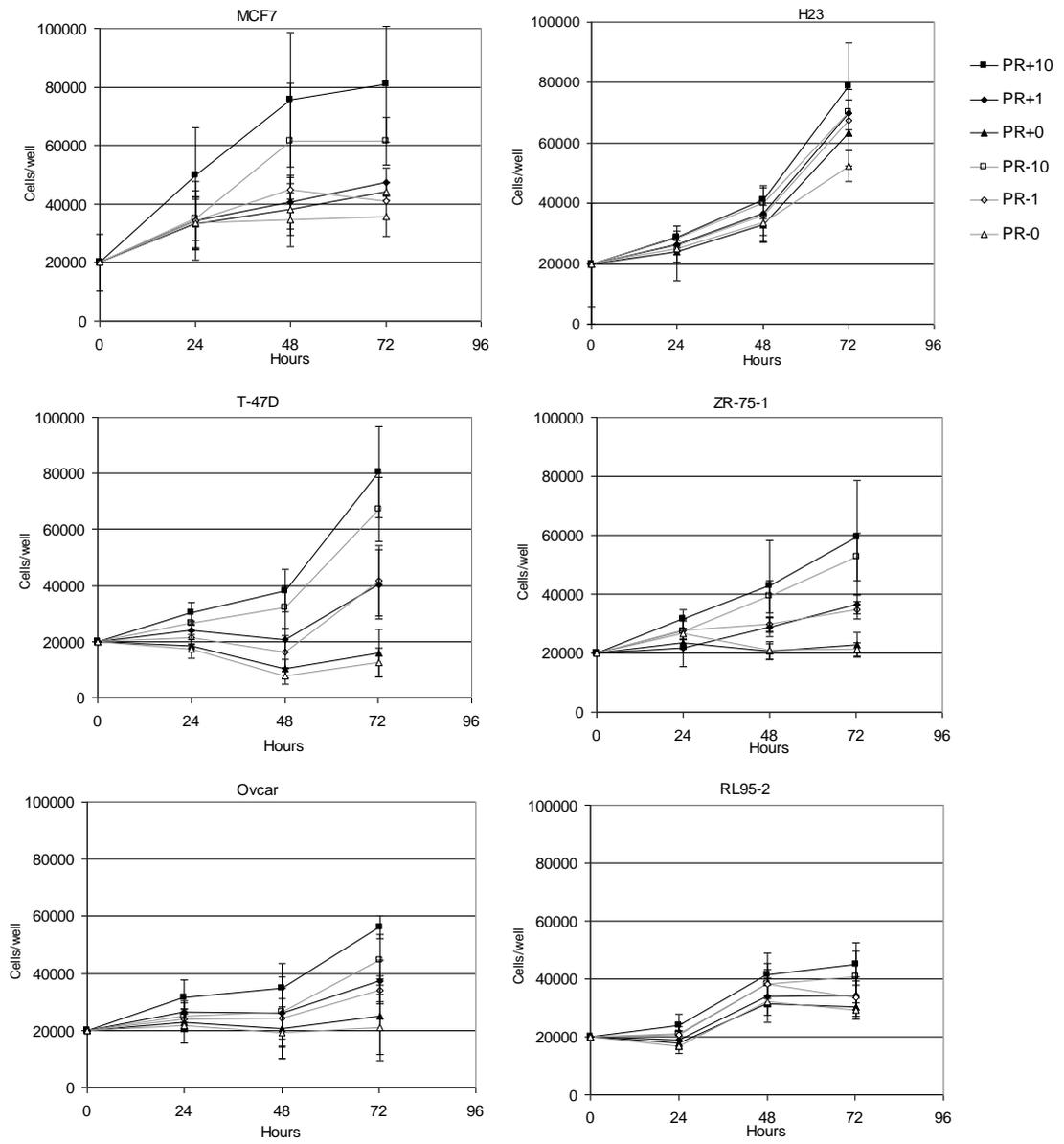
Figure 4.3: Proliferation of 20000 cells/well MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL95-2 for 72 hours in 10% FCS, 1% DC-FCS or 0% FCS with or without PR.

In a 96 well plate, 20000 cells per well were left to attach to the growth surface in the cell's respective medium supplemented with 10% FCS. To produce a standard curve, the MTT assay was applied after 4 or 6 hours. At the same time the media in the remaining plates was aspirated and replaced with media supplemented with 10% FCS, 1% DC-FCS or 0% FCS with or without PR. The equations that described the relationship between cells/well values and absorbance were used to convert absorbance derived after 72 hours to actual cell number. The experiment was repeated on three separate occasions (n=3) and the means ± standard deviations are represented for 72 hours. A significance difference in cell number to PR+ media supplemented with 10% FCS after 72 hours are represented by (   ) (p<0.05).
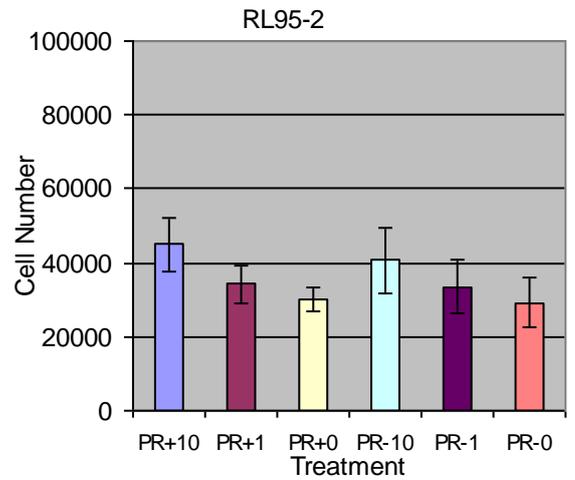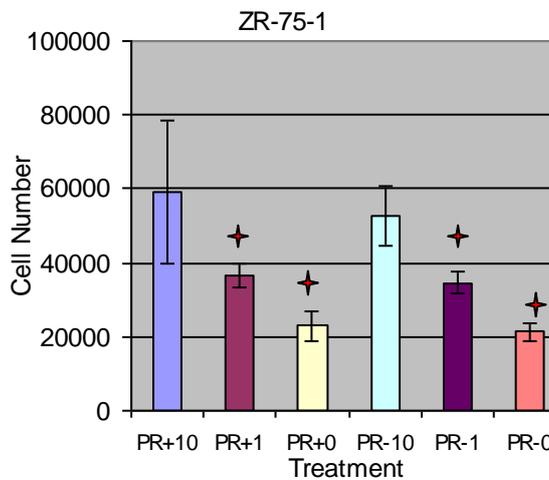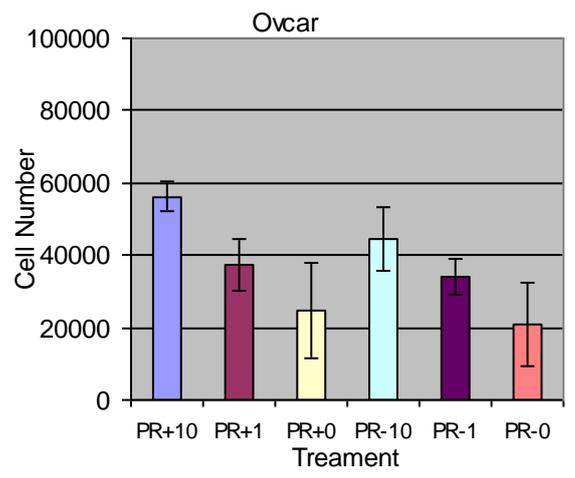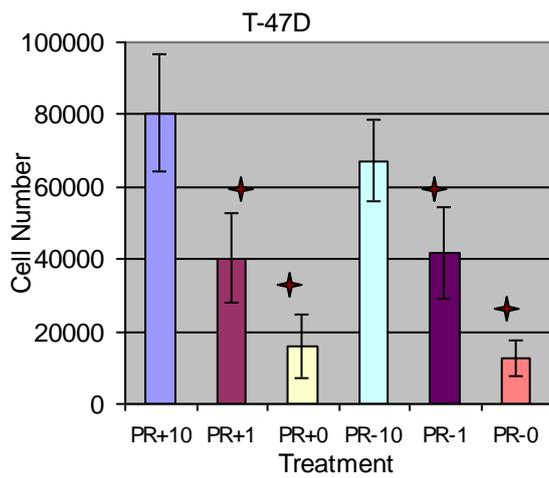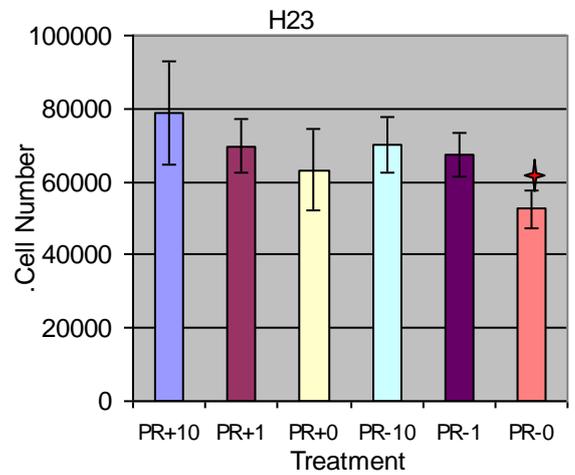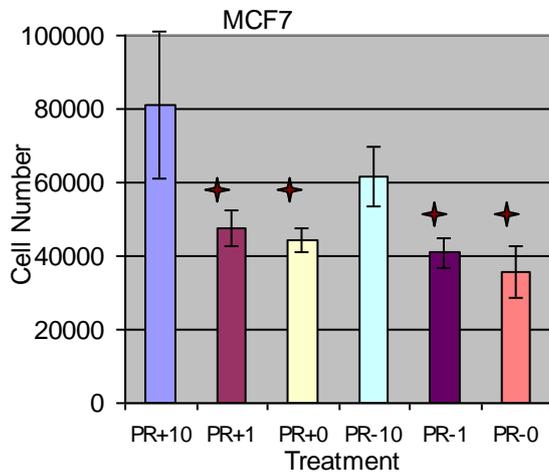
4.3.2 ERα protein expression in PR-free medium supplemented with 1% DC-FCS*:*

There was no significant difference (p<0.05) in the expression of the ERα protein in any of the cell lines tested after 48 hours in PR-free medium supplemented with 1% DC-FCS compared to PR+ medium supplemented with 10% FCS (Figure 4.4). In PR-free medium supplemented with 1% DC-FCS, the ER increased 1.6% (±25.6%) and 3.1% (±2.7%) for MCF7 and ZR-75-1 respectively and decreased 5.0% (±7.6%) in T-47D cells.
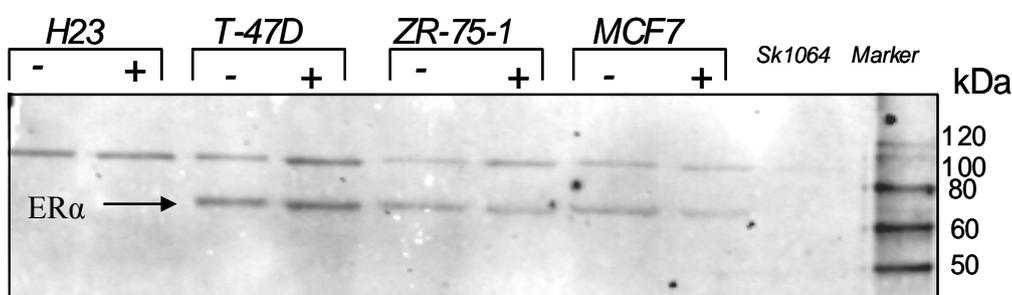
Figure 4.4*: Western blot analysis monitoring the expression of ERα after 48 hours growth of H23, T-47D, ZR-75-1, MCF7 in PR-free medium supplemented with 1%DC-FCS or PR+medium supplement with 10%FCS.*

Total protein was extracted from monolayers of H23, T-47D, ZR-75-1, MCF7 and 1064Sk cells in T25cm^2 flasks exposed to PR-free medium supplemented with 1% DC-FCS (-) or PR+ medium supplemented with 10% FCS (+) for 48 hours. Protein concentrations were quantified using a BCA Protein Determination Kit and BSA standards. 2µg of protein per cell line was resolved using SDS/PAGE. Protein was transferred to a PVDF membrane. A rabbit polyclonal anti-ERα (HC-20) identified the 66kDa protein in T-47D, ZR-75-1 and MCF7 protein lysate only. Sk1064 was used as a negative control. The western blot analysis was performed on 3 separate occasions and a representative blot shown.

4.3.3 Method 1: 6 hour attachment, 48 hour pre-treatment, 72 hour E$_2$ exposure, media changed every 24 hours.

The DC-FCS used in this procedure was prepared using Method A. Cells were pre-treated for 48 hours in PR-free medium supplemented with 1% DC-FCS and then exposed to 2.8x 10^{-13} to 2.8 x 10^{-7}M E$_2$ in PR-free medium supplemented with 1 % DC-FCS for 72 hours with daily media changes. The E$_2$ did not significantly stimulate the proliferation of MCF7, T-47D, ZR-75-1 or H23 cells. A response trend can be seen in T-47D cells, however, large standard deviations negate significance (Figure 4.5).

The use of the ER antagonist, Fulvestrant, generally caused lower cell numbers overall but the differences were not significant.

Fulvestrant was not used with H23 cells, as they were shown earlier to not have the ERα receptor, and were present in this experiment as a control only. There was no dose response shown by H23 cells.

A response to E$_2$ when media was not changed for 72 hours was included for comparison purposes only. The line does not represent actual cell numbers as only OD was measured in that instance (which was multiplied by 10^5) (see section 4.#). The comparative line shows that T-47D cells in both cases are showing a dose dependant increase in cell number/OD, which perhaps may have been more apparent in the cells with media changes every 24 hours, if the E$_2$ range had extended to lower concentrations.

Figure 4.5: Proliferative response of MCF7, T-47D, ZR-75-1 and H23 cells after 72 hours exposure to E2 with a 48 hour pre-treatment and daily media changes.

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in the cell's respective media supplemented with 10% FCS. To produce a standard curve, the MTT assay was applied after 6 hours. At the same time the media in the remaining plates was aspirated and replaced with PR-free media supplemented with 1% DC-FCS and left for 48 hours. Cells were treated for 72 hours with daily media changes with E_2 ($2.8x10^{-7}M$ to $2.8x10^{-13}M$) with or without Fulvestrant ($10^{-7}M$). The MTT assay was applied after 72 hours exposure. The equations that described the relationship between cells/well values and absorbance were used to convert absorbance derived after 72 hours to actual cell number. The experiment was repeated on three separate occasions (n=3) and the means ± standard deviations represented. (E_2) represents cells treated with the corresponding E_2 treatment and (E_2 Fulv) represents cells treated with the corresponding E_2 treatment and $10^{-7}M$ Fulvestrant. **For comparative purposes only**, *cell proliferation after 72 hours in an E-screen assay where media was not changed every 24 hours was included (see Section 4.3). The position of the line is not indicative of actual cell number and is a direct indication of OD values ($x10^5$). Standard deviations have not been represented on this line for clarity.*

MCF7

T-47D

ZR-75-1

H23
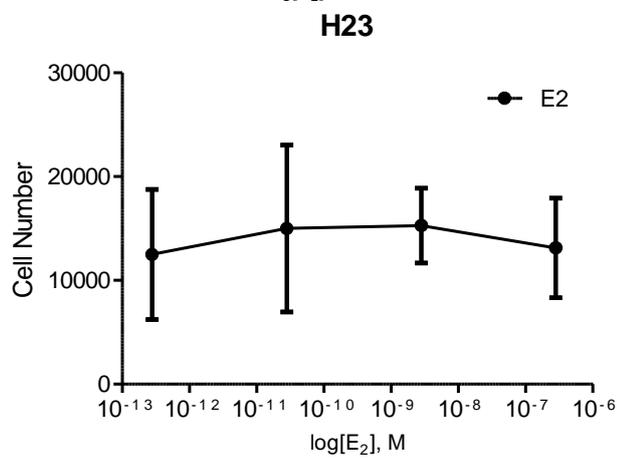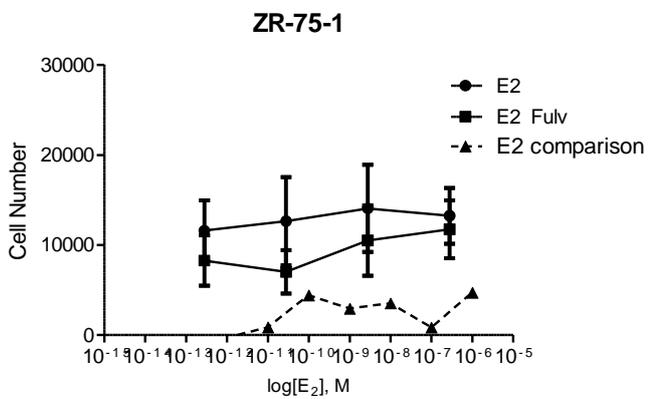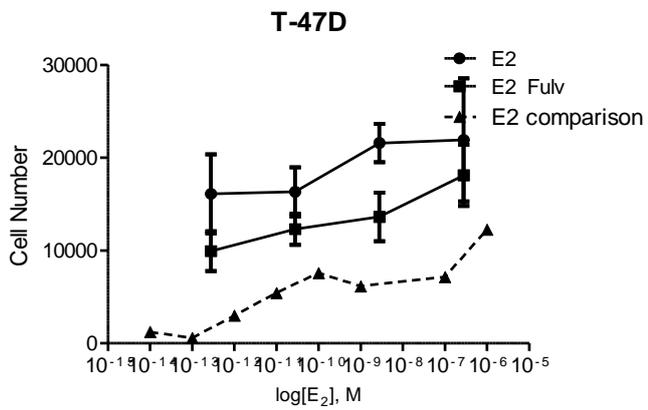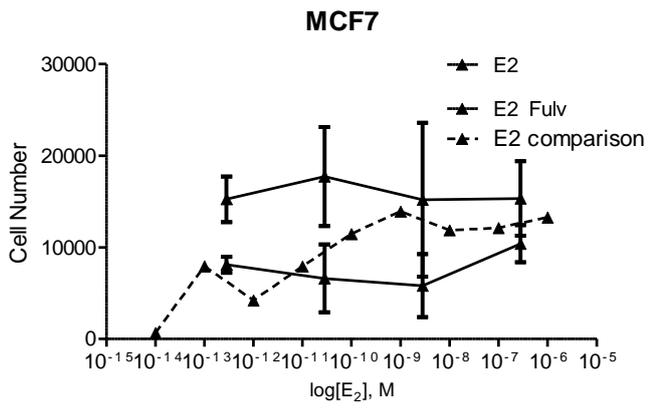
4.3.4 E-Screen: 16-24 hour attachment/pre-treatment, and 3, 5 or 7 day E_2 exposure (Passage <18)

MCF7, T-47D and ZR-75-1 cell lines were used in an E-screen assay (Method 2), inclusive of an overnight attachment/pretreatment period (24 hours) in PR-free medium supplemented with 10% DC-FCS. Cells were then exposed to E_2 (10^{-14} to 10^{-6}M) for 3, 5 or 7 days after which cell proliferation was determined by absorbance of the cell stain, crystal violet (Figure 4.6). MCF7, T-47D and ZR-75-1 cell lines all responded to increased concentrations of E_2 with a dose-responsive increase in cell number. T-47D cells gave a clear time as well as dose-dependent response; there were more cells after 7 days than 5 days and more cells after 5 days than 3 days. MCF7 and ZR-75-1 cells had more cells after 5 days culture than after 3 days culture. After 7 days culture however, approximately the same number of cells were present per well as after 5 days. Typically all cell lines responded to E_2 concentrations between 10^{-14} to 10^{-10}M . E_2 concentrations higher than this (up to 10^{-6}M) generated no further increase in cell number. MCF7 and ZR-75-1 cells showed a small decrease in cell number at E_2 concentrations at 10^{-8}M or higher. The 10^{-6}M E_2 data point was removed from analysis of the T-47D and ZR-75-1 curve as it was below the maximal plateau of cell number and skewed the best-fit line.

Analysis of the proliferative response of MCF7 cells to E_2 shows very large SEM as previously described. The EC_{50} values had large corresponding 95% CI values (Table 4.2). Day 3 had the smallest 95% CI, indicated in brackets, with an EC_{50} of 1.14 pM (0.024 to 52 pM). The technique whereby the data is normalised on a plate by plate basis, can reduce inter-experiment variability, is represented for Days 5 and 7 (Figure 4.6). MCF7 cells, however, still have large 95% CI range with an EC_{50} of 1.14pM (0.095pM to 12pM). After 7 days proliferation, the data is deemed non-converged, that is, a best fit line can not be fitted to the data set within reason. The exclusion of E_2 concentrations from 10^{-6} to 10^{-9} M allowed a best fit line representation of the data which has an EC_{50} of 0.35pM (0.049pM to 2.26pM). MCF7 cells showed a maximal average 2.2-fold proliferation above control.

The T-47D cell E-screen has the lowest SEM of the three cell lines and greatest increase in cell number after 3, 5 and 7 days. The highest concentration of 10^{-6} M E $_2$ was

removed from analysis because it clearly deviated from maximum response i.e. the upper plateau, and skewed the best-fit line. Day 5 and 7 have similar EC_{50} values: Day 5, 6.36pM (2.19 to 11.8pM) and day 7, 9.6pM (6.35pM to 14pM) (Table 4.2). T-47D cells showed a maximal average 3.4-fold proliferation above control.

ZR-75-1 cells proliferated to half the extent of T-47D cells. After 3 days exposure, the cell numbers had barely increased and the best-fit line was deemed non-convergent. After Day 5, EC_{50}=17.9pM (3.9pM to 80pM) or Day 7, EC_{50}=43pM (19.7pM to 94 pM). ZR-75-1 cells showed a maximal average 1.7-fold proliferation above control.

E_2 stimulated proliferation of the cell lines was also performed with the addition of Fulvestrant (10^{-7}M), an ER antagonist, to the medium (Figure 4.7). After 3, 5 or 7 days, the concentration of E_2 required to stimulate proliferation was much higher than without the addition of Fulvestrant. Proliferation occurred only at the highest E_2 concentrations (>10^{-8}M). The non-linear regression curves were deemed by GraphPad Prism as ambiguous because an upper plateau of proliferation is no longer reached. Hence, the EC_{50} values were ~10^{-7}M with 'very wide' 95% CI reported. Nevertheless, the fact that Fulvestrant blocked proliferation until enough E_2 was added to overcome its effect, shows that the proliferative response seen was a response to E_2 via the ER.

Cell Line	5 Days	7 Days
T-47D	5.1pM (1.6-16pM)	7.4pM (4.6-11.8pM)
MCF7	0.15pM (0.009-2.7pM)	0.03pM (0.002-1.1pM)
ZR-75-1	6.9pM (0.8-61pM)	22.1pM (6.9-71.1pM)

Table 4.2. EC_{50} value for each cell line after an overnight attachment and exposure to E_2 for 5 or 7 days.
The EC_{50} value was determined from best-fit linear regression of data. The 95% CI was also determined and is indicated in brackets.

Figure 4.6: E-screen using 5000 cells/well MCF7, T-47D, ZR-75-1 (Passage < 18).

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in PR-free medium supplemented with 10% DC-FCS. After 24 hours, a further 100μl of PR-free medium supplemented with 10% DC-FCS was added, giving final E_2 concentrations from 10^{-14}M to 10^{-6}M. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with best fit log linear plot using linear regression in GraphPad Prism. Normalised values after 5 or 7 days exposure to E_2 are also shown.
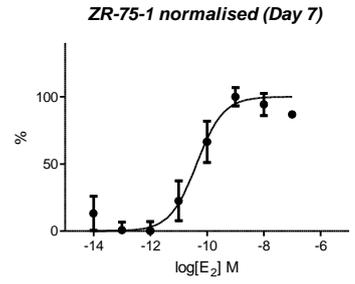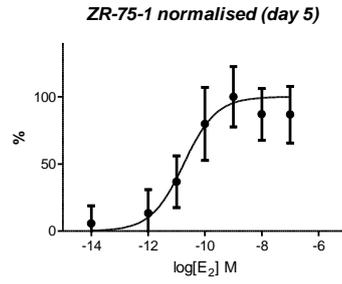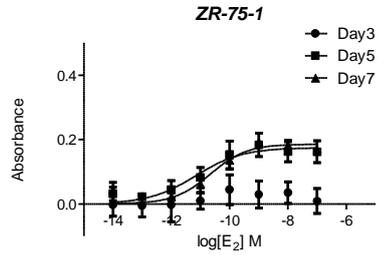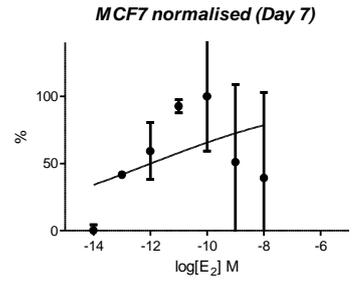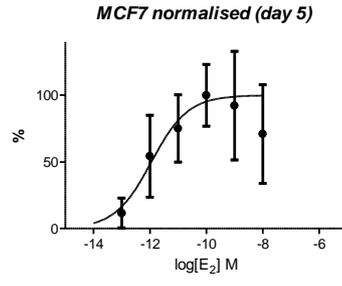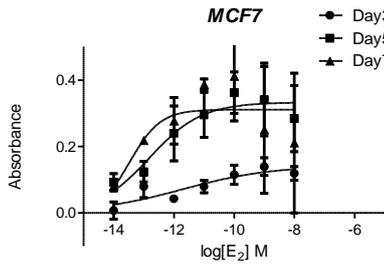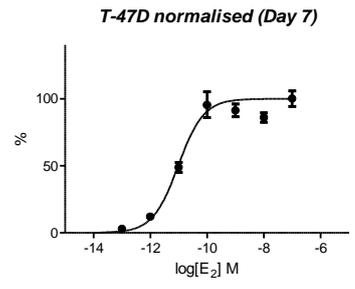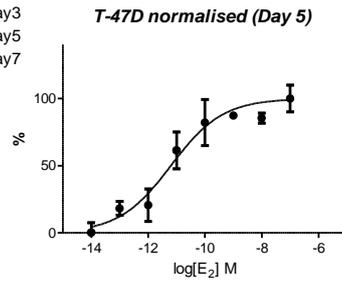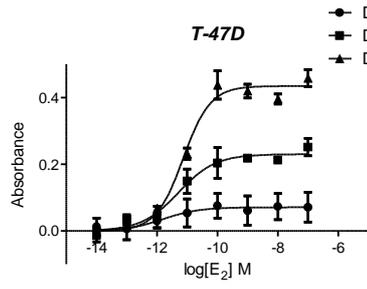
Figure 4.7: E-screen using 5000 cells/well MCF7, T-47D, ZR-75-1 with the inclusion of the E₂ antagonist, Fulvestrant (Passage < 18).

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in PR-free medium supplemented with 10% DC-FCS. After 24 hours, a further 100µl of PR-free medium supplemented with 10% DC-FCS was added, giving final E_2 concentrations from $10^{-14}M$ to $10^{-6}M$ with the ER antagonist, Fulvestrant, applied at $10^{-7}M$. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with best fit log linear plot using linear regression in GraphPad Prism.

T47D + Fulvestrant

Absorbance

0.6
0.4
0.2
0.0

-16 -14 -12 -10 -8 -6 -4

log[E$_2$], M

- Day3
- Day5
- Day7

MCF7 + Fulvestrant

Absorbance

0.6
0.4
0.2
0.0

-16 -14 -12 -10 -8 -6 -4

log[E$_2$], M

- Day3
- Day5
- Day7

ZR-75-1 + Fulvestrant

Absorbance

0.6
0.4
0.2
0.0

-16 -14 -12 -10 -8 -6 -4

log[E$_2$], M

- Day3
- Day5
- Day7

130
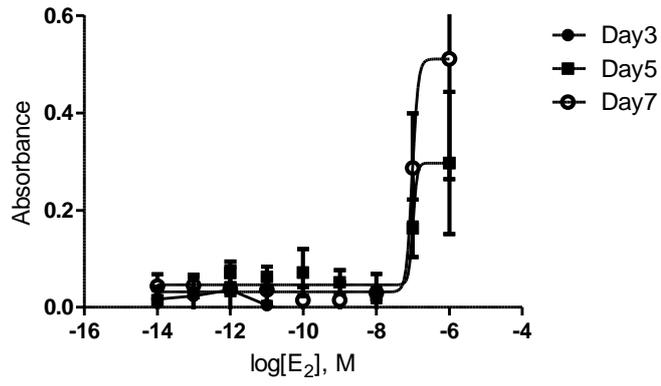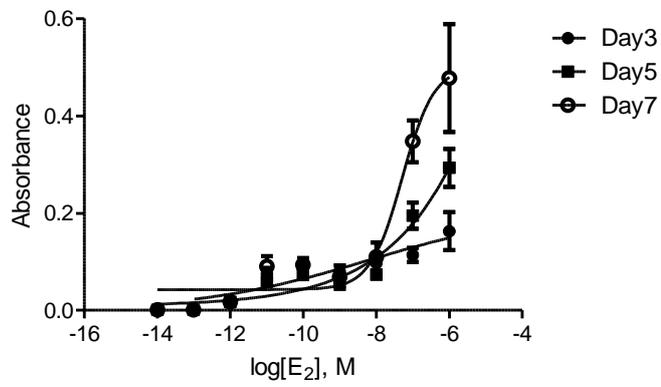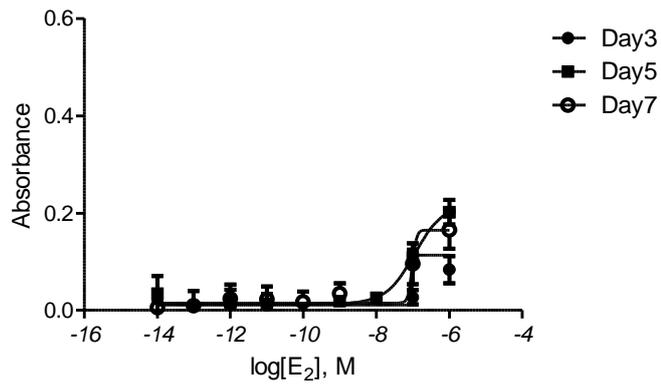
4.3.5 E-screen: 6 hour attachment, and 3, 5 or 7 day E_2 exposure (Passage <18)

The E-screen assay was performed with the modification of using 6 hours attachment before E_2 (10^{-6} to 10^{-14} M) was added to the PR-free medium supplemented with 10% DC-FCS (Figure 4.8).

The proliferation of MCF7 cells, after Day 7, was greatly increased from a maximal absorbance of ~0.3 AU with an overnight night attachment (Figure 4.6), to 0.6 AU (Figure 4.8). After 5 and 3 days there was slightly less proliferation than in the cells attached overnight. EC_{50} values for Day 7 were 10.9pM (6.7 to 17.9pM) and 13.5pM (7.8 to23.1 pM) at Day 5. MCF7 cells had a maximal average 2.7-fold proliferation above control.

T-47D cells, after 7 days, proliferated to a similar level as when an overnight attachment was used. After 5 and 3 days there was less proliferation. EC_{50} values are comparable to the overnight attachment; Day 5 - 7.6pM (2.1 to 27.1pM) and Day 7 - 11.2pM (7.8 to 16.1 pM). T-47D cells had a 3.1-fold proliferation above control, slightly lower than the overnight attachment.

ZR75-1 cells performed similarly in these conditions compared to the overnight incubation. EC_{50} values of; Day 5 – 6.8pM (0.9 to 49.9pM) and Day 7 – 10.5pM (4.6 to 23.4pM). At the higher concentrations of E_2 (10^{-8} to 10^{-6}M) the cell number was below the maximum response i.e. the upper plateau, however, the data was not removed from analysis in this case as the best-fit line of non-linear regression was not substantially skewed with its inclusion. ZR-75-1 cells had a maximal average 1.8-fold proliferation above control.

Changes in Significance between overnight and 6 hour attachment

All data sets, from the E-screens performed after a 6 hour attachment, rather than overnight, were characterised by greatly reduced SEMs. There was also a reduction in the concentration at which there was a significant difference in absorbance to the control wells.

After an overnight attachment period, the lowest concentration at which MCF7 cells were significantly different to the control was at an E_2 concentration of 10^{-10}M after 3 and 5 days. After 7 days with an overnight attachment period, due to the high variability, no treatments were significantly different to the control.

After a 6 hour attachment period of MCF7 cells, the LOD was reduced to 10^{-12}M (Day 3) and 10^{-11}M E_2 (Day 5 and 7). Interestingly by reducing the attachment period to 6 hours the reduction in variation in the Day 7 MCF7 cell population now generated a significant difference to control.

There was no change in the LOD for T-47D cells which were significantly different to control after both attachment periods at 10^{-11}M E_2 after Day 5 and 7. Neither attachment period generated significant differences after the Day 3 incubation.

ZR-75-1 cells, after an overnight attachment period, had an LOD of 10^{-10}M E_2. With 6 hours attachment after Day 5 this concentration increased to 10^{-9}M but decreased to 10^{-11}M at Day 7. Neither attachment period generated significant differences after the Day 3 incubation

Cell Line	Day 5	Day 7
T-47D	7.6pM (2.1-27.0pM)	11.1pM (7.8-16.1pM)
MCF7	13.5pM (7.8-23.1pM)	11.0pM (6.7-17.9pM)
ZR-75-1	6.8pM (0.93-50.0pM)	10.5pM (4.7-23.5pM)

Table 4.3: EC_{50} values for each cell line after a 6 hour attachment and exposure to E_2 for 5 or 7 days.
The EC_{50} value was determined from best-fit linear regression of data. The 95% CI was also determined and is indicated in brackets.

Figure 4.8: E-screen using 5000 cells/well MCF7, T-47D, ZR-75-1 with a 6 hour attachment.

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in their respective PR-free medium supplemented with 10% DC-FCS. After 6 hours, a further 100μl of PR free medium supplemented with 10% DC-FCS was added giving final E_2 concentrations from $10^{-14}M$ to $10^{-6}M$. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with a best fit log linear plot using linear regression in GraphPad Prism. Best fit log-linear plots after data normalisation after 5 or 7 days exposure to E_2 are also shown with the means ± SEM indicated

T-47D

Absorbance vs log[E$_2$] M
- Day3
- Day5
- Day7

T-47D normalised (Day 5)

% vs log[E$_2$] M

T-47D normalised (Day 7)

% vs log[E$_2$] M

MCF7

Absorbance vs log[E$_2$] M
- Day 3
- Day 5
- Day 7

MCF7 normalised (Day 5)

% vs log[E$_2$] M

MCF7 normalised (Day 7)

% vs log[E$_2$] M

ZR-75-1

Absorbance vs log[E$_2$] M
- Day3
- Day5
- Day7

ZR-75-1 normalised (Day 5)

% vs log[E$_2$] M

ZR-75-1 normalised (Day 7)

% vs log[E$_2$] M

134
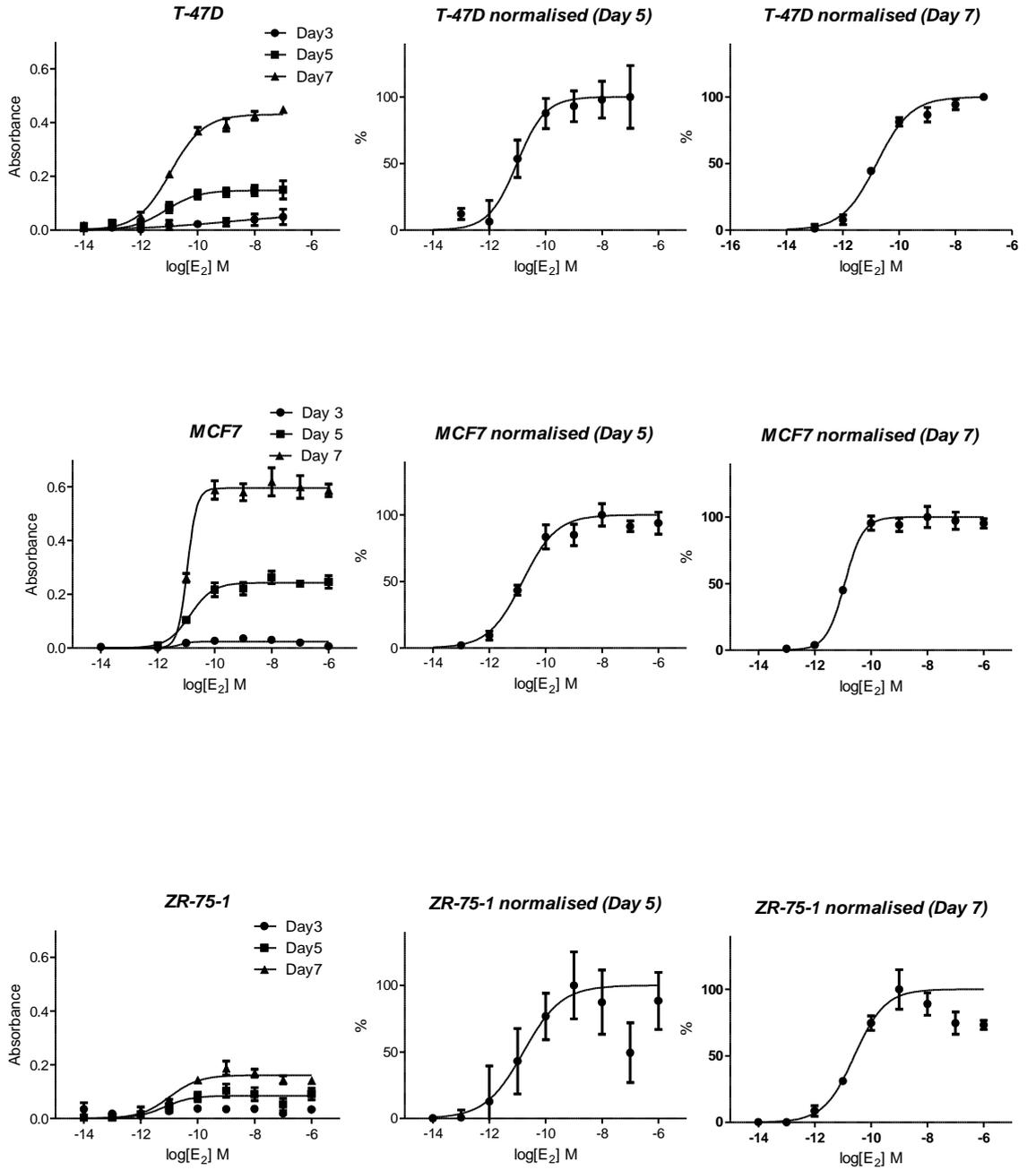
4.3.6 E-Screen of MCF7, T-47D and ZR-75-1 cells (Passage >18) overnight attachment

MCF7, T-47D and ZR-75-1 cell lines responded to E_2 with a dose dependent increase in cell numbers. 10^{-14}M stimulated T-47D and MCF7 proliferation whereas, 10^{-12}M E_2 was required to stimulate ZR-75-1 cell proliferation (Figure 4.9).

The E_2 stimulated increase in cell numbers was also clearly time-dependent in the T-47D and ZR-75-1 cell lines, with cell numbers being highest after 7 days exposure and lowest after 3 days exposure.

In the MCF7 cell line, 5 Days exposure to E_2 resulted in higher cell numbers than 3 day exposure, but 7 Day exposure to E_2 resulted in cell numbers similar to those obtained after 5 Days exposure. An additional 2 days exposure to E_2 post Day 5 did not stimulate additional proliferation.

At Day 7, exposure to 10^{-14}M E_2 resulted in OD values of 0.28 AU, 0.02 AU and 0.0 AU for T-47D, MCF7 and ZR-75-1 cell lines respectively. The same 7 day exposure to 10^{-6}M E_2 resulted in OD values of 0.60 AU, 0.29 AU and 0.33 AU for T-47D, MCF7 and ZR-75-1 respectively. The proliferative response of T-47D cells was much higher than that of MCF7 or ZR-75-1 cells.

Normalisation of Day 5 data, so that the lowest value was designated 0% and the highest value designated 100%, resulted in the sigmoidal curves also shown in Figure 4.4. It can be seen from the normalised data that T-47D cells maintained their proliferative response to the full range of E_2, up to a concentration of 10^{-6}M. MCF7 and ZR-75-1 did not increase cell number above 10^{-10}M and 10^{-11}M E_2 respectively, and actually decreased to approximately 60% of the maximum proliferation. Although MCF7 cells responded to much lower concentrations of E_2, variation between replicate wells was much higher than that of ZR-75-1 and T-47D.

T-47D cells, passaged at least 18 times, after Day 7 have absorbance values that are much higher than expected and do not approach 0 at the lowest concentrations of E_2.

Day 3 and 5 also show this trend of starting above the baseline. A reported fold increase of 5.64 above control may therefore not be accurate as there is such a large difference in OD between the lowest E_2 concentration (10^{-14}M) and the negative control, from which the fold proliferation is calculated. This is unlikely to be due to plating errors as the experiment was repeated on three separate occasions. The normalisation of data overcame this distortion of the best-fit log-normal curve. The regression on the normalised data had very low EC_{50} values but very large 95% CI; for day 5 - 0.3pM (0.003 to 32pM) and day 7 – 0.6pM (0.001 to 35.4pM).

Likewise for MCF7 cells, the EC_{50} value decreased, but the 95% CI greatly increased, reflected in the large SEMs. The EC_{50} values were; Day 5 – 0.06 pM (0.008 to 0.5 pM) and day 7 – 1.0 pM (0.01 to 58pM). MCF7 cells had a 2.3-fold proliferation above control.

Late passage ZR-75-1 cells, after Day 7 of exposure to E_2, had the same 1.7 fold proliferative response as did the early passage cells. Proliferation after Day 3 and 5 remained very low. As seen previously, cell numbers decreased in response to E_2 concentrations above 10^{-10}M. The EC_{50} values were; Day 5 – 29.2 pM (6.8-125pM) and for Day 7 – 32.49pM (18.5 to 56.6pM). (1.7 –fold).

Cell Line	Day 5	Day 7
T-47D	0.31 pM (0.004-32.6 pM)	0.6 pM (0.01-35.4 pM)
MCF7	0.063 pM (0.008-0.5 pM	1.0 pM (0.02-58.7 pM)
ZR-75-1	29.2 pM (6.8-125 pM)	32.4 pM (18.5-56.6 pM)

Table 4.4: EC_{50} value for each cell line (passage>18) after an overnight attachment and exposure to E_2 for 5 or 7 days.

The EC_{50} value was determined from best-fit linear regression of data. The 95% CI was also determined and is indicated in brackets.

Figure 4.9: E-Screen of MCF7, T-47D and ZR-75-1 cells (Passage > 18) overnight attachment.

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in PR-free medium supplemented with 10% DC-FCS. After 24 hours, a further 100µl of PR-free medium supplemented with 10% DC-FCS was added, giving final E_2 concentrations from 10^{-14}M to 10^{-6}M. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with best fit log linear plot using linear regression in GraphPad Prism. Normalised values after 5 or 7 days exposure to E_2 are also shown.
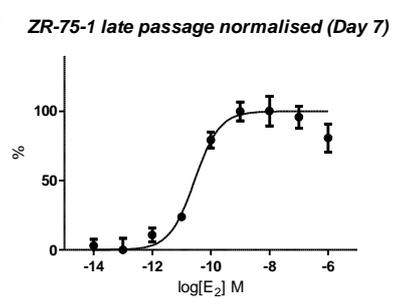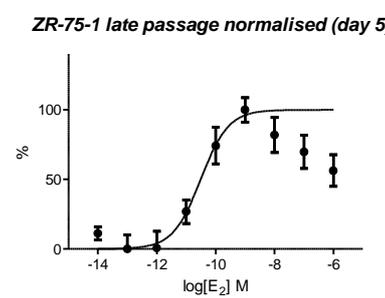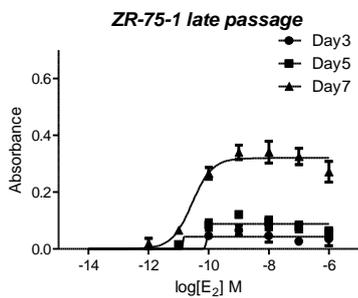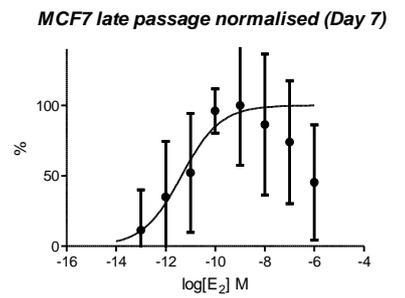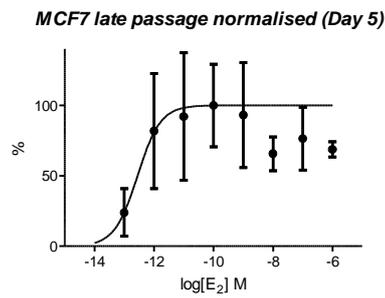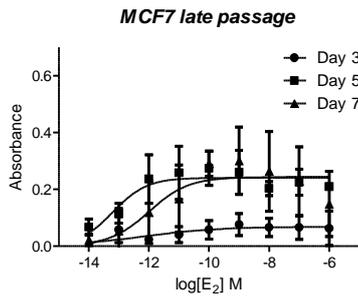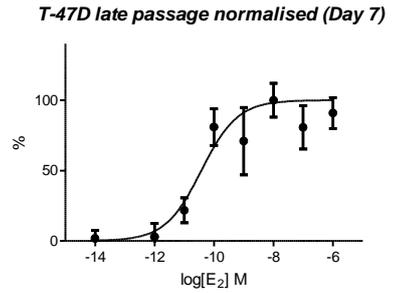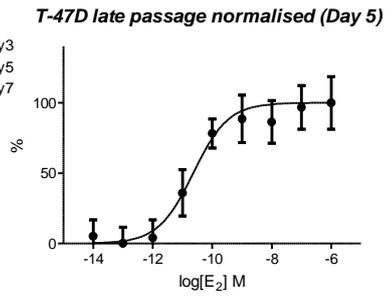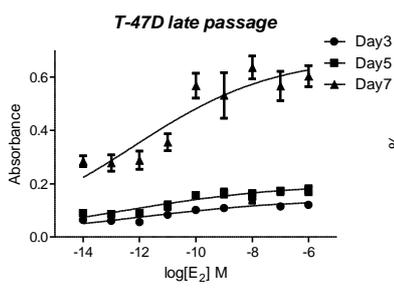
4.3.7 E-Screen of MCF7, T-47D and ZR-75-1 cells (Passage > 18) 6hr attachment.

After a reduced attachment time of 6 hours, MCF7, T-47D and ZR-75-1 cell lines responded to E_2 with a dose dependent increase in cell numbers. 10^{-14}M stimulated T-47D proliferation whereas, 10^{-10}M and 10^{-11}M E_2 was required to stimulate MCF7 and ZR-75-1 cell proliferation (Figure 4.10).

The E_2 stimulated increase in cell numbers was also time-dependent in the MCF7 cell lines, with number being highest after 7 days exposure. Cells did not have a measurable increase in proliferation after 3 days exposure.

In the T-47D and ZR-75-1 cell lines, 5 Days exposure to E_2 resulted in higher cell numbers than 3 day exposure, but 7 Day exposure to E_2 resulted in cell numbers similar to those obtained after 5 Days exposure. ZR-75-1 cells had a slightly higher increase in levels of proliferation after 7 days compared to 5 days however, the difference was not significant.

Normalisation of Day 5 data, resulted in the sigmoidal curves also shown in Figure 4.10. It can be seen from the normalised data that T-47D cells maintained their proliferative response to E_2, up to a concentration of 10^{-10}M. MCF7 and ZR-75-1 did not increase cell number above 10^{-9}M and 10^{-8}M E_2 respectively. T-47D cells in this case responded to much lower concentrations of E_2, however variation between replicate wells was much higher than that of ZR-75-1 and T-47D. The normalised data of ZR-75-1 cells after 5 days showed to lowest SEMs of any of the normalised data sets, compared to Day 7 which showed very large SEMs

Reducing the attachment time to 6 hours decreased proliferation of the late passage T-47D cells, which proliferated to less than half than after an overnight attachment period. Again the best fit curve shows greater sensitivity to estradiol, however, large SEMs exist. A reduction in proliferative response is caused by concentrations above 10^{-8}M E_2. The EC_{50} values were; Day 5 - 0.31pM (0.005 to 32.6 pM) and Day 7 − 0.6pM (0.01 to 35.4 pM). T-47D cells had a maximum 2.4-fold proliferation above control.

After 7 days, the late passage MCF7 cells proliferated approximately half the extent that the early passage cells did using the same protocol. There was very little proliferation after 3 or 5 days. EC_{50} values were; Day 5 - 0.063pM (0.008 to 0.5pM) and Day 7 – 1.0 pM (0.02 to 58pM). MCF7 cells had a maximum 1.9-fold proliferation above control.

ZR-75-1 proliferated to a greater extent than the high passage overnight attachment cells but only after 5 or 7 days. (With removal of the 10^{-6}M E_2 due to reduced proliferation at this point) EC_{50} values for were; Day 5 – 29.1pM (6.8 to 125pM) and Day 7 – 32.3 pM (18.5 – 56.5pM). ZR-75-1 cells had a maximum 1.9-fold proliferation above control.

Changes in Significance between overnight and 6 hour attachment

Passage 18 and above MCF7 cells, after a 6 hour or overnight attachment period, were at no time or E_2 concentration significantly different to the control.

T-47D cells were significantly different to control at 10^{-9}M E_2 and above after Day 3, 5 and 7 but only after an overnight attachment period. After a 6 hour attachment period, after Day 5 concentrations of 10^{-11}M and above are significantly different to the control, however, after day 7 there were no significant differences.

ZR-75-1 cells, after an overnight attachment period, were significantly different to the control at concentrations of 10^{-10}M E_2 and above but only after day 7. After 6 hours attachment, Day 5 concentrations of 10^{-10}M E_2 and above were significantly different to the control, however, after day 7 there were no significant differences.

Cell Line	Day 5	Day 7
T-47D	0.064pM (0.008-0.5 pM)	1.0 pM (0.02-58.7 pM)
MCF7	~74.4pM (very wide)	52.4 pM (6.8-0.04 pM)
ZR-75-1	15.8 pM (6.0-41.2 pM)	32.4 pM (2.3 -455.6pM)

Table 4.5. EC_{50} value for each cell line (passage>18) after a 6 hour attachment and exposure to E_2 for 5 or 7 days.
The EC_{50} value was determined from best-fit linear regression of data. The 95% CI was also determined and is indicated in brackets.

Figure 4.10: E-Screen of MCF7, T-47D and ZR-75-1 cells (Passage > 18) 6 hour attachment.

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in PR-free medium supplemented with 10% DC-FCS. After 6 hours, a further 100µl of PR-free medium supplemented with 10% DC-FCS was added, giving final E_2 concentrations from 10^{-14}M to 10^{-6}M. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with best fit log linear plot using linear regression in GraphPad Prism. Normalised values after 5 or 7 days exposure to E_2 are also shown.

T47D late passage

Day 3
Day 5
Day 7

Absorbance

log[E$_2$] M

T47D late passage normalised (Day 5)

%

log[E$_2$] M

T47D late passage normalised (Day 7)

%

log[E$_2$] M

MCF7 late passage

Day 3
Day 5
Day 7

Absorbance

log[E$_2$] M

MCF7 late passage normalised (Day 5)

%

log[E$_2$] M

MCF7 late passage normalised (Day 7)

%

log[E$_2$] M

ZR-75-1 late passage

Day3
Day5
Day7

Absorbance

log[E$_2$] M

ZR-75-1 late passage normalised (day 5)

%

log[E$_2$] M

ZR-75-1 late passage normalised (Day 7)

%

log[E$_2$] M
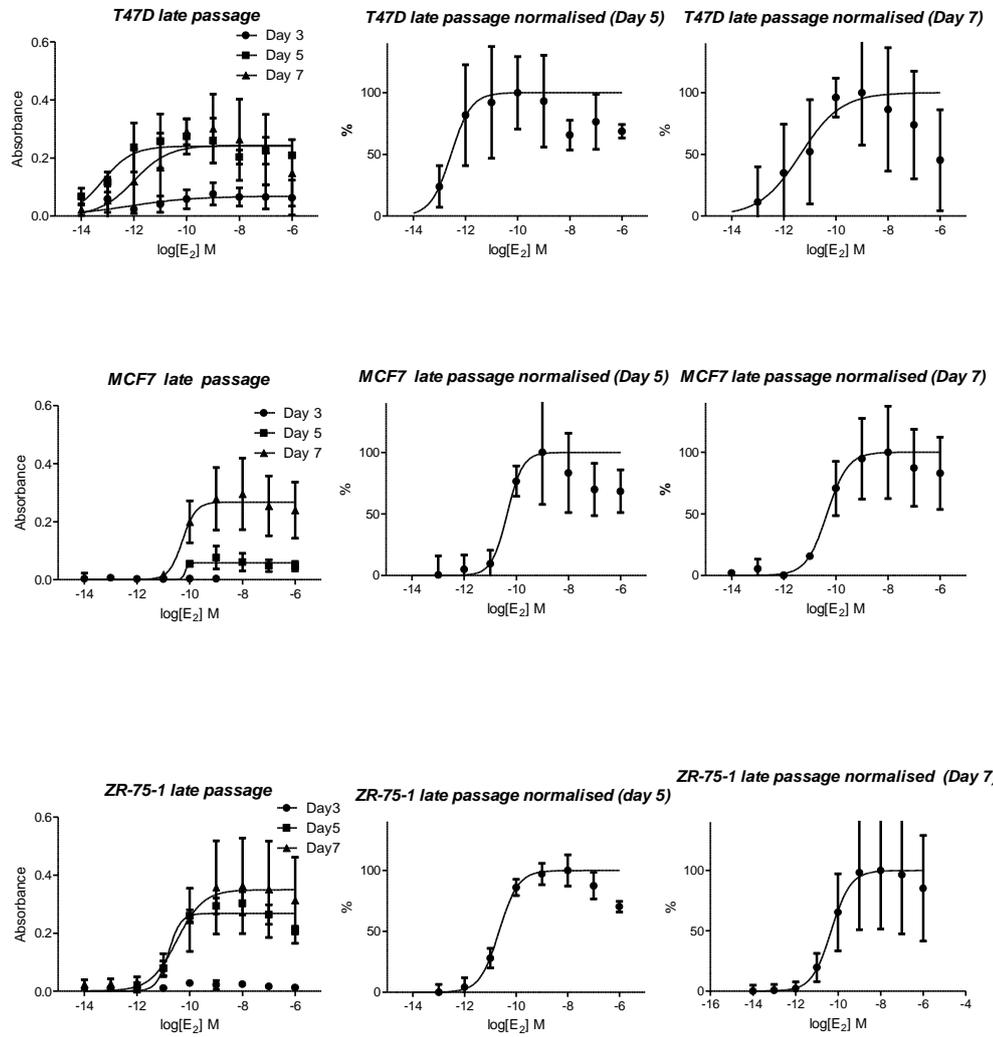
4.3.8 ERα protein expression in ZR-75-1 cells adapted to 5% and 2.5 % FCS:

ZR-75-1 cells that were grown over successive passages (up to 65 passages) in reduced amount of serum showed an increase in the 66 kDa ERα protein expression compared to those grown in 10% FCS. Cells grown in 2.5% FCS supplemented medium appear to express ERα to a greater level that those in 5% FCS supplemented media.
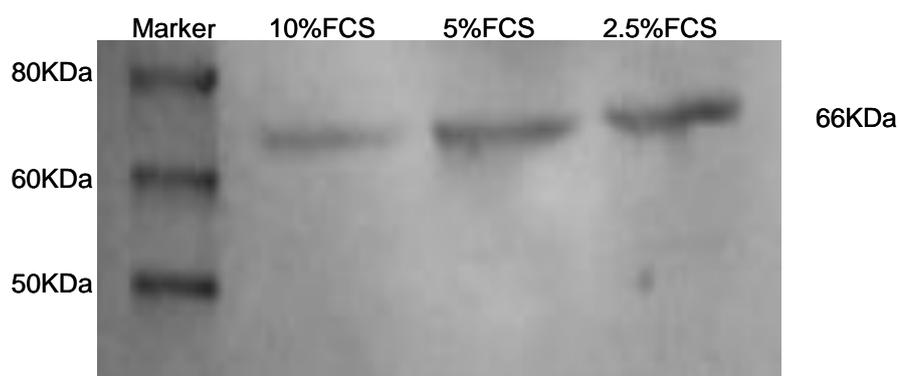
Figure 4.11: Western blot analysis of ERα expression after adaptation of ZR-75-1 cells to growth in medium supplemented with 10%FCS, 5%FCS or 2.5%FCS. Total protein was extracted from monolayers of ZR-75-1 cells adapted to growth in medium supplemented with 10% FCS, 5% FCS or 2.5% FCS. Protein concentrations were quantified using a BCA Protein Determination Kit and BSA standards. 10μg of protein per cell line was resolved using SDS/PAGE. Protein was transferred to a PVDF membrane. A rabbit polyclonal anti-ERα (HC-20) was used to identify the 66kDa ERα protein. Bound antibody was detected using an ECF substrate.

4.3.9 Effect of FCS supplementation on ZR-75-1 cells' response in the E-screen:

The AU results displayed are a result of a subtraction of the average absorbance of the control (0.5% EtOH control) wells (Figure 4.12). The data sets in this set of experiments fall below 0 AU at E_2 concentrations below 10^{-11}M E_2, which of course is not realistic. There may have been estrogenic contamination in the EtOH control media that was added to the control well, causing a higher number of cells in these wells than the cells exposed to E_2 levels below 10^{-11}M. Although this has caused all data sets here to be shifted down (below the 0 AU line), the dose-response curves are still an accurate representation of the cells' response to estrogen.

Both cell lines, adapted to growth in medium supplemented with 2.5% or 5% FCS, were exposed to E_2 either in their respective 2.5 or 5% DC-FCS, and 10% DC-FCS. In all cases cells showed a dose-dependant and time-dependant increase in cell number in response to E_2.

When cells adapted to growth in 2.5% FCS supplemented medium were exposed to estrogenic stimuli in 2.5% DC-FCS supplemented medium the maximal proliferation after 7 days was 1.5-fold. The fold proliferation of cells exposed to E_2 in medium supplemented with 10% DC-FCS increased to 2.1-fold. The EC_{50} at Day 7 for 2.5% DC-FCS and 10% DC-FCS supplemented media were 10.9pM (4.3-27.8pM) and ~11.0pM (very wide), respectively.

The cells adapted to growth in 5% FCS showed a similar trend, however, the fold-increase was only 1.6 when the cells were exposed to E_2 in the presence of 5% DC-FCS and 1.7 in the presence of 10% DC-FCS. The EC_{50} at Day 7 for 5% DC-FCS and 10% DC-FCS supplemented media were 9.3pM (3.3 – 25.7pM) and 11.9pM (6.0 - 26.0pM) respectively.

The EC_{50} values are similar between the ZR-75-1 cells grown in 10% FCS supplemented medium and the two low serum adapted sub-lines. ZR-75-1 cells had the same fold increase in response to E_2 as the ZR-75-1 (5%) cells (1.7-fold). ZR-75-1 (2.5%) grew best (2.1-fold). The cells that were exposed to E_2 in either 2.5% or 5% DC-FCS had slightly lower proliferative response to E_2 (1.6- and 1.7-fold).

The cells grown in 5% FCS and exposed to E_2 in either 5% or 10% DC-FCS were both significantly different from control at 10^{-10}M E_2 and above after day 5 and 7. The cells grown in 2.5% FCS and exposed to E_2 in 10% DC-FCS were significantly different from control at 10^{-11}M and above after Day 5 and 7. The cells exposed to E_2 in 2.5% DC-FCS were significantly different at E_2 concentrations of 10^{-11}M and 10^{-10} M after Day 5 and 7 respectively.

Cell line	E-screen DC-FCS concentration	Day 5	Day 7
ZR-75-1 (2.5)	2.5	12.6pM (6.2- 25.7 pM)	10.9pM (4.3-27.8pM)
ZR-75-1 (2.5)	10	11.5pM (5.6 – 23.7pM)	~11.0 pM (very wide)
ZR-75-1 (5)	5	13.5pM (2.4 – 74.3pM)	9.3pM (3.3 – 25.7 pM)
ZR-75-1 (5)	10	12.9pM (very wide)	11.9 pM (6.0-26.0pM)
ZR-75-1	10	29.22pM (6.8 –125pM)	32.4 pM (18.5-56.6pM)

Table 4.6. EC_{50} value for ZR-75-1 cells adapted to medium supplemented to 5 or 2.5 % FCS, after a 6 hour attachment, and exposure to E_2 for 5 or 7 days.

The EC_{50} value was determined from best-fit linear regression of data. The 95% CI was also determined and is indicated in brackets.

Figure 4.12: E-Screen of ZR-75-1 cells adapted to 2.5 and 5% FCS supplemented medium exposed to E$_2$ in the corresponding % DC- FCS or 10% DC-FCS .

In a 96 well plate, 5000 cells/well were left to attach to the growth surface in the cell's respective media and % DC-FCS supplementation. To produce a standard curve, the crystal violet assay was applied after 6 hours. At the same time the media in the remaining plates were aspirated and replaced with media supplemented with 2.5, 5 or 10% DC-FCS without PR and Estradiol concentrations from 10^{-6}M to 10^{-14} M. A) ZR-75-1 cells adapted to 2.5% DC-FCS with 2.5% DC-FCS supplementing media during the E-screen. B) ZR-75-1 cells adapted to 2.5% DC-FCS with 10% DC-FCS supplemented media during the E-screen C) ZR-75-1 cells adapted to 5% DC-FCS with 5% DC-FCS supplemented media during the E-screen, D) ZR-75-1 cells adapted to 5% DC-FCS with 10% DC-FCS supplemented media during the E-screen E) ZR-75-1 cells with 10% DC-FCS supplemented media during the E-screen The equations that described the relationship between cells/well values and absorbance were used to convert absorbance derived after 72 hours to actual cell number. The experiment was repeated on three separate occasions (n=3) and the means non linear regression used to fit sigmoidal curves to the means represented here ± standard deviations. The experiment was repeated on three separate occasions (n=3) and the means ± SEM represented with best fit log linear plot using linear regression in GraphPad Prism

ZR-75-1 (2.5%) - E₂ & 2.5% DC-FCS exposure

ZR-75-1 (2.5%) - E₂ & 10% DC-FCS exposure

ZR-75-1 (5%) - E₂ & 5 % DC-FCS exposure

ZR-75-1 (5%) - E₂ & 10% DC-FCS exposure
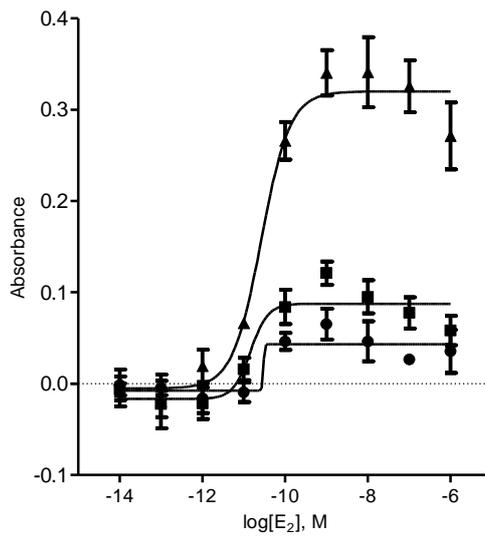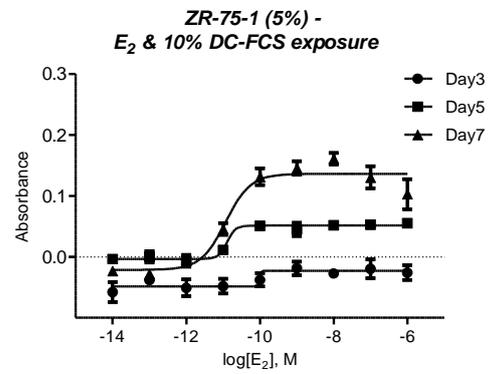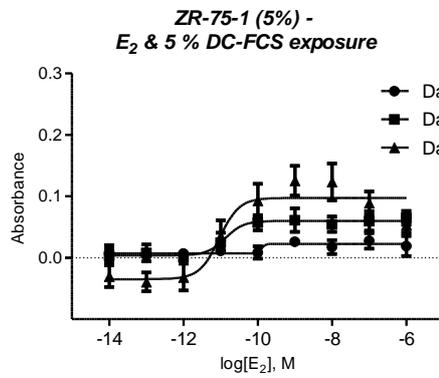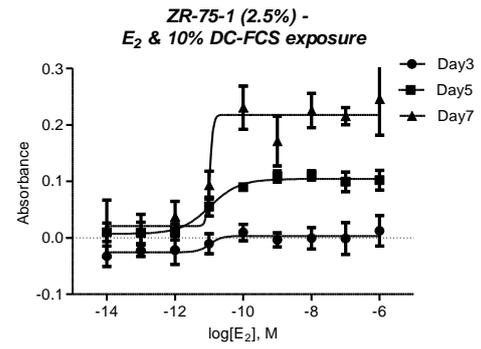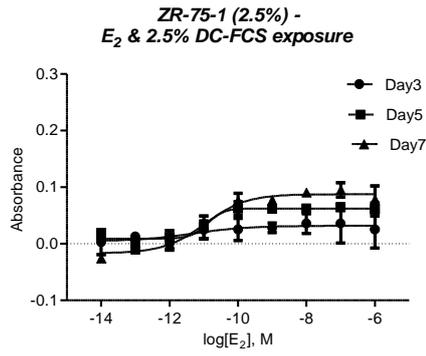
4.4 Discussion:

The E-screen is a highly sensitive assay which uses human breast cancer cell lines to detect (xeno)estrogens at concentrations often in the picomolar range. Interestingly, an exposure medium consisting of 10% DC-FCS, which is referred to as 'estrogen-free', potentially has an estrogen content of approximately 0.8pM from the DC-FCS alone (compared to 27.5pM in 10% FCS) (Fortunati et al., 1999). Rasmussen et al (2002) however found commercial preparations of DC-FCS to have no detectable amount of E_2. Excessive costs of commercially prepared DC-FCS and unstable supply (during the period of this study) made batch preparation a viable alternative. Although the process is relatively labour intensive and batch-to-batch variations may occur it was used with success in this study. The estrogen content of the DC-FCS we prepared was not directly tested however it would be interesting to optimise the procedure and monitor the batch variations that do occur.

Characteristics of a cell line that make it amenable for the E-screen include the ability of a cell population to cease, or significantly decrease, proliferation in an 'estrogen-free' medium. This is due to the E-screen being a comparison between the cells that have been stimulated by the estrogenic test sample to those that have not. If a cell population continues to proliferate in the absence of estrogenic stimuli, the relative effect of estrogenic stimuli on the cells is reduced. Secondly, the cell must be highly responsive to small increases in the estrogen concentration, which will allow for weakly estrogenic compounds or mixtures to be identified (Jones et al., 1997).

It was previously shown by Villalobos et al (1995) that MCF7 (ATCC) cells continue to proliferate regardless of a reduction in the estrogen content of the medium, seeming to make it less amenable for the E-screen than other sub-lines that do cease or reduce proliferation such as MCF7 BOS cells (Villalobos et al., 1995). However, the MCF7 (ATCC) cell line tested here did significantly reduce proliferation in medium supplemented with 0% and 1% DC-FCS compared to 10% FCS. Also, the other ERα positive cell lines, T-47D and ZR-75-1, did cease proliferation, having the same number of cells per well after 72 hours in medium supplemented in 0% or 1% DC- FCS. The cell lines that did not contain ERα, H23 and RL95-2, proliferated to the same extent regardless of serum concentration in the medium. These cell lines appear to have no or

little regulation by estrogen or indeed other component of FCS within the medium. The response of the clone of MCF7 (ATCC) cells used by Villalobos et al (1995) is consistent with one that had lost expression of the ERα. The ERα expression was however, quantified by Villalobos et al (1995), so the loss of E_2 regulation may have been a result of their maintenance of the cell line in Dulbecco's modification of Eagle's medium (DME) supplemented with 5% FCS. Alternatively, we maintained the cells in Eagle's minimum essential medium, supplemented with 0.01 mg/ml insulin and 10% FCS as recommended by ATCC.

Ovcar, shown to express ERα mRNA only, responded in the same way as the cells that express ERα protein, that is they did not proliferate in the absence of FCS. The addition of 1% DC-FCS was able to stimulate proliferation, however, cell numbers were greatest in the presence of 10% FCS. This suggests that either the ERα is post-transcriptionally modified in these cells, so that it remains active, but is not detectable by the ERα antibody we used in western blotting or there is another pathway substituting for the ERα mediating E_2 responsiveness. Stimulation by non-estrogenic mitogens in the serum may also contribute to this. Ovcar cells were not chosen for further characterisation in the E-screen assays because the ERα protein was apparently not expressed, however, the reduction in growth displayed by Ovcar, T-47D and ZR-75-1 in estrogen-free medium make them all good candidates for characterisation and comparison to MCF7 in an E-screen assay and for studies into the mechanisms underlying the estrogen response.

The presence of Phenol Red, a pH indicator, had little effect on proliferation over the tested 72 hour period of growth. Phenol red in the presence of 1% DC- FCS did not stimulate proliferation, although it is likely that 1% DC- FCS restricts estrogen-stimulated proliferation, whereas a higher DC-FCS concentration may facilitate proliferation (Villalobos et al., 1995). The absence of Phenol Red from the medium supplemented with 10% FCS, resulted in slightly lower cell numbers after 72 hours. The absence of a weak estrogen in medium supplemented with 10% FCS would not be expected to cause any effect, as the cells are already in optimal growth conditions. The non-significant reduction in cell numbers seen may therefore be due to slight variations in the composition of the medium with or without PR, which were produced separately, rather than the actual presence or absence of Phenol Red. Although a significant

proliferative effect was not seen, it has been shown that phenol red is slightly estrogenic (0.001% the potency of E_2) (Berthois et al., 1986) and this effect may have been seen using a longer incubation period. The inclusion of phenol red in a pre-treatment period for the E-screen assay, potentially may have implications for the sensitivity of the cells by subtle regulation of ERα and cellular components by a weak estrogen, with changes to the subsequent proliferative response of the cell.

Regulation of the ERα protein content of MCF7, T-47D, ZR-75-1 and H23 was assessed after a 48 hour pre-treatment period in PR-free medium supplemented with 1% DC-FCS compared to 48 hours incubation in medium supplemented with 10% FCS. It was found that the changes in protein expression were minimal and non significant in all the cell lines tested.

An increase in ERα protein expression when cells are grown in a low serum medium has been shown previously in MCF7 sub-lines (Welshons, 1987; Katzenellenbogen et al., 1995), with an almost two-fold up-regulation after 3 days in estrogen-free medium. Katzenellebgoben et al (1995) and Welshons et al (1987) showed this using whole cell [^{3}H]estradiol uptake in MCF7 cells sourced from the Michigan Cancer Foundation and an undisclosed source, respectively.

T-47D cells have also been shown to have up-regulated ERα expression in the absence of estrogens, also by whole cell [^{3}H]estradiol uptake (Reddel et al., 1984). Further experimentation, including a time-course of exposure to estrogen free media would be required to elucidate the molecular process.

The proliferative ability of MCF7, T-47D, ZR-75-1 and H23 cells in response to a range of E_2 concentrations, with daily media/treatment changes was assessed. Initially we hypothesised that after a brief period in estrogen-free media to up-regulate the cellular ERα, followed by exposure to E2, replenished every day, that cell proliferation would rapidly occur. It was thought that daily changes of E_2 would provide a constant supply of E_2 at the desired concentration and it would not be subject to degradation in culture. However, it was shown that after a 72 hour period, none of the cell lines were able to significantly proliferate in response to E2 or show a dose-dependant increase in cell numbers when the medium was changed every 24 hours.

A 48 hour pre-treatment in PR-free medium supplemented with 1% DC-FCS followed by 72 hours exposure to E_2 with daily media/treatment changes would also remove any autocrine and paracrine compounds being produced by the cells in response to E_2. There are however examples of this in the literature where daily media/treatment changes have still supported proliferation (Wang and Lou, 2004; Oh et al., 2006) and so it may be appropriate to do this in situations where the compound being tested is known to be unstable, but would need to be validated.

Although a small non-significant increase can be seen in this experiment after 72 hours proliferation of T-47D cells, the length of exposure was not enough to generate significant results. For comparison purposes, the dose-response of T-47D cells after 72 hours exposure to E_2, without media changes every 24 hours was also displayed on Figure 4.5. The two can not be directly compared as one is in AU scale and the other is cell number, however the dose-response is similar, that is, the higher concentrations of E_2 have caused the T-47D cells to proliferate more than the lower concentrations of E_2. The proliferation of the T-47D cells when the Fulvestrant antagonist is included with the E_2 treatment shows the same small dose-response increase. The antagonist prevents E_2 from interacting with ER, and we have determined from later experiments that approximately 10^{-7} M E_2 is required to overcome the antagonist effect of 10^{-7} M fulvestrant, so it is unknown why the cells exposed to this treatment would show the same increase in cells.

MCF7, ZR-75-2 and H23 did not show this small increase. Soto et al (1995) only found significant differences in growth rates between control and exposed cells after 4 days in the highly E_2-responsive MCF7 BOS cell line. Perhaps 1% DC-FCS was not able to support E_2 stimulated growth because the concentrations of required serum components are not high enough.

The use of method A for dextran-charcoal treatment of FCS comes into question as the Fulvestrant was still able to block the proliferation of cells slightly. There may still have been a residual estrogen content in the serum, and so this led us to use an alternative 'in-house' cell-culture validated method (Method B).

The DC-FCS produced using Method B, was used in an E-screen assay using T-47D, MCF7 and ZR-75-1 cells. A GWRC standard operating procedure for the E-screen was acquired which was used in modified form as described. The cells were attached to the growth surface of a 96 well plate for 16-24 hours in PR-free medium supplemented with 10% DC-FCS. A range of E_2 concentrations (10^{-6} to 10^{-14}M) were then added and cell proliferation measured after 3, 5 and 7 days. All cell lines showed a dose-dependant increase in cell number. T-47D was the only cell line that also showed a time-dependent increase in cell number over the full 7 days, meaning that MCF7 and ZR-75-1 cells had stopped proliferating after day 5. MCF7 and ZR-75-1 cells may have reached confluence faster than T-47D cells, although this is not consistent with calculated doubling rates (in complete media) of 41.6, 36.9 and 36.0 hrs respectively. The percentage confluence of the wells was not visualised at this time which may have helped elucidate the differences observed. Alternatively, MCF7 and ZR-75-1 cells may have become non-responsive to E_2 after the five days exposure, whereas T-47D maintained their E_2-responsiveness for the 7 day period.

Inter-experimental variability has been shown in the E-screen assay to be as high as 60%, and normalisation of the proliferation is an effective way of reducing this(Rajapakse et al., 2002). By converting the proliferation data from each plate to a percentage value with 0% being the negative (solvent) control and 100% being the maximum proliferative response of the cells, the inter-experimental variability is reduced considerably. When this technique was applied to the data here, it also gave a clearer indication of the dose-response of cells, especially a reduction in proliferative response to the higher concentrations of E_2 used. T-47D, MCF7 and ZR-75-1 cells all reduced their proliferative response to E_2 at concentrations above 10^{-7}, 10^{-10} and 10^{-10}M respectively.

Using this particular method of overnight attachment/pre-treatment it is clear that T-47D cells have small SEMs and respond to E_2 from 10^{-14}M to 10^{-7}M, that is, the proliferative response to 10^{-6} M E_2 was lower than that of 10^{-7}M E_2. MCF7 cells and ZR-75-1 cells have large SEMs and reduce their proliferative response above 10^{-10}M after 5 Days.

The range of E_2 concentrations tested arbitrarily went from 10^{-14}M up to 10^{-6}M, so a reduction in response above 10^{-10}M is not necessarily a disadvantage. The concentration of environmental samples would often be lower than the equivalent of 10^{-10}M E_2 and are applied to the E-screen in serial dilutions(Tan et al., 2007). This means that the actual concentration of the test samples would still be within a useable range of the cell lines.

Rasmussen et al (2002) found that a pre-treatment period of MCF7 BOS cells in estrogen-free media (0 - 6 days) did not alter the LOD (the limit of detection, $p < 0.05$) but did reduce the fold proliferation after 7 days by 25% if cells had a 1 day pre-treatment period and up to a 50% reduction in fold-proliferation if the cells had 3 to 6 day pre-treatment period.

The response of cells in an E-screen assay was therefore also compared between an overnight attachment/pre-treatment period and the optimised 6 hour attachment time. Using the 6 hour attachment period there was a decrease in the lowest concentration of E_2 that was significantly different from control in MCF7 and ZR-75-1 cells from 100pM to 10pM. The fold-proliferation of the MCF7 cells increased from 2.2 to 2.7 fold. The ZR-75-1 had a small increase of 1.7 to 1.8 fold.

T-47D cells were sensitive to 10pM E_2 with either attachment/pre-treatment time and fold proliferation slightly decreased from 3.4 to 3.1 fold. The EC_{50} values after the 6 hour attachment time were all in the range of 10pM for all 3 cell lines. Interestingly MCF7 cells, after an overnight attachment, appeared to have a lower EC_{50} value (1pM), although because data variability meant there was a lack of significance below 100pM, this finding will need confirmation.

A reduction of attachment/pre-treatment period to just 6 hours instead of overnight is consistent with the study by Rasmussen et al (2002) where an elimination of a pre-treatment time caused an increase in fold proliferation. A 6 hour attachment time, however, has not previously been reported.

Many studies where E-screen assays have been used have placed limitations on the number of passages within which cells are used. A comparison of the sensitivity of cells at high passage (greater than 18 passages) has not been quantitatively reported

previously for MCF7 cells or any other cell line. None of the cell lines tested in this study in the E-screen after 18 passages were able to maintain the same level of responsiveness to E_2 as the cells tested at earlier passage number. Here we found that MCF7 cells had similar fold responsiveness at high passage number, however, due to large SEMS there was no concentration at which proliferation caused by an estrogenic response was significantly different to the control. The ZR-75-1 cell line, at late passage number had a similar fold proliferative response to E_2 and the same EC_{50} of 100pM. The passaging of T-47D cells on more than 18 occasions increased the LOD from 10^{-11}M E_2 to 10^{-9}M E_2.

Decreased cellular response with increasing passaging raises the question of which cell components apart from the commonly described 'genetic instability' causes the cell to maintain a response to estrogen but with a lesser fold proliferation. A comparison of ER levels in the late and early passage cells would be a good indicator of why there may be an effect.

The ZR-75-1 cells that were adapted to a low serum environment had upregulated ERα protein expression. It has been shown that adaptation of MCF7 cells to a low serum media led to less proliferative cells in an E-screen(Villalobos et al., 1995). This had not previously been tested in ZR-75-1 cells, but we found that there was little change in the proliferative response or LOD of these cells. However, given how low the fold proliferation was (~1.7) the cells are of minimal use to E-screen testing and have no advantage over the 'wild-type' ZR-75-1 cell line in the E-screen assay.

A fast transference of the cells from normal media to test conditions seems to be preferable. In fact, further testing could include a 6 hour attachment time in PR+ medium with 10% FCS. If pre-treatment of cells in a low serum environment leads to up-regulation of the ERα and a lower responsiveness in the E-screen further testing could assess the outcome of hyper-stimulation with E_2 immediately prior to an E-screen assay to perhaps temporarily reduce the ERα expression.

An improvement in sensitivity and fold proliferation was seen when using the 6 hour attachment/pre-treatment period instead of the commonly used 24 hour attachment/pre-treatment period. In some cases, results were significant after 3 days' proliferation,

however at this time the fold proliferation was very low and accurate determination of the estrogenicity of test chemicals would be limited. A high proliferative response to E_2 is preferable for the detection of weak or very low concentrations of (xeno)estrogens, which can occur in environmental samples.

The characteristics of a cell line that makes it a good candidate for the E-screen are: the expression of ERα – the receptor primarily responsible for estrogen-dependant proliferative response (Penot et al., 2005); no expression of ERβ or the 46kDa splice variant of ERα – both these receptors have been shown individually to be detrimental to the proliferative effect originating from the ERα receptor (Strom et al., 2004; Penot et al., 2005; Hartman et al., 2006); a significant reduction or cessation of growth in estrogen-free media – as the endpoint of the E-screen is a measure of the induction of cell growth by a test sample relative cell growth in an estrogen-free control, a cell line that can reduce or stop proliferating in estrogen-free media can generate higher relative fold responses; a high proliferative response to E_2 – cells need to respond to estrogen by significantly increasing proliferation.

The ZR-75-1, MCF7 and T-47D cell lines satisfy these characteristics, such as expression of 66 kDa ERα, no detected expression of ERβ, and a cessation of proliferation (MCF7 had significant reduction) in estrogen-free media makes them potentially good candidates for use in the E-screen assay.

Whilst the proliferative response of ZR-75-2 cells to E_2 has been documented (Marth and Daxenbichler, 1985; Daly and Darbre, 1990; Ruedl et al., 1990), a quantitative and comparative description of their use in the E-screen has not previously been reported. The ZR-75-1 cells had high SEMs and low proliferative response. A reduction in attachment/pre-treatment time from 16-24hrs to 6hrs did not increase the fold-proliferation or reduce the SEMs. The E-screen assay has already been described as having a high inherent variability (Rajapakse et al., 2002; Vanparys, 2006). The combination of this, high SEMs and low fold proliferative response to E_2, make the use of the ZR-75-1 cell line a poor choice in the E-screen. An adaptation to low serum/estrogen media increased the ERα content, but did not increase the proliferative response of the cells to E_2.

The MCF7 (ATCC) cell line used in this study also had the characteristics to be useful in an E-screen assay and had been widely used previously in E-screen assays (Table 1). MCF7 had the highest ERα expression of the cell lines tested, no detectable ERβ, and a significant reduction of growth in estrogen-free media. The cells were found to have a fold proliferation in response to E_2 of 2.2, this was greater than previously described for MCF7 (ATCC) possibly a result of the sub-optimal conditions Villalobos et al (1995) maintained the cells in. The most significant finding presented here is the vast reduction in SEMs, increase in fold proliferation (2.2 to 2.7 fold), and broader range of concentrations of E_2 at which MCF7 cells maintain there proliferative response to, as a result of reducing the pre-treatment/attachment period from the commonly used 24 hours period to the optimised 6 hour pre-treatment/attachment period.

T-47D cells have been used less commonly than MCF7 cells in the E-screen assay (Table 1). Soto et al (1999) compared the two cells lines, demonstrating that T-47D cells had an EC_{50} to E_2 of 30pM± 9.0 whereas MCF7 cells had 7.3pM±1.2 when cells were attached/pre-treated for 24 hours and exposed to E_2 for 6 and 5 day respectively. It is not clear which sub-line of MCF7 Soto et al (1999) used in this study but we would assume that it was MCF7 BOS cells. T-47D cells were incubated for one day more than MCF7 cells due to a slower doubling time (contrary to what we found - discussed in Chapter 3). In this study we found that the EC_{50} values to E_2 were actually very similar after a 6 hours attachment/pre-treatment period and 7 days exposure to E_2 (with 95% CI represented in brackets) [T-47D: 11.1pM (7.8-16.1) and MCF7: 11.0pM (6.7-17.9pM)]. The T-47D cells did have a larger fold proliferation than MCF7 cells in the same experiment (3.1 and 2.7 fold respectively).

MCF7 and T-47D cells are both good candidates for use in the E-screen. Further studies and comparisons between the two cell lines could show how they perform against not only various single chemicals, but also with complex mixtures and extracts from water samples. It is possible that one cell line may be more robust in its proliferative response to estrogenic compounds when there are also potentially cytotoxic compounds present. It would also be interesting to use the highly responsive MCF7 BOS cell line to see if the reduction in attachment/pre-treatment time to 6 hours would increase the fold proliferation possible.

CHAPTER 5

Estrogen Activated Gene-linked Luciferase Expression

5.1 Introduction

Estrogens exert effects on organs and tissues and are regulators of growth, proliferation, differentiation and metabolism through the transduction of other hormones, growth factors and cytokines (Tang et al., 2004; Terasaka et al., 2004). Estrogens primarily interact with estrogen receptors, estrogen response elements or cross-signalling pathways. Regardless of the pathway, an estrogenic effect is one that has an effect on the expression of estrogen responsive genes (Tang et al., 2004; Moggs, 2005). This response depends on the tissue type, the type of estrogen receptor present, regulators of expression and the type and concentration of agonist/antagonist (McDonnell and Norris, 2002).

A holistic approach to the *in vivo* effects of estrogenic chemicals can be found in the transcriptional cellular responses (Terasaka et al., 2004; Moggs, 2005). Jorgensen et al (2000) argue that the endpoint of all animal and cellular assays to determine estrogenicity originate from changes in gene expression and provided that the correct set of genes are identified then measuring changes in endogenous gene expression would be as useful as other endpoints. Activated ERs primarily interact with cis regions of genes by either directly associating with the 15bp palindromic estrogen response element (ERE) which has a conserved sequence of 5'- GGTCANNNTGACC-3' (Klein-Hitpass et al., 1988), or with distal elements such as AP-1 (Webb et al., 1999) or Sp1 transcription factor complexes and their respective binding sites.

5.1.1 Microarray Expression Analysis

Cellular responses to estrogen can be analysed by using the hybridisation based technology of DNA microarray. The use of a DNA microarray platform to perform genome-wide analysis was first reported by Schena et al in 1995. Microarray analysis is an assay whereby a large numbers of genes can be simultaneously analysed for up-regulation or down-regulation compared to a control (Schena et al., 1998).

The microarray concept has been applied to many areas of genomics and is based on the printing or spotting of thousands of pre-synthesised cDNAs (500-2000 bases) or oligonucleotides (20-50 bases) to glass or a membrane by mechanical spotting, bubble jet applicator or by photolithography. The sequence data is sourced from a collection of

expressed sequence tags (ESTs) corresponding to mRNA of unknown genes or from fully sequenced genes (Lobenhofer et al., 2001). Reverse transcribed cDNA from a sample and a control, using two different fluorescent labels, are hybridised to the array. This provides a fluorescent point on the array at a point of complementary cDNA. The use of two colours allows for scanned arrays to be analysed with image analysis software and a significant difference in signal intensity corresponding to an up or down regulated gene can be identified (Lobenhofer et al., 2001).

The genes that are up-regulated or down-regulated have been collated by the Dragon Estrogen Response Gene Database (ERGBD) by analysing all data available on PubMed and currently has a total of 1069 human genes largely derived from microarray data (http://defiant.i2r.a-star.edu.sg/projects/Ergdb-v2/index.htm) (Tang et al., 2004).

A number of studies have used microarrays to assess the gene expression profile in human cell lines after exposure to estrogen or xenoestrogens. DNA microarray allows novel toxicant-induced gene expression to be analysed (Lobenhofer et al., 2001).

The molecular response of genes to estrogenic stimuli is a dynamic process, whereby activity and expression change over time with variations seen not only between tissue type but also cell line (Mutarelli et al., 2008)

5.1.2 MCF7 expression profiling

When estrogen is presented to MCF7 cells, microarray shows an increase in genes associated with proliferation and inhibition of apoptosis (Frasor et al., 2003). A study by Teresaka et al. (2004) used MCF7 cells to determine the gene expression response when cells were exposed to 10nM E_2 for 72 hours. Using two microarray chips totalling 21807 genes, 1150 genes showed at least a 2 fold response. A subset of 172 genes were identified that showed reproducible up-regulation or down-regulation after exposure to estrogen. One hundred and eight genes were up regulated and 64 genes, including the gene for ERα, were down-regulated. The results were in part confirmed with the use of real-time quantitative RT-PCR for 11 of the genes. Using this 'concentrated' group of estrogen responsive genes provides a highly sensitive tool for assessing estrogen responsiveness. This study did, however, only examine the cellular response at 72 hours, meaning that many of the identified genes would be a result of secondary

responses and gene signalling cascades. Considering the response to estrogen can be seen in MCF7 cells after 2-8 hours (Jorgensen et al., 2000) many of the early events may have been overlooked.

A comprehensive study by Frasor et al (2003) assessed over 12000 genes for estrogen regulation in MCF7 cells by microarray. The regulation of genes was assessed after 4, 8, 24 and 48 hours of exposure to 10nM E_2. Over 400 genes were found to be consistently responsive to E_2, 50 of which were further confirmed by quantitative PCR. Interestingly, although 70% of the regulated genes were down-regulated, the majority of up-regulated genes were seen after 4 hours exposure to E_2.

A study by Jorgeson (2000) used four endogenous "marker" genes in MCF7 cells that were characterised for their estrogenic response. The genes Trefoil Factor 1 (pS2) and α1-antichymotrypsin (α1-ACT) were up-regulated and monoamine oxidase A (Mao A) and transforming growth factor β3 (TGβ3) were down-regulated. MCF7 cells were incubated for 6 days in estrogen-free medium, allowing estrogen response genes to return to basal levels, before exposure to 10nM E_2. A response was seen in each of the four genes within 2-8 hours. The relatively labour intensive process of differential display of reversed transcribed mRNAs technology (DDRT-PCR) and phosphorimaging were used to assess the difference in specific gene expression after exposure to estrogens and 11 common xenoestrogens. α-1 ACT was shown to be less responsive to the xenoestrogens than the other genes requiring 10-100 times higher doses to elicit a response.

5.1.3 T-47D expression profiling

Lin et al. (2004) used microarray analysis of 18,912 human genes to determine the response of T-47D cells to 1nM E_2 every hour for the first 8 hours and then every second hour up to 24 hours. Three hundred and eighty six genes were found to be estrogen responsive, 226 of which were up-regulated. To increase the specificity of the set of estrogen response genes the anti-estrogen ICI 182780, an ER antagonist, was added to the cells in a repeat experiment. This showed that 137 genes (36%) were directly transduced by the estrogen receptor. Treatment with CHX, a protein synthesis inhibitor, was also used to ensure the effects seen were a primary response. After this round of selection, only 89 genes were defined as being responsive to estrogen. This set

of genes was very similar to the estrogen response genes identified in MCF7 cells (Lin et al., 2004).

5.1.4 ZR-75-1 expression profiling

A similar study by Soulez and Parker (2001a) used microarray to analyse gene expression in ZR-75-1 cells, using the four different ligands E_2, ICI 182780 and the selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene. Cycloheximide was used as a protein synthesis inhibitor to ensure that only primary gene responses were analysed and MCF7 cells were used as a control for known genes. Out of 5600 genes, 53 were identified as estrogen responsive. ZR-75-1 cells showed a 100 fold up-regulation of the cytochrome P450-IIB gene, responsible for oxidation of steroids, an effect not detectable in MCF7 cells.

5.1.5 Gene Specific Studies

Five genes were identified from the available literature, selected on the basis of being responsive to estrogen in more than one cell line and also *in vivo* (Watanabe et al., 2002). Further to this, they were identified as being highly up-regulated or down-regulated within hours of estrogenic exposure. On this basis, the following five genes were selected for use in this study:

5.1.5.1 IGFBP4

Insulin-like growth factor binding protein 4 (IGFBP4) binds Insulin-like growth factors I and II, prolongs their half life and alters the way they interact with cell surface receptors (Durai et al., 2006). IGFBP4 has been shown, by this mechanism, to inhibit cell growth *in vitro* and *in vivo* (Mazerbourg et al., 2004).

IGFBP4 is reported as being up-regulated 9.1 fold in MCF7 cells after 4 hours (Frasor et al., 2003), reducing to 4 fold up-regulation after 72 hours (Inoue et al., 2002).

Soulez and Parker (2001a) showed a 4.8 fold down-regulation of IGFBP4 in ZR-75-1 cells after 6 hours, whereas Mutarelli et al (2008) showed a slight up-regulation in ZR-75-1 cells after 1 hour which continued until 32 hours (when their measurement period ceased). In both cases cells were treated with 10nM E_2. The discrepancy may be related to the source of the cells, a ZR-75-1 sub-line being used, or different culture conditions of the cells.

The promoter region of IGFBP4 has a TATA box at 299 nucleotides before the ATG transcription initial site (-299) and an AP1 site at -869 (Dai et al., 1997). Qin et al (1999) found GC rich regions at -559 to -553 and -72 to -64 that were important for E_2 induced activation. Qin et al (1999) inserted the promoter sequence, spanning -1214/+18, upstream of a firefly luciferase reporter (pGL2) and transiently transfected it into MCF7 cells. The promoter was found to increase luciferase expression 10-fold when exposed to 10n M E_2. An ERE in the IGFBP4 promoter is located at -4124 (Bourdeau et al., 2004) and was not included in the promoter fragment used by Qin et al (1999).

5.1.5.2 pS2

Trefoil Factor 1 (pS2) up-regulation after cellular exposure to estradiol is a primary transcriptional event (Brown et al., 1984) and is widely used as an marker of estrogenic stimulation (Jorgensen et al., 2000). pS2 is involved in the regulation of proliferation and its expression promotes migration (Soulez and Parker, 2001b).

Up-regulation after estrogenic exposure has been shown in MCF7 (Brown et al., 1984; Jorgensen et al., 2000; Inoue et al., 2002), T47-D (Recchia et al., 2004), ZR-75-1 (Mutarelli et al., 2008), HepG2 (Barkhem et al., 2002) cell lines, and human breast and gastrointestinal tissue (Ribieras et al., 1998).

Rechhia et al (2004) showed that Bisphenol A and 4-nonylphenol significantly increased pS2 expression in MCF7 cells (Soulez and Parker, 2001a). Non-estrogenic compounds have not been found to induce pS2 expression in MCF7 cells (Jorgensen et al., 2000).

The pS2 promoter has a TATA box at -30 to -24 (Sewack et al., 2001) . A non-palindromic ERE at -405 to -393 which differs by one base pair to a perfect ERE (Berry et al., 1989) and an AP1 site at -332 to -338 (Barkhem et al., 2002).

5.1.5.3 VEGF

Vascular endothelial growth factor (VEGF) is key to angiogenic growth and is induced by estrogen in many human and animal tissues (Charnock-Jones et al., 1993; Mueller et

al., 2000). Frasor et al (2003) showed a significant 2.8 fold induction of VEGF at 4 hours post E_2 exposure in MCF7 cells.

Buteau-Lozano et al (2002) mapped estrogen responsive elements and an AP-1 site between -1.2kb and -2.3kb and successfully used a -2275/+75 promoter fragment with a luciferase reporter to show up-regulation when tested in MCF7 cells after 24 hours of E_2 exposure.

In animal models, VEGF reached maximum induction in rat uterus 1 to 3 hours after E_2 treatment (Long et al., 2001). Bisphenol A also significantly induced VEGF in rat uterus, vagina and pituitary at 37.5mg/kg body weight (Long et al., 2001).

5.1.5.4 EGR1

Early Growth Response 1 (EGR1) mediates gene events after injury to the vasculature (Fu et al., 2003) and is up-regulated in response to E_2. It responds via the extracellular signal-regulated kinase, ERK1/2 (de Jager et al., 2001). Myocardium cells isolated from rat show very high up-regulation of EGR1 within 15 minutes of exposure to E_2 (de Jager et al., 2001). Further studies in which the EGR1 promoter was linked to luciferase showed a 21 fold increase in luciferase expression when co-transfected with an ERα in primary neonatal cardiomyocytes (de Jager et al., 2001). Mutarelli et al (2008) showed a significant 1.2 fold up regulation in ZR 75 cells after 1hr (1.2). After 6 hours, the same study showed an increase of 3.3 fold (microarray) or 5.5 fold increase by qRTPCR. Watanabe (2002) showed up-regulation in mouse uterine tissue after 6 hours exposure. EGR1 has been shown to have two half EREs and four AP-1 sites within -1200 of the TSS (de Jager et al., 2001).

5.1.5.5 Mao-A

Monoamine Oxidase A is an enzyme that degrades amine neurotransmitters, elevated expression of which has been linked to major depression and violent anti-social behaviour in humans (Meyer et al., 2006). MaoA is consistently shows up to 90% down regulation in MCF7 cells when exposed to E_2 and numerous xenoestrogens such as DES, α zearanol, nonylphenol, genistein, methoxychlor, endosulfan, o,p,-DDE, bisphenol A, dibutylphthalate (Jorgensen et al., 2000). Down regulation of MaoA has

also been demonstrated in rats exposed to E_2 (Ortega-Corona BG, 1994). The promoter of MaoA has been mapped with two imperfect EREs at approximately -200 and -1500 (Jorgensen et al., 2000).

5.1.6 The reporter vector pGL4.21 (luc/2P)

Fluorescence and chemiluminescence are both essentially photon emitting events that can be measured, differing only in how the transition in energy state occurs (Fan and Wood, 2007) . Reporter genes such as luciferase, enhanced green fluorescent protein (EGFP), placental alkaline phosphatase (PAP), or lac Z are commonly used (Denison et al., 2004). Fluorescence assays tend to give higher signal intensity, however the use of photons for excitation also leads to a higher background, making fluorescence better for macroscopic application (Fan and Wood, 2007). Chemiluminescence, such as luciferase, out-performs fluorescence in a multiwell plate format due to a better signal-to-noise ratio giving greater sensitivity (O'Brien, 2006).

pGL4.21 (luc/2P) uses an optimised synthetic luciferase based on the *Photinus pyralis* gene. This optimised Rapid Response^{TM} luciferase gene has a protein degradation sequence attached. This reduces the half-life of the destabilised luciferase to less than 3 hours generating less accumulation of the luciferase (http://www.promega.com/paguide/chap8.htm). The outcome of this is a faster response time, with greater magnitude changes than the more stable version of luciferase. The promoter sequences are inserted directly upstream of the luc2P gene as shown in figure 5.1

The ER-CALUX assay is the most comparable assay to the assay proposed in this study. The ER-CALUX assay, as described previously, uses a triple perfect ERE sequence preceding a TATA minimal promoter driving the expression of a luciferase gene (http://www.biodetectionsystems.com/; Legler et al., 1999b). The stably transfected T-47D cell line is exposed to the ant/agonist for 24 hours before measurement of luciferase induction. As seen from microarray data, many genes are up-regulated in a fraction of this time (Frasor et al., 2003). Although the time required for a significant response from ER-CALUX could probably be reduced, a perfect triplet ERE is not necessarily physiologically relevant. The complex interactions within cells from myriad ant/agonists may be better identified using endogenous promoters. A set of genes that

respond to estrogenic stimuli in a similar manner in MCF7, T-47D and ZR-75-1 cell lines were identified with responses to estrogen demonstrated within 4 hours. The promoter regions from these genes will be cloned into pGL4.21(luc2P/Puro) and transfected into cell lines. This will potentially create reporter cell lines that have a semi-physiological response to the presence of estrogen with a measurable increase or decrease in the expression of luciferase after less time than the ER-CALUX. Unlike the ER-CALUX an arbitrary number of genes would need to be used to determine estrogenicity requiring multiple cell lines.

Figure 5.1 Amplification of the promoter sequence and the pGL4.21(luc2P/Puro) vector

A. Promoter regions of estrogen responsive genes will be amplified from genomic DNA using primers (→) purified and ligated to B. pGL4.21 (luc2P/Puro) major features of which include puromycin resistance, poly(A) sequence prior to the introduced promoter sequence to prevent background expression and the luc2P destabilised protein luciferase gene.

A. Promoter — Estrogen regulated gene

B. pGL4.21(luc/2P)

Synthetic poly(A)
ori
Amp^r
Poly(A) block (for background reduction)
Puromycin Resistance
Upstream Element
– Multiple cloning region
– Promoter/ response elements
SV40 early enhancer/ promoter
SV40 late poly(A) signal
Luciferase Gene
– Firefly (luc2)
Promoter
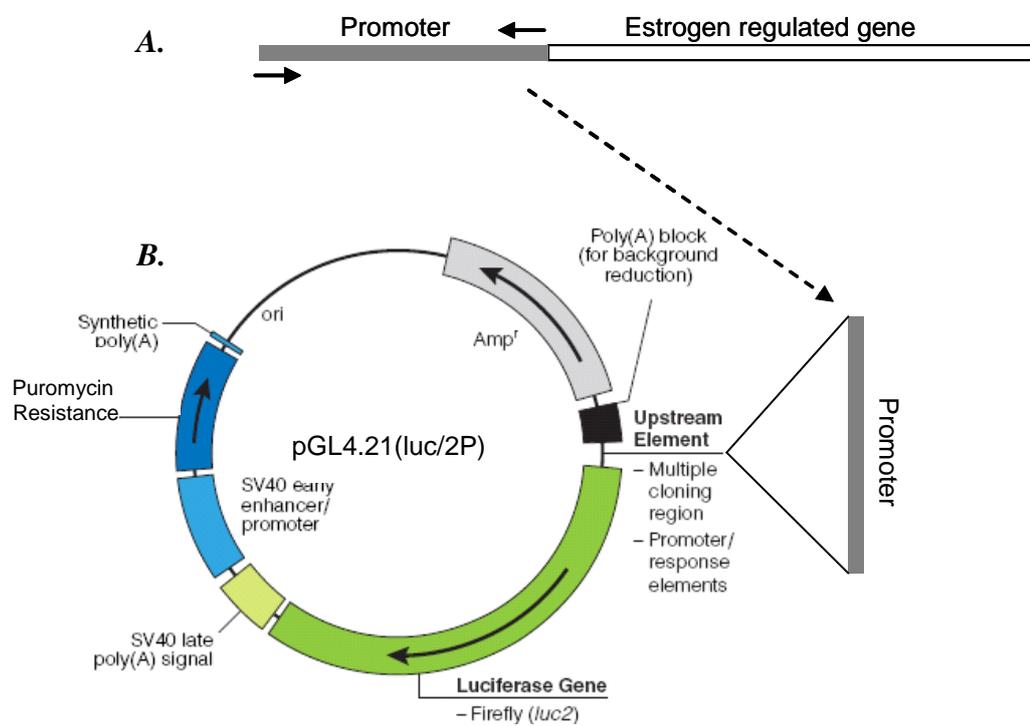
5.2 Methods:

5.2.1 pGL4.21-promoter vector construction

5.2.1.1 Primer design for amplification of promoter sequences

Forward primers were designed with a 5' flanking EcoRV restriction site including one nucleotide prior to the restriction enzyme (RE) binding site for efficient cleavage (G GAT*ATC). A HindIII restriction site and a 3 nucleotide flanking region were added to the 5' end of the reverse primer (GAG A*AGCTT), again for efficient RE cleavage.

The promoter sequences immediately upstream from the transcription start site (TSS) to approximately 100 nucleotides past the TSS (as determined by the UCSC Genome Browser (Kent et al., 2002) and NCBI) were determined. Each promoter length was chosen as described. The defined region was imported to NCBI/Primer BLAST or Primer3 and the best primer match for the defined region determined.

The amplicon sequences were imported to NEBCutter2 (NEB) to check that the EcoRV and HindIII restriction sites were not present in the sequence.

The primers, including appropriate flanking region, were entered to Primer3 and *in silico* PCR blast (UCSC Genome Browser) was performed to determine the salt-adjusted melting temperature of each primer and for any other possible matches with human genomic DNA.

Numbers in brackets below describe the position of the forward and reverse primer with respect to the TSS.

pS2 (Accession number: NM_003225):
1634 bp amplicon (-1590 /+43)
Forward: 5'- GGATATC CGAAAACCACCGTCACTATG -3'
Reverse; 5'- GAGAAGCTT CATTGCCTCCTCTCTCTC-3'

VEGF (Accession number: NM_003376):

2438 bp amplicon (-2343 /+94)

Forward: 5'- GATATC CAGAGGGAAACACAAACAGG -3'

Reverse: 5'- GAGAAGCTT GCTGACCGGTCCACCTA -3'

EGR1 (Accession number: NM_001964):

1259 bp amplicon (-1086 /+162)

Forward 5'- GATATC GGAAAGTGACCCCTCACC -3'

Reverse 5'- GAGAAGCTT AACACTGAGAAGCGTGCAG -3'

MAO A (Accession number: NM_000240):

1435 bp amplicon (-1283 /+125)

Forward: 5'- GATATC CCGGCCTAGAGTCACTT -3'

Reverse: 5' - GAGAAGCTT GGAAGGACCCTTCTATCA -3'

IGFB4 (*Accession number: NM_001552):*

1139 bp amplicon (-988 /+150)

Forward: 5'- GATATC GCCACCACACCTGGCTA -3'

Reverse: 5' - GAGAAGCTT ACGTAGCGGGGGAAGTTA -3'

All primers were ordered from Geneworks (Australia) and were resuspended in water (Baxter) to a stock concentration of 100µM. Before use and for short term storage primers were diluted 1:20 with water (Baxter) to 5µM and stored at -20°C.

5.2.1.2 Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from a T-47D cell monolayer grown in a 25 cm^2 flask. Cells were trypsinised as described and resuspended in 2 ml complete medium, 1 mL of which was used in a Qiagen FlexiGene DNA Kit according to the manufacturer's protocol. Additional isopropanol was used during the final precipitation due to incomplete precipitation as suggested by the manufacturer's protocol. The gDNA was resuspended in water (Baxter) and after an extended resuspension period (o/n at 4°C and 1hr at 65°C) was spun at 6000 xg for 3 minutes and the supernatant retained. gDNA was quantified in triplicate using a GeneQuant (Amersham Biosciences).

5.2.1.3 PCR amplification of promoters

A high fidelity *pfu* Taq DNA Polymerase (Promega) with proof-reading ability was used throughout to minimise errors during amplification. Gradient PCR reactions were initially used to determine the optimal annealing temperature of the primer pairs. Reactions were assembled with the following components; 2.5µl 10x *Pfu* Buffer (Promega), 0.5µl dNTP mix (10mM each) (Qiagen), 0.5µl each primer (5µM), 0.1µg gDNA, 0.165 µl *Pfu* Polymerase and water to a final volume of 25 µl.

8 annealing temperatures were tested in the following cycle conditions; 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 55-64°C for 30 seconds, 75°C for 4 minutes, finished with 75°C for 10 minutes.

5µl of the amplification products were resolved on a 1.2% agarose gel using a 100bp and 1kb marker (NEB) and visualised as described (Figure 5.2).

5.2.1.4 PCR 'clean-up'

In the case of a single product being amplified in the reaction (pS2 and MaoA), the remaining PCR reaction volume was passed through a Qiagen Qiaquick Gel Extraction Kit. The initial buffer used in the extraction, Buffer QC, was increased to 5x volume (instead of 3x) as suggested in the manufacturer's protocol and the agarose melting procedure omitted. The isolated amplicon was eluted in 40µl water (Baxter). 2µl of the purified product was resolved on a 1.2% agarose gel to check for recovery.

5.2.1.5 Gel Extraction

After the initial 5µl of amplification product was resolved on a 1.2% agarose gel, in the case of multiple bands arising from the amplification, the entire remaining volume, 20µl, was resolved on a 1.2% agarose gel and the correct band excised under UV light. The gel slice was weighed and the amplicon extracted using the QiaQuick Gel extraction kit using the manufacturer's protocol.

Figure 5.2 Gradient PCR of the five promoters

Genomic DNA from T-47D cells was used as a template in PCR to amplify the promoter region of the five chosen estrogen responsive genes: (A) pS2 (1634bp), (B) MaoA (1367bp), (C) IGFBP4 (1139bp), (D) EGR1 (1085bp) and (E) VEGF (2438bp). Each lane corresponds to an annealing temperature as described.
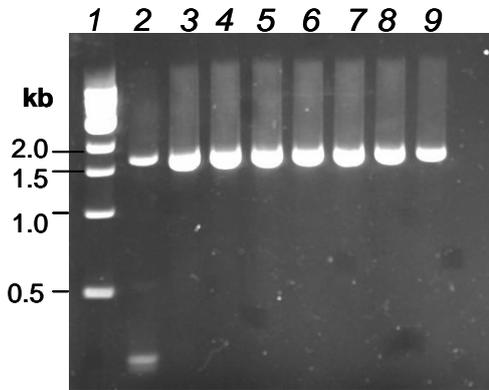
M - 1kb Marker
1 - 55.0°C
2 - 55.5°C
3 - 56.0°C
4 - 57.2°C
5 - 58.5°C
6 - 59.8°C
7 - 60.4°C
8 - 62.6°C
9 - 63.4°C
10- 64.0°C

A. PS2 (1634bp)
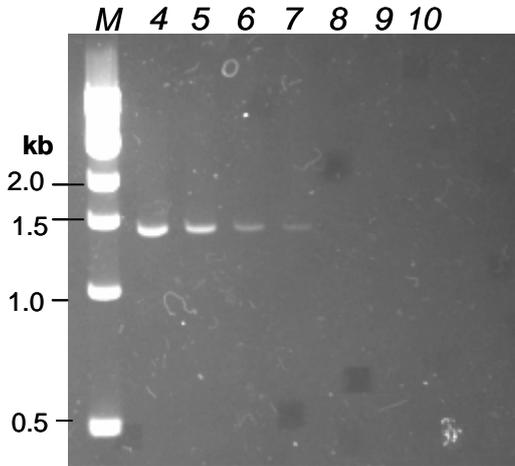
B. MaoA (1367 bp)
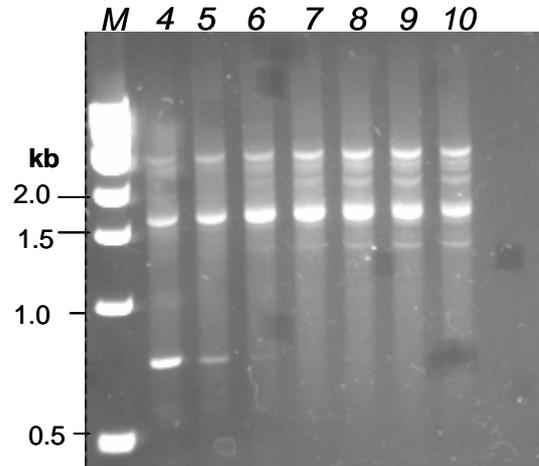
C. IGFBP4 (1139 bp)
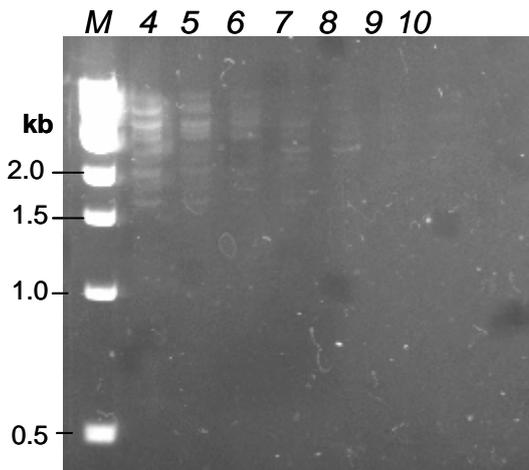
D. EGR1 (1085 bp)
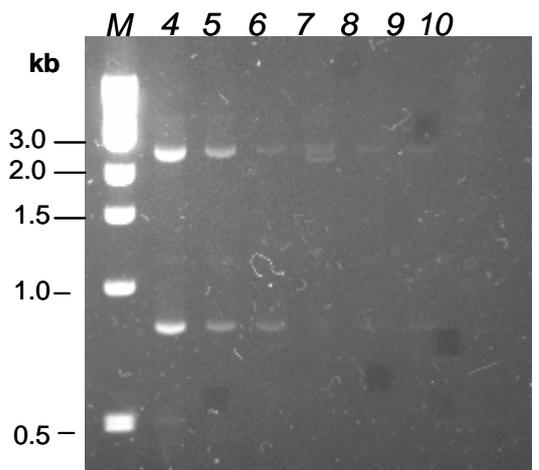
E. VEGF (2438 bp)

5.2.1.6 Double Digestion

Promoter fragments were digested in a 20 µl reaction using 2µl Buffer 2, 2µl 10x BSA, 1µl EcoRV, 1µl HindIII and 14µl promoter fragment for 2 hours at 37°C. The extended incubation time was to ensure efficient cleavage of fragment ends.

The double cut fragment was isolated from the reaction mixture using the QIAquick Gel Extraction kit (using 5x volume Buffer QC). Again 2µl was resolved on a 1.2% agarose gel to check for recovery.

5.2.1.7 pGL4.21 Digestion

1 µg of the 5532bp pGL4.21 vector was prepared in a restriction digest of 20 µl, with 2 µl Buffer 2, 2µl 10x BSA, 1µl EcoRV and 1µl HindIII made to 20µl with water (Baxter). The reaction was incubated at 37°C for 2 hours. Control single digests using HindIII or EcoRV were performed simultaneously, in which case 1µl of RE was used and the final volume adjusted with water.

2µl of each reaction was resolved on a 1.2% agarose gel and visualised as described (Figure 5.3).The 5508bp cut vector was purified from the remaining reaction (and 24 bp fragment) mix using the QIAquick Gel cleanup kit.

M – 1 kb Marker
1 – EcoRV digest
2 – HindIII digest
3 – EcoRV/HindIII digest
4 – uncut pGL4.21
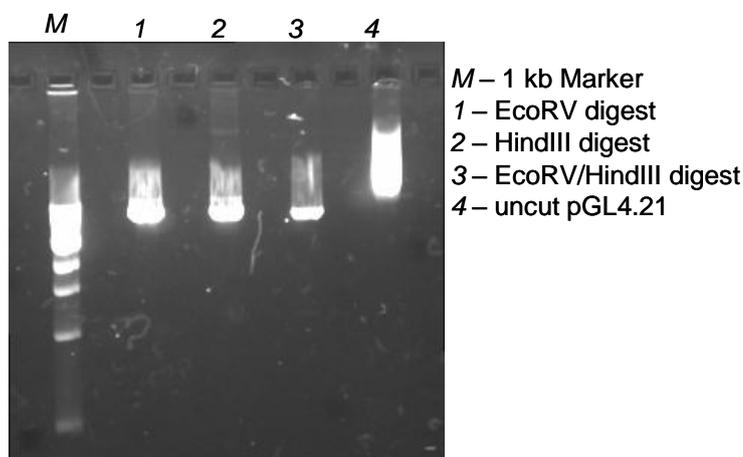
Figure 5.3 Preparation of pGL4.21 for insert ligation

pGL4.21 was prepared for insert ligation by double restriction digest reaction using EcoRV/HindII. Single digests with EcoRV and HindII alone were also performed as controls.

5.2.1.8 Ligation

The purified and cut pGL4.21 vector and promoter insert were ligated in a reaction using; 1µl T4 DNA Ligase (NEB), 0.5µl vector, 3µl insert, 2µl 10x Buffer and water to 20µl. The reaction was incubated at 16°C overnight.

5.2.1.9 Transformation

Chemically competent JM109 cells (a strain of *Escherichia coli*) were thawed on ice for 15-20 minutes. 0.5µl, 2µl, 5µl of ligation mix or 1µl pGL4.2 (positive control) was added to four separate tubes containing 50µl competent cells each and the tubes flicked 4 times to mix. Tubes were returned to ice for 5 minutes followed by heat shock at 42^0C in a water bath for 50 seconds. Tubes were then returned to ice for 10 minutes. 450µl SOC media was aseptically added to each tube before incubation at 37^oC in a shaking incubator for 30 minutes. The entire tube content was placed onto a pre-warmed and dried LB/Amp agar plate and spread over the surface until dry. The plate was inverted and incubated overnight at 37^0C.

5.2.1.10 Screening colonies for successful ligation

Successful ligations resulted potentially in colony formation. Colonies were picked using a sterile tip from each plate, touched to another LB/Amp plate and placed in 2mL LB/Amp liquid media and incubated overnight at 37°C with shaking. LB/Amp agar plates were incubated at 37°C overnight.

5.2.1.11 Plasmid isolation from JM109 cultures

A GenElute Five minute plasmid Miniprep kit (Sigma-Aldrich) was used to isolate plasmid from 2mL overnight cultures following the manufacturer's protocol. 2µl of isolated plasmid was analysed in a single restriction enzyme digest using 0.5µl HindIII, or a double restriction enzyme digest using 0.5µl each of HindIII and EcoRV, 1µl 10X BSA, and 1µl Buffer 2, made to a final volume of 10µl with water. Reactions were incubated at 37°C for 20 minutes and analysed on a 1.2% agarose gel using a 100bp and 1kb marker.

5.2.1.12 Sequencing promoter inserts

Primers were designed specific to the pGL4.21 vector, 5' and 3' to the promoter insertion site.

Forward: 5'- CTAGCAAAATAGGCTGTCCC -3'

Reverse: 3' - GCTGGGCCCTTCTTAATGTT -3'

With the provision of plasmid at 10ng/μl (10μl) and each primer at 5μM (2μl), sequencing reactions were performed by SouthPath and Flinders Sequencing Facility, Department of Haematology & Genetic Pathology, Flinders Medical Centre using both the vector specific primers and promoter specific primers.

5.2.1.13 Sequencing Alignment

Sequencing results were viewed using Sequence Scanner v1.0 (Applied Biosciences). The four overlapping sequences (2 forward and 2 reverse sequences per promoter fragment) were aligned using EMBOSS Pairwise Alignment Algorithms (KAlign) (http://www.ebi.ac.uk/Tools/emboss/align/index.html). The alignment resulted in one consensus sequence which was checked for correct insertion into the vector and against published mRNA sequence using BLASTn to check for any mutations (www.ncbi.nlh.nih.gov).

5.2.2 Generation of stable cell lines expressing the pGL4.21-promoter construct

5.2.2.1 Kill curve - determination of the effective concentration of puromycin (G418)

T-47D cells were placed in all wells of two 6 well plates at $1x10^5$ cells/well (approximately 30% confluence) and incubated overnight in complete media. The following day, medium from each well was aspirated and replaced with complete medium supplemented with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10μg/ml Puromycin. After 4 days the cells were visualised under an inverted microscope. The optimal concentration was selected as the lowest concentration of puromycin that was able to kill all cells within the well after 4 days.

5.2.2.2 Transfection of T-47D cells with pGL4.21-promoter construct

T-47D cells were trypsinised and resuspended in OptiMEM (Invitrogen) supplemented with 10% FCS (without antibiotics). Cells were counted and $1x10^6$ cells placed into one well of a 6 well plate.The following day, in two separate 1.5mL tubes (Sarstedt), 250μl OptiMEM was combined with 4μg plasmid, and 250μl OptiMEM was combined with

10µl LipofectAmine2000 (Invitrogen), and left at room temperature for 5 minutes after gentle mixing. The two solutions were then combined, mixed gently and left at RT for ~20 mins to allow lipid dendrimer-plasmid DNA complex formation. The mixture was then added drop-wise across the surface of the media in the well, containing the T-47D cells. After 5 hours, medium and complex were aspirated and replaced with OptiMEM supplemented with 10% FCS and left for 24 hours.

On the third day, cells were trypsinised and the whole cell population placed in a 75cm^2 flask in normal complete medium and left overnight to attach. The following day puromycin was added at 5ug/ml and left for 4 days to kill the untransfected cells.

5.2.2.3 Colony isolation and growth

Transfected cells were left to grow in the flask for two weeks, allowing single cells to form colonies. Medium was supplemented with 1 µg/ml puromycin and changed every 4-5 days during this time to encourage stable incorporation of the plasmid and clonal growth of transfected cells. After two weeks, the flask was placed on an inverted microscope inside the laminar flow and single colonies removed individually using sterile pipette tips.

Colonies were transferred to one well of a 96 well plate, each containing 100µl complete media. Colonies were gently repeat pipetted to encourage single cells to be released from the colony. Replicate plates of each clonal population were made when the colonies had expanded sufficiently.

5.2.3 Screening colonies for luciferase expression

5.2.3.1 pGL4.21-pS2 transfected cells

Media from wells of one replicate 96 well plate was aspirated and replaced with PR-free media supplemented with 10% DC-FCS and incubated for 5 days. Wells were each scored for confluence by visual inspection. Media was then aspirated and replaced with PR-free media supplemented with 10%DC-FCS and $1x10^{-8}$ M E$_2$ for 4 hours before cells were tested for luciferase activity using the one glow assay described below.

5.2.3.2 pGL4.21-MaoA transfected cells

As the MaoA linked luciferase would be down-regulated with exposure to estrogen clones were screened for luciferase expression after 5 days incubation in PR-free media supplemented with 10% DC-FCS cells. Luciferase activity was assessed using the

ONE-Glo assay described below. Colonies that showed luciferase expression were expanded for further testing.

5.2.3.3 ONE-Glo assay

ONE-Glo^{TM} (Promega), a lysis buffer and substrate solution, was prepared and utilised as per manufacturer's instructions with minor adjustments. Briefly, media volume in wells was adjusted to 50µl using a multichannel pipette. 50µl RT ONE-Glo was dispensed to each well and left for 3 minutes to allow cell lysis. Visual inspection after 3 minutes often revealed intact cells. A multichannel pipette was used not only to disrupt cells but also to transfer the solution to a black 96 well plate (Greiner). Luminescence was detected using an Ω Plate Reader (BMG) set to: Emission- Lens, Gain- 4095, Measurement interval- 1 second; or a Wallac Victor Multilabel plate reader using the same settings.

5.2.3.4 Dose response of the clonal populations to E$_2$

Isolated clones were then tested for their ability to respond to E$_2$ in a dose-dependant manner. Clonal cell populations were incubated in PR-free medium supplemented with 10% DC-FCS for 5 days. Cells were trypsinised, resuspended in PR-free medium supplemented with 10% DC-FCS and 5000 cells/well placed into wells of a 96 well plate. 16 hours later medium from each well was aspirated and PR-free medium supplemented with 10% DC-FCS and E$_2$ at concentrations of 10^{-8} to 10^{-13}M was added to each well for 4 hours. Luciferase was then measured using the ONE-Glo assay as described.

5.2.3.5 Down-regulation of pS2-luciferase expression in estrogen-free media

Clone 3 and 13 of theT-47D cells stably transfected with pGL4.21-pS2 construct, were tested for a down-regulation of pS2-luciferase expression over 5 days in estrogen-free media. This was the pre-treatment time used before testing for induction and was also shown by Jorgenson et al (2000) to be an effective time for pre-treatment before measuring for induction of the gene response when the cells were exposed to E$_2$.

Clonal cell populations were resuspended in complete media, counted and placed into replicate wells of two 96 well plates at a density of 5000 cells/well. Cells were placed into another plate at densities of 1250-80000 cells/well which was used as a standard

curve (section 3.2.2). Cells in the standard curve plate were left to attach for 6 hours before the crystal violet assay was applied (Section 2.2.5).

The remaining two 96 well plates (with 5000 cell/well) were left to incubate for 5 days. Media was changed in replicate wells on each plate, after each 24 hour period from complete media supplemented with 10% FCS to PR-free media supplemented with 10% DC-FCS. This generated two replicate plates with cells exposed to estrogen-free media for 0 to 5 days. After the 5 day time period, one plate was used for a crystal violet assay and the other plate was used for a ONE-Glo luciferase assay.

This allowed comparison of luciferase counts on a per cell basis for 5 days. The experiment was performed in this format so that the data would come from a single assay, rather than 5 separate assays, which inherently would have high variability.

5.2.3.6 Time-response of T-47D-pS2-luciferase cells to E_2

Clone 3 and 13 of the T-47D cells stably transfected with pGL4.21-pS2 construct, were tested to determine if the 4 hour exposure to E_2 that we had previously been testing them with was in fact missing the point of induction of the luciferase. The cells were therefore tested at 0,1 4,8 and 24 hours post addition of E_2.

5000 cells/well were placed in replicate wells of a 96 well plate and left to attach for 6 hours. The media was then changed to PR-free media supplemented with 10% DC-FCS and left for 5 days. PR-free media supplemented with 10% DC-FCS and 10nM E_2 was then added to the cells after 0, 16, 20 and 23 hours effectively generating cells exposed to 10nM E_2 for 0, 1, 4, 8 and 24 hours. A solvent control (PR-free media supplemented with 10% DC-FCS and 0.5% EtOH) was added after 20 hours (effectively 4 hours exposure to the EtOH control). After 24 hours, the ONE-Glo assay was applied to all cells.

5.2.3.7 Testing a non-clonal pool of selected T-47D-pS2-luciferase cells

As estrogen-inducible luciferase expression was not occurring in the clonal populations, the remaining selected, non-clonal population of cells from which the colonies were initially isolated were tested for luciferase induction by exposure to E_2. These cells had been grown in selective media for >4 weeks. Cells were pre-treated in PR-free media supplemented with 10% DC-FCS for 5 days. Cells were then thoroughly resuspended (by repeat pipetting to homogenise the population) in PR-free media supplemented with

10% DC-FCS. 5000 cells per well were placed into replicate wells of a 96 well plate and left to attach for 24 hours.

PR-free media supplemented with 10% DC-FCS and; 10nM E_2, 10nM E_2 and 100nM Fulvestrant, and 0.5% EtOH were added to the cells. Additional controls were included of PR-free media supplemented with 10% DC-FCS and complete media supplemented with 10% FCS.

After 4 hours, luciferase expression was determined using the ONE-Glo assay as described.

5.2.3.8 Response of T-47D-pGL4.21-MaoA to E_2

Clones 1-4 of the T-47D cells stably transfected with pGL4.21-MaoA construct were tested for a down-regulation of luciferase expression after exposure to E_2. The clonal populations were pre-treated in PR-free media supplemented with 10% DC-FCS for 5 days. Cells were then placed in wells of a 96 well plate at 5000 cells/well and left to attach for 24 hours. PR-free media supplemented with 10% DC-FCS and 10nM E_2 or 0.5% EtOH were added to wells for 24 hours before testing luciferase expression using the ONEGlo assay as described.

5.2.4 Transient transfection and exposure to E_2

Clonal isolation and characterisation lead to non-inducible luciferase expression. Cells were therefore tested for luciferase response after transient transfection of the vector and to exposure to E_2. This was done to test the estrogenic response of the vectors independent of factors such as the position of genomic integration, which may have influence over the control of luciferase expression.

5.2.4.1 Exposure to E_2 in a 96 well plate format

T-47D cells were trypsinised and resuspended in OptiMEM (Invitrogen) supplemented with 10% FCS (without antibiotics) and $8x10^4$ cells dispensed into the inner wells of a 96 well plate. The following day in two separate 1.5mL tubes (Sarstedt) 250µl OptiMEM was combined with 4µg plasmid (pGL4.21 or pGL4.21-pS2 or pGL4.21-MaoA) and 250µl OptiMEM was combined with 10µl LipofectAmine2000 (Invitrogen) and left at room temperature for 5 minutes after gentle mixing. The two solutions were then combined, mixed gently and left at RT for ~20 mins to allow complex formation,

after which time the mixture was added drop wise across the surface of the well containing the T-47D cells and media.

After 5 hours media and complex were aspirated and replaced with OptiMEM supplemented with 10% DC-FCS and 10nM E_2, 10nM E_2 + 100nM Fulvestrant, or supplemented with 0.5% EtOH. Cells were then incubated for 24 hours before using the ONE-Glo assay.

5.2.4.2 Exposure to E_2 in a 24 well plate format

The same procedure was repeated in a 24 well plate format instead of a 96 well plate format using 2×10^5 T-47D cells per well. After exposure to 10nM E_2, medium from each well was collected in sterile tubes, cells were trypsinised, resuspended in 100µl of their corresponding medium and centrifuged in a bench top microcentrifuge at 800rpm (100xg) for 5 minutes. Medium was gently aspirated from the pellet and the cells were resuspended in 50µl of their corresponding media and placed into wells of a 96 well plate before use of the ONE-Glo assay (Section 5.2.3.3).

5.3 Results:

5.3.1 PCR amplification of promoters

The amplification of the pS2 promoter, using a primer annealing temperature of 56 to 63.4°C, and MaoA promoters, using annealing temperature of 57.2 to 60.4°C, resulted in single amplicons which were isolated directly from the PCR reactions (Figure 5.2).

The amplification of the VEGF promoter resulted in two major bands, one of which was at the correct size of 2438bp. Optimisation of annealing temperature, use of *pfu* Taq and Taq mastermix (QuantiTECT and Platinum PCR supermix) all resulted in two major amplicons. Second round PCR of the gel extracted product was attempted but not successful, suggesting the excised fragment may not have been the VEGF promoter.

The EGR1 and IGFBP4 promoter amplification reactions were also optimised, without successful isolation of a suitable amplicon.

5.3.2 pGL4.21- pS2 and pGL4.21- MaoA Vector Construction

The 1634bp pS2 and 1367bp MaoA promoters were successfully ligated into the pGL4.21 vector. The plasmids were linearised to using a single digest (HindIII) or the promoter fragment was excised at the insertion points using a double digest (HindIII/EcoRV) (Figure 5.4). The plasmids were called pGL4.21 – pS2 and pGL4.21-MaoA.

M -1kb Marker
1 - pGL4.21-MaoA HindIII digest
2 - pGL4.21-MaoA EcoRV/HindIII digest
3 - pGL4.21-PS2 HindIII digest
4 - pGL4.21-PS2 EcoRV/HindIII digest
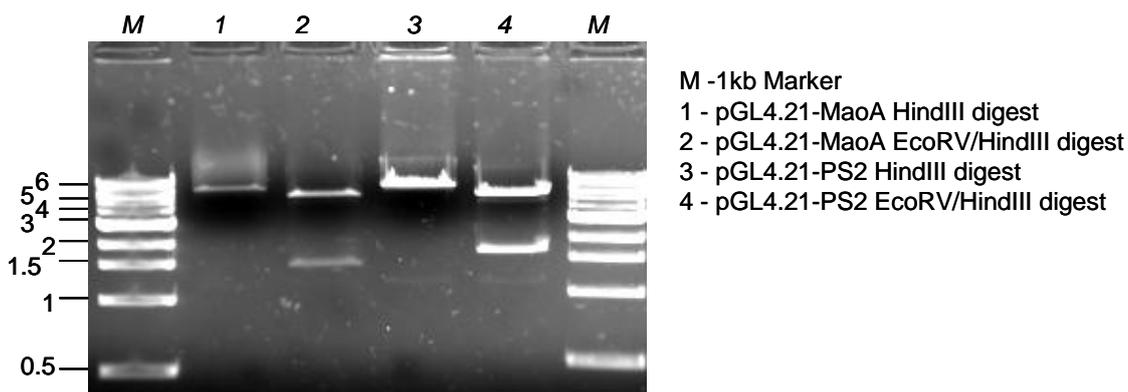
Figure 5.4 Restriction digest analysis of plasmid isolated from transformed competent cells following ligation.

Following ligation of the 1367bp MaoA or 1634bp pS2 inserts to pGL4.21 ,and the transformation of competent cells, colonies were isolated from agar plates and grown overnight in LB broth with ampicillin. Plasmid was isolated from the culture and analysed using EcoRV and/or HindIII restriction digests. Lane 2 and 4 show the isolation of the insert from successfully ligated vectors pGL4.21-MaoA and pGL4.21-pS2 respectively.

5.3.3 Sequencing

5.3.3.1 pGL4.21-PS2 sequencing and promoter key features

Sequencing of the pGL4.21-pS2 vector revealed that the pS2 promoter fragment had been successfully inserted into the pGL4.21 vector. The sequencing was done using forward and reverse primers specific to the pGL4.21 vector and forward and reverse primers specific to the insert/promoter. The four sequences were successfully aligned to a single consensus sequence (Figure 5.5). A C $\rightarrow$ T base pair change at position -918 relative to the transcription start site was confirmed by sequences from both of the forward primer reactions. The base pair change was not in any known regulatory region of the promoter, and may be unique to T-47D cells, or the mutation was introduced during the initial PCR amplification of the promoter fragment.

TTCTCTGGCCTAACTGGCCGGTACCTGAGCTCGCTAGCCTCGAG GAT˘ATC

PS2 insert

CGAAAACCACCGTCACTATGAGCACCACCCTGGGCCTGGTGCCCCCGTCC

TGTTTTCAATAAACTTAAGCTGACCACAGAGGACACCAGGCCTCGGCCCC

TCCCTGGGTCCTGCGGTCAGGCCCCTCCTGGAGACCCTGGCCCAGGAGTG

GAGACCCCGGTGCAGGAGTGGCAGCCCTGGAGGAGGGGCTGGGTCCTGGG

ATGGAGCGAAGAGGAACATGGCCGCCCCCATTCTGGCCCAGGCTCCCCTC

CCAGAGGGGCTGCAGAAATGTACTGACTAGGTCACCCAAGAAAAGATAGT

ACCTTTGTTAGGCTAGCATGCATTTTCCTCAGGGCCTAATTTCTCACTGA

AGAAGAAAGATTTCTCCTGTTGTCCATTTCTTCATGCAGCTCTTCAACA

GCTGTTTCTCGAATGCCAATCAAAGCCACTGTTCTAGGGTCTGGGGCCAC

CTCAAGGCACTAGGAGATGAGGACTTCTGCTCCCATGCGCCCTCCCGTCT

GCTGCAGGGAGGAGTGCAATGAATAAATAACCAACATAATGTGTCAGTCA

CTTGTTTTATCCACCAGGAGGTAATAAGAGCTATGAAAGAGAAAGCTCCG

AGCAGGGGAGGGGAGTGAGGCATGGTACAGGAGAGCAGGAGGCTGTCCTC

                         C➔T change

TAAAATACAGGAGTCCAGGGGAC**T**AACTGGGAAGGTGTGGGAGGGGGAGG

GAGGGAGCCCCATAGACACAGGGGAGTGAACCACGTTCACTTTGTCAGTT

TTTGATGGCAGCTCGTATATACTATTTTTTTCTCCCTCCTGCCCCCAGCT

CCTCCCAGAAGGAGACTTAATCTGTCGCTCAGGCTGGAGTGCAGTAGGGT

GATCTCGACTCACTGCAACCTCCGCCTCCCAGGTTCAAGTGATTCTCCTG

ACTTAACCTCCAGAGTAGCTAGGATTACAGGCACCCGCCACCATGCCTGG

```
CTAATTTTTGTATTTTTTTTTTTTTGTAGAGACGGGGTTTCGCCATGTTGG


CCAGGCTAGTCTCAAACTCCTGACTTTAAGTGATCCGCCTGCTTTGGCCT


CCCAAAGTGTTGGGATTACAGGCGTGAGCCACTGCGCCAGGCCTACAATT


TCATTATTAAAACAATTCCACTGTAAAGAATTAGCTTAGGCCTAGACGG
                                      Non-palindromic ERE
AATGGGCTTCATGAGCTCCTTCCCTTCCCCCTGCAAGGTCACGGTGGCCA


CCCCGTGAGCCACTGTTGTCACGGCCAAGCCTTTTTCCGGCCATCTCTCA
    AP-1 site
CTATGAATCACTTCTGCAGTGAGTACAGTATTTACCCTGGCGGGAGGGCC


TCTCAGATATGAGTAGGACCTGGATTAAGGTCAGGTTGGAGGAGACTCCC


ATGGGAAAGAGGGACTTTCTGAATCTCAGATCCCTCAGCCAAGATGACCT


CACCACATGTCGTCTCTGTCTATCAGCAAATCCTTCCATGTAGCTTGACC


ATGTCTAGGAAACACCTTTGATAAAAATCAGTGGAGATTATTGTCTCAGA


GGATCCCCGGGCCTCCTTAGGCAAATGTTATCTAACGCTCTTTAAGCAAA
           TATA Box                             TSS
CAGAGCCTGCCCTATAAAATCCGGGGCTCGGGCGGCCTCTCATCCCTGAC
                                   HindIII       Vector
TCGGGGTCGCCTTTGGAGCAGAGAGGAGGCAATG A^A GCTT GGCAAT


CCGG
```

Figure 5.5 Sequence of pGL4.21-pS2 promoter insert and vector boundaries including major features of the promoter.

Primers specific to the insert and the vector were used to partially sequence pGL4.21-pS2. The sequences were aligned using KAlign. A C→T change at position -918 was confirmed from both forward primer sequencing reactions. The major estrogenic response elements of the pS2 promoter including an AP-1 and ERE site and a TATA box are highlighted.

5.3.3.2 pGL4.21-MaoA promoter sequencing and key features

Sequencing of the pGL4.21-MaoA vector revealed that the MaoA promoter fragment had been successfully inserted into the pGL4.21 vector. The sequencing was done using forward and reverse primers specific to the pGL4.21 vector and forward and reverse primers specific to the insert/promoter. The four sequences were successfully aligned to a single consensus sequence (Figure 5.6). The MaoA contained no point mutations and had 100% alignment with reference mRNA for the promoter region of MaoA. The partial ERE, as described in Jorgensen et al (2000), could not be located.

Vector

TTTCTCTGGCCTAACTGGCCGGTACCTGAGCTCGCTAGCCTCGAGGAT**GA**

EcoRV MaoA insert

T˅ATCCCGGCCTAGAGTCACTTCTCCCCGCCCCTGACTGGGCCGGGAGCCC

GGGGCTGGTCTCTAAGAGTGGGTACCGAGAACAGCCTGACCGTGGAGAAG

GGCTGCGGGAAGCAGAACACCGCCCCCAGCGCCCAGCGTGCTCCAGAAAC

ATGAGCACAAACGCCTCAGCCTCCTTCCCCGGCGGCACCGGCACCGGCAC

CAGTACCCGCACCAGTACCGGCACCGGCACCAGTACCCGCACCAGTACCG

GCACCGGCACCAGTACCCGCACCAGTACCGGCACCGGCACCGAGCGCAAG

GCGGAGGGCCCGCCCGAAGCCGGGGGCACAACTGCCCAGGTCCCGAACCC

GGACTCCAGCTTGGACGACACCTCCTACAGCCTGTCCGAATGGAGCGTCC

GTTCTGAGTGGCGGTCCGTCTCGGATCCGCTAGCCAGTTCCCAGTGGAGC

ACGTCCTCAACTGCCGAGGCCGCCTCCTGGAGCTCCAGCATACACTCCCC

AATCAGCACTACCGGTCTTAGCGAGAGTACTGACTCCGACTCCAAGAGTG

GCCTCCGGGGTTTCAGCGCTTACAACCCGAGCAGTCGGATCCCCAAGTCT

ACCACCAGCTCGAACTCCTCCGATGGGGCCGTCACAGCCTCCAATCAGGA

CACCGGCATTCCCTGGGTATTAGTAACAGGACCTACCCCGCCCGTAAACT

```
CCCCCGTAGAGTCATTGCAAGGGTCTGCCTTCTCCTCAGGGTTCAGCACC

CCACGGGGTTTGGTAAAAGGACCGACCCTGCCCCCGGATTCCAACCTGAC

CTCAGTGTCCGACTACACTTGGATATTTGTACGGGGACCTCCTATACCCA

ATGACCTTTCGCAAGTGTCAATACAAGCACCTCCTACACCCAGTAACACC

CCCGAGTGTCAGTACAAGGGTCTGCCGCATCCTCAGTGTCCAGCTTCCCC

TGGGGTTTGGTACCAGGACCACCTCTACCCAATAACATTTCCCCAGTGTC

GCCACAAGCACCTCCTGCACCCCATAACATCCCCCCAGTGTCAAGGCAGG

CGTCTACCCCCACCTCAGTGCCTGACACTCCGCGGGGTTCAATACAAGAA

CCTCCTGCACCCAGTAATCCTTTCCAGCTGCCGACACAAGGACATTCTAA

ACCTAATAACTCTCGCCGAGTGTCAGTACAAGGGTCCGCCCCGCTCTCAG

TGCCCAGCTCCCCCCGGGTATCAGCTGAAACATCAGCTCCGCCCCTGGGC

GCTCCCGGAGTATCAGCAAAAGGGTTCGCCCCGCCCACAGTGCCCGGCTC

CCCCCGGGTATCAAAAGAAGGATCGGCTCCGCCCCCGGGCTCCCCGGGGG
```
```
                         HindIII      Vector
AGTTGATAGAAGGGTCCTTCC A^A GCTT GGCAATCCGGTAC
```

Figure 5.6 Sequence of pGL4.21-MaoA promoter insert and vector boundaries including major features of the promoter.

Primers specific to insert and vector were used to partially sequence pGL4.21-MaoA. The sequences were aligned using KAlign. The major estrogenic response elements of the MaoA promoter are highlighted.

5.3.4 Stable Colony screening

5.3.4.1 T-47D- pGL4.21 -pS2

30 colonies were initially isolated from T-47D cells transfected with pGL4.21-pS2 and grown in selective medium in a 96 well plate for 2 weeks. 21 colonies continued growth and were tested for luciferase expression. Two clones, designated clones 3 and 13, after exposure to 10nM E_2 (section 5.2.3.1), were found to have luciferase expression when exposed to 10 nM E_2, of 580 and 56 counts per second (CPS) respectively when using the ONE-Glo reagent.

Further isolation of colonies from the same flask, which remained in selective medium resulted in 60 more colonies being isolated. None of these showed luciferase expression, despite maintaining puromycin resistance.

A second round of transfection and selection of T-47D cells led to a further 60 colonies being isolated and tested for luciferase expression. One clone displayed luciferase activity of 76 CPS (compared to negative control activity of ~20 CPS).

5.3.4.2 T47D- pGL4.21-MaoA

72 colonies resistant to puromycin were isolated and tested for luciferase expression. As the gene is down-regulated in the presence of E_2, cells were screened in complete media. Of the 72 clones screened, 4 clones showed luciferase expression of up to ~280 CPS.

5.3.4.3 Dose Response of pGL4.21-pS2 Clone 3 and 13 to E_2

T-47D-pS2 clones 1 and 13 were exposed to 0.1 to 10000 nM E_2 for 4 hours after 5 days in PR-free medium supplemented with 10% DC-FCS to determine if the cells responded in a dose-dependant manner (section 5.2.3.4). Data was represented as a relative % of luciferase expression compared to that of the 0.5% EtOH control. There was no induction of luciferase activity in either of the clones (Figure 5.6). The presence of fulvestrant did not have any ER antagonist activity as measured by luciferase induction.

The clone isolated from the second round transfection lost significant luciferase expression when tested in a dose response experiment (data not shown).

Dose Reponse of pGL4.21-pS2 Clone 3 and 13
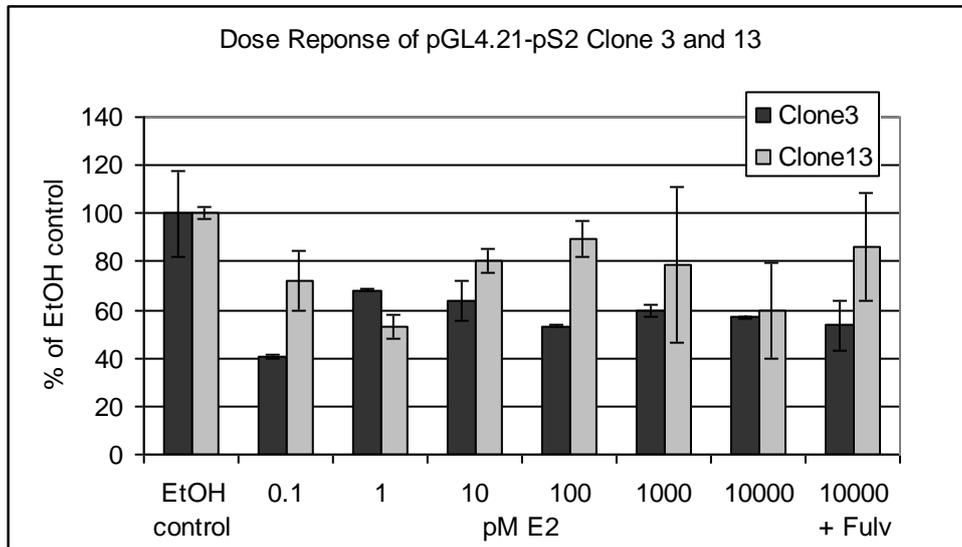
Figure 5.7: Dose response of T-47D-pGL4.21-pS2 clone 3 and 13 to E_2

Cells were incubated in PR-free medium supplemented with 10% DC-FCS for 5 days. Cells were then resuspended, counted and 5000 cells per well placed into a 96 well plate and left to attach overnight. Serial dilutions of E_2 or 10 nM E_2 and 100 nM Fulvestrant were added to the medium and left to incubate for 4 hours. 50μl medium was removed from each well and 50μl ONEGlo added to the remaining 50μl in each of the wells. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader with 1 second exposure time per well. The mean and standard deviations as a percentage of the solvent (EtOH) control are represented here (n=1).

5.3.4.4 Down-regulation of luciferase expression in E_2 free medium

Prior to cells being tested for an E_2 induced up-regulation of luciferase expression, the clonal cell populations were incubated in estrogen-free medium for 5 days. It was hypothesised that during this period, the estrogen-related cellular activity and the expression of the pS2 gene would reduce to a basal level. Without this reduction of pS2 to a basal level it was thought that it would not be possible to see an up-regulation when the cells were exposed to E_2.

Figure 5.7 shows clones 3 and 13, that were exposed to estrogen-free media for 5 days then luciferase expression measured every 24 hours throughout the period (method section 5.2.3.5). Clone 3 has the highest luciferase expression at Day 0 which decreased after the first day and third day and was approximately 82% lower after Day 5 than Day 0.

Clone 13 reduced luciferase expression by 40% after 1 day. After 5 days in estrogen-free medium, the luciferase expression was approximately 55% lower than Day 0. A large standard deviation of the mean luciferase expression of clone 13 on Day 3 negates significance of this large increase.
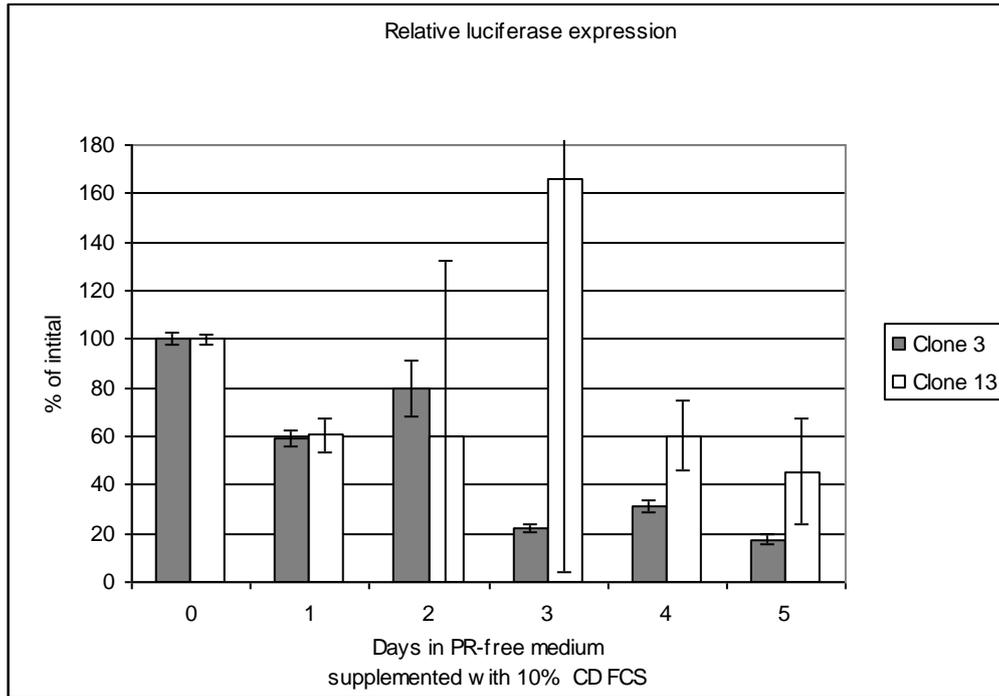
Figure 5.8: T-47D-pGL4.21-pS2 clone 3 and 13 luciferase down-regulation.

In duplicate plates, 5000 cells/well were placed into 6 wells duplicate wells in PR+ medium supplemented with 10% FCS. On consecutive days, duplicate wells were rinsed gently with PBS and the medium replaced with PR-free medium supplemented with 10% DC-FCS. After 5 days, 50μl medium was removed from each well and 50μl ONEGlo added to the remaining 50μl per well. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate. Using the same cell suspension at the time of placing cells into the test plate, 1250 to 80000 cells/well were placed into triplicate wells of a 96 well plate. After 6 hours attachment, a crystal violet assay was applied as described. An equation describing the relationship between cell number and absorbance was determined. The crystal violet assay was applied also to the replicate test plate after 5 days and cell number determined by comparison with the standard curve. The luciferase CPS was then adjusted to a count per cell basis and the data displayed as a percentage of the initial (day 0) luciferase CPS/cell (n=1).

5.3.4.5 Time-responsive activity of T-47D-pGL4.21-pS2 clone 3 and 13 to E_2

As a response to E_2 was not seen after 4 hours exposure, it was hypothesised that the up-regulation of luciferase linked to the pS2 promoter was potentially occurring either earlier or later than the 4 hour exposure. Cells were therefore tested for luciferase up-regulation in response to 10nM E_2 after 1,4 ,8 and 24 hours (method section 5.2.3.6). There was no up-regulation of luciferase in either clone when exposed to 10 nM E_2 after 1, 4, 8 or 24 hours (Figure 5.8). There was a ~30% increase in luciferase expression after 1 hour compared to the EtOH (4 hour) control, however, compared to the 0 hr luciferase there was no significant increase, that is, cells in the EtOH control treatment wells have reduced luciferase expression compared to the 0hr control. There was a significant reduction in luciferase expression in Clone 3 after 8 hours only.
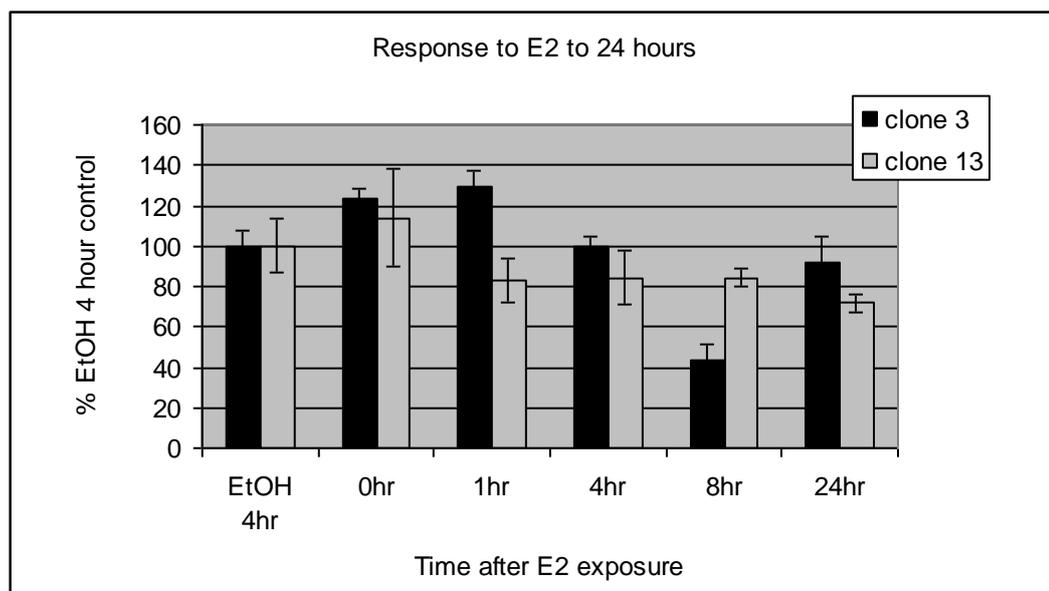
Figure 5.9: Time-response of T-47D-pGL4.21-pS2 clone 3 and 13.

Cells were incubated in PR-free medium supplemented with 10% DC-FCS for 5 days before 5000 cells/well were placed in a 96 well plate in the same medium overnight to attach. Cells were then exposed to 10 nM E_2. After 0, 1, 4, 8 and 24 hours, 50μl medium was removed from each well and 50μl per well ONEGlo added to the remaining 50μl. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader. CPS were adjusted relative to the EtOH control expressed here as a mean ± SD (n=1).

5.3.4.6 Effect of E_2 on luciferase expression by a non-clonal pool of selected T-47D-pGL4.21-pS2 cells.

As the two clonal populations of cells did not show an up-regulation of luciferase in response to E_2, the entire population of puromycin selected but non-clonal cells, (the original source of the colonies), was tested for E_2 induced luciferase expression. The cell population was thoroughly resuspended after trypsinisation to aid homogenisation of the population. After 5 days pre-treatment in estrogen-free media the cell population was exposed to 10nM E_2, 10nM + 100nM Fulvestrant, or an EtOH control. No significant differences were seen compared to the control treatments (Figure 5.9). Despite the maintenance of puromycin resistance by the cells, very low luciferase CPS indicated the loss of luciferase expression.
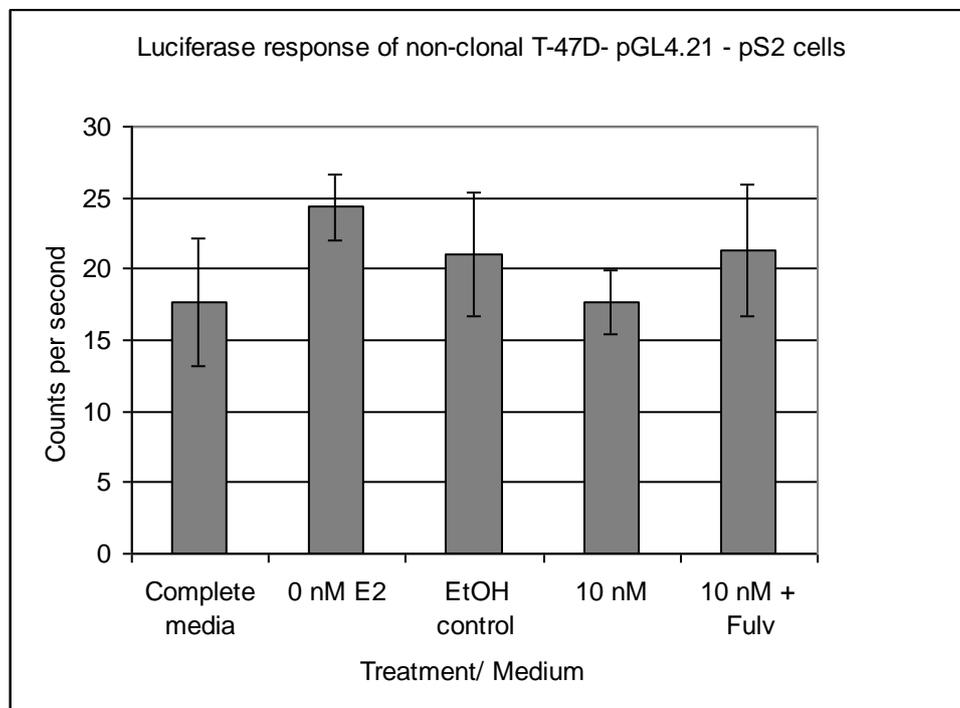
Figure 5.10: Luciferase response of non-clonal T-47D-pGL4.21-pS2 cells.

Transfected cells were grown in selective medium for at least 4 weeks before being incubated in PR-free medium supplemented with 10% DC-FCS for 5 days before cells were resuspended and 5000 cells/well placed in a 96 well plate in the same medium. Cells were left to attach overnight before the addition of 0nM E_2 (PR-free medium supplemented with 10% DC-FCS), 10 nM E_2, 10nM E_2 + 100nM Fulvestrant, EtOH control or complete media. After 4 hours, 50μl medium was removed from each well and 50μl per well ONEGlo added to the remaining 50μl. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of

the cells. Luminescence was determined using a Wallac Victor plate reader with 1 second exposure time per well. The mean and standard deviation of three replicate wells is shown here (n=1).

5.3.4.7 Response of T-47D- pGL4.21-MaoA clones 1 to 4 to E_2

Four clonal populations of T-47D-pGL4.21-MaoA were tested for down-regulation of luciferase when exposed to 10nM E_2. As this was a screening stage, cells were tested on one occasion only. Clone 1 and 4 showed a slight decrease in luciferase expression when exposed to 10nM E_2 (Figure 5.10). Luciferase expression by clones 2 and 3 was increased by 10nM E_2 in comparison to the EtOH control.
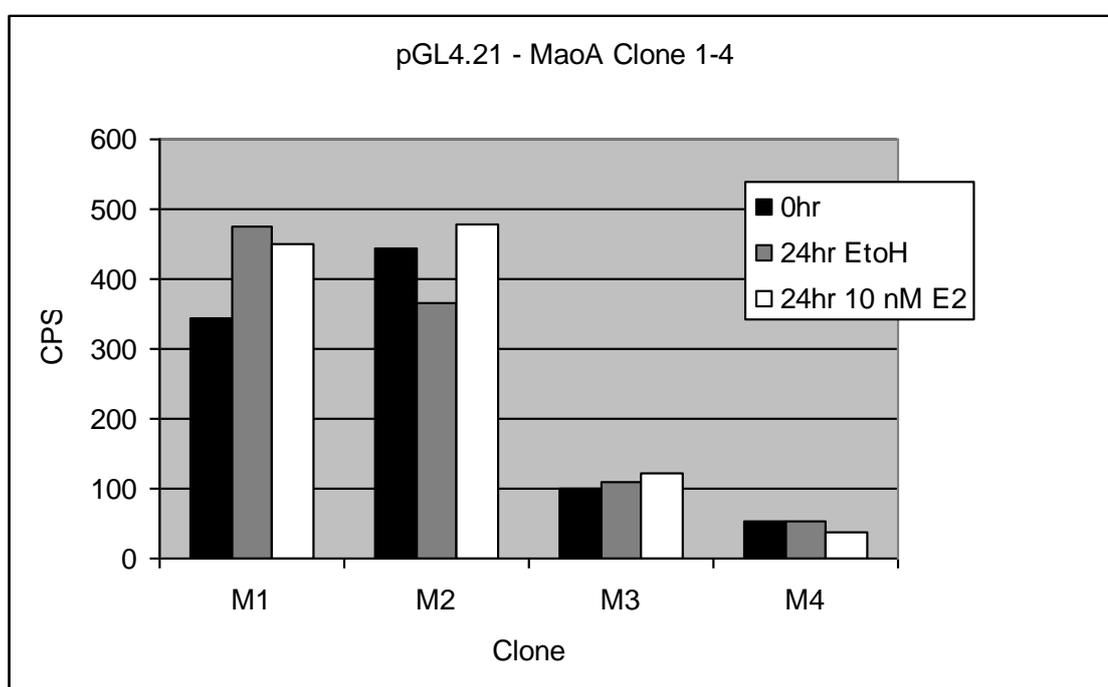
Figure 5.11: Response of T-47D-pGL4.21-MaoA clones 1 - 4 to E_2

Cells were incubated in PR-free medium supplemented with 10% DC-FCS for 5 days. Clones 1 to 4 were designated as M1 to M4. Cells were then resuspended, counted and 5000 cells per well placed into a 96 well plate and left to attach for 24 hours. 10 nM E_2 (24hr 10 nM E_2) or solvent (24hr EtOH) was added to the medium and cells left to incubate for 24 hours. 50μl medium was removed from each well and 50μl ONEGlo added to the remaining 50μl. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader with 1 second exposure time per well. The experiment was repeated on one occasion (n=1).

5.3.4.8 Response of pGL4.21-MaoA Clone 1 to E_2

T-47D-pGL4.21 MaoA clone 1 was exposed to 10 nM E_2 for 24 hours after 5 days in PR-free medium supplemented with 10% DC-FCS. Luciferase activity was up-regulated in both the EtOH control and 10 nM treatments compared to the 0 nM media control treatment. There was very little down-regulation caused by 10nM E_2 compared to the EtOH control (Figure 5.11).
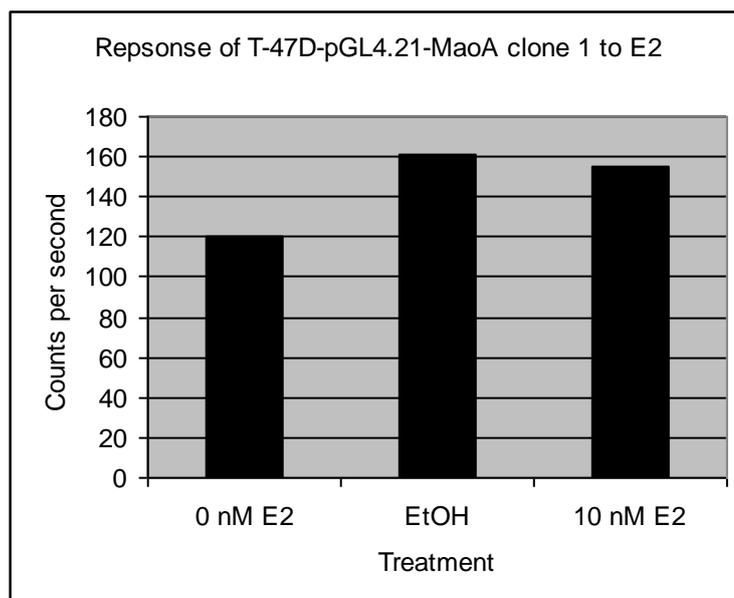
Figure 5.12: Response of T-47D-pGL4.21-MaoA clone 1 to E_2

Cells were incubated in PR-free medium supplemented with 10% DC-FCS for 3 days. Cells were then resuspended, counted and 5000 cells per well placed into a 96 well plate and left to attach. 10 nM E_2 was added to the medium and cells left to incubate for 24 hours. 50µl medium was removed from each well and 50µl ONEGlo added to the remaining 50µl. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader. The experiment was repeated on one occasion only (n=1).

5.3.5 Transient transfection of pGL4.21-pS2 and pGL4.21-MaoA

5.3.5.1 Exposure to E_2 in a 96 well plate format

The production of stable cell lines did not generate clonal cell populations in which the expression of luciferase linked to estrogen responsive promoters was driven or reduced by the presence of 10nM E_2. We hypothesised that this may be due to integration of the plasmid in regions of the cellular genome influenced by other regulatory factors. The response of a cell line that is transiently transfected with the plasmid instead would allow for a more accurate response profile of the plasmid. Although this would not generate a stable cell line that could be characterised, it was the best option for characterising the response of the plasmid.

T-47D cells were transiently transfected with pGL4.21-pS2 or pGL4.21-MaoA, and treated with 10 nM E_2, 10nM E_2 + 100nM Fulvestrant or an EtOH control.

There was no difference between any of the treatment groups when pGL4.21-pS2 was transfected into cells (Figure 5.12). The luciferase count was however higher than that of the promoterless vector, pGL4.21, showing at least some basal activity of the promoters.

pGL4.21-MaoA transfected cells displayed luciferase up-regulation after 24 hours by 10nm E_2. In the presence of 100nM Fulvestrant, luciferase expression was also up-regulated but not to such an extent as 10nM E_2 alone.

As counts per second were generally low, the assay was only repeated once in this format and instead repeated in a 24 well format.
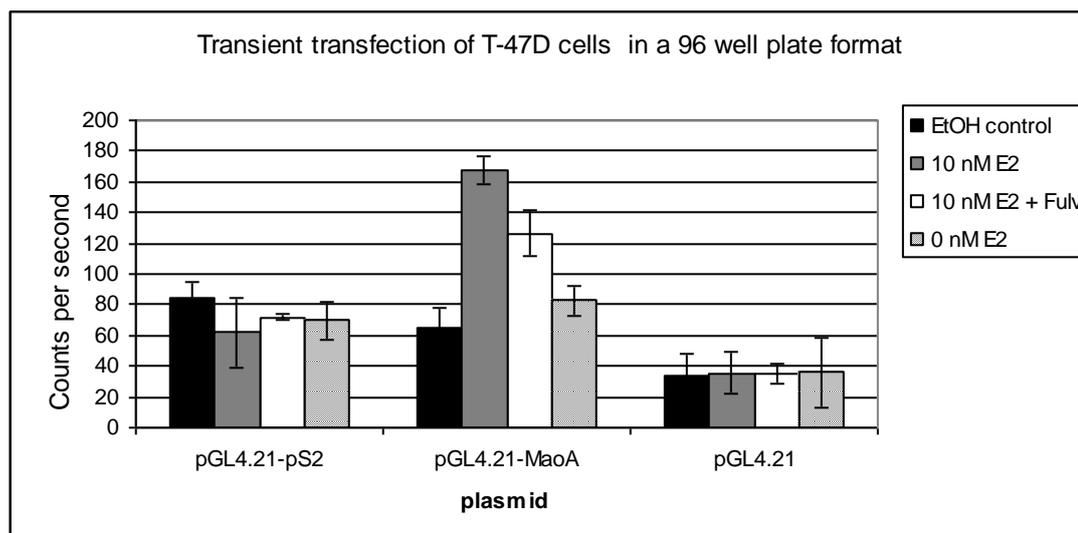
Figure 5.13: Transient Transfection of T-47D cells with pGL4.21-pS2 and pGL4.21-MaoA

T-47D cells were incubated in PR-free medium supplemented with 10% DC-FCS for 4 days before being resuspended, counted and 80000 cells /well placed in a 96 well plate in the same medium type. Cells were transfected with pGL4.21-pS2, pGL4.21–MaoA or pGL4.21 (promoterless vector control) the following day. Transfection complex was removed 5 hours later and replaced with media containing EtOH control, 10 nM E_2, 10nM E_2 + 100nM Fulvestrant all in PR- free medium supplemented with 10% DC-FCS or 0nM E_2 (PR-free medium supplemented with 10% DC-FCS) as indicated. 50µl medium was removed from each well and 50µl ONEGlo added to the remaining 50µl media in each well. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader. The experiment was repeated on one occasion (n=1).

5.3.5.2 Exposure to E_2 in a 24 well plate format

Luciferase expression was determined 48 hours after transient transfection and 4 hours after exposure to treatments. The luciferase expression under the control of the pS2 and MaoA promoter was not significantly up- or down-regulated in T-47D cells in a 96-well plate format, and had relatively low CPS. We hypothesised that by increasing the number of transfected cells per tested population, the luciferase counts would also increase. This was done using a 24-well plate format in preference to the 96 well plate.

MCF7 and ZR-75-1 cells were also used to determine if the lack of response seen until now was cell line specific.

T-47D cells transfected with pGL4.21-pS2 in a 24-well plate format showed no significant increase in luciferase expression when exposed to 10nM E_2 (Figure 5.13 A). Alternatively, it was found that in ZR-75-1 and MCF7 cells, the luciferase expression decreased slightly with E_2 exposure (Figure 5.13 B & C). This however was also the case in MCF7 cells transfected with the promoterless pGL4.21.

Under the control of the MaoA promoter, the luciferase expression was significantly up-regulated in T-47D cells, contrary to its predicted down-regulation. Although the luciferase expression was decreased when Fulvestrant is present, the difference was not significant. Luciferase expression decreased after exposure to E_2 in MCF7 and ZR-75-1 cells transfected with the pGL4.21-MaoA plasmid. Again, luciferase expression from cells transfected with the promoterless pGL4.21 vector showed the same response.

A

Transient transfection of T-47D cells (n=3)

- EtOH
- 10nM E2
- 10 nM E2 and Fulv

% of EtOH control

Plasmid: pGL4.21- pS2, pGL4.21-MaoA, pGL4.21

B

Transient transfection of ZR-75-1 cells (n=1)

- EtOH
- 10 nM E2
- 10nM E2 and Fulv

% of EtOH control

Plasmid: pGL4.21 -pS2, pGL4.21 -MaoA, pGL4.21
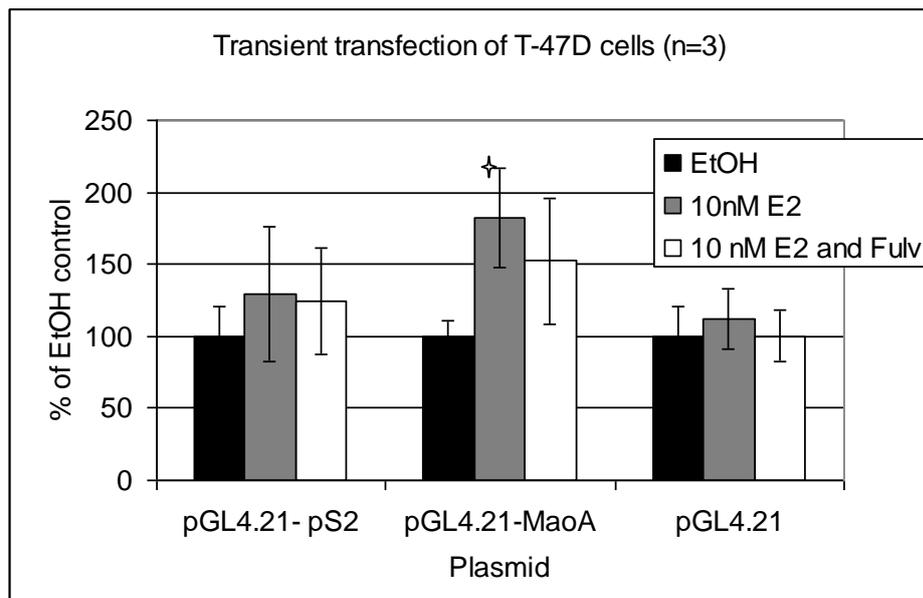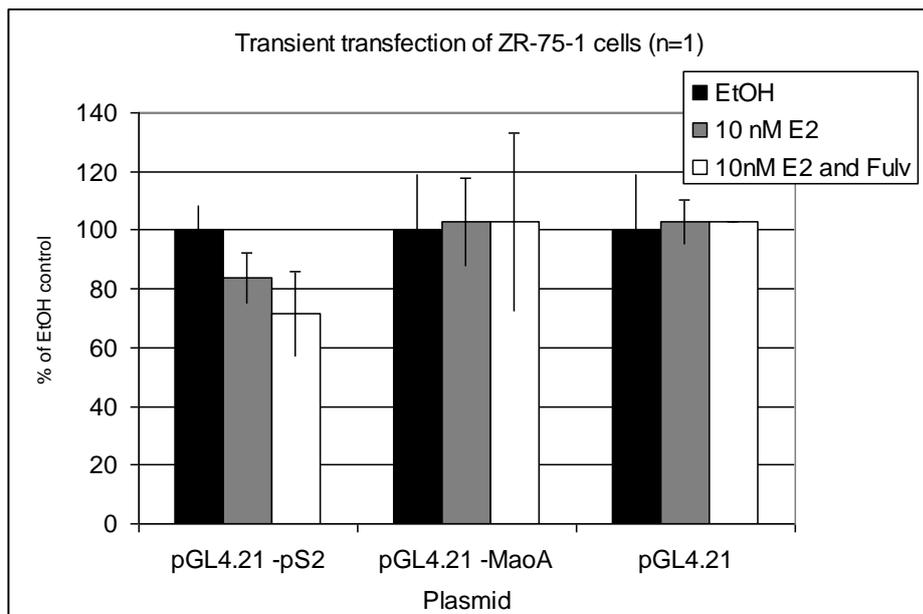
C
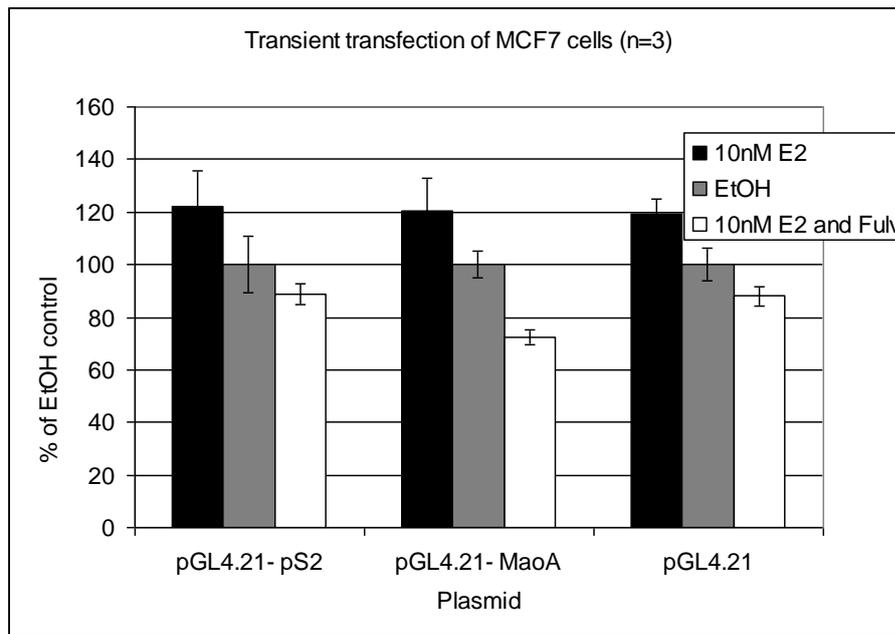
Figure 5.14: Transient Transfection of human breast cancer cell lines with pGL4.21-pS2 and pGL4.21-MaoA

T-47D (A), MCF7 (B) or T-47D (C) cells were incubated in PR-free medium supplemented with 10% DC-FCS for 4 days before being resuspended, counted and $2x10^5$ cells/well placed in a 24 well plate in the same medium type. Cells were transfected with pGL4.21-pS2 or pGL4.21–MaoA the following day. Transfection complex was removed 5 hours later and replaced with media containing EtOH control, 10 nM E_2 or 10nM E_2 + 100nM Fulvestrant all in PR- free medium supplemented with 10% DC-FCS as indicated for 24 hours. Media were collected from each well and 50µl used to resuspend the cells after trypsinisation and centrifugation. 50µl ONEGlo added to the 50µl cell suspension and the whole volume dispensed to one well of a 96 well plate per sample. After 3 minutes incubation at room temperature, cells were repeat pipetted with a multichannel pipette to aid lysis of the cells. Luminescence was determined using a Wallac Victor plate reader with 1 second exposure time per well. The experiment was repeated on one occasion only and the mean and standard deviation represented here (n=1).

5.4 Discussion

Stable integration of the pGL4.21- pS2 and pGL4.21- MaoA vectors into T-47D cells resulted in non estradiol-inducible expression of the luciferase reporter gene. To overcome any effect that stable cellular integration of the vector may have had, transient transfection was also used, which similarly resulted in non estradiol-inducible expression of luciferase.

The pS2 and MaoA promoters were successfully amplified, isolated and ligated into the pGL4.21 vector. Sequencing of the pGL4.21- pS2 plasmid demonstrated that the promoter fragment (-1590/+43) from T-47D genomic DNA, showed a C to T mismatch at position -918 relative to the TSS. This was confirmed with two separate sequencing reactions. The base pair change is not within proximity to any known promoter feature such as the ERE, TATA box or AP1 site, all involved in estrogenic activation(Berry et al., 1989). It is not known if this mismatch is unique to T-47D cells or if the base pair change was generated during amplification from the T-47D genomic DNA. The use of a *pfu* polymerase with proofreading ability would suggest that the base pair change was unique to T-47D cells.

The MaoA promoter fragment (-1283/+125) sequencing did not show any such mismatch. The imperfect ERE at approximately position -200 as described by Jorgensen et al (2000) could not be found in the promoter sequence, even though the accession numbers were in agreement. The actual location of the TSS that Jorgensen et al (2000) describes is unknown, however the TSS the we chose was as defined by NCBI and UCSC databases and confirmed with other sources (such as SwitchGear Ltd's database of TSS).

The VEGF promoter was thought to be isolated and amplified by PCR (one of two PCR amplicons) but could not be successfully ligated to the vector. Multiple repeat isolations and ligations using different vector:insert ratios were attempted. Second round PCR of the isolated fragment, thought to be the correct amplicon, by others within the laboratory was not successful, which may indicate that the PCR product was not actually the expected VEGF promoter template. The inability of the amplicon to ligate to the vector also indicates, regardless of the sequence, that the primers and incorporated RE sites were not present at both ends of the amplicon. The use of nested

PCR may have been appropriate to help with specific amplification of the promoter region as performed by Oduro et al (2008). The use of nested PCR can help increase the specificity of the final PCR amplification when amplifying regions from a complex template such as genomic DNA (Sambrook et al., 1989).

The EGR1 and IGFBP4 promoters were not isolated. The use of various polymerase master mixes by others, T-47D cell genomic DNA extractions, and primer annealing temperatures did not generate a band of the correct size that could be isolated. The PCR reaction using EGR1 primers produced many amplicons whereas the IGFBP4 primers produced 3 to 4 major amplicons, none of which were at the correct size of 1085bp and 1139 bp respectively. Again, the use of nested primers in this case may have helped with isolating the promoter from genomic DNA.

Subculturing of a clone, grown from a single cell, results in a homogenous population of cells that responds in a cohesive and reproducible way. During ER-CALUX development, Legler et al (1999b) transfected the construct pEREtata-Luc into ECC-1 and MCF7 cells, but were then plagued with clonal populations that had constitutive, non-estradiol- inducible luciferase expression. Through en masse screening and use of the T-47D cell line they were able to isolate clones that were estradiol inducible. Other similar studies where reporter genes are under the control of estrogen-inducible promoters such as Jausons-Loffreda et al (1994) (reported in Legler et al (1999b)) have encountered the same problem of non-inducible constitutive expression. Colleagues using similar estrogen or androgen responsive reporter systems with the pGL4 vector and its predecessor pGL3 vector have also encountered similar problems (*personal communication* with Anne Rogers and Yabin Zhou).

The transfection of mammalian cell lines with a lipid based transfection reagent, such as LipofectAmine 2000, is based on the uptake of the plasmid by the cell and subsequent integration into the cells genome under selective pressure. The integration of the plasmid, unless site directed, is a random integration event and the plasmid construct may integrate to regions under the influence of other promoters which can lead to extraneous regulation of the reporter gene (Legler et al., 1999b). Mutations of the construct during integration can also lead to hormone independent expression of luciferase.

Initial testing of puromycin selected, clonal populations of the cells did not show the expected up-regulation of luciferase driven by the pS2 promoter nor down-regulation of luciferase driven by the MaoA promoter when the cells were exposed to E_2. Cells were pre-treated in estrogen-free media for 5 days prior to any testing of luciferase induction. This was done to return the estrogen responsive genes to a basal expression level. Luciferase expression in T-47D-pGL4.21-pS2 clones 3 and 13 was down-regulated over 5 days. This could be attributed to two factors; a direct down-regulation of pS2 expression over 5 days as the cells were in an E_2 free environment, or a decrease in the overall metabolism of the cells as they entered stationary phase. This would result in a whole subset of genes being down-regulated (not pS2 alone). In either case, the demonstrated down-regulation of luciferase driven by the pS2 promoter in estrogen-free media did demonstrate some regulation of the promoter-reporter construct. Jorgenson et al (2000) also showed that pS2 expression decreased over a period of 5 days.

With this knowledge, T-47D-pGL4.21-pS2 Clone 3 was therefore again tested to see if the reason for the apparent constitutive expression of luciferase was a result of the induction point of luciferase being missed. The determination of this time point is more critical with the use of luc/2P destabilised luciferase given that a 'pooling' of luciferase within the cell is not occurring to the extent it may with luciferase that is not tagged with protein destabilisation sequences (www.promega.com). The monitoring of luciferase expression 0, 1, 4, 8 and 24 after the addition of 10nM E_2 indicated that there was no induction of luciferase expression at any time point. After 8 hours, there was a significant 55% reduction in luciferase expression which returned to control levels after 24 hours. The experiment would need to be repeated to verify if this reduction 8 hours post-addition of E_2 is an actual event. As no vehicle (EtOH) control was used for each time point we can only compare the 4hr EtOH control point, at which time luciferase was down-regulated compared to 0 hours. This experiment was performed on one occasion only and would need to be repeated to confirm what is occurring. It would appear that the EtOH is causing a down-regulation in luciferase. In either case, the down regulation of pS2 in T-47D cells when exposed to E_2 is probably an unrealistic event with no indication to the contrary reported in literature (Brown et al., 1984; Berry et al., 1989; Barkhem et al., 2002; Oduro et al., 2008). No up-regulation of luciferase expression was seen, indicating non-inducible expression.

Another study using the pS2 promoter similarly encountered constitutive expression. The promoter region of -1587/+38 was found in the study by Oduro et al (2008) to drive constitutive luciferase expression despite the pS2 mRNA being greatly up-regulated. There are elements of the pS2 gene known to give constitutive expression but others that are estrogen dependent (Oduro et al., 2008). Although some studies have successfully used pS2-luc constructs it may be the influence of distal promoter elements and co-activators that consistently show up-regulation of pS2 mRNA (Oduro et al., 2008) but not necessarily the isolated and potentially truncated pS2 promoter segment attached to luciferase reporter gene. There are no previous reports of a MaoA-luciferase reporter construct found in the literature.

Lipid-based transient transfection of the pGL4.21-pS2 and pGL4.21-MaoA constructs into MCF7, T-47D and ZR-75-1 cells allowed examination of luciferase induction in the absence of integration into the genome, as the plasmid remains in the cytoplasm (Legler et al., 1999b).

The expression of pS2-luciferase in the transiently transfected cells was non-inducible in MCF7, T-47D and ZR-75-1 cells and no significant differences were seen. The expression of pS2-luciferase in MCF7 cells decreased (in order of) 10 nM E_2 > EtOH control > 10nM E_2 + 100 nM Fulvestrant. This however also occurred in the promoterless pGL4.21 transfected cells, negating the significance of the changes of luciferase expression. Luciferase under the control of the MaoA promoter showed significant up-regulation compared to the EtOH control in T-47D cells when the cells were exposed to 10nM E_2. The difference between the 10nM E_2 treated cells and those with the inclusion of the E_2 antagonist, however, was not significant indicating that the effect was not a direct result of E_2 stimulation.

The testing of luciferase in the transiently transfected cells only took place either 24 or 48 hours post-transfection. E_2 was added to the cells 5 hours after transfection, at the same time that transfection complexes were removed from the cells. This was done in keeping with other protocols, whereby luciferase induction was tested after this time. The window of up-regulation of pS2 promoter or down-regulation of MaoA promoter, given they are early response genes, would need to be taken into consideration when

interpreting these results. Given that pS2 and MaoA have been shown to respond within 4 hours of E_2 exposure (Jorgensen et al., 2000; Frasor et al., 2003), the assessment of their expression after 48 hours may be too late. Jorgenson et al (2000) showed that pS2 and MaoA mRNA expression was actually greatest and lowest respectively after 48 hours. It is unknown what correlation there may be between the pS2 promoter driven mRNA expression and truncated pS2 promoter driven luciferase protein expression.

A 24 well plate format was used to increase cell numbers and the luciferase CPS. The increase in cell numbers was thought to increase the luciferase signal which was relatively low in the 96-well plate format, however was not successful. It is unknown what the effect of an excess of cells per volume of ONE-Glo may have and was not tested.

Further transient transfection experimentation may be able to determine the time-response of the luciferase protein expression after the addition of an estrogenic stimulus. However, uncertainty still remains as to the amount of time taken for uptake and expression of the transgene after transient transfection. An alternative approach would be to wait 48 hours post-transfection and then add E_2 stimuli and monitor expression of luciferase over a time course.

The isolated pS2 and MaoA promoters driving the expression of a destabilised luciferase were not successfully used in this study to detect the presence of estrogen. The complex cellular regulation of promoter sequences may have impacted on this result. The inclusion or exclusion of slightly different promoter elements (by excising the pS2 and MaoA promoter fragment at the chosen sites) and source material may impact the actual outcome. The concept of reporter cell lines incorporating endogenous estrogen response elements and activators makes this assay more physiologically relevant to the responses likely to be an outcome of (xeno)estrogen exposure and if explored further would provide a valuable assay.

CHAPTER 6

General Discussion

6.1 General Discussion

A large number of *in vitro* bioassays have been developed with the ability to screen individual chemicals and water samples, sometimes containing complex mixtures, for estrogenicity (reviewed by (Zacharewski, 1997; Fang et al., 2000; Soto et al., 2006; Leusch et al., 2010)).

There has been a collaborative assessment by those including the Global Water Research Coalition and others, to select *in vitro* estrogenicity bioassays that are best suited to tiered evaluation of water quality (Leusch et al., 2010). This work was done not only to determine which assays correlated best with chemical analysis but also to attempt to bring about standardisation of assay methodology and data analysis techniques (Andersen et al., 1999; Leusch et al., 2010).

In this study, the human cell lines; MCF7, T-47D, ZR-75-1, Ovcar, H23 and RL-95, were assessed for characteristics which made them amenable for use in estrogenic response assays. This work was done to provide relevant information on cell lines that can be used for bioassays, or for the development of new assays; or to expand on the knowledge base of the E-screen assay; and to develop a rapid estrogenic response assay, similar to the ER-CALUX, but using a set of endogenous gene promoters to give a more physiologically realistic response to potentially estrogenic compounds and mixtures.

Mammalian cell line bioassays provide a physiologically relevant response to the total estrogenic load of water samples. Chemical methods based on chromatography and related identification techniques are very sensitive and accurate for detection of already identified chemicals or subsets of chemicals. They are however, a blinkered approach for routine testing as they can only report on specific targeted chemicals.

The six cells lines were chosen because reports in the literature suggested that they were estrogen responsive. Using western blotting with an antibody specific to the C-terminus of the ERα protein it was found that of the cell lines chosen, only MCF7, T-47D and ZR-75-1 expressed the endogenous 66kDa ERα protein. A major band at approximately 90kDa in the cell lysates was also detected by the antibody. It was initially thought that

the extra band may be due to dimerisation, phosphorylation or a splice variant of ERα. Often incomplete western blots are presented in literature to present only the band of interest and so the relevance and nature of this band was unknown for some time. It was later determined from literature that the extra band present was a consequence of the particular antibody being used, and was probably not related to actual ERα expression (Pavao and Traish, 2001). Importantly it was not the 46kDa isoform of ERα which has been shown to negate the proliferative effect that the 66kDa isoform has (Penot et al., 2005). The levels of ERα protein expression was MCF7>T-47D> ZR-75-1.

This same result was obtained when qPCR was used. Primers in the 3'UTR were used which are able to detect all splice variants. The major difference between results of the two techniques used was that Ovcar expressed ERα mRNA (but not ERα protein). The difference may be due to translational modifications of the mRNA preventing the mRNA from being translated to protein

The unexpected absence of ERα protein from the remaining cell lines, was confirmed by using reverse transcription PCR. It is unknown why the ERα mRNA was not detectable in H23 or RL-95-2. The cell lines were both received directly from ATCC, and grown in the optimal conditions described by ATCC. Culture conditions, such as serum content of medium, have been shown to effect the expression of the ER (Marth and Daxenbichler, 1985; Villalobos et al., 1995). This, along with the individual 'history' of the cell line's maintenance potentially may be the reason that others have found these cell lines to express the ERα mRNA (Dechering et al., 2000) . The insistence of publishers for the karyotyping of cells or use within a small time frame from delivery (from such sources as ATCC), may increase the accuracy of reported cell line ER status. Further to this fact, is the variation in doubling time of the cells determined in this study and those previously reported.

Although the majority of estrogenic bioassays use ERα, endogenous or otherwise, ERβ has a different physiological role and has been shown to have different interactions with potential ligands (Koehler et al., 2005). ERβ was not detected in any cell line. Previous studies have also expected to detect ERβ in MCF7 cells and not been able to detect expression (Dechering et al., 2000; Jorgensen et al., 2000). Whilst cell line models that do have ERβ expression would be useful to expand knowledge on the actions of an

activated ERβ, for the scope of this study it was preferable to have only ERα present, as ERβ has been shown to be detrimental to estrogen-stimulated proliferation.

In all the mammalian cell bioassays reviewed, an overnight or 24 hour attachment period was used for attachment combined with a pre-treatment period in estrogen free media (that is phenol red free media supplemented with 10% FCS which had been treated with dextran-charcoal to remove steroids). The cell lines were assessed for their attachment times to new growth surfaces i.e. 96-well plate. Our studies showed that cells that were attached for 4 or 6 hours gave more accurate and reproducible results, based on lower variability between replicates, than after a 24 hour attachment period. The production of a standard curve was also therefore more accurate at this time (relating absorbance to cell number). No reports of this having been assessed before were found. In fact all the assays reviewed allowed the cells to attach overnight before the commencement of any manipulations or measurements. In some cases this may be through following procedure without optimisation of an overnight attachment period that is considered as routine.

MCF7, MCF7 sub-lines and T-47D cells have all been used in E-screen assays. In this study MCF7, T-47D and ZR-75-1 cells were used in a side-by-side comparison to assess their suitability for use in the assay. This was based on their proliferative response and sensitivity to E_2, the longevity of their response after multiple passaging, and the effect of reducing an attachment/pre-treatment time to the determined 4 or 6 hours (although for consistency 6 hours was used throughout).

The E-screen response was analysed as a fold increase in cell number above that of the control cell number (cells grown in estrogen-free medium). Cells, when placed in estrogen-free medium, are best suited to an E-screen assay if they stop proliferating or slow proliferation significantly. The growth rate of MCF7 (ATCC) cells has been shown to not be affected by estrogen-free medium (Villalobos et al., 1995). The line of MCF7 cells we used did significantly ($p < 0.05$) decrease growth. T-47D or ZR-75-1 cells, both ceased proliferating in these conditions, potentially making them better candidates for the E-screen.

Initially, it was hypothesised that upregulation of the ER would lead to more receptors being available to respond to lower concentrations of E_2 with a greater magnitude of cellular response. This up-regulation would be achieved by a pre-treatment of the cells in estrogen-free medium. Although western blotting demosntrated that the ERα of ZR-75-1 cells was up-regulated after extended culture (more than 65 passages) in medium supplemented with a lower FCS concentration of 5% and 2.5%, there was no up-regulation of ERα in ZR-75-1, T-47D or MCF7 cells after 48 hours in PR-free medium supplemented with 1% DC-FCS. A longer exposure period may have been required in this case to show a change in expression levels of the protein. We also hypothesised that changing the medium every day throughout the exposure period would cause a higher cellular response, as the E_2 may otherwise be degraded/depleted. Media replacement every 24 hours resulted in a loss of expected growth which we now believe to be associated with a loss of autocrine and paracrine factors. This would need to be tested again after 5 or 7 days exposure to E_2, and also with the inclusion of media supplemented with 10% DC-FCS. In this experiment we only supplemented media with 1% DC-FCS, which may help up-regulate the ER, but may not be enough to support cellular proliferation (Villalobos et al., 1995). This may also be the case when assessing gene expression changes, whereby 1% DC-FCS may not provide all the necessary serum components for rapid cell signalling.

The E-screen assay was performed using the three selected cell lines MCF7, T-47D and ZR-75-1. This was done to directly compare their proliferation in response to estrogenic stimulation. MCF7 or MCF7 sub-lines are most commonly used in the E-screen assay. The cells are commonly attached and pre-treated for 24 hours (up to 72 hours) before the addition of E_2. Rasmussen et al (2002) showed that doing this had no effect on the concentration of E_2 at which proliferation became significantly different to the control, $p<0.05$ (LOD) but did decrease the fold proliferation. The fold proliferation is important for the detection of weak or low concentration estrogens. Not only would a reduction of the pre-treatment/attachment time reduce the overall period of the assay, it may also increase the fold proliferation.

Firstly, it was determined that the reduction of attachment time from 24 hours to 6 hours before exposure to E_2, decreased the large SEMs found between experimental replicates. The E-screen has been shown to have very large (60%) inter-experimental

variability (Rajapakse et al., 2002). Large SEM values increased the value at which the proliferative response became significantly different to the control. The MCF7 BOS cell line typically has LOD in the $\leq$ 1pM range (Korner et al., 1999; Rasmussen and Nielsen, 2002). In this study the cells showed sensitivity generally in the 10pM range. Although these cells had a higher concentration of E_2 LOD and lower proliferative response than MCF7 BOS, MCF7 and T-47D cells are still commonly used in E-screen assays.

T-47D cells have been shown to be far less responsive to non-estrogenic growth-promoting factors than MCF7 cells (Karey and Sirbasku, 1988). This would make them a valuable alternative if a T-47D sub-line was isolated that had an equal or greater proliferative response to estrogens as MCF7 BOS cells.

The cell lines assessed in this study were far below the 10 fold proliferation achievable in MCF7 BOS cells (Rasmussen and Nielsen, 2002). The fold proliferation, may impact on sensitivity, given that smaller increments of E_2 generate larger changes in cell number, equating to greater significant difference and statistical certainty.

Long term culture of ZR-75-1 cells in serum-reduced medium did not increase their responsiveness to E_2, similar to that found in MCF7 BB-104 cells (Villalobos et al., 1995). ZR-75-1 cells, although having similar EC_{50} and LOD to the other cell lines tested, had a very low fold proliferation and therefore would be of limited use in the E-screen.

Five highly estrogen responsive genes; pS2, MaoA, IGFBP4, VEGF and EGR1, were selected from multiple microarray analyses (Jorgensen et al., 2000; Inoue et al., 2002; Frasor et al., 2003; Terasaka et al., 2004) and *in vivo* data (Watanabe et al., 2002). The genes were selected on the basis that they were highly up-regulated or down-regulated in a short period of time. It was hypothesised that if the promoter regions of these genes were used to drive the expression of a reporter gene in the selected cell lines, it would be possible to generate a bioassay that responded quickly to estrogenic substances in a physiologically relevant way. This may have the advantage over similar assays such as the ER-CALUX, which uses a triplet ERE with possibly less physiological relevance. The ER-CALUX assay concept has expanded to include DR-CALUX and AR-CALUX for detection of dioxins and androgen respectively. These have an associated licensing

fee which would need to be considered depending on the scale of application and resources available. Similar assays have used the Vitellogenin promoter, to drive the expression of a reporter gene, however, this gene is not endogenous to humans, is better for assessment of exposure in fish and other similar species (Cosnefroy et al., 2009).

Initially we entered negotiations with SwitchGear Genomics Ltd to provide the qualified promoters regions in vectors with a luciferase reporter gene. Due to the costs involved and proprietary limitations on the use and applications of the vectors, we instead chose to amplify and clone the promoter regions ourselves. Interestingly, SwitchGear Genomics Ltd provides promoters in the pGL4.21 (luc2P/Puro) vector, which we had previously selected as the expression vector of choice. This expression vector was chosen after a review of the available reporter vectors available. Initially, enhanced GFP was thought to be the best option; no costly reagents are required for detection, and live cells can be analysed without cellular disruption. Also, the advent of mutations of the eGFP gene have allowed for emission and detection of the protein at various wavelengths (Yang et al., 1998). This feature would allow for multiple promoter activity detection simultaneously, driving the expression of different coloured fluorescent proteins, within the same cell. A destabilised luciferase reporter gene was chosen in preference to eGFP due to increased sensitivity of detection. Luciferase is based on emission only rather than excitation and emission , as required for eGFP, which brings with it higher background and less sensitivity (Denison et al., 2004). The advent of a destabilised luciferase protein was also an exciting advance in luciferase chemistry, as the half-life of the protein had been reduced, allowing for faster detection of changes in expression levels of the luciferase.

The promoter region of only pS2 and MaoA were successfully amplified, which included the ERE and other activation sites. The amplification of the other promoters may have been more successful if a nested-PCR approach was used. This approach was used by Oduro et al (2008) and is useful for isolating amplicons from large, complex templates such a gDNA. The promoter amplicons were inserted into pGL4.21 (luc2P/puro) and the sequence analysed to determine the complete insertion of the promoter to the plasmid without mutation. The promoter constructs were transfected into T-47D cells and stable clonal populations isolated. Clones were selected that expressed luciferase. Of the clones isolated, they were all found have non-inducible

luciferase expression. As no estrogen-inducible response was seen from the stable cell lines, transient transfection was then used to test the reporter constructs. Albeit a temporary incorporation and expression by the host cell of the plasmid, transient transfection allows for the vector to be tested without the complications of integration of the vector to the genome, potentially under the regulation of other promoters (Legler et al., 1999a). This, however, also resulted in non-inducible expression of the reporter gene. The reasons why this occurred are still unknown, the induction time of the destabilised luciferase were accounted for as were potential problems with integration. Further studies could include isolation of the promoters at the exact points relative to the TSS that have been shown in previous studies to drive the expression of a reporter gene , however, it is unknown if this would resolve the problems being faced here as all the regulatory elements required for induction of the reporter gene would still be included. Whilst other studies have successfully used a luciferase reporter gene, based on its sensitivity of detection, the development of estrogenic assays whereby the expression of eGFP (and its variants) are driven by estrogen-responsive promoters may be beneficial. The use of eGFP, a substrate free detection system, would be highly beneficial for routine, high volume testing scenarios. Once regulatory guidelines are determined, it may be that the levels of detection possible with eGFP systems are sufficient, eliminating the need for costly substrates and reduce the amount of handling required.

The use of endogenous promoters linked to a reporter gene to create a series of cell lines that express (or down-regulate) reporter gene activity in response to estrogens could be a valuable tool for the assessment of estrogenic responses. A response profile would be created showing different regulation of particular genes in response to different types of xenoestrogens, which may help with not only assessing estrogenicity of a sample or individual chemical but assist with predictions of what organism-level effects the chemical(s) may have.

6.2 Conclusions

With regard to the initial hypotheses:

- **Cell lines express the estrogen receptor alpha and beta to different levels.** MCF7, T-47D and ZR-75-1 cells express ERα mRNA and protein (MCF7>T-47D>ZR-75-1). Ovcar express ERα mRNA only. ERβ was not detected in any of the cell lines tested

- **Cell lines have different growth characteristics such as attachment time, doubling time and experimental variability associated with both of these.** A 4 or 6 hour attachment time was sufficient time to allow attachment and was shown to have lower inter-experimental variability in cell number than after 24 hours. Doubling times were reported which were not only different to those previously reported but varied with initial cell density.

- **The E-screen assay is currently performed with several different cell lines, one of which may be best suited.** T-47D had greater fold proliferation than MCF7 in response to E_2 but had a comparable EC_{50} of 11.0pM and 11.1pM respectively. The SEM was reduced by using a 6 hour attachment period rather than 24 hours. Significant proliferation in response to E_2 was achieved after 5 days. None of the cell lines tested maintained their sensitivity to E_2 after extended time in culture (> 18 passages). Long term culture of ZR-75-1 cell in a low estrogen media did increase ER α expression but did not increase their sensitivity in an E-screen assay.

- **Exposure to Estrogen in mammalian cell culture rapidly induces endogenous genes.** We were not able to show induction or reduction in a luciferase reporter gene coupled to the pS2 or MaoA promoter fragments. Non-inducible expression of the promoter-luciferase complex resulted when the constructs were transfected into MCF7, T-47D and ZR-75-1 cells.

Appendix 1

A1.1 Phosphate Buffered Saline (10x)

10x PBS solution

In a 1Litre Schott Bottle

KCl $\qquad$ 2g

KH_2PO_4 $\qquad$ 2g

NaCl $\qquad$ 80g

Na_2HPO_4 $\qquad$ 11.5g

Make up to 1 Litre with Water (Baxter water for irrigation) and pH to 7.4

Autoclave on liquid cycle.

To make 1 Litre of 1XPBS

100ml of 10X stock

900ml water (Baxter water for irrigation)

Filter sterilise using 250ml vacuum filters

Appendix 2

Western Blotting Solutions

A2.1 mRIPA

50mM Tris-HCl, pH 7.4

1% NP-40

0.25% sodium-deoxycholate,

150mM NaCl,

1mM EDTA

Solution stored at 4°C and supplemented immediately before use with 1mM NaF, 1mM activated Na_3VO_4, 1mM phenylmethanesulphonylfluoride (PMSF) (solubilised in isopropanol) and 2% Cocktail of Protease Inhibitors (Sigma- Aldrich).

A2.2 Reducing Sample Buffer

1M Tris-Cl pH 6.8 2.4 ml

20% SDS 3 ml

Glycerol (100%) 3 ml

Bromophenol blue 0.006g

make to 8.4 mL with milli Q water (store 4°C)

Solution stored at 4°C and supplemented immediately before use with 16% β-mercaptoethanol

A2.3 Running Buffer

10X Running buffer routinely made:

Tris base 30.3 g

Glycine 144 g

SDS 10 g

make to 1L with milliQ water

Dilulte 1:10 before use.

A2.4 Transfer Buffer

25mM Tris, pH 8.3

192mM glycine

20% methanol (v/v)

A2.5 Blocking Buffer

40mM Tris pH 7.4

0.1% (v/v) Tween20

5% (w/v) skim milk powder

A2.6 Tris-Tween

40mM Tris pH 7.4

0.1% (v/v) Tween20

A2.7 Wash Buffer

20mM Tris, pH 7.4

150mM NaCl

5% (w/v) skim milk powder

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