Understanding the Impact of Estrogens in Mammary Gland Formation using an *in vitro* Three-Dimensional Model

Submitted by Stephanie Donata Marchese

for the degree of Doctor of Philosophy

UCL School of Pharmacy, University of London

Supervisor: Dr Elisabete Silva

Second Supervisor: Prof Andreas Kortenkamp

Declaration

This thesis describes research conducted in the UCL School of Pharmacy between Oct 2008 and Oct 2011 under the supervision of Dr Elisabete Silva and Prof Andreas Kortenkamp. I certify that the research described is orginal and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation th has already appeared in publication.

Abstract

Increased ERα expression correlates with increased breast cancer risk, suggesting that the ER and estrogens play a role in cancer initiation and progression. Nonetheless, a clear understanding of the involvement of the ER in breast epithelium formation, carcinogenesis and cancer progression is lacking. In addition to this, the role of other estrogen-responsive receptors, such GPER-1 remains unknown.

The mammary gland is comprised of milk ducts terminating in secretory units termed acini. These are comprised of a layer of epithelial cells surrounding a hollow lumen. These cells are in turn surrounded by a layer of myoepithelial cells attached to an underlying basement membrane. 3D cultures of immortalised breast cells recapitulate many of the features of acini *in vivo*, such as spherical growth-arrested acini with a layer of epithelial cells attached to an underlying basement membrane, surrounding a hollow lumen. We aimed to use 3D cultures of ERα, ERβ and GPER competent MCF-12A cells to assess the effects of estrogenic compounds on acini formation.

MCF-12A cells were cultured in Matrigel with etOH (solvent control), E2, bisphenol A (BPA) or n-propylparaben. To assess the role of the ER or GPER-1, cells were also pre-treated with the ER and GPER -1 antagonists, as well as inhibitors of the MAPK and PI3K signalling cascades. Immunocytochemical staining using antibodies against activated caspase-3 and laminin-V was performed. Image acquisition was achieved by laser confocal microscopy.

Control acini were encapsulated by a basement membrane with central cells either cleared or undergoing apoptosis. In comparison, cells treated with E2, BPA or propyparaben possessed filled lumen with little or no sign of apoptotic central cells and were larger and disorganised. Co-incubation with ER or GPER-1 antagonists, or PI3K and MAPK inhibitors reverted some of the effects of the test compounds resulting in growth-arrested, spherical acini with evidence of luminal clearing.

We have successfully established an *in vitro* 3D model using immortalised ER competent cells to study the effects of estrogens on mammary gland formation. We have demonstrated that estrogenic compounds disrupt acini by inducing luminal filling and preventing growth arrest. Moreover, these effects seem to be mediated by both estrogen responsive receptors ER and GPER-1.

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List of Abbreviations

AF-1	Activation function-1
AF-2	Activation function-2
AIF	Apoptosis-inducing factor
APAF1	apoptotic protease activating factor-1
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
Bad	Bcl-2-antagonist of cell death
Bax	Bcl-2 associated X protein
BBP	Butyl benzyl phthalate
Bcl-2	B-cell lymphoma-2
BH-2	Bcl-2 homology 2
BH-3	Bcl-2 homology 3
Bim	Bcl-2 interacting protein
BMI	Body mass index
BP	Benzoapyrene
BPA	Bisphenol A
BSA	Bovine serum albumin
β-HCH	β-hexachlorocyclohexane
cAMP	Cyclic adenosine monophosphate
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
CDS	Charcoal-dextran stripped
CK5/6	Cytokeratin 5/6
CK2	Casein kinase 2
COMT	Catechol-*O*-methyltransferase
CpG	Cytosine and guanine dinucleotide
Ct	Cycle threshold
DAPI	4', 6-diamidino-2-phenylindole

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
-d(RFU) / dT	Rate of change in relative fluorescence with time
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EF2	Elongation actor-2
EHS	Engelbreth-Holm-Swarm
EMT	Epithelial–mesenchymal transition
ERE	Estrogen response element
ERK1/2	Extracellular regulated kinase ½
etOH	Ethanol
DDE	Dichlorodiphenyldichloroethylene
DED	Death effector domain
DES	Diethylstilbestrol
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen Receptor
E1	Estrone
E2	17β-estradiol
E3	Estriol
FACS	Fluorescence activated cell sorting
FADD	Fas-associated protein with death domain
FasL	Fas ligand
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehydes-3-phosphate
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor

GPER-1	G-protein coupled estrogen receptor-1
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
hDlg	Disks large
HER2	Human epidermal growth factor receptor 2
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRT	Hormone replacement therapy
Hsp90	Heat shock protein 90
IAP	Inhibitor of apoptosis protein
IF	Immunofluorscence
IGF-1	Insulin/insulin-like growth factor-1
JNK	c-Jun N-terminal
LCM	Laser confocal microscopy
LH	Luteinising hormone
LOH	Loss of heterozygosity
MAC	Mitochondrial apoptosis-induced channel
MAPK	Mitogen-activated kinase
MDB	Membrane desalting buffer
MDM2	Murine double minute 2
mER	Membrane bound ERα
MIBE	ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1Hindol-3-yl] but-2-enoate
miRNA	microRNA
mRNA	Messenger RNA
NLS	Nuclear localization signal
o,p'-DDT	Dichlorodiphenyltrichloroethane
P-AKT	Phosphorylated AKT
PALS1	Protein associated with lin-7 1
PAR3	Proteinase-activated receptor-3
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDGF	Platelet derived growth factor
P-ERK	Phosphorylated ERK
PFA	Paraformaldehyde
PhIP	2-amino-1-methyl-6-phenylimidazol [4,5-*b*] pyridine
PIP_2	Phosphatidylinositol diphosphate
PIP_3	Phosphatidylinositol triphosphate
PI3K	Phosphatidylinositol 3-kinases
PKA	Protein kinase A
PKC	Protein Kinase C
PLCβ	Phospholipase C β
PR	Progesterone receptor
PTB	Phosophotyrosine binding
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma
REST	Relative Expression Software Tool
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RTK	Receptor tyrosine kinase
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulfate
SERD	Selective estrogen receptor disrupter
SERM	Selective estrogen receptor modulator
SH2	Src-homology 2
siRNA	Small interfering RNA
SMAC	Second mitochondria-derived activatator of caspase
SMT	Somatic mutation theory
Sos	Son of sevenless
SRB	Sulforhodamine B

TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEB	Terminal end bud
TDLU	Terminal ductal lobuloalveolar unit
TGF-β	Transforming growth factor-β
Tm	Melting temperature
TMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor
TOFT	Tissue organization field theory
TRADD	Tumour necrosis factor receptor type 1-associated DEATH domain protein
TRAIL	TNF-related apoptosis-inducing ligand
UHQ	Ultra-high quality
VDAC	Voltage-dependent anion channel
ZO-1	Zonula occludens-1
3D	Three Dimensional

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CHAPTER I: Introduction: The Breast and Cancer

1.1 Epidemiology of Breast Cancer

Since the 1970's, breast cancer incidence rates have been on the rise and the chance of developing breast cancer in the United Kingdom stands at 1 in 9, making it the most commonly diagnosed cancer in women (Cancer Research UK, 2009). Despite improvements in detection and survival rates, breast cancer remains the most common cause of cancer-related deaths in women.

Breast cancer incidence rates can be influenced by many epidemiological characteristics such as age, genetic variation, familial history, ethnicity, socio-economic status, and geographical location. Increasing age correlates with increased breast cancer incidence (Kelsey & Gammon, 1991), and approximately 80% of cases occur in women aged over fifty years of age. The majority of cases in women aged 50 and over appear to coincide with the years leading up to menopause. After the age of 65, breast cancer incidence rates decrease and then increase again after the age of 70 (**Figure 1.1**). This dip in breast cancer incidence is known as "Clemmensen's hook" (Clemmensen, 1948) and could indicate two different forms of the disease being more prevalent at different ages. In fact, it has been demonstrated that the molecular characteristics of the cancers differ at each "peak" (Anderson *et al*, 2002; Kravchenko *et al*, 2011).

In 1988, a breast cancer screening programme was started in the UK for women between the ages of 50-64 and this is now being extended to women in their late 40's up until the age of 70. This has consequently led to higher incidence rates, as new cases are being detected, but has also had a positive effect on survival rates, as breast cancer is being diagnosed at earlier stages and treated accordingly (**Figure 1.2**).

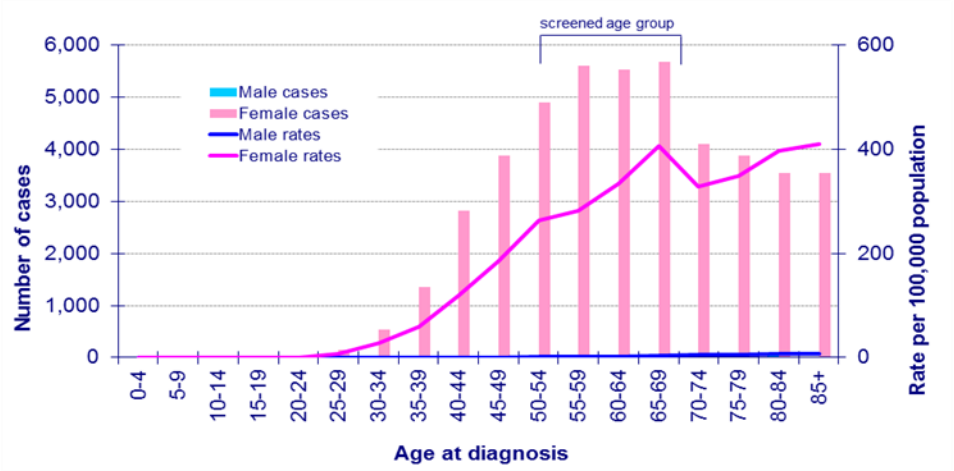

Figure 1.1: Age specific breast cancer incidence rates in the UK in 2006. Breast cancer incidence increases up until the ages of 65-69 and then falls, before gradually increasing again (Breast Cancer UK, 2009).

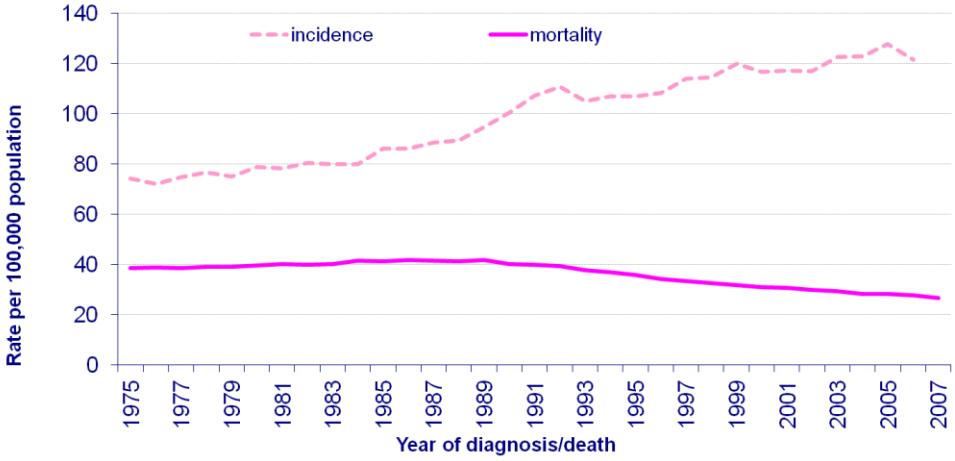

Figure 1.2: Age standardised breast cancer incidence and mortality rates in Europe. In the last 30 years, the incidence of breast cancer has increased and since 1989 the mortality rate of breast cancer has decreased (Cancer Research UK, 2009).

A family history of breast cancer has been linked to increased breast cancer risk in cases where the tumour suppressor genes, BRCA1 and BRCA2 are mutated and this accounts for 10% of all breast cancer cases (Castilla *et al*, 1994). Mutations in the BRCA1 and BRCA2 genes can result in an increase of breast cancer of up to 80% (van Garderen & Schalken, 2002). Other rarer genetic mutations can also increase an individual's risk of developing breast cancer, such as inherited mutations in p53 and phosphatase and

tensin homolog (PTEN). Familial history, where no inherited genetic mutation has been identified, also appears to increase the risk of breast cancer by 2 to 3-fold.

Caucasian women have a marginally increased risk of developing breast cancer compared to African women, although the mortality rate for African woman is higher. Geographical variation of breast cancer incidence exists, with more cases being diagnosed in developed western countries, such as the UK and North America and low incidence rates in Japan, India and Africa (**Figure 1.3**) (Cancer Research UK, 2008). However, in the countries of South America and Asia, breast cancer incidence rates are on the rise and it is thought to be due to the adoption of westernised lifestyles.

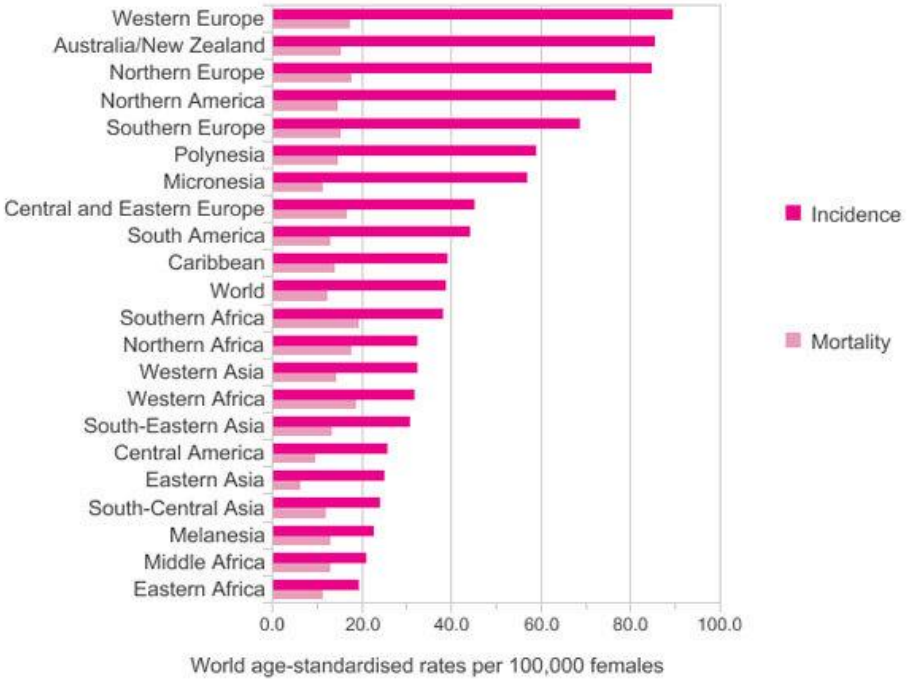

Figure 1.3: Incidence and mortality rates across the globe. The highest incident rates of breast cancer are found in western Europe. Mortality rates for breast cancer are also very high in western Europe with only southern and western Africa having a higher breast cancer mortality rate (Cancer Research UK, 2008).

Socio-economic variation also exists for breast cancer, although unlike many other cancers, it is the more affluent groups that show a higher incident rate and have a 20% increase in risk of developing the disease (National Cancer Intelligence network, 2008). This variation could be due to later first pregnancies and decreased parity in more affluent groups, two factors associated with increased breast cancer risk, as will be discussed next.

1.2 Risk Factors

Several risk factors, in addition to epidemiological characteristics, for developing breast cancer have been identified including: exposure to ionizing radiation, previous breast disease, age at menarche, age at first pregnancy (Kelsey *et al*, 1993), parity, age at menopause onset, breast density, smoking (Band *et al*, 2002), diet, obesity, alcohol consumption, use of hormone replacement therapy (HRT) (Beral *et al*, 1997), and the use of oral contraceptives (Pike *et al*, 1993).

Reproductive factors, such as age at menarche, first pregnancy and menopause, appear to have an impact on the risk of developing breast cancer. Early menarche can increase breast cancer incidence by up to 22% (Garcia-Closas *et al*, 2006) and breast cancer risk increases by approximately 3% a year for every delayed year of menopause (Collaborative Group on Hormonal Factors in Breast Cancer, 1997). On the other hand, age at first pregnancy and increased parity have a protective effect from breast cancer, with a risk reduction of 7% per pregnancy (Ma *et al,* 2006).

The use of HRT and to a much lesser extent, oral contraceptives, increases the risk of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 1997), with women who use HRT having up to a 66% increased risk of developing the disease (Beral, 2003). The link between oral contraceptive use and breast cancer remains of debate, but it has been shown that people using oral contraceptive have a slightly increased risk of breast cancer. Stopping their use reduces the risk back to levels seen in women who have never used oral contraceptives (Collaborative Group on Hormonal Factors in Breast Cancer, 1996).

In addition to reproductive and medicinal factors, various lifestyle factors can impact upon breast cancer risk. Post-menopausal women who are overweight have a 10-20% increased risk, with increasing body mass index (BMI) correlating with increased breast cancer risk (Reeves *et al,* 2007). Increased alcohol consumption also correlates with increased breast cancer risk, with each 10g (1 unit) a day alcohol consumption correlating to an increased relative risk of 12% (Allen *et al*, 2009). Smoking also increases a women's risk by 10-20% (Luo *et al*, 2011). Diet may also influence an individual's risk of developing breast cancer. People with a high fat intake have a 13% increased risk of breast cancer compared to people on a lower fat diet (Boyd *et al*, 2003). Phytoestrogen consumption may have a protective effect against breast cancer in Asian populations and a reduction in breast cancer risk by 15% in post-menopausal women with a high phytoestrogen intake has been observed (Velentzis *et al*, 2009; Dong & Qin, 2011), however, there are also studies that show no protective influence of phytoestrogens (Keinan-Boker *et al*, 2004; Hedelin *et al*, 2005).

Many of the risk factors described above contribute to an individual's cumulative lifetime exposure to the endogenous hormones, estrogens. Estrogens are physiologically important in females, where they function to induce reproductive tract and secondary sexual characteristics development, as well as regulate menstruation and reproductive processes. In addition to sexual differentiation, estrogens have an important role in the development and maintenance of bone tissue (Rickard *et al*, 1999), cardiovascular protection (Chambliss *et al*, 2010) and are capable decreasing circulating cholesterol levels (Liu & Bachmann, 1998). Paradoxically, estrogens have also been shown to have a role in breast cancer development and progression. This will be discussed in this chapter, after a brief revision of the breast development processes and morphology.

1.3 Breast Development

Development of the mammary gland is a highly complex and dynamic process, where proliferation, apoptosis and cellular differentiation all play essential roles that culminate in the formation of a network of milk ducts, which will eventually function to provide milk for breast feeding.

As the breast undergoes morphological development, lobule classification alters from type 1 to type 4 (**Figure 1.4**). Lobules classified as type 1 are present in the immature breast prior to the onset of puberty and are the least differentiated. Evolution of type 1 lobules forms type 2 lobules with increased differentiation and an increased number ducts. Type 3 lobules are present during pregnancy, in response to hormonal stimulation or following regression of type 4 lobules, after a cessation in lactation. Finally, during lactation, the lobules develop into type 4 and secrete milk proteins. Following menopause, regression of type 2 and type 3 lobules results in the presence of type 1 lobules, with both parous and nulliparous women predominately possessing type 1 lobules (Russo & Russo, 1994).

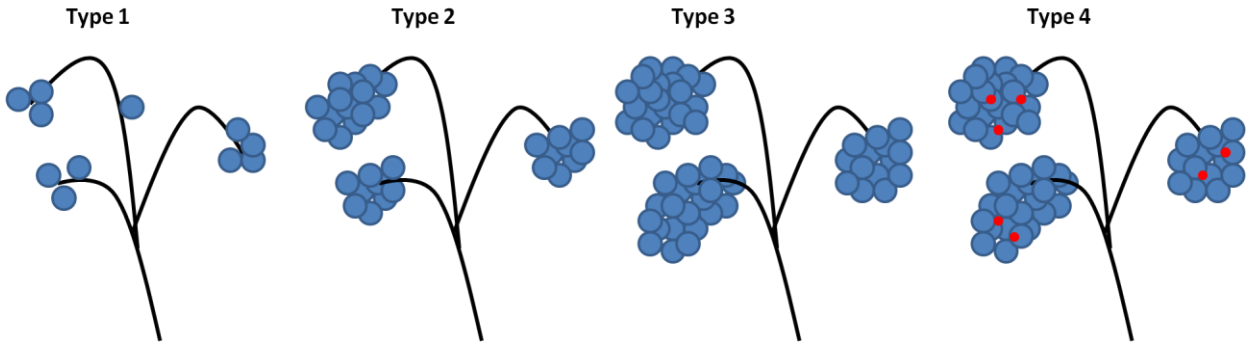

Figure 1.4: Lobule differentiation. During morphological development of the breast, the lobules progress from a Type 1 classification prior to the onset of puberty, to a Type 4 classification wherein milk is produced. Regression to a Type 3 classification then occurs as lactation ceases (Red notes milk production) (adapted from Russo & Russo, 1994).

Breast development begins at the embryonic stage, where cellular branching builds the foundations for the future glands and ducts. Between birth and the age of two, breast involution occurs resulting in a ductal system lacking the terminal end buds (TEBs) (Howard & Gusterson, 2000), structures comprised of epithelial cell layers.

Breast development then remains dormant until the onset of puberty, a period when the cells of the TEBs are most proliferative. Although the precise mechanism for breast development initiation remains unclear, it is believed to coincide with the activation of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axis. This activation results in a surge of follicle-stimulating hormone (FSH) from the pituitary, which in turn stimulates the secretion of estrogen from primordial follicles in the ovaries. Estrogen is then able to circulate around the body and induce cellular proliferation, elongation and branching of the mammary ducts.

During the pubertal development stage of the breast, a network of between 10 and 15 branching ducts elongate and develop from the nipple. These transverse through the mammary gland terminating in TEBs, which are specialized structures responsible for driving the growth of the ductal network (Anbazhagen *et al*, 1998). The cells of the TEBs will eventually give rise to both the luminal and basal cell layers of the ducts. It is hypothesised that within these terminal end buds are populations of stem cells. These stem cells are thought to play a pivotal role in normal breast development and breast cancer initiation (Russo & Russo, 1996). In addition, the terminal buds exhibit the highest proliferative activity, highest estrogen receptor (ER) abundance and highest carcinogen-DNA binding rate of that found in the breast (Russo & Russo, 2008). Each duct is associated with a specific breast lobule and

these lobules are separated from one another by connective tissue, in which nerves, blood vessels and lymphatics are located.

The ductal network develops further, resulting in the formation of terminal ductal lobuloalveolar units (TDLUs) consisting of ducts and acini. At this stage, the lobules of the breast are of the classification type 1. Following pregnancy and during lactation these structures will become the functional milk-secretory units (**Figure 1.5**) where they are of the classification type 3 and 4.

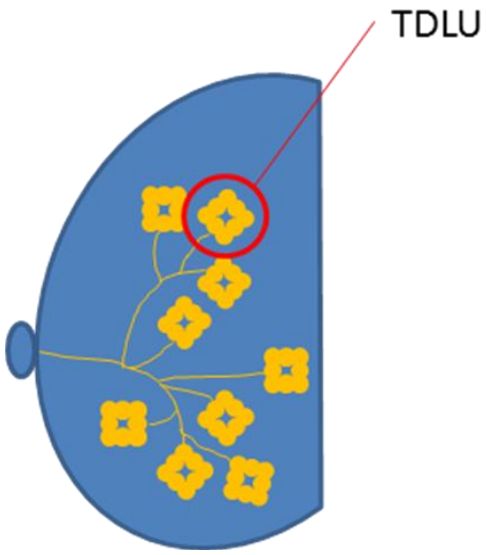

Figure 1.5: The structure of the breast. The breast consists of a highly branched ductal network extending from the nipple and terminating in TDLUs.

Further maturation of the breast occurs during pregnancy and lactation. During this time, TDLUs, in the presence of hormones, develop further until the fully mature breast is comprised of a network of milk ducts terminating in spherical milk-producing glands, termed acini (Russo & Russo, 1998). Once offspring have been weaned, involution causes mammary epithelial cell death and the regression of the TDLUs. These cells are replaced by adipose tissue and mesenchyme that function to support breast lobules (Russo & Russo, 1993). The mammary gland, however, remains more differentiated than that present in nulliparous women (Cardiff & Wellings, 1999).

The acini of the TDLU are highly organised structures (**Figure 1.6**), comprising of a basement membrane which encapsulates a layer of myoepithelial cells, that surrounds a layer of epithelial cells, which in turn surround a hollow lumen. Surrounding the entire structure is the stroma, comprised of fibroblasts, immune cells, adipocytes, blood vessels, lymphatic vessels and extracellular matrix (ECM).

The basement membrane of acini functions to provide mechanical support and to generate signals for cell survival to the cell attached to it, via transmembrane receptors called integrins. At the centre of the structure is a hollow lumen, the size of which is dependent on whether the acini is in a state of rest, in which case the lumen is small, or is secreting milk, in which case the lumen is much larger (Plachot *et al*, 2009).

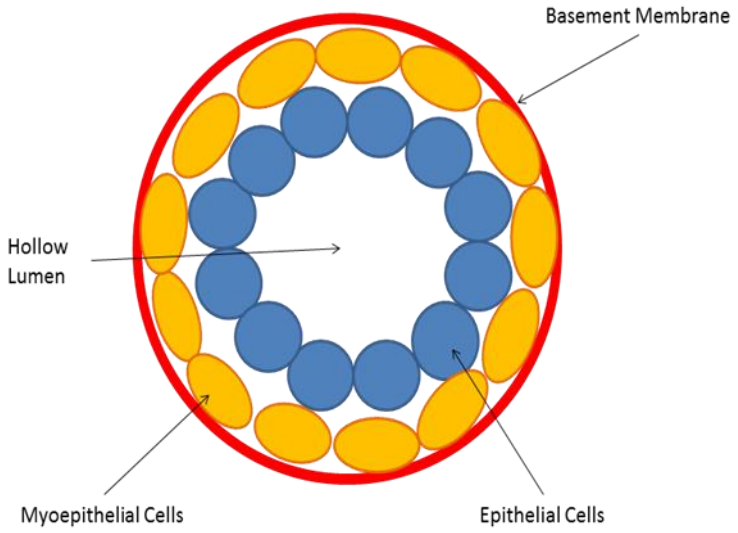

Figure 1.6: *In vivo* structure of a TDLU. A basement membrane encapsulates an acinus of a TDLU. Within this structure is a layer of myoepithelial cells and a layer of epithelial cells, surrounding a central hollow lumen.

The myoepithelial and epithelial cells of acini possess cell-cell contacts with neighbouring cells, via adhesion proteins, such as E-cadherin and β-catenin (Debnath *et al*, 2002), and have basal polarisation of α6 integrin, collagen IV and laminin V. Collagen IV and laminin V are basement membrane components that are secreted by cells. Laminin via its integrin receptor is involved in ECM attachment, polarization differentiation and morphogenesis *in vivo* (Rodriguez-Boulan & Nelson, 1989; Sorokin *et al*, 1990). Collagen IV provides structural support to the basement membrane (Pöschl *et al*, 2004). Disks large (hDlg) and the Golgi apparatus are apically located in these polarised cells (Debnath *et al*, 2003) and function in the establishment of polarity and protein modification respectively. Work in three-dimensional (3D) cultures has demonstrated that acini initially form as complete spheres of cells and the hollow lumen is achieved and maintained through caspase-mediated apoptosis, autophagy and anoikis of the centrally located cells (Mills *et al*, 2004). The formation of the TDLUs *in vivo*, occurs in a very similar fashion to that observed in 3D *in vitro* models, in that the TDLUs form initially as filled structures,

and then the central cells undergo apoptosis and are cleared to form a hollow lumen (Humphreys *et al*, 1996; Mailleux *et al*, 2007). However, the exact mechanism for this i.e. whether the outer cells receive pro-survival signals or are protected from death signals, remains unclear.

1.4 Breast Cancer

Factors like genetic aberrations and epigenetic changes can result in tumour development in which losses or gains of function of various proteins, collectively induces alterations in cell behaviour. These alterations have been classified as "The Hallmarks of Cancer" by Hanahan and Weinberg (2000) who propose six criteria that are auspicious for tumour growth: enhanced proliferative potential, resistance to apoptosis, independent growth signal production, resistance to inhibitory signals, angiogenic potential and the capacity to migrate and invade surrounding tissues. Given that the breast undergoes extensive morphological changes through a women's lifetime, due to a vast array of proliferative and hormonal influences, events like mutations and epigenetic modifications often target the cells of the mammary gland.

1.4.1 Breast Cancer Subtypes

Breast cancers arising from mesenchymal tissues are known as sarcomas, whereas the more common form of cancer, carcinoma, arises from epithelial tissues. Specifically, when the cancer is derived from glandular epithelial tissue, it is referred to as an adenocarcinoma. Amongst breast malignancies, tumours arising in the TDLUs are the most common (Wellings *et al*, 1975; Petersen *et al*, 2003).

Various subtypes based on the molecular profile have been identified by Perou *et al* (2000). These finding were bases on the expression of the estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2) and whether tumours were basal-like or luminal-like. Luminal-like tumours tend to express the ER and genes associated with luminal and glandular cells, whereas basal-like tumours tended to lack ER expression and express genes more associated with myoepithelial cells. Further work demonstrated that the luminal sub-type could be further characterized into two groups: luminal A (HER2-) and luminal B (HER-2+) (Sorlie *et al*, 2003). More recent studies have further charterized the sub-types of breast cancer. After determining whether a tumour is luminal or non-luminal and confirming the HER2 status, tumours can be further cauterized depending on whether they express basal markers (**Figure 1.7**) (Blows *et al*, 2010).

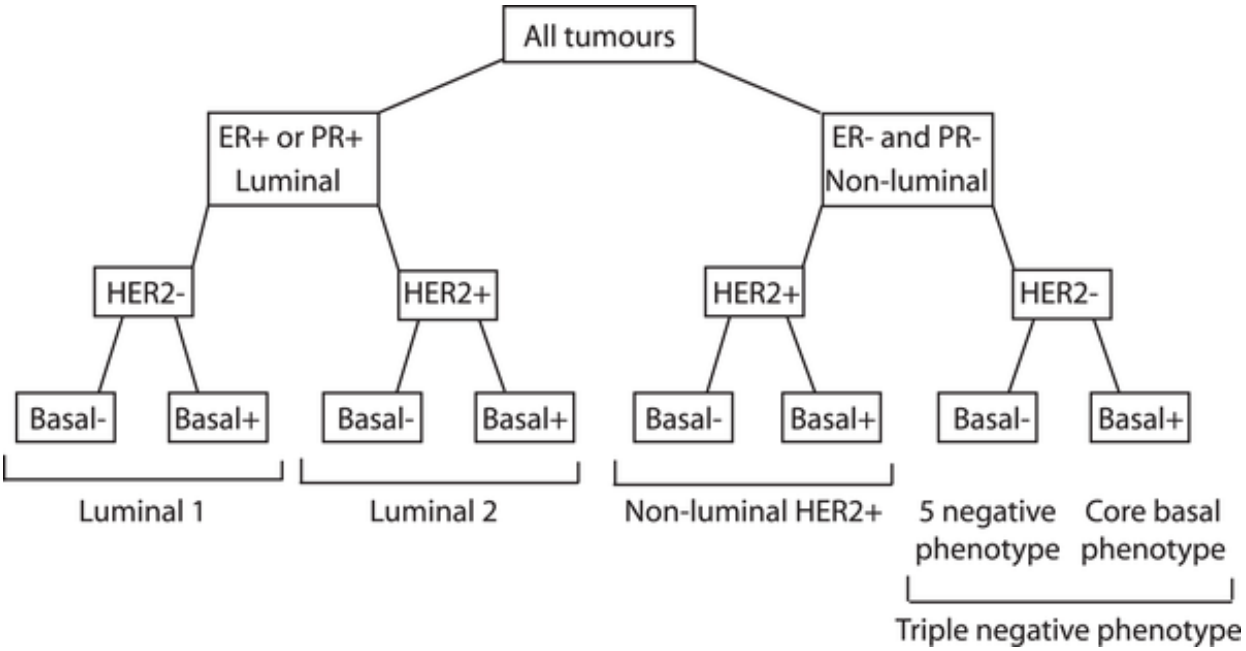

Figure 1.7: Characterization of breast cancer. Luminal-like tumours were divided into HER2- (luminal 1 which is roughly equilivant to luminal A) and HER2+ (luminal 2 which is actually a subset of luminal B classified tumours). Non-luminal-like tumours were divided into two sub-groups based on HER2 expression. All sub-groups were then characterized based on the expression on basal markers such as cytokeratin 5/6 (CK5/6) and epidermal growth factor receptor (EGFR). Image taken from Blows *et al*, 2010)

Of these subtypes, the luminal A subtype is the most prevalent and has the best prognosis, and tumours of this class tend to have be smaller, well differentiated and there is less likely to be lymph node involvement. Triple negative subtypes appear to present as larger tumours, be poorly differentiated and have the poorest prognosis (Onitilo *et al*, 2009).

Luminal breast cancer subtypes tend to express higher expression levels of ERα and cytokeratins or HER2. HER2 is a commonly amplified oncogene in breast cancer that is related to EGFR. It is a receptor tyrosine kinase that is over-expressed in 20-30% of metastatic breast cancer samples, and in patients this correlates with a poorer prognosis.

The basal-like subtype tends to express cytokeritin 5/6/14/17, p-cadherin, caveolin-1 and p53 and frequently lacks ER, PR and HER2 (Li *et al*, 2002; van der Rijn *et al*, 2002). This subset is usually very aggressive in patients and presents with a high histological grade.

1.4.2 Models of Breast Carcinogenesis

As with any malignancy, tumours of the breast possess distinctive hallmarks of cancer, such as invasiveness, metastatic ability, and can ultimately cause death in patients. Advances in cancer research over recent decades have shed light on to the causes of cancers and suggested that mutations involving the loss of tumour suppressor genes and overexpression of oncogenes are the foundations of cancer initiation and progression. The initiation and progression of breast cancer is no different and arises as a result of genetic mutation accumulation, increased proliferation and disruption of tissue architecture, although more recently theories involving tissue organisation, epigenetic modification and aneuploidy have been proposed for the initiation and progression of cancer.

The somatic mutation theory (SMT) is based on the assumption that cancer arises from DNA mutations within cells, which, if unrepaired, lead to either loss or gain of the affected proteins. These mutations accumulate over time, affecting cellular proliferation, survival, differentiation, migration and apoptosis and, eventually, induce a cancer phenotype (**Figure 1.8**). Two models for SMT carcinogenesis and progression have been hypothesised: a linear and non-linear model. The linear model is based on the theory that the cell of origin of the cancer is the same, regardless of tumour subtype. Here, mutations in genes involved in proliferation and survival accumulate and only then the subtype, is determined. The basis for alternative the non-linear model of breast carcinogenesis is that each breast cancer subtype has a different cell of origin (Jeselsohn *et al*, 2010).

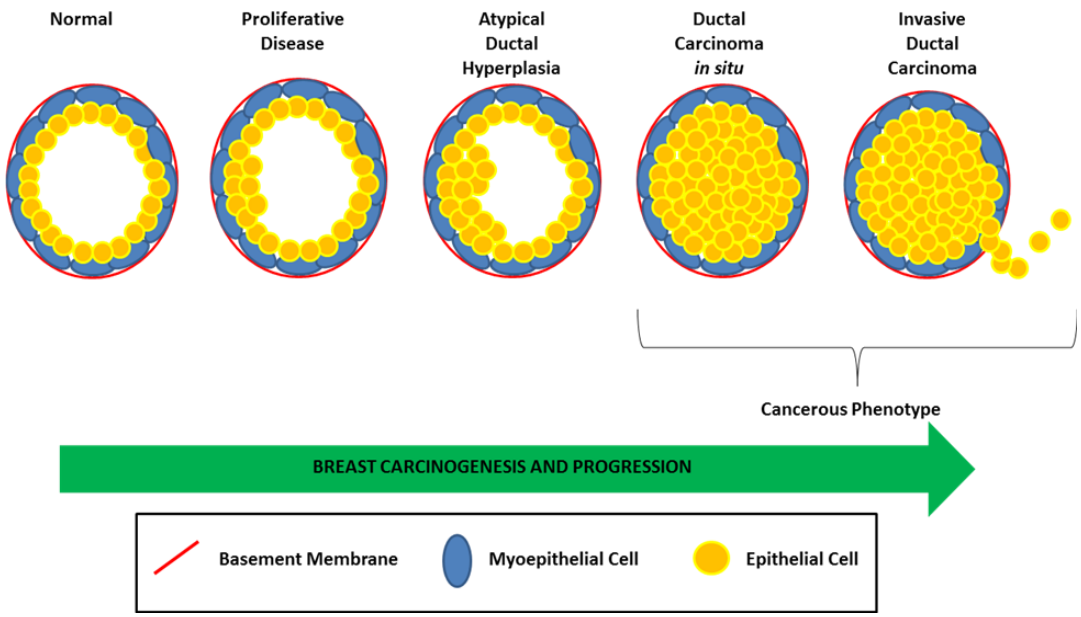

Figure 1.8: The progression to invasive ductal carcinoma. The progression to an invasive ductal carcinoma from a normal state occurs as a consequence of over-proliferation and apoptotic suppression (adapted from Myal *et al*, 2010).

More recently, the tissue organization field theory (TOFT) has been proposed. In this model, carcinogenesis occurs as a result of tissue disorganization, increased proliferation and inter-cellular communication. These alterations to the microenvironment result in the loss of control of tissue organisation upon cellular proliferation and survival, and, as a consequence, cells are able to proliferate, migrate and evade apoptosis (Soto & Sonnenschein, 1991; Soto & Sonnenschein, 2011). In this context, the presence of mutations is random and does not occur in every cell of the tumour.

1.5 Estrogens

Estrogens, such as 17β-estradiol (E2), estriol (E3) and estrone (E1), are a family of steroid hormones that promote proliferation of both normal and cancerous cells. They function as the primary sex hormone in females and are, hence, involved in a variety of physiological processes, such as the menstrual cycle and breast acini development. Estrogens are also involved in cardiovascular and nervous system function and the regulation of bone remodelling (Venkov *et al*, 1996; Sims *et al*, 2002; Maggi *et al*, 2004; Kim *et al*, 2010). However, estrogens may also have a detrimental effect on health and have been implicated in breast cancer development by both experimental and epidemiological data.

Estrogen synthesis is regulated by follicle stimulating hormone (FSH) and luteinising hormone (LH), which are both secreted from the anterior pituitary gland (**Figure 1.9**). Upon stimulation by these hormones, structures such as the ovaries, corpus luteum, placenta and adrenal cortex synthesise estrogen. The majority of this synthesis occurs in the ovaries and begins with synthesis of the precursor, progesterone from pregnolone, a derivative of cholesterol. The most abundantly synthesised estrogen is E2 although E1 and E3 are also formed. Biologically active estrogens account for just 5% of the total amount of circulating estrogens. The remainder is bound to plasma proteins, such as sex hormone binding globulin (SHBG) and albumin.

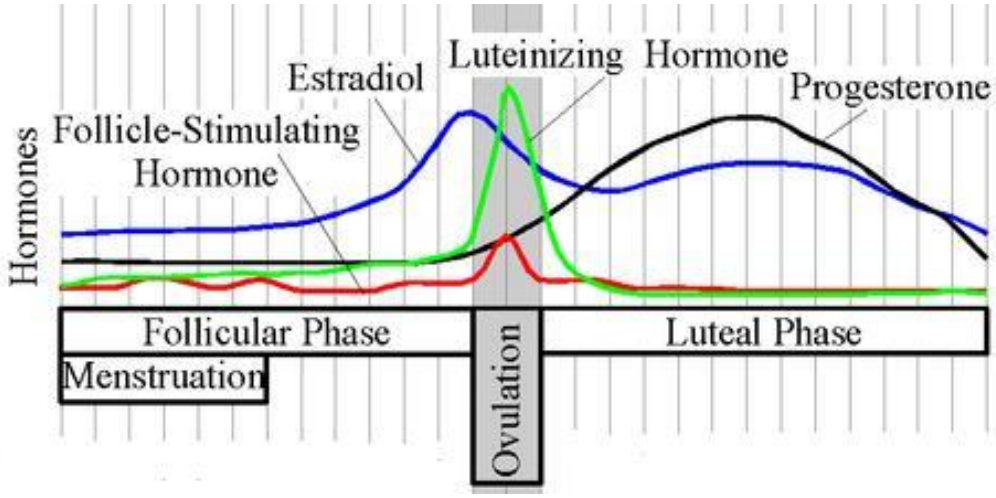

Figure 1.9: The ovarian cycle. E2 levels increase prior to ovulation and then decrease following the release of an egg from the ovaries, although a slightly higher level of E2 exists to maintain uterus lining until menstruation (Image from Psychology Wiki http://psychology.wikia.com/wiki/File:Estradiol.Cycle.JPG)

Natural estrogens, such as E2 and E1, undergo metabolism and conjugation in the liver to less potent derivatives. Conjugation reactions, such as glucuronidation, sulfonation and *O*-methylation results in increased water solubility, allowing the derivatives to be more easily excreted in bile or urine.

1.5.1 Endogenous Estrogens and Breast Cancer

Many of the risk factors associated with breast cancer contribute to an individual's cumulative lifetime exposure to the endogenous hormone, estrogen and, therefore, this increased cumulative estrogen exposure is hypothesised to contribute to increased breast cancer risk. Both clinical and animal studies have reinforced this idea.

Early menarche, late menopause, late age at first pregnancy, increased weight or the use of HRT post-menopause have all been linked to an increase in the risk of developing breast cancer (Kelsey *et al*, 1993). Breast cancer risk for those with early onset of menarche, and, hence, greater lifetime estrogen exposure, is 1.2-2.2 times greater than individuals with a later menarche onset (Titrus-Ernstoff *et al*, 1998). Similarly, late onset of menopause, again contributing to increased estrogen exposure, correlates with an increased risk of breast cancer development. Statistically, women in which menopause onset occurs before 45 have half the risk of breast cancer malignancy than women where onset occurs after the age of 55 (Trichopoulos *et al*, 1972).

Another factor believed to be responsible for higher exposure to endogenous estrogens is obesity. This has been demonstrated, in post-menopausal women, to also contribute towards breast cancer risk (Le Marchand *et al*, 1988), although in pre-menopausal women, obesity may provide some level of protection against pre-menopausal breast cancer (Verla-Tebit & Chang-Claude, 2005). The increase in breast cancer risk in obese post-menopausal women could be attributed to increased estrogen production in adipose tissues, thus, linking increased body weight with estrogen exposure. The reasoning as to why increased weight has a protective effect against breast cancer in early life remains unclear, although it is hypothesised that obese women with abnormal ovulatory cycles have a reduced estrogen exposure (Pike *et al*, 1993).

Animal studies have substantiated the hypothesised link between exposure to ovarian hormones and breast cancer: administration of estrogens has been shown to promote mammary carcinogenesis, an effect that can be reversed by ovariectomy (Mackenzie, 1955) or co-administration of estrogen with estrogen antagonists (Dao, 1981). Ovariectomy has also been observed to decrease the risk of both ER positive (Press *et al*, 2011) and ER negative breast cancers in women carrying *BRCA1* mutation (Rebbeck *et al*, 1999). Further to *in vivo* studies, epidemiological studies have shown that breast cancer patients exhibit higher estrogen serum and urine levels in comparison to their healthy counterparts (Key & Pike, 1988; Toniolo *et al*, 1995; Dorgan *et al*, 1996). A role for estrogens in the initiation of cancers other than that of the breast, such as the kidney and liver, has also been demonstrated in animal models (Li *et al*, 1995; reviewed in Yager, 2000).

Interestingly, early first pregnancies in life and increased parity, which would increase estrogen exposure, have been found to have a protective effect against breast cancer, with parity conferring a 25% reduction in the risk of breast cancer compared to nulliparous women (Kelsey *et al*, 1993; Lambe *et al*, 1996; Hinkula *et al*, 2001; Sivaraman & Medina, 2002; Ma *et al*, 2010). This protective effect

however, is limited to ER positive breast cancers (Ma *et al*, 2006) and is not immediate. In fact, a comparison of uniparous and nulliparous women has shown that uniparous women have an elevated breast cancer risk for approximately 10 years following delivery, with more notable increases in risk in women aged 30 years or over at the time of delivery (Lambe *et al*, 1994; Schedin, 2006).

Many hypothesises have been put forwarded to explain this. The protective effect of pregnancy may be mediated by changes in the ratios of circulating estradiol, prolactin and growth hormone (Henderson & Feigelson, 2000). It has also been proposed that the hormone surge during pregnancy induces changes in the stem cells of the terminal end buds of the breast ducts, resulting in these cells to differentiate, proliferate and respond to DNA damage. Ultimately, these modified stem cells may be able to repair DNA damage and metabolise carcinogens unlike their unmodified counterparts in nulliparous females (Russo & Russo, 1997). Similarly, the differentiation of breast epithelial cells and breast morphology may render cells less susceptible to carcinogenesis (Britt *et al*, 2007). Comparisons of breast morphology of nulliparous with women who had undergone at least one full-term pregnancy, have shown that pregnancy causes maturation of breast lobules, resulting in increased alveoli and a decrease in the percentage of proliferating cells (Russo *et al*, 1994; Russo & Russo, 1994; Russo *et al*, 2006). This decrease in the percentage of proliferating cells could, therefore, reduce the probability of mutations occurring and, consequently, of a cancerous phenotype.

It has also been hypothesised that *in utero* or early postnatal hormone exposure is the determining factor as to whether breast epithelial cells proliferate in response to estrogen or are estrogen independent in terms of proliferation (Trichopoulos ,1990; Nandi *et al*, 1995). This idea is supported by epidemiological observations which demonstrated that factors associated with increased estrogen, increased size of foetus, obesity and mother's age, result in increased breast cancer risk of female offspring (Thompson & Janerich, 1990; Ekbom *et al*, 1992; Vatten *et al*, 2002, Park *et al*, 2008). With pre-eclampsia, a condition associated with decreased estrogen levels, the risk of female offspring developing breast cancer appears to be reduced (Ekbom *et al*, 1992). Animal studies further support this theory as in rats increased estrogen exposure *in utero* leads to an increase in the number of TEB in the mammary gland (Hilakivi-Clarke *et al*, 1997) and it is these structures that have been identified as the structures from which mammary tumours arise (Russo & Russo, 1987; Russo *et al*, 1990).

1.5.2 Exogenous Estrogens and Breast Cancer

Exogenous estrogens, such as pharmaceutics, may also have implications for breast cancer onset. Exogenous estrogens are commonly administered in the form of oral contraceptives, hormone replacement therapy (HRT) and drugs to prevent miscarriage and, overall, their use has increased over recent years.

Statistically, a third of women in the UK between the ages of 50 and 64 have used HRT to treat the symptoms associated with menopause since the 1990's. However, the dramatic effects of its use only became apparent when a very large epidemiological study (The Women's Health Initiative study) in 2002 was prematurely stopped after a correlation was observed between combined estrogen-progesterone HRT use and an increase in breast cancer development amongst the women enrolled in the study (Writing Group for the Women's Health Initiative Investigators, 2002, 2003). The study also showed that estrogen-only HRT decreased the risk of breast cancer, however another similar study has shown the opposite (Million Women Study Collaborators, 2003). Meta-analysis conducted for HRT studies has shown that there is a positive correlation between estrogen-only HRT use and breast cancer (Greiser *et al*, 2005).

Incidence rates of breast cancer according to age has been increasing in women over the age of 40 (**Figure 1.10**). Although not the only factor in breast cancer incidence, the incidence rate in age groups over 40 years old does correspond to the age at which HRT is administered. This observation must however be treated with caution as other factors, such as breast cancer screening may play a role. Due to the outcomes of the Million Women and the Women's Health Initiative studies, HRT use since 2000 has decreased significantly. It now remains to be seen whether this will coincide with a decrease in breast cancer incidence.

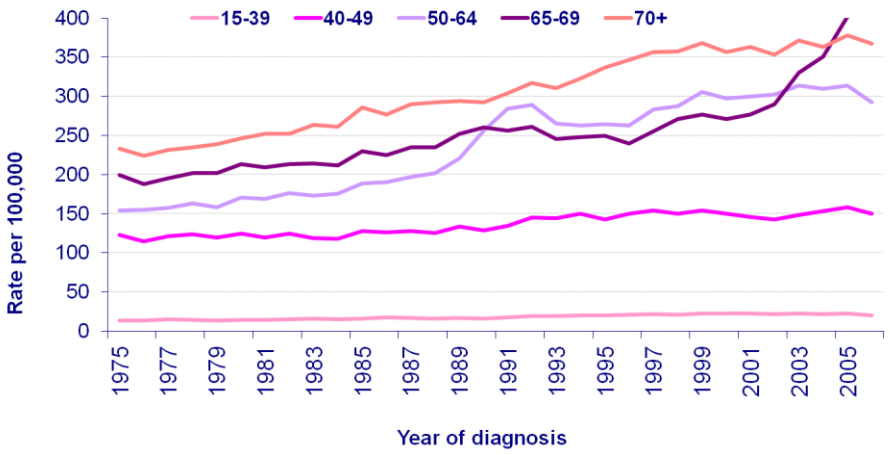

Figure 1.10: Age-specific breast cancer incidence rates in the UK. The increase in breast cancer incidence in 40-49 year olds corresponds to the increase in HRT use in the 1990's (Breast Cancer UK, 2009).

Although the link between HRT use and cancer risk is substantiated by most reports, the link between oral contraceptive use and breast cancer risk is less clear and still the cause of much debate. Many studies have found no correlation between the two (Marchbanks *et al*, 2002), however, several studies have identified a possible risk in women who use oral contraceptives for several years at younger ages, prior to first pregnancy (Olsson *et al*, 1989), after the age of 45, (Hennekens *et al*, 1984) and nulliparous women with early onset of menarche (Stradel *et al*, 1988). Other studies have found that women using the pill have a slight, but insignificant, increased risk of developing breast cancer and this risk returns to that of non-users after stopping the use of oral contraceptives (Collaborative Group Study, 1996).

Diethylstilbestrol, (DES), a synthetic estrogen and a drug prescribed to pregnant women between the 1940's and 1970's, has been associated with breast, vagina, and cervical cancer development in the daughters of mothers who took it (Palmer *et al*, 2006). This highlights that pre-natal exposure to estrogenic compounds may impact upon offspring later in life. However given that these individuals are only just reaching their 50s, the true impact of DES exposure during development remains to be seen. Further to this, the mothers prescribed DES, have experienced a 30% higher rate of breast cancer incidence (Greenberg *et al*, 1984; Colton *et al*, 1993; National Cancer Institute, 1999).

1.5.3 Xenoestrogens and Cancer

Since World War II, a multitude of chemicals have been synthesised and incorporated into people's daily lives such, as pesticides, preservatives and other industrial products. These chemicals were intended to

improve living standards, however, at the time, their endocrine disrupting properties were not known. Following evidence linking xenoestrogen exposure and reproductive health in wildlife, the endocrine disrupting properties of these compounds came to light. The impact on wildlife exposed to xenoestrogens, such as *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT) and dichlorodiphenyldichloroethylene (DDE), became clear, as population declines in a variety of species (including alligators, bald-eagles and marine birds) were observed and reproductive disorders reported (Colborn *et al*, 1993; Guillette Jr. *et al*, 1995; 1996). These observations have also been reproduced in controlled *in vivo* studies involving a variety of laboratory models, such as rat models (Fusani *et al*, 2007).

Endocrine disruptors have been defined as exogenous substances that alter functions of the endocrine system and, consequently, cause detrimental health effects in an organism, its progeny, or within populations. Such adverse effects can include cancer, birth defects and developmental disorders. The commonality amongst those chemicals that disrupt the endocrine system by mimicking the action of estrogenic hormones (so-called xenoestrogens) is the presence of a 4-OH group on the lipophillic phenyl A ring with a hydrophobic moiety present at the 2-position of propane (**Figure 1.11**) (Kitamura *et al*, 2005).

Based on the endocrine disrupting properties of environmental chemicals, it has been hypothesised that an individual's lifetime exposure to estrogens may be further increased by the presence of these pollutants, many of which exhibit estrogenic properties. It has been argued that the increased prevalence of breast cancer in developed countries could be attributed to the increased presence of endocrine disrupting chemicals.

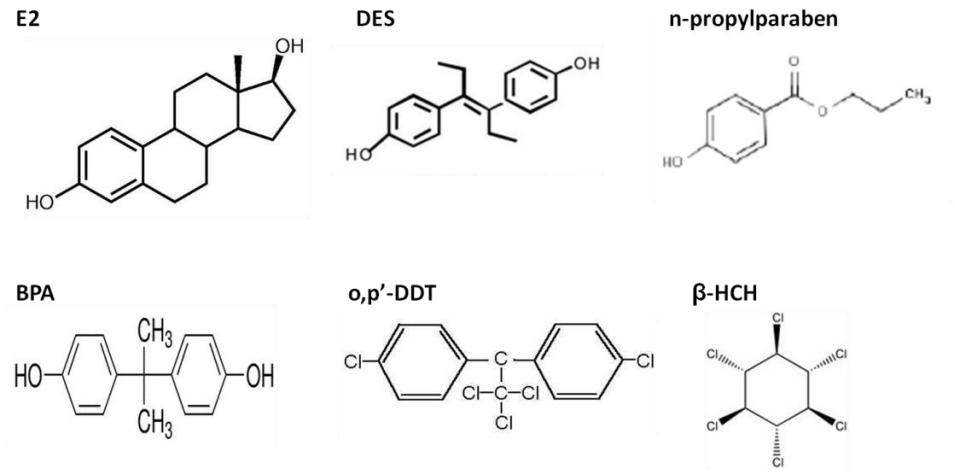

Figure 1.11: The chemical structures of E2 and various estrogens. Estrogenic compounds containing a phenyl ring and with structurally similarity to endogenous E2 are able to elicit responses through the ER.

As mentioned above, xenoestrogens are those endocrine disruptors (either synthetic or naturally occurring) that exhibit estrogen-like activities through interaction with the ER. However, it remains unclear whether these endocrine disrupters are able to elicit carcinogenic effects. Contact with xenoestrogens in everyday life is common, as many are found in cosmetics (parabens and phthalates) or in the case of butyl benzyl phthalate (BBP) and bisphenol A (BPA), food packaging. The latter of these is of current interest as it leaches from food packaging, such as water bottles (Brotons *et al*, 1995; Le *et al*, 2008) and the lining of metallic food containers (Vandenberg *et al*, 2007), and into food products. Worryingly, until recently, BPA was also present in feeding bottles and equipment for babies, and there are concerns over the impact this may have (Maragou *et al*, 2008). It can also be absorbed through the skin and has been found in 95% of urinary samples in an American survey (Calafat *et al*, 2005).

In regards to its estrogenic activity, BPA has been shown to bind to the ER and induce proliferation in MCF-7 cells, and in *in vivo* studies in ovariectomized mice, BPA exposure led to increased uterine weight (Krishnan *et al*, 1993; Papaconstantinou *et al*, 2000). More recent studies have focused on the link between xenoestrogens and carcinogenesis. In one such study, BPA treatment of MCF-10F cells (a non-tumourigenic mammary epithelial cell line) resulted in increased expression in genes involved in DNA repair, such as *BRCA1* and *BRCA2*. BPA also down-regulated the expression of the pro-apoptotic gene *BIM* and induced hypermethylation of various other genes involved in cancer initiation and progression (Fernandez *et al*, 2012). Further to this, epidemiological studies have found a positive correlation between BPA levels in urine and the development of meningioma, a tumour associated with

the nervous system (Duan *et al*, 2012). Phthalates (chemicals used in the manufacture of plastics with endocrine-disrupting properties) have also been demonstrated to exhibit properties that are involved in the progression of liver cancer in rats (Rusyn *et al*, 2006). Overall, these studies, and many more besides, have shown a link between the exposure to individual estrogenic compounds, present in our environment, and the initiation, and progression of cancer,

1.6 Estrogens and the Estrogen Receptor

In approximately 70% of cases, breast cancers are ER positive and hormone dependent at some point during their development (Masood, 1992; Musgrove & Sutherland, 2009)). The presence, or indeed absence, of the ER is used to assess the prognosis of a patient and to establish the most suitable form of treatment. ER expression is a characteristic of many breast cancers, which is exploited during treatment with anti-estrogens such as tamoxifen and fulvestrant (also known as ICI 182,780). Unfortunately, 25% of patients are unresponsive to tamoxifen (the most common form of treatment) and in addition, many patients develop acquired hormone independence. For this reason, it is importance to investigate the development of hormone-dependent cancer of the breast, and to more thoroughly understand the mechanisms of action exerted by estrogens. In doing this, we, may in the future, be able to identify new targets for cancer therapy, which would result in a better outcome for patients.

Estrogenic action is mediated by the nuclear receptors ER α and β. ERα and ERβ are encoded for by separate genes on different chromosomes, but do still share significant sequence homology. ERα is encoded for by the ESR1 gene and ERβ by ESR2 on chromosomes 6 and 14, respectively. They are composed of five domains denominated A-F (**Figure 1.12**). At the N-terminal are the A and B domains, which together make up the transcriptional activation function (AF-1) region. This is the region of the receptor that can activate transcription of target genes in the absence of bound estrogen, following its activation by signalling pathways in response to growth factors (Bunone *et al*, 1996). This transcriptional activation, however, is comparatively weak to that activated when estrogen is bound to the receptor. The C domain is the region of the receptor that actually binds to the estrogen response elements (EREs) located in target genes. The D domain acts as a hinge connecting the C domain to the E domain, the latter being the region where estrogen binds to activate gene transcription. This hinged domain allows the receptor to undergo conformational changes upon ligand binding. Also, it is the location of nuclear localization signal (NLS) that permits activated receptor entry into the nucleus. The E domain, in addition to acting as a binding site for estrogen, also makes up the ligand-inducible

transcription activation function (AF-2) region where co-activators and co-repressor proteins can bind and has a dimerization interface for interaction with other ERs. Finally at the C-terminal is the F domain although the function of this domain remains to be discovered.

Figure 1.12: The structure of ERα, consisting of five domains. Ligand binding occurs in the E domain and the C domain is responsible for binding of the ER to the ERE. The A/B domain contains the AF-1 region, which is responsible for the transcriptional activation of estrogen-responsive genes. The F domain contains the AF-2 region that allows association with co-regulatory proteins required for the activation or cessation of transcription.

Within the structure of ERα and ERβ, the greatest sequence homology is located within the DNA-binding domain, where the ER proteins are 96% similar and, hence, binding to the ERE of target genes is identical for the two subtypes. As the sequence homology of the other regions ranges between 30 and 54%, association with Sp-1 or Ap-1 (factors that enable activated ER to interact with genes lacking an ERE) is different for ERα and ERβ and hence the transcription of some genes is exclusive to one receptor form and more intriguingly, the same ligand can elicit both agonistic and antagonistic effects depending on which ER form it binds to (reviewed in Katzenellenbogen & Katzenellenbogen, 2000). The sequence homology in the ligand binding domain does not impact greatly upon the binding of estrogens and estrogen antagonists but does, however, have an effect on phytoestrogen binding affinities, meaning that these bind to ERβ in preference in relation to ERα (Kuiper *et al*, 1998). Despite significant sequence homology between ERα and ERβ, the receptors display different tissue distribution patterns and, possibly, mediate different effects. ERβ expression is also often down-regulated in cancer (Bardin *et al*, 2004; Ström *et al*, 2004), whereas ERα expression is often increased in cancer patients (Holst *et al*, 2007).

In recent years, a membrane associated receptor for estrogen has been widely debated. Various groups propose differing views as to the exact location and structure of this receptor, with the main trains of thought eluding it to be: a membrane bound ERα (mER) (Pappas *et al*, 1995; Watson *et al*, 1999; Powell *et al*, 2001; Zivadinovic & Watson, 2004; Kim *et al*, 2008), a G-protein-coupled receptor, G-protein coupled estrogen receptor 1 (GPER-1, formally known as GPR30) (Filardo *et al*, 2002; Razandi

et al, 2003; Kumar *et al*, 2007) or a cooperative signalling through a membrane associated ERα and G-proteins (Wyckoff *et al*, 2001).

A number of studies have shown that the classical ERα can reside in the plasma membrane, although only to a level 2-3% of that in the nucleus, and that the effects of E2 mediated by this membrane ER are reversed by anti-estrogens, such as ICI 182,680 (Kim *et al*, 1999; Razandi *et al*, 1999; Haynes *et al*, 2000; Russell et *al*, 2000). Treatment of MCF-7 cells has been shown to result in the translocation of ERα to the membrane, where it resides in caveolae, small cholesterol-rich invaginations of the plasma membrane (Levin, 2002; Razandi *et al*, 2002; Figtree *et al*, 2003; Acconcia *et al*, 2004; Zivadinovic & Watson, 2004; Acconcia *et al*, 2005), or interacts through its N-terminal region with adapter proteins containing SH2 domains, such as Src (Pelicci *et al*, 1996; Song *et al*, 2002; Song *et al*, 2004). As ERα does not contain any hydrophobic domains capable of expanding the cell membrane, the receptor must be modified or associate with other proteins in order to localize at the cell membrane. Various mechanisms have been identified for the association of the ER with the plasma membrane, such as post-translational palmitoylation of ER's ligand binding domain (Acconcia *et al*, 2005) and association of the ER with caveolin-1 (Pedram *et al*, 2007). Caveolin-1 is a structural component of the caveolae and functions to recruit components of the signalling pathway at all levels such as receptors, G-proteins, Src kinases and phosphatidylinositol 3-kinases (PI3K) (Levin & Pietras, 2008). These ER associations do not occur in the absence of estrogens and are disrupted by anti-estrogen treatment or small interfering RNA (siRNA) against ERα (Song *et al*, 2004).

In addition to the classical form of the ERα, truncated forms of this receptor have been identified in the plasma membrane, which are capable of mediating the non-genomic effects of estrogens (Flouriot *et al*, 2000; Li *et al*, 2003; Wang *et al*, 2005; Wang *et al*, 2006; Kim *et al*, 2008). The 46 kDa isoform of 66 kDa ERα lacks 173 amino-acids present at the N-terminus, contains no A/B domain and is encoded by the ERα gene lacking exon 1A. It can also mediate non-genomic estrogenic actions in addition to forming heterodimers with the ER to mediate transcriptional responses (Flouriot *et al*, 2000; Denger *et al*, 2001). A 36 kDa (ERα36) isoform has also been identified and this lacks both the AF-1 and AF-2 domains, and contains an additional 27 amino acids at the C-terminal (Wang *et al*, 2005; Wang *et al*, 2006; Lin *et al*, 2010; Shi *et al*, 2010; Tong *et al*, 2010). The lack of AF-1 and AF-2 domains renders ERα36 unable to mediate transcriptional responses to estrogens, but still allows the receptor to play a role in mediating non-genomic responses (Chaudhri *et al*, 2012).

Conversely, many other studies have demonstrated the presence of a mER that is distinctive from ERα or ERβ and E2-induced non-genomic signalling has been observed in cells lacking ERα or ERβ expression or following treatment with ER antagonists (Gu *et al*, 1999; Filardo *et al*, 2000). A G-protein-coupled receptor, GPER-1 has been proposed as a mediator for these ER-independent effects of E2 and has been identified in the cell membrane and will be discussed later (Razandi *et al*, 1999; Benten *et al*, 2001; Filardo *et al*, 2002).

1.6.1 GPER-1

GPER-1 is hypothesised to mediate some of the rapid non-genomic effects of estrogens. It is a G-protein coupled receptor (GPCR), structurally distinct from ERα or ERβ, which spans the membrane seven times starting with an extracellular N-terminus and ending in an intracellular C-terminus. G-proteins function in a variety of physiological roles, such as sensory perception, mood and immune system regulation, autonomic nervous system transmission and cell density determination. The exact localization of GPER-1 remains the centre of some debate, with some groups showing its localization to the plasma membrane (Filardo *et al*, 2000, Filardo *et al*, 2007) and others to the membranes of the endoplasmic reticulum (Revankar *et al*, 2005; Otto *et al*, 2008).

Specifically, GPER-1 is part of the chemokine receptor-like 2 subfamily within the class A rhodopsin-like family. It shares a 28% sequence homology with the angiotensin II 1A and interleukin 8A receptors (Carmeci *et al*, 1997). GPER-1 has been shown to couple to G-proteins and its activation by E2 results in adenylyl cyclase activation, consequent increases in cyclic adenosine monophosphate (cAMP) (Thomas *et al*, 2005) and activation of the PI3K signalling cascade, with consequent AKT activation. Additionally, SRC-like tyrosine kinase activation resulting in fibronectin matrix assembly and activation of metalloproteases was also observed.

GPCRs are inactive until ligand, which may be a peptide, neurotransmitter or hormone, binding occurs. This induces a conformational change at the extracellular binding site that in turn induces a conformational change of the loops on the cytoplasmic side of the membrane. This increases the affinity of the receptor for heterotrimeric G-proteins, which, bind to the receptor forming the receptor-G protein complex. G-proteins are composed of Gα, Gβ and Gγ subunits and their targets are determined by the type of Gα subunit, although Gβγ can also elicit intracellular effects. Gα is palmitoylated and Gγ has a glycosylphosphatidylinositol covalently bound to it rendering them anchored to the membrane. They remain inactive with a reversibly bound guanosine diphosphate (GDP) to the α

subunit or lacking any guanine nucleotide association, but upon binding to the GPCR and undergoing a conformational change, GDP is released and guanosine triphosphate (GTP) binds to the α subunit. Gα is then able to dissociate from the Gβγ dimer and bind to its effectors. This effector is determined by the form of Gα activated; G_s forms activate adenylyl cyclase, G_q activates phospholipase C β (PLCβ), G_i inhibits adenylyl cyclase and $G_{12/13}$ potentially activates Ras, Src, phospholipase D and protein kinase C. For E2-induced GPER-1 activation, immunoprecipitation studies have shown that the G_s form mediates E2 responses (Thomas *et al*, 2005).

1.6.2 Modes of Action of the Estrogen Receptor: Genomic Actions

The classical mode of estrogenic action is that mediated by nuclear ERα and ERβ. Upon diffusion into the cell, estrogen binds to the ligand binding domain of the ER, inducing a conformational change in the receptor and, liberating it from its cytosolic chaperone, heat shock protein 90 (Hsp90) (Tanenbaum *et al*, 1998). These estrogen-induced conformational changes also expose nuclear localization signals present in the DNA binding domain and, so, once free from its chaperone, the ER-estrogen complex is transported into the nucleus, where it can form dimers with other ER-estrogen complexes (Métivier *et al*, 2002). These dimers bind to estrogen response elements (EREs) within the promoter regions of target genes and induce or inhibit the transcription of these genes (**Figure 1.13**). In the absence of an ERE, estrogen is still able to induce transcriptional activity changes of target genes via ER interactions with AP-1 and Sp1 (Krishnan *et al*, 1994; Porter *et al*, 1996; Paech *et al*, 1997; Duan *et al*, 1998). These are transcription factors that, when bound to own their response elements, can bind to ER complexes. This interaction recruits further co-activator or chromatin remodelling complexes, facilitating the formation of the transcription initiation complex, which permits RNA polymerase II interaction with the DNA and hence results in transcription. Interestingly, ERα can also be activated in the absence of estrogens following the phosphorylation of ser118 in the A/B domain and, thus, activating AF-1 (Lannigan, 2003).

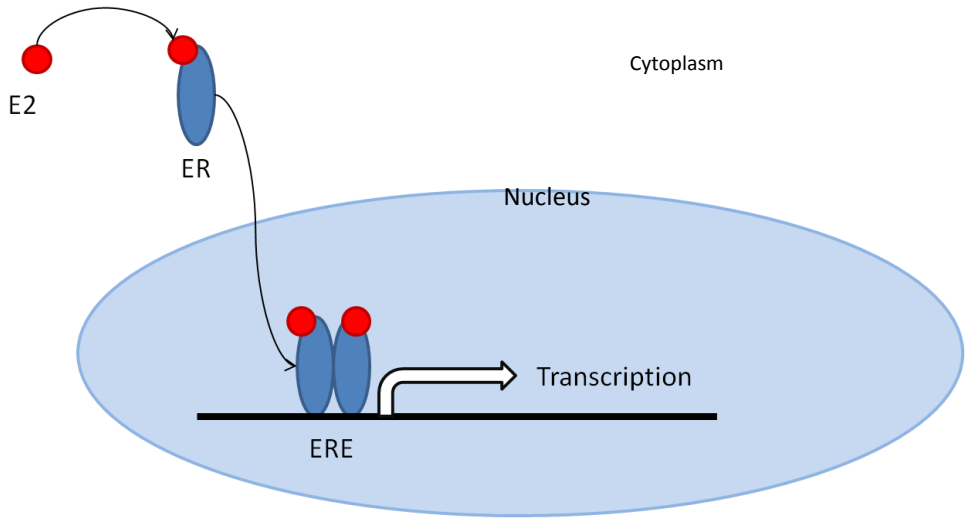

Figure 1.13: The genomic mechanism of E2 action. E2 enters the cells and binds to the ER. The E2-ER complex translocates to the nucleus, dimerises and binds to the ERE of estrogen-responsive genes to activates transcription.

Estrogens have also been found to regulate the expression of microRNA (miRNA), small, non-coding sequences of RNA that block translation or promote degradation of target messenger RNA (mRNA) (Klinge, 2009; Wickramasinghe *et al*, 2009). miRNA are short, non-coding RNA sequences that, when incorporated into the RNA-induced silencing complex (RISC), bind to complimentary or near-complimentary target mRNA and prevent RNA translation or target it for degradation. If miRNA binds to target mRNA imperfectly, the result is translational repression, whereas perfect or near-perfect binding results in mRNA degradation (Rhoades *et al*, 2002; Zeng *et al*, 2002; Doech & Sharp, 2004; Cuellar & McManus, 2005). Either mechanism can have implications for proliferation, apoptosis and cell differentiation and, therefore, the dysregulation of miRNA expression may be involved in carcinogenesis.

1.6.3 Non-Genomic Estrogenic Actions

In recent years, studies have demonstrated that, in addition to the effects mediated by the nuclear ER, estrogens also induce other, short-term effects, which, occur so soon after exposure that cannot be attributed to the classical genomic pathway. In this instance, estrogens are now known to act via a non-genomic mechanisms, involving the activation of secondary messenger signalling pathways, such as cAMP production following adenylate cyclise activation (Aroncia *et al*, 1994), inositol phosphate production (Le Mallay *et al*, 1997) leading to phosphatidylinositol 3-OH kinase (PI3K) activation (Haynes

et al, 2003; Simoncini *et al*, 2000), increased intracellular Ca^{2+}(Improta-Brears *et al*, 1999; Migliaccio *et al*, 1996; Wozniak *et al*, 2005; Zhao & Brinton, 2007; Szatkowski *et al*, 2010; Yan *et al*, 2011), Src and mitogen-activated kinase (MAPK) activation (Migliaccio *et al*, 2003) and protein kinase C (PKC) activation (Picard *et al*, 2003).

The mechanism by which E2 is able to elicit its non-genomic effects is highly complex and involves a variety of different estrogen-responsive receptors and cross-talk between signalling pathways. It has been demonstrated that E2-ERα complexes are able to associate with Src tyrosine kinase and the PI3-K's regulatory subunit (Castoria *et al*, 2001; Razandi *et al*, 2003; Simoncini *et al*, 2003), which has implications for proliferation and cell survival. On the other hand, estrogens can also activate the G-protein-coupled receptors, GPER-1, leading to adenylate cyclase and phosphlipase C stimulation and subsequent activation of protein kinases involved in MAPK signalling. E2-activated membrane-associated receptors, such as mER and GPER-1, can also result in EGF receptor activation (reviewed in Filardo, 2002).

Cross-talk between estrogen receptors and various signalling pathways exists and this can result in hormone independent ER activation. It has been observed that the ER can become activated as a result of MAPK and PI3K signalling pathway activation, despite the lack of estrogen presence (Kato *et al*, 1995), leading to transcription of E2-inducable genes. Additionally, E2 can rapidly activate the PI3K and MAPK pathways, normally associated with growth factor receptors, such as that of epidermal growth factor (EGF).

1.7 Growth Factor Signalling Pathways

Signalling pathways are governed by the presence of receptors on cell membranes. Different classes of receptors mediate different signalling pathways that have implications for cell growth, survival, migration, proliferation, differentiation and apoptosis. Disruption of signalling pathways, such as overexpression of receptors or constitutive activation of key kinases, is implicated in many cancers, including that of the breast. This can be exemplified by the overexpression of the EGF receptor, Her-2. Activation of this receptor by its ligands activates PI3K and MAPK signalling pathways, leading to increased proliferation and survival. It has been demonstrated that estrogen action can result in the rapid activation of Src and Erk, key components in Akt and MAPK signalling (Wozniak *et al*, 2005; Tong *et al*, 2010).

1.7.1 PI3K/AKT Signalling

The PI3/AKT pathway is involved in cellular growth, proliferation, cell motility and survival in normal and cancerous cells, however in cancer, it often becomes dysregulated (Vivanco & Sawyers, 2002). During tumour formation, it is also implicated in angiogenesis and EMT as a result of increased AKT activation (Yao *et al*, 2008). The pathway can be mediated by receptor tyrosine kinases (RTKs) and the binding of ligands, such as EGF.

RTKs exist as inactive monomers in the cell membrane and contain Src-homology 2 (SH2) or phosophotyrosine binding (PTB) domains at their cytoplasmic ends. Ligand binding causes receptor dimerization and activation. The active dimers then undergo trans-auto phosphorylation at their cytoplasmic regions allowing the binding of target proteins to the SH2 or PTB domains. The binding of adaptor or docking proteins to these domains link signalling proteins to the receptor or provide additional binding sites for signalling molecules respectively. Transcription factors, such as STAT's can bind to and become activated by RTKs, as well as signalling molecules, such as PI3K, which become activated by binding to RTKs phosphorylation sites. The signals from RTKs is terminated upon receptor internalisation.

PI3K is a lipid kinase comprised of a p85 regulatory subunit and a p110 catalytic subunit. PI3K is responsible for the reaction that synthesises the secondary messenger, phosphatidylinositol triphosphate (PIP_3), a membrane phospholipid, from phosphatidylinositol diphosphate (PIP_2). PIP_3's function is to recruit Akt to the plasma membrane so that it can be phosphorylated and, hence, activated (**Figure 1.14**). Upon activation, Akt translocates to the nucleus and is able to phosphorylate and activate various targets that are involved in cell cycle progression, apoptosis and epithelial–mesenchymal transition (EMT) pathways (Yao *et al*, 2008). Many of these targets are enzymes involved in the modulation of the Akt pathway, such as murine double minute 2 (MDM2), E3 ligase, α6β4 integrin and PTEN (Tokunaga *et al*, 2008; reviewed in Tokunaga *et al*, 2010). Ultimately, Akt pathway activation leads to increased cell cycle progression due to the inhibition of the cell cycle inhibitors p21 and p27 (Zhou *et al*, 2001), apoptotic inhibition due to caspase-3 and pro-apoptotic factor inhibition and increased cell survival, as a result of p53 inhibition.

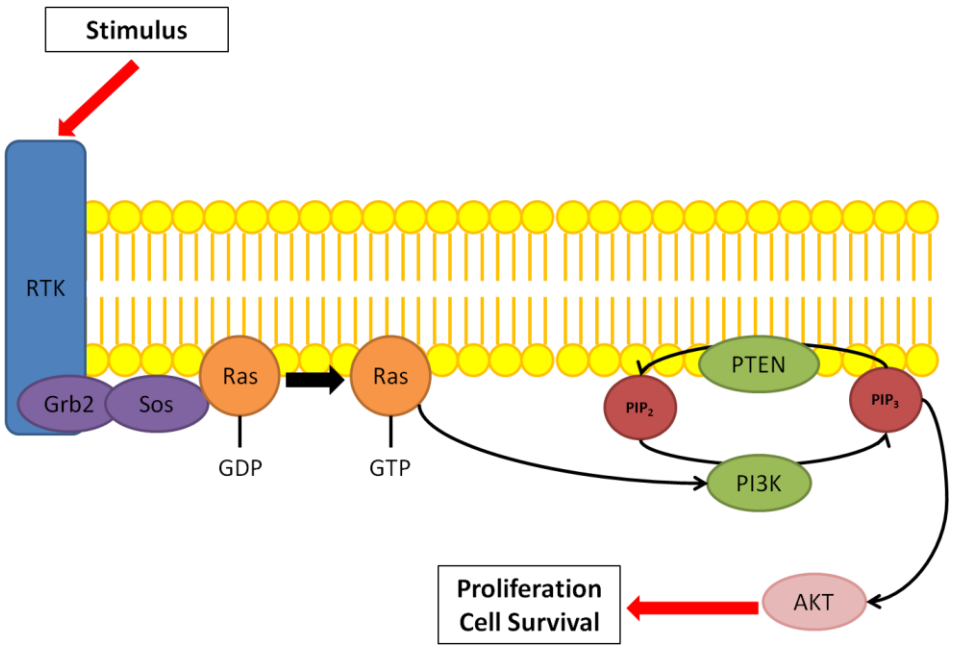

Figure 1.14: The PI3K/AKT signalling pathway. Following stimulation, Grb2 and Sos are recruited to the activated receptor and Ras GDP is converted to Ras GTP. PI3K is then activated and converts PIP_2 to PIP_3, which activates AKT. AKT then functions by phosphorylating and activating or inhibiting proteins involved in proliferation and survival, or apoptosis, respectively.

PTEN phosphatase removes the 3-phosphate from PIP_2 and PIP_3, thus rendering these secondary messengers inactive and, consequently, causing a cessation in PI3K signalling. Hence, the down-stream targets of PI3K can negatively regulate the PI3K pathway via a feedback loop.

The PI3K pathway has been implicated in a variety of cancers, including that of the breast (Zinda *et al*, 2001) with increased Akt activity identified in up to 40% of breast cancer samples (Tokunaga *et al*, 2006). The pathway can be activated by the HER2, which is over-expressed in ~30% of breast cancers and ER, which is present in ~60% of breast cancers (Dancey & Sausville, 2003). Additionally, mutations in PTEN, often observed in breast cancers, render it functionless resulting in hyper-activation of the pathway (Cantley & Neel, 1999). Finally, gene mutations in both the regulatory p85 and catalytic p110 subunits of PI3K have also been identified in a variety of cancers, including that of the breast and are present in approximately 8% of breast cancer tumours (Ma *et al*, 2000; Philp *et al*, 2001; Bachman *et al*, 2004; Campbell *et al*, 2004; Samuels *et al*, 2004; Levine *et al*, 2005). Regardless of the mechanism, dysregulation of the PI3K pathway is associated with cancer and has implications for proliferation,

metabolism, cellular adhesion, apoptosis, migration and arrangement of the cytoskeleton (Levine *et al*, 2005; Liu *et al*, 2010).

1.7.2 Mitogen Activated Protein Kinase Signalling

The MAPK signalling pathway is involved in the regulation of apoptosis, proliferation, survival, differentiation and motility. MAPK's are serine-threonine kinases that are activated by phosphorylation and function to transduce extracellular signals to targets within a cell, such as enzymes, components of the cytoskeleton and transcription factors. Ultimately, activation of the MAPK pathway results in increased cell proliferation and survival. This pathway is often dysregulated in cancer (Hilger *et al*, 2002). The MAPK pathway can be divided into four cascades: the extracellular regulated kinase 1/2 (ERK1/2), the c-Jun N-terminal (JNK), the p38 and the ERK 5 pathway. The ERK1/2 cascade has proved to be of significant relevance for the effects of estrogens and throughout this thesis will remain the primary focus for investigation (Santen *et al*, 2002; Whyte *et al*, 2009).

As with PI3K signalling, one mechanism by which MAPK signalling can be activated is via RTKs. Ligand binding induced activation of transmembrane receptors results in the recruitment of the SH2 domain containing adapter protein, growth factor receptor-bound protein 2 (Grb2) to the receptor. This SH2 domain is present in many proteins involved in intracellular signal transduction. Its function is to allow proteins containing an SH2 domain to dock with phosphorylated tyrosine residues present on other proteins (**Figure 1.15**).

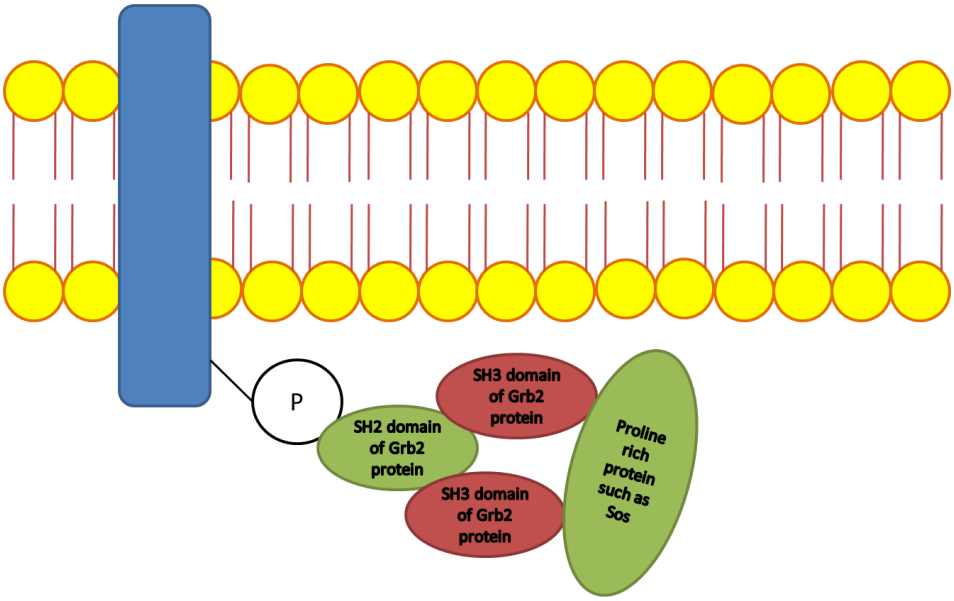

Figure 1.15: The activation of SH2 domains and their role in signal transduction. The SH2 domain of a protein binds to the phosphorylated tyrosine residues of a receptor. The SH3 domains of the same protein are then able to bind to proline-rich proteins to form a protein complex required for the downstream signal transduction events

Following recruitment of Grb2, the guanine nucleotide exchange factor, son of sevenless (Sos) is then sequestered via the SH3 domains of Grb2. The role of these SH3 domains is to allow proteins containing them to interact with other proteins which are proline-rich. This results in the assembly of protein complexes, such as those involved in intracellular signal transduction (**Figure 1.15**). Sos then stimulates the dissociation of GDP and binding of GTP to Ras, thus activating it. This activation triggers a kinase cascade involving Raf, MEK, and ERK's. ERK then phosphorylates and activates the transcription of genes such as *c-FOS* and *c-JUN*. Additionally the transcription of MKP-1 is also induced which leads to the dephosphorylation and deactivation of ERK, thus terminating the signal (**Figure 1.16**).

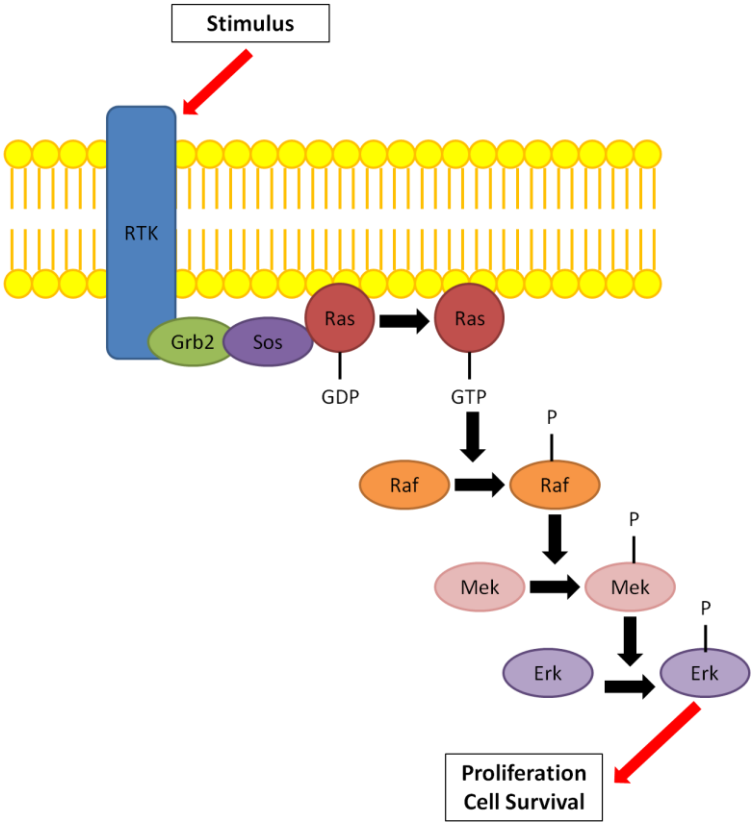

Figure 1.16: The MAPK signalling pathway ERK cascade. Following stimulation, Grb2 and Sos are recruited to the activated receptor and Ras GDP is converted to Ras GTP. A phosphorylation cascade involving Raf, Mek and Erk subsequently induces the induction of genes involved in proliferation and cell survival.

E2 treatment can result in the activation of MAPK signalling and a variety of mechanisms have been postulated regarding how this is achieved. The first proposed mechanism is via ER interaction at the membrane with components of signalling pathways. ERα can associate with SH2 domain containing adapter proteins, such as Shc, in the presence of E2 and growth factor receptors resulting in formation of the Shc-Grb2-Sos complex and activation of the MAPK pathway (Song *et al*, 2002). If ERα or SH2 are knocked down by siRNA, or in the case of co-incubation with ER antagonists, MAPK signalling is not observed (Song *et al*, 2004). Secondly, estrogens are able to induce MAPK signalling via GPER-1, independently of the presence of ER. As with the aforementioned mechanism involving ERα association with Shc, activation of GPER-1 in response to E2 results in the formation of Shc-EGF receptor complexes and subsequent MAPK signalling (Filardo *et al*, 2000). The role of GPER-1 in mediating E2's induction of MAPK signalling are further supported by the observation that increased levels of Ca^{2+} following

treatment with E2 are not prevented by co-incubation with ER antagonists, but are abolished upon co-incubation with inhibitors of Gα (Doolan & Harvey, 2003).

Disruption of MAPK signalling is associated with cancer and can occur at many levels within the pathway. At the receptor level, receptor overexpression or mutation, such as that seen with EGFR, can lead to increased activation of the MAPK pathway. In normal cells, the GTP attached to Ras is hydrolysed to GDP making Ras inactive, however, mutations in Ras, which occurs in 30% of all cancers, prevent this hydrolysis of Ras, which becomes constitutively activated and MAPK signalling becomes dysregulated (Cox & Der, 2002; Davies *et al*, 2011).

1.7.3 Crosstalk Between Estrogen and Growth Factor Pathways

It has been demonstrated that two of the pathways through which estrogens elicit rapid non-genomic effects are the PI3K and MAPK signalling cascades. For example, E2 treatment of cells increases MAPK activation to approximately 20% of the level observed with EGF treatment, within 5 minutes of exposure and this effect lasts for an hour, before returning to normal levels, even in the continued presence of E2 or EGF (Improta-Brears *et al*, 1999). Also E2-treated MCF-7 cells have been shown to have increased Shc phosphorylation, an indicator that E2 is causing Shc/GRB2/Sos complex formation (Migliaccio *et al*, 1996) an early event in the PI3K and MAPK signalling cascades.

Estrogen action can also interact with growth factor signalling in a process known as cross-talk. Cross-talk is the interaction between different signalling pathways and results in the integration of various signals and the different cellular responses that they elicit. Various classes of cross-talk have been identified: receptor function, signal flow, substrate availability, gene expression and cellular communication (**Figure 1.17**) (Donaldson & Calder, 2011). Receptor function cross-talk is when a receptor's ability to bind to its ligand and elicit a response in one signalling pathway is affected by another, such as the increase in EGF receptor expression in response to E2 (Das *et al*, 1994) resulting in increased EGF-initiated signalling pathways, or MAPK pathway-induced ER phosphorylation (Tang *et al*, 2004). The phosphorylation of the ER has implications for the receptor's localization, dimerization and transcriptional activity (Chen *et al*, 1999; Le Goff *et al*, 1994; Lee & Bai, 2002) and can also induce its degradation via the ubiquitin-proteasome pathway (Marsaud *et al*, 2003). Signal flow cross-talk occurs when a component of one pathway affects the activation of a protein in another pathway. Substrate availability cross-talk is when two pathways compete for the same protein, for example EGF activation of MAPK and E2-induced activation of MAPK. Gene expression cross-talk concerns the activation or

suppression of the same target genes in conflicting ways when two pathways are activated simultaneously. Cellular communication cross-talk is when the activation of one pathway results in the release of a ligand that activates another pathway, such as the increased release of EGF in response to E2 (Das *et al*, 1994). Estrogens can also cross-talk with insulin/insulin-like growth factor-1 (IGF-1) signalling pathways, resulting in increased insulin signalling (Stewart *et al*, 1990).

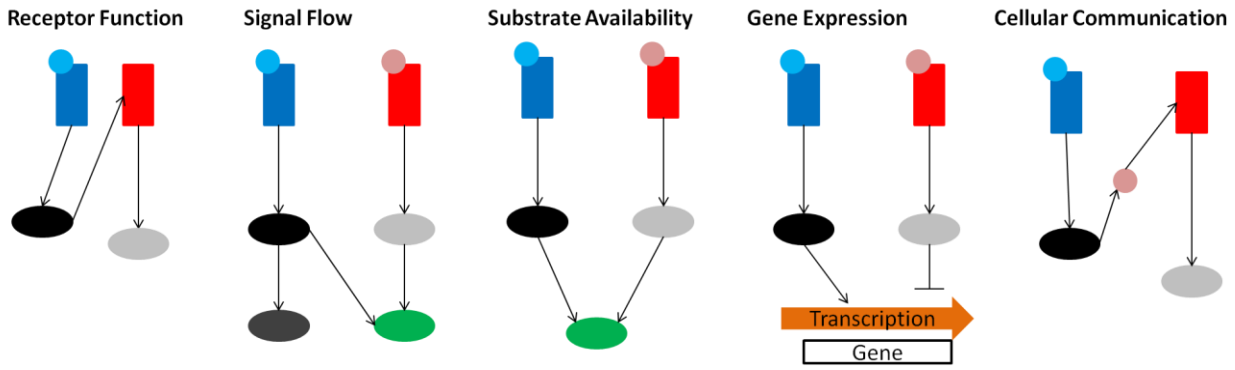

Figure 1.17: Different types of cross-talk. Cross-talk between estrogen (blue) and growth factor (red) signalling pathways may have impacts upon the availability of substrates to perform certain processes, gene transcription and the activation of other receptors (Adapted from Donaldson & Calder, 2011).

1.8 Theories Surrounding Hormonal Carcinogenesis

Hormonal carcinogenesis is the initiation of carcinogenesis or the promotion of tumour growth in response to hormones, such as estrogens. The process is not fully understood although various theories have been proposed to explain the relationship between estrogen and cancer. Firstly, estrogen is thought to stimulate proliferation of receptive cells (Holland & Roy, 1995) and, as a result of this increased proliferation, DNA mutations accumulate and a malignant phenotype may result (Travis & Key, 2003). Indeed, increased proliferation induced by E2 *in vivo*, results in increased lobular size of the mammary gland in rats (Kovalchuk *et al*, 2007). The theory of proliferation-induced hormonal carcinogenesis differs from the chemical or genotoxic model of carcinogenesis in that no initiating event, such as genetic mutations, are required.

More recently, it has been hypothesised that estrogens and their can cause genotoxicity through DNA adduct formation (Cavalieri *et al*, 1997; Lu *et al*, 2007; Saeed *et al*, 2008). Estrogens and their metabolites can also induce gene mutations (Liehr, 2001), loss of heterozygosity (Russo *et al*, 2002), single strand DNA breaks (Yared *et al*, 2002; Rajapakse *et al*, 2005) and aneuploidy (Li *et al*, 2004; Kabil

et al, 2008), thus highlighting the complexity as to the role of estrogens in carcinogenesis that is not entirely contemplated by the more traditional theory described previously.

E2 is metabolised via oxidations at the C-2. C4 and C16 positions to form the active metabolites: 2-hydroxyestradiol and 4-hydroxyestradiol. These then undergo methylation or oxidation to form electrophilic semiquinones and catechol estrogen quinone. Conjugations via O-methylation by catechol-*O*-methyltransferase (COMT) results in metabolite inactivation (**Figure 1.18**). If however, this inactivation does not occur, the active metabolites can form stable adducts with DNA resulting in DNA strand breaks. They can also induce depurination, processes that increase the chance of mutations, which, in turn, may result in carcinogenesis (Dwivedy *et al*, 1992; Cavalieri *et al*, 1997; Chakravarti *et al*, 2001; Li *et al*, 2004). The failure for an organism to completely metabolise estrogens could occur for a variety of reasons, such as polymorphisms of the genes encoding for cytochrome P450 enzymes (CYP17 and CYP1A1, and of COMT), all of which are key enzymes involved in estrogen metabolism (Huang *et al*, 1999; Dawling *et al*, 2001). Interestingly, in addition to this, estrogen and its metabolites may down-regulate or inhibit COMT (Roy *et al*, 1990; Xie *et al*, 1999).

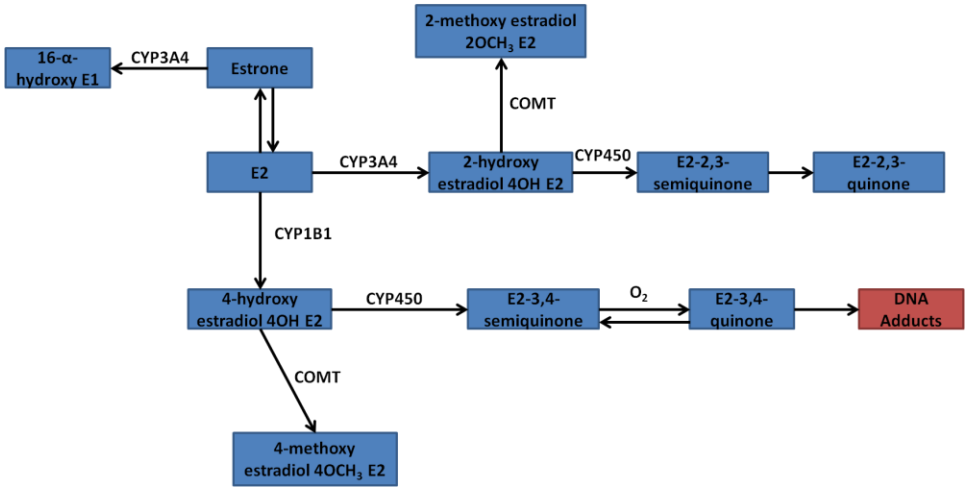

Figure 1.18: The metabolism of E2. E2 metabolism can lead to the formation of reactive metabolites that are capable of forming adducts with the DNA. (Adapted from Medscape http://www.medscape.com/viewarticle/449725_2)

Loss of heterozygosity in parts of chromosome 11 has also been observed in E2-treated cells *in vitro* (Russo *et al*, 2002). Loss of heterozygosity is the loss of the normal functioning of a gene allele in a cell where the second allele is already inactive. In the context of cancer, this could result in the suppression

of tumour suppressor genes and, consequently, tumourigenesis. Chromosome 11 frequently exhibits loss of heterozygosity in breast cancer and loss of heterozytm'sgosity at the 11q23.3 loci specifically has been reported in approximately 35% of breast tumour samples (Tomlinson *et al*, 1996), suggesting the presence of a tumour suppressor gene close to this locus. In a model used by Russo and Russo (2006), E2-treated transformed cells were injected into severe combined immunodeficient (SCID) mice and the result was tumour formation. Using this method, other genomic gains and losses in response to estrogen treatment both *in vitro* and *in vivo* were identified. Most notably was the genomic gain of 8q24.1 and deletion of chromosome 4 (Fernandez *et al*, 2005; Fernandez *et al*, 2006; Russo & Russo, 2006).

Estrogens can affect the expression miRNAs and abnormal miRNA expression patterns have been observed in breast cancer (Iorio *et al*, 2005; Blenkiron *et al*, 2007; Tavazoie *et al*, 2008). In rats, following E2-induced mammary carcinogenesis, numerous miRNAs were up- or down-regulated including mi RNA's that target E2F1 and BCL6 (Kovalchuk *et al*, 2007). In MCF-7 cells, E2 treatment affected the regulation of multiple miRNAs involved in apoptosis, proliferation, metastasis and drug resistance (Miller *et al*, 2008; Yu *et al*, 2008). Notably, E2 up-regulates the miRNA that targets the caspase-3 and down-regulates the miRNA that targets Cyclin D, and, thus, has implications for caspase-mediated apoptosis and proliferation (Tsang & Kwok, 2008; *Yu et al*, 2008)

Estrogens can also have an impact upon epigenetic modifications, heritable changes within chromatin that do not alter the DNA sequence, but do nevertheless affect gene expression due to chromatin remodelling (Kovalchuk *et al*, 2007; Mello *et al*, 2007; Starlard-Davenport *et al*, 2010). This can occur by DNA methylation or histone modification. DNA methylation occurs predominately at cytosine and guanine dinucleotide (CpG) rich regions of DNA, termed CpG islands. During this process a methyl group is transferred to the 5' carbon of the cytosine residue resulting in the repression of gene expression, due to chromatin remodelling. Whilst hypermethylation results in transcriptional repression, hypomethylation has the opposite effect and promotes transcription. Both of these processes dysregulate gene expression and are implicated in breast cancer and can be affected by E2 (Kovalchuk *et al*, 2007). During histone modifications, the structure of chromatin is modified to either promote, in the case of histone acteylation, or repress, in the case of histone deacetylation, gene transcription. In any case, the result of epigenetic modifications can lead to the repression of tumour suppressor genes, the increased expression of oncogenes or chromosomal instability, all of which can be involved in the initiation and progression of cancer. Estrogens have been implicated in modification of

chromatin structure through the histone acteylation or deacetylation of and histone methylation or demethylation of the ERα gene promoter regions (Glass *et al*, 1997; Garcia-Bassets *et al*; 2007). The deregulation of the enzymes involved in these processes could therefore have a role in epigenetic modification-induced carcinogenesis (Mann *et al*, 2011).

The impact of estrogens on epigenetic modifications can be exemplified by DES, a pharmaceutical estrogen, mention before in this chapter. Daughters of mothers who took DES during pregnancy are more likely to develop cancer (Palmer *et al*, 2006). A similar outcome was also observed in mice studies, where animals were shown to develop breast tumours and to present demethylated estrogen-responsive genes, such as lactoferrin and c-fos (Newbold *et al*, 1990; Li *et al*, 1997[b]). These findings suggest that *in utero* estrogen exposure can result in a predisposition for cancer later in life.

Further to this, E2 can disrupt the dividing of stem cells (Russo *et al*, 2010) or enhance stem cell proliferation in a paracrine fashion (Fillmore *et al*, 2010). Also, increased *in utero* E2 exposure correlates with increased number of stem cells (Savarese *et al*, 2007), indicating that estrogens may increase the stem cell populations, which some hypothesise to be the origin of cancer (Sell, 2004; Martinez-Climent *et al*, 2006). Stem cell populations within the mammary gland are able to give rise to a variety of different cell types and have the ability to divide asymmetrically, producing one stem cell and one cell which will differentiate into a ductal epithelial, alveolar epithelial or myoepithelial cell (Kordon & Smith, 1998; Shackleton *et al*, 2006). During pregnancy, this enables the stem cells to maintain a stem cell pool whilst populating the breast in preparation for breast feeding. Studies have shown that 1-10% of the total cell population displaying markers for stem cells are able to form tumours in severe Combined Immunodeficiency (SCID mice), contrasted to non-stem cells being unable to elicit the same tumour growth (Al-Hajj *et al*, 2003; Ponti *et al*, 2005; Ginestier *et al*, 2007).

Additionally, estrogens can enhance remodelling of tissues within in the body through interactions between the stroma and epithelium (Soto & Sonnenschein, 2010), increasing the probability of tissues structure becoming disorganised and, hence, initiate cancer. Nevertheless, although the intracellular mechanisms involved in hormonal carcinogenesis have been widely studied, little is known about the effects of estrogens on the glandular structure of the breast.

1.9 Hypothesis

The disorganisation of tissue structures, such as the ducts of the breast, is associated with cancer progression and, despite advances in research, it still remains unclear if this disorganisation leads to a

cancerous phenotype or if it is, in fact, a result of aberrations of the genome and loss of proliferative control, which occur as a consequence of carcinogenesis. The role of estrogens, which are required for the normal development of the breast, in this disorganisation also remains unknown.

We propose that estrogenic chemicals, such as E2, interact with estrogen-responsive receptors and key signalling pathways during acini formation which, ultimately, has an impact on the development and architecture of breast acini. We aim to use a 3D *in vitro* model (which will be explained in detail in **Chapter III**) to investigate the effects of estrogens on the morphogenesis of the mammary gland, in addition to the role that estrogen-responsive receptor play in this.

1.10 Scope of the Thesis

Much of the work published in the literature concerning 3D cultures has focused on the ER negative, non-tumourigenic MCF-10A cell line and has revealed mechanisms and genes involved in the formation and maintenance of the hollow lumen of acini. Limited work concerning the effects of 17β-estradiol on acini formation using the ERα negative, but ERβ positive MCF-10F cell line has demonstrated that E2 treatment results in cells losing their ability to form acini in collagen matrices, but suggests that this effect is ER-independent (Russo *et al*, 2002; 2003; 2009; 2010). In order to investigate the full spectrum of estrogenic effects on acini formation and the potential involvement of the ERα in these processes, a different cell line was, therefore, required. For that, a non-tumorigenic, ERα and ERβ positive cell line was selected: the MCF-12A cell line.

In **Chapter II**, we sought to confirm the receptor status of the cell lines to be used to further study the impact of estrogens upon proliferation and apoptosis in a 3D model. In addition to confirming the receptor status of MCF-12A cells, the receptor status of MCF-10A cells was investigated to confirm its ER negative phenotype and to ascertain whether this cell line was GPER-1 competent. Growth curves were also constructed for these cell lines in the presence of E2 and receptor antagonists for both the ER and GPER-1 to investigate the impact of E2 upon proliferation and whether this was mediated by the ER or GPER-1. Finally, the well-studied MCF-7 and MDA-MB-231 cell lines were used as comparison to investigate the effects of E2 upon proliferation in an ER competent and ERα negative tumourigenic cell line.

In **Chapter III**, we proceeded to optimise an established 3D model for use with MCF-12A cells which has previously been used with MCF-10A cells. After optimising the conditions required to successfully perform 3D culture of MCF-12A cells in reconstituted basement membrane (Matrigel), we

established a time-course for the morphogenesis of MCF-12A acini. This was done so that a comparison was available between normal MCF-12A acini formation and acini formation following treatment with estrogenic test compounds.

After establishing the normal morphogenesis of MCF-12A cells in 3D culture and selecting the estrogenic test compounds to perform subsequent 3D studies, a time-course for E2, BPA and n-propylparaben was established in **Chapter III**. Further to this, in **Chapter IV**, we decided investigate whether the malformations induced by the estrogenic test compounds were mediated by the ER or GPER-1, using antagonists for these receptors. This work has been submitted for publication (Marchese & Silva, 2012).

After the observation that both of these receptors are implicated in the estrogen-induced disruption of MCF-12A acini, in **Chapter V**, we proceeded to investigate the non-genomic effects of estrogens using inhibitors of the PI3K and MAPK signalling pathways.

Treatment of 3D cultures with the estrogenic test compounds resulted in deformed acini of increased size and displaying filled lumen. These effects were mediated by both the ER and GPER-1, and were, in part, a consequence of activation of the PI3K and MAPK pathways. We decided then, to investigate how estrogens impacted upon the expression of key apoptotic factors in 3D culture. The first step in doing this was to optimise the primers for future real-time polymerase chain reaction (PCR). This had to be conducted in monolayer due to technical and financial constraints. We then decided in **Chapter VI**, to investigate first, whether in monolayer culture, E2 affected the gene expression of four key apoptotic factors: *BCL-2*, *BCL-XL*, *BAX* and *BAD* in four mammary cell lines. After the observation that in monolayer, E2 did not greatly impact upon a single apoptotic factors, but did instead affect the expression of multiple apoptotic factors, we calculated the Bax/Bcl-2 ratio and found that estrogenic treatment did appear to induce an anti-apoptotic influence. Finally, protein analysis of the PI3K and MAPK were performed to investigate whether in monolayer culture, E2 was capable of inducing non-genomic effects, which was previously noticed in 3D cultures of MCF-12A cells in **Chapter V**.

In order the take the analysis of gene expression one step further, this was then conducted in cells isolated from 3D cultures. Initially, whole MCF-12A acini were extracted from Matrigel to investigate the impact of estrogens upon apoptotic factors and *CCND1* (the gene encoding cyclin D1) expression. We observed that E2 does have an impact on gene expression of whole acini, in that *CCND1* expression was increased in E2-treated acini. In **Chapter VII**, we developed a method by which the outer and inner cell populations of MCF-12A acini could be separated based on their integrin β4 expression by

fluorescence activated cell sorting (FACS). This enabled us to clearly see that E2 treatment did impact upon gene expression in a way that would promote proliferation of both inner and outer cell populations, and suppress apoptosis, particularly in the case of the centrally located cell population.

Overall, throughout this work we have two main aims. Firstly we aim to investigate whether estrogens can impact upon the 3D architecture of the breast, by using a 3D *in vitro* model of epithelial cells cultured in reconstituted basement membrane. In the possibility that estrogens, in fact, affect acini formation of MCF-12A cells, our second aim is to dissect the mechanisms through which estrogens act in 3D cultures of this non-tumourigenic epithelial cell line. It is the hope, that the work conducted in this study will enable us to understand the role of both the nuclear ER and transmembrane receptors (GPER-1) in estrogen-induced malformations of acini.

CHAPTER II: Characterization of Immortalised Cell Lines - Confirmation of Receptor Status and Determination of the Effect of Estrogens on Proliferation in Monolayer Culture

A common hallmark of cancer is the ability of cells to proliferate with unlimited potential replication, even in the presence of anti-proliferative signals, or absence of growth signals (Hanahan & Weinberg, 2000). Malignancies of the breast are no different and cancer cells have been demonstrated to have a higher level of proliferation in comparison to non-malignant breast cells (reviewed in Lari & Kuerer, 2011).

Normal mammary gland growth and differentiation requires the actions of various hormones, such as estrogen, and growth factors. Paradoxically, estrogens also play a role in breast carcinogenesis. As discussed in **Chapter I**, in this study, we propose to study the actions of estrogens and the role of the ER in mammary gland formation (and potentially carcinogenesis) by using *an in vitro* 3D model that can recapitulate the features of the normal breast epithelium. For that, it was essential to use a human, immortalised, non-tumourigenic cell line, which expressed the ER and responded to estrogens. The cell line selected was the MCF-12A.

2.1 Cell Lines of Choice

MCF-10A and MCF-12A are two non-transformed immortalised breast cell lines. The MCF-10A cell line was originally derived from a patient with fibrocystic breast disease, and upon culture, spontaneously became immortalised. It is reported as being of luminal origin and is ER and HER2 negative (Soule *et al*, 1990; Tait *et al*, 1990; Paine *et al*, 1992). In addition to this, MCF-10A cells also have a stable and close to diploid karyotype and express wild type p53, but not oncogenic ras (Soule *et al*, 1990; Merlo *et al*, 1995). The MCF-10A cell line is commonly used as a control for "normal" cells in breast cancer investigations (Hsieh *et al*, 2005; Spink *et al*, 2006). MCF-10A cells provide a model in which to study cancer progression and the role of oncogenic gene expression, as well as loss of tumour suppressor function (Zientek-Targosz *et al*, 2008). MCF-10A cells have also been used extensively in three dimensional cultures to investigate the process of mammary gland development in an *in vitro* model (Muthuswamy *et al*, 2001; Debnath *et al*, 2002; Debnath *et al*, 2003).

The MCF-12A cell line was also derived from a patient with fibrocystic breast disease, but is ER status remains controversial with conflicting studies claiming it is ER negative (Thompson *et al*, 2004)

and ER positive(Eisen & Brown, 2004; Engel *et al*, 2011). The MCF-12A cell line is often used to study the progression of cancer and the role of oncogenic gene expression (Schedin *et al*, 2004). The cell line is used comparatively less than MCF-10A cells as a non-tumourigenic cell line and, therefore, the impact of estrogens on this cell line remain unknown.

MCF-7 cells are derived from a human adenocarcinoma of the breast and are ERα, ERβ and GPER-1 positive, but HER2 negative (Soule *et al*, 1990; Carmecci et al, 1997; Filardo *et al,* 2000). MCF-7 cells have a karyotype of 69 chromosomes (the normal human karyotype is 46). They also express wild-type p53 but possess mutations in CDKN2A and phosphatidylinositol 3-kinase catalytic 110-KD alpha (PIK3CA) (Cancer Genome Project). MCF-7 cells also display further genetic aberrations including deletions, translocations, chromosomal rearrangements and chromosomal loss or amplification (Wang *et al*, 2009).

MDA-MB-231 cells are negative for ERα (Filardo *et al,* 2000), GPER-1 (Carmecci et al, 1997,) but do, however, express ERβ and HER2 (Dotzlaw *et al*, 1997; Okubo *et al*, 2004). They express mutant p53, *BRAF*, *CDKN2A* and *KRAS* (Cancer Genome Project; Olivier *et al*, 2002). Mutations in the ras gene lead to constitutive PI3K signalling, whereas BRAF mutations result in increased MAPK signalling, which in turn, results in increased proliferation and survival. In addition to these specific mutations, microarray studies have shown that MDA-MB-231 cells over-express genes commonly associated with EMT (Kao *et al*, 2009).

The MCF-7 and MDA-MB-231 cell lines are two of the most commonly used cell lines for the study of breast cancer and investigations of the signalling pathways and molecular mechanisms involved in cancer (Tobin *et al*, 2001).

Long-term *in vitro* culture and immortalization of cells can result in genetic and epigenetic changes, such as the loss of the gene locus encoding cyclin-dependent kinase inhibitor 2A (CDKN2A) and the prevention of telomere shortening (Li *et al*, 2007[b]). The loss of the CDKN2A locus in particular, is one mechanism through which mammary epithelial cells overcome retinoblastoma (Rb)-mediated cell cycle regulation and lose their finite lifespan to become immortalized. (Brenner *et al*, 1998; Li *et al*, 2007[b]). Despite these aberrations, MCF-10A and MCF-2A cells are still commonly used in studies as non-tumourigenic controls, due to their near diploid karyotype, dependence on exogenous growth factors, and their inability to induce tumour growth when implanted into nude mice (Soule *et al*, 1990; Paine *et al*, 1992). Aberrant behaviour of MCF-10A and MCF-12A cells, such as the adoption of a spindle-like morphology and increased expression of actin stress fibres and focal adhesion complexes has been

observed in monolayer cultures (Sarrió *et al*, 2008) and, therefore, passage number needs to be carefully monitored for these cell lines, in order to avoid such changes.

The majority of the work in this thesis will concentrate of the MCF-12A cell line, as the primary aim is to characterize the effects of estrogens on 3D cultures of an ER competent non-tumourigenic cell line. The remaining cell lines will be used for the purpose of comparison. The MCF-10A cell line was selected as it is another example of a non-tumourigenic breast cell line, although does not express the ER. The MCF-7 and MDA-MB-231 cell lines on the other hand, are both tumourigenic cell lines, with different ER statuses, in which a lot of work has been conducted in. These cell lines will provide us with the opportunity to compare the response to estrogens in cell lines that differ according to their ER status, in addition to the tumourigenic state.

2.2 ER and GPER-1 Receptor Status

As discussed in **Chapter I**, estrogens interfere with the growth and maintenance of normal and breast cancer cells, and the majority of these effects have been shown to be mediated by the ER. As the main aim of our work is to investigate the effects of estrogens in normal MCF-12A cells and the mechanisms involved in these effects, it is important that these cells express a fully functional ER.

Due to conflicting reports concerning the ER status of the MCF-12A cell line (Soule *et* al, 1990; Carmecci et al, 1997; Filardo *et al,* 2000; Eisen & Brown, 2004; Thompson *et al*, 2004; Moyano *et al*, 2006; Engel *et al*, 2011) and to confirm that the remaining cell lines possess the same ER status as those reported in the literature, it was necessary to confirm the ERα, ERβ and GPER-1 status of all four cell lines. This was achieved by analysing both the gene and protein expressions by reverse transcription real-time polymerase reaction (RT-real-time PCR) and immunoblotting, respectively. Confirmation of ER and GPER-1 status was performed at both the gene and protein level, as, due to post-translational modifications, gene expression does not always correlate with protein expression. Although the GPER-1 status has been characterised in the MCF-7 and MDA-MB-231 cell lines, it is unknown whether MCF-10A or MCF-12A cells express this estrogen-responsive receptor (Migliaccio *et al*, 2003; Pedram *et al*, 2006). As the majority of our work will primarily involve the MCF-12A cell line, it is necessary to know if this cell line expresses GPER-1, as the presence of this receptor may have implications for the responses of these cells to estrogens and estrogen antagonists. Furthermore, ascertaining the GPER-1 status of the remaining cell lines will shed some light onto the function of GPER-1 in breast cells and whether estrogens are able to induce effects through this receptor.

2.3 Principle of Reverse Transcription (RT) Real-Time Polymerase Chain Reaction (PCR)

Various techniques are available in order to conduct gene expression analysis, such as, PCR, real-time PCR and microarrays. For the purposes of this thesis, we decided to conduct RT real-time to investigate gene expression and the impact of estrogens upon this. Although a relatively new technique, RT real-time PCR is well established and widely used to quantify DNA, and for the investigation of gene expression and gene expression regulation (Logan *et al*, 2009). Real-time PCR has multiple advantages over standard PCR. For example, in standard PCR, the detection of the presence of target products is performed at the end of the PCR reaction, by additional time-consuming steps, such as through the use of gel electrophoresis. Analysis is, therefore, achieved based on the size of the amplified product. This presents a potential disadvantage as different fragments of similar molecular weights cannot be distinguished from one another, and sample variability is common. Real-time PCR on the other hand allows the simultaneous amplification, detection and quantification of target DNA sequences and this means that real-time PCR is more sensitive to small changes in PCR product. Also, unlike standard PCR, real-time PCR allows DNA content to be quantified after each reaction cycle, rather than just following the completion of the reaction, as in the case of standard end point PCR. Therefore, real-time PCR provides us with quantitative, rather than qualitative (as in the case of standard end point PCR) data and permits data analysis during the exponential phase of the reaction, which is a far more accurate point at which to analyse target amplification.

Although DNA microarrays would allow for the investigation of multiple genes, the technique is very expensive. Furthermore, cross hybridisation can occur with microarrays due to sequence similarity between genes that are closely related. This leads to the failure to specifically detect target genes that instead hybridise to spots corresponding to different genes. For the reasons discussed here: cost sensitivity and ease of detection and quantification, RT real-time PCR will be used to study the gene expression of *ERα*, *ERβ* and *GPER-1*, and for further experiments in later chapters.

In terms of the reaction process itself: Briefly, during the reverse transcription step, complementary DNA (cDNA) is produced from mRNA, and this is then amplified using real-time PCR, which enables us to study small changes in gene expression. Changes in gene expression can be assessed via various detection methodologies, but SYBR Green is commonly used, due to its ease of use, accuracy and low cost. In terms of its mode of action, SYBR Green specifically binds to the minor groove of newly synthesized double-stranded DNA and thus fluorescence increases as the reaction continues to

synthesise new cDNA strands with each cycle (**Figure 2.1**). However, SYBR Green also has its disadvantages, such as the detection of non-specific double-stranded DNA, which may lead to false positives and increased background. Other methods are available for mRNA quantification in real-time PCR, such as probes. Probes consist of a primer sequence with a fluorescent and a quencher molecule attached. The quencher acts to suppress the fluorescent molecule. When the probe binds to its target DNA sequence, the quencher molecule is cleaved by DNA polymerase, and fluorescence is emitted. The use of probes, although highly specific and with the ability to reduce background, is more expensive and labour intensive, relative to SYBR Green. There are instances when the use of SYBR Green is not advisable. These are in the case of multiplex quantitative PCR, in which multiple gene products can be amplified, detected and quantified in a single plate. In this example, SYBR Green would bind to all the double stranded DNA of the separate target genes and quantification would not be possible. In this specific case, other multiple fluorescent primers are used with quenchers and fluorescent molecules. Regardless, we will not be implementing this technique, and will instead use a plate-based methodology, in which a single gene target is amplified in each well. To ensure accurate results whilst using SYBR Green, primers for the target sequence of a high specificity will be designed and optimised, and this will be discussed further in the **Materials and Methods** section.

SYBR Green specifically binds to the minor groove of newly synthesized double-stranded DNA and thus fluorescence increases as the reaction continues to synthesise new cDNA strands with each cycle (**Figure 2.1**).

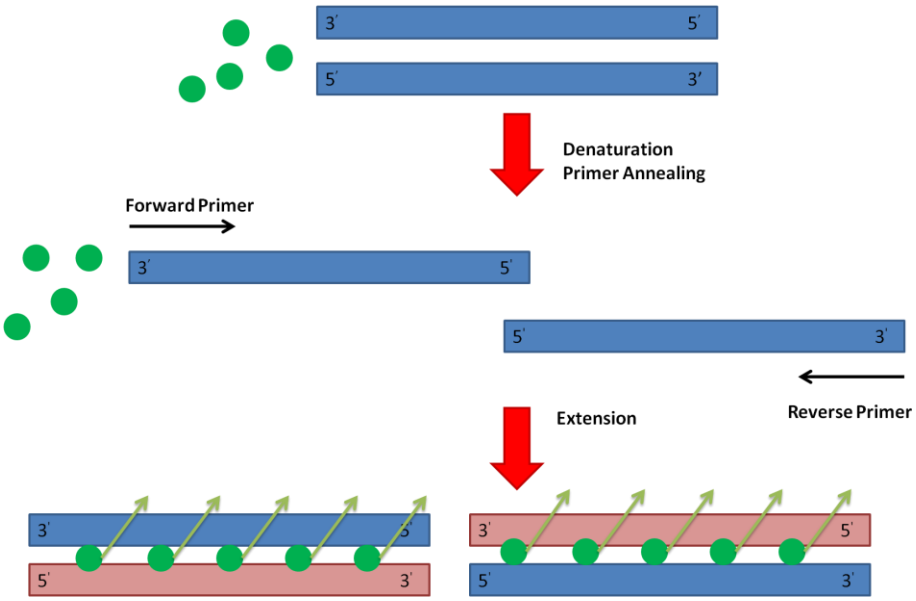

Figure 2.1: The principle of SYBR Green-based detection in real-time PCR. Following denaturation, primer annealing and extension, SYBR Green (green circles) binds to the newly synthesised complimentary DNA strand. Only when complexed with double-stranded DNA, does SYBR Green absorb blue light and emit green light with a wavelength of 520 nm.

Following real-time PCR, amplification, standard and melt curves are generated. As fluorescence is recorded after each cycle, the exponential phase of the PCR experiment can be analysed, and this is important, as this is the phase where the first changes in PCR product are seen, and corresponds to the amount of target gene being amplified. The more target sequence that is present initially in the sample, the fewer cycles it takes for the reaction to produce a detectable fluorescent signal, and hence, reach the exponential phase. In respect to how changes in gene expression are detected using SYBR Green: SYBR Green incorporates itself into the minor grove of double-stranded DNA, and once bound is able to emit fluorescence upon excitation. Unbound SYBR Green, on the other hand, cannot fluoresce. As cycle number during the reaction process increases, the amount of replicated double-stranded DNA increases. Hence, more SYBR Green binding occurs and more fluorescence is emitted and detected. In the next amplification cycle, during the denaturation process, SYBR Green is released and no longer fluoresces. However, it is then incorporated into the newly synthesised double-stranded DNA and the process is repeated again.

Amplification curves depict cycle number (x-axis) at which the level of fluorescence (y-axis) crosses the cycle threshold (Ct). This is the point at which the amplification curve crosses the baseline

of fluorescence. To calculate the Ct value, the baseline, is adjusted to the point where the increase in PCR product, as detected by fluorescence, becomes exponential (i.e. the amount of target cDNA is doubled following each cycle of the reaction) (**Figure 2.2**). It is important that for each primer pair, this is kept consistent, so that the Ct value can be determined accurately, and so that gene expression between samples can be compared.

Changes in gene expression can be seen from the shift of a Ct value to the left or the right of the initial curve. For instance, in the event that more target cDNA is present, the curve will shift to the left. This is because the Ct value will be lower, as the point at which the target sequence is detected by florescence occurs at earlier cycles. In the case that a sample expresses less of a target gene, the curve will shift to the right and a higher Ct value will be observed. This is due to more cycles being required for the detection of the target cDNA to be achieved (**Figure 2.2**).

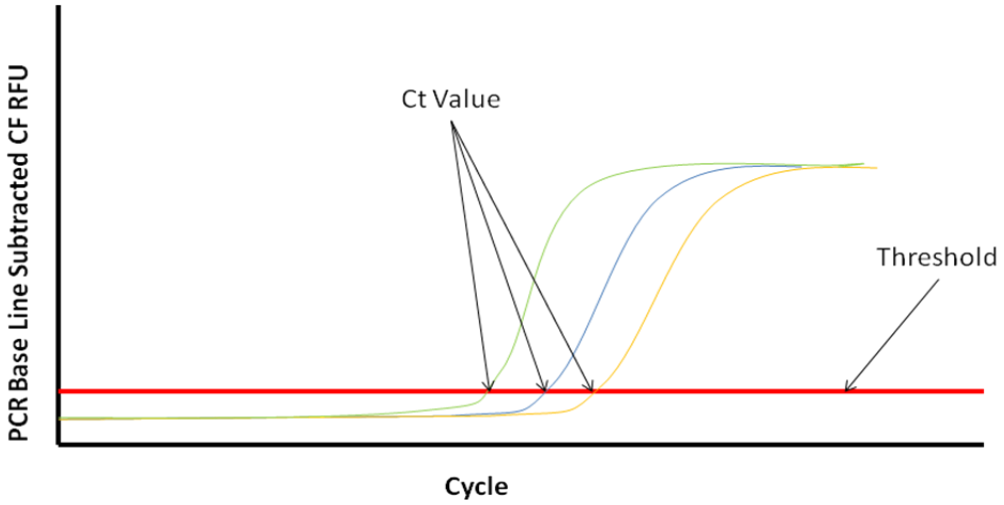

Figure 2.2: A hypothetical amplification curve obtained following real-time PCR. Cycle number is plotted against arbitrary fluorescence. The threshold is shown in red and is set at the point at which fluorescence becomes exponential. The cycle at which the curve crosses the threshold is known as the Ct value. In this example the blue line shows the gene product of a sample. If, under specific conditions, expression levels of the gene of interest increases, more gene product is present and the curve shifts to the left (green). If, under the same conditions, gene expression decreases, less gene product is present and the curve shifts to the right (orange).

As increases in fluorescence could be potentially generated by SYBR Green binding to non-target amplification fragments, such as primer dimers, products of mis-priming and genomic DNA, melting

curves are used to ensure that these increases in fluorescence are a result of increases in the target product. PCR products of individual primer pairs have specific melting temperatures (Tm), which is determined by the base composition and length of the target sequence, hence the Tm of target product should match that estimated for each primer pair. The Tm is determined by increasing the temperature a fraction of a degree at a time and measuring changes in fluorescence. When the Tm is reached, the double stranded DNA separates and the fluorescence rapidly decreases.

The melting curve depicts temperature (x-axis) against the relative fluorescence units over time (dRFU/dT) (y-axis) with peaks corresponding to PCR product Tm. Primer dimers or miss-priming resulting in amplification of DNA fragments other than the gene of interest results in extra peaks, which are normally present to the left of the actual Tm. This is because primer dimers and alternative amplification products are usually short in length and consequently have a lower Tm than the gene of interest. Contamination with genomic DNA would result in increased Tm values, as genomic DNA is longer than cDNA (as it contains both introns and exons), and would be depicted as peaks to the right of the actual Tm on melting curves (**Figure 2.3**).

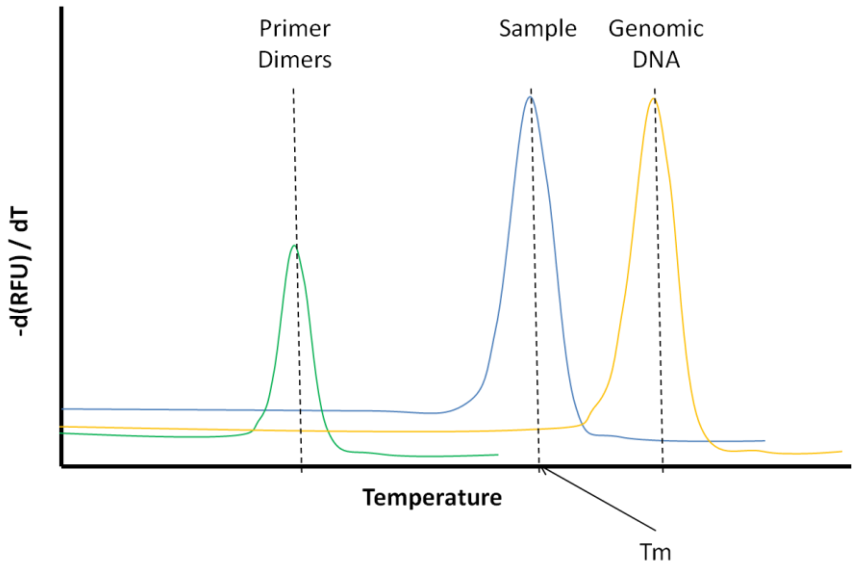

Figure 2.3: A hypothetical of a melt curve following real-time PCR. The rate of change in relative fluorescence with time (-d(RFU) / dT) is plotted against temperature. The various peaks show the melting temperature (Tm). The blue peak shows the actual Tm for a sample. Primer dimers produce peaks at lower temperatures (green), whereas genomic DNA contaminations produces peaks at higher temperatures (orange).

2.4 Assessing the Effects of Estrogens and Anti-Estrogens on Proliferation

The effect of estrogens on the proliferation of ER-competent non-tumourigenic MCF-12A cell lines is, to our knowledge, not documented. We, therefore, decided to investigate how estrogens impact upon the proliferation of these cells and whether this effect was mediated by the ER or GPER-1. This will enable us to more thoroughly understand how these cells respond to estrogens. In future chapters, it will also allow us to compare the effects of estrogens on MCF-12A cells in monolayer and 3D cultures. Furthermore, in order to optimise the optimal seeding density and maintenance of MCF-12A cells in 3D cultures, it is important to investigate whether these cells proliferate in the presence of estrogens. Proliferation studies will also be conducted in MCF-10A, MCF-7 and MDA-MB-231 cells for the purposes of comparison of the impact of estrogens in non-tumourigenic and cancer cell lines and in ER positive and ER negative cells.

Estrogens have previously been shown to induce proliferation in both normal and cancerous cell lines, and these effects have been associated with the presence of the ER. The ER-mediated effects elicited by estrogens are antagonised by ER antagonists, such as tamoxifen and ICI 182,780. More recently, however, it has been reported that tamoxifen and ICI 182,780 are capable of activating signalling pathways in some tissues, including that of the breast (Cabot *et al*, 1997; Wang *et al*, 2006). This may have consequences for the responsiveness to anti-estrogen therapy, as the non-genomic effects of these antagonists may counteract the desired effect of abrogating estrogen responsiveness to suppress tumour growth.

Tamoxifen is classed as a selective estrogen receptor modulator (SERM) and it is only capable of inhibiting the transcription of estrogen-inducible genes under the control of the AF-2 domain of the ER. The term, SERM, is applied to tamoxifen because in different tissues, the dominance of AF-1 and AF-2 differs, and thus, tamoxifen is selectively antagonistic to tissues where AF-2-activated transcription is dominant. For example, in the breast, AF-2 mediated transcription is dominant, so tamoxifen acts as an antagonist. In contrast, in the uterus, AF-1 is dominant, and therefore, tamoxifen acts as an agonist. ICI 182,780, on the other hand, is classed as a selective estrogen receptor disrupter (SERD) and inhibits the activity of both AF-1 and AF-2 of the ER in addition to promoting proteasome-mediated degradation of the ER-ICI 182,780 complex (Giamarchi *et al*, 1999; Wijayaratne & McDonnell, 2001; Pearce & Jordan, 2004). Due to tamoxifen only blocking AF-2 activity, it can only antagonise the effects of E2 where this domain is dominant and thus leaves the AF-1 domain of the ER susceptible to activation. This means that the use of tamoxifen may only prevent the estrogen-induced transcription of genes under the

control of the AF-2 domain. Furthermore, tamoxifen is postulated to act as an agonist for the transcription of genes under the control of the AF-2 domain (Tzukerman *et al*, 1994; McDonnel *et al*, 1995). Therefore, the pure ant-estrogen, ICI 182,780 will be used for all experiments to abolish both functioning domains of the ER and circumvent any agonist activity towards the ER.

2.5 Materials and Methods

2.5.1 Test Agents and Other Chemicals

E2 (98% pure) was obtained from Sigma (Dorset, UK) and prepared in absolute ethanol as 1 mM stock solutions. Subsequent dilutions were prepared using absolute ethanol also. ICI 182,780 was a kind gift from Dr. Ian White (MRC Molecular Endocrinology Group, Leicester, UK) and was prepared in absolute ethanol (etOH), as 1mM stocks. Subsequent dilutions were prepared using absolute ethanol. Dilutions were prepared so that the final ethanol concentration would not exceed 0.5%, to ensure that the solvent would have no detrimental effects upon cells (Payne *et al*, 2000).

The GPER-1 agonistG-1 and GPER-1 antagonist G-15 were purchased from Sigma and prepared as per manufacturers instruction as 1 mM stocks in dimethyl sulfoxide (DMSO). Subsequent dilutions were also prepared using DMSO. All stock solutions and dilutions were stored in critically cleaned glass vials and stored in a -20^{o}C spark free freezer.

2.5.2 Routine Culture of Cells

MCF-10A and MCF-12A cells (obtained from the American Type Culture Collection) were maintained in 75 cm^2 flasks (Greiner, Gloucestershire, UK) flasks in supplemented Dulbecco's Modified Eagle Medium and Ham's F-12 nutrient mixture (DMEM/F12, Invitrogen, Paisley, UK) (**Table 2.1**) at 37^{o}C in the presence of 5% CO_2. Upon 70% confluence, cells were sub-cultured using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Invitrogen, Paisley, UK).

Briefly, media was aspirated and the surface of the flask was washed gently with 10 ml Hanks balanced salt solution (HBSS). This was aspirated and 2 ml 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) was added, which was then aspirated so that only a thin film covered the surface of the flask. Cells were then incubated at 37^{o}C in 5% CO_2 for approximately 10 minutes until cells were completely detached from the flask upon gentle tapping to avoid clonal selection. 4 ml of re-suspension media was added and cells were re-suspended and transferred to a 15 ml conical tube. A further 4 ml or

re-suspension media was added to rinse the flask and this was transferred to the conical tube. It is important at this point to use re-suspension media containing 20% horse serum, as this is necessary to inactivate the trypsin and allow re-attachment of cells in new flasks. Cells were centrifuged at 1000 rpm for 5 minutes and the re-suspension media was carefully aspirated so that only the cell pellet remained. The pellet was then re-suspended in 10 ml growth media and seeded into fresh 75 cm^2 flasks containing 13 ml fresh growth media at a dilution of 1:5 or 1:6. Upon 70% confluence, this process was repeated for a maximum of 10 subcultures.

Table 2.1: Media components for MCF-10A and MCF-12A media (Soule *et al*, 1990; Debnath *et al*, 2003).

Component	Growth Medium	Re-Suspension Medium
DMEM/F12 (Invitrogen, Paisley, UK)	500 ml	500 ml
Horse Serum (Invitrogen, Paisley, UK)	5%	20%
Epidermal Growth Factor	20 ng/ml	20 ng/ml
Cholera Toxin (Sigma-Aldrich, Dorset, UK)	100 ng/ml	100 ng/ml
Insulin (Sigma-Aldrich, Dorset, UK)	10 μg/ml	10 μg/ml
Hydrocortisone (Sigma-Aldrich, Dorset, UK)	0.5 μg/ml	0.5 μg/ml
Pen/Strep (Sigma-Aldrich, Dorset, UK)	5000 μg/ml	5000 μg/ml
L-Glutamine (MCF-10A media only)	109.5 mg/2.8 ml	109.5 mg/2.8 ml

EGF (Sigma-Aldrich, Dorset, UK) was prepared as a 100 μg/ml stock in 0.2 μm-filtered 10 mM acetic acid with 0.1% bovine serum albumin (BSA, Sigma-Aldrich, Dorset, UK).

MCF-7 cells were obtained from M. Dufresne (University of Windsor, Ontario, Canada). MDA-MB-231 cells were a kind gift from Dr. Michael Johnson (Lombardi Cancer Centre, Washington DC, USA).

MCF-7 and MDA-MB-231 cells were maintained in 75 cm^2 flasks in supplemented MEM-α (Invitrogen, Paisley UK) with 5% fetal bovine serum (FBS) at 37°C in the presence of 5% CO_2. Upon 70% confluency, cells were sub-cultured using trypsin-EDTA. This was achieved by aspirating the media and washing the surface of the flask gently with 10 ml HBSS. This was then aspirated and 2 ml 0.25% trypsin-EDTA was added. Flasks were placed in an incubator at 37°C in 5% CO_2 for approximately 3 minutes until cells were completely detached from the flask upon gentle tapping to avoid clonal selection. Cells were then re-suspended in 10 ml growth media and seeded into fresh 75 cm^2 flasks containing 13 ml growth full media at a dilution of 1:10 or 1:20. Upon 70% confluence, this process was repeated for a maximum of 10 subcultures. In a similar fashion to the previously described cell lines, MCF-7 and MDA-MB-231 cells were regularly tested for mycoplasma infection.

Due to batch variation amongst serum lots concerning fat and protein levels, which may impact upon experimental results, serum of the same batch number was used throughout and stored at -20°C before use.

2.5.3 Preparation of Charcoal-Dextran Stripped (CDS) Serum

Charcoal-dextran stripping removes the hormones in serum that would otherwise be present as free or protein bound, and could potentially interfere with our experimental outcomes. This procedure was performed as described by Soto *et al* (1995), as follows: a suspension of 5% charcoal and 0.5% Dextran T70 (Amersham, Buckinghamshire, UK) was prepared in 500 ml ultra-high quality (UHQ) water and was left to mix by rolling at 10 cycles/min for 30 min at room temperature. The suspension was then centrifuged for 10 min at 1000g, the supernatant removed and the serum added to the pellet. This was mixed by rolling at 10 cycles/min for 1 h at room temperature. Finally, the mixture was centrifuged at 50,000 g for 20 min at 4°C and the CDS serum was filtered through a 0.2 µm nylon filter (Nalgene, Dorset, UK). CDS serum was stored at -20°C until use.

2.5.4 Cell Culture for Gene Expression Analysis

80,000 cells per ml were seeded in growth media in 25 cm^2 flasks and, upon 70% confluence, the media was aspirated and replaced with D-MEM/F12 (MCF-10A and MCF-12A) or MEM-α (MCF-7 and MDA-MB-231) phenol-red free media (Invitrogen, Paisley, UK) containing 0.5% CDS horse serum for MCF-10A and MCF-12A cells or 0.5% FBS for MCF-7 and MDA-MB-231 cells. The use of phenol red-free media ensures that the estrogenic properties of phenol red do not influence ER expression and levels of activation, as it

has been shown to act as a weak estrogen (Berthois *et al*, 1986). After 24 hours, cells were treated with 1 nM E2 (98% pure, Sigma-Aldrich, Dorset, UK) or 0.5% etOH for a further 24h. Cells were incubated at 37^{o}C in 5% CO_2 throughout.

2.5.5 Selection of Suitable Reference Genes

In order to quantify gene expression, data needs to be normalised to a reference gene to allow for inter-PCR variation, such as sample loading errors, RNA integrity, and the efficiency of the RT. Commonly used reference genes, such as *GLYCERALDEHYDES-3-PHOSPHATE* (*GAPDH*), *β-ACTIN* and *HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE* (*HPRT*), are present in most cell types, as they are essential for cell survival. Moreover, their expression is stable and usually unaffected by treatment conditions (Bustin, 2002). In order to be a suitable reference gene, this must be unaffected by the treatments to which samples are exposed to, and thus prior to work on gene expression, the effects of E2 on the expression of the potential reference genes: *GAPDH*, *β-ACTIN* and *HPRT* were assessed in all cell types.

2.5.6 RNA Extraction and RNA Quantification

Following 24 h treatment, cells were trypsinized using 1 ml 0.25% Trypsin-EDTA. The cells were re-suspended in a total of 7 ml growth media and centrifuged at 0^{o}C for 15 minutes. RNA extraction was performed using Nucleospin RNA II (Machery-Nagel, Düren, Germany) as per manufacturer's instructions.

Following the removal of media to leave just the cell pellet, the cells were lysed by vortexing with the addition of 350 µl RA1 buffer and 3.5 µl β-mercaptoethanol. The lysates were filtered at 11,000 g for 1 minute to reduce viscosity and the binding conditions were adjusted with the addition of 350 µl 70% ethanol to the filtrate. This was loaded to RNA binding columns which were spun at 11,000 g for 30 sec. The silica membranes of the binding columns were desalted using 350 µl membrane desalting buffer and centrifugation at 11,000 g for 1 min. To digest contaminating genomic DNA, samples were incubated with 95 µl deoxyribonuclease (DNase) reaction mixture for 15 min, at room temperature followed by washing and drying of the silica membrane with buffers and centrifugation at 11,000 g 3 times. Finally, RNA was eluted in 40 µl RNase (ribonuclease)/DNase free water at 11,000 f for 1 min into RNase/DNase free collection tubes.

RNA concentration and purity was assessed using the ND-1000 Spectrophotometer (NanoDrop, Thermo Scientific, Delaware, USA). 2 µl of extracted RNA was pipetted onto the pedestal of the instrument and absorbance was read at 230, 260 and 280 nm. Absorbance at 260 nm measures RNA concentration and RNA purity is measured using the ratio of 260/280 nm absorbance with a value between 1.8 and 2 being a generally acceptable value. Measuring the purity ensures that the measured concentration is of RNA only and not of contaminants, such as proteins, carbohydrates and phenol, or chemicals used in the RNA extraction process. Values lower than the generally accepted range may give an indication of contaminates that absorb wavelengths of approximately 280 nm, such as those aforementioned. The 260/230 nm ratio provides a second measurement of RNA purity. Accepted values range between 1.8 and 2.2, however this value will be less if co-purified contaminates are present in the sample, such as proteins.

2.5.7 Reverse Transcription (RT)

2.5 µg isolated total RNA corresponding to a final concentration of 0.5 µg/µl were used for each reaction. For each sample: 7 µl 5x RT buffer (Promega, Southampton, UK), 4 µl 10 mM deoxynucleotide triphosphates (dNTPs) mix (Stratagene, Cheshire, UK), 1 µl RNAse inhibitor (Promega, Southampton, UK) and 1 µl random hexamer primers (Invitrogen) were added to a volume of 20 µl comprising of 2.5 µg total RNA and RNAse/DNAse-free water (Promega, Southampton, UK).

Samples were heated to 65^{o}C for 10 minutes and cooled on ice for 2 minutes prior to 42^{o}C incubation with 2 µl Reverse Transcriptase (Promega, Southampton, UK) for 90 minutes. cDNA samples were stored at -80^{o}c until use.

2.5.8 Real-Time Polymerase Chain Reaction (Real-Time PCR)

Primer pairs were prepared as 10 µM stocks using RNase, DNase free water, except in the case of the HPRT primers, which were prepared as 1 µM stocks. All primer pairs were optimised and only those that yielded an amplification efficiency of > 98% were used for further work.

For each reaction: 0.8 µl cDNA from a 1:10 stock, 10 µl SYBR Green mixture (Biorad, Herts, UK), forward and reverse primers at the concentrations listed in **Table 2.2** and RNase, DNase free water to make a final volume of 20 µl was added.

Table 2.2: Primers used for real-time PCR. Primers were designed based on their Genebank ascension number using Beacon Designer 5 (Premier Biosoft, California, USA) and purchased as high quality oligos from Eurogentec (Hampshire, UK).

cDNA	Genebank Ascension Number	Primer Sense	Primer Sequence (5'-3')	Concentration (nM)	Target Size (bp)
β-ACTIN	X00351	Forward	TGCTATCCAGGCTGTGCTAT	300	97
		Reverse	GATGGAGTTGAAGGTAGGTT	300	
GAPDH	NM_002046	Forward	TCTCTGCTCCTCCTGTTC	900	120
		Reverse	GCCCAATACGACCAAATCC	900	
HPRT	M31642	Forward	CCTTGGTCAGGCAGTATAATCC	100	135
		Reverse	GGGCATATCCTACAACAAACTTG	100	
ERα	NM_000125	Forward	GCCCTCCCTCCCTGAAC	250	146
		Reverse	TCAACTACCATTTACCCTCATC	250	
ERβ	AF051A27	Forward	TCCTCCCAGCAGCAATCC	200	138
		Reverse	CCAGCAGCAGGTCATACAC	200	
GPER-1	BC011634	Forward	GTTCCTCTCGTGCCTCTAC	200	130
		Reverse	ACCGCCAGGTTGATGAAG	200	

The real-time PCR reaction was performed using the iCycler IQ real-time PCR detection system (Biorad, Herts, UK). cDNA was denatured at 95°C for 3 minutes and then cooled to 55°C to allow primer binding. 40 subsequent cycles of heating to at 95°C for 1 minute and cooling to 55°C for 1 minute were performed to allow denaturing of the newly produced strands, further annealing and cDNA amplification (**Figure 2.4**). Amplification and melt curves were generated upon real-time PCR protocol completion.

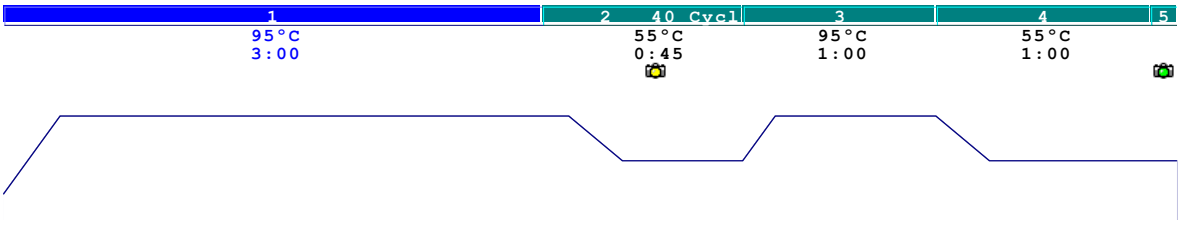

Figure 2.4: Real-time PCR cycle protocol (iCycler, Biorad).

2.5.9 Protein Level Confirmation

As the presence of a gene does not always correlate with protein expression, western blots were performed to confirm the presence of ERα, ERβ and GPER-1.

1 million cells per ml were seeded onto 55 mm petri dishes in growth media and maintained at 37^{o}C in 5% CO_2. Upon 80% confluence, the media was aspirated and replaced with phenol red-free media containing 0.5% CDS stripped serum for 24h at 37^{o}C in 5% CO_2. Following 24 h incubation, samples were placed on ice, the media removed and the dish was rinsed twice with cold HBSS.

Lysis buffer was prepared consisting of 2.5 ml 1 M Tris pH 6.8, 2 ml 20% sodium dodecyl sulfate (SDS), 4 ml glycerol and 2 ml β-mercaptoethanol. 200 µl was added to each sample and a cell scraper was used to dislodge the cells. The viscous cell solution was then transferred to pre-chilled eppendorf tubes and the samples were boiled for 10 minutes at 95^{o}C. After brief centrifugation, the supernatant was transferred to a fresh pre-chilled eppendorf tube.

To determine the protein concentration, the Bradford assay was conducted. For that, standards were prepared using bovine serum albumin (BSA, Sigma-Aldrich, Dorset, UK) from a 2 mg/ml stock (**Table 2.3**) to prepare a calibration curve in order to quantify protein level of the samples. For the samples: 500 µl water, 2 µl sample and 500 µl Bradford reagent were combined in eppendorf tubes. 250 µl of standard and sample preparation were loaded in duplicate onto a 96 well plate and absorbance was read to measure protein amount. Following quantification, 6 µl of bromophenol blue was added to each sample in order to facilitate loading onto acrylamide gels.

Table 2.3: Preparation of standards to generate a calibration curve in order to quantify protein concentration in samples.

Protein Concentration (µg/µl)	Water Volume (µl)	BSA Standard Sample Volume (µl)	Bradford Reagent Volume (µl)
0	500	0	500
2	500	2	500
4	500	4	500
6	500	6	500
8	500	8	500
10	500	10	500

12% SDS-acrylamide running gels (**Table 2.4**) were prepared and allowed to polymerize. To prevent bubble formation, a layer of saturated butanol was added on top of the gel and then removed using

blotting paper. A 5% SDS-acrylamide gel (**Table 2.4**) was loaded above this and combs inserted to create wells. Upon polymerization, the combs were removed.

Table 2.4: Running and stacking gel compositions.

Gel	Component	Volume (ml)
12% Running Gel	Water	3.3
	30% acrylamide mix	4
	1.5 M Tris-buffer (pH 8.8)	2.5
	10% SDS	0.1
	10% ammonium persulfate	0.1
	Tetramethylethylenediamine (TEMED)	0.004
5% Stacking Gel	Water	2.1
	30% acrylamide mix	0.5
	1.5 M Tris-buffer (pH 6.8)	0.38
	10% SDS	0.03
	10% ammonium persulfate	0.03
	TEMED	0.003

The electrophoresis tank was assembled and the wells washed thoroughly to remove all traces of residual acrylamide. 10 µl protein marker (Cell Signalling, Hertfordshire, UK) was added. An equal amount of total protein was loaded for every sample into the wells of the prepared gels (to allow for comparisons between samples) and separated by SDS-PAGE for 2 h at 80 V. The proteins were then transferred onto nitrocellulose membranes (Amersham, Buckinghamshire, UK) in transfer buffer consisting of 25 mM Tris, glycine, 0.1% SDS and 20% methanol at 40 V for 1 h.

Unspecific binding was blocked using 5% milk in Tris buffered saline (TBS) containing 0.05% Tween (TBS-T) for 1 h. The membranes were then washed twice with TBS containing 0.05% Tween for 5 minutes and incubated overnight at 4^{o}C with the primary antibody (antibody dilutions are presented in **Table 2.5**).

Table 2.5: Primary antibodies used for western blot analysis.

Primary Antibody	Source	Dilution
ERα (Sigma-Aldrich, Dorset, UK)	Rabbit	1:3000 in 5% BSA in TBS-T
ERβ (Epitomics, Insight Biotechnology Ltd, Wembley, UK)	Rabbit	1:1000 in 5% BSA in TBS-T
GPER-1 (Abcam, Cambridge, UK)	Rabbit	1:1000 in 5% BSA in TBS-T
β-actin (Cell Signalling, Hertfordshire, UK)	Mouse	1:1000 in 5% milk in TBS-T

Samples were then washed twice with TBS containing 0.05% Tween for 5 minutes and incubated with 1:2000 mouse anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling Technology, New England Biolabs, Hertfordshire, UK) in TBS containing 0.05% Tween and 5% milk for 1h. Blots were then washed three times for 5 minutes with TBS containing 0.05% Tween.

Enhanced chemiluminescence (ECL) detection was used to visualise results in a dark room. In brief, 50 µl luminol (250 mM) and 25 µl coumaric acid (90 mM) were added to 10 ml ECL buffer (100 mM Tris-Hcl pH 8.5) and 4 µl H_2O_2. Membranes were drained, placed on a flat surface and incubated with ECL solution for 1 min. The excess solution was drained and the membranes placed between two sheets of acetate. Membranes were exposed onto Amersham Hyperfilm™ ECL (GE Healthcare, Buckinghamshire, UK) in a hypercassette (GE Healthcare, Buckinghamshire, UK) for 60/30/20/10 seconds. The autorads were developed in 1:5 developer (Kodak, Sigma-Aldrich, Dorset, UK) rinsed in distilled water and fixed in 1:5 fixer and replenisher solution (Kodak, Sigma-Aldrich, Dorset, UK) for 2 min.

2.5.10 Cell Proliferation Studies

To investigate the effects of estrogens on cellular proliferation, cells were treated with 1 nM E2 alone or with a combination of E2 and the ER antagonist ICI 182,780. In addition to this, cells were incubated with and without EGF, to investigate the necessity of this growth factor for cell growth.

10,000 cells per ml were seeded onto 24 well plates daily for 7 days in phenol red-free assay media with 0.5% CDS serum to eliminate any sources of contaminating estrogens. Samples were treated with the following: phenol red-free media and 0.5% CDS serum + 0.5% etOH (Control), 20 ng/ml EGF + 0.5% etOH, 1 nM E2 + 20 ng/ml EGF, 1 nM E2 + 100 nM ICI 182,780 + 20 ng/ml EGF, 100 nM ICI 182,780 + 20 ng/ml EGF. For cells co-treated with ICI 182,780, 30 minute pre-incubation with ICI 182,780 was carried out prior to addition of E2.

The sulforhodamine B (SRB) assay was used to assess cell proliferation of each cell line in response to various treatments, using the protocol previously described (Silva *et al*, 2006): After 7 days, the plates were placed on ice for 1 minute and the media was removed. To each well, 100 µl 10% trichloroacetic acid (TCA) was added and the plates were left on ice for 20 minutes. The plates were then washed 5 times in cold water and left to air dry. 100 µl 4% SRB solution was added to each well and the samples were left for 20 minutes. The plates were then washed with 1% acetic acid to remove unbound dye and left to air dry in the dark. The bound dye was solubilised using 200 µl 10 mM Tris-base (pH 10.5), the solution transferred to a 96-well plate and absorbance was read at 510 nm to quantify cell number.

2.5.11 The Role of GPER-1 in Cell Proliferation

In addition to investigating the impact of E2 on cell proliferation and the role of the ER, the role of GPER-1 was also investigated. G-1 was used as an agonist for GPER-1 (Albanito *et al*, 2007) and G-15 was used as a GPER-1 antagonist (Dennis *et al*, 2011). G-1 and G-15 were prepared as described previously.

Cells were seeded as described previously and treated with the following: phenol red-free media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO (control), 0.5% etOH, 1 nM E2, 1 nM E2 + 10 nm G-15, 1 nM E2 + 10 nM G-15 + 100 nM ICI 182,780, 100 nM G-1 or 100 nM G-1 + 10 nM G-15. All samples other than controls were also treated with 20 ng/ml EGF as we observed that this was required for growth in the MCF-10A and MCF-12A cell lines. For samples treated with G-15 or ICI 182,780, a 20 minute pre-incubation with the antagonists was performed prior to addition with E2 or G-1.

2.5.12 Statistical Analysis of Real-Time PCR Data

Experimental variation between samples can be controlled and real-time PCR results quantified through the use of a reference gene. This allows you to see the changes of expression of target genes relative to

a reference gene using the relative expression software tool (REST©) (Pfaffl *et al,* 2002). REST allows the comparison of two data sets and permits the statistical analysis of these results.

A reference gene, that showed no changes in expression following E2 treatment, was used to normalise gene expression for all further experiments. The relative expression ratio (R) of the target gene was calculated using the Pfaffl's mathematical equation (Pfaffl, 2001) that uses the amplification efficiency (E) and threshold cycle (Ct) to determine whether a target gene's expression is up-regulated or down-regulated in response to a treatment. The model also incorporates the expression of the reference gene under control and test conditions (**Equation 2.1**).

$$R = \frac{E_{target}^{\Delta Ct_{target}\,(Mean_{control}-Mean_{treatment})}}{E_{reference}^{\Delta Ct_{reference}\,(Mean_{control}-Mean_{treatment})}}$$

Equation 2.1: The Pfaffl equation used to determine the relative expression ratio (R), where E is amplification efficiency and Ct is threshold cycle (Pfaffl, 2001).

To determine whether up- or down-regulations of *ERα*, *ERβ* or *GPER-1* were statistically significant, the pair wise fixed reallocation randomisation test was performed (REST, Pfaffl *et al,* 2002). This statistical test makes no assumptions concerning the distribution of data, which is necessary given the quantities being tested are ratios and subject to variability. The result is the calculation of a *p-value* (the chance that a data point occurred randomly, and not as a consequence of a treatment). This is achieved by repeatedly and randomly pairing the sample data for target and reference genes of control and sample conditions, and then calculating the expression ratios based on the mean. A *p-value* of < 0.05 was considered statistically significant.

2.5.13 Statistical Analysis of Proliferation Curve Data

As we wanted to make comparisons between multiple treatments at various time points, we applied the 2-way analysis of variance (ANOVA) test to the proliferation data using the GraphPad Prism software. 2 way-ANOVA works by calculating the mean square for both variables (in this case, the time point and the various treatments), a total mean square of a group (the mean for each treatment), and a remainder mean square (total mean square minus both mean squares for each variable). The F-statistic (the

expected level of variation in a data set) for the treatments was determined by dividing the effect mean square for treated samples by the remainder mean square. Finally the p-value was calculated and a value of < 0.05 was considered statistically significant.

2.6 Results

2.6.1 Selection of A Suitable Reference Gene

In order to select an appropriate reference gene for the experimental work, three genes, regularly used as references were tested for response to estrogen treatment in all cell lines. These were *GAPDH*, *β-ACTIN* and *HPRT*. The aim of this work was to indentify one gene which, in terms of expression, was not altered by estrogen treatment in any of the cell lines used. Changes in gene expression would be observed as shifts of the curve, following E2 treatment, to the left or to the right of the curve for that of solvent controls. A shift of the curve to the left would indicate a gene up-regulation, whereas, a shift to the right would be indicative of a gene down-regulation, relative to solvent controls.

GAPDH expression was down-regulated in MCF-10A cells and up-regulated in MDA-MB-231 cells in response to E2 treatment and was, therefore, an unsuitable candidate for use as a reference gene.

HPRT expression was expressed at low levels in MCF-10A and MCF-12A cells and E2 treatment down-regulated its expression even further. Additionally, expression was barely detectable in the MCF-7 and MDA-MB-231 cell lines and, therefore, *HPRT* was also unsuitable for use as a reference gene.

β-ACTIN was the only reference gene that was unaffected in terms of gene expression in all four cell lines and was selected as the reference gene for all further experiments (**Figure 2.5; 2.6; 2.7; 2.8**).

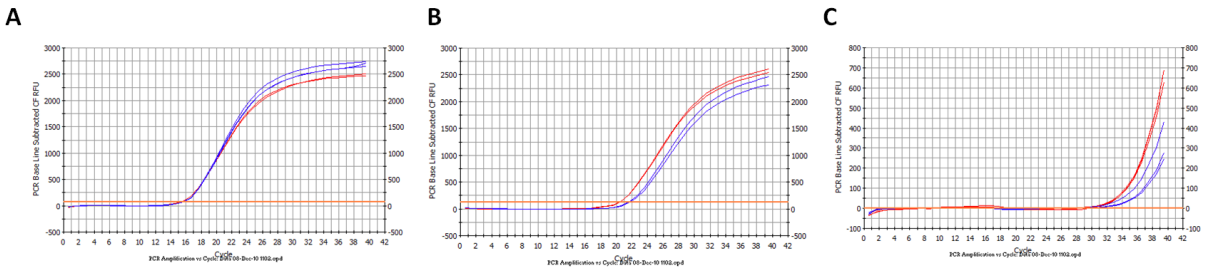

Figure 2.5: Reference gene PCR amplification curves for MCF-10A cells. (A) *β-ACTIN* **(B)** E2-induced down-regulation of *GAPDH* **(C)** E2-induced down-regulation of *HPRT*. Red lines are solvent control samples and blue lines are 1 nM E2-treated samples.

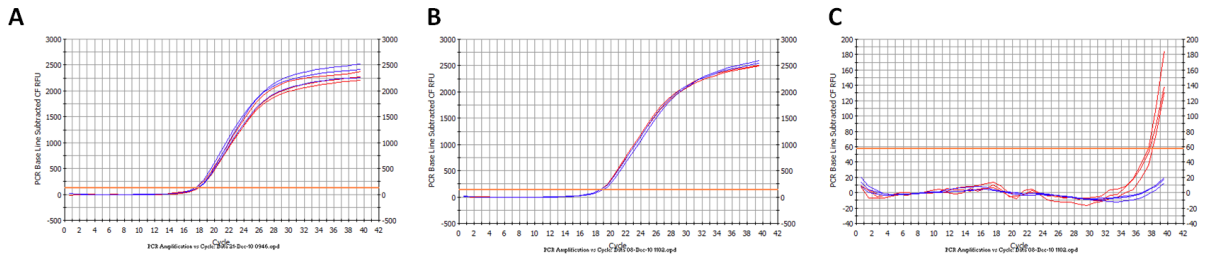

Figure 2.6: Reference gene PCR amplification curves for MCF-12A cells. (A) *β-ACTIN* **(B)** *GAPDH* **(C)** E2-induced down-regulation of *HPRT*. Red lines are solvent control samples and blue lines are 1 nM E2-treated samples.

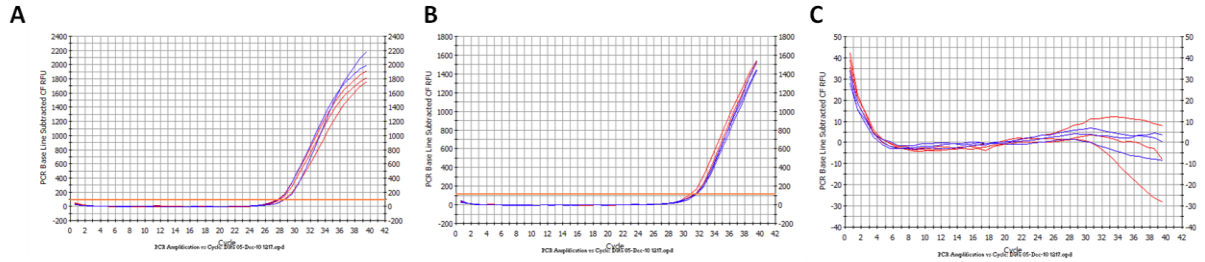

Figure 2.7: Reference gene PCR amplification curves for MCF-7 cells. (A) *β-ACTIN* **(B)** *GAPDH* **(C)** Lack of *HPRT* expression. Red lines are solvent control samples and blue lines are 1 nM E2-treated samples.

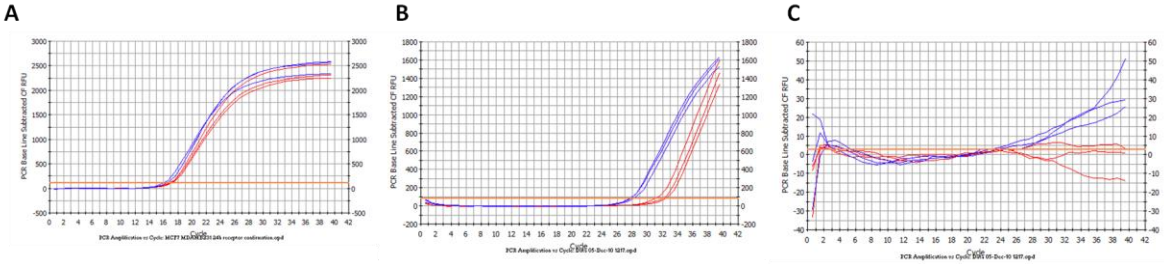

Figure 2.8: Reference gene PCR amplification curves for MDA-MB-231 cells. (A) *β-ACTIN* **(B**) E2-induced up-regulation of *GAPDH* **(C)** No *HPRT* expression detected. Red lines are solvent control samples and blue lines are 1 nM E2-treated samples.

2.6.2 Confirmation of Receptor Status at the Gene Level and the Effects of 17β-Estradiol

The ER status of the MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines has been characterized previously (Soule *et* al, 1990; Carmecci et al, 1997; Filardo *et al,* 2000; Engel *et al*, 2011). However, due to gene expression variation between cell batches, it was imperative to confirm the ERα, ERβ and GPER-

1 status of each cell line. First, this was achieved by detecting the corresponding genes by real-time PCR. Subsequently, western blots were also used to confirm the receptor status at the protein level.

Amplification curves for *ERα*, *ERβ* and *GPER-1* are shown below for solvent controls and E2-treated samples. Although some E2-treated samples show deviation from the solvent control curve, at this point, changes in gene expression, as a result of E2, cannot be confirmed. These could instead indicate that a different amount of cDNA was present in each sample. To confirm E2-induced up and down-regulation of the target genes, normalisation to the reference gene will be carried out and the REST analysis software tool will be used, and this will be discussed later. Nevertheless, as the levels of *β-ACTIN* did not change in the samples analysed, we present here the amplification curves for the different receptors, previous to normalisation, as well as their melt-curves, in order to provide a visual account of what was observed during gene expression detection.

As can be seen in **Figure 2.9**, *ERα* and *ERβ* could not be detected in MCF-10A cells. *GPER-1* was, however, expressed in MCF-10A cells and the amplification curve was shifted to the right upon E2 treatment, relative to solvent controls. This may indicate a down-regulation in *GPER-1* expression in response to E2.

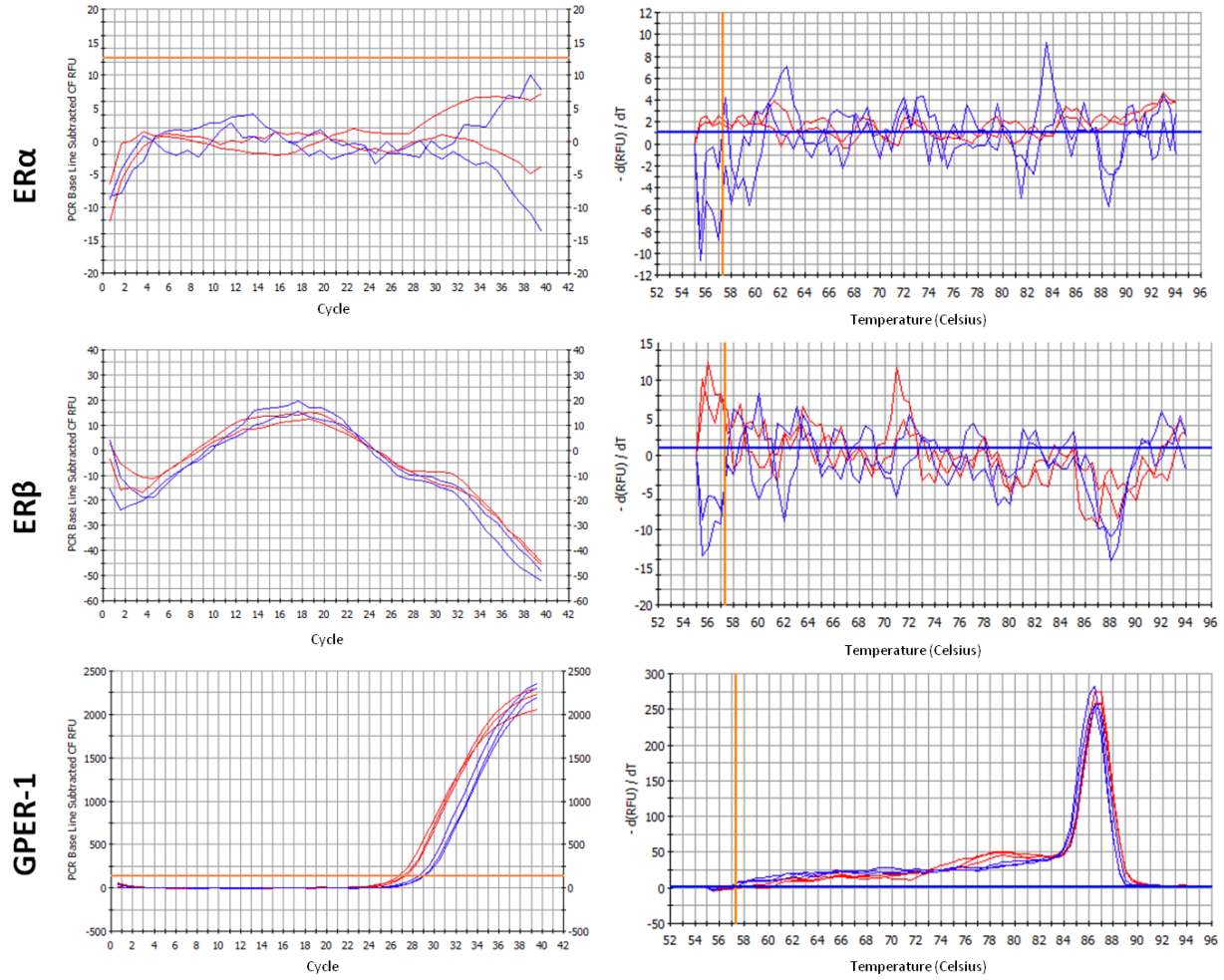

Figure 2.9: Real-time PCR amplification curves (left) and melt curves (right) for *ERα*, *ERβ* and *GPER-1* in etOH controls (red) and 1 nM E2-treated (blue) in MCF-10A cells. No *ERα* or *ERβ* was detected, and hence, no amplification or melt curves were generated. *GPER-1* was expressed in MCF-10A cells and E2 treatment down-regulated its expression, as indicated by a shift of the curve to the right. The melt curves for solvent controls and E2-treated samples for *GPER-1* are identical and show no indications of contamination or mis-priming.

MCF-12A cells expressed both *ERα* and *ERβ* (**Figure 2.10**) and E2 treatment did not seem to have a great effect on gene expression levels, although a slight decrease in ERα was noted upon E2 treatment. *GPER-1* was also expressed in MCF-12A cells and 24h treatment with 1 nM E2 shifted the curve to the right, relative to solvent controls. This may be indicative of a down-regulation in the expression of *GPER-1* following E2 treatment.

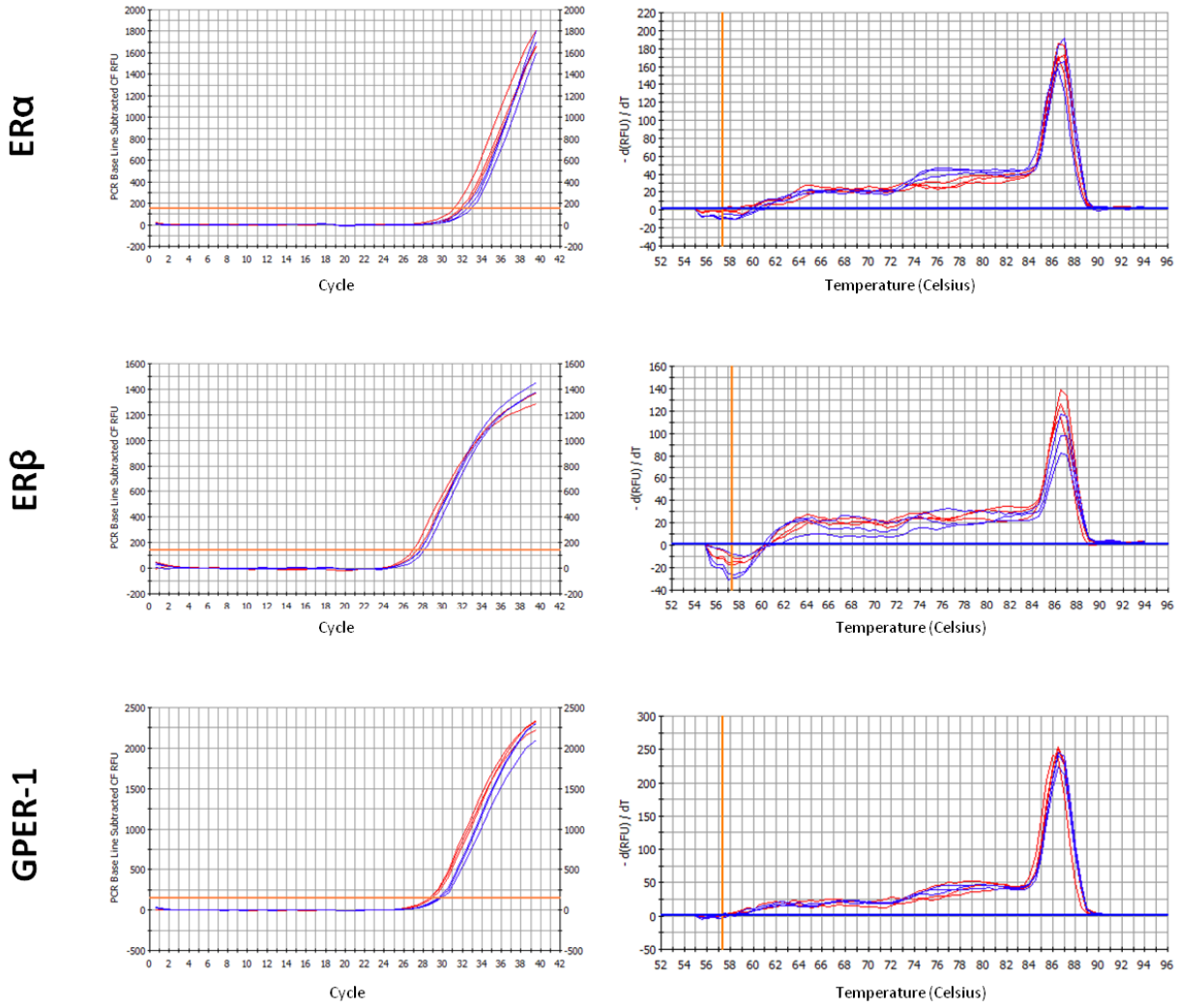

Figure 2.10: Real-time PCR amplification curves (left) and melt curves (right) for *ERα*, *ERβ* and *GPER-1* in etOH controls (red) and 1 nM E2-treated (blue) in MCF-12A cells. No shift of the curve was observed upon E2 treatment for *ERα* or *ERβ*. A slight shift to the right of the *GPER-1* amplification curve was observed for E2-treated cells, relative to controls. Melt curve analysis indicates that only the target product was amplified and there was no genomic DNA contamination, primer dimers or mis-priming.

At the gene level, it was observed that MCF-7 cells express *ERα*, *ERβ* and *GPER-1* at relatively high levels (**Figure 2.11**). 24 h treatment with 1 nM E2 led to a shift of the amplification curve of *ERα* and *ERβ* to the right and left, respectively. This may mean that *ERα* is down-regulated and *ERβ* is up-regulated in response to E2. *GPER-1* was expressed but appeared to be unaffected by E2 treatment (**Figure 2.11**).

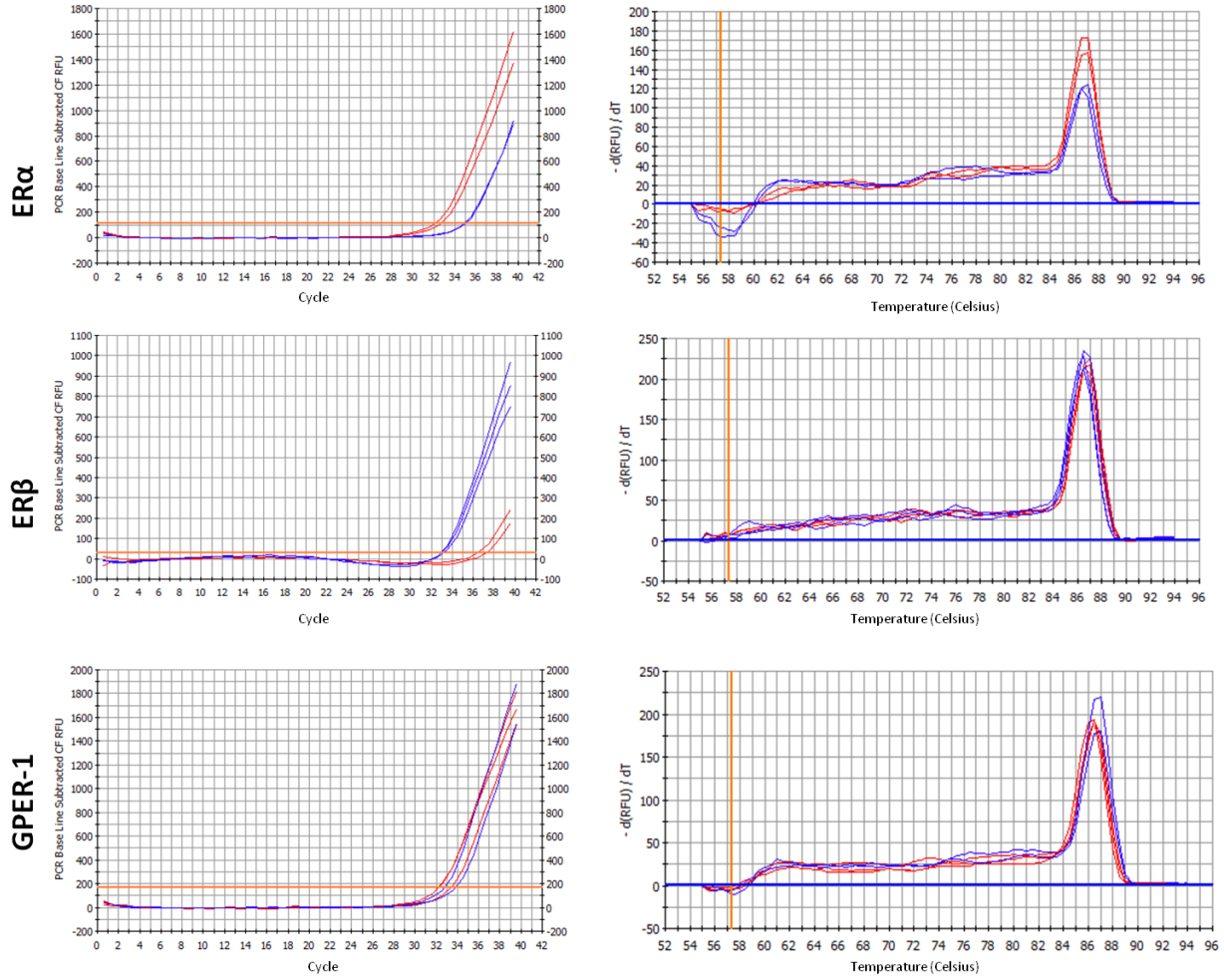

Figure 2.11: Real-time PCR amplification curves (left) and melt curves (right) for *ERα*, *ERβ* and *GPER-1* in etOH controls (red) and 1 nM E2-treated (blue) in MCF-7 cells. The amplification curve for *ERα* of E2-treated cells was shifted to the left relative to controls. The curve of *ERβ* was shifted to the left following E2 treatment, in comparison to the curve for solvent controls. The data for *GPER*-1 was more variable but it did not appear that E2 affected the amplification curve for this gene. Melt curve analysis showed no mis-priming, primer dimers or contamination with genomic DNA.

MDA-MB-231 cells express *ERβ* and *GPER-1* and upon E2 treatment, the amplification curves for these genes shifted to the left, relative to controls. This may indicate an up-regulation in these genes. *ERα* was not detected at the gene level in this cell line under control conditions. However, after treatment with 1 nM E2, *ERα* gene expression was observed (**Figure 2.12**).

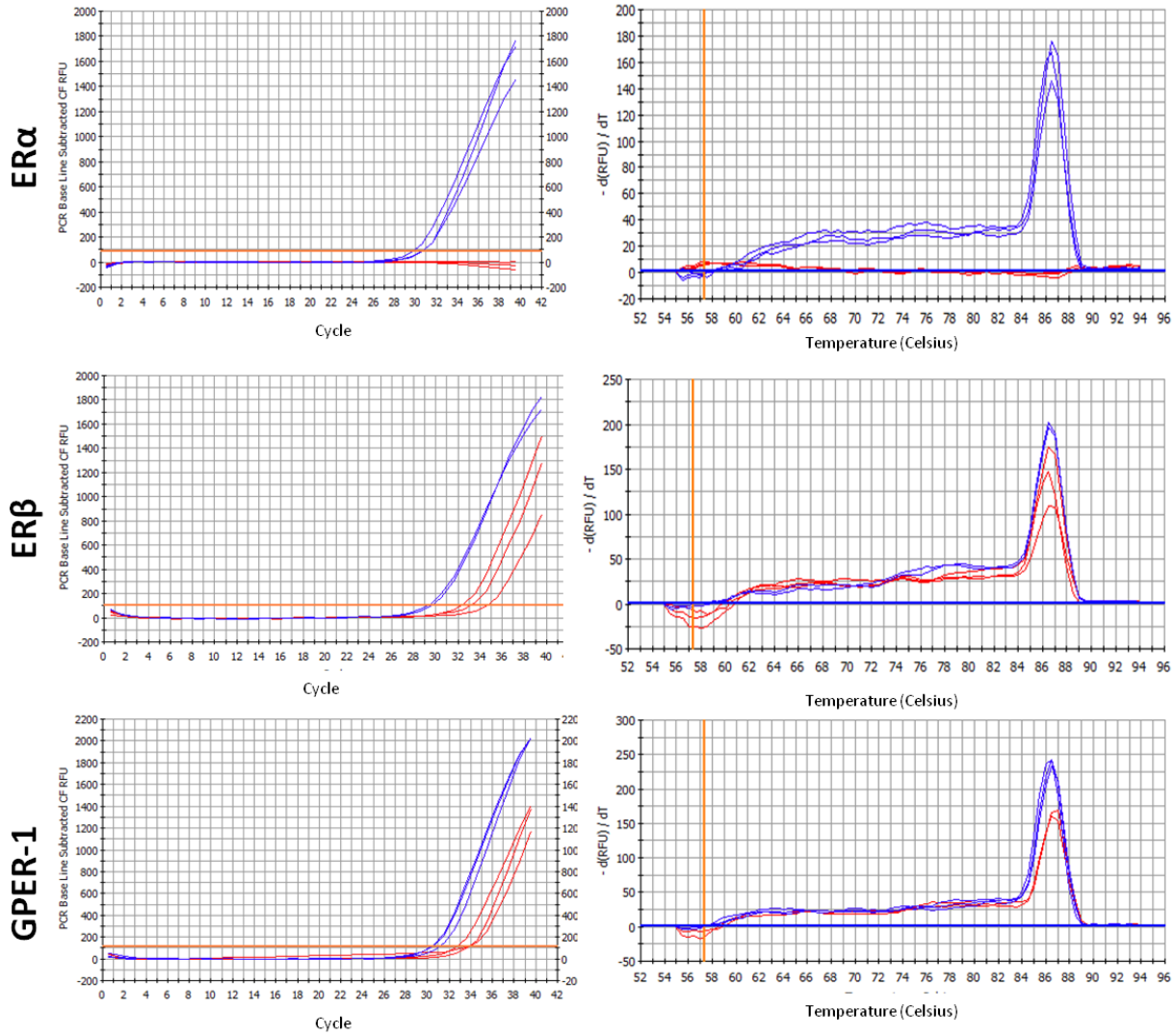

Figure 2.12: Real-time PCR amplification curves (left) and melt curves (right) for *ERα*, *ERβ* and *GPER-1* in etOH controls (red) and 1 nM E2-treated (blue) in MDA-MB-231 cells. *ERα* expression was only detected in E2-treated cells. E2 treatment shifted the amplification curves of *ERβ* and *GPER-1* to the left, relative to solvent controls, indicating an increase in gene expression. Melt curve analysis shows no signs of primer-dimers, mis-priming or genomic DNA.

2.6.3 Relative Expression Software Tool Analysis

After normalisation to the reference gene and according to the REST analysis, there was no significant change in the expression of ERα, ERβ or GPER-1 in MCF-10A and MCF-12A cells in response to E2. In MCF-7 cells, E2 decreased the gene expression of *ERα* and increased the expression of *ERβ*, but had no

effect on GPER expression. As MDA-MB-231 did not express *ERα* under solvent control conditions, treatments could not be compared with controls and, therefore the data is not presented on the graph below. E2 treatment, in addition to inducing the gene expression of *ERα*, increased the gene expression of *ERβ* and *GPER-1* in the MDA-MB-231 cell line (**Figure 2.13**).

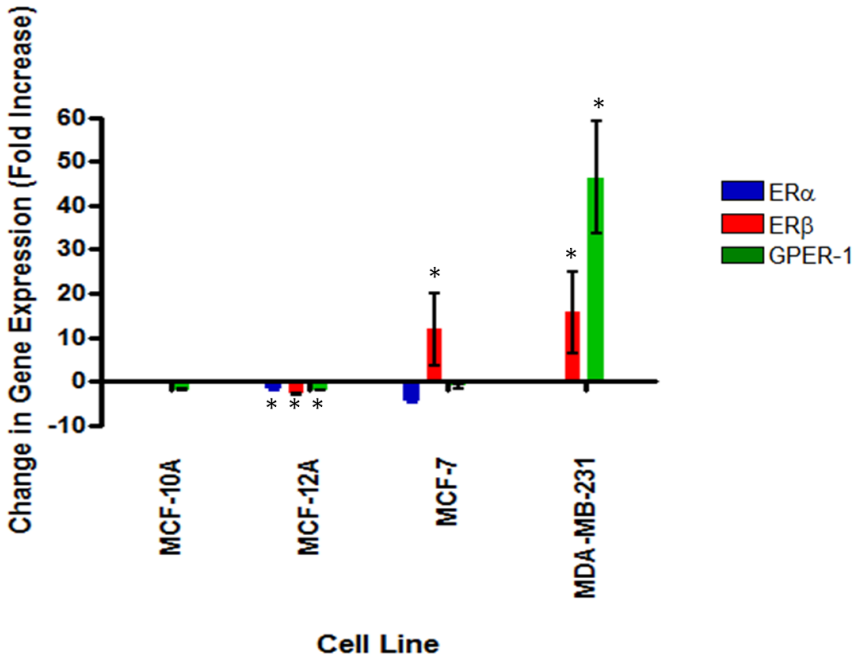

Figure 2.13: Fold change in gene expression in response to 1 nM E2 of *ERα*, *ERβ* and *GPER-1* for all four cell lines. As MDA-MB-231 cells did not express *ERα* under solvent control conditions, the fold change cannot be shown here. EtOH controls are set to 0 (x-axis). Error bars represent standard error of the mean (n = 6). A *P-value* of 0.05 was considered statistically significant and is indicated by an *.

Results for real-time PCR analysis of receptor status and the impact of E2 are summarised in **Table 2.6**.

Table 1.6: Table summarising gene expression of *ERα*, *ERβ* and *GPER-1* in the cell lines studied and the impact of E2 on gene expression

Gene	MCF-10A	MCF-12-A	MCF-7	MDA-MB-231
ERα	X	Yes	Yes-Decreased by E2	No-Expressed when E2-treated
ERβ	X	Yes	Yes-Increased by E2	Yes-Increased by E2
GPER-1	Yes-Decreased by E2	Yes	Yes	Yes-Increased by E2

2.6.4 Receptor Confirmation at the Protein Level

Antibodies against ERα and ERβ were optimised. However, problems occurred with the antibodies against GPER-1, due to high levels of background. This has, thus, impacted upon the quality of the images obtained. This could have been due to the quality or specificity of the GPER-1 antibody.

At the protein level, GPER-1 was expressed in all four cell lines, even though not at very high levels. ERα was expressed in MCF-12A and MCF-7 cells, but was not detectable in MCF-10A or MDA-MB-231 cells. ERβ was expressed in MCF-12A, MCF-7 and MDA-MB-231 cells at significant levels, but was not expressed in the MCF-10A cell line (**Figure 2.14**).

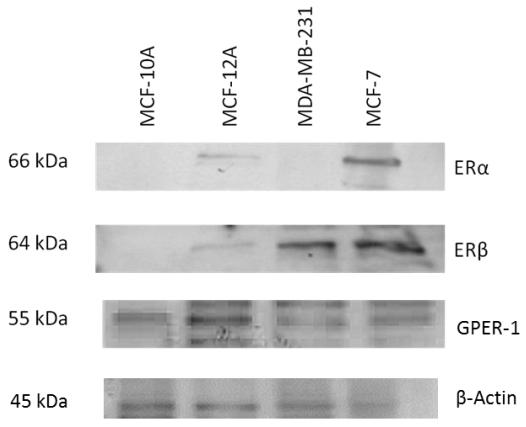

Figure 2.14: Western blot image confirming receptor status of MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines.

2.6.5 Cell Proliferation Studies in Response to E2

The SRB assay was used to assess cell proliferation of each cell line in response to various treatments. Controls consisted of phenol red free media with 0.5% CDS-stripped serum, 0.5% etOH and had no EGF. All other samples were treated with 20 ng/ml EGF, as this was shown to be essential for cell maintenance and proliferation of the non-tumourigenic cell lines.

MCF-10A cells did not proliferate in the absence of EGF. In solvent controls, or upon the addition of E2, cell proliferation gradually increased over time, peaking after 6 days and then decreased again and these curves were almost superimposed on one another. This suggests that this corresponded just to the normal doubling of cells (i.e. the basal level of cell proliferation without treatment), which is normally observed in *in vitro* cultures, and E2 induced no significant proliferative effects. The same pattern was seen with cells treated with ICI 182,780 alone or E2 + ICI 182,780. However, the peak in growth at day 6 was not as great as that seen with solvent controls or E2-treated samples. This suggests that in this ER negative cell line, ICI 182,780 treatment exerts a small anti-proliferation effect (**Figure 2.15**).

Statistical analysis showed that there were no significant differences between the proliferation data for solvent controls and samples treated with E2, E2 + ICI 182,780 or ICI 182,780.

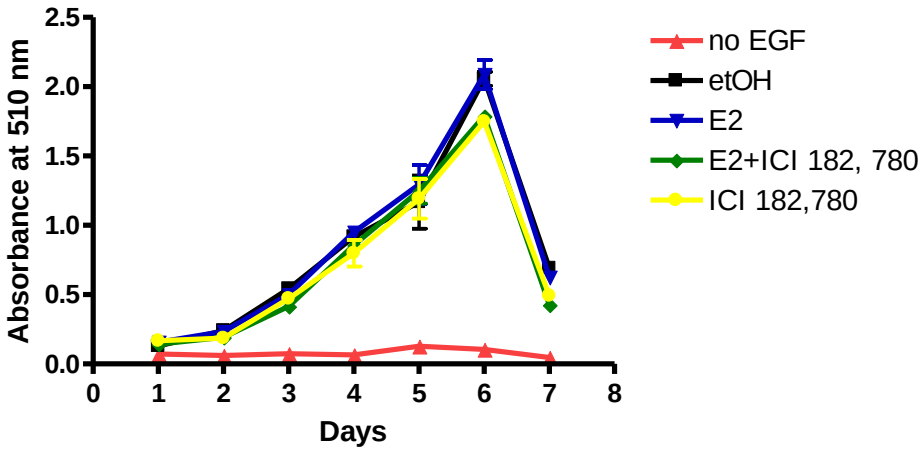

Figure 2.15: MCF-10A proliferation analysis. E2 had no impact upon proliferation. ICI 182,780, either alone or in combination with E2, appears to have a small anti-proliferative effect. Cells where no EGF was present in the assay media did not proliferate. Error bars show standard error of the mean (n = 6). Solvent controls consisted of phenol red free media with CDS serum + 0.5% etOH. All samples were treated with 20 ng/ml EGF, in addition to the indicated treatment, unless otherwise stated.

EGF was also necessary for the proliferation of MCF-12A cells. For cells treated with etOH, E2, E2 + ICI 182,780 or ICI 182,780, proliferation increased until day 5 and then started to decrease. For samples treated with ICI 182,780, however, proliferation increased until day 6 to a greater level than observed in other treatments. This could be indicative of delayed proliferation or due to the increase in cell number compared to other treatments. E2 treatment of the ER positive MCF-12A cell did not appear to stimulate proliferation (**Figure 2.16**).

Statistical analysis showed that there was no significant difference between the proliferation of solvent controls and cells grown in the presence of E2, E2 + ICI 182,780 or ICI 182,780.

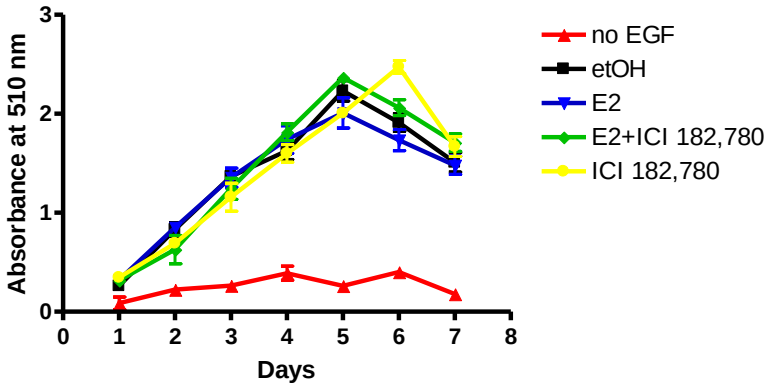

Figure 2.16: MCF-12A proliferation analysis. Only ICI 182,780 appeared to have an impact upon MCF-12A proliferation as shown by the increased cell number at Day 6. Error bars show standard error of the mean (n = 6). Solvent controls consisted of phenol red free media with CDS serum + 0.5% etOH. All samples were treated with 20 ng/ml EGF in addition to the indicated treatment, unless otherwise stated.

In ERα, ERβ, GPER-1 positive tumourigenic MCF-7 cells, cells grown in the lack of EGF, did not proliferate. This was unexpected, as these cells normally proliferate in the absence of EGF (Silva *et al*, 2001). However, as cells were cultured in 0.5% serum in phenol red-free media, the lack of any growth factors probably accounts for this. For all samples, with the exception of those where ICI 182,780 was present, cells proliferated until day 7 and then cell number declined. E2 treatment induced a slight increase in proliferation. Treatment with ICI 182,780, whether alone or with E2, resulted in no cell proliferation (**Figure 2.17**).

Statistical analysis demonstrated that E2 did significantly increase the proliferation of MCF-7 cells, and this was inhibited by the presence of ICI 182,780 ($p = 0.002$).

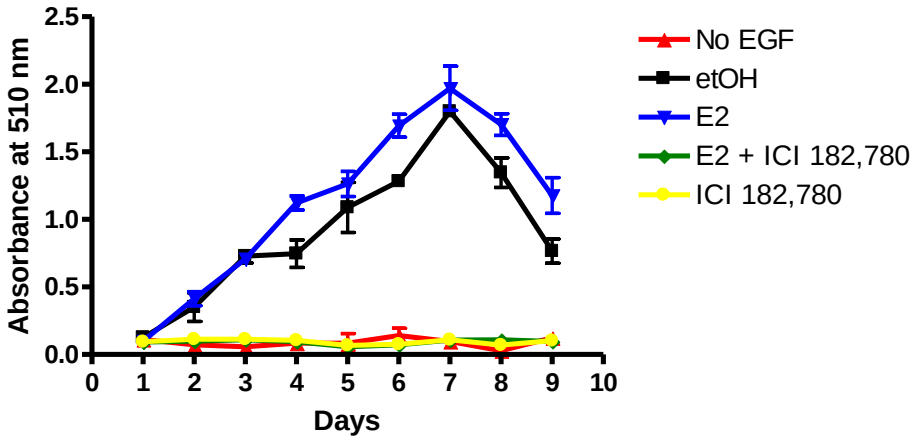

Figure 2.17: MCF-7 proliferation analysis. Proliferation increased slightly in response to E2 was this not observed upon co-incubation with ICI 182,780. Cells grown the presence of ICI 182,780 did not proliferate. Error bars show standard error of the mean (n = 6). Solvent controls consisted of phenol red free media with 0.5% CDS serum + etOH. All samples contained 20 ng/ml EGF in addition to the indicated treatment, unless otherwise indicated.

In ERβ, GPER-1 positive, ER negative tumourigenic MDA-MB-231 cells, cells were still able to proliferate in the absence of EGF. All samples proliferated in the same fashion and to the same extent, with cell number increasing until day 6 and then declining. etOH-treated samples showed increasing cell number at day 3 as opposed to day 4 when cell number increases in all other treated samples were observed. The second difference was induced by E2 treatment, these cells had a greater cell number at day 4 compared to cells treated with ICI 182,780, E2 + ICI 182,780 or media lacking EGF (**Figure 2.18**). However, statistical analysis showed no significant differences between samples at these, or any other time points.

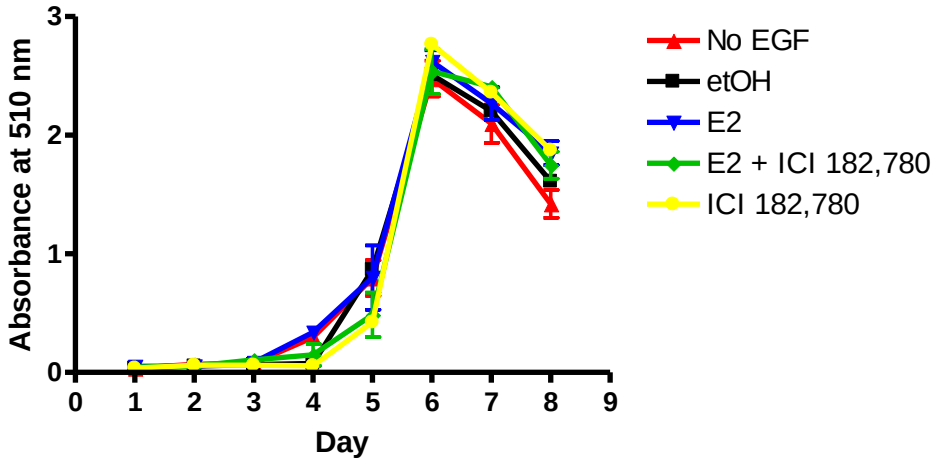

Figure 2.18: MDA-MB-231 proliferation analysis. E2, alone or in combination with ICI 182,780, did not have an impact upon cell proliferation. Error bars show standard error of the mean (n = 6). Solvent controls consisted of phenol red free media with 0.5% CDS serum + 0.5%. All samples contained 20 ng/ml EGF in addition to the indicated treatment, unless stated otherwise.

2.6.6 The Role of GPER-1 in Proliferation

E2 only appeared to have an impact upon MCF-7 proliferation and co-incubation with ICI 182,780 prevented MCF-7 proliferation. In MCF-12A cells, ICI 182,780 appeared to impact upon proliferation, which may be due to it agonistic properties for GPER-1 (Filardo *et al*, 2002). It was therefore decided to investigate whether GPER-1 could mediate any of E2's proliferative effects using the GPER-1 antagonist, G-15 (Dennis *et al*, 2011). It was also decided to see if GPER-1 was involved in cellular proliferation through the use of GPER-1 agonist, G-1 (Albanito *et al*, 2007).

In MCF-10A cells, E2, either alone or in combination with ICI 182,780 or G-15, had no impact on proliferation (**Figure 2.19**). Therefore, these data suggests that despite being GPER-1 positive, MCF-10A cells do not proliferate in response to estrogen.

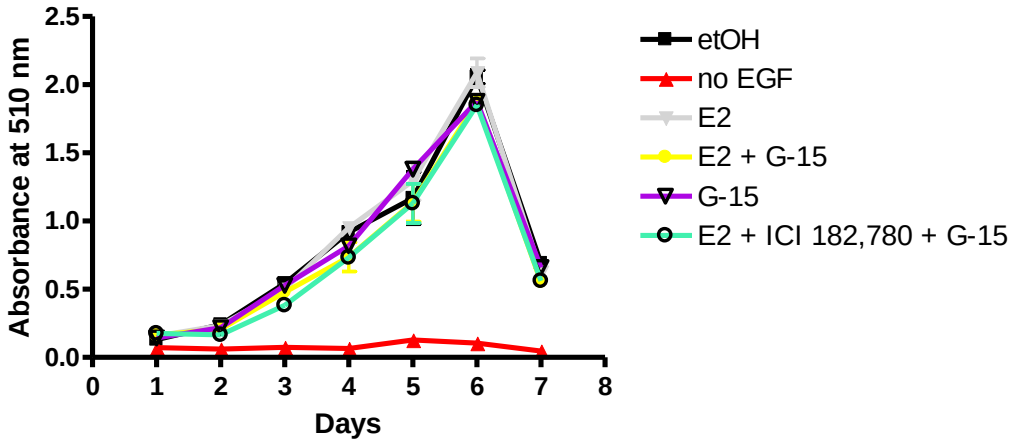

Figure 2.19: E2 and the role of GPER-1 in the proliferation of MCF-10A cells. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF, unless otherwise stated. Error bars represent standard error of the mean (n = 6).

In MCF-10A cells G-1 did not appear to increase proliferation. However, G-15 alone and G-15 in combination with G-1, did decrease proliferation compared to etOH controls (**Figure 2.20**). These changes, however, were not statistically significant.

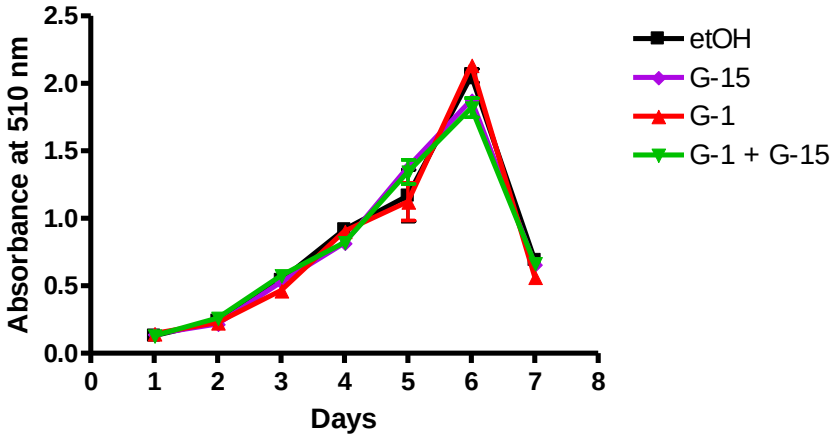

Figure 2.20: The role of GPER-1 on MCF-10A proliferation. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF. Error bars represent standard error of the mean (n = 6).

The MCF-12A proliferation curves were similar for E2 + ICI 182,780 + G-15, E2 + ICI 182,780 and E2 + G-15 (**Figure 2.21**). There were no statistically significant differences between these growth curves.

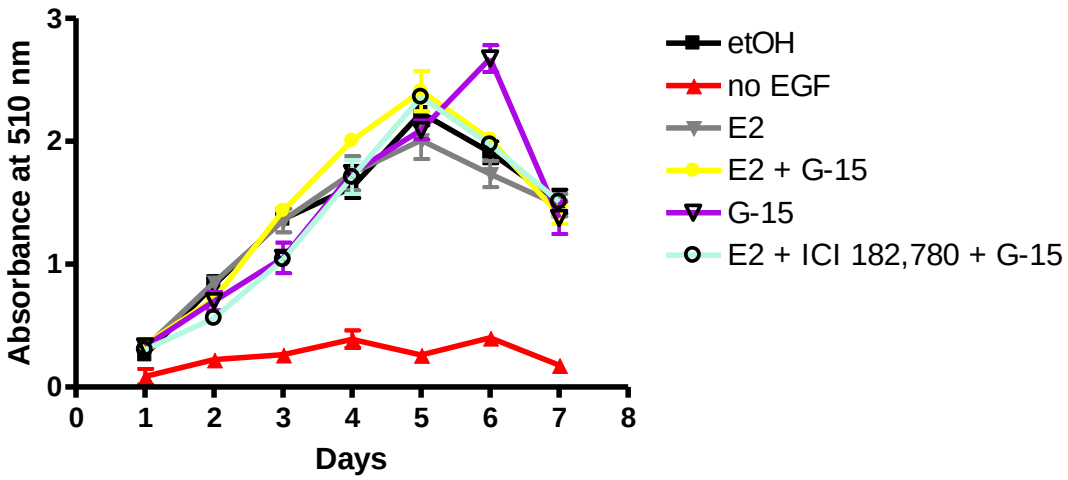

Figure 2.21: E2 and the role of the GPER-1 in proliferation of MCF-12A cells. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF, unless otherwise stated. Error bars represent standard error of the mean (n = 6).

In MCF-12A cells, G-1 increased proliferation and cells continued to proliferate to day 6, as opposed to day 5, as observed in solvent controls. However, this was not statistically significant. Co-incubation of G-1 with G-15 decreased this effect-induced by G-1, but again, this was not statistically significant. (**Figure 2.22**). This suggests that in ER and GPER-1 positive MCF-12A cells, GPER-1 does not play a great role in proliferation.

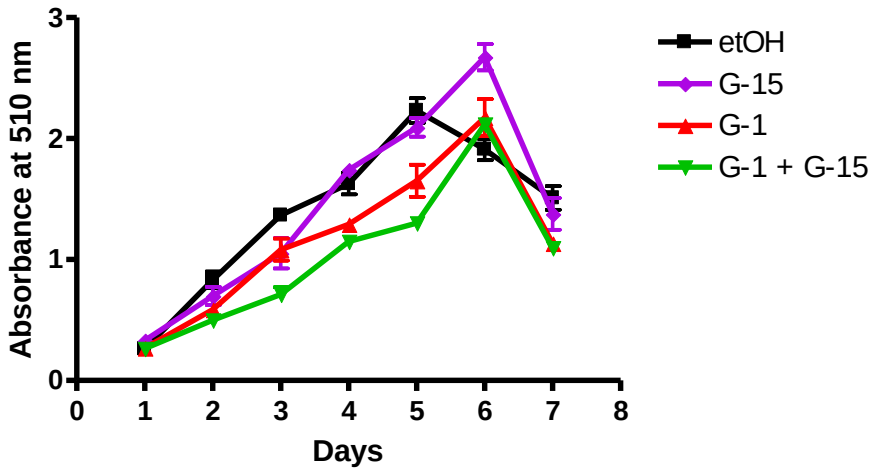

Figure 2.22: Involvement of the GPER-1 on MCF-12A proliferation. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF. Error bars represent standard error of the mean (n = 6).

E2 slightly increased MCF-7 proliferation and co-incubation with G-15 appeared to reduce this at several time points (**Figure 2.23**). When statistical analysis was performed, a significant difference between E2-treated samples and E2 + G-15 treated samples ($p = 0.02$). This suggests that the GPER-1 may play some role in mediating the E2-induced proliferation of E2 in MCF-7 cells.

Combined G-15 and ICI 182,780 pre-treatment prior to E2 addition resulted in lack of proliferation. As this is very similar to the data seen with ICI 182,780 either alone or in combination with E2 (**Figure 2.23**), it suggests that the reduction of the effect of the hormone was due to the presence of the ICI 182,780 and not G-15. Therefore, the data also suggests that the small E2-induced increased in proliferation in the MCF-7 cell line is mediated by the ER and not GPER-1.

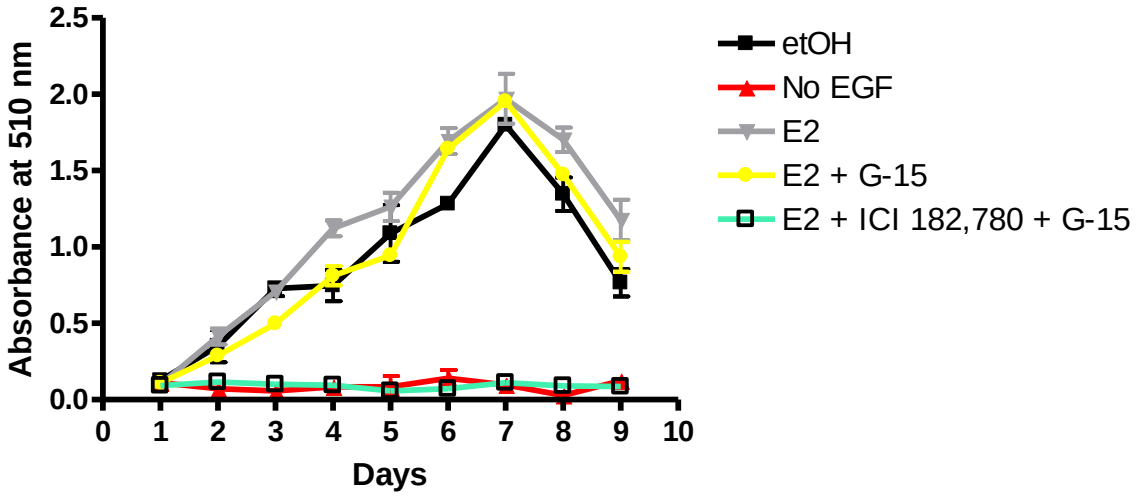

Figure 2.23: E2 and the role of the GPER-1 in the proliferation of MCF-7 cells. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF, unless otherwise stated. Error bars represent standard error of the mean (n = 6).

G-1 treatment of MCF-7 cells appeared to slightly decrease cell proliferation, relative to solvent controls, and this was considered statistically significant ($p = 0.0048$) (**Figure 2.24).** This may mean that the activation of GPER-1, by G-1, has an anti-proliferative effect. G-1 + G-15 appeared to further decrease MCF-7 proliferation, relative to both solvent controls and G-1 treated cells. This was only significant relative to solvent controls ($p = 0.0057$).

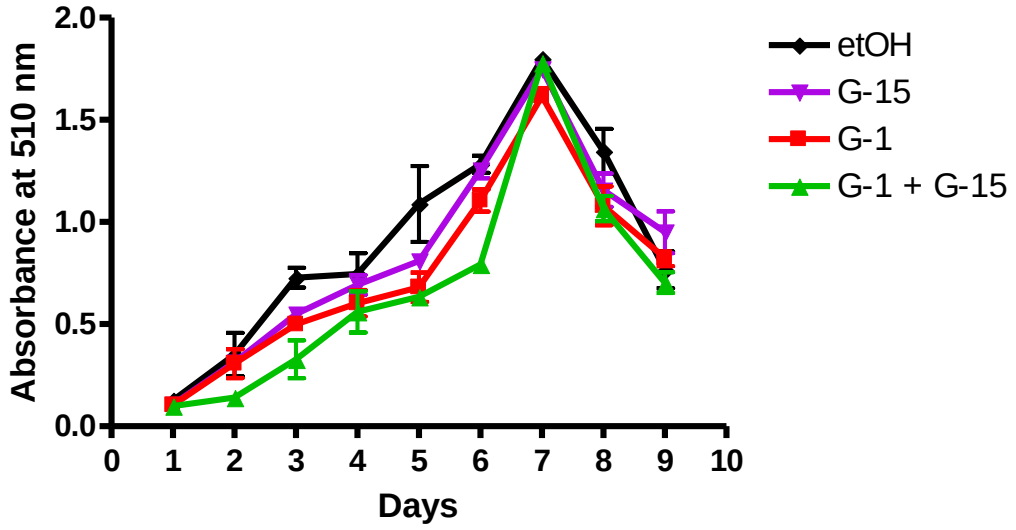

Figure 2.24: The involvement of the GPER-1 on MCF-7 proliferation. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF. Error bars represent standard error of the mean (n = 6).

Co-incubation of G-15 or ICI 182,780 G-15 with E2 did not significantly effect upon MDA-MB-231 proliferation (**Figure 2.25**).

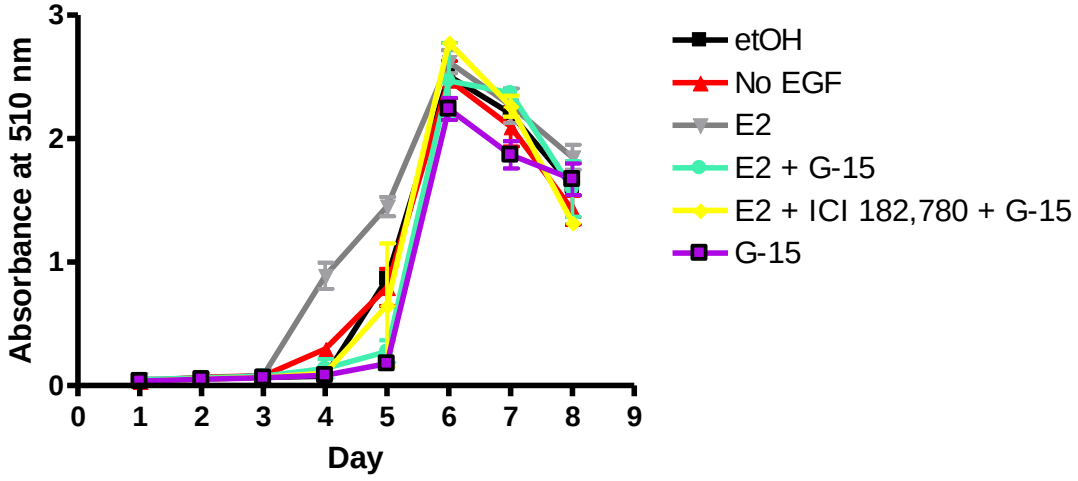

Figure 2.25: E2 and the role of GPER-1 in proliferation in MDA-MB-231 cells. Solvent controls consisted of media and 0.5% CDS serum + 0.5% ethanol + 0.2% DMSO. All treatments contained 20 mg/ml EGF, unless otherwise stated. Error bars represent standard error of the mean (n = 6).

G-1, G-1 + G-15 and G-15 had no significant impact upon MDA-MB-231 proliferation (**Figure 2.26**). This may indicate that not only does E2 not increase proliferation in this cell line, but GPER-1, is not involved in MDA-MB-231 proliferation, even though it is expressed at, albeit at low levels.

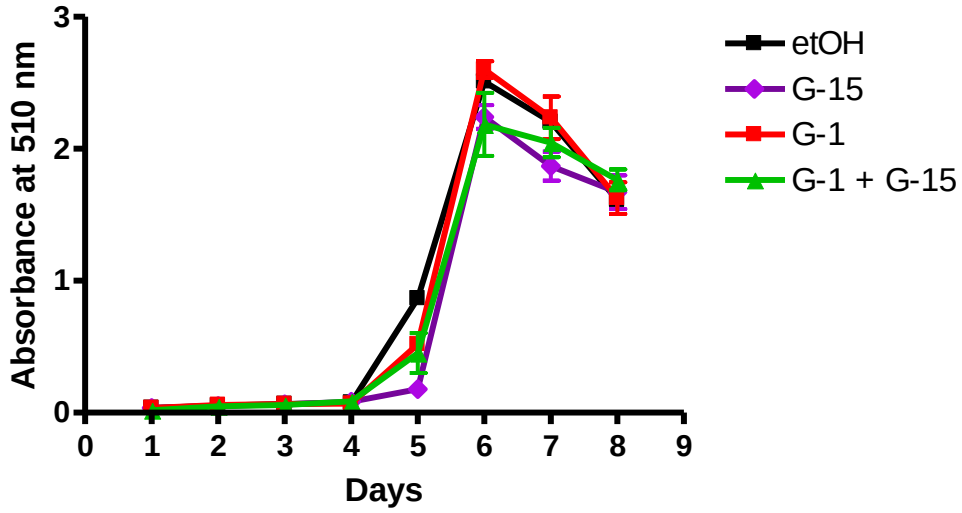

Figure 2.26: The involvement of GPER-1 on MDA-MB-231 proliferation. Solvent controls consisted of media and 0.5% CDS serum + 0.5% etOH + 0.2% DMSO. All treatments contained 20 mg/ml EGF. Error bars represent standard error of the mean (n = 6).

2.6.7 Summary Table for Cell Proliferation Studies

Table 2.7: Summary of proliferation analysis of cell lines in response to estrogen and ER or GPER-1 antagonists

Treatment	MCF-10A	MCF-12A	MCF-7	MDA-MB-231
E2	No effect	No effect	Increased	No effect
E2 + ICI 182,780	No effect	No effect	Decreased	No effect
E2 + G-15	No effect	No effect	Decreased relative to E2-treated cells	No effect
G-1	No effect	No effect	Increased	No effect
G-1 + G-15	No effect	No effect	Decreased	No effect
ICI 182,790	No effect	No effect	Decreased	No effect
G-15	No effect	No effect	No effect	No effect

2.7 Discussion

2.7.1 Receptor Confirmation

The ER status of the MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines at both the gene and protein level has been confirmed. It is widely accepted that cells vary greatly between batches (Herold *et al*, 1994; Payne *et al*, 2000). As the primary aims of the work to be conducted was to investigate the impacts of estrogens in ER and GPER-1 competent, MCF-12A cells, in a 3D culture model, it was first necessary to confirm the presence of these receptors in this cell line.

In the cell batches tested here, MCF-10A cells were found to be negative for ERα and ERβ, whilst MCF-12A cells expressed both ERα and ERβ. Both "normal" cell lines expressed measurable levels of GPER-1. On the other hand, the tumourigenic MCF-7 cells were positive for all three receptors and MDA-MB-231 cells expressed ERβ and GPER-1, but were ERα negative, at both the protein and gene level. Upon E2 treatment, however, ERα was detected at the gene level in this cell line.

The ERα negative phenotype observed in the MCF-10A cell line tested has previously been observed by other groups (Spychala *et al*, 2004) and, hence, this cell line can be used for further experiments as an example of a non-tumourigenic ER negative breast cell line.

The ERα status of the MCF-12A cell line, however, has been reported as both positive (Eisen & Brown, 2004; Engel *et al*, 2011) and negative (Thompson *et al*, 2004). The American Type Culture Collection (ATCC) MCF-12A cell line used in this study was proven by us to express both ERα and ERβ in measurable amounts, at both the gene and protein level. The precise reason for discrepancies between research groups is unknown; however, it has previously been shown that the ER negative MCF-10A cell line can spontaneously become ER positive and the same could, therefore, be true for the MCF-12A cell line (Lane *et a*l, 1999). It is not clearly understood how this may have occurred, although other groups have found that ectopic expression of oncogenic Ha-ras can result in ERα expression in previously ER negative cell lines (Shekhar *et al*, 1998). Other factors that may account for the variability in responses to estrogens, or indeed their ER status, can include seeding density, length of estrogen-deprivation prior to E2 treatment and cytogenetic changes (Payne *et al*, 2000). For instance, it has been demonstrated that in E2-deprived MCF-7 cells, ERα is up-regulated, leading to increased responses when estrogens are added (Katzenellenbogen et al, 1987). Furthermore, low seeding densities appear to increase estrogen responsiveness in MCF-7 cells (Payne *et al*, 2000).

Although small, the decrease in *ERα* gene expression in response to E2 in MCF-12A cells was statistically significant ($P < 0.05$). This decrease in *ERα* expression may lead to decreased estrogen responsiveness, and this possibly presents a negative feedback mechanism through which estrogen responsiveness is controlled.

As the focus of the work described in this thesis is to investigate the effects of estrogens in an ER positive non-tumourigenic mammary epithelial cell line, as shown here, MCF-12A cells are the best candidates for this purpose as the gene and protein analysis clearly demonstrates that these cells express a functional ER.

GPER-1 was also found to be present at both the gene and protein level in both MCF-10A and MCF-12A cell lines. GPER-1 gene expression decreased upon E2 treatment in the ER negative MCF-10A cell line and ER competent MCF-12A cell line. This E2-induced down-regulation of *GPER-1* gene expression was however only statistically significant in MCF-12A cells ($P < 0.05$).

ERα gene expression was down-regulated upon E2 treatment in MCF-7 cells as, at the gene level, suggesting that the down-regulation of the ER in response to estrogen occurs at the transcriptional

level. This agrees with previous reports, where estrogen down-regulated ERα expression, not only at the gene level, but also at the protein level (Jenesen *et al*, 1999; Marsaud *et al*, 2003; Kocanova *et al*, 2010). In terms of the down-regulation of ERα gene expression, it has been proposed that activated ERα is able to bind to various regions located within the promoter region of the ER. This negative feedback mechanism prevents any further transcription of the gene, but because the ER lacks an ERE, other co-activators must be involved, such as AP-1 and SP-1 (Saceda *et al*, 1989; Kaneko *et al*, 1993; Santagati *et al*, 1997).

In MDA-MB-231 cells, *ERα* gene expression was only detected upon E2 treatment. Previous studies have shown that MDA-MB-231 cells are negative for ERα (Rai *et al*, 2005; Zhang *et al*, 2011). An increase in ERα gene expression in response to E2 has been observed in other cell lines that express the gene at very low levels (Read *et al*, 1989). Therefore, a similar effect may be occurring in MDA-MB-231 cells following E2 treatment.

2.7.2 Proliferation in Response to Estrogen

It is well established that the development of the breast is influenced by estrogens (Russo *et al*, 1999) and that E2 can stimulate the proliferation of breast cancer cell lines and may influence breast cancer growth *in vivo* (Lippman *et al*, 1976; Russo *et al*, 2002). To study this further, non-tumourigenic, and tumourigenic cell line proliferation studies were performed in the presence of E2, ICI 182,780 and a combination of the two. Additionally the role of GPER-1 in mediating cellular proliferation was studied using an agonist and antagonist for this receptor and co-incubation of the GPER-1 antagonist, G-15 with E2.

It was observed that in MCF-10A, MCF-12A and MCF-7 cells, the lack of EGF, resulted in a lack of proliferation. A dependence on EGF has been noted previously in the MCF-10A and MCF-12A cell line (Soule *et al*, 1990; Paine *et al*, 1992; Wang *et al*, 1997), but not in MCF-7 cells. This discrepancy may be due to the very low concentration of serum used and the phenol red-free media, rather than a necessity for EGF itself.

E2 does not appear to greatly influence proliferation of the cell lines studied, although a small increase in cell number was observed in MCF-7 cells in response to E2 treatment. This lack of proliferation in response to the steroid hormone would be expected in the ERα negative MCF-10A and MDA-MB-231 cell lines and has been observed previously (Spink *et al*, 2006), but it is also common for ER positive non-transformed mammary epithelial cells, such as MCF-12A to be non-proliferative in

response to stimulus (Clarke *et al*, 1997; Russo *et al*, 1999). Additionally, it is important to remember that MCF-7 cells, although estrogen-responsive, do not always show significant proliferation in response to estrogens, and proliferation in response to these compounds, varies significantly between cell batches (Payne *et al*, 2000).

The lack of proliferative effect of E2 in MCF-12A cells suggests that E2 does not induce proliferation in non-transformed cell lines, regardless of their ER status, and supports previous work demonstrating that non-transformed mammary epithelial cells do not increase DNA synthesis in response to E2 treatment (Haslam, 1986). Alternatively, it has been hypothesised that in cell cultures, and even in *in vivo* models, two populations of epithelial cells exist; one which is ER positive and the other which is ER negative. It is the ER negative cells that proliferate in response to paracrine signals from ER positive cells and cells in the surrounding stroma, after stimulation by E2 (Nandi *et al*, 1995; Clarke *et al*, 1997; Russo *et al*, 1999; Mallepell *et al*, 2006; Gupta *et al*, 2007). This theory adds an additional level of complexity to the model of hormonal carcinogenesis, whereby the events leading to breast cancer are governed not just by the presence of E2, but also the interactions of the various cell types in the mammary gland and surrounding tissue.

Another possibility is linked to the expression of ERβ. It has been demonstrated that increased expression of this receptor correlates with decreased cell proliferation (Sotoca *et al*, 2008). Furthermore, in breast tumours, the ratio of ERα to ERβ is higher than though of normal breast tissue (Lazennec *et al*, 2001). These observations have led to the hypothesis that ERβ suppresses the proliferative effects of ERα, and would mean that reduced ERβ expression correlates with increased proliferation (Covaleda *et al*, 2008). Indeed, Covaleda *et al* (2008) demonstrated this effect in breast cancer cell lines. However, the exact mechanism for how ERβ mediates the actions of ERα, to our knowledge, remains elusive. The small stimulatory effect of ICI 182, 780, either alone or in combination with E2, on MCF-12A proliferation could be explained by the confirmed presence of GPER-1 in MCF-12A cells, as both E2 and ICI 182,780 can act as agonists for this receptor (Kamanga-Sollo *et al*, 2008; Kuhn *et al*, 2008). Indeed, when MCF-12A cells were treated with the GPER-1 agonist, G-1, increased proliferation was observed. This means that, in MCF-12A cells at least, GPER-1 activation can induce proliferative effects. In addition to this, when the GPER-1 antagonist, G-15 was added to E2 and ICI 182,780 co-incubated MCF-12A cells, the proliferation curve resembled that of solvent controls.

Our observations agree with previously published work showing that E2 has a mitogenic effect on the MCF-7 cell line (Lewis *et al*, 2005; Spink *et al*, 2006; Wang *et al*, 2008[b]). In fact, out of all four cell

lines analysed, MCF-7 cells were the only cells to show increased proliferation in response to E2 treatment. The impact of estrogens on MCF-7 proliferation has been widely studied. Several factors are believed to contribute to the mitogenic effect of E2 in this cell line. Firstly, E2 is able to induce the transcription of the proto-oncogenic transcription factor, *c-fos* via genomic and non-genomic mechanisms (Prall *et al*, 1997; Sabbah *et al*, 1999; Duan *et al*, 2002). C-fos proteins form dimers with members of the Jun transcription factor family to form the AP-1 transcription factor that consequently mediates the transcription of various genes involved in differentiation and proliferation. Consequently, the expression of cyclin D1, a protein involved in cell cycle progression, is up-regulated in response to E2 in the MCF-7 cell line and this correlates with increased proliferation (Prall *et al*, 1997; Planas-Silva *et al*, 2001). Estrogens also down-regulate the expression of the cyclin dependent kinase (CDK) inhibitor $p27^{kip1}$ thus preventing the interactions of cyclins and CDKs which is necessary for cell cycle progression (Foster *et al*, 2003). Finally, in terms of the cell cycle, E2 treatment induces the hyperphosphorylation of the Rb protein. In its hyperphosphorylated state Rb becomes liberated from elongation actor-2 (EF2), allowing the transcription of genes required for passage through the G1/S phase checkpoint and consequently, increased proliferation (Foster & Wimalasena, 1996; Hurd *et al*, 1997; Dinda *et al*, 2002). These E2-induced alterations in gene expression or phosphorylation status present plausible mechanisms by which estrogens induce their proliferative effects. As stated above, MCF-7 cells were the only cells to have a proliferative response to E2. Co-incubation of E2 with ICI 182,780 or G-15 in this cell line, seemed to significantly reduce this effect. It, therefore, would appear that both the ER and GPER-1 are capable of mediating the proliferative effects of estrogens in MCF-7 cells. Strangely however, G-1, a GPER-1 agonist, appeared to have an anti-proliferative effect. The reason for this remains unclear and warrants further investigation, although is not in the scope of this thesis.

As described in the results section, ICI 182,780 treatment of MCF-7 cells, either alone or in combination with E2, prevented cell proliferation, which agrees with previous findings that ICI 182,780 and other ER antagonists induce growth arrest in this cell line (Lippman *et al*, 1976; Carroll *et al*, 2000; Lewis *et al*, 2005; Martin *et al*, 2005; Wang *et al,* 2008[b]).

The lack of response of ERα negative MDA-MB-231 to E2 and ICI 182,780 is not surprising, considering the absence of ERα in this cell line. These cells do, however, express ERβ, and given the lack of response to E2, this may suggest that ERβ does not mediate the proliferative effects of estrogens. Similar observations with E2, ICI 182,780 and even tamoxifen have been demonstrated previously (Lippman *et al*, 1976; Rai *et al*, 2005; Wang *et al*, 2008[b]). These cells were the only cell line that

proliferated in the absence of growth factors, which suggests that they have become self-sufficient in terms of growth signals, a common hallmark of cancer (Hanahan & Weinberg, 2000). Indeed, Ras mutations, as displayed in MDA-MB-231 cells, results in increased production of TGFα (Ciardiello *et al*, 1988; Cancer Genome Project). This means that MDA-MB-231 are capable of producing growth factors, and may, therefore, mean that they are capable of surviving in an environment where growth factors are limited.

Finally, the role of GPER-1 in proliferation was investigated. All of the cell lines tested are competent for this receptor. In MCF-7 cells, the only cell line in which E2 increased proliferation, GPER-1 antagonism did not reverse this effect. This suggests that, at least in the case of MCF-7 cells, GPER-1 does not mediate the mitogenic influences of estrogens. In MCF-12A cells however, ICI 182,780 and G-1, both GPER-1 agonists, did increase proliferation. This may implicate that GPER-1 in this cell line can mediate changes in proliferation, although E2 itself does not increase induce this effect. It remains to be seen whether GPER-1 is implicated in the apoptotic process in the cell lines studied or indeed in three-dimensional (3D) cultures of MCF-12A cells, which will consist of work in later chapters. Nevertheless, it would appear that GPER-1 in the non-tumourigenic ER-competent MCF-12A cell line may be implicated in cellular proliferation.

The data presented here suggests that estrogens may not impact greatly on cellular proliferation in these cell lines, and, therefore, may be exerting their effects via other mechanisms. However, as discussed above, these studies were performed using individual cell lines. In the human mammary gland, multiple cell types are present, and these signal to one another to induce alterations in cell growth and behaviour. Therefore, it is plausible that the lack of significant proliferation in response to E2 is due to the absence of paracrine signalling mechanisms with other cell types. Indeed, as mentioned earlier, there is evidence that suggests that responses of both normal and malignant mammary epithelium to estrogens is not as a result of estrogens directly, and that estrogens instead induce the release of growth factors, such as EGF and TGFα, which, in turn proceed to induce proliferation (Vonderhaar, 1987; Salomon *et al*, 1992). Further experiments, such as the co-culture of ER positive and ER negative cells, or of epithelial cells with cells of the stroma, would shed further light on this, however for the purpose of the work to be described in later chapters this is not necessary at this stage.

The work presented here has not conclusively shown that E2 acts as a mitogen in ER-competent cell lines or that the primary function of GPER-1 is associated with proliferation. It has, however, established an understanding of how the cells lines tested respond to estrogens and will be important,

particularly in the case of the MCF-12A data, for future work. The observations here will allow us to compare the differences between monolayer cultures of MCF-12A cells, and those grown in 3D. This is important to investigate as we are aiming to utilise a 3D model of mammary epithelial MCF-12A cells, to more accurately characterise the impact of estrogens in a more physiologically relevant setting, and highlight the failings of only conducting experiments in traditional monolayer cultures.

In the proceeding chapters we will investigate the impact of estrogenic compounds on 3D cultures of MCF-12A cells and try to elude the role of the ER and GPER-1 in mammary carcinogenesis and the mechanisms involved using a 3D model.

CHAPTER III: Establishing Three Dimensional Cultures of Non-Tumourigenic Breast Cells

3.1 Principles of Three Dimensional Culture

Monolayer culture of normal and breast cancer cells has provided valuable knowledge regarding cancer, in respect to oncogenic events, molecular mechanisms of cancer progression and cancer therapy (Darbre *et al*, 1983; Santen *et al*, 1987). Additionally, these monolayer cultures provide insight into the biological processes and responses to treatments, such as apoptosis, proliferation and drug resistance (Normanno *et al*, 2006). However, the monolayer culture system has limitations. Previous reports have shown that the effects of cytotoxic compounds, potential therapies, oncogenes and tumour suppressor genes in traditional monolayer cultures often do not translate to the same effects observed in three-dimensional (3D) cultures, in *in vivo* models or in humans (Dhiman *et al*, 2005; Horning *et al*, 2008; Yang *et al*, 2009). For example, in monolayer cultures of breast cancer cells, HER2 (a receptor that has implications for breast cancer treatment and prognosis) forms heterodimers with HER3 resulting in PI3K signalling, but, in 3D, HER2 forms homodimers and MAPK signalling is favoured (Pickl & Ries CH, 2009). Hence, monolayer cultures often give a poor representation of how possible carcinogens, genetic aberrations and treatments affect cancer initiation and progression *in vivo*. Indeed, analysis has revealed that cells cultured in monolayer and 3D differ in their gene and protein expression patterns and in the integration of signalling pathways (Gaedtke *et al*, 2007; Kenny *et al*, 2007; Härmä *et al,* 2010; Weigelt *et al*, 2010). For example, global gene expression analysis has revealed that in monolayer cultured hepatocytes, genes associated with the extracellular matrix (ECM), cytoskeleton and adhesion are up-regulated, whereas in 3D cultured cells, genes associated with metabolism are up-regulated instead (Chang & Hughes-Fulford, 2009). At the protein level, 3D cultures, compared to monolayer cultures, have down-regulated levels of prohibitin and up-regulated levels of annexin 4, suggesting a decrease in proliferation and increased apoptosis, respectively (Grun *et al*, 2009). These findings suggest that cells can have dramatic and fundamental functional differences, depending on the means of culture.

Also of extreme importance, is the inability of monolayer cultures to recapitulate the micro-environment found *in vivo*. In an *in vivo setting*, signals from the micro-environment exist, originating from the surrounding stroma, immune cells, endothelial cells and the ECM, and these impact upon the development of the mammary gland (Bissell & Radisky, 2001). However, in monolayer cultures, these

external cues are not present. Consequently, cellular organisation into functional structures and ECM interactions cannot be studied. As mentioned previously, one way to overcome the limitations of monolayer cultures is by using 3D models of immortalised cell lines, where the signals from the ECM are restored, and *in vivo* architectural and physiological features recapitulated (Lawrenson *et al*, 2009). As described in detail in **Chapter I**, 3D culture using reconstituted basement membrane matrix (Matrigel) allows the organisation of non-malignant epithelial cells, such as those of the breast, into structures that resemble the glandular architecture of the breast (**Figure 3.1**) (Debnath *et al*, 2002; 2003). In Matrigel, non-tumourigenic breast cells form polarised, basement membrane encapsulated acini that undergo growth arrest and luminal hollowing. The 3D cellular organisation also allows cells to form cell-cell attachments, such as gap junctions, and to communicate with one another, in a similar fashion to what occurs in the human breast tissue.

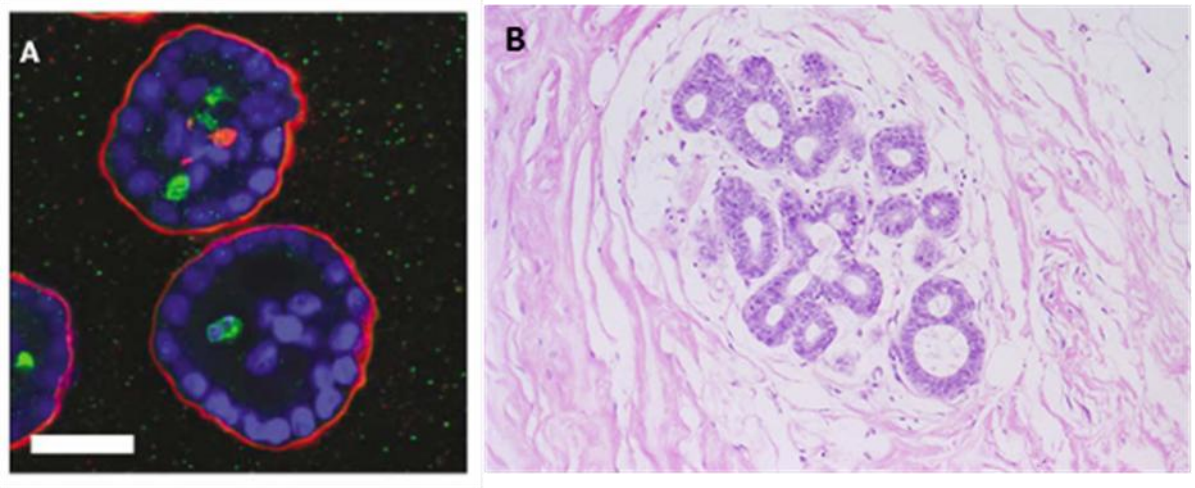

Figure 3.1: Comparison of acini *in vitro* and in histological samples from humans. (A) An *in vitro* MCF-10A acinus. A single layer of cells surrounds a hollowing lumen and is surrounded by basement membrane (Debnath *et al*, 2003) **(B)** Human mammary acini. A bilayer of cells surrounds a hollow lumen and is encapsulated by a basement membrane (Duke University, 2011)

In addition to allowing the study of normal features of the mammary gland, 3D cultures of breast cells can also be used to investigate the mechanisms implicated in the neoplastic transformation of the breast. For instance, treatment of MCF-10A cells cultured in Matrigel, with the carcinogen, 2-amino-1-methyl-6-phenylimidazol [4,5-*b*] pyridine (PhIP), results in increased proliferation and irregular acinar shape (Choudhary *et al*, 2012). Furthermore, luminal filling and excess proliferation, common hallmarks of breast cancer, can be induced through the over-expression of oncogenes, such as cyclin D1 and Bcl-2,

or suppression of tumour suppressor genes (Muthuswamy *et a*l, 2001; Debnath *et al*, 2002). However, it is worth noting that the ectopic overexpression of cyclin D1 or Bcl-2 alone, results in delayed, rather than inhibited lumen formation. Likewise, suppression of pro-apoptotic Bim expression delays lumen formation and this has been substantiated in *in vivo* models (Reginato *et al*, 2005; Mailleux *et al*, 2007). As described previously, and will be discussed in more detail in further chapters, profound and permanent dysregulation of acini morphogenesis requires the over-activation and inhibition of multiple pathways. This can be exemplified by the formation of acini lacking hollow lumen if both cyclin D1 is up-regulated and PI3K hyperactivated, or if *BIM* expression is reduced with simultaneous up-regulation of *BCL-2* expression (Debnath *et al*, 2002; Mailleux et al, 2008).

3D studies have also been conducted using tumourigenic cell lines in order to attempt to revert cancerous phenotypes. Antibodies blocking β1 integrin or the EGFR, reverted some of the abnormal acini structures of malignant cells and resulting in organised acini similar to non-tumourigenic breast morphology (Weaver *et al*, 1997).

Given the aforementioned observations, it is clear that 3D culture, provides an opportunity to investigate a more physiologically relevant context in which to study, not only functions involved in mammary gland formation and maintenance, such as signalling pathways, polarity, apoptosis and gene expression, but also the processes involved in the early stages of carcinogenesis and cancer progression.

It should be noted, however, that the 3D culture system has limitations in itself. For example, most simple 3D culture experiments reported in the literature do not recapitulate interactions with the stroma, as they are comprised of epithelial cells only. To achieve this, it would be necessary to culture various cell types together, such as epithelial, myoepithelial, fibroblast and immune cells. Techniques for the heterotypic 3D co-cultures of these various cell types of the breast have recently been developed, such as co-cultures of epithelial cells with fibroblasts, endothelial cells or adipocytes (reviewed in Wang & Kaplan, 2012). At the beginning of this work, many of these techniques were in their infant stages and not widely used. Also, some issues concerning variability had been reported with heterotypic 3D co-cultures due to the different scaffolds required for the different cell types and batch variation in creating these scaffolds (reviewed in Weigelt & Bissell, 2008), which led to some concerns about using this set-up for our work. Finally, from the perspectives of cost and complexity, it was not possible to implement these models in our laboratory within the time-frame of this PhD. In spite of being unable to utilise heterotypic 3D cultures of mammary epithelial cells, it is our belief that the single cell type model we implemented to be sufficient to address the issue of whether estrogens have an

impact on acini formation, and to dissect the mechanisms behind these effects. Furthermore, at this stage, little work has been performed using 3D cultures of MCF-12A cells, and, therefore, a more focused approach using one cell type was deemed more appropriate. Obviously, however, in the future heterotypic cultures with these cells and other cell types (i.e. fibroblasts) would be needed to more entirely understand the full impact of estrogens on the formation of mammary acini.

3.2 3D Culture of Non-Tumourigenic Breast Cell Lines

As mentioned previously, non-malignant breast cells grown in Matrigel, such as MCF-10A mammary epithelial cells, form polarised spheroid acini consisting of a single layer of epithelial cells (outer cells) attached to a basement membrane surrounding a hollow lumen (Debnath *et al*, 2002; Debnath *et al*, 2003; Underwood *et al*, 2006; Imbalzano *et al*, 2009). This basement membrane consists of a sheet of ECM at the basal surface, is composed of laminin, type IV collagen, entactin, proteoglycans and heparin sulphate (Debnath *et al*, 2003) and functions to provide structural support and regulate the organisation and proliferation of acini.

Previous work conducted in 3D models using MCF-10A cells has provided knowledge concerning some of the processes involved in mammary duct formation. In this cell line, cells proliferated for 14 days in Matrigel before undergoing growth-arrest. During acini morphogenesis, initially structures consist of complete spheres of cells, but from day 8 apoptosis, as shown by activated caspase-3 staining, was observed, until complete formation of a hollow lumen after 25 days. This hollowing achieved through a combination of apoptosis, autophagy and anoikis in cells that lack contact with the basement membrane (inner cells) (Debnath *et al*, 2002; Mills *et al*, 2004; Fung *et al*, 2008). The deposition of the basement membrane, as indicated by laminin V and polarised organization of hDlg and the Golgi apparatus was observed from day 3 and maintained throughout acini morphogenesis (**Figure 3.2**) (Debnath *et al*, 2003). The final structures have basal polarisation of α6 integrin, collagen IV, laminin V and Disks large and apical polarisation of the Golgi apparatus (Debnath *et al*, 2003). E-cadherin and β-catenin are also localised at cell-cell junction (Debnath *et al*, 2002). In the following sections a brief overview of the different processes involved in the formation of acini will be presented.

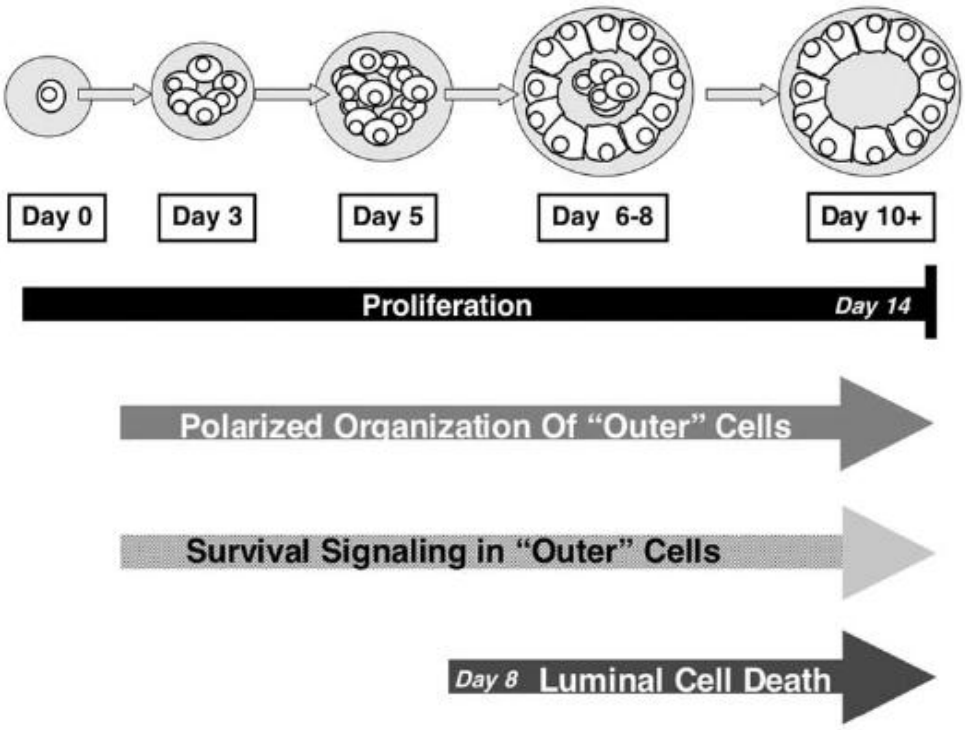

Figure 3.2: Schematic representation of the morphological programme of MCF-10A cells grown in Matrigel (taken from Debnath *et al*, 2003).

Despite extensive research into the morphological changes acini go through, the precise mechanisms and signalling pathways necessary for acini morphogenesis, remains elusive. Although much work has been conducted to investigate the role of individual oncogenes upon acini formation, there is little research into the implications of the dysregulation of these processes and pathways by compounds, such as estrogens.

This 3D culture of mammary epithelial cells provides a way to study glandular epithelium morphogenesis, as demonstrated by Debnath *et al* (2003). Non-malignant mammary epithelial cells grown in 3D form acini-like hollow spheroids that recapitulate the *in vivo* features of the breast. These acini display apico-basal polarization with basal deposition of collagen IV and laminin V. This model has provided the opportunity to study both the structural and functional implication of cancer genes in a physiologically relevant model, in which control conditions can be regulated. It also presents a model in which to study the early indications of carcinogenesis: loss of polarity, luminal filling and loss of proliferative control.

3.3 Luminal Clearing

The exact mechanisms and signalling pathways involved in lumen formation remain unclear, and it is still unknown whether this is achieved through pro-survival signals to the outer cells or protection of these cells from apoptotic signals.

As mentioned previously, the clearance of the central cells of acini, has been shown to occur by a combination of apoptosis, anoikis and autophagy (Debnath *et al*, 2002; Mills *et al*, 2004; Fung *et al*, 2008). Failure of one of these mechanisms delays lumen formation, rather than inhibiting it. However, if both autophagy and apoptosis are inhibited simultaneously, the formation of the hollow lumen is impeded and luminal filling is observed (Mills *et al*, 2004). Suppression of these pathways is a common event in cancer *in vivo*, and luminal filling is an early indication of carcinogenesis (Debnath *et al*, 2002; Russo *et al*, 2010.

The apoptotic process can be extrinsically or intrinsically triggered. Extrinsic apoptosis involves the activation of receptors within the cell membrane by pro-apoptotic ligands, such as Fas ligand (FasL), tumour necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL). Various proteins are recruited which, ultimately, leads to the activation of caspase-8 and down-stream caspases, such as caspase-3 (**Figure 3.3**).

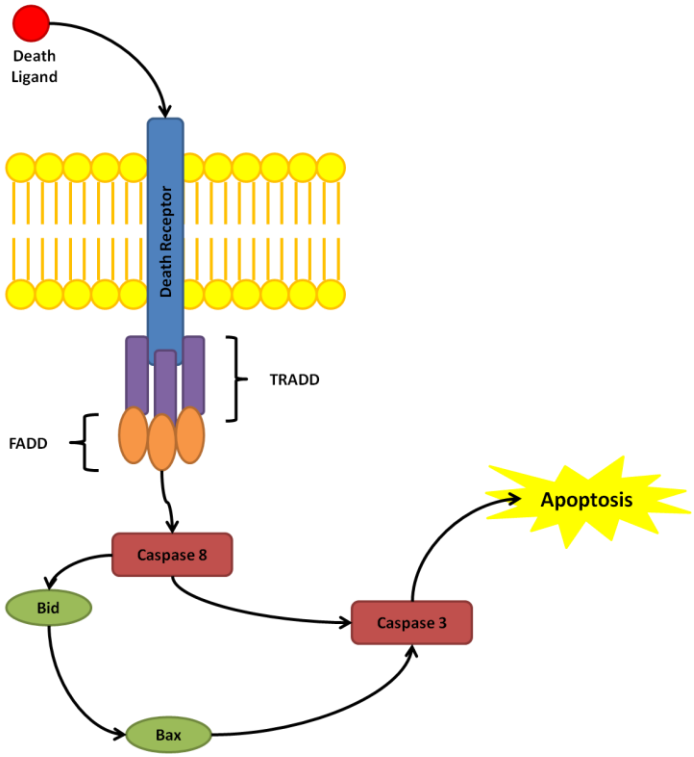

Figure 3.3: Extrinsic apoptosis pathways. Death receptor-mediated signalling upon binding of death ligands such as FasL, TNF or TRAIL and recruitment of TNF receptor type 1-associated DEATH domain protein (TRADD) and Fas-associated protein with death domain **(**FADD) results in the activation of caspases and subsequent apoptosis.

Intrinsic apoptosis (**Figure 3.4**) occurs under conditions that induce cellular stress such as heat, radiation, deprivation of nutrients, hypoxia, increased Ca^{2+} concentration or viral infections. The intrinsic apoptotic mechanism is executed by members of the B-cell lymphoma (Bcl-2) protein family and caspases. Members of the bcl-2 family can be either pro-apoptotic or anti-apoptotic and whether a cell enters apoptosis or not is dependent on the ratio, localization and phosphorylation status of these factors. For example, if there are more available pro-apoptotic factors than anti-apoptotic ones, cells are more likely to undergo apoptosis. Transcriptional regulation can affect the amount of these factors present. However, phosphorylation events, which can be mediated by AKT or MAPK signalling, can also impact on whether pro- or anti-apoptotic factors are able to exert their functions (Datta et al, 1997). Important anti-apoptotic factors include Bcl-2 homology (BH) domain containing: Bcl-2 and Bcl-xl, and important pro-apoptotic factors include Bcl-2 interacting protein (Bim), Bcl-2 associated X protein (Bax), p53 upregulated modulator of apoptosis (PUMA) and Bcl-2-antagonist of cell death (Bad). These pro-apoptotic factors can be divided into two categories: multi-domain Bax-like, or Bcl-2 homology 3 (BH3)-

only. Members of the first category include Bax and members of the latter include Bad, Bim and Puma. These BH3 domain containing factors bind to anti-apoptotic factors such as Bcl-2 and Bcl-xl, thus preventing their association with pro-apoptotic Bax. This liberates Bax, enabling it to dimerise with other Bax proteins on the outer mitochondrial membrane.

The role of Bax is to form oligomers on the outer mitochondrial membrane surface that promote the release of factors, such as cytochrome c and second mitochondria-derived activatator of caspases (SMAC's) from the mitochondria, which in turn, promote the activation of executers of apoptosis. In normal conditions, binding to Bcl-2 or Bcl-xl renders Bax inactive and, hence, apoptosis does not occur. In the event of cellular stress, the pro-apoptotic factors such as, Bad bind to the anti-apoptotic factors, preventing their binding to Bax and allowing the initiation of apoptosis. Bax dimers interact with mitochondrial voltage-dependent anion channels (VDAC's), causing the formation of a channel known as the mitochondrial apoptosis-induced channel (MAC), a decrease in mitochondrial membrane potential and triggering the release of cytochrome c, apoptosis-inducing factor (AIF) and SMAC's (Candé *et al*, 2002; Saelens *et al*, 2004; Dejean *et al*, 2006). These factors proceed to promote induce chromatin condensation, DNA fragmentation and activate the caspases (Susin *et al*, 1999; Loeffler *et al*, 2001). Activated caspase 3 then proceeds to cleave and activate the effectors of apoptosis resulting in proteolysis of components of the cytoskeleton, degradation of the nuclear envelope, DNA fragmentation and nuclear shrinkage (Rao *et al*, 1996; Lee *et al*, 1997; Chan *et al*, 1999).

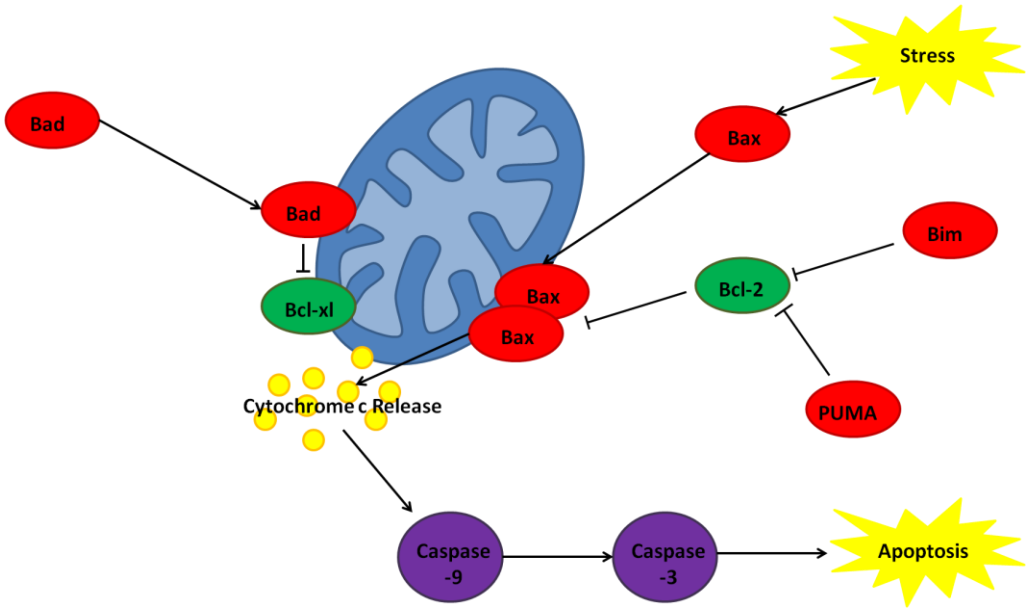

Figure 3.4: Intrinsic apoptosis pathways. Cellular stress results in the dimerization of pro-apoptotic Bax, which results in cytochrome c release from mitochondria and subsequent activation of caspases, leading to apoptotic onset.

In the context of 3D cultures of mammary epithelial cells, activated caspase-3 is observed in the centrally located cells prior to luminal clearance, implicating apoptosis in this process. Furthermore, activated AKT is only observed in the outer cells attached to the basement membrane (Debnath *et al*, 2002; Debnath *et al*, 2003[b]). Activation of this pathway inactivates Bad and the caspases, which results in decreased apoptosis and increased survival (Datta *et al*, 1997; Polo *et al*, 2010). In 3D cultures, it would appear that detachment from the basement membrane determines the fate of the inner cells of acini, and this would imply that the intrinsic mechanism is more likely to be implicated in luminal clearing.

As mentioned earlier, apoptosis alone is not solely responsible for hollow lumen formation (Debnath *et al*, 2005) and other mechanisms of cell death seem to play a role in this process. Studies have shown that autophagic vesicles are present in centrally located acini cells (Mills *et al*, 2004; Fung *et al*, 2008), suggesting that autophagy is also involved in formation of the hollow lumen. Autophagy is a catabolic process in which components of a cell are degraded by lysosomal action. The process involves a membrane forming around cellular organelles to form a vesicle, referred to as an autophagosome. This fuses with a lysosome containing acidic lysosomal hydrolases that degrades the contents of the vesicle (**Figure 3.5**).

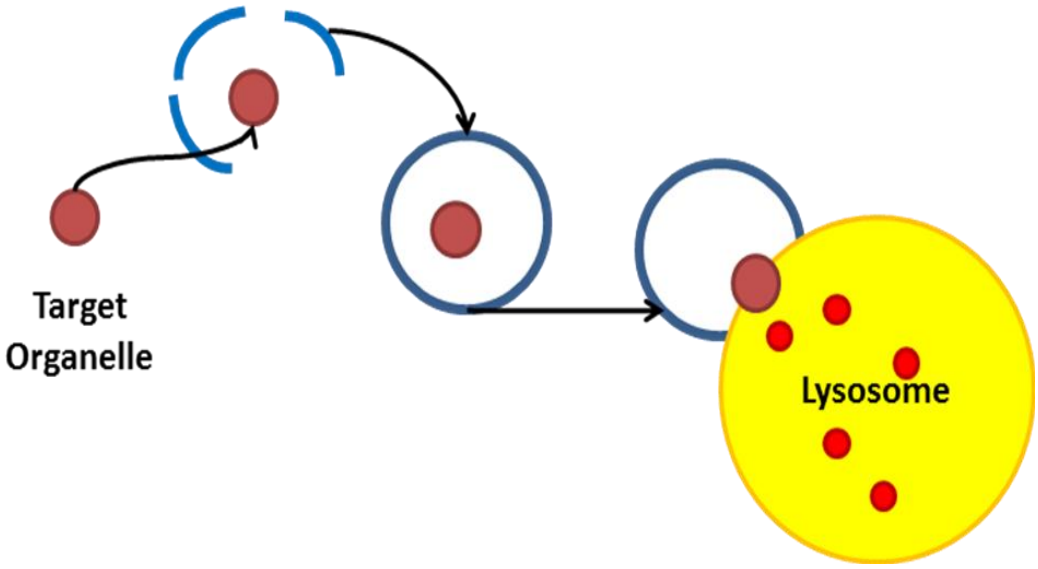

Figure 3.5: The autophagic process. Target organelles are surrounded by a membrane, and this fuses with a lysosome, where the contents are degraded by lysosomal hydrolases.

Luminal filling is a characteristic of the early stages of breast cancer, and this suggests that apoptosis is an important process involved in the development and maintenance of the mammary gland, and highlights that apoptotic onset is a very valuable step in acini morphogenesis. Therefore, it is important to understand the mechanisms behind apoptotic onset and how these are disrupted.

During tumourigenesis, cells evade the processes of apoptosis, anoikis (death as a result of detachment from the ECM) and autophagy, leading to an increase in cell number. One way in which cancer cells acquire resistance to apoptosis and anoikis is through the constitutive activation of signalling pathways, due to oncogenic Ras and c-myc activation (Frisch & Francis, 1994; Rosen *et al*, 2000). This can result in the deactivation of pro-apoptotic factors and caspases, which prevents the apoptotic process. Anti-apoptotic factors, such as Bcl-2 and Bcl-xl, are also commonly over-expressed or mutated in cancer, which results in increased sequestering of pro-apoptotic Bax and an inhibition of mitochondria-mediated apoptosis (Tsujimoto *et al*, 1985; Krajewska *et al*, 1996). In the case of autophagy, this process can be inhibited through the actions of the PI3K pathway, which is often dysregulated in cancer (Bellacosa *et al*, 1995). Often, dysregulation of the apoptotic and autophagic processes alone does not result in carcinogenesis, and other mutations in genes involved in processes, such as metabolism and proliferation, are required for a cancerous phenotype.

3.4 Proliferation

The cell cycle is the process by which cells divide and is composed of two phases: interphase, where cells prepare to divide and mitosis, the production of two daughter cells. Interphase itself is also composed of several phases: G_1, S and G_2. Additionally, non-dividing cells remain in a state of rest denoted G_0 (**Figure 3.6**). Normally, cells cannot just pass through the cell cycle and divide uncontrollably. The process is regulated at three checkpoints by cyclins and cyclin dependent kinases (CDKs) to prevent mutated cells or cells lacking an adequately assemble mitotic spindle to divide.

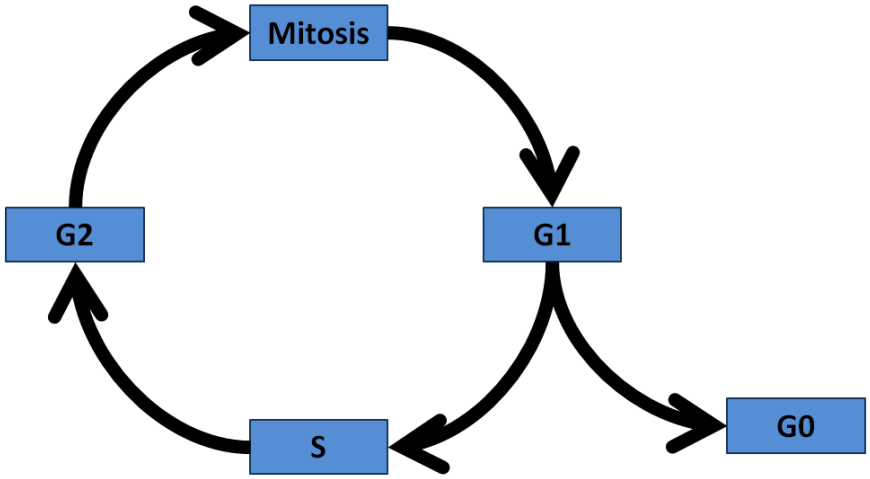

Figure 3.6: The cell cycle. Cells progress through the cycle passing through interphase (G_1, S & G_2), before undergoing division during mitosis to produce two daughter cells.

The G_1/S restriction point is regulated by cyclin D, which binds to and activates CDK4 and CDK6. CDK4 and CDK6 go on to phosphorylate the retinoblastoma (Rb) protein, liberating it from the transcription factor, E2F. E2F can then activate the transcription of genes required for transition into S phase. Further regulation is applied to progression through the cell cycle by the presence of CDK inhibitors, such as $p21^{CIP1/WAF1}$ and $p16^{ink4}$, which function to inhibit progression through G1. The dysregulation of the cyclins, CDKs and CDK inhibitors is common in cancer and results in uncontrolled progression through the cell cycle and increased proliferation (Tsihlias *et al*, 1999).

Immunocytochemistry (ICC) of 3D cultures of mammary epithelial cells showed that the expression of CDK inhibitor, $p27^{kip1}$ gradually increased throughout morphogenesis, implying that during acinar morphogenesis, proliferation is initially high, but with time cell cycle progression is inhibited, which results in decreased cellular proliferation and, hence, growth-arrest. Simultaneously, Ki-67 (a

marker of proliferation) was expressed at high levels during acini formation, but following growth-arrest, its expression declined (Coppock *et al*, 2007). In a different study it was reported that AKT was only present in the basement membrane-attached outer cells of acini (Debnath *et al*, 2002; Debnath *et al*, 2003[b]). Given the activation of this kinase is still maintained following a cessation of proliferation, this implies that during the later stages of acinar morphogenesis, AKT functions as a pro-survival mechanism, rather than an anti-apoptotic one (Hutchinson *et al*, 2001). Previous work has also demonstrated that in 3D cultures, mammary epithelial cells proliferate excessively when cyclin D1 is over expressed, or Rb is inactivated and that increased proliferation is involved in loss of growth arrest and luminal filling (Muthuswamy *et al*, 2001; Debnath *et al*, 2003[b]). Finally, it has also been shown that excessive proliferation, in itself, is not sufficient to induce luminal filling and that aberrations in other processes, such as apoptosis are required (Debnath *et al*, 2002).

3.5 Polarity

Cellular polarity is defined by the asymmetrical distribution of proteins in cellular domains. The control of proliferation, survival, migration, asymmetric stem cell division and, ultimately, correct functioning of epithelial tissues is dependent on the establishment of baso-apical polarity, and abnormal cell polarity is the hallmark of many cancers.

In the acini of the breast, as the hollow lumen forms, apical polarity is established, as seen by the apical formation of tight junctions markers, such as proteinase-activated receptor-3 (PAR3) and PALS1 (Plachot *et al*, 2009). Hemidesmosome formation of α6β4 integrins at the basal surface (Koukoulis *et al*, 1991) and apical localization of Zonula Occludens-1 (ZO-1) (Fogg *et al*, 2005) is also established during acini formation. Disruption of acinar apical polarity, such as the redistribution of tight junctions, can force cells into the cell cycle (Chandramouly *et al,* 2007) and, therefore, may have implications upon acini formation and correct mammary gland function (Itoh & Bissell, 2003).

It would appear that the correct establishment and maintenance of polarity is essential for acini development and, thus, it is important to know the signalling pathways involved in establishing polarity. Src kinases are activated early in the AKT signalling cascade and these phosphorylate PAR-3 resulting in delayed tight junction formation (Wang *et al*, 2006). Polarity proteins can also influence signalling pathways. In Drosophila, PAR-3 recruits PTEN to developing junctions and promotes the formation of PIP_2 from PIP_3 (Pinal *et al*, 2006), which reduces Akt signalling.

3.6 Considerations for 3D Culture

Matrigel is extracted from the Engelbreth-Holm-Swarm (EHS) mouse tumour and is essentially basement membrane in a solubilised form, with a composition of laminin, collagen and entactin (Kleinman *et al*, 1986). Although variation exists from batch to batch, the approximate composition consists of 60% laminin, 30% collagen IV and 8% entactin, with the remainder consisting of heparin sulphate proteoglycans, growth factors and matrix metalloproteinases (BD Biosciences, 2011). It is the presence of these structural proteins that allow cells cultured in 3D matrices to form interactions with proteins which they would encounter *in vivo*, but are, otherwise, not present in traditional monolayer cultures (Streuli *et al,* 1995; Hughes *et al*, 2010). These interactions then result in the activation of various signalling cascades and can regulate the transcription of various genes, in addition to providing structural support for 3D structures (Streuli *et al*, 1995; reviewed in El-Hamamsy & Yacoub, 2009).

The use of 3D culture systems presents many considerations and requires optimisation of conditions for varying cell lines. Firstly, the cells themselves can behave differently within Matrigel, depending on the number of times they have undergone trypsinisation and re-seeding after resurrection (passage number), morphology in monolayer culture prior to seeding onto Matrigel and seeding density. After resurrection from stocks stored in liquid nitrogen, mammary epithelial cells, such as MCF-10A and MCF-12A, require approximately 2 passages to fully recover and to adapt to the culturing conditions. If cells have only undergone a single passage following resurrection, acini are usually smaller in size and lumen formation does not occur (Benton *et al*, 2009). Similarly, MCF-10A and MCF-12A cells that are over-confluent or under stress have a spindle-like morphology in monolayer culture, and will not produce acini in 3D culture (Debnath *et al*, 2003). Finally, regarding the seeding density, optimisation of the correct seeding density is essential for good acini formation. If the number is too low, acini do not form or those formed are small in size and slow in growth. On the other hand, if too many cells are seeded, as acini grow, they overlap with each other, and this affects proper acini formation and acini visualisation at the end of the experiment.

The source, composition, and thickness of Matrigel, as well as whether it is combined with other 3D matrices, also impacts upon successful 3D culture (Swamydas *et al*, 2010). The presence of growth factors, such as fibroblast growth factor, transforming growth factor-β (TGFβ), EGF or insulin-like growth factor can impact upon epithelial growth in Matrigel (Vukicevic *et al*, 1992) and, therefore, growth factor-reduced versions are typically used for 3D studies. Despite the manufacturer's quality control measures, Matrigel composition varies from batch to batch and this lot variation can severely affect

acini morphogenesis. It is, therefore, necessary to test different Matrigel lots to determine the best one for further experiments. The most appropriate Matrigel batch will produce spherical acini of similar sizes that arrest in growth and produce hollow lumen. Another consideration when working with Matrigel, is that it rapidly polymerizes at temperatures above 4^{o}C. For this reason, it must be handled and stored appropriately to avoid untimely polymerization, which may affect acini formation or hinder subsequent analysis by immunocytochemistry and microscopic visualisation.

The way in which cells are seeded when using Matrigel also impacts upon acini formation. In initial published studies using the 3D Matrigel matrix, cells were completely embedded in the Matrigel. However, a more recent method involving an overlay procedure (termed the "overlay method") has been developed, whereby cells are seeded onto a layer of polymerised Matrigel and grown in media supplemented with 2% Matrigel (Debnath *et al*, 2003). The latter method, not only has the benefit of using significantly less Matrigel, and, hence, being far more economical, but the resultant acini are larger in size. This is advantageous as the lumen of these acini are easily identified.

3.7 Materials and Methods

All reagents were purchased from Sigma-Aldrich (Dorset, UK), unless otherwise stated.

3.7.1 Cell Lines and Routine Culture

MCF-12A cells were cultured as described in **Chapter II**, in the **Materials and Methods** section.

3.7.2 3D Cultures of Immortalised Mammary Epithelial Cells: The Overlay Method

45 µl growth factor reduced Matrigel® (BD Biosciences, Bedford, MA USA) was spread evenly over the surface of each well of an eight-well chamber slide (Lab-Tek® Chamberslide™ System Nalge Nunc International, Naperville, IL, USA) and incubated at 37^{o}C for a minimum of 15 minutes, to allow the Matrigel to set. Confluent cells were trypsinized as per routine culture, resuspended in a total of 7 ml re-suspension medium and centrifuged for 5 minutes at 150 g. The re-suspension media was aspirated and cells were re-suspended in 2 ml assay media, before pipetting several times through a 1000 µl Gilson pipette (P1000) to obtain a single-cell suspension. Additional assay media was added so that the final volume of cell suspension was approximately 8 ml. Cells were counted and diluted in assay media (**Table 3.1**) in order to obtain a final concentration of 20,000 cells per ml. The cell suspension was added in a 1:1 ratio to assay media containing 4% Matrigel and 10 ng/ml EGF. 400 µl of this cell suspension

was plated per well, on top of the Matrigel in the chamber slides. This ensured that 8000 cells were seeded into each well. Media changes consisting of 400 µl assay media containing 2% Matrigel and 5 ng/ml EGF were carried out every four days. Cells were maintained at 37^{o}C in 5% CO_2. The morphology of the acini was assessed throughout the incubation period by light microscopy. Following completion of 20 days, (although this was later reduced to 16 days), incubation in 3D culture, immunocytochemistry was performed and acini were imaged using the Zeiss LSM 510 META confocal microscope (Carl Zeiss Ltd, Hertfordshire, UK).

Table 3.1: Assay media composition for MCF-12A cells (Debnath *et al*, 2003)

Component	Assay Medium
DMEM/F12 (Invitrogen, Paisley, UK)	500 ml
Horse Serum (Invitrogen, Paisley, UK)	2%
Epidermal Growth Factor	-
Cholera Toxin	100 ng/ml
Insulin	10 µg/ml
Hydrocortisone	0.5 µg/ml
Pen/Strep	5000 µg/ml
L-Glutamine (MCF-10A media only)	109.5 mg/2.8 ml

3.7.3 Immunocytochemistry

At the end of the incubation period, the media was aspirated from individual wells and cells were fixed with freshly prepared 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4 for 20 minutes, at room temperature. Cells were permeabilised to allow antibody entry across the plasma membrane, using 0.5% Triton-X, (to avoid disruption of the tertiary structure of proteins), in PBS for 10 minutes, at 4^{o}C. Samples were then washed three times with 100 mM glycine solution (130 mM NaCl, 7 mM Na_2HPO_4, 3.5 mM NAH_2PO_4, 100 mM glycine in PBS) for 10 minutes at room temperature, with gentle rocking, to quench the cross-linking induced by the fixation procedure. Samples were incubated

with the primary blocking solution (130 mM NaCl, 7.7 mM NaN_3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, 10% goat serum) for 1 hour at room temperature, to prevent unspecific primary antibody binding. The primary block solution was aspirated and the secondary block (130 mM NaCl, 7.7 mM NaN_3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, 10% goat serum, 20 µg/ml goat anti-mouse F(ab')2 fragment (Jackson Immuno Research, Suffolk, UK) was applied for 40 minutes at room temperature, prior to 18 h primary antibody incubation at 4^{o}C, to prevent unspecific antibody binding through the Fc receptor and reduce background staining as a result. Primary antibodies and concentrations are listed in **Table 3.2** (antibodies were diluted in secondary block solution). Antibodies were centrifuged at 5000 rpm for 5 minutes, to prevent aggregate formation prior to dilutions being prepared in the secondary block solution.

Table 3.2: Primary antibodies used for immunocytochemistry to investigate apoptosis and polarity in 3D culture of mammary epithelial cells

Primary Antibody	Species	Source	Purpose	Dilution
Cleaved caspases-3	Rabbit	Cell Signalling, Hertfordshire,UK	Apoptotic marker	1:200
Laminin	Mouse	Abcam, Cambridge, UK	Basement membrane	1:200

Samples were washed three times with immunofluorescence buffer (IF) (130 mM NaCL, 7 mM Na_2HPO_4, 3.5 nM NaH_2PO_4, 7.7 NaN3, 0.1% BSA, 0.2% Triton X-1000, 0.05% Tween-20) at room temperature, with gentle rocking for 20 minutes. Secondary antibodies (**Table 3.3**) were prepared in IF buffer with 10% goat serum and applied to samples for 50 minutes, at room temperature. As with primary antibodies, secondary antibodies were centrifuged at 5000 rpm for 5 minutes, prior to dilutions preparation with IF buffer + 10% goat serum.

Table 3.3: Secondary antibodies used for immunocytochemistry to investigate apoptosis and polarity of mammary epithelial cells

Secondary Antibody	Species	Source	Dilution
Alexa Fluor® 488 anti-rabbit IgG	Goat	Molecular Probes (Invitrogen, Paisley, UK)	1:200
Alexa Fluor® 555 anti-mouse IgG	Goat	Molecular Probes (Invitrogen, Paisley, UK)	1:200

After this, samples were washed three times with IF buffer at room temperature with gentle rocking for 20 minutes and then counterstained with 5 µM TOPRO-3 (Molecular Probes, Invitrogen, Paisley, UK) in PBS for 15 minutes at room temperature. Samples were rinsed with PBS for 5 minutes with gentle rocking and mounted with Prolong Antifade Reagent (Molecular Probes, Invitrogen, Paisley, UK). Samples were left to dry at room temperature overnight and image acquisition was conducted using the Carl Zeiss MicroImaging, Inc. LSM510 confocal microscopy system with LSM version 4.20 (Carl Zeiss Ltd, Hertfordshire, UK). All images are representative of three independent experiments performed in duplicate.

3.8 Results

3.8.1 Acini Morphogenesis: Optimisation of the Overlay Method for 3D Cultures of MCF-12A Cells Matrigel Composition Impacts on Acini Formation

Debnath *et al* (2003), describe in their protocol that better 3D of cultures MCF-10A were obtained from Matrigel lots where protein concentrations ranged between 10 and 12 mg/ml and where endotoxins levels did not exceed 2 EU/ml. Because of this, various batches of Matrigel with varying protein concentrations and endotoxin levels were tested. It was observed that these characteristics of Matrigel did, in fact, dramatically influence acini morphogenesis and morphology.

Based on the literature, normal acini grown using the methodology described here and by Debnath *et al*, (2003), are spheroid and comprised of a single layer of cells, observed by staining with a nuclear counterstain (blue) surrounding a hollow, or hollowing lumen with the presence of apoptotic cells (green). This structure is surrounded by a basement membrane (red) as shown in **Figure 3.7**.

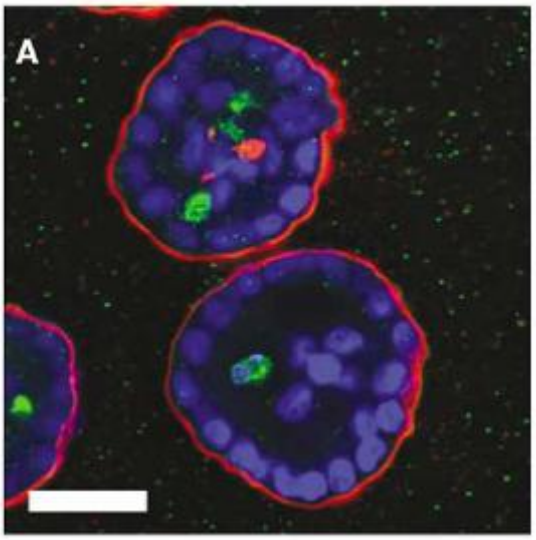

Figure 3.7: Confocal image of acini from 3D cultures of MCF-10A cells. Cells are shown in blue through the use of a nuclear counterstain. Green staining shows activated caspase-3 as a marker for apoptosis and red staining shows laminin V as a marker for the basement membrane. (Image from Debnath *et al*, 2003.)

After 20 days in 3D culture, cells cultured in Matrigel batches that contained a protein concentration below 10 mg/ml and endotoxin level of 2.0 EU/ml, or above, formed acini of varying sizes. The resultant acini were also not hollow and some cells in contact with the basement membrane were apoptotic, as indicated by the presence of activated caspase-3. Laminin was also observed to be connecting adjacent acini (**Figure 3.8**). The extent of luminal filling decreased as protein concentration increased, and at 10.4 mg/ml protein, acini were almost hollow with continuing signs of apoptosis. The laminin connections between acini were not observed in higher protein concentrations and, thus, the image quality in terms of background staining greatly improved upon seeding in higher protein concentrations. Finally, cells cultured onto Matrigel where the protein composition was 10.3 mg/ml and the endotoxin level was less than 1.5 EU/ml formed spherical acini exhibiting hollow lumen after 20 days in culture.

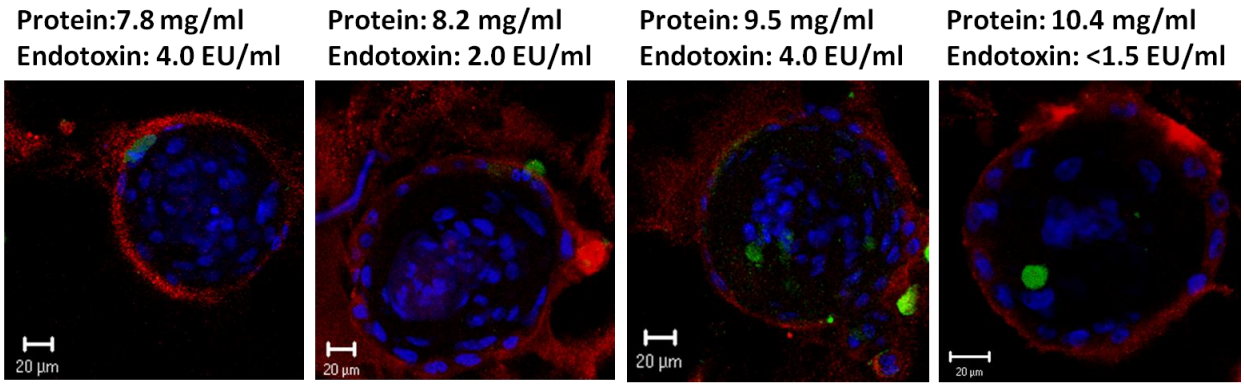

Figure 3.8: Acini grown in Matrigel with different protein and endotoxin concentrations. Acini were immunostained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain.

In terms of seeding density, 20,000 cells per ml was sufficient to produce an adequate number of acini and seeding densities greater than 25,000 cells per ml resulted in overlapping and interconnecting structures that impeded analysis of LCM images. Also, it was observed that using 45 µl as opposed to the 40 µl Matrigel suggested by Debnath (2003), made Matrigel spreading easier and produced a more even distribution of acini, less overlapping structures, and importantly, more acini that fit the characteristics described above (hollow lumen and intact basement membrane). In initial experiments, monolayer growth of cells underneath the Matrigel proved to be an issue, as it obscured acini during analysis. Modification of the amount of Matrigel used appeared to resolve this issue, as did ensuring that Matrigel was evenly spread across the whole surface of the chamber wells, with no bubbles.

3.8.2 3D Culture: Time-Course

To characterise the development of acini with ER, GPER-1 competent MCF-12A cells, a time-course was set up with 20,000 cells being seeded every four days, as described previously. Following 4, 8, 12 and 16 days in 3D culture, acini were fixed and immunocytochemistry was performed (**Figure 3.9**). The aim of this was to establish the timing of key events during morphogenesis, such as the formation of the basement membrane, induction of apoptosis and growth arrest. This provides the opportunity to study the impact of estrogens on these events, in future experiments.

After 4 days in Matrigel, MCF-12A cells had formed small, spherical cellular clusters clearly surrounded by a basement membrane, as shown by the presence of laminin V around the spheroid structures. At this point, there was little or no evidence of caspases-mediated apoptosis of the centrally

located cells and organisation of the cells was absent. Between days 8 and 16, it became evident that acini consisted of two populations of cells: a single cell layer of cells attached to the basement membrane and a population of cells not in contact with the basement membrane. This was inferred by the observation that only the cells lacking contact with the basement membrane showed evidence of caspase-mediated apoptosis.

Between 8 and 12 days, the acini continued to grow in size and remained spherical. The cells in contact with the basement membrane organised themselves into a uniform arrangement during this time and extensive apoptosis, as indicated by the presence of activated-caspase-3, was observed within the central cells of the acini. At 16 days, complete or near complete, spherical acini with hollow lumen surrounded by a single layer of epithelial cells were observed with an approximate size of 120 ± 6.9 µm and the surviving outer layer of cells showed no signs of apoptosis. In acini where the formation of the hollow lumen was not yet complete, signs of apoptosis were still present (**Figure 3.9; 3.10**).

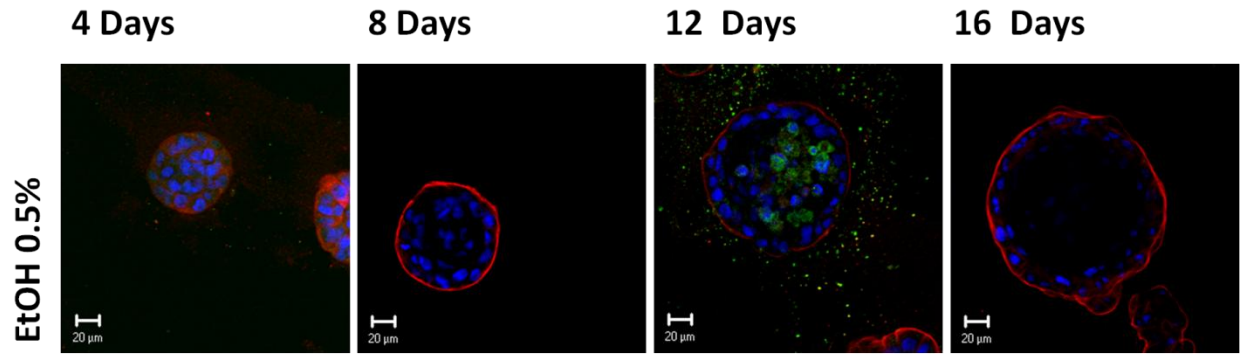

Figure 3.9: Time-course of MCF-12A acini over 16 days. Acini were stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain.

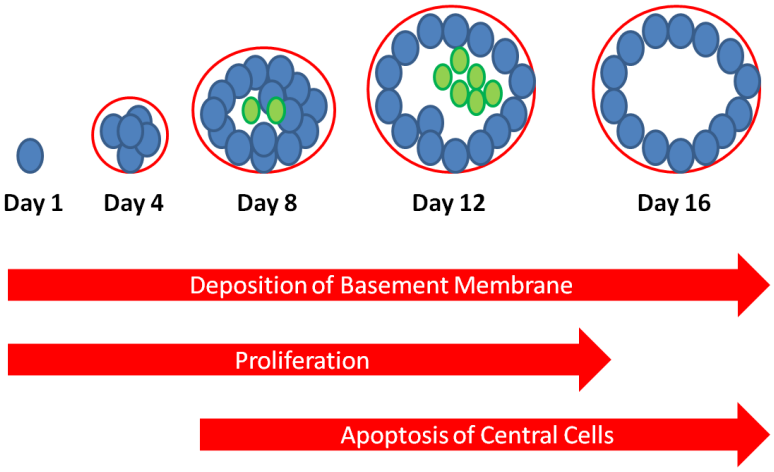

Figure 3.10: Schematic of MCF-12A morphogenesis in 3D culture. Basement membrane deposition (red) occurs as early as 4 days. Proliferation and increase of acinar size terminates between days 12 and 16 and apoptosis (green) of the central cells occurs from day 8. Finally at day 16 a hollow lumen is observed, surrounded by a layer of organised epithelial cells attached to a basement membrane.

3.9 Discussion

The composition of Matrigel clearly has an effect on acini formation. Based on our observations, the factors that significantly impact on the development of these structures are protein and endotoxin concentrations.

Among the various proteins present in Matrigel, collagen, laminin and fibronectin are key constituents (Kleinman *et al*, 1986), although the levels of these and other constituents vary from batch to batch.

During breast development or carcinogenesis, in humans and *in vivo*, there are changes in the levels of collagen, laminin and fibronectin, and this aids breast morphogenesis (Woodward *et al*, 2001; Bissell *et al*, 2005; Wolf *et al*, 2009). Fibronectin stimulates cellular proliferation and increased levels of fibronectin result in increased proliferation and increased acinar size (Williams *et al*, 2008). Laminin also promotes proliferation, as well as cell adhesion and its absence in 3D matrices results in acini lacking lumen and displaying aberrant polarity (Gudjonsson *et al*, 2002). Collagen facilitates cell growth, differentiation and attachment and regulates matrix modelling (Karamichos *et al*, 2006; Karamichos *et al*, 2007) and its presence stabilises the basement membrane during embryonic development (Pöschl *et al*, 2004). Increased collagen, however, increases cellular proliferation resulting in larger acini (Provenzano *et al*, 2009).

Although the supplier provides information regarding the approximate total protein concentration of each Matrigel batch, the precise concentration of individual components is not provided. Under our experimental conditions, acini grown in Matrigel with lower protein concentrations had normal shape and size, but displayed filled lumen. It has previously been reported that the absence of laminin or high concentrations of collagen, produce acini that lack hollow lumen (Gudjonsson *et al*, 2002). However, the exact reason for this, and indeed, for our observations presented above remains unclear.

Endotoxins are lipopolysaccharides present in the membranes of gram-negative bacteria. Structurally, they are comprised of a hydrophobic lipid group and a polysaccharide tail. It is the lipid group that is responsible for anchoring the endotoxin to the membranes of bacteria and mediating the effects of endotoxins. Endotoxins are present in water, serum, resins and culture additives and, can be released upon the destruction of gram-negative bacteria. Therefore, endotoxin contamination of Matrigel can potentially occur as a result of the use of detergents, or during the Matrigel extraction process.

Endotoxins have previously been shown to interact with cell membranes resulting in surface ruffling and cell membrane disruption (Csaba *et al*, 1984). This membrane disruption, however, was not the only effect observed in Matrigel with higher endotoxin levels, suggesting that endotoxins may interact with other cellular processes. Indeed, it has been shown that endotoxins can influence the production of various cytokines, such as tumour necrosis factor alpha (TNFα) in various cell types including those of the breast (Adams & Czuprynski, 1990; Bienhoff *et al*, 1992; Safieh-Garabedian *et al*, 2004) and other cellular products such as cyclooxygenase-2 (COX-2), collagenases and prostaglandins (Barker & Holt, 1983; Morales *et al*, 1984; Cury *et al* 1988; Huang *et al*, 2008). TNF is usually associated with induction of apoptosis, however, it has also been reported to promote the growth of both normal and breast cancer cells via MAPK and Akt signalling pathways (Rivas *et al*, 2008). This mitogenic action and dysregulation of cell signalling pathways could account for the lack of hollowing observed in acini grown in Matrigel containing larger amounts of endotoxins.

Additionally, endotoxins can have mitogenic effects in some mammalian cell lines and have consequences for cell differentiation (Brunette, 1984; Meghji *et al*, 1996; Dawson, 1998; Putnins *et al*, 2002). It could, therefore, mean that increased endotoxin levels in Matrigel contribute to increased proliferation, as characterized by luminal filling.

Overall, in our work, it became clear that the Matrigel composition greatly affects acini formation and for this reason it was ensured all subsequent experiments were conducted using Matrigel with a protein concentration of 10 mg/ml or above and an endotoxin level not exceeding 1.5 EU/ml.

Following optimisation of the 3D overlay method described by Debnath (2003) and selection of the appropriate Matrigel batch and seeding density, a time-course to investigate the formation of acini from 3D cultures of MCF-12A cells was performed. This was necessary to identify when key events (deposition of the basement membrane, proliferative arrest and formation of the hollow lumen) occurred, so that in future work, we could assess the effects of estrogens upon 3D cultures of MCF-12A cells. During these initial experiments, it was observed that MCF-12A cells grown in 3D formed acini with a hollow, or near hollow, lumen after 16 days, and following this observation, subsequent experiments were performed for 16 days. This is a similar timeframe to what was observed with 3D cultures of MCF-10A cells (Debnath *et al*, 2003). This may infer that the morphogenic programme of non-transformed mammary epithelial cells cultured in Matrigel occurs in the same manner across cell lines, although this would have to be studied further.

Whilst establishing the time-course for acini morphogenesis in MCF-12A cells, it became evident that there are two cell populations within acini: cells at the periphery of the spheroid structures, attached to an underlying basement membrane, and those that are centrally located. The two populations have very different properties (e.g. polarity and attachment to the basement membrane) and fates; the outer peripheral cells are attached to a basement and organise themselves into a single layer of organised cells, whereas the centrally located cells are initially disorganised and then undergo apoptosis and are cleared as acini formation progresses. Given that this luminal clearance coincides with the expression of activated caspase-3, it can be inferred that mitochondria-meditated apoptosis plays a role in the formation of the hollow lumen. This observation is in agreement with the data reported by Debnath and co-workers in ER negative MCF-10A cells grown in 3D culture (Debnath *et al*, 2002; Debnath *et al*, 2003).

Basement membrane formation appears to be an early occurrence in acini development and was clearly visible after 4 days in 3D culture. The observation that basement membrane deposition occurs so early during acini morphogenesis suggests that this process is fundamental for subsequent stages in acini formation, such as the establishment of apico-basal polarity, proliferation and luminal clearing.

We observed that MCF-12A acini underwent growth arrest, as inferred by the cessation of increased acinar size, indicating decreased proliferation, at approximately 12 days, as acini did not grow greatly in size between days 12 and 16 days in 3D culture. Studies with the proliferative marker, Ki-67, have shown that during MCF-10A acinar morphogenesis proliferation increases between day 1 and 12 and then decreases again (Coppock *et al*, 2008).

By establishing a time-course, the morphogenic process of single MCF-12A cells to hollow acinar structures can be modelled and is comparable to that described for MCF-10A cells (**Figure 3.10**) (Debnath *et al*, 2003; Coppock *et al*, 2007). This, in addition to optimising the conditions for 3D culture of MCF-12A cells, will enable to study the impact of estrogens upon key stages in the morphogenic programme: deposition of the basement membrane, proliferation, apoptosis and hollow lumen formation. The model, therefore, provides us with an opportunity to model the effects of estrogenic compounds on mammary gland formation, using an *in vitro* methodology.

To conclude, in this chapter we describe the work carried out to establish a 3D model, in which to study the morphogenesis of non-transformed ERα, ERβ, GPER-1-competent MCF-12A cell acini. The structures formed under these conditions present features resembling those found in the human mammary gland, such as a layer of epithelial cells encapsulating a hollow lumen, surrounded by basement membrane. Once this preliminary work was carried out we could proceed to study the impact of estrogens on the formation and maintenance of the epithelial acini in our 3D Matrigel model.

Chapter IV: 3D Culture: The Effects of Endogenous and Exogenous Estrogens on Acini Formation

In **Chapter III**, we studied and characterised the morphogenesis of MCF-12A in 3D culture. Defining the normal behaviour of these cells when grown in Matrigel *in vitro* provides a baseline, control reference to which treated acini can be compared against. As discussed previously, estrogen exposure has been associated with breast cancer risk and although the molecular mechanisms involved have been extensively studied, little work has been carried out on the impact of estrogen on the architecture and formation of the mammary gland. The Matrigel 3D model provides an opportunity to study this in an *in vitro* setting.

4.1 Involvement of the ER and GPER-1 in Breast Carcinogenesis

Both clinical and epidemiological data have demonstrated that there is a link between exposure to female ovarian hormones, such as E2, and breast cancer risk ([b]Pike *et al*, 1993). These effects, as well as the normal physiological function of estrogens, are mediated by ERα and ERβ, although more recently, estrogens have been demonstrated to also activate growth factor signalling pathways and associate with GPER-1 (Filardo *et al*, 2002). These effects are also responsible for some down-stream effects of E2 (Migliaccio *et al*, 1996; Filardo *et al*, 2002). The exact mechanism through which estrogens contribute to breast epithelium formation, breast carcinogenesis and cancer progression remains unclear, although various theories have been developed.

Firstly, estrogens promote cellular proliferation (Russo *et al*, 1999). In **Chapter II**, however, increased proliferation was not observed in ER competent MCF-12A cells. Nevertheless, increases in proliferation, provides an increased chance for mutations to occur which may result in a cancerous phenotype. Furthermore, it remains to be seen whether estrogen treatment of 3D cultures of MCF-12A cells results in increased proliferation.

An alternative hypothesis is that, independently from the ER, genotoxicity induced by estrogens and their metabolites may lead to tumourigenesis. In this model, the presence of estrogens and their metabolites increases the rate at which mutations occur due to the production of electrophiles capable of forming DNA adducts (Ashburn *et al*, 1993). This carcinogenic effect has been demonstrated in the ER negative mammary epithelial, MCF-10F cell line, whereby treatment with E2 induced DNA adduct formation and caused loss of heterozygosity (LOH), the loss of normal function of one gene allele where

the second allele was previously inactivated, in chromosome 11 (Russo *et al*, 2002; Lu *et al*, 2007). In their corresponding experiments, Russo *et al* (2002; 2003) also assessed the ductulogenic potential of MCF-10F cells treated with E2. In these experiments, it was shown that in MCF-10F cells cultured in 3D collagen matrices and treated with E2, increased proliferation was observed and cells lost their capability to form hollow ductules, resulting in the formation of large solid masses. These data may implicate ERβ in mediating some of E2's effects in terms of increased proliferation, but the authors attributed these effects to genotoxic events, rather than ER-mediated actions. These observations further demonstrate the importance of studying estrogens in a more physiologically relevant context, although it remains to be seen whether E2 impacts upon MCF-12A cells cultured in Matrigel in a similar fashion.

As described in **Chapter III**, 3D cultures of breast epithelial cells, such as MCF-10A and MCF-12A have been utilized for their ability to recapitulate some of the characteristics of breast epithelium *in vivo*. These features include the formation of highly organised, polarised, growth-arrested structures with hollow lumen and basal deposition of the basement membrane. This 3D model has also been used to study the transformation of cells to a malignant state, by allowing the detection of changes, such as the disruption of polarity, increased proliferation and luminal filling (Debnath *et al*, 2003). The work presented in this chapter aims to further investigate the impact of estrogens on acini formation using ER competent MCF-12A cells, in an *in vitro* Matrigel model. Our work differs from that conducted and described by Russo and colleagues (2002; 2003) in that MCF-12A cells express both ERα and ERβ, whereas MCF-10F cells only express ERβ at low levels. Our methodology allows us to investigate whether the ER mediates the actions of estrogens on the 3D epithelial architecture of acini.

In **Chapter II**, we confirmed the expression of the estrogen-responsive receptor, GPER-1 in the MCF-12A cell line, in addition to ERα and ERβ. As described previously, the precise function of GPER-1 in the mammary gland is unknown, but it may be associated with tamoxifen resistance in the MCF-7 cell line (Ignatov *et al*, 2010). To date, there is little published data regarding the role of GPER-1 in acini morphogenesis. Therefore, in addition to investigating the role of the classical ER, we will also assess whether this receptor is also involved in mediating the impact of estrogens upon MCF-12A acini morphogenesis in 3D culture.

4.2 Xenoestrogens

As mentioned in **Chapter I**, xenoestrogens, such as *o,p'*-DDT, β-hexachlorocyclohexane (β-HCH), BPA and n-propylparaben can exert estrogenic actions via interactions with the ER.

BPA is able to bind to ERα, ERβ, mER and GPER-1 (Matthews *et al*, 2001; Watson *et al*, 2005; Thomas & Dong, 2006). Although its affinity for the ER is 10,000-fold less than that of E2 (Kuiper *et al*, 1998), studies have shown that it may be just as efficacious as E2, in respect to the detrimental effects of E2 *in vivo*, and may also be equally potent in terms of non-genomic calcium ion secondary messenger generation (reviewed in Vanderberg *et al*, 2007).

In vitro, BPA is able to stimulate the proliferation of MCF-7 breast cancer cells, although not to the same extent as E2 (Krishnan *et al*, 1993; Miyakoshi *et al*, 2009). Perinatal exposure to BPA in rats has been shown to affect breast development and increase the risk of developing breast cancer later in life. These effects are observed as undifferentiated structures, delayed hollow formation, increased sensitivity to estrogen and increased incidence of preneoplastic lesions after birth (Muñoz-de-Toro *et al*, 2005; Vandenberg *et al*, 2007[2]; Moral *et al*, 2008). BPA also may reduce the sensitivity of some tumours to chemotherapy treatment (LaPensee *et al*, 2010).

As with E2, BPA is able to elicit genomic effects. Gene microarray PCR analysis has revealed that BPA appears to impact upon genes involved in differentiation, proliferation, cell death and immune responses (Moral *et al*, 2008). The consequence of this change in genomic profile was undifferentiated structures and delayed lumen formation *in vivo*. At the molecular level, BPA has been documented to decrease DNA methylation (Dolinoy *et al*, 2007), which suppresses the expression of certain genes, and induce DNA adduct formation (De Flora *et al*, 2011). Additionally, BPA has been linked to aneuploidy in breast cancer cells, an effect believed to be mediated by extranuclear signalling cascades (Kabil *et al*, 2008). BPA can also generate non-genomic actions, such as the activation of MAPK signalling in rat sertoli cells, and more importantly, in human breast cancer cell lines (Dong *et al*, 2011; Pupo *et al*, 2012). The consequence of these non-genomic effects may present itself as increased cellular proliferation or survival, and these may, in turn, result in the morphological effects on breast epithelium described above.

Compared to E2, the affinity of BPA to the ER is very low and so it has been classed as a weak estrogen, along with many other xenoestrogens. However, the low estrogen activity elicited by these compounds individually is something that should not be ignored, as low levels of individual xenoestrogens can have an additive effect with other xenoestrogens and endogenous estrogens (Silva *et*

al, 2002). This leads to a stronger response to estrogenic compounds in combination, than observed individually.

Although naturally occurring, propylparabens, are also synthetically manufactured and used in cosmetics as preservatives and in food products as additives. Upon absorption into the body, parabens are metabolized and excreted rapidly. They are weakly estrogenic and the presence of parabens has been found in breast cancer tumours at low concentrations and exhibit weak estrogenic activity (Darbre *et al*, 2004; Harvey & Everett, 2004). These observations led to suggestions that parabens may be associated with breast cancer risk. Although this correlation remains to be proven, treatment with parabens has been demonstrated to increase proliferation of MCF-7 cells in an ER-dependent fashion (Byford *et al*, 2002).

o,p'-DDT and β-HCH, were used as pesticides worldwide until being banned by much of the developed world. As they are highly lipophillic, resist degradation and have a very high half-life, they accumulate in the environment and the food chain, and are present in human samples many years after a ban in its use (Ahlborg *et al*, 1995; Rivas *et al*, 2001). β-HCH has been detected in breast cancer samples at higher levels than in normal tissue samples, suggesting that there may be a correlation between β-HCH levels and breast cancer incidence (Mussalo-Rauhamaa *et al*, 1990; Ahlborg *et al*, 1995). β-HCH treatment of mammary epithelial cells causes changes in the gene expression of various oncogenes, including cyclin D1 and it enhances tumour development *in vivo* (Mussalo-Rauhamaa *et al*, 1990; Wong & Matsumura, 2007). Similarly, *o,p'*-DDT, is capable of inducing the transcription of the progesterone receptor, TFF1 and various E2-responsive genes (Soto *et al*, 1995; Dees *et al*, 1997). Additionally, both β-HCH and *o,p'*-DDT have been reported to stimulate the proliferation of ER competent mammary epithelial cell lines (Steinmetz *et al*, 1996) and to activate growth factor signalling cascades in human cancer cells (Silva *et al*, 2010).

Given that the xenoestrogens described above exert similar actions to E2 and have long been hypothesised to contribute to breast cancer risk, we considered it important to study the impact of xenoestrogens on 3D cultures of MCF-12A cells with the aim of elucidating if and how these compounds affect acini morphogenesis.

4.3 The Effect of Receptor Antagonists

As mentioned previously, the endogenous hormone, E2 is involved in both normal physiological processes and disease, and this effect is primarily mediated by the ER. Approximately two thirds of

breast cancers are dependent on E2 at some point during cancer progression (Musgrove & Sutherland, 2009) and thus antagonists that target the ER, such as the SERM, tamoxifen and pure antagonist, fulvestrant (ICI 182,780), have been used in the treatment of the disease (Katzenellenbogen *et al*, 2000; Jordan, 2009). ICI 182,780 is an analogue of E2 and its structure differs from that of other ER antagonists, such as tamoxifen and raloxifene, in that it possesses a steroidal-like structure (**Figure 4.1**). ICI 182,780 acts as a competitive inhibitor of the ER and has a binding activity of 89% of E2, compared to 2.5% of tamoxifen (Wakeling & Bowler, 1988; Wakeling *et al*, 1991). ICI 182,780 acts by binding to the ER and thus impairing ER dimerisation and translocation to the nucleus. Moreover, it also promotes ER protein degradation (Fawell et al, 1990; Nicholson *et al*, 1995; Salazar *et al*, 2011). Consequently, ICI 182,780 not only inhibits the activity of the ER but also down-regulates ER at the protein level.

G-15, as discussed in **Chapter II**, is a GPER-1 antagonist, that selectively binds to GPER-1 and exhibits little affinity towards ERα and ERβ. G-15 binds to GPER-1 with an affinity of approximately 20 nM, whereas, the affinity of E2 for this receptor is between 3-6 nM, thus E2 displays a higher affinity for GPER-1 (Thomas *et al*, 2005; Dennis *et al*, 2009).

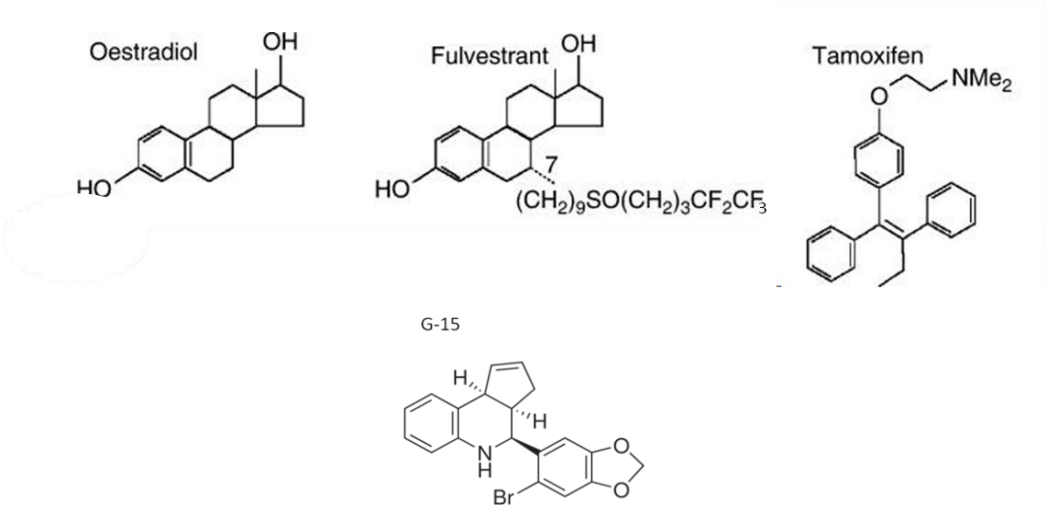

Figure 4.1: The chemical structures of E2, ICI 182,780, tamoxifen and G-15. ICI 182,780 is structurally more similar to E2 that selective estrogen receptor modulators (SERM's), such as tamoxifen (Adapted from Osborne *et al*, 2004 and www.sigmaaldrich.com).

The co-incubation of estrogens with the ER antagonist ICI 182,780 or the GPER-1 antagonist, G-15, or indeed a combination of the two, will help to evaluate whether the malformations induced by estrogens are mediated by the ER, GPER-1 or both. Additionally, the 3D cultures of MCF-10A cells in the presence

of the estrogenic test compounds will provide us with an example of an ER negative non-tumourigenic cell line.

In this chapter, we study the impact of estrogens upon the morphogenesis of ERα, ERβ, GPER-1 competent MCF-12A acini in a 3D model. The use of this model offers an opportunity to study the involvement of these receptors in breast morphogenesis, as well as the impact of ER agonists, such as estrogens and estrogen-like chemicals, on mammary gland formation, disruption and, potentially, carcinogenesis.

In order to achieve this, MCF-12A acini were first treated with a range of E2 concentrations to ensure that any observed effects in response to E2 were due to the presence of estrogens. We then progressed to investigate the role impact of E2 and xenoestrogens upon acini morphogenesis and the role of the ER and GPER-1 through co-incubation studies with ICI 182,780 and G-15. Finally, the ER negative, but GPER-1 competent MCF-10A cell line was cultured in 3D in the presence of E2 and select xenoestrogens to further investigate and confirm the role of the ER in mediating the effects of E2 in our 3D model.

4.4 Materials and Methods

4.4.1 Dose Responses Studies with E2

Varying concentrations of the natural hormone, E2, were tested on MCF-12A acini grown in Matrigel for 16 days. Cells were seeded onto Matrigel as described previously and samples were treated with increasing concentrations (1 pM, 10 pM, 100 pM 1 nM and 10 nM) of E2. Media changes were carried out as described before and after 16 days, acini were fixed and immunocytochemistry performed. Controls consisted of 0.5% ethanol. All estrogen concentrations were prepared using absolute ethanol and final ethanol concentration did not exceed 0.5% as this concentration was shown in previous work to not cause observable cytotoxicity (data not shown). Experiments were performed in duplicate twice.

4.4.2 Effect of Estrogenic Compounds on Acini Formation

MCF-10A and MCF-12A cells were seeded onto Matrigel, as described previously, and samples were treated with 0.5% etOH (solvent control), 1 nM E2, 10 µM BPA (>99% Sigma-Aldrich, Dorset, UK), 10 µM n-propylparaben (>99%, Sigma-Aldrich, Dorset, UK), 10 µM β-HCH (>99% Sigma-Aldrich, Dorset, UK) or 10 µM *o,p'*-DDT (>99% Sigma-Aldrich, Dorset, UK). The only exception was in the case of the assay

media for MCF-10A cells, which contained L-glutamine (**Chapter II**, **Table 2.1**). Media changes, consisting of 400 µl assay media with 2% Matrigel, 5 ng/ml EGF with the above treatments, were carried out every four days. Cells were maintained at 37^{o}C in 5% CO_2. Experiments were performed in duplicate three times and were double-blinded.

4.4.3 Effects of E2 and Estrogen-Like Compounds on Acini Formation: Time-Course Analysis

Time-course experiments were performed for E2, BPA and n-propylparaben-treated acini at 4, 8, 12 and 16 days, as described previously. Experiments were performed in duplicate three independent times and were double-blinded. Acini size was measured using the LSM 5 Image Browser (Zeiss) and mean and standard error calculated from 6 randomly selected acini for each treatment.

4.4.4 The Effect of Receptor Antagonists

Cells were seeded as before with or without a 1h pre-incubation with 1 µM ICI 182,780 (a kind gift from Dr. Ian White at the MRC Molecular Endocrinology Group, Leicester, UK), 10 nM G-15 (Sigma-Aldrich, Dorset, UK) or a combination of ICI 182,780 and G-15 for 12 and 16 days. Cells were treated as described before, with 1 nM E2, 10 µM BPA or 10 µM n-propylparaben, following pre-incubation with the receptor antagonists. Solvent controls consisted of 0.5% ethanol with 0.2% DMSO. Experiments were performed in duplicate, three independent times.

4.4.5 Acici Quantification and Statistical Analysis

Acini formation and disruption by the test compounds were quantified by analysing confocal images of acini and measuring the size, in addition to enumeration of the total number of cells and number of apoptotic cells within representative acini. Additionally, the percentage of apoptotic cells were calculated as the number of activated (cleaved) caspase-3 positive cells (shown in green) over the total number of cells in each acini. A minimum of 10 randomly selected acini were analysed for each independent experiment for each treatment.

For experiments involving co-incubation with ICI 182,780 or G-15, the ratio of cell number to acini size was also calculated to allow for the differentiation between reduction in cell number due to lumen formation (i.e. increased apoptosis) or decreased acini size (i.e. proliferative arrest). If the presence of

either inhibitor leads to a reduction in cell number as a consequence of lumen formation, then the ratio of cell number to acini size would decrease. If, however, the number of cells decreased solely due to smaller acini size, then this ratio would be unaffected.

Data analysis was performed using the Prism Software (GraphPad Software, version 5.04, La Jolla, CA, USA). ANOVA followed by Dunnett's post-hoc test was applied in order to compare treatments against controls, whilst ANOVA and Bonferroni's post-hoc test was used to compare paired samples (treatment with inhibitors).

4.5 Results

4.5.1 Effects of E2 on Acini Formation in MCF-12A Cells: Dose Response

MCF-12A acini under control conditions produced spherical acini that underwent growth arrest at 16 days. These acini consisted of a single layer of cells attached to a basement membrane, surrounding a near-hollow central lumen and were on average 121 µm in diameter. Upon treatment with E2, however, acini became progressively larger, misshapen and disorganised (the outer cells of acini did not form a uniform single layer), and presented filled lumen in a concentration-dependent manner (**Figure 4.2**).

Even at concentrations as low as 1 pM, the effects of E2 on acini formation were observed and as the concentration of the hormone was increased, these effects become more pronounced. At 1 pM E2, MCF-12 acini were generally similar to controls, both in morphology and size (average diameter 128 µm ± 7.9), but some structures displayed less luminal clearing. At 10 pm E2, however, acini were slightly larger than those formed under control conditions, measuring on average 136 µm ± 6.7. Here, the acini possessed completely filled lumens with little or no sign of apoptosis, as indicated by the reduction in activated caspase-3 expression (shown throughout in green).

As the E2 concentration was increased to 1 nM and 10 nM, MCF-12A acini become considerably larger, being 182 ± 12.6µm and 217 ± 15.6 µm in diameter on average, respectively. At these higher concentrations, the acini were clearly misshapen and disorganised with completely filled lumen. The increased acinar size and irregular shape are both indicators of uncontrolled proliferation (Debnath *et al*, 2002).

As the concentration of E2 increased, however, the abundance of malformed acini increased. For example: at E2 concentrations of 1 nM, over 70% of acini were misshapen and at 10 nM, acini were

no longer spherical and are greatly increased in diameter (**Figure 4.2**). This data suggests that estrogens induce acini malformations and do so in a dose-dependent manner. It should be noted at this point, that E2 treatment resulted in acini of varying size and shape and the images presented are representative of the majority of acini presented in each sample.

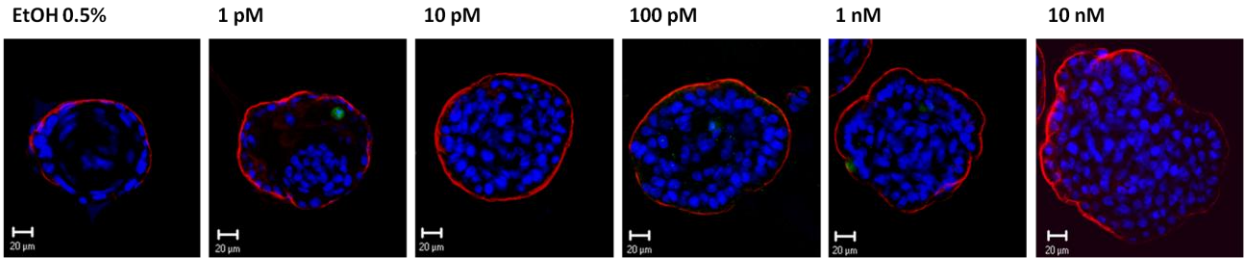

Figure 4.2: Dose response analysis of MCF-12A cells in 3D culture with increasing E2 concentration after 16 days incubation. Cells were either treated with solvent (0.5% ethanol) as a negative control, or increasing concentrations of E2 ranging from 1 pM to 10 nM. Acini were stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain. As E2 concentration increases the extent of acini disruption also increases in respect to luminal filling and acini size and shape. Experiments are representative of duplicate experiments performed twice.

4.5.2 Impact of E2 and Other Estrogenic Chemicals on MCF-12A Acini Formation

After 16 days incubation, acini treated with the test compounds were larger in diameter, exhibited filled lumen and were of an irregular shape. Basement membrane deposition was observed in all treatments, as this did not seem to be disrupted, even in the most affected structures. The malformations induced by n-propylparaben were similar to those seen with 1 nM E2 in respect to shape, luminal filling and size. Treatment with BPA, however, resulted in acini that were more spherical than E2 or n-propylparaben-treated samples. Treatment with *o,p'*-DDT or β-HCH resulted in acini that were malformed in a similar fashion to acini treated with n-propylparaben. (**Figure 4.3**).

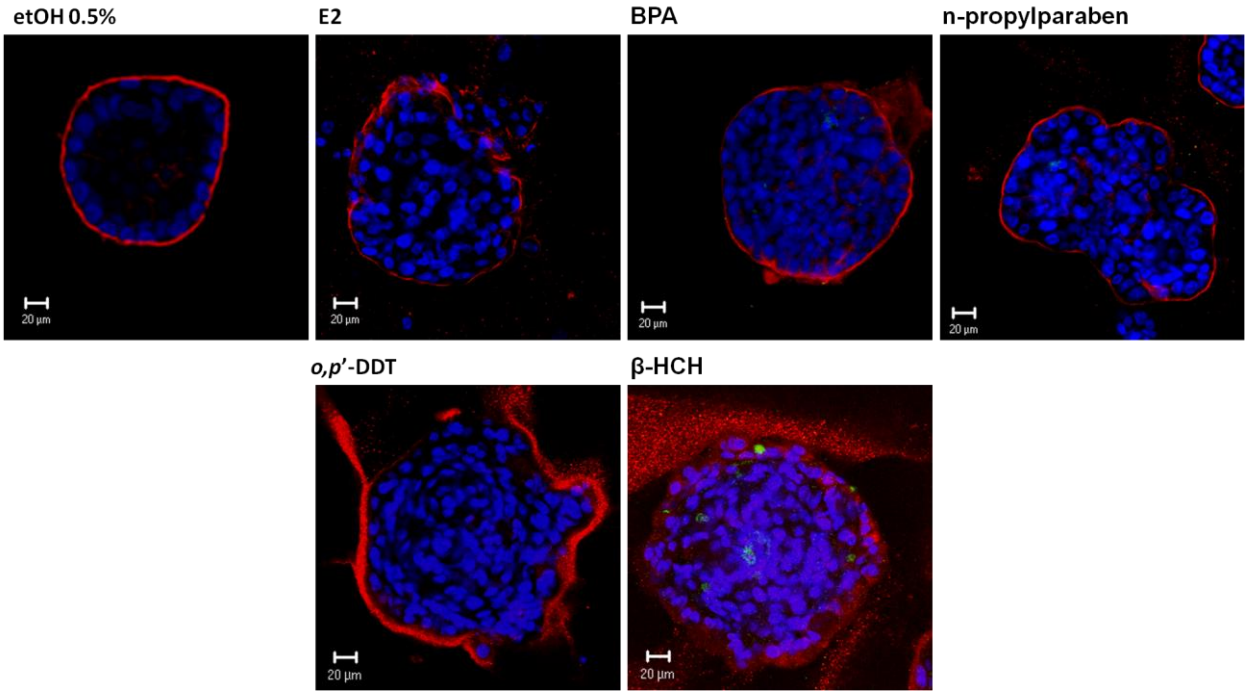

Figure 4.3: MCF-12A cells grown in 3D culture for 16 days incubation with estrogenic compounds. Cells were treated with solvent (0.5% ethanol) as a negative control, 1 nM E2, 10 µM BPA, 10 µM n-propylparaben 10 µM *o,p'*-DDT, or 10 µM β-HCH and acini stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain. Experiments are representative of duplicate experiments performed three times.

4.5.3 Effects of E2 and Estrogen-Like Compounds on Acini Formation – Time-Course Analysis

A time-course was conducted for MCF-12A cells grown in 3D culture treated with the test compounds: E2, BPA and n-propylparaben. This would allow comparison between controls and treated acini to be investigated in terms of key events and stages in MCF-12A acini morphogenesis, such as the formation of the basement membrane, apoptotic onset, growth arrest and luminal clearance (**Figure 4.4**).

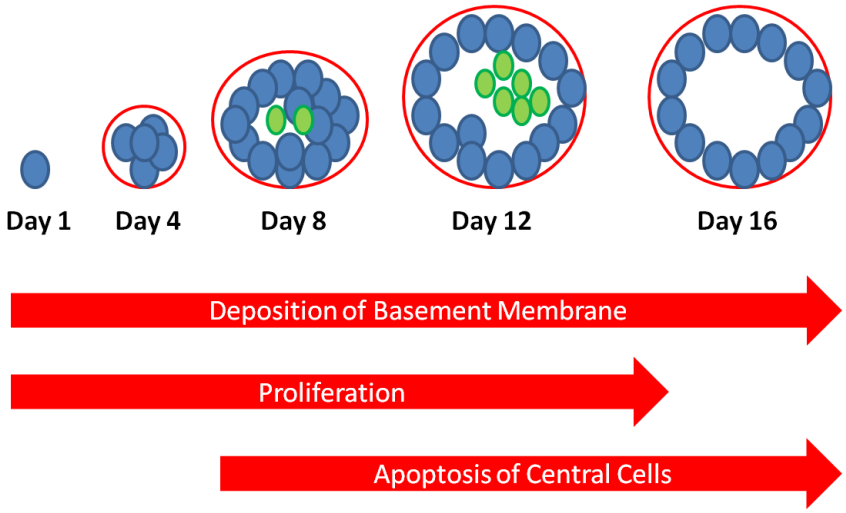

Figure 4.4: Schematic of MCF-12A morphogenesis in 3D culture. Basement membrane deposition (red) occurs as early as 4 days. Proliferation and increase of acinar size terminates between days 12 and 16 and apoptosis (green) of the central cells occurs from day 8. Finally at day 16 a hollow lumen is observed, surrounded by a layer of organised epithelial cells attached to a basement membrane.

Additionally, it was of interest to see if all test compounds exerted their actions on MCF-12A acini at the same time points, in the same manner and to the same extent. The apoptotic marker, activated (cleaved) caspase-3 was used to investigate the impact of the test estrogenic compounds upon apoptotic onset, which was evident from day 8 in control 3D cultures, as demonstrated in **Chapter III**. Antibodies against laminin V were used to investigate whether estrogens affected basement membrane deposition, which under control conditions in **Chapter III**, was observed as early as day 4 in 3D culture.

E2, BPA and n-propylparaben were carried forward to perform a 16 day time-course and *o,p'* DDT and β-HCH were omitted. Although it would have been ideal to investigate all five test compounds from the initial 16 day incubation experiments, time and financial constraints limited the amount of estrogenic compounds that could be tested. E2 was selected, as it is the most relevant endogenous estrogen and is known to be implicated in hormonal carcinogenesis of the breast. In terms of xenoestrogens, BPA and n-propylparaben were selected, because, as mentioned in the introduction, they are currently of great public, scientific and regulatory interest, whereas *o,p'* DDT and β-HCH have been already banned from use in agriculture, making them less interesting and relevant chemicals to study.

As with E2, BPA and n-propylparaben treatments resulted in large, deformed, non-spherical acini with filled lumens after 16 days in 3D culture (**Figure 4.5**). The increase in acini size compared to

controls was evident as early as 4 days incubation, with etOH controls measuring 61 ± 4.2 µm in diameter on average, as opposed to 72 ± 5.2 µm in the case of estrogen-treated acini, meaning that treated acini were approximately 18% larger than controls. At the 4-day time point, the various estrogenic treatments induced similar responses in respect to increased acinar size. After 8 days incubation, etOH controls measured 99 ± 6.2 µm in diameter on average and, as before, estrogenic treatment resulted in larger acini, although there was no great variation between the treatments. On average, following 8 days incubation with E2, BPA or n-propylparaben, acini were 111 ± 7.2, 128 ± 8.9 and 109 ± 10.2 µM in diameter, respectively. This made E2 and n-propylparaben treated acini approximately 12% larger than controls, and BPA-treated acini almost 30% larger on average than controls. At this time point, it should also be noted that there was variability in the size of acini treated with BPA, with some acini reaching 141 µM in diameter. Estrogen-treated acini continued to increase in diameter, unlike untreated acini which underwent growth arrest after approximately 12 days. At this time point, solvent controls were, on average, 110 µM in diameter and estrogen-treated acini were on average 17% larger than controls. For the treated acini, size was variable with E2, BPA and n-propylparaben treated acini measuring 129 ± 8.8, 134 ± 9.4 and 130 ± 12.4 µm in diameter on average, respectively. At 16 days, solvent control acini were on average 112 µm ± 6.9 in diameter whereas 1 nM E2, BPA and n-propylparaben-treated acini were 159 ± 14.9, 186 ± 12.2 and 244 ± 14.6 µm in diameter respectively (45%, 69% and 121% larger than controls respectively).

As well as luminal filling, decreased apoptosis was also observed with all treatments, most notably through days 12-16. This was inferred through the reduction in activated caspase-3 detection following estrogen treatment, in comparison to solvent controls. The reduction in apoptosis was more pronounced upon treatment with BPA or n-propylparaben, which displayed fewer apoptotic cells, despite containing a greater number cells where the lumen would form, throughout the entirety of incubation in 3D culture. None of the treated acini showed any signs of cellular organisation, in respect to the uniform arrangement of the cells in contact with the basement membrane, which was observed in the solvent controls.

Basement membrane formation, as shown by the deposition of laminin, appeared to be unaffected by treatment with E2, BPA or n-propylparaben. Of all the treatments, the most deformed acini resulted from n-propylparaben and BPA treatments following 16 days incubation in Matrigel. These acini showed increased indications of disruption in comparison to solvent controls and 1 nM E2-treated acini. Most notably, acini treated with BPA or n-propylparaben showed no organisation of the

outer layer of cells in contact with the basement membrane and there appeared to be more cells present in the luminal space. They were also, on average, larger in diameter than both their etOH and E2-treated counterparts (**Figure 4.5**).

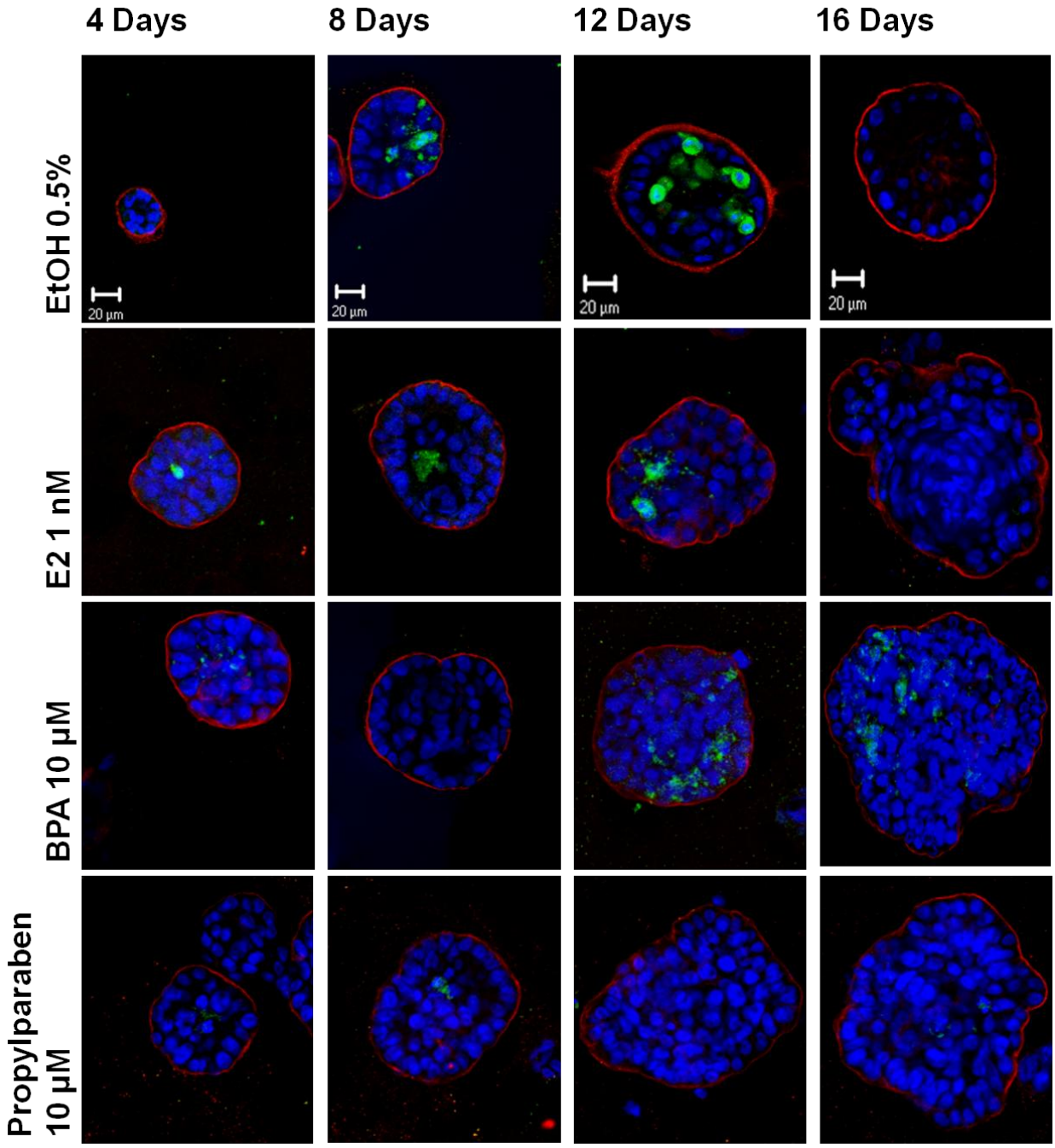

Figure 4.5: Confocal images for time-course of MCF-12A estrogen-treated acini in 3D culture. Samples were incubated with solvent (0.5% ethanol) as a control, 1 nM E2, 10 µM BPA or 10 µM n-propylparaben for 4, 8, 12 and 16 days. Acini were stained with antibodies against laminin V (red) to identify the basement membrane. Anti-

bodies against activated caspase-3 (green) were used a marker of apoptosis. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain. Images are representative of duplicate experiments performed three independent times.

An interesting observation made with the samples treated with E2, was that in some acini, cells appeared to be moving out of the acini structure, through the basement membrane (**Figure 4.6**). This seems to indicate that the cells acquired some invasive potential, being able to disrupt the basement membrane and migrate into the environment outside the acini. The development of invasive characteristics is an indication of neoplastic transformation (Hanahan & Weinberg, 2000), and the implications of this will be discussed further in the discussion section.

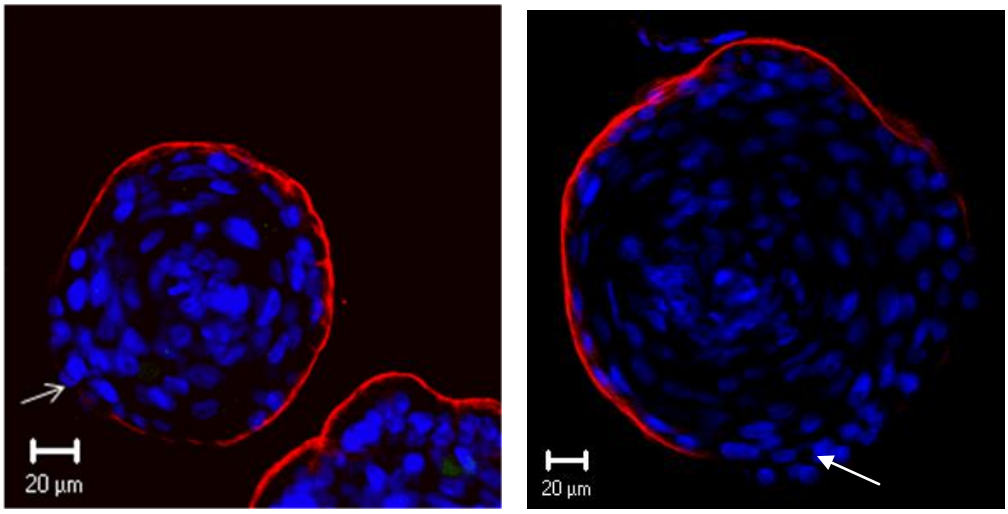

Figure 4.6: Confocal image of 1 nM E2-treated MCF-12A acini after 12 and 16 days in 3D culture. Acini stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells respectively. Nuclear staining was achieved by counterstaining with TOPRO-3 (blue). The arrows indicate cells migrating through the basement membrane and invading the extra-acinar environment.

Finally, in addition to the observations described before, it was also observed that a small proportion of treated MCF-12A cells grown in Matrigel formed multi-acinar structures. This was more evident in samples treated with n-propylparaben (**Figure 4.7**). These multi-acinar structures were comprised of a larger, filled acini with smaller filled acini "budding off" from the central mass. These have also been observed in 3D cultures of MCF-10AT cells, a breast cell line that has been transformed by induced expression of oncogenic Ha-Ras (Imbalzano *et al*, 2009). The entire structure was surrounded by

basement membrane, although there were indications of laminin expression within the structure itself. This may, however, be due to the acini being comprised of a 3D structure and so some of the buds from the main structure may be in a different plane, resulting in the surface of this bud being imaged inside the main structure.

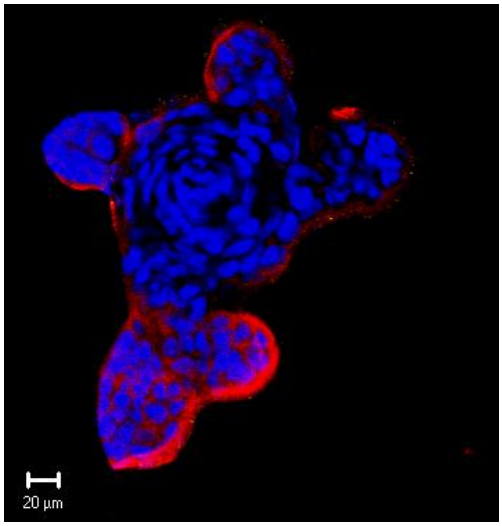

Figure 4.7: Confocal image of n-propylparaben MCF-12A acini after 16 days in incubation. Laminin V, an indicator for basement membrane deposition, is shown in red. Activated caspase-3, a marker for apoptosis is shown in green, although was not detected in the sample shown. Nuclear counterstaining (TOPRO-3) is shown in blue.

4.5.4 The Impact of ER & GPER-1 Antagonists on Estrogen-Induced Acini Disruption

One of the main characteristic of most estrogenic compounds, including E2, BPA and n-propylparaben, is their ability to bind to and activate the ER and GPER-1. We, therefore, were interested in investigating whether receptor antagonists for the ER or GPER-1 would prevent the acini disruption induced by estrogenic treatment, revealing a potential involvement of these receptors in estrogen-induced acini malformation. 3D cultures of MCF-12A cells were treated with the test compounds E2, BPA or n-propylparaben in combination with the ERα antagonist ICI 182,780, GPER-1 antagonist G-15 or a combination of both antagonists, for 16 days. The inhibitors were also tested in the absence of estrogens. From this work, it became apparent that the antagonists alone exerted a small impact upon MCF-12A acini morphology and induced a small degree of luminal filling. Nevertheless, we observed that the presence of both antagonists reverted the extent of acini disruption caused by the three test

compounds and, when used in combination, the extent of reversion was greater than with either antagonist alone.

As shown in **Figure 4.8**, co-incubation with ICI 182 780 allowed E2-treated acini to recuperate some of the features displayed by solvent control acini. This is exemplified by partial luminal clearance, in addition to an overall increase in acini organisation. This increased organisation was inferred by the presence of a uniform layer of outer cells in contact with the basement membrane surrounding a nearly hollow lumen. Although at 16 days activated caspase-3 was absent in E2 samples pre-treated with ICI 182,780, the resulting acini were smaller, more spherical and displayed less luminal filling than samples treated with E2 alone. Similar effects were observed upon co-incubation with the GPER-1 antagonist, G-15, although G-15 also resulted in acini of a much smaller size compared to those treated with E2 + ICI 182, 780. Co-incubation with both antagonist simultaneously had a greater impact on the reversion of E2-induced malformations and produced organised, spherical, growth-arrested acini with partial luminal filling. This strongly suggests that both the ER and GPER-1 are involved in mediating the effects of E2 on 3D MCF-12A structures.

Both antagonists also reverted some of the BPA and n-propylparaben-induced acinar malformations. However, this reversion was more notable with BPA-treated samples than with n-propylparaben. Co-incubation of BPA with ICI 182, 780 or G-15 resulted in a reduction in acini size. These acini were also more spherical and organised, and presented partially cleared lumen, features that were absent when acini were treated with BPA alone. Upon co-incubation of both antagonists with BPA, the acini were even smaller and the extent of luminal clearing was greater than that observed upon co-incubation of the estrogen with ICI 182, 780 or G-15 individually.

On the other hand, when used alone, neither antagonist, greatly reverted the effects of n-propylparaben in terms of luminal filling or overall organisation. Acini were, however, smaller in size and spherical. Interestingly, G-15 co-incubation with n-propylparaben resulted in larger nuclei, as indicated by TOPRO-3 staining. This was not observed when the antagonists were used in combination, however co-incubation with both ICI 182, 780 and G-15 did appear to increase the extent of luminal clearance compared to n-propylparaben treatment alone.

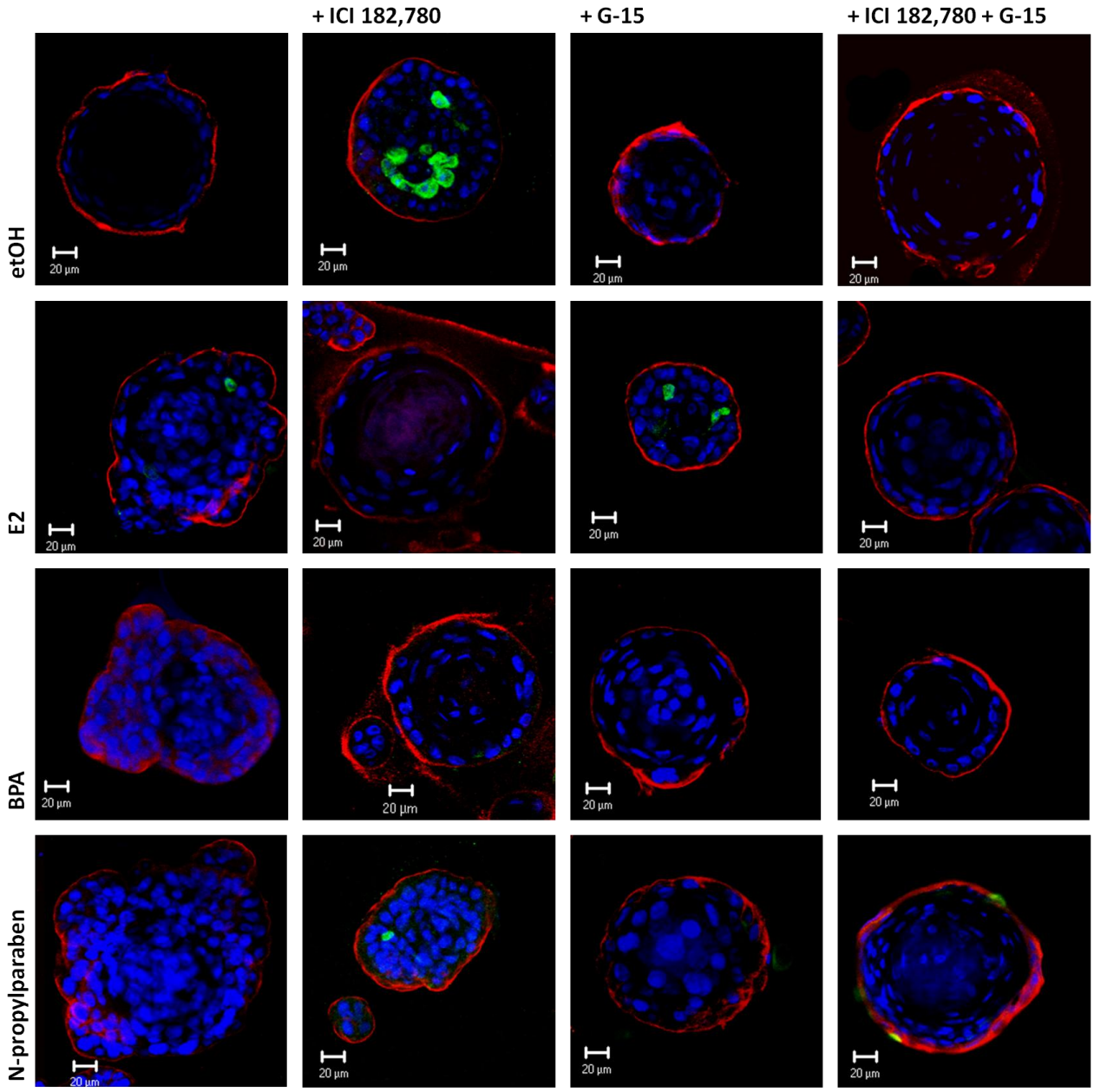

Figure 4.8: The role of ER and GPER-1 in estrogen-induced MCF-12A acini disruption. Samples were treated with 1 nM E2, 10 µM BPA or 10 µM n-propylparaben, alone, or in combination with 1 µM ICI 82, 780, 10 nM G-15 or 1 µM ICI 82, 780 + 10 nM G-15, for 16 days. Acini were then stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear marker. Experiments are representative of duplicate experiments performed two independent times.

4.5.5 The Effect of Estrogens on ERα-Negative MCF-10A Cells in 3D Culture

We then proceeded to investigate the impact of E2, BPA and n-propylparaben in MCF-10A cells, which in **Chapter II** were shown to be negative for ERα and ERβ. This was performed to ascertain whether the ER is required for estrogens to induce acini disruption, as the previous results in the ER positive MCF-12Acell line seem to indicate. MCF-10A cells do, however, express GPER-1, as demonstrated in **Chapter II** and, as this receptor has been shown to influence the effects of estrogens in MCF-12A cells, it is possible that the same may be true in MCF-10A cells. The MCF-10A cell line has been commonly used in 3D culture investigations (Debnath *et al*, 2002: Debnath *et al*, 2003) to characterize acini formation in Matrigel.

Under solvent control conditions, we observed that following 16 days in Matrigel, MCF-10A cells formed growth-arrested, spherical acini with hollow lumen. Here, acini were round and well organised, with a single layer of cells surrounded by a basement membrane. The lumen was completely formed with no residual apoptotic cells. However, in contrast to untreated cells, a small proportion of acini presented some slight filling of the lumen (**Figure 4.9**), evident by the presence of a second layer of cells attached to the outer cells, which seems to indicate a mild disruption of acini formation or maintenance. The MCF-10A acini were characteristically similar to those reported previously (Debnath *et al*, 2003) and also bore resemblance to the etOH control acini of MCF-12A cells.

Treatment with E2, BPA or n-propylparaben did not greatly impact upon acini formation. Additionally, n-propylparaben-treated samples did not have a smooth basement membrane structure that was observed with controls and other treatments (**Figure 4.9**).

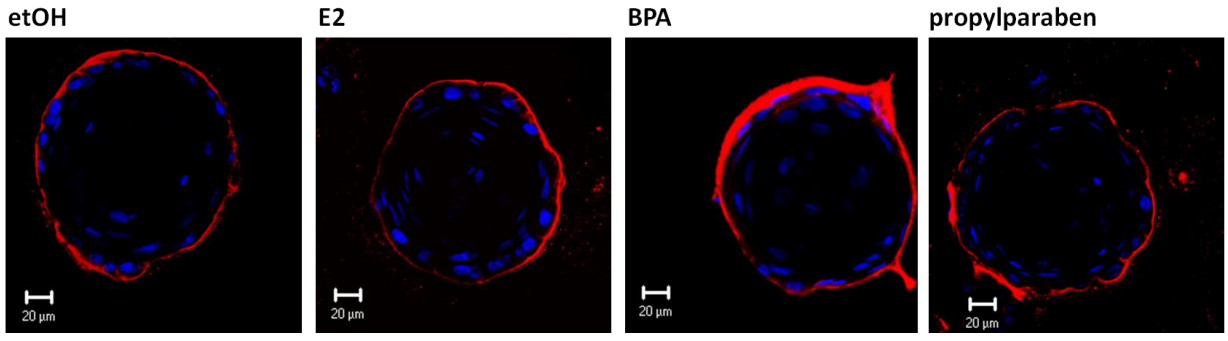

Figure 4.9: LCM images of normal epithelial breast MCF-10A cells cultured in Matrigel for 16 days. Samples were treated with solvent controls (0.5% ethanol), 10 nM E2, 10 µM BPA or 10µM n-propylparaben. Acini Anti-bodies against laminin V (red) and activated caspase-3 (green) were used to identify the basement membrane and

apoptotic cells respectively. Cells were counterstained with TOPRO-3 (blue) as a marker for the nucleus. Experiments are representative of experiments performed in duplicate on three independent times.

4.5.6 Acini Quantification and Statistical Analysis

After acini size was measured using the Carl Zeiss MicroImaging, Inc. LSM510 confocal microscopy system with LSM version 4.20 (Carl Zeiss Ltd, Hertfordshire, UK), ANOVA was performed to compare treatments against controls and treatments against treatment + inhibitor.

Although estrogen-treated MCF-12A acini were larger throughout, at 8 days, only acini incubated with n-proplparaben were significantly larger in size. After 12 days incubation, acini treated with BPA or n-propylparaben were significantly larger than solvent controls. Finally, after 16 days, treatment with all compounds (E2, BPA or n-propylparbaben) resulted in acini that were significantly larger in diameter in comparison to controls (**Figure 4.10**).

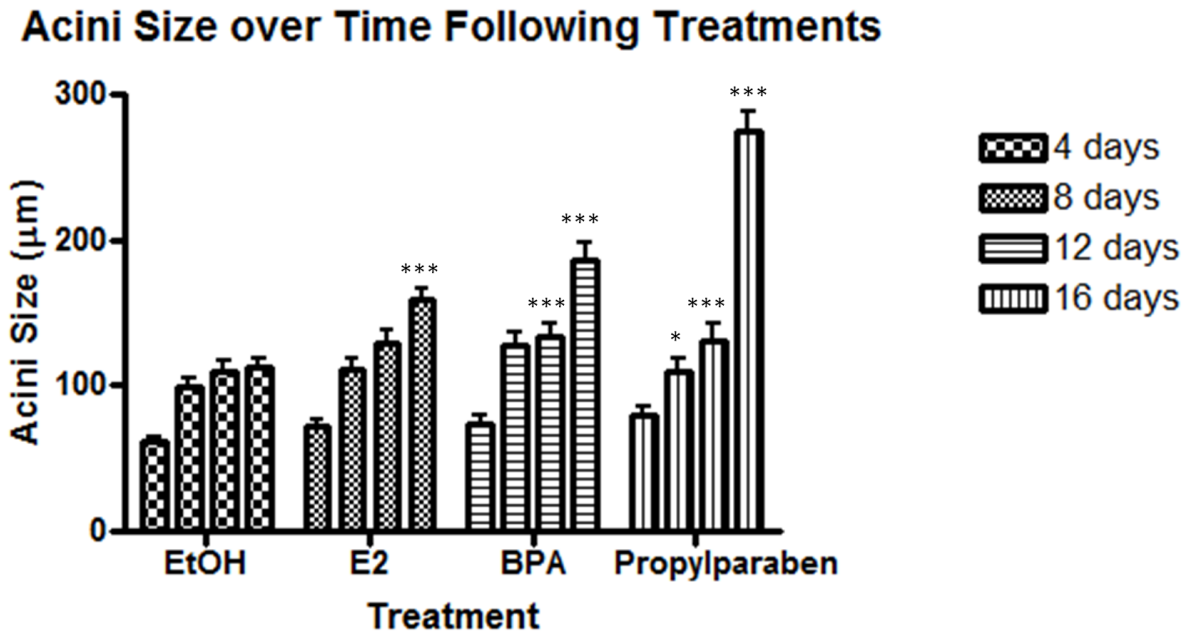

Figure 4.10: Quantification of acini size. Acini size was determined by analysing confocal images following 4, 8, 12 and 16 days incubation. Data corresponds to mean ± SEM and results from three independent experiments, where a minimum of 10 representative acini were analysed per experiment. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between treatments and controls.

When the percentage of apoptotic cells per acini was calculated we found that this was significantly decreased in all treatments and at all time points, indicating that these estrogenic compunds have an anti-apoptotic impact on MCF-12A acini (**Figure 4.11**).

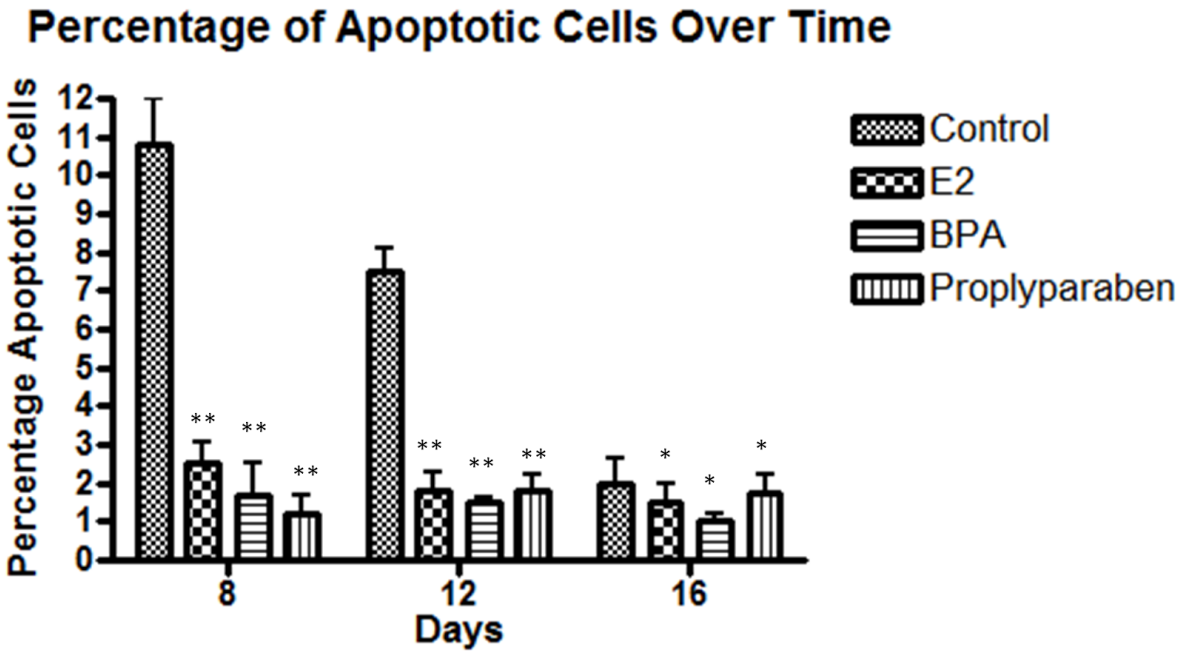

Figure 4.11: Quantification of percentage of apoptotic cells per acini. The percentage of apoptotic cells (those positive for activated caspase-3 staining) was calculated for 8, 12 and 16 days incubation in Matrigel. Data corresponds to mean ± SEM and results from three independent experiments, where a minimum of 10 representative acini were analysed per experiment. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between treatments and controls.

We next sought to investigate whether the reversion of estrogen-induced malforamtions by ER or GPER-1 antagonists were statistically significant following 16 days incubation. The reduction in acini diameter in E2-treated sample was only significant upon co-incubation with ICI 182,780. In BPA-treated samples, either antagonist, or a combination of the two, significantly reduced acinar size. In n-propylparben-treated samples, a significant reduction in MCF-12A acini size was observed only when ICI 182,780 was present. Neither inhibitor had a significant impact on the average number of cells per acini in n-propylparaben-treated samples, however the dat here was most variable for controls and this may account for the lack of significant decreases in cell number. In E2-treated samples, ICI 182,780 or G-15 alone reduced the average number of cells, however in combination this reduction was no longer significant. Only when BPA-treated samples were co-incubated with ICI 182,780, either alone, or in combination with G-15, did cell number significantly reduce (**Figure 4.12**). Taking these observations

into account along with the LCM images, suggests that the various estrogenic test compounds utilse the ER and GPER-1 differently in the MCF-12A cell line.

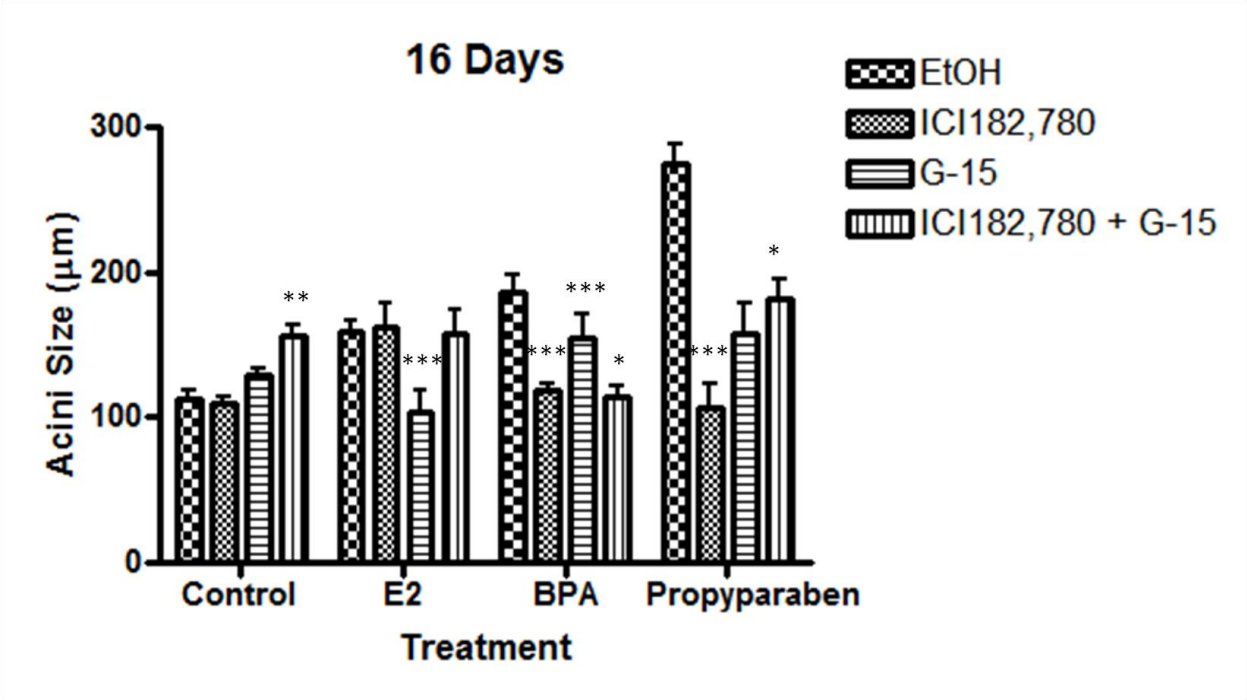

Figure 4.12: Acini Size after 16 days upon co-incubation with ER and GPER-1 antagonists. Data corresponds to mean ± SEM and results from three independent experiments, where a minimum of 10 representative acini were analysed per experiment. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between treatments and controls.

4.6 Discussion

Following on from the results presented in **Chapter III**, where we established a time-course for the formation of acini by MCF-12A cells, in this chapter, we proceeded to establish a time-course for MCF-12A acini formation in the presence of both endogenous and exogenous estrogens, namely: E2, BPA and n-propylparaben.

The data presented in this chapter shows that under control conditions (0.5% ethanol), MCF-12A cells cultured in Matrigel form growth arrested, spherical, hollow acini after 16 days incubation. In comparison, MCF-12A cells treated with estrogens form malformed acini. The malformations observed, such as loss of the single layer of cells in contact with the basement membrane, luminal filling, misshapen acini, over-proliferation and invasion through the basement membrane resemble those observed in early breast cancer lesions (Debnath *et al*, 2003; Hebner *et al*, 2008). Furthermore, the estrogen-treated malformed acini also resemble those produced by malignant cells cultured in 3D

(Petersen *et al*, 1992). It is important to note at this point, however, that whilst these estrogen-treated acini resemble breast cancer lesions, they have not necessarily undergone neoplastic transformation. This would have to be confirmed by genotyping of estrogen-treated acini.

4.6.1 Proliferation of MCF-12A Acini in Response to Endogenous Estrogen and Xenoestrogens

The test compounds led to increased acinar size and this effect could be seen as early as 4 days in 3D culture. This suggests that the presence of estrogenic compounds increases cellular proliferation. As noted previously, estrogen-treated were misshapen and in some cases multi-acinar structures were present, characterised by what appeared to be multiple acini that were encapsulated by a single surrounding basement membrane. These multi-acinar filled structures have been observed previously in 3D cultures of MCF-10A cells with ectopic expression of HER-2, a receptor associated with increased responses to growth factors, and hence, increased proliferation (Muthuswamy *et al*, 2001; Debnath *et al*, 2002) (**Figure 4.13**). These observations also suggest that estrogens induced an increase in the proliferation of MCF-12A cells cultured in Matrigel. It is clear that in this 3D model, estrogens have an impact upon acini size and shape, thus indicating uncontrolled cellular proliferation, although this was not the only estrogen-induced effect observed. These observations are supported by other studies that report cellular proliferation in response to estrogens in rats (Holland & Roy, 1995). Holland & Roy (1995) observed that in female rats, proliferation of mammary epithelial cells was increased following treatment with estrone.

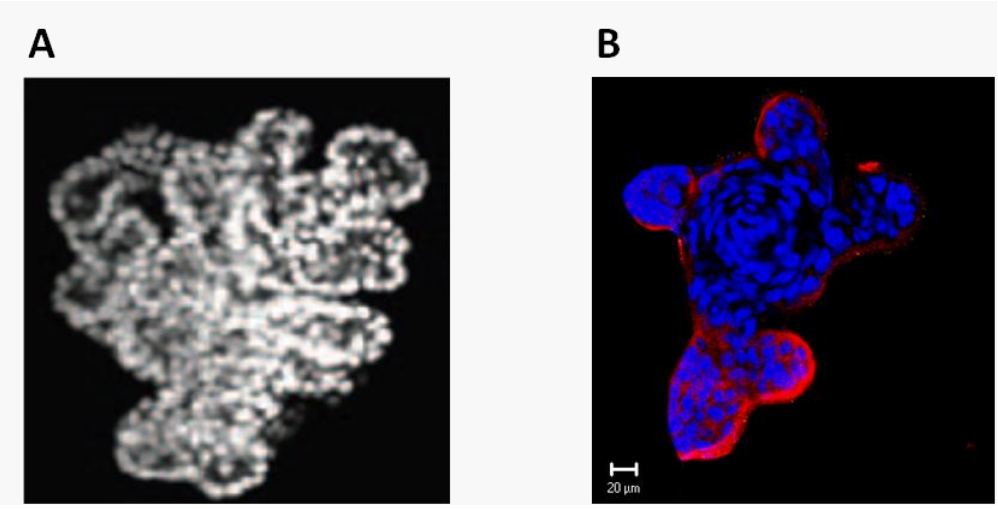

Figure 4.13: Comparison of multi-acinar structures grown in Matrigel. (A) Phase contrast image of a MCF-10A multi-acinar structure where HER-2 was ectopically expressed. Staining for DAPI is shown (Taken from Debnath *et*

al, 2002). **(B)** Confocal image of a n-propylparaben treated MCF-12A multi-acinar structure. Basement membrane was visualised by staining with antibodies against laminin V (red). Anti-bodies against activated caspase-3 were used as an indicator of apoptosis (green) but were not observed in this sample. Nuclear counterstaining was performed using TOPRO-3 (blue).

Previously published data has demonstrated that E2-treated MCF-10F acini formed in 3D collagen matrices, have increased Ki-67, a marker of proliferation (Kocdor *et al*, 2009). This supports the idea that the estrogen-induced increased acinar size and irregularity of shape were, to some extent, due to increased cellular proliferation.

The disruption of acini formation at 16 days shown in the 3D model has also been replicated *in vivo* in mouse and rat models and this both supports our results and adds validation to the model used (Hilakivi-Clarke *et al*, 2006). In the aforementioned study, *in utero* estrogen treatment resulted in lack of apoptotic cells in the TEBs of rat mammary glands compared to in untreated animals where apoptotic cells were present. Additionally, E2 can induce hyperplasia, demonstrated by increased number of ducts and alveoli size, and irregular shaping of the lumen and luminal filling of the mammary gland in rats (Kovalchuk *et al*, 2007; Starlard-Davenport *et al*, 2010). In other *in vivo* studies, animals underwent perinatal exposure to BPA and the results were alterations in mammary gland organization during puberty and adulthood, reduced levels of apoptosis and increased number of proliferative cells (Markey *et al*, 2001; Munoz de Toro *et al*, 2005; Murray *et al*, 2007). Interestingly, some of these alterations mirrored known risk factors associated with breast cancer risk in humans, such as increased responses to estrogen, increased epithelial density and carcinomas (Vandenberg *et al*, 2009).

It has been reported that when proliferation becomes unregulated in 3D cultures, the cause of this may be that signalling pathways influencing survival become over-activated, or that signals that would normally induce cell death are blocked. Indeed, ectopic expression of HER2, an oncogenic receptor involved in growth factor signalling, results in large, malformed multi-acinar structures (Debnath *et al*, 2002). Furthermore, activated AKT is localised to the outer cells of acini that possess contact with the basement membrane. Cells destined for apoptosis, i.e. the central cells, do not express activated AKT and disruption of this dichotomy disrupts proliferation and the apoptotic process (Debnath *et al*, 2003[b]; reviewed in Debnath & Brugge, 2005). This supports the idea that acinar disruption occurs as a result of disruption of key signalling pathways and this will be studied further in relation to estrogens in later chapters.

4.6.2 Apoptosis and Lumen Formation Following Estrogen-Treatment of MCF-12A Acini

In addition to increasing proliferation, estrogens appear to have an inhibitory impact upon apoptosis, and indeed, it has been demonstrated that E2 is able to suppress apoptosis (Murdoch & Kirk, 2002). The exact mechanism for this is unclear, however, it has been demonstrated that E2 increases Mdm2 expression (Brekman *et al*, 2011). This would ultimately lead to the inhibition of p53, thus preventing p53-mediated apoptosis. In addition to this, E2 treatment of synovial fibroblasts has been shown to have a suppressive effect upon apoptosis and this was mediated by the MAPK pathway (Yamaguchi *et al*, 2012).

Under control conditions, activated caspase-3 was observed after 8 days in 3D culture and was still evident at 12 days. Upon formation of the hollow lumen after 16 days, activated caspase-3 was no longer observed as no, or very few, cells were left to be cleared. As activated caspase-3 is an indicator of apoptosis, it can be assumed that through days 8 to 16, apoptosis occurs, leading to the formation of the hollow lumen. When cells were treated with the test compounds, less activated caspase-3, and hence less apoptosis, was observed throughout, and there was little evidence of luminal clearance. Further to this, the presence of apoptotic cells in some of the estrogen-treated samples was not specific to the inner cell population within acini. This would suggest that despite signs of apoptosis in estrogen-treated samples, as indicated by the presence of activated caspase-3, apoptosis is dysregulated and is not sufficient to induce luminal clearance. This may be due to reduced apoptosis, but the increased proliferation discussed previously may also contribute to this observation. The estrogen-induced reduction in apoptosis indicates that the central cells of the acini, which lack direct contact with the basement membrane, had acquired the ability to survive without signals from the ECM. It is already known that these signals can be transmitted from the surrounding environment via cell surface receptors, such as integrins and the EGFR, and promote survival and proliferation (reviewed in Shaw *et al*, 2004). In order to proliferate, normal cells require growth signals, such as activation of receptors by growth factors or components of the ECM. It is known that some oncogenes exert their pro-survival influence by mimicking these normal growth signals or enhancing them. In this way, cancer cells become self-sufficient from growth signals, and hence, do not rely on an external cue to enter into a proliferative state. A plausible explanation for estrogen-treated cells forming malformed acini may be that these cells have become self-sufficient, and thus do not require external growth cues in order to proliferate or survive, or that estrogens are able to activate the pathways involved in survival.

Nevertheless, the acquisition of self-sufficiency for growth signals remains a hallmark of cancer (Hanahan & Weinberg, 2000) and is involved in the initiation and progression of cancer *in vivo*.

In normal tissues, homeostasis is achieved not only by the stimulatory effect of growth signals, but also through anti-growth signals. These anti-growth signals can induce their anti-proliferative effects by forcing cells into a quiescent state. It may therefore be that estrogen-induced acinar malformations were also as a result of an acquired insensitivity to anti-proliferative signals. Indeed, it has been shown that estrogens are able to suppress the transcription of the anti-growth factor, TGFβ, in MCF-7 cells (Matsuda *et al*, 2001).

A role for estrogens in suppressing apoptosis, and thus impacting upon acini formation is supported by the observation that E2 and BPA can increased the expression of anti-apoptotic Bcl-2 and Bcl-xl via both genomic and non-genomic mechanisms of action (Perillo *et al*, 2000; Gompel *et al*, 2004; Bratton *et al*, 2010; LaPensee *et al*, 2010). In the aforementioned studies, the disruption of single genes associated with apoptosis did not induce luminal filling. When, however, apoptosis was suppressed and proliferation enhanced simultaneously, luminal filling was observed (Debnath *et al*, 2002; Reginato *et al*, 2005). This would suggest that the disruption of acini requires a combination of uncontrolled hyper-proliferation and apoptotic suppression, both of which were observed in the estrogen treated 3D cultures of MCF-12A cells described in this chapter.

Similar aberrations with E2 transformed MCF-10F cells grown in 3D collagen matrices have also been previously reported (Russo *et al*, 2002; Russo *et al*, 2006; Tiezzi et al, 2007; Russo *et al*, 2010). In these 3D studies, treatment of MCF-10F cells with E2 induced neoplastic phenotypes. Normally, these immortalised breast cells form duct-like structures in collagen gels, similar to the acini formed in Matrigel. However, in the presence of E2, these cells experience a decrease in ductulogenic potential and an increase in invasive potential. Additionally, when injected into immunodeficient mice, these cells are capable of forming tumours, which is indicative of neoplastic transformation. The xenoestrogens, BPA and BBP resulted in similar effects to those induced by E2, in a similar way to the data presented in this chapter. Russo and colleagues argue that these E2-induced effects are as a result of genotoxicity of estrogens and their metabolites, and are independent of the ER (Russo *et al*, 2002; Russo *et al*, 2010). The E2-transformed MCF-10F acini in these studies were found to have undergone various genetic alterations and epigenetic modifications, such as the loss or amplification of chromosomes and DNA methylation (Russo *et al*, 2010). Contrary to the published data by Russo *et al*, only minimal malformations were observed in Matrigel cultures of ER negative MCF-10A cells treated with estrogens.

This suggests that genotoxicity may not be the determining factor in E2-induced acini malformations and the ER does, in fact, play a role in mediating the effects of estrogens.

It remains unclear whether the acinar malformations observed in response to estrogen treatment were a consequence of increased proliferation, decreased apoptosis, or indeed, a combination of both. Given that estrogen-treated acini were larger in size and possessed filled lumens with reduced levels of apoptosis, it would suggest that increased proliferation and decreased apoptosis are both implicated, respectively. Several mechanisms are involved in the development and maintenance of mammary acini for proliferation and apoptosis. It is thought that RTKs, such as the EGF receptor, mediate signalling pathways, such as PI3K and MAPK. Additionally, there also appears to be a key role in genes involved in the cell cycle and apoptosis, including cyclin D1 and Bcl-xl (Debnath *et al*, 2002; Reginato *et al*, 2005; Yanochko & Eckart, 2006). In the event that signalling pathways become over-activated, the resultant acini become malformed. This has been shown in the MCF-10A cell line, where hyper-activation of the PI3K pathway disrupted acini polarity and led to increased proliferation and consequently increased acini size (Liu *et al*, 2004).

4.6.3 The Basement Membrane of MCF-12A Acini and the Impact of Estrogens

Despite the apparent impact of estrogens upon proliferation and apoptosis during the morphogenesis of MCF-12A acini, estrogen treatment did not appear to impact upon the deposition of basement membrane. The basement membrane forms a separation between acini and the stroma of the breast and provides a physical barrier for tumours that arise within these structures. However, a characteristic of cancer progression is the migration and invasion of cancer cells across this boundary (Hanahan & Weinberg, 2000). The observation that estrogenic treatment resulted in cells invading through the basement membrane suggests that the invasive potential of the cells had been increased. Indeed, Matrigel invasion studies with non-tumourigenic MCF-10F cells and the breast cancer cell line, T47-D have demonstrated that E2 treatment results in a higher potential for migration and invasion (Huang *et al*, 2007; Zheng *et al*, 2011). It is worthy to note that normal cells do not have the capability to invade, and thus, the data presented here and by Huang *et al* (2007) suggests that estrogenic treatment of normal breast cells seems to result in the acquisition of invasive potential. The exact mechanism for invasion appears to be highly complex and is not completely understood. Various changes in expression or activation status of proteins have been found to be associated with increased invasive potential. For example, estrogens have been reported to down-regulate E-cadherin expression (Oesterreich *et al*,

2003), increase matrix-degrading protease production and secretion (Briozzo *et al*, 1988), induce the phosphorylation of ezrin, impact upon cytoskeleton remodelling (Giretti *et al,* 2008; Zheng *et al*, 2011) and alter the expression of various integrins (Li *et al*, 2000; Kimmins *et al*, 2003). Given that estrogens can impact on so many of the mechanisms involved with invasion, it is plausible that the observed invasion of cells across the basement membrane was as a result of estrogen treatment.

4.6.4 Both the ER and GPER-1 are Implicated in Estrogen-Induced Acini Disruption

Both ICI 182, 780 and G-15 induced a small degree of acini disruption. ICI 182,780 is an agonist for GPER-1 (Filardo *et al*, 2000) and this could imply that its effects were a result of GPER-1 activation. Activation of GPER-1 can interfere with EGF-mediated signalling and this could account for the disruption. In this model, GPER-1 ligands, such as E2 or ICI 182,780, activate GPER-1 and cause the subsequent activation of the Gβγ subunit. This activates Src and Src consequently activates metalloproteinase (MMP) which cleaves heparin-bound EGF from pro-heparin-bound EGF complexes and thus liberates it to activate the EGF receptor. The result is the activation of EGFR-mediated signalling pathways, such as the MAPK cascade (**Figure 4.14**) (Filardo *et al*, 2002).

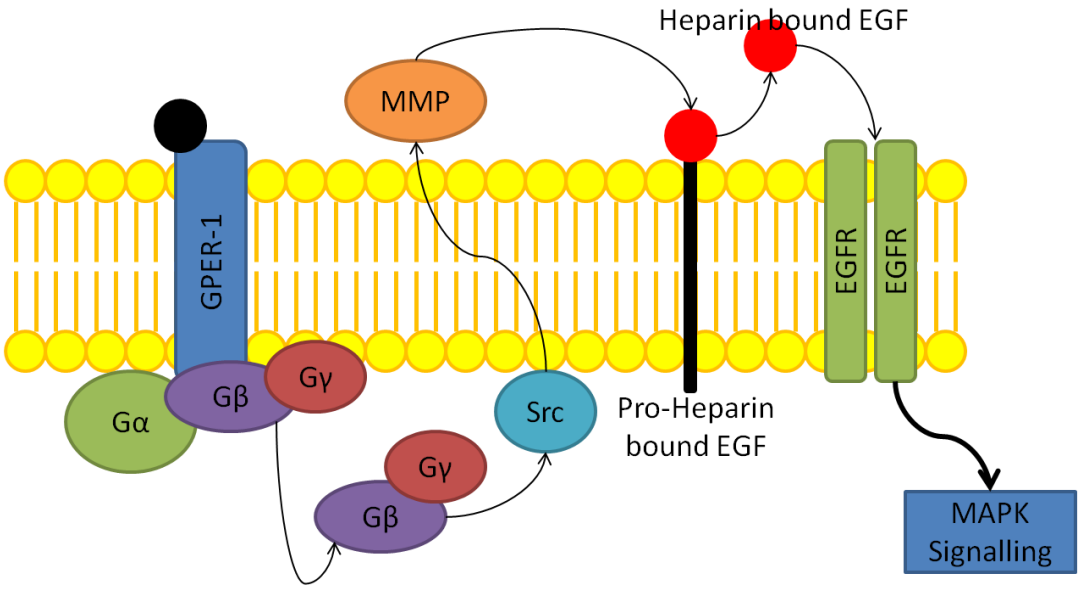

Figure 4.14: Cross-talk between GPER-1 and MAPK pathways. Ligand binding to GPER-1 activates the Gβγ subunit which activates Src. Src mediates the activation of metalloproteinase (MMP) which releases heparin-bound EGF. The heparin-bound EGF can then activate the EGFR causing MAPK signalling.

Further to the model above (**Figure 4.14**), GPER-1 agonists, such as ICI 182,780, can mobilise intracellular calcium and induce rapid PI3K signalling (Stenoien *et al*, 2001; Wei *et al*, 2012). Thus, ICI 182,780 potentially disrupts key signalling pathways involved in acini formation. It may, therefore, be the case that when acini were treated with ICI 182,780, the aberrations to acinar structure were due to ICI 182,780 induced activation of GPER-1.

Interestingly, treatment with G-15 alone, but not ICI 182,780 alone resulted in smaller acini, an effect that was reversed with the etOH controls were treated with both ICI 182, 780 and G-15. This suggests that G-15 may have an impact upon acini, and it has recently been demonstrated that G-15 is capable of inducing effects through the ER (Dennis *et al*, 2011). One important point to consider is that in controls co-incubated with both ICI 182, 780 and G-15, acini were more similar to controls than samples treated with each of the antagonists individually. This suggests that if it is the case that G-15 is activating the ER, and ICI 182,780 is activating GPER-1, the combination of the two prevents the activation induced by either G-15 or ICI 182,780. Although from the results, the reason for ICI 182, 780 or G-15's small effects on acini formation cannot be completely determined, toxicity can be ruled out as neither antagonist induced toxic effects in the monolayer MCF-12A cultures described in **Chapter II**.

Acinar malformations induced by estrogenic treatment were partially reverted upon co-incubation with ICI 182, 780 or G-15. Most notably, co-incubation of the estrogenic test compounds with ICI 182,780 or G-15 resulted in acini that were similar in size to ethanol controls, or smaller in the case of E2 co-incubation with G-15. The presence of the antagonists also reverted the irregular acinar shape induced by the test compounds, although in the case of n-propylparaben, ICI 182,780 only partially reverted the impact of n-propylparaben upon acinar shape. The observation that ICI 182,780 and G-15 were capable of reverting some of the effects of the test compounds upon MCF-12A acini suggests that the activation of the ER and GPER-1 resulted in increased proliferation. This further supports previous observations, showing that in a 3D model, estrogens induce acinar malformations by increasing cellular proliferation.

In terms of apoptosis and luminal clearance, no significant increase in activated caspase-3 was observed upon co-incubation of the test compounds with either ICI 182,780 or G-15. Increased luminal clearance was however observed when the test compounds were co-incubated with ICI 182,780 or G-15. The exception to this was following n-propylparaben treatment of acini, where neither ICI 182,780 nor G-15 increased the expression of activated caspase-3 or the extent of luminal clearance. This suggests that the ER and GPER-1 may be implicated in the estrogen-induced luminal filling of MCF-12A acini, but a

mechanism other than apoptosis may be responsible for the formation of the hollow lumen. Autophagy, the process by which cells break down their own organelles has been proposed to be at least partially responsible for luminal clearance in acini. The presence of autophagic vesicles in the central cells of MCF-10A acini has been demonstrated (Debnath *et al*, 2002; Underwood *et al*, 2006). Furthermore, TRAIL is involved in the initiation of the autophagic process during the morphogenesis of MCF-10A acini and combined inhibition of autophagy and apoptosis results in luminal filling (Debnath *et al*, 2002; Mills *et al*, 2004). This data supports the idea that perhaps apoptosis was not solely responsible for the luminal clearance of MCF-12A acini upon co-incubation of the estrogen test compounds with ICI 182,780 or G-15. It does, however, raise the question as to why antagonism of ERα and GPER-1 did not result in the same levels of activated caspase-3 observed in etOH controls. A role for GPER-1 in mediating the E2-induced acinar malformations observed in MCF-12A acini is also suggested by the observation that ICI 182, 780 and G-15, when used in combination, reverted more of the estrogen-induced malformations than with either antagonist alone. Indeed it has been demonstrated that E2 and BPA can bind to GPER-1 and activate various signalling cascades resulting in increased proliferation that is ER-independent (Razandi *et al*, 1999; Bouskine *et al*, 2009; Sheng & Zhu, 2011). The observation that ER antagonism with ICI 182,780 did not fully revert the treatment-induced malformations suggests the involvement of ER-independent mechanisms of action, and is supported by findings that E2 and BPA are able to mediate non-genomic signalling via a membrane-associated receptor not related to ERα or ERβ (Nadal *et al*, 2000; Ropero *et al*, 2002).

When estrogen-treated MCF-12A acini were co-incubated with a combination of ICI 182,780 and G-15, the reversion of estrogen-induced malformations was greater. In particular, estrogen treatment combined ICI 182,780 + G-15 treatment resulted in acini that were very similar to controls. These were the same size as control structures, were spherical in shape and the outer cells in contact with the basement membrane were organised into a single uniform layer. The lumen was not, however, completely hollow, suggesting that not all of the effects of E2 had been prevented by ERα and GPER-1 antagonism.

One possible receptor present in our cell line is a 36 kDa isoform of ERα that resides in the plasma membrane. This receptor is able to mediate the non-genomic effects of estrogens and its activity is not prevented by either tamoxifen or ICI 182,780 (Wang *et al*, 2006). It is currently not known whether MCF-12A cells express this receptor, and it would be of interest, in the future, to investigate the presence and role of this receptor in this cell line. Moreover, the bi-phasic activation of the PI3K

pathway is not completely abrogated by ICI 182,780 co-incubation with estrogens and it is proposed that an estrogen-response membrane receptor could be responsible for this effect (Marino *et al*, 2003). It is, therefore, feasible that some of the effects elicited by the estrogens in the MCF-12A cells are mediated through this receptor although further experiments are required to confirm this.

Unlike MCF-12A cells, estrogen-treated MCF-10A acini, on the whole, formed growth-arrested, spherical, well organised acini with a small increase in cell numbers within the lumen. The primary difference between MCF-10A and MCF-12A cells is that the latter express ERα and ERβ. This, in addition with the partial reversion of E2-induced acinar malformations upon ICI 182,780 co-incubation, suggests that the disruption elicited by E2 is strongly dependent of the expression of the ER. Other estrogen-responsive receptors, such as GPER-1, which both cell lines express, may, however, play a role in the E2-induced acinar malformations and this might be behind the slight acinar disruption seem with E2-treated MCF-10A cells. *In vivo*, ovariectomy, which would prevent the presence of endogenous estrogen, not only prevents the development of ER positive tumours, but also that of ER negative tumours (Early Breast Cancer Trialists' Collaborative Group, 1992). This, and giving that E2 can induce PI3K signalling in ER negative breast cancer cells (Friedl & Jordan, 1994; Tsai *et al*, 2001), does indeed support the hypothesis that estrogen may induce effects independently of the ER, via estrogen-responsive receptors, such as GPER-1, truncated ERα isoforms or even a receptor with the pharmacological profile of γ-adrenergic receptor (Nadal *et al*, 2000). In contrast to the data shown, other groups have shown that the ER negative MCF-10F cell line form acini comparable to benzoapyrene (BP)-treated acini in response to E2 (Soule *et al*, 1990; Russo *et al*, 2002; Russo *et al*, 2002[b]; Mello *et al*, 2007). This was not observed in the MCF-10A cell line and may be due to differences in receptor expression or culture conditions.

4.6.5 Xenoestrogens Appear to Induce Greater MCF-12A Acinar Malformations than Endogenous E2

Although all of the compounds tested induced larger mis-shapened acini possessing filled lumens, the malformations induced by BPA and n-propylparaben were greater than those induced by E2. The affinities of these compounds to the ER are between 1000 and 2000 times less than that of E2 (Bouskine *et al*, 2009), which suggests that the affinity of these estrogenic compound for the ER does not necessarily correlate with biological response. It has been demonstrated that different ER ligands induce different conformation of ER-ligand complexes and hence the recruitment of co-factors and

subsequent gene transcription could differ between different estrogens. Additionally, different ER ligands impact upon the stability of ERα in different ways. This indicates that different estrogens may have varying effects upon ER degradation and could therefore induce increased responses to E2 through decreasing the level of activated ER degradation (Wijayaratne & McDonnell, 2001). It is also noteworthy that xenoestrogens, such as BPA, may exert actions via pathways independent of the ER that E2 does not itself utilize. For example, it has already been shown that BPA is capable of binding to the thyroid hormone receptor (Zoeller, 2007). Therefore, some of the effects induced by BPA or n-propylparaben may have been mediated by another receptor. Interestingly, different ER ligands have been shown to induce different transcriptional responses. For example, although the transcriptional response for *BCL-2* was the same for E2 and BPA-treated MCF-7 cells, BPA induced a greater up-regulation of *JUN* and *FOS*, which are involved in proliferation and apoptosis, (Tilghman *et al*, 2012. The differences in the extent of acinar malformations observed in response to the different compounds tested may, therefore, be due to differences in the transcriptional responses that each of these compounds elicits. BPA, like E2, has also been shown to form adducts with DNA and this may lead to mutations (Atkinson & Roy, 1995; Izzotti *et al*, 2009). It may, therefore, also be plausible that the genotoxicity induced by endogenous estrogens and xenoestrogens differs and this may account for the differences in acinar malformations.

The malformations induced by n-propylparaben treatment of acini were not reverted to the same extent, in terms of luminal clearing and organisation, as E2 or BPA treated samples, following co-incubation with either antagonist. This could suggest that the mode of action of n-propylparaben is different from the other tested estrogens. Indeed, it has been demonstrated that n-propylparaben can induce chromosome aberrations and sister-chromatid exchange, whereas E2 only induced chromosomal aberrations in the Chinese hamster ovarian cell line, CHO-K1 (Tayama *et al*, 2008). It has also been demonstrated that the regulation of genes induced by parabens differs from the transcriptional responses induced by E2 (Das *et al*, 1998; Ghosh *et al*, 1999; Pugazhendhi *et al*, 2007). Whether these are mediated through ERα, ERβ, GPER-1, or indeed by another mechanism, remains unresolved, although it has been demonstrated that environmental estrogens, such as chlordecone, induce gene expression changes through a mechanism that is independent of ERα (Das *et al*, 1997).

Interestingly, mammary epithelial cells treated with parabens or other xenoestrogens are able to evade the effects or the tamoxifen and ICI 182,780 (Das *et al*, 1998; Ghosh *et al*, 1999; Goodson *et al*, 2011). This is similar to what we observed upon co-incubation of n-propylparaben treated MCF-12 acini with ICI 182,780 and G-15 and suggests that the effects of parabens, such as n-propylparaben, are at

least in part mediated by receptors other than the ER or GPER-1. The hypothesis that the ER is not solely responsible for the impact of estrogenic compounds upon acini morphogenesis is further supported by the observations that estrogen treatment induced mild malformations of MCF-10A acini and this was most notable upon treatment with n-propylparaben. In this instance however, a role of GPER-1 in mediating the effects of n-propylparaben is a possibility, as MCF-10A cells are GPER-1 competent as shown in **Chapter II**.

4.6.6 MCF-12A Cells Cultured in 3D Respond Differently to Estrogens than Cells Grown in Monolayer

Strangely, in **Chapter II**, increased proliferation was not observed in the MCF-12A cell line in response to E2 treatment, suggesting that cells respond differently to estrogens and other extracellular signals under more physiologically relevant 3D conditions than in monolayer (Dhiman *et al*, 2005; Horning *et al*, 2008; Yang *et al*, 2009). The response of breast cancer cells to the HER-2 antibody, trastuzumab, exemplifies the culture-dependent proliferative changes associated with monolayer and 3D cultures. In one study, 2D cultures of SKBR-3 cells showed only a limited decrease in proliferation following incubation with trastuzumab. However, following 3D culture, trastuzumab treatment decreased proliferation by 48% (Pickl & Ries, 2009). Another study using primary cultures of mammary epithelial cells has demonstrated that the functionality of the ER, and, hence, a cell's ability to respond to estrogen, is compromised in monolayer cultures. When these cells were cultured in 3D, however, they regained their responsiveness to estrogenic treatment (Novaro *et al*, 2003). Additionally it has also been shown that cells cultured in 3D express higher levels of ERα in comparison to the same cells cultured in monolayer, which may result in increased estrogen responsiveness (Yang *et al*, 2000). In monolayer cultures of MCF-12A cells, described in **Chapter II**, E2 induced no change in proliferation, however, upon 3D culture, estrogen-treated acini were larger than controls and misshapen, both indicators of uncontrolled, increased proliferation. These observations, taken together with the reported literature, suggest that the regulation of cellular proliferation of mammary epithelial cells extends beyond the presence of growth factors or hormones and that the micro-environment (monolayer or 3D culture) governs the responses to these mitogens. It remains unclear, however, at this point if this was due to increased ERα expression in 3D cultures or increased functionality of the ER in response to estrogens.

4.6.7 Conclusions

In conclusion, estrogens are able to induce luminal filling and disruption of acinar growth in ERα-competent MCF-12A cells and this effect is not observed to the same extent in ERα negative MCF-10A cells, although mild effects are observed. This alone suggests that the ER is involved in mediating some of the effects elicited by estrogens on acini formation. We conducted further experiments using ER and GPER-1 antagonists to elucidate the role of estrogen-responsive receptors, and found that both ICI 182,780 and G-15 alone were able to revert some of the induced malformations. Furthermore, this reversion was greater when the antagonists were used in combination. This further supports the hypothesis that estrogens are able to disrupt acini formation and do so via the ER, and to some extent through GPER-1 and emphasises a role for these receptors in mediating the actions of estrogens during glandular morphogenesis and maintenance of breast tissue.

Chapter V: Signalling Pathways Implicated in Acini Formation

In **Chapter IV**, we observed that estrogens were capable of disrupting the formation of MCF-12A acini, and to a much smaller extent, MCF-10A acini. As discussed in **Chapter I**, estrogens are capable of eliciting both genomic and non-genomic responses. In this chapter we will try to dissect the mechanisms through which estrogens disrupt acini morphogenesis, by focusing on the potential role of extracellular signalling cascades, more specifically, the PI3K and MAPK signalling pathways on these effects.

The work described in **Chapters III and IV**, and in studies conducted with MCF-10A, cells has demonstrated the importance of proliferation and apoptosis for the correct organisation of 3D acinar structures (Debnath *et al*, 2002; Debnath *et al*, 2003; Mills *et al*, 2004; Murtagh *et al*, 2004; Debnath *et al*, 2005; Fung *et al*, 2008). Several reports have demonstrated that correct acini formation in 3D cultures requires a fine balance between cellular proliferation and cell death. If these processes are dysregulated, acini become deformed and display luminal filling, irregular shapes and loss of apico-basal polarity (Debnath et al 2003[b]; Reginato *et al*, 2005; Haenssen *et al*, 2010).

To date, a number of mechanisms responsible for cell proliferation and apoptosis have been identified as playing an important role in the development and maintenance of mammary acini. These include signal transduction pathways associated with the EGFR, such as the PI3K and MAPK cascades, as well as proliferation and apoptosis-regulating genes such as *CCND1* and *BCL-XL* (Debnath *et al*, 2002; Liu *et al*, 2004; Yanochko & Echhart, 2006). Often, dysregulation of acinar structures arises when one or more of these pathways are activated or blocked. For example, in 3D cultures of MCF-10A cells, hyperactivation of the PI3K cascade disrupts acinar polarity, acinar size and proliferation, resulting in deformed acini of increased size (Irie *et al*, 2005). When hyperactivation of the PI3K cascade or EGF signalling is combined with ectopic up-regulation of the oncogene, cyclin D1, MCF-10A acini escape proliferative arrest and present with filled lumen (Debnath *et al*, 2003). Transfection of oncogenes involved in signalling pathways, such as B-Raf of the MAPK cascade, into MCF-10A cells, which are then grown in 3D results in the loss of acinar organisation and an invasive cell phenotype (Herr *et al*, 2011). Furthermore, 3D cultures of the malignant breast cell line, HMT-3522, have revealed that increased MAPK pathway activity results in the development of abnormal acinar structures (Beliveau *et al*, 2010).

In this chapter, we will explore the role of the PI3K and MAPK pathways in MCF-12A acini formation, using inhibitors specific for these pathways. Estrogens have been demonstrated to activate

these pathways and it would, therefore, be of interest to investigate whether the estrogen-induced acinar malformations seen in **Chapter IV** are a consequence of hyperactivation of these signalling cascades (Wozniak *et al*, 2005; Tong *et al*, 2010).

5.1 The PI3K Signalling Pathway

As mentioned previously, the PI3K and MAPK pathways are involved in proliferation, survival, apoptosis, motility, differentiation, tumour invasion and angiogenesis. The activation of the PI3K signalling pathway occurs in the cytoplasm, in response to the stimulation of RTKs, such as the IGF-1 receptor or G-protein-coupled receptors. Following PI3K activation, the secondary messenger, PIP_3 is generated from the phosphorylation of PIP_2 (Vanhaesebroeck *et al*, 2001). Targets of PIP_3 include AKT, which, in turn, phosphorylates various target proteins. PI3K signalling has implications for cyclin D1, cyclin D2, mdm2-p53 axis, Bcl-2 phosphorylation, Bad, caspase-9, Myc, and the regulation of $p27^{kip1}$, amongst many others (**Figure 5.1**) (Datta *et al*, 1997; Mayo *et al*, 2002; Wanzel *et al*, 2005).

The cyclins, on the other hand, are required for progression through the cell cycle; cyclin D1 and D2 aid progression through the G1/S checkpoint following binding with CDK2 and CDK4. Thus, increased expression of these proteins by PI3K action can result in increased cell cycle progression and increased proliferation (Bouchard *et al*, 2004). Cip and kip proteins, such as $p27^{kip1}$, are CDK inhibitors that function to regulate the formation of cyclin-CDK complexes and, thus, modulate progression through the cell cycle. Mutations or activation by AKT prevent their inhibitory effect upon cyclin-CDK complex formation and, hence, result in increased progression through the cell cycle and increased proliferation (Banerji *et al*, 2001; Yuan *et al*, 2007).

Apoptotic proteins are also targeted by activated AKT: the pro-apoptotic factor Bad is phosphorylated by this kinase, causing it to dissociate from anti-apoptotic Bcl-2 and Bcl-xl. These in turn, are liberated and bind to pro-apoptotic Bax, preventing its action (Datta *et al*, 1997; Cardone *et al*, 1998).

p53, a tumour suppressor gene that functions to initiate DNA repair, regulate the cell cycle and induce apoptosis, is another target of AKT. Specifically, AKT inhibits transcription and apoptosis mediated by p53 and promotes its degradation, at least in part through Mdm2 protein (Ogawara *et al*, 2002). Mdm2 is an ubiquitin ligase, that upon phosphorylation and activation by AKT, ubiguinates p53, thus targeting it for proteasome degradation, and inhibiting its pro-apoptotic function. Ultimately, activation of AKT effectors results in increased cell cycle progression, cellular proliferation, survival and

decreased apoptosis (**Figure 5.1**) (Tokunaga *et al*, 2008). We showed that AKT can become activated in response to estrogens in ER competent MCF-12A and MCF-7 cells, and given that its activation promotes cell survival and suppresses apoptosis, estrogen-induced activation of this kinase may contribute to the malformations observed in estrogen-treated MCF-12A acini.

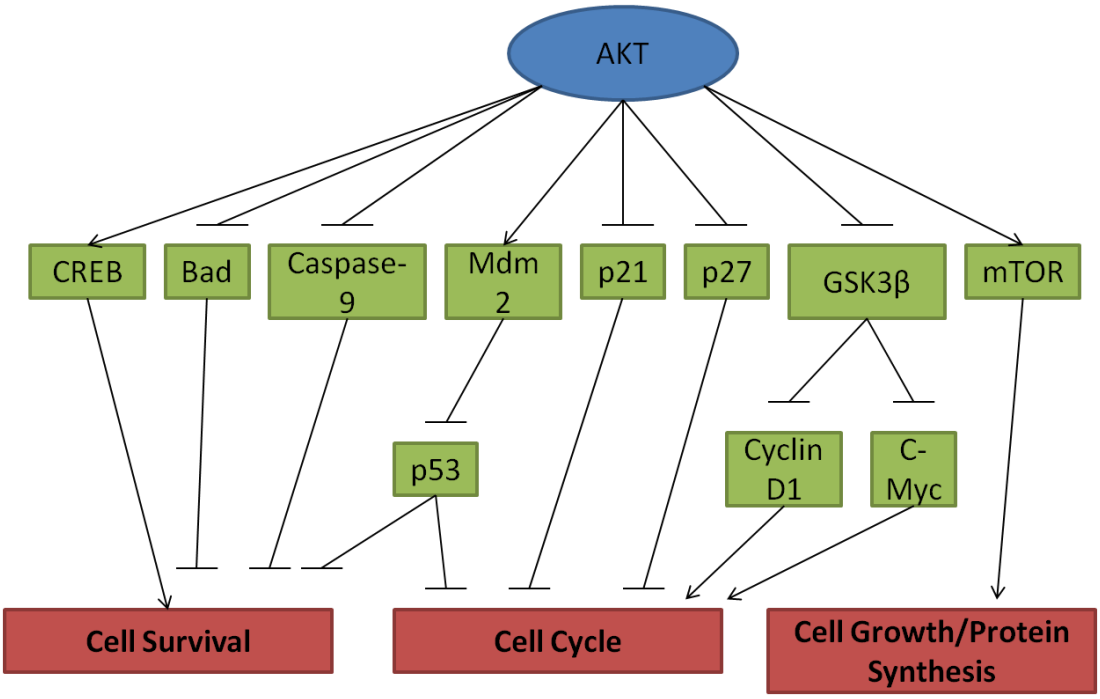

Figure 5.1: Targets of activated PI3K/AKT signalling. AKT activation leads to increased survival, cell cycle progression, cell growth and decreased apoptosis via the activation or inhibition of various proteins (Figure adapted from Tokunaga *et al*, 2008).

5.2 The MAPK Signalling Pathway

Activation of RTKs or G-protein-coupled receptors in the cell membrane initiates the MAPK signalling cascade. Receptor activation leads to the recruitment of the GRB2-SOS complex and activation of Ras upon the binding of GTP. Ras-GTP then proceeds to phosphorylate and activates Raf kinase which, in turn, phosphorylates and activates the serine/threonine protein kinases MEK1/2. Phosphorylated MEK1 and MEK2 phosphorylate tyrosine residues within ERK1/2 regulatory sites and activate them, permitting them to activate or inhibit their target proteins. The MAPK pathway shares some common effectors with the PI3K pathway such as $p27^{kip1}$ and c-Myc. In addition to these, the MAPK cascade also has

implications for Bim, ZO-1 and β-catenin (**Figure 5.2**), among many others (Pinkas & Leder, 2002; Ewings *et al*, 2007).

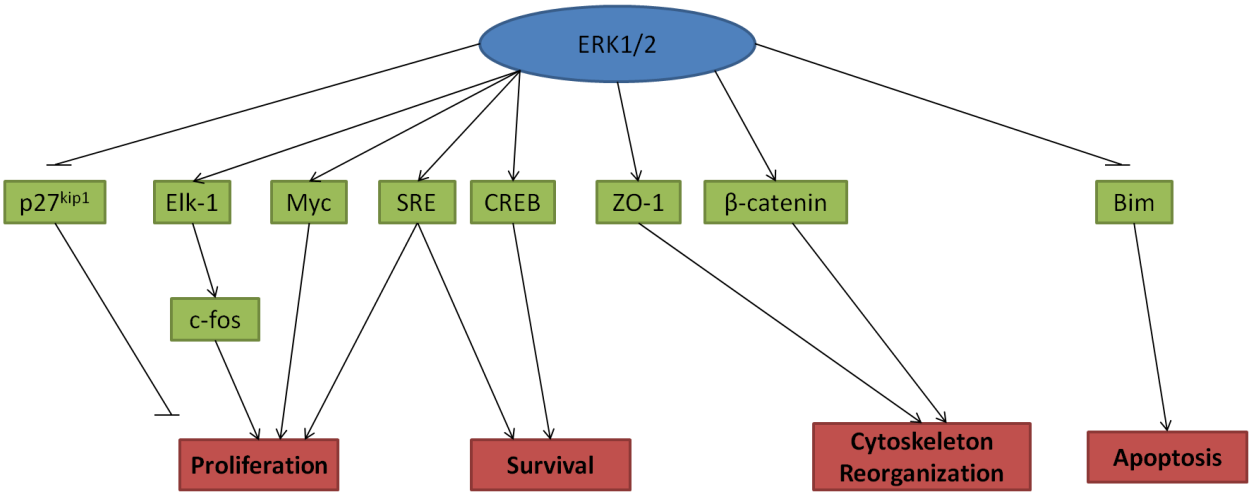

Figure 5.2: Implications of activated ERK1/2 as a result of MAPK signalling. ERK1/2 activation leads to increased survival, cell cycle progression and cell growth and decreased apoptosis via activation or inhibition of various proteins and transcription factors (Pinkas & Leder, 2002; Davis, 2003; Ewings *et al*, 2007).

The dysregulation of MAPK and PI3K signalling pathways is a recurring hallmark of cancers (Bellacosa *et al*, 1995; Hilger *et al*, 2002; Di Cosimo *et al*, 2007). Both these pathways frequently undergo genetic alterations during cancer initiation and progression through all tiers of the signal transduction pathway, from the receptor level to the effectors.

Because of the over-activation of signalling pathways in tumours, inhibitors of PI3K and MAPK signalling are being investigated for their use in cancer therapy. Such drugs used or in development for therapy include gefitinib, trastuzumab, wortmannin derivatives and LY 294002 conjugates (Tokunaga *et al*, 2008; Maria *et al*, 2009). To date, the wortmannin derivative, PX-866 (ProlX Pharmaceuticals) and the LY 294002 Arg-Gly-Asp-Ser-conjugated pro-drug, SF1126 (Semafore Pharmaceuticals) have shown promising results in early studies in ovarian, colon, lung, prostate and breast cancer and are in the process of undergoing phase 1 clinical trials (Ihle *et al*, 2004, 2005; Garlich *et al*, 2008).

Estrogens are able to induce rapid non-genomic effects, such as the activation of the PI3K and MAPK pathways via the ER and GPER-1 (Improta-Brears *et al*, 1999; Wozniak *et al*, 2005; Tong *et al*, 2010). Consequently, this has implications for proliferation, apoptosis and treatment response. We

hypothesised, therefore, that the MAPK and PI3K cascades may be implicated in the malformations induced in MCF-12A acini by estrogens. In order to test this, the role of PI3K and MAPK signalling during acini morphogenesis was investigated using specific inhibitors for the ERK1/2 or PI3K/AKT cascades. LY 294002 is a commonly-used, potent, specific inhibitor of PI3-kinase that is derived from quercetin, a plant-derived flavanoid. It functions in a reversible, competitive fashion to inhibit the binding of ATP to the catalytic subunit of PI3K and, thus, prevents PI3K activation, but does not affect the activities of other signalling proteins including EGFR, Src tyrosine kinases, protein kinase A (PKA), protein kinase C (PKC) or MAPK (Vlahos *et al*, 1994). There are reports, however, that LY 294002 can interact with mTOR, casein kinase 2 (CK2), glycogen synthase kinase 3β (GSK3β), Hsp90 and Ca^{2+}-mediated signalling (Brunn *et al*, 1996; Davies *et al*, 2000; Tolloczko *et al*, 2004; Gharbi *et al*, 2007). For the purpose of the data presented here, however, this is not an issue as the aim of using LY 294002 was to abrogate PI3K signalling, and many of these proteins are components of this pathway. PD 184352 is a commonly used specific MAPK pathway inhibitor that acts by preventing the activation of MEK1 (the kinase responsible for phosphorylating ERK 1/2), and, consequently preventing the activation of both ERKs (Davies *et al*, 2000). We proceeded to co-incubate the previously tested compounds with the MAPK and PI3K pathway inhibitors to selectively block each cascade individually. The main aims of this were to investigate the impact of these signalling pathways on the formation of MCF-12A acini and whether they are involved in the estrogen-induced disruption of these acinar structures.

5.3 Materials and Methods

LY 294002 (Promega, Southampton, UK) and PD 184352 (Promega, Southampton, UK) were resuspended in DMSO to produce 50 mM stock solutions. Subsequent dilutions to make working stock concentrations of 10 µM and 25 µM, respectively, were also prepared using DMSO. Dilutions were stored at -20^{o}C and allowed to reach room temperature prior to use.

For incubation with the inhibitors, cells were pre-treated for 1 hour with 10 µM PI3K inhibitor, LY 294002 or 25 µM MEK inhibitor, PD 184352 and seeded onto Matrigel with 0.5% etOH, 1 nM E2, 10 µM BPA or 10 µM n-propylparaben for 8 and 12 days, as described previously, as these were the time-points where activated caspase-3 was observed most strongly (See **Materials and Methods** in **Chapter III** and **IV**). Controls consisted of 0.5% etOH + 0.2% DMSO. Inhibitor-free samples were also prepared, containing the test estrogenic compound at the same concentration as before in combination with 0.2% DMSO.

In order to confirm that the changes observed were due to interaction of estrogens with the studied cascades, cells were seeded and maintained as described previously for 16 days and treated with PD 184352 or LY 294002 in combination with varying concentrations of E2 (1 pM, 10pM, 1 nM). Controls consisted of 0.5% etOH or E2 + 0.2% DMSO.

5.4 Results

5.4.1 The Effect of MAPK and PI3K Pathway Inhibitors on Acini Disruption by Estrogenic Compounds

One of the first observations made in our experiments was that the presence of the inhibitors affected the formation of acini, not only in estrogen-treated samples, but also in controls. Most notable, was the observation that both inhibitors seemed to have a weak effect on proliferative control, as they reduced the overall number of acini formed in controls with inhibitor by approximately 50%, relative to controls without inhibitor. A similar decrease in acini abundance was also observed in samples treated with treatment + inhibitor in comparison to treatment alone.

More importantly, however, were the differences between MCF-12A acini treated with the selected estrogenic test compounds and the test compounds in combination with a MAPK or PI3K pathway inhibitor, which were observed after 8 days incubation in 3D culture (**Figure 5.3**). The impact of the inhibitors was seen, primarily, in the size and shape of acini. As can be seen in **Figure 5.3 & 5.4**, the presence of the PI3K inhibitor, LY 294002, resulted in acini that were spherical and notably smaller in diameter in relation to samples treated with the test compounds alone. Interestingly, this effect, although observed with all the estrogenic compounds tested, was more marked in samples co-incubated with E2. This could be because BPA and n-propylparaben exert greater malformation than E2, and hence, not all of their effects could be reverted to the same extent as the endogenous hormone. Alternatively, it could be that the effects of BPA and n-propylparaben are mediated by other pathways, which are not blocked by LY 294002. Similarly, when estrogen-treated MCF-12A acini were co-incubated with the MAPK pathway inhibitor, PD 184352, acini were again spherical and smaller in size, although larger than those observed upon co-incubation with LY 294002.

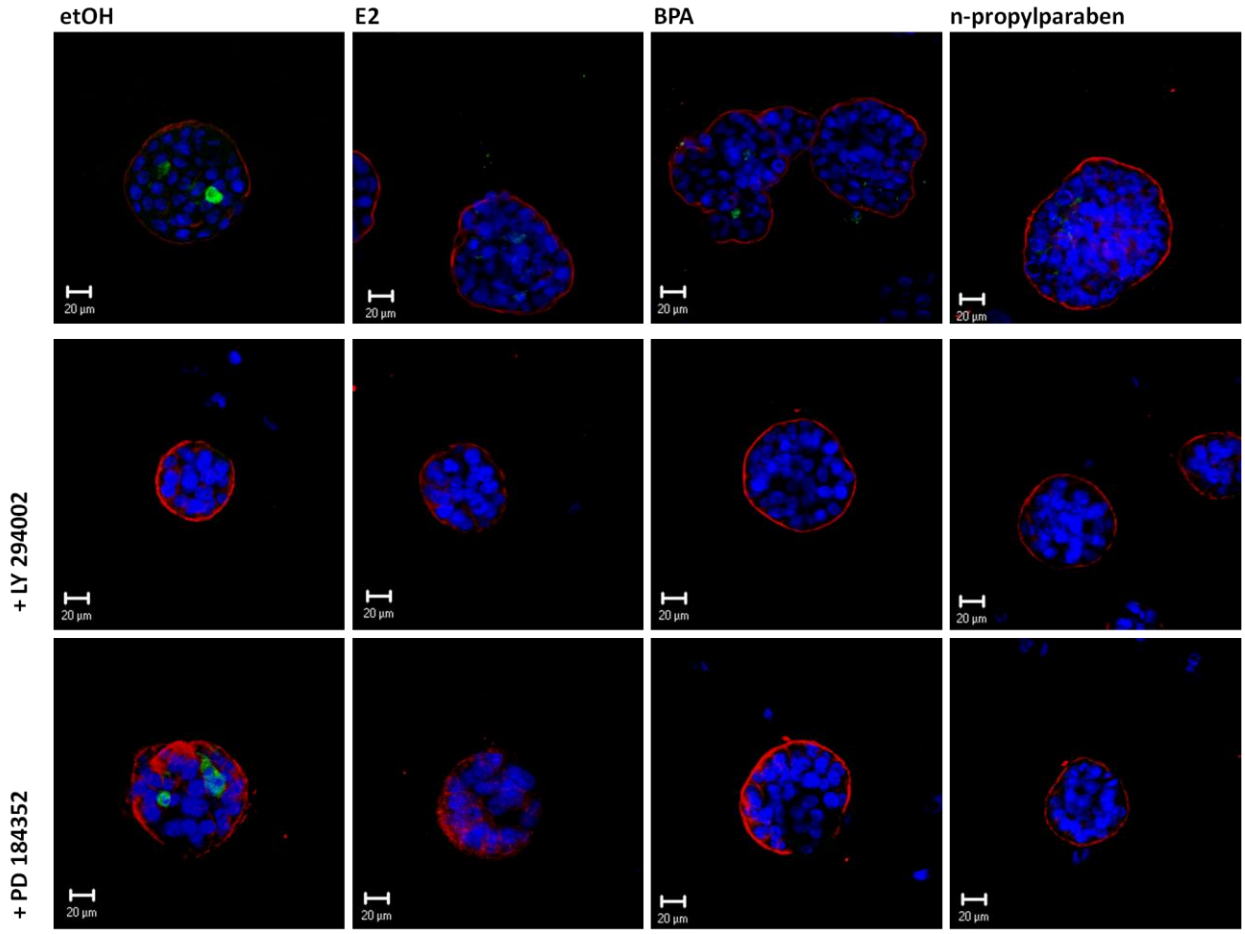

Figure 5.3: Confocal images of MCF-12A acini after 8 days in 3D culture with MAPK and PI3K pathway inhibitors: Samples were treated with 1 nM E2, 10 µM BPA or 10 µM n-propylparaben, alone or in combination with 10 µM LY 294002 or 25 µM PD 184352, for 8 days. Acini were then stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain. Controls consisted of 0.5% ethanol with 0.2% DMSO, 10 µM LY 294002 or 25 µM PD 184352.

12 day co-incubation of treated samples with LY 294002 resulted in spherical acini that were slightly smaller in diameter than those observed in solvent controls (**Figure 5.4**). Notable luminal clearing was also observed in E2-treated samples co-incubated with LY 294002 at the same time-point. However, this effect was not observed with BPA or n-propylparaben, highlighting the possibility that these compounds may act through pathways distinct from those of E2.

After 12 days in 3D culture, treatment with PD 184352 alone, had an impact on acini formation, as these exhibited some luminal filling (**Figure 5.4**). Given that PI3K and MAPK signalling pathways are

implicated in cell survival, proliferation and apoptosis, it is hardly surprising that the inhibition of these pathways affected acini.

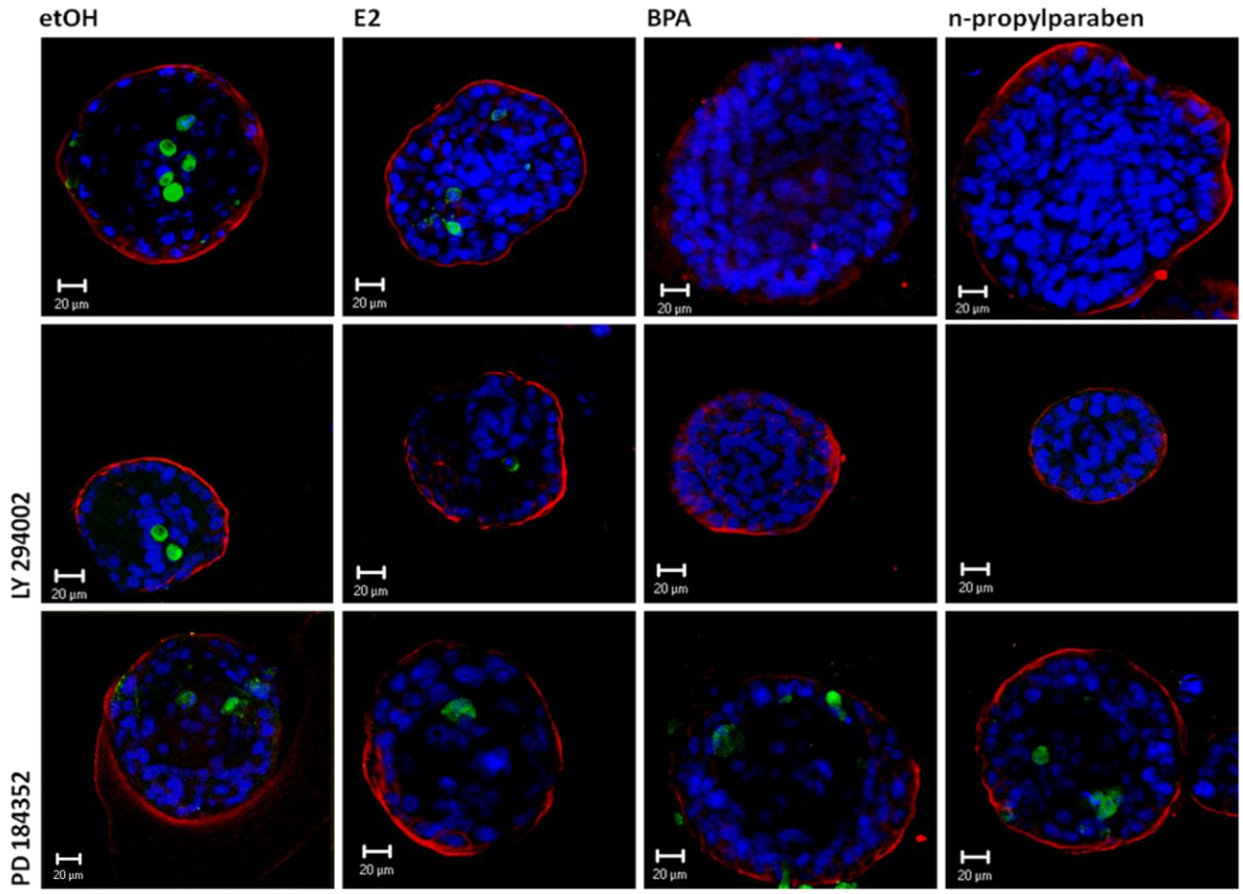

Figure 5.4: Confocal images of MCF-12A acini after 12 days in 3D culture with MAPK and PI3K pathway inhibitors. Samples were treated with 1 nM E2, 10 µM BPA or 10 µM n-propylparaben, alone or in combination with 10 µM LY 294002 or 25 µM PD 184352, for 12 days. Acini were then stained with antibodies against laminin V (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain. Controls consisted of 0.5% ethanol + 0.2% DMSO, 10 µM LY 294002 or 25 µM PD 184352.

The results presented above suggest that in this 3D model, the PI3K and MAPK pathways are involved in proliferation, and to a smaller extent, luminal clearance. Furthermore, the estrogenic test compounds may be eliciting their effects via these pathways, particularly in the case of overcoming growth arrest to induce malformations of MCF-12A acini, characterised by increased acinar size, irregular shape and luminal filling. It would also appear that the effects of LY 294002 and PD 184352 differ suggesting that

the PI3K and MAPK pathways and individual specific roles in acini formation. Co-incubation of treatments with LY 294002 gives rise to acini that are smaller in size and may indicate the main role of the PI3K pathway is concerned with proliferation. On the other hand, co-incubation with PD 184352 appeared to have more of an impact on lumen formation, suggesting that the MAPK pathway is more involved in apoptosis.

5.4.2 Acini Quantification and Statistical Analysis

MCF-12A acini cultured for 8 and 12 days in Matrigel were quantified as described previously.

After 8 days incubation, LY 294002 with 0.5% ethanol resulted in acini that were smaller in diameter and contained less cells per acini. PD 184352 did not have a significant impact on acini size or number of cells per acini in rthanol controls. Although not statistically significant, a small reduction in acini size of E2-treated acini was observed when cells were co-incubated with LY 294002. However, this observation was not made when cells were co-incubated with PD 184352. The average number of cells was reduced upon co-incubation of E2 with LY 294002 or PD 184352 and strangely, the number of apoptotic cells was also reduced upon co-incubation with both pathway inhibitors. Neither LY 294002 or PD 184352 were able to reverse the impact of BPA on acini size, or have a significant impact upon the average number of cells per acini or percentage of apoptotic cells. LY 294002 and PD 184352 both significantly reduced acinar size and the number of cells per acini in n-propylparaben-treated samples, and this reversal of these characteristics was more pronounced than those observed with E2, suggesting that n-proplyparaben may utilise the MAPK and PI3K pathways to a greater extent than endogenous E2 when eliciting its effects in these cells. There was, however, no change in the number of apoptotic cells at this timepoint.

Following 12 days incubation, ethanol control acini were of a similar size, and contained a similar number of cells, to those treated with LY 294002 or PD 184352. The presence of PD 184352 did however result in a reduced percentage of apoptotic cells per acini. In the case of BPA and n-propylparaben, co-incubation with LY 294002 or PD 184352 resulted in reduced acini size and a decrease in the average number of cells per acini. An increase in the percentage of apoptotic cells was also observed relative to cells treated with BPA or n-propylparaben alone. LY 294002 or PD 184352 did not have a great impact on acini size of E2-treated acini, however, they did result in less cells present per acini. This supports the findings from the LCM images where partial luminal clearing was observed with

the presence of pathway inhibitors in estrogen-treated samples (**Figure 5.4**). As with BPA and n-propylparaben, co-incubation of E2-treated acini with LY 294002 or PD 184352 significantly increased the percentage of apoptotic cells, and thus would indicate that inhibition of apoptosis through activation of the MAPK and PI3K pathways is one mechanism through which the test compounds were able to induce luminal filling (**Figure 5.5**).

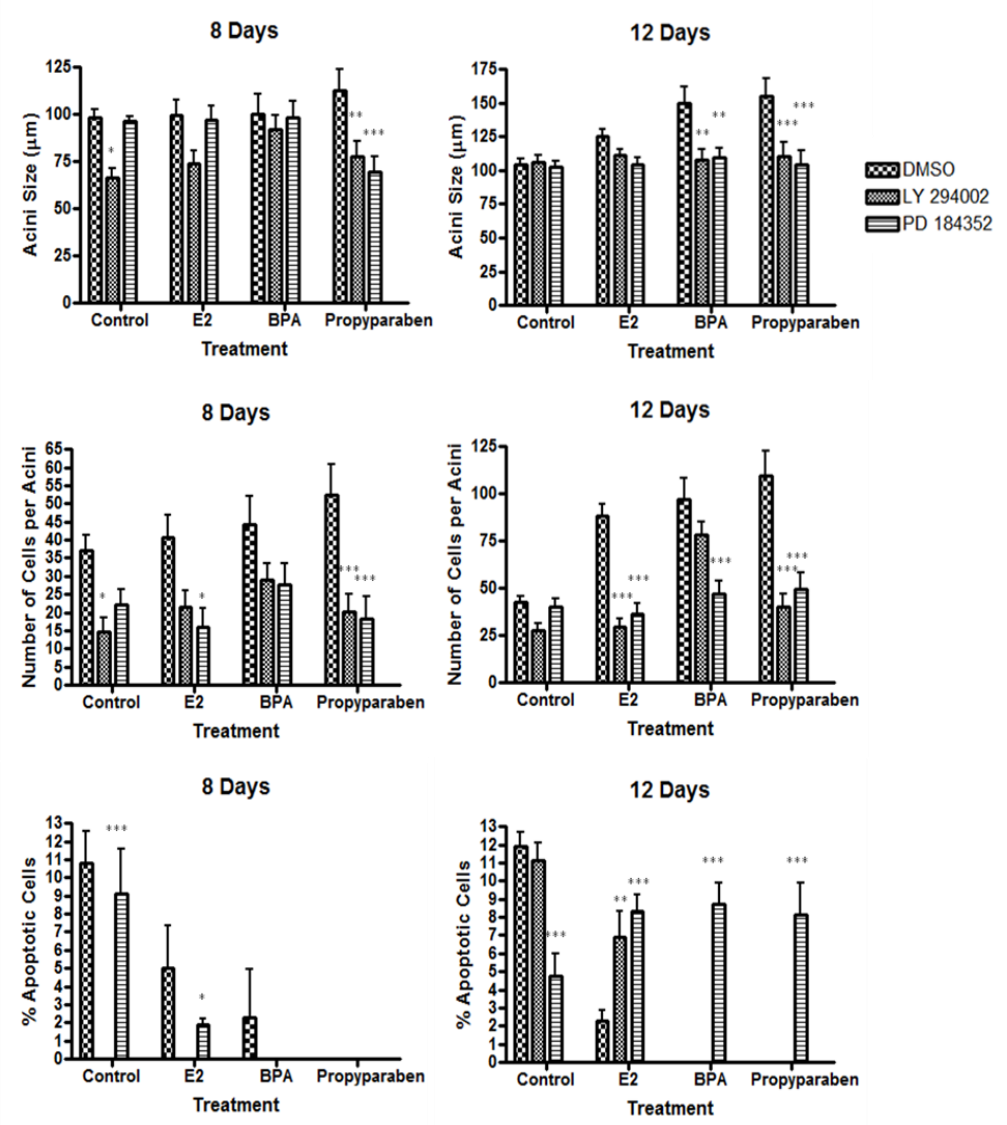

Figure 5.5: Quantification of acini size, number of cells per acini and percentage of apoptotic cells. The size of acini (µm) and the number of cells per acini were determined by analyzing confocal images of the incubation periods of 8 and 12 days. Data corresponds to mean ± SEM and results from three independent experiments, where a minimum of 10 representative acini were analysed per experiment. * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between treatments and controls.

5.5 Discussion

In **Chapter IV**, the estrogenic test compounds disrupted the architecture of MCF-12A acini and this was mediated by both the ER and GPER-1. Perturbations in the structure of the mammary gland *in vivo*, such as luminal filling and loss of organisation are indicative of a cancerous phenotype.

Using a previously established 3D model, our results in this chapter indicate that the E2-induced malformations of MCF-12A acini involve, at least to some extent, the PI3K and MAPK signalling pathways. This was concluded, as inhibition of these pathways partially reverted some of the malformations induced by the estrogenic test compounds, such as the loss of proliferative control and luminal filling. PI3K and MAPK signalling are often hyper-activated in cancer (Bellacosa *et al*, 1995; Hilger *et al*, 2002; Di Cosimo *et al*, 2007) and are often associated with resistance to anti-hormone therapy (McGlynn *et al*, 2009). Previous work has shown that in 3D cultures of mammary epithelial cells, the increased expression of activated AKT is associated with mis-shapened acini and increased proliferation (Debnath *et al*, 2003[b]; Isakoff *et al*, 2005). In addition to these 3D culture observations, monolayer culture has provided insights into the consequences of hyper-activation of the PI3K pathway, such as anchorage-independent growth, independence from growth factors and evasion from anoikis (Isakoff *et al*, 2005). All of these characteristics could have implications for 3D cultures in terms of acinar size and shape as well as the formation of the hollow lumen.

As a large number of studies have now demonstrated that extranuclear signalling cascades, in particular the PI3K and Erk pathways, are targets for estrogenic action, it is plausible that some of the effects induced by estrogens on acini formation involve interactions with these secondary messenger cascades. The results presented in this chapter indicate that, in fact, PI3K and MAPK signalling are involved in MCF-12A acini formation and that estrogens interact with these pathways to exert their effect on these spheroid structures.

5.5.1 The PI3K Signalling Pathway is Involved in Estrogen-Induced Acinar Malformations

Treatment with the PI3K inhibitor, LY 294002, in combination with the test compounds, resulted in a decrease in the number of acini formed and acini size at all time points, and thus reverted some of the effects induced by the estrogenic compounds. In, E2, but not BPA or n-propylparaben-treated samples, there was also increased luminal clearing and activation of caspase-3, when samples were co-incubated with this inhibitor.

The PI3K pathway, and in particular the activation state of AKT, is already known to be important for the morphogenesis of MCF-10A acini. AKT in its active form is present only in the outer cells of MCF-10A acini under normal conditions (Debnath *et al*, 2003[b]). This could explain why, normally, outer cells in contact with the basement membrane survive, whereas the central cells undergo apoptosis and are cleared to form the hollow lumen. Aberrations in PI3K signalling have, however, been shown to impact upon the formation of growth-arrested, spherical acini. The effect of LY 294002 on acini size in 3D cultures has also been observed in cell derived from hamster mammary tumours and cultured in Matrigel (Polo *et al*, 2010). This suggests that during acini formation, the PI3K pathway plays a role in the control of cellular proliferation. These findings are further supported by other groups that have observed decreased proliferation, decreased EGFR, cyclin D1 and β1 integrin, increased PTEN and reversion of some malignant phenotypes in response to PI3K inhibition in 3D culture (Wang *et al*, 2002; Isakoff *et al*, 2005; Provenzano *et al*, 2009; Eritja *et al*, 2010). Furthermore, it has been demonstrated that PI3K signalling is a target for estrogenic action (Watson *et al*, 2007; Yin *et al*, 2007) and is implicated in the E2-induced proliferation of various cell types. PI3K pathway inhibitors, such as LY 294002 have been shown to inhibit E2-mediated transcription and progression into the S phase of the cell cycle (Lobenhofer *et al*, 2000; Marino *et al*, 2003; Gao *et al*, 2004).

Overall, our data is in agreement with previous publications by suggesting that the PI3K signalling pathway is involved in MCF-12A acini morphogenesis, especially by controlling cellular proliferation.

It was clear, from our results that the effect of PI3K pathway inhibition was more marked with E2 treatment than BPA or n-propylparaben. This suggests that the mechanism of action of the xenoestrogens differs from that of E2. Similar observations were also made upon MAPK inhibition of E2, BPA and n-propylparaben treated MCF-12A acini. Indeed, it has been shown that ERK signalling activation patterns, i.e. the time points at which ERK is phosphorylated and de-phosphorylated following treatment of E2, differ from BPA (Wang *et al*, 2005). At this point is noteworthy to discuss briefly the interactions of xenoestrogens, such as BPA, with estrogen-responsive receptors in terms of their elicited genomic and non-genomic responses. Many studies have shown that xenoestrogens exert very weak genomic responses, and only at concentrations that are at least 1000 times that observed with E2 (reviewed in Bulayeva & Watson, 2004). It is generally accepted that these genomic response are mediated by the classical ER receptors: ERα and ERβ. However, as described in **Chapter I**, estrogens are capable of non-genomic events, which is what we aimed to study in this chapter. Unlike genomic

responses, the exact mechanism underlying the non-genomic effects of estrogens remains of much debate, although it is commonly accepted that a membrane-associated estrogen-responsive receptor is probably involved. When Bulayeva & Watson (2004) investigated the patterns of ERK phosphorylation of pituitary tumour cells in response to 1 nM E2 they observed a bi-modal ERK activation pattern that occurred three times after treatment application. When, however, xenoestrogens at a concentration of 1 nM were applied, the patterns of ERK activation differed depending on the xenoestrogen tested. These responses ranged from no response at all, in the case of BPA, to single sustained ERK activation, in the case of the coumestrol. Although in this instance, BPA did not induce activation of the ERK pathway, the data clearly shows that E2 and xenoestrogens induce different patterns of non-genomic responses. Other data has shown that even at 1 nM concentrations, BPA is capable of stimulating the release of intracellular calcium, a key secondary messenger implicated in the PI3K pathway (Wozniak *et al*, 2005). Although our data demonstrated that endogenous estrogens and xenoestrogens are capable of inducing distinctly different non-genomic responses, it has not yet shed light on as to whether the mechanism of action is different, or how.

A possibility that remains is that the difference in responses to the cascade inhibitors is not associated with a different mechanism of action, but due to a potency or concentration issue. As shown in previous chapters, we have shown that in 3D cultures of MCF-12A cells, the effects of the exogenous estrogens are more marked than those of the endogenous E2. Although the concentrations selected for these studies were chosen based on previous publications, showing a similar level of effect for all chemicals in cells such as MCF-7 (Kabil *et al*, 2008; Silva *et al*, 2011), it is possible that, in this experimental setting, chemicals have different potencies, and BPA and n-propylparaben are more potent than E2. In this case, the same concentration of LY 294002 would have potentially a weaker impact on a xenoestrogens' effects than on E2, even if all chemicals acted via the same mechanisms. In the future, it would therefore be of interest to perform the experiments described here with lower concentrations of both BPA and n-propylparaben to ascertain whether their effects could be reverted further by LY 294002.

Taken together with the reported literature, the data presented in this chapter and in **Chapter IV**, implies that estrogens promote cellular proliferation in 3D cultures of MCF-12A cells. It would also appear that the activation of the PI3K pathway by estrogens is, in part, responsible for the MCF-12A malformations associated with increased and dysregulated proliferation. As described previously, hyperactivation of the PI3K pathway in 3D culture models results in large misshapen acini with increased

cellular proliferation (Liu *et al*, 2004; Brummer et al, 2006; Schweppe *et al*, 2009), similar to those observed upon E2 treatment. This observation is comparable to the process of tumourigenesis in which, PI3K signalling is often hyper-activated. This has consequences for proliferation, apoptosis, adhesion, migration, metastasis, angiogenesis and the correct arrangement of the cytoskeleton (Levine *et al*, 2005; Yin *et al*, 2007; Liu *et al*, 2010). It is therefore plausible that the estrogen-induced activation of the PI3K pathway, as inferred by the reversal of estrogen's effects upon co-incubation with LY 294002, may have implications for tumourigenesis and cancer progression *in vivo*.

5.5.2 The MAPK Signalling Pathways are Involved in E2-Induced Acinar Malformations

Acini treated with the test compounds in combination with the MAPK pathway inhibitor, PD 184352, became spherical in shape and formed growth-arrested structures with some evidence of luminal clearing. This suggests that the actions of estrogens are not solely mediated by the PI3K pathway, but also by the MAPK cascade. The MAPK pathway involves a kinase cascade and is involved in proliferation, apoptosis, survival, differentiation and motility in response to mitogens and growth factors in normal cells. However during carcinogenesis, overexpression, amplification or mutations in the various components of the pathway result in the dysregulation of these processes, leading to hyper-activation of the pathway.

Inhibition of the MAPK pathway by PD 184352 initially produced acini that were smaller in size when compared to etOH controls. This decrease in acinar size, however, was not observed after 12 days incubation. This suggests that inhibition of the MAPK pathway delays acinar proliferation, thus the smaller acinar size, however, other mechanisms may overcome this proliferative suppression. This could, for example, be achieved by activation of the PI3K pathway, which as described above, estrogens are capable of achieving in our 3D model.

In addition to a reverting the estrogen-induced increased acinar size, MAPK pathway inhibition also resulted in spherical acini. This suggests that proliferation in the presence of the MAPK inhibitor, PD 184352, acini were able to recuperate the proliferative control that was lost in response to estrogen treatment. These observations indicate that estrogens seem to illicit their proliferative effects on 3D cultures of MCF-12A cells, at least in part, via activation of the MAPK pathway. A plausible mechanism for this may be increased progression through the cell cycle in response to estrogens. Indeed, one of the effectors of the MAPK pathway is cyclin D1 (Lobenhofer *et al*, 2000; Marino *et al*, 2002), a key

regulator of cell cycle progression and importantly, cyclin D1 expression can also be induced by E2 (Planas-Silva *et al*, 2001). The role of this protein is to facilitate the passage of cells through the G1/S boundary of the cell cycle, and thus an increase in cyclin D1 expression correlates with increased proliferation. Importantly E2-induced progression through G1 to S phase of the cell cycle in response to E2 is inhibited by MAPK inhibition (Lobenhofer *et al*, 2000). The results presented above do not elude to whether the levels of cyclin D1 were affected by estrogen or pathway inhibitor treatment, although the possibility of a role for this key regulator of cell cycle progression remains plausible and warrants further investigation.

After 12 day co-incubation with PD 184352, increased luminal clearance and activated caspase-3 expression was observed, suggesting reduced cell survival and increased apoptosis in response to MAPK signalling inhibition, in addition to the decreased proliferation described previously. In **Chapter III**, we defined a time-course for the morphogenesis of MCF-12A acini . In this time-course, the onset of apoptosis, as inferred by the expression of activated caspase-3, occurred after 8 days incubation. This suggests that the MAPK cascades are not solely responsible for the reduction in apoptosis in response to the test compounds but that other, earlier signalling pathways may be involved. Nevertheless, estrogens appear to reduce apoptosis and inhibit lumen formation, in part via activation of the MAPK pathway. Indeed, it has previously been shown that activation of MEK protects cells from apoptosis and autophagy by inhibiting the expression and activation of pro-apoptotic Bim (Thaker *et al*, 2001; Reginato *et al*, 2005; Wickenden *et al*, 2010). Bim phosphorylation by ERK1/2 prevents its association with Bcl-2 and Bcl-xl, which enables these anti-apoptotic factors to bind to pro-apoptotic Bax and prevent apoptosis (Ewings *et al*, 2007; [b]Ewings et al, 2007). Furthermore, Bim's phosphorylation promotes its degradation (Ley *et al*, 2003). This presents one mechanism through which estrogen-activated MAPK signalling could induce luminal filling, although this malformation was not completely reverted by MEK or PI3K inhibition, implying that a combination of signalling cascades are involved or that other estrogen-activated pathways or compensating upon inhibition of one pathway, as demonstrated by Pearson & Hunter (2009).

The data presented in this chapter adds to the existing body of evidence that shows that E2, and xenoestrogens, such as BPA and n-propylparaben are capable of eliciting non-genomic effects such as activation of the MAPK pathway (Migliaccio *et al*, 1996). This ultimately leads to increased cellular proliferation and decreased apoptosis (Chen *et al*, 2004; Watson *et al*, 2007; Sheng & Zhu, 2011).

5.5.3 Conclusions

Over-activation of signalling pathways is one mechanism in which breast tumours acquire resistance to anti-hormone therapy (Isakoff *et al*, 2005; Liu et al, 2007; McGlynn *et al*, 2009; Musgrove & Sutherland, 2009). The results above indicate that estrogens act through both PI3K and MAPK signalling to increase cellular proliferation and cell survival, resulting in deformed, filled acini that evade growth-arrest. It was observed before, that the effects of n-propylparaben were not reverted by treatment with ER or GPER-1 antagonists. Interestingly, the results presented in this chapter show that PI3K and MAPK signalling mediate, to some extent, the effects of n-propylparaben, particularly in relation to proliferation. This was inferred from the decrease in acinar size and reversion to a more spherical shape when n-propylparaben-treated MCF-12A acini were co-incubated with the PI3K pathway inhibitor, LY 294002. Further to this, the MAPK pathway inhibitor, PD 184352 also resulted in smaller, more spherical acini with increased signs of apoptosis and luminal clearing. Consistent with these results, increased AKT activity in tumourigenic MCF-7 cells has been shown to result in ICI 182780 resistance and incubation of 3D cultures of MCF-7 cells with LY 294002 result in spherical acini displaying hollow lumen (Beeram *et al*, 2007; Vahidnezhad *et al*, 2009). This highlights that the non-genomic mechanisms of estrogens, such as increased proliferation due to the activation of the PI3K or MAPK pathways, may play a role in breast carcinogenesis and resistance to anti-hormone therapy. Although not conclusive, (and more work is required) this could indicate that complete reversion of a cancer-like phenotype both *in vitro* and *in vivo* requires therapy involving a combination of anti-hormone treatment and MAPK and PI3K signalling inhibition.

Finally, the data presented here supports the hypothesis that endogenous estrogens and xenoestrogens are capable of inducing non-genomic responses. Through the activation of signalling pathways, such as that of the PI3K and MAPK cascades, estrogenic compounds may *in vivo*, interfere with the normal actions of endogenous estrogen. Activation of these pathways may lead to cross-talk between estrogen and growth factor signalling, as described in **Chapter I** and this may have implications for physiological signalling pathways, causing them to become hyper-activated. In this way therefore, estrogens are capable of inducing rapid cellular responses, such as increasing cellular proliferation, that are normally performed in response to growth factor stimulation.

It cannot be determined whether the mediator of the non-genomic actions of estrogens is a membrane bound form of ER, GPER-1 or perhaps another undefined receptor. However, given that both ER and GPER-1 antagonists were able to revert some of the effects induced by estrogens, both may

be implicated in the progression to a cancer-like phenotype. In any case, the data presented in this chapter and in **Chapter IV**, clearly shows that estrogens are capable of disrupting MCF-12A acini morphogenesis to some extent via the ER and GPER-1, and possibly other receptors. Furthermore, the disruption of acini by estrogens also appears to involve the PI3K and MAPK pathways, implying that the mode of action of the disruptive effects of estrogens is at least in part non-genomic. These findings may have implications for how breast cancer is treated, as common ER antagonists, such as Fulvestrant and Tamoxifen do not target GPER-1. This would allow GPER-1 competent cells to continue to respond to estrogens through activation of signalling pathways, such as PI3K and MAPK. It would therefore be of interest to investigate whether a combination of receptor and pathway antagonism is capable of further reverting the estrogen-induced MCF-12A acinar malformations in our 3D culture model.

Chapter VI: The Role of Estrogen-Receptive Receptors in Mediating the Effects of Estrogens

From the data presented in **Chapter IV** and **V**, it became apparent that both endogenous and exogenous estrogens have the ability to disrupt the morphogenesis of MCF-12A acini in 3D culture. The described structural changes in acini, i.e. luminal filling; increased size; and irregular shape, have been previously associated with a dysregulation of apoptosis and proliferation processes (Debnath *et al*, 2002; Shaw *et al*, 2004). In addition, the data presented in **Chapter V**, shows that by blocking pathways known to mediate such processes (such as PI3K and ERK), some of the detrimental effects of the test compounds can be reverted. These observations provide indirect evidence that estrogens interact with cell growth and death to induce MCF-12A acini malformations. However, direct evidence of the impact of estrogens on these cellular functions in MCF-12A acini is still missing.

To date, the vast majority of evidence gathered concerning the intracellular targets of estrogens *in vitro* has been obtained from breast cancer cells, such as the MCF-7 cell line (Lucas *et al*, 2010). The same wealth of knowledge, however, is lacking for the non-tumourigenic MCF-12A cell line that has been utilised in this study. In **Chapter II**, we showed that estrogen treatment of MCF-12A cells had no significant impact upon cell number. This indicated that estrogens, in monolayer cultures of this cell line, neither increased proliferation nor suppressed apoptosis. However, in 3D cultures, estrogens clearly promoted MCF-12A cell proliferation and had an inhibitory effect upon apoptosis (See **Chapter IV**), as indicated by the increased acinar size and irregular shape of estrogen-treated acini with filled lumens.

These conflicting observations between monolayer and 3D cultures of breast cells have been described before (Horning *et al*, 2008; Pickl & Ries CH, 2009). Several reports have demonstrated that cells behave differently depending on whether they are cultured in monolayer or 3D, and that the latter has the closest resemblance to the arrangement of cells *in vivo* and, therefore, provides information that is more representative of what actually occurs in the human breast. The differences between the behaviour of cells cultured in monolayer and in 3D models are exemplified by observations that cells cultured in 3D are more resistant to chemotherapy than monolayer-cultured cells; and the gene expression profiles of these cells differ depending on culture methodology (Horning *et al*, 2008). Furthermore, the organisation of the membrane receptor, HER2 differs depending on culture conditions

and this has an impact the activation of various signalling pathways, most notably, an increase in MAPK signalling in 3D cultures (Pickl & Ries CH, 2009).

In order to compare the outcomes from monolayer and 3D cultures, and further understand the interaction of estrogens with the mechanisms involved in glandular architecture formation, we selected a number of well characterised apoptotic genes, as well as a few cytoplasmic kinases for both cellular death and survival, for the studies described in this chapter.

6.1 Apoptosis: The Apoptotic Factors

As discussed previously, apoptosis is the biological process by which a cell undergoes programmed cell death, and, along with proliferation, regulates tissue homeostasis. In the context of breast development, the formation of the hollow mammary ducts and TDLUs is dependent on the fine balance between proliferation and apoptosis. If apoptosis is dysregulated, such as during cancer, cells become resistant to apoptosis, which then results in the accumulation of cells. The dysregulation of apoptosis in cancer cells can occur as a result of inhibition of tumour suppressor genes, the activation of oncogenes, impaired function of the apoptotic executioners or over-activation of pro-survival signalling pathways. This apoptotic resistance, in addition to contributing to tumour formation, can also impact upon a patient's response to therapeutic strategies. The data shown in **chapters II, III** and **IV** clearly demonstrates that estrogens impact upon mammary gland formation in a 3D model. The estrogen-treated acini in these experiments exhibited decreased levels of activated caspase-3 and filled lumens. This is indicative of a reduction in apoptosis, a process that, in part, is regulated by the ratio of pro-apoptotic factors to anti-apoptotic factors.

The interaction between pro- and anti-apoptotic factors can determine whether a cell enters apoptosis or not and has implications for prognosis (**Figure 6.1**). This ratio between intracellular Bax and Bcl-2 has a significant impact upon a cell's ability to respond to apoptotic stimuli, such as the detection of irreparable genetic aberrations: cells with a high Bax/Bcl-2 ratio are more sensitive to an apoptotic stimulus than cells with a lower Bax/Bcl- ratio. For example, breast cancer patients with higher ratios of Bax/Bcl-2 respond better to chemotherapy. On the other hand, in patients where this ratio is lower, Bcl-2 is able to suppress therapy-induced apoptosis (Orlandi *et al*, 1999; Zhang *et al*, 1999). If the expression of only a single apoptotic gene is dysregulated, the effect upon apoptosis may be negligible. However, the cumulative dysregulation of multiple apoptotic factors could impact upon the apoptotic process.

Pro-apoptotic factors include Bax and Bad, and members of the anti-apoptotic factor family include Bcl-2 and Bcl-xl. The pro-apoptotic factors can be classified as Bax-like or BH3-only. Bax-like apoptotic factors, such as Bax, contain the Bcl-2 homology domains BH1, BH2 and BH3. Members of the BH3-only family contain just the BH3 domain and members include Bad, Bim and PUMA (reviewed in Adams & Cory, 2007). The anti-apoptotic factors Bcl-2 and Bcl-xl are members of the Bcl-2 subfamily of anti-apoptotic factors and contain BH1, BH2, BH3 and BH4. It is the presence of the BH3 domain that promotes the formation of homodimers and heterodimers of the apoptotic factors. Specifically, it is thought that the C-terminal membrane anchor domains located in the BH3 binding pocket permits the dimerization process via the two terminal charged residues (Suzuki *et al*, 2000; Jeong et al, 2004). The dimerization of the apoptotic factors can either promote apoptosis or have an inhibitory effect upon it, depending on the apoptotic factors involved. For example, homodimerization of Bax promotes apoptosis; whereas, dimerization of Bcl-2 or Bcl-xl with Bax, inhibits apoptosis. Both the levels and the activation status of these apoptotic factors are regulated by transcription, protein modification and degradation.

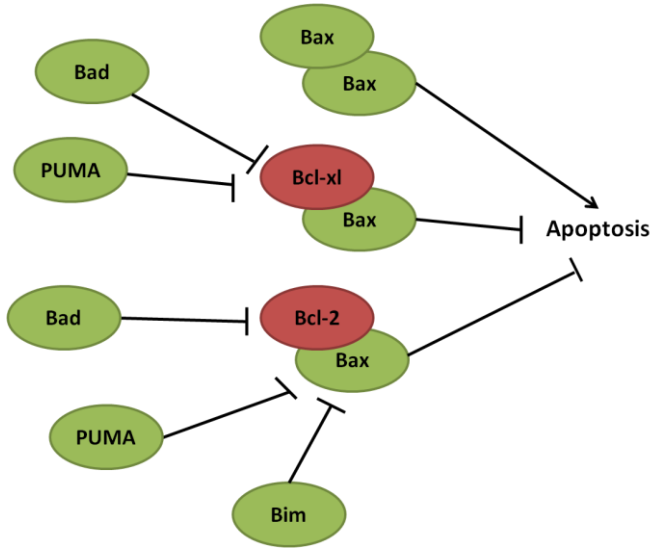

Figure 6.1: Apoptotic Factors. The interaction of pro- (green) and anti- (red) apoptotic factors and the impact upon apoptotic onset. Bax dimerization induces apoptosis by promoting cytochrome c release from mitochondria. Bcl-2 or Bcl-xl binding to Bax inhibits the pro-apoptotic actions of Bax. On the other hand, pro-apoptotic factors, such as Bad, PUMA and Bim, can bind to, and inhibit the actions of Bcl-2 and Bcl-xl.

6.1.1 Bcl-2

The function of the anti-apoptotic factor, Bcl-2, is to sequester Bax, and inhibit apoptosis. In normal breast tissue, Bcl-2 expression varies depending on the development stage of the mammary gland. For example, in the developing breast tissue of the foetus, Bcl-2 expression is observed in the basal cell layer of the budding mammary gland (Nathan *et al*, 1994). Furthermore, Bcl-2 expression shows cyclic variation through the menstrual cycle (Sabourin *et al*, 1994). These observations have led to the idea that Bcl-2 is necessary for the growth and morphogenesis of the breast.

Cell line studies have demonstrated that the basal expression of *BCL-2* is higher in tumourigenic cells, such as in the MCF-7 and MDA-MB-231 cell lines, in comparison to non-tumourigenic cell lines, such as MCF-10A and MCF-12A (Zapata *et al*, 1998; Amundson *et al*, 2000). Given that Bcl-2 is also commonly over-expressed in breast cancer, this may suggest that Bcl-2 is implicated in malignant progression (Nahta & Esteva, 2003). This idea is further supported by observations that in breast cancer, Bcl-2 is expressed in 80% of tumours and this is associated with decreased levels of apoptosis, resistance to chemotherapy , increased tumour size and ER expression (Orlandi *et al*, 1999; Zhang *et al*, 1999; Kymionis *et al*, 2001). In 3D cultures, caspase-mediated apoptosis is associated with the formation of the hollow lumen of acini. In a similar fashion to what occurs *in vivo* and in the human mammary gland, ectopic expression of Bcl-2, or indeed Bcl-xl, in 3D cultures of mammary epithelial cells results in the delayed formation of the hollow lumen (Debnath *et al*, 2002; Humphreys *et al*, 2006; Underwood *et al*, 2006). This seems to indicate that Bcl-2 plays an important role in lumen formation during acini morphogenesis, but, given that its overexpression *in vitro* delays lumen formation, rather than inhibit it, suggests that other factors are also involved.

In terms of its mode-of-action, Bcl-2 binds to Bax, localises to the outer mitochondrial membrane, nuclear membrane and endoplasmic reticulum, and functions to prevent Bax from forming homodimers and initiating mitochondria-mediated apoptosis (Krajewska *et al*, 1993; Jeong *et al*, 2004). The expression and activity of Bcl-2 can be regulated through transcription, microRNA (miRNA) binding and phosphorylation events (Cheema *et al*, 2003; Deng *et al*, 2004; Cimmino *et al*, 2005). The activation of signalling pathways by growth factors, such as vascular endothelial growth factor (VEGF), increases the expression of Bcl-2 at the protein level (Pidgeon *et al*, 2001) and *Bcl-2* transcription can be induced by the signal transducers and activation of transcription (STAT) transcription factor family (Lord *et al*, 2000). In the case of post-transcriptional gene regulation by miRNA, these single-stranded moieties bind to target sequences in mRNA, preventing mRNA translation and decreasing the corresponding protein level. Hence, the presence of miRNAs that target Bcl-2 mRNA results in less Bcl-2 protein, which,

consequently, results in the promotion of apoptosis. From the perspective of estrogen-mediated regulation of the Bcl-2 gene, miRNAs may also play a role. Indeed, it has been demonstrated that E2 induced a down-regulation in the expression of the miRNA, mi-R21, which targets *BCL-2* (Wickramasinghe *et al*, 2009). Also important, for the scope of this thesis, is the fact that it has been established that E2 is capable of up-regulating *BCL-2* expression in breast cancer cell lines (Wang & Phang, 1995; Lucas *et al*, 2010). This up-regulation of *BCL-2* expression potentially increases the ratio of pro- to anti-apoptotic factors and in particular the Bax/Bcl-2 ratio, favouring an anti-apoptotic effect. Indeed, Wang & Phang (1995) demonstrated that E2 increased *Bcl-2* expression in MCF-7 cells, thus decreasing the Bax/Bcl-2 ratio, and this in turn resulted in decreased cell death.

6.1.2 Bcl-xl

Bcl-xl, an anti-apoptotic factor, localises to the cytosol or outer mitochondrial membrane. If present in the form of homodimers, Bcl-xl resides in the cytosol, however, following the formation of heterodimers with Bad or Bax, it localises to the outer mitochondrial membrane (Jeong *et al*, 2004). Bcl-xl exerts its anti-apoptotic function through the formation of heterodimers with Bax and, thus, preventing the homodimerisation of this factor and the apoptotic process that follows it (Billen *et al*, 2008). It has been proposed that Bcl-xl binds to proteins involved in Bax activation, and this prevents the activation of Bax ([b]Kim *et al*, 2006).

The activities of Bcl-xl can be regulated at both the transcriptional and post-translational level. At the transcriptional level, *BCL-XL* transcription is activated by extracellular growth factors, such as EGF or VEGF. These growth factors activate the AKT and MAPK signalling pathways, which, in turn, induce the expression of c-Fos and c-Jun, transcription factors involved in the transcription of *BCL-XL* (Pidgeon *et al*, 2001; Takeuchi *et al*, 2006). Post-translational events, such as the phosphorylation of Bcl-xl results in its inactivation and permits Bax dimerization with other Bax proteins (Scatena *et al*, 1998).

The over-expression of Bcl-xl in cancer is associated with resistance to p53-mediated apoptosis, increased metastasis and resistance to chemotherapy (Schott *et al*, 1995; Amundson *et al*, 2000; Fernández *et al*, 2000). Although there is limited data available concerning the correlation between the ER and Bcl-xl expression, or the correlation between tumourigenic state and Bcl-xl, the basal expression of *BCL-XL* is up-regulated the MDA-MB-231 cell line, compared to non-tumourigenic cell lines (Amundson *et al*, 2000).

Based upon what is known about Bcl-2 and Bcl-xl, and their role in apoptosis, it is plausible that in our MCF-12A 3D model, the expression of these anti-apoptotic factors will be up-regulated in response to E2 treatment. This could explain some of the anti-apoptotic effects (luminal filling and reduced activated-caspase-3) induced by estrogen treatment described in **Chapter IV**. As mentioned above, it has already been documented that E2 is capable of up-regulating *BCL-XL* expression in MCF-7 cells (Gadd et al, 2002). There is, however, little reported data on the MCF-12A cell line. For these reasons, it is important to investigate whether E2 can impact on *BCL-2* and *BCL-XL* expression in the non-tumourigenic MCF-10A and MCF-12A cell lines, in a similar fashion to those described in cancer cell lines.

It is important to note that a splice variant of Bcl-xl exists, known as Bcl-xs. This apoptotic factor is pro-apoptotic and its presence would therefore favour the initiation of the apoptotic process. It is known to contain a transmembrane region in addition to BH3 and a BH4 domain (Boise *et al*, 1993). Bcl-xl on the otherhand contains the BH1, BH2, BH3 and BH4 domains. Bcl-xs is hypothesised to be implicated in cancer cell apoptosis, based upon the observation that loss of Bcl-xs expression correlates with decreased survival in patients with myelogenous leukemia (*Yamaguchi et al*, 2002). Bcl-xs localises to the mitochondria and is implicated in caspase-dependent and caspase-independent cytochrome c release and its activity is inhibited by the presence of Bcl-xl and Bcl-2, although its exact mechanism of action remains elusive (Chang *et al*, 1999; Lindenboim *et al*, 2001).

6.1.3 Bax

The pro-apoptotic factor, Bax is cytosolic and bound to anti-apoptotic factors, such as Bcl-xl. However, upon its activation, Bax localizes to the outer mitochondrial membrane where it signals to the mitochondria to release cytochrome c and activate the effectors (the caspases) of apoptosis (Wolter *et al*, 1997; Suzuki *et al*, 2000). Bax activation and its translocation to the mitochondrial membrane can occur as a result of phosphorylation via AKT or MAPK signalling pathways, however, depending on the site, phosphorylation can have the opposite effect and inactivate Bax (Gardai *et al*, 2004; Kim *et al*, 2006). In addition to phosphorylation events, the activity of Bax can be regulated at the level of transcription: wild type p53 (but not mutant p53) and c-Myc are able to initiate the transcription of the *Bax* gene (Miyashita & Reed, 1995; Mitchell *et al*, 2000).

It has been demonstrated that the protein levels of Bax are reduced in invasive tumours of the breast (Krajewski *et al*, 1995; Baccouche *et al*, 2003). Similar findings have also been identified in cell

lines: normal mammary cell lines have a higher basal expression of *BAX* in comparison to breast cancer cell lines (Bargou *et al*, 1995; Amundson *et al*, 2000). This lower level of *BAX* expression reduces the ratio of pro-apoptotic to anti-apoptotic factors and is associated with increased metastasis and resistance to chemotherapy (Krajewski *et al*, 1995). However, in cases of breast cancer where *BAX* expression is not decreased, patients tend to have a good prognosis and respond well to chemotherapy (Kapranos *et al*, 1997). Literature concerning the influence of E2 upon *BAX* expression has demonstrated that in MCF-7 cells, E2 does not impact upon *BAX* expression, but may instead disrupt the apoptotic factor ratio by increasing *BCL-2* expression (Wang & Phang, 1995). It remains to be seen if this is also the case in the MCF-12A cell line and whether if, in a 3D culture system estrogens have a similar impact upon the expression of apoptotic factors such as *BAX*.

6.1.4 Bad

Bad is a cytosolic, pro-apoptotic factor, which in its inactive state is hyperphosphorylated. However, in the presence of an apoptotic stimulus, Bad is dephosphorylated, migrates to the mitochondria and forms heterodimers with Bcl-2 and Bcl-xl. Transcriptionally, *BAD* expression is controlled by p53, which acts to initiate transcription. Bad, when in excess, then acts to negatively regulate the entry of p53 to the nucleus through the formation of Bad/p53 complexes and, thus, prevents further Bad transcription (Jiang *et al*, 2006). At the post-translational level, Bad is inactivated by phosphorylation at specific serine residues: Ser112, Ser136 and Ser 155, via the AKT pathway and this can be induced by EGF (Chattopadhyay *et al*, 2001; Ezzoukhry *et al*, 2011). This phosphorylation results in the formation of a complex comprised of Bad and 14-3-3 proteins in the cytosol and prevents Bad's interaction with Bcl-2 or Bcl-xl (Zha *et al*, 1996), which leaves these anti-apoptotic factors free to form heterodimers with Bax and prevent apoptosis.

In non-malignant breast tissue, the basal levels of *Bad* are higher than those present in other tissues and this implies that Bad may have a specialized function in the breast (Kitada *et al*, 1998). *BAD* expression is documented to be unaffected by E2 treatment in neuronal cells (Dubal *et al*, 1999), but there is little literature available concerning the effects of estrogens on *Bad* expression in breast cells or if GPER-1 has any potential role in the regulation of *BAD*. However, it has been shown that the expression of Bad in patients with breast carcinoma is reduced, compared to normal mammary tissue, suggesting that the suppression of Bad could be involved in the progression to a malignant phenotype in the breast (Yu *et al*, 2010).

6.2 Impact of Estrogens on the Expression Profile of Apoptotic Genes: Evaluating the Mechanisms Behind Estrogen Disruption During Acini Morphogenesis

It is clear that the literature concerning the impact of estrogens upon apoptotic gene expression and the role of GPER-1 in these transcriptional responses, is limited. This lack of data is particularly notable for the MCF-12A cell line. However, with an important role for apoptosis in the formation of lumen in acini (**Chapter IV**), the observed impact of estrogens on MCF-12A acini morphogenesis and the indication from other cell lines that gene expression of apoptotic genes can be modulated by estrogens, it is likely our test chemicals also influence expression of these genes in MCF-12A cells, and this is an issue that requires further investigation. In **Chapter IV**, the co-incubation of E2 with either ER or GPER-1 antagonists partially reverted some of the estrogen-induced MCF-12A acini malformations, in terms of luminal clearance, suggesting an anti-apoptotic role for estrogens, mediated by both the ER and GPER-1. In order to take this work further and develop an understanding of the mechanisms and genes involved in such effects, we decided to follow a similar approach to investigate whether the ER or GPER-1 were involved in the regulation of expression of apoptosis-related genes following E2 treatment. For that, MCF-12A cells were treated with both ER and GPER-1 agonists alone and in combination with specific receptor inhibitors, prior to gene expression analysis.

Ideally, the gene expression analysis experiments would have been conducted in 3D cultures of MCF-12A cells, as these are believed to be more physiologically relevant, as discussed previously. However, financial, time and technical constraints hindered this. It was decided, instead, to optimise primers and perform initial experiments in monolayer cultures of MCF-12A and other cell lines. Following these preliminary experiments, where primer pairs would be optimised and initial expression profiles obtained, experiments were to be repeated with 3D cultures of MCF-12A cells.

In the interest of investigating whether the tumourigenic status, or indeed the ER status, of a cell line had an impact on changes in gene expression in response to E2, the same four cell lines in **Chapter II** were treated, and real-time PCR was used to analyse gene expression. The data obtained for monolayer cultures of MCF-12A cells following the various treatments will be valuable for comparison with 3D cultures of estrogen-treated MCF-12A cells in future gene analysis experiments. Primarily, this will enable us to determine whether the genomic responses to estrogen in monolayer are an accurate representation of what occurs in a more physiologically-relevant 3D setting.

6.3 Non-Genomic Effects of Estrogens in MCF-12A cells: An Alternative Mechanisms for Acini Disruption

Estrogens do not just impact upon transcription, and, as discussed in previous chapters, the non-genomic responses to estrogen have been explored in detail (Improta-Brears *et al*, 1999; Sun *et al*, 2001). As described in **Chapter I** and **Chapter V**, estrogens can exert their effects via non-genomic mechanisms. This is exemplified by the activation of the PI3K and MAPK cascades and has been reported in various cell lines (Castoria *et al*, 1999; Improta-Brears *et al*, 1999; Sun *et al*, 2001). However, similarly to the expression profiles of apoptosis-related genes, there is little data available concerning the non-genomic actions of estrogens in the MCF-12A cell line. As observed in **Chapter V**, in 3D cultures of estrogen-treated MCF-12A cells co-incubated with PI3K or ERK1/2 inhibitors, the malformations induced by estrogens were partially reverted. Similar observations have been made in previous publications, where the PI3K and MAPK pathways appeared to be implicated in acini formation of mouse mammary tumour cells and in 3D cultures of MCF-10A cells (Debnath *et al*, 2002; Yanochko & Eckhart, 2006; Polo *et al*, 2010). Similarly, work by Schedin and colleagues (Schedin *et al*, 2004) has shown that 3D cultures of MCF-12A cells, which have been transformed through ectopic expression of Ras, present filled lumens, suggesting that over-activation of Ras signalling (a component of the MAPK pathway) impacts upon apoptosis and proliferation (**Figure 6.2**).

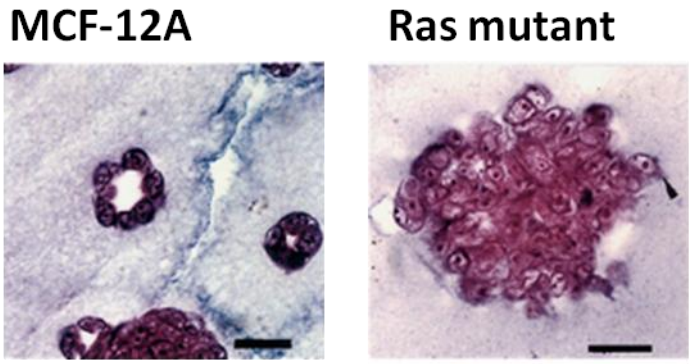

Figure 6.2: Ras mutations induce luminal filling in 3D cultures of MCF-12A cells. Wild-type MCF-12A cells form spherical hollow acini in 3D culture whereas Ras-transformed MCF-12A cells form solid masses. Size bars equal 25 µm (Image from Schedin *et al*, 2004).

The data presented in **Chapter V** adds to this knowledge and indicates that the MAPK and PI3K pathways seem to mediate the effects of estrogens in 3D cultures of MCF-12A cells. This could potentially mean that estrogens exert non-genomic effects via activation of signalling cascades in 3D

cultures of non-transformed breast epithelial cells, to induce a phenotype that resembles that of breast cancer. To date, there is no direct evidence that estrogens activate PI3K or MAPK signalling cascades in monolayer, or indeed 3D cultures, of MCF-12A cells. As discussed previously, responses to treatments can differ depending on whether cells are cultured in monolayer or in 3D. In order to investigate if estrogens activate MAPK and PI3K signalling in a 3D setting, it was first necessary to establish whether this occurred in MCF-12A monolayer cultures and optimise antibodies for future work. Unfortunately, due to time and technical constraints, it was not possible to progress and investigate the impact of E2 on MAPK and PI3K signalling by protein analysis in 3D cultures. Nevertheless, the monolayer culture of estrogen-treated MCF-12A cells has still provided us with some knowledge regarding the non-genomic responses to estrogens in this cell line.

As discussed previously, GPER-1 is capable of mediating some of the non-genomic effects of estrogens, and its activation can have implications for PI3K and MAPK signalling (**Figure 6.3**).

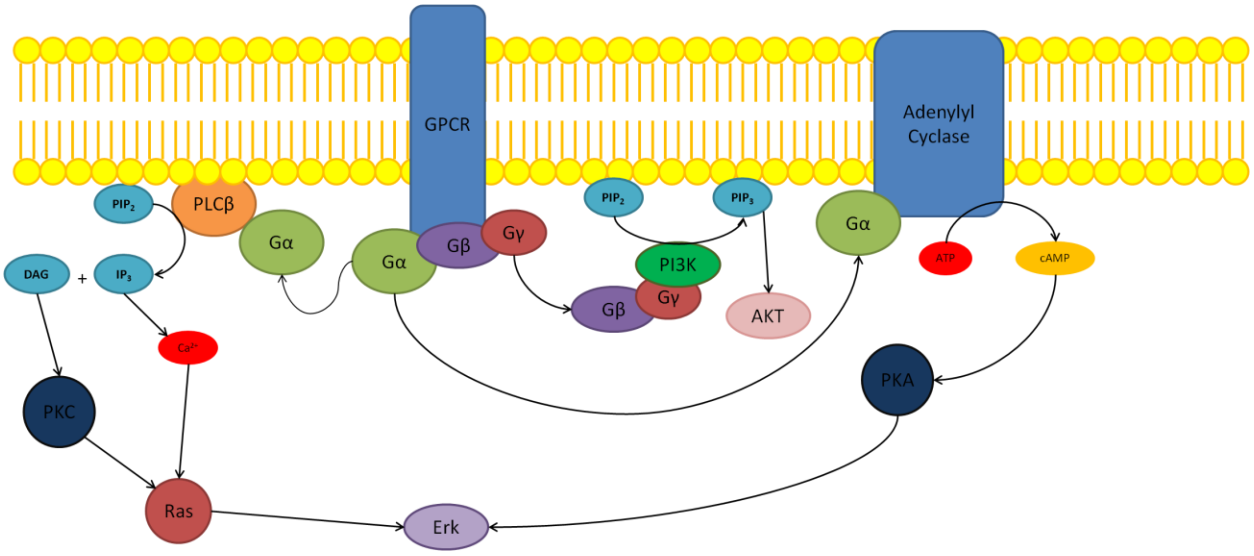

Figure 6.3: GPCR-mediated signalling. Activation of G proteins following ligand binding to the GPCR induces the MAPK and PI3K signalling pathways through the formation of secondary messengers.

As for its role in cancer, GPER-1 gene expression has found to be decreased in breast cancer samples and its expression correlates with ERα expression (Kuo *et al*, 2007). In breast cancer GPER-1 is reported to stimulate the proliferation, migration, metastatic potential and drug resistance cumulating in a poorer prognosis (Maggiolini *et al*, 2004; Filardo *et al*, 2006/2008; Pandey *et al*, 2009). Previous work has demonstrated that E2 and BPA, are able to induce ERK1 and ERK2 activation in various human

breast cancer cell lines via the activation of GPER-1, including the ER negative, but GPER-1 positive cell line, SKBR3 (Dong *et al*, 2011). This observation is contrary to the hypothesis that GPER-1 requires the ER to mediate the actions of E2.

The role of GPER-1 in the regulation of apoptotic genes remains poorly understood, as is the role of this receptor in mediating in the non-genomic actions of estrogens in the MCF-12A cell line. Gene analysis by real-time PCR following treatment with E2 in combination with G-15 was performed to investigate the impact of E2 upon the expression of key apoptotic factors. Further to this protein analysis was also performed to study the activation of key proteins involved in PI3K and ERK signalling.

6.4 Materials and Methods

6.4.1 Preparation of Cultures for Apoptotic Gene Expression Studies

The MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines were seeded into 6-well plates at a density of 25,000 cells per ml in full media and allowed to reach 70% confluency. The media was then replaced with phenol red-free assay media containing 0.5% CDS serum. After 24 h of growth factor and estrogen depletion, cells were treated with E2 (1 nM) with or without 30 min pre-incubation with 100 nM ICI 182,780 or 10 nM G-15, for 12 hours. In addition, cells were also treated with 100 nM G-1 with or without 30 min pre-incubation with 100 nM ICI 182,780 or 10 nM G-15 for 12 hours. RNA extraction and RT real-time PCR were performed, as described previously. Solvent controls consisted of 0.5% ethanol + 0.2% DMSO.

6.4.2 Primer Design

Primers for the apoptotic factors: *BCL-2*, *BCL-XL*, *BAX*, and *BAD* were designed based on their mRNA sequence (Genebank), using Beacon Designer 5^{TM}. To ensure good quality primers for real-time PCR, these were designed with a GC content higher than 50%, a T_m of 50-65^{o}C, no repeating sequences longer than 3 bases, and no complimentary pairing of primers (Bustin, 2002).

To optimise the designed primer pairs, a 10-fold cDNA dilution series (5 concentrations) was prepared and amplified in the presence of several concentrations of each specific primer pair. The concentrations of primers tested were (forward:reverse): 100:100, 150:150, 200:200, 250:250 and 300:300 nM. A standard curve was constructed plotting the threshold cycle against cDNA dilution for each primer set concentration (**Figure 6.4**).

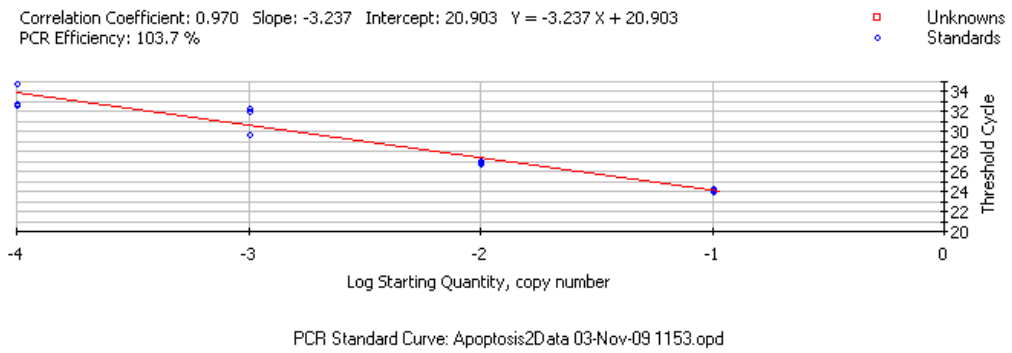

Figure 6.4: Example of a standard curve obtained during primer optimisation (BioRad, iCycler). Threshold cycle is plotted against cDNA dilution (-1 corresponds to neat cDNA, -2 to 1:10 etc). From the calculated standard curve, the correlation coefficient and PCR efficiency are automatically calculated.

The calculated standard curve allows the correlation coefficient and PCR efficiency to be determined and, hence, allows the appropriate primer concentration to be selected. In a perfect reaction, the total amount of target cDNA should double following each PCR cycle. To assess this, various characteristics of the standard curve provide information regarding to how efficient the amplification reaction was. The slope of a PCR standard curve is an indicator of PCR efficiency. A slope of -3.3 for a 10-fold cDNA dilution series correlates to sample detection 3.3 cycles apart and corresponds to an approximate PCR efficiency of almost 100%. This is an accepted parameter for optimised primers following real-time PCR of a 10-fold cDNA dilution and would indicate that each cycle of amplification resulted in the doubling of the amount of cDNA. In addition to this, correlation coefficients greater than 0.99 indicate an optimised reaction, again, where cDNA content doubled during each PCR cycle for each cDNA dilution.

For all reactions, a total volume of 20 µl reaction volume consisting of primer pairs at the concentrations listed in **Table 6.1**, 0.8 µl cDNA and 10 µl SYBR Green (Bio-Rad, Herts, UK) was added to each well of a 96-well PCR plate (Bio-Rad). Amplification was performed using the iCycler (Biorad) and a 2-step PCR protocol as described in **Chapter II**. Briefly, cDNA was denatured at 95^{o}C for 3 minutes and then cooled to 55^{o}C to allow primer binding. 40 subsequent cycles of heating to 95^{o}C for 1 minute and cooling to 55^{o}C for 1 minute were performed to allow denaturing of the newly produced strands, further annealing and cDNA amplification. Amplification curves were generated to calculate Ct values. Melt curves were also generated upon real-time PCR protocol completion to ensure that there was a single amplification product. Experiments were performed in duplicate three independent times. As before,

β-ACTIN was used as a reference gene, as it is unaffected by estrogen treatment in the cell lines of choice.

6.4.3 Real-Time Polymerase Chain Reaction (Real-Time PCR)

Table 6.1: Primers for real-time PCR. All primer pairs were ordered from Eurogentec.

cDNA	Genebank Ascension Number	Primer Sense	Primer Sequence (5'-3')	Concentration (nM)	Target Size (bp)
β-ACTIN	X00351	Forward	TGCTATCCAGGCTGTGCTAT	300	97
		Reverse	GATGGAGTTGAAGGTAGGTT	300	
BCL-2	EU287875	Forward	CCTGGTGGACAACATC	100	160
		Reverse	GAGCAGAGTCTTCAGAG	100	
BCL-XL	BT007208	Forward	TGAACAGGTAGTGAATGAAC	100	105
		Reverse	TCCTTGTCTACGCTTTCC	100	
BAX	BC014175	Forward	CCTCCTCTCCTACTTTGG	150	103
		Reverse	GCCTCAGCCCATCTTC	150	
BAD	BT006678	Forward	GATGAGTGACGAGTTTGTG	150	133
		Reverse	GCCCAAGTTCCGATCC	150	

6.4.4 Data and Statistical Analysis

As described previously, the relative expression of the target genes were calculated using the Pfaffl equation (**Chapter II**, **Equation 2.1**). Gene expression in treated and control samples was normalised against the reference gene, *β-ACTIN*. Results were expressed as fold increase over controls (ethanol + DMSO) with the values for controls being set to 1. In this case, an up-regulation in gene expression would show as a value greater than 1, and a down-regulation in gene expression would correspond to a decreased deviation from control conditions.

Quantification of gene expression and REST were performed on the data obtained from the real-time PCR. The Pfaffl equation, as described previously, standardises the expression of target genes to the reference gene. The mathematical model utilises PCR efficiencies for individual primer pairs and the mean Ct deviations between controls and sample groups. The average expression ratio ± standard error

of the mean (SEM) was calculated for each gene and tested for statistical significance by a Pair Wise Fixed Reallocation Randomisation Test (REST© Relative Expression Software Tool) (Pfaffl *et al*, 2002). With this, comparisons were made between both controls and treatments; and between treatments and treatments with inhibitors. *P-values* were also calculated using the REST© tool and a value of 0.05, or less, was considered statistically significant.

Finally, to ascertain whether changes in *BCL-2* and *BAX* expression would convey a pro- or anti-apoptotic influence, the relative gene regulation values were used to calculate the Bax/Bcl-2 ratio relative to *β-ACTIN*. This was calculated by dividing the relative gene regulation values (normalised to *β-ACTIN*) of *BAX* by that of *BCL-2*. A value of 1 was set for controls (solvent-treated samples). Anything above this value indicates an increase in the Bax/Bcl-2 ratio which would favour apoptosis. A value lower than 1 would indicate a decrease in the Bax/Bcl-2 ratio, and this would favour survival. Statistical analysis was performed using the unpaired *t*-test and a value of < 0.05 was considered statistically significant.

6.4.5 Activation of MAPK and PI3K Signalling Pathways

For the MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines, 1 million cells per ml were seeded in full media into 65 mm petri dishes and maintained at 37^{o}C in 5% CO_2. Upon 80% confluency, the media was removed and replaced with phenol red-free media containing 0.5% CDS serum for 24 h. Cells were treated with 20 ng/ml EGF, 1 nM E2 or 100 nM G-1 for 20 minutes with or without a 20 min pre-incubation with 10 nM G-15. Controls consisted of 0.5% etOH with 0.2% DMSO in phenol red-free media containing 0.5% CDS serum. In these initial experiments, the ER antagonist, ICI 182,780 was not included as our aim was to investigate whether GPER-1 was capable of mediating the non-genomic responses to E2, in respect to PI3K and MAPK activation.

Lysate preparation and western analysis were performed as described previously using the antibodies in **Table 6.2**. Following image acquisition, membranes were stripped of the phosphorylation-specific antibodies and re-probed with the corresponding total-protein antibody.

To strip membranes after probing with phosphorylated antibodies, membranes were incubated at 50^{o}C for 45 minutes with 4 ml 10% SDS, 2.5 ml 0.5 M Tris HCl pH 6.8, 13.5 ml water and 160 µl β-mercaptoethanol. The membranes were then washed three times for 10 minutes with TBS-Tween and blocked using 5% milk before probing with total AKT or total ERK antibodies (**Table 6.2**). Detection was performed by ECL as described in Chapter II.

Table 6.2: Primary antibodies used for western blot analysis of AKT and MAPK signalling.

Antibody	Source	Dilution
P-AKT (Cell Signalling)	Rabbit	1:1000 in 5% BSA
Total AKT (Cell Signalling)	Rabbit	1:1000 in 5% BSA
P-ERK 1/2 (Cell Signalling	Rabbit	1:3000 in 5% BSA
Total ERK 1/2 (Cell Signalling)	Rabbit	1:3000 in 5% BSA

6.5 Results

6.5.1 The Effects of E2 on Apoptotic Factor Expression

In the MCF-12A cell line, E2 treatment significantly up-regulated *BCL-2*, *BCL-XL* and *BAX* expression relative to controls. G-15 alone also induced an up-regulation in *BCL-XL* expression. Co-incubation of E2 with G-15 significantly reversed the E2-induced up-regulation of *BAX*, but failed to impact upon the effect of E2 on the remaining genes. Treatment with G-1 led to an up-regulation of *BCL-2* expression and this was strongly reverted upon co-incubation with G-15. Strangely, ICI 182,780 also inhibited the effect of G-1 upon *BCL-2* expression (**Figure 6.5**).

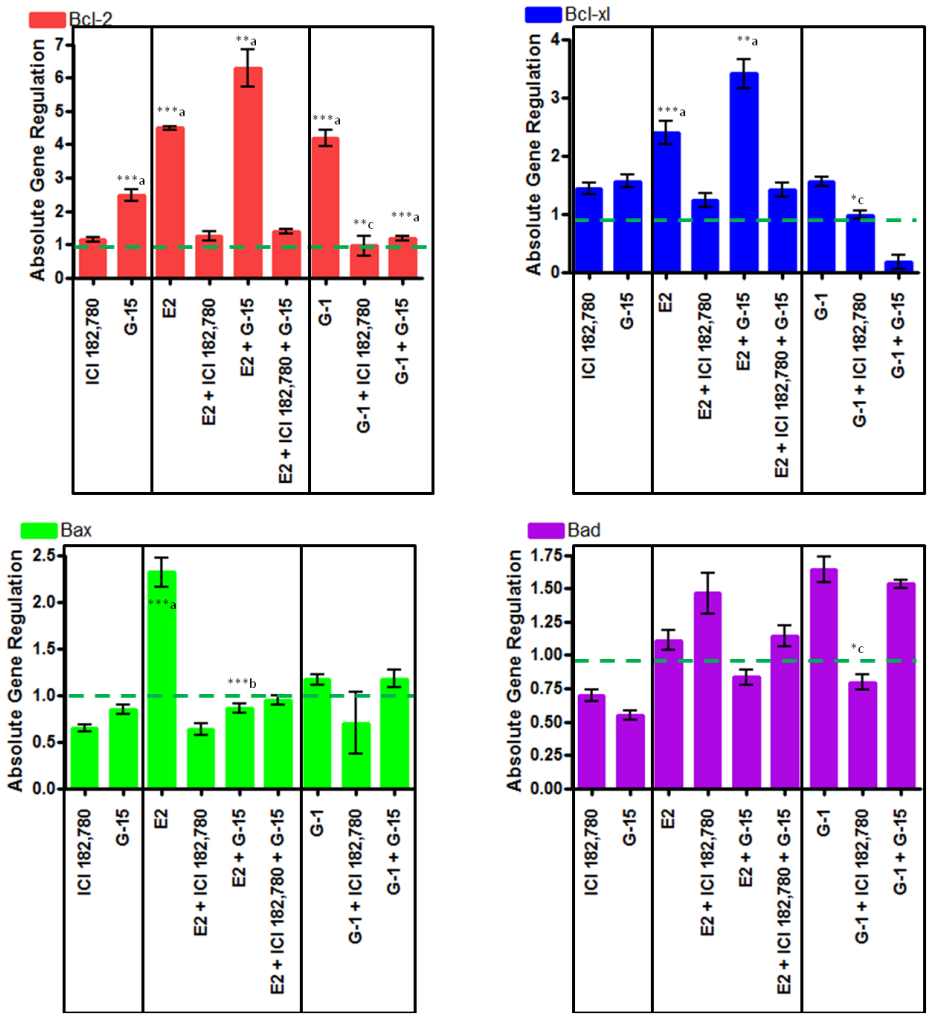

Figure 6.5: Fold changes in gene expression of apoptotic genes in MCF-12A cells. Negative controls (etOH/DMSO) have been set to 1 and are shown as a horizontal dashed line. A significant changes in expression in treatment relative to controls are referred to as (a). Significant changes in gene expression relative to E2 (b) and G-1 (c) are also indicated. *P*-values of 0.001 (***). 0.01 (**) and 0.05 (*) are indicated. Error bars represent +/- standard error or the mean. Data is representative of three experiments performed in duplicate.

In MCF-10A cells, E2 only significantly decreased the expression of *BAD*. Both combinations of E2 with ICI 182,780 and ICI 182,780 + G-15 led to a down-regulation of *BAD* expression relative to samples treated with the steroid alone. An up-regulation of *BCL-2* in response to G-15 and a down-regulation in *BAX* expression following ICI 182,780 treatment was also shown to be statistically significant.

The up-regulation of *BCL-2* expression in response to G-1 or G-1 with ICI 182,780 relative to etOH controls was deemed statistically significant. G-1 treatment also significantly up-regulated *BAX*

expression relative to controls and the down-regulation in *BAX* expression upon co-incubation with G-15 was deemed statistically significant relative to G-1 treated cells (**Figure 6.6**).

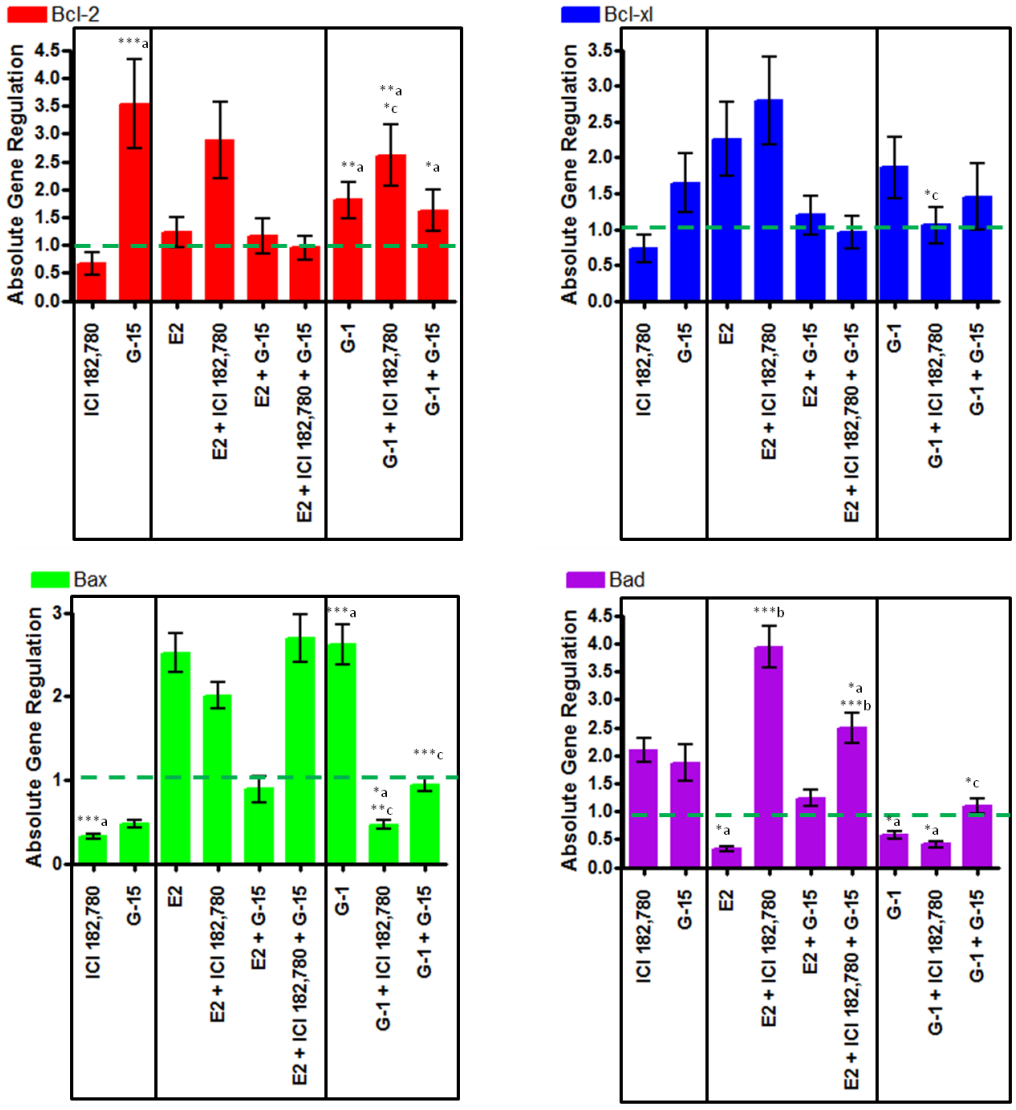

Figure 6.6: Fold changes in gene expression of apoptotic genes in MCF-10A cells. Negative controls (etOH/DMSO) have been set to 1 and are shown as a horizontal dashed line. A significant changes in expression in treatment relative to controls are referred to as (a). Significant changes in gene expression relative to E2 (b) and G-1 (c) are also indicated. *P*-values of 0.001 (***). 0.01 (**) and 0.05 (*) are indicated. Error bars represent +/- standard error or the mean. Data is representative of three experiments performed in duplicate.

In MCF-7 cells, E2 treatment significantly down-regulated the gene expression of *BAX* ($p < 0.001$) and *BAD* and up-regulated *BCL-2* expression. G-15 alone also appeared to down-regulate the expression of

BAX. Co-incubation of E2 with ICI 182,780 or with both ICI 182,780 and G-15 significantly reverted the E2-induced up-regulation of *BCL-2* expression. This was despite the E2-induced *BCL-2* up-regulation being considered statistically insignificant. Although the up-regulation of *BCL-XL* expression in response to E2 was also considered insignificant, co-incubation with G-15 significantly increased gene expression relative to controls and E2-treated samples. G-15 and ICI 182,780 together caused a significant decrease in gene expression relative to controls and E2-treated samples. Co-incubation of E2 with G-15 also increased *BAX* expression significantly, completely reversing the down-regulation induced by E2.

G-1 treatment induced a down-regulation in the expression of *BAX* and neither ICI 182,780 or G-15 had a significant impact upon this. Strangely, G-1 in combination with either ICI 182,780 or G-15 caused a significant up-regulation in *BAD* expression relative to controls (**Figure 6.7**).

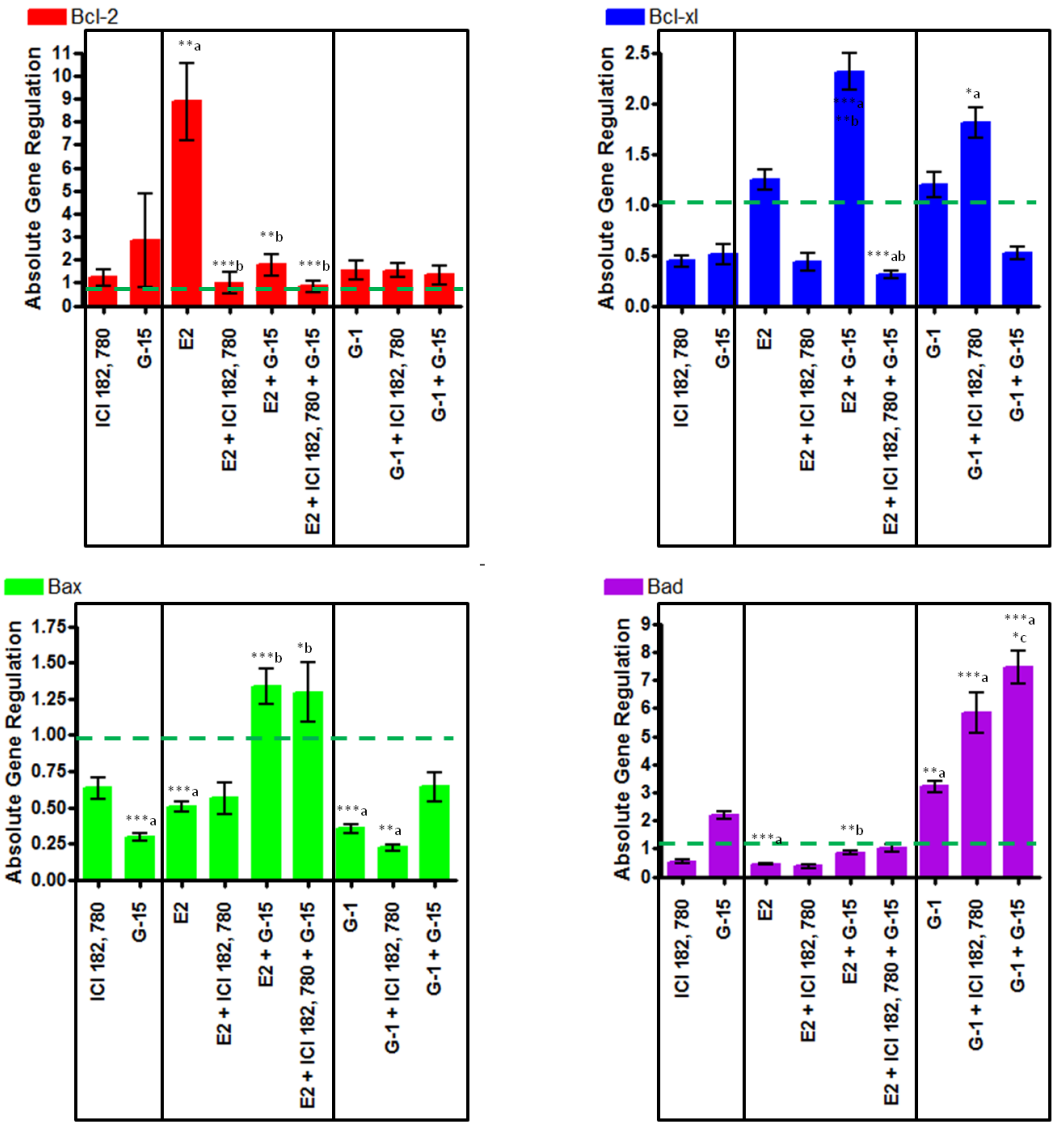

Figure 6.7: Fold changes in gene expression of apoptotic genes in MCF-7 cells. Negative controls (etOH/DMSO) have been set to 1 and are shown as a horizontal dashed line. A significant changes in expression in treatment relative to controls are referred to as (a). Significant changes in gene expression relative to E2 (b) and G-1 (c) are also indicated. *P*-values of 0.001 (***). 0.01 (**) and 0.05 (*) are indicated. Error bars represent +/- standard error or the mean. Data is representative of three experiments performed in duplicate.

In MDA-MB-231 cells, ICI 182,780 alone induced a significant down-regulation in *BAX* expression. The increase in expression of *BCL-2* induced by E2 was insignificant. The E2-induced down-regulation of *BCL-XL* expression was, likewise, insignificant. It remains to be seen, whether these seemingly insignificant changes in gene expression impact upon the Bax/Bcl-2 ratio, or indeed, if the cumulative effect of E2 on

apoptotic factor expression is more important than the impact of E2 on an individual gene's level of expression. E2 also caused an insignificant down-regulation in the expression of *BAX*. This was reversed upon co-incubation with G-15. Only *BCL-2* expression was significantly influenced by treatment with G-1. In this case, an up-regulation in gene expression was observed that was unaffected by co-incubation with either G-15 or ICI 182,780 (**Figure 6.8**).

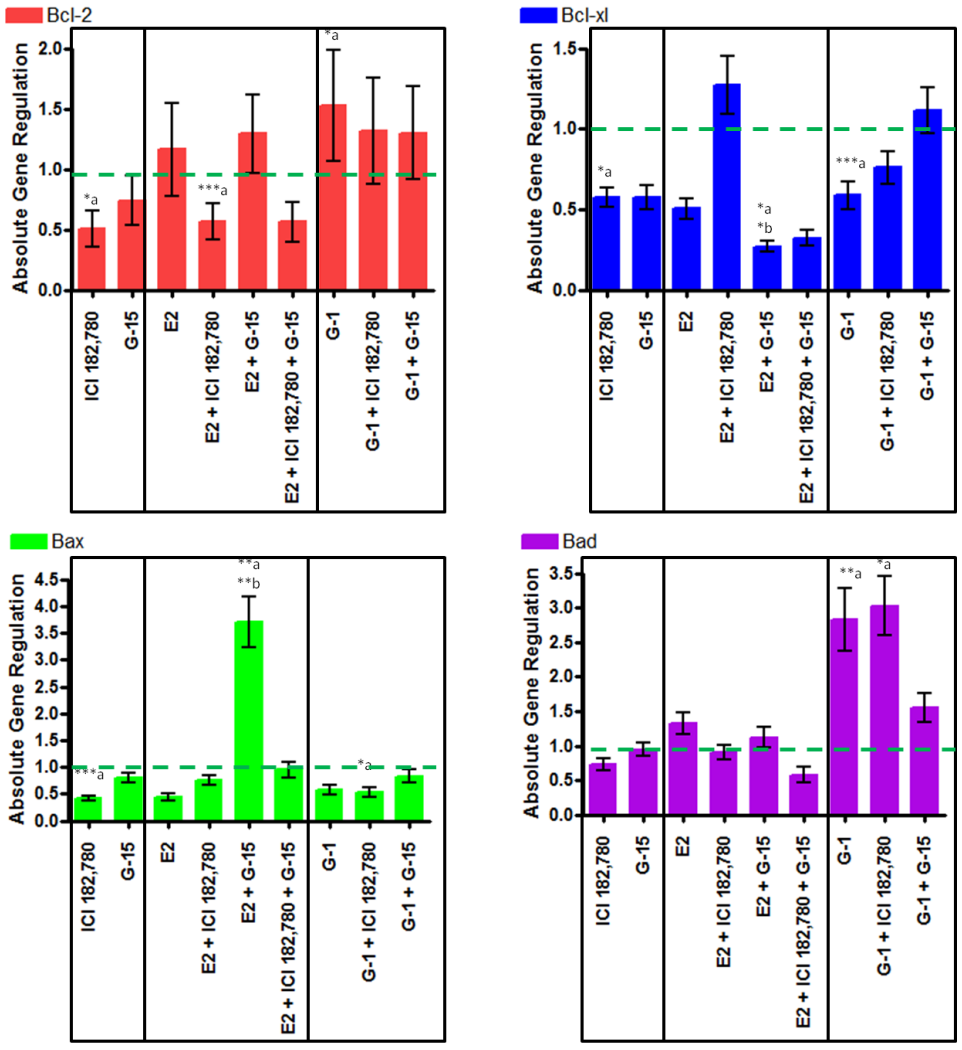

Figure 6.8: Fold changes in gene expression of apoptotic genes in MDA-MB-231 cells. Negative controls (etOH/DMSO) have been set to 1 and are shown as a horizontal dashed line. Significant changes in expression in treatment relative to controls are referred to as (a). A significant changes in gene expression relative to E2 (b) and G-1 (c) are also indicated. *P*-values of 0.001 (***). 0.01 (**) and 0.05 (*) are indicated. Error bars represent +/- standard error or the mean. Data is representative of three experiments performed in duplicate.

Table 6.3: Summary of gene expression in response to E2 and antagonists to the ER and GPER-1. ↑ indicates an up-regulation in gene expression and ↓indicates a down-regulation. a denotes relative to controls, b and c denote relative to E2 and G-1 treated cells, respectively

Treatment	*Bcl-2*				*Bcl-xl*				*Bax*				*Bad*			
	MCF-12A	MCF-10A	MCF-7	MDA-MB-231	MCF-12A	MCF-10A	MCF-7	MDA-MB-231	MCF-12A	MCF-10A	MCF-7	MDA-MB-231	MCF-12A	MCF-10A	MCF-7	MDA-MB-231
ICI 182,780				↓a						↓a		↓a				
G-15	↑a	↑a									↓a					
E2	↑a		↑a		↑a				↑a		↓a				↓a	↓a
E2 + ICI 182,780			↓b	↓a										↑b		
E2 + G-15	↑a		↓b		↑a		↑a ↑b	↓a ↓b			↑b	↑a ↑b		↑a ↓b	↑b	
E2 + ICI 182,780 + G-15			↓b				↓a ↓b				↑b			↓a		
G-1	↑a	↑a		↑a				↓a		↑a	↓a			↓a	↑a	↑a
G-1 + ICI 182,780	↓c	↑a ↑c			↓c	↓c	↑a			↓a ↓c	↓a	↓a	↓c	↑c	↑a	↑a
G-1 + G-15	↓a	↑a								↓c					↑a ↑c	

6.5.2 Bax/Bcl-2 Ratio

The Bax/Bcl-2 ratio was calculated by dividing the relative gene regulation values obtained from the gene analysis data of *BAX* by that of *BCL-2* for each treatment. A value of 1 was set for etOH controls to enable comparison of Bax/Bcl-2 ratios following the treatments.

In MCF-12A cells, treatment with E2 caused a decrease in the Bax/Bcl-2 ratio, which remained the same in the presence of ICI 182,780. On the other hand, E2 in combination with G-15, decreased the Bax/Bcl-2 ratio further than observed with E2 or E2 with ICI 182,780. Co-incubation of MCF-12A cells with both ICI 182,780 and G-15 seems to have slightly reverted the effects of E2 upon the Bax/Bcl-2 ratio (**Figure 6.9**). In all cases, this decrease in this Bax/Bcl-2 ratio in response to E2 would have an anti-apoptotic influence upon MCF-12A cells, which does not appear to be antagonised by either of the tested inhibitors.

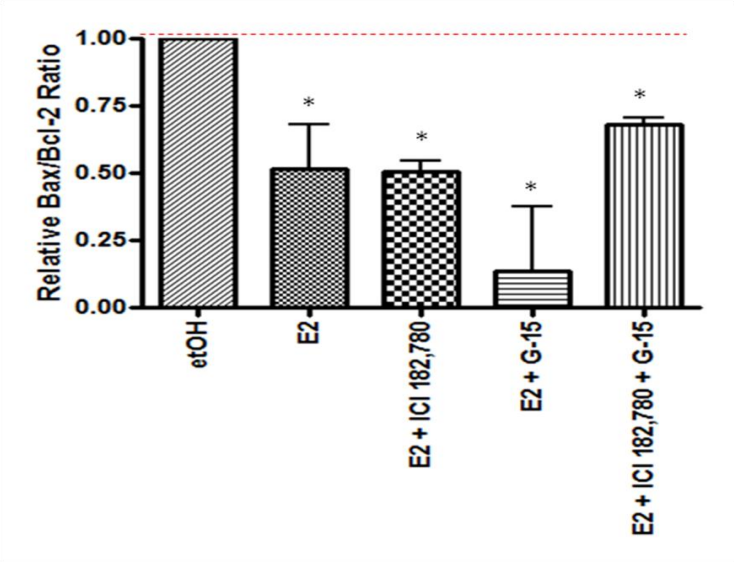

Figure 6.9: Relative Bax/Bcl-2 ratio for MCF-12A cells following incubation with E2 and ER or GPER-1 antagonists. A value of 1 was set as the Bax/Bcl-2 ratio for etOH controls and treatment ratios are relative to this. Data is normalised to *β-ACTIN*. Error bars represent standard error of the mean. D A *P-value* of < 0.05 was considered statistically significant and is denoted by *. Data is from three experiments performed in duplicate.

Conversely to what was observed in MCF-12A cells, in MCF-10A cells, E2 treatment increased the Bax/Bcl-2 ratio. Co-incubation of E2 with ICI 182,780 or G-15 decreased the effect of the hormone, reducing the Bax/Bcl-2 ratio below control levels. However, when E2 was combined with both ICI 182,780 and G-15 an increase in the Bax/Bcl-2 ratio was observed (**Figure 6.10**). The increase in Bax/Bcl-2 ratio in response to E2 or E2 in combination with both ICI 182,780 and G-15 potentially signifies a pro-apoptotic influence. However, the decreased Bax/Bcl-2 ratio seen with the other treatments would favour an anti-apoptotic effect.

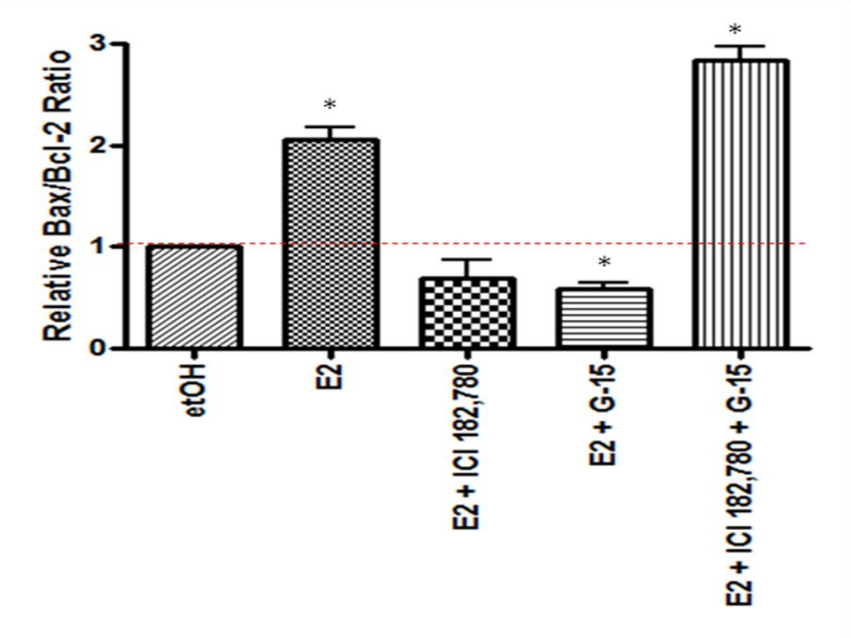

Figure 6.10: Relative Bax/Bcl-2 ratio for MCF-10A cells following incubation with E2 and ER or GPER-1 antagonists. A value of 1 was set as the Bax/Bcl-2 ratio for etOH controls and treatment ratios are relative to this. Data is normalised to *β-ACTIN*. Error bars represent standard error of the mean. A *P-value* of < 0.05 was considered statistically significant and is denoted by *. Data is from three experiments performed in duplicate.

E2 treatment of MCF-7 cells decreased the Bax/Bcl-2 ratio relative to etOH controls, which would suggest an anti-apoptotic influence of E2. Co-incubations of E2 with ICI 182,780 or G-15 strongly reversed this effect, even if not to control levels. When cells were treated with E2 in combination with ICI 182,780 and G-15 simultaneously, an increase in the Bax/Bcl-2 ratio was observed which may contribute to a pro-apoptotic influence, and revert the anti-apoptotic effect of E2 (**Figure 6.11**).

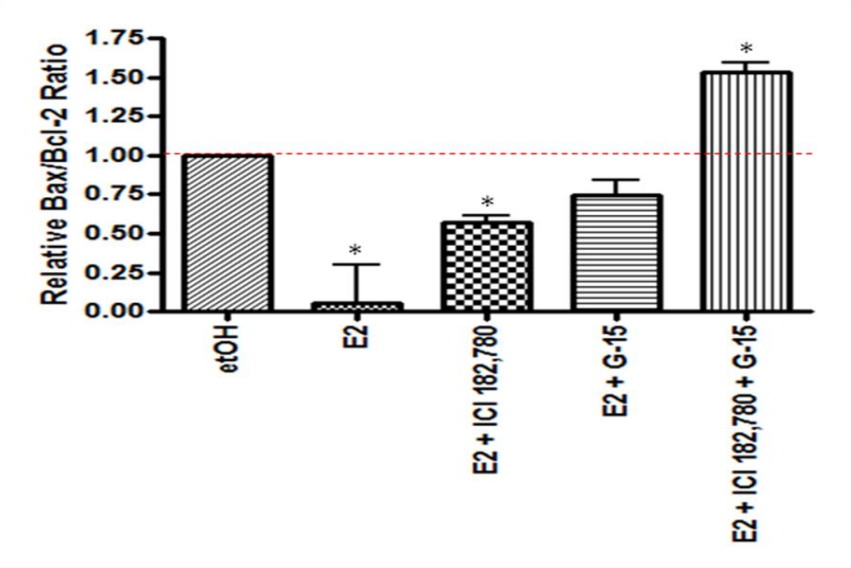

Figure 6.11: Relative Bax/Bcl-2 ratio for MCF-7 cells following incubation with E2 and ER or GPER-1 antagonists. A value of 1 was set as the Bax/Bcl-2 ratio for etOH controls and treatment ratios are relative to this. Data is normalised to *β-ACTIN*. Error bars represent standard error of the mean. A *P-value* of < 0.05 was considered statistically significant and is denoted by *. Data is from three experiments performed in duplicate.

In MDA-MB-231 cells, E2 treatment only slightly decreased the Bax/Bcl-2 ratio and probably therefore had little impact upon apoptosis. Interestingly, ICI 182,780 slightly reduced the effect of the hormone by inducing a small increase in the Bax/Bcl-2 ratio. E2 in combination with G-15 or ICI 182,780 and G-15, caused a more marked increase in the Bax/Bcl-2 ratio (**Figure 6.12**). This would suggest that antagonism of GPER-1 following E2 treatment has a pro-apoptotic influence relative to solvent controls.

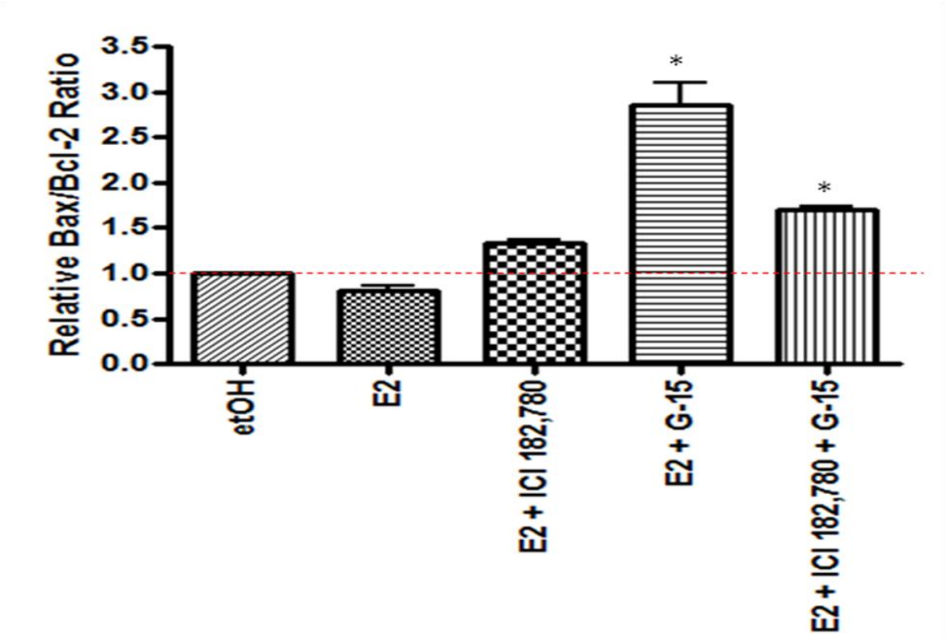

Figure 6.12: Relative Bax/Bcl-2 ratio for MDA-MB-231 cells following incubation with E2 and ER or GPER-1 antagonists. A value of 1 was set as the Bax/Bcl-2 ratio for etOH controls and treatment ratios are relative to this. Data is normalised to *β-ACTIN*. Error bars represent standard error of the mean. A *P-value* of < 0.05 was considered statistically significant and is denoted by *. Data is from three experiments performed in duplicate.

6.5.3 Signalling Pathway Activation

Activation of the PI3K and MAPK signalling pathways by estrogens is well documented (Tsai *et al*, 2001; Song *et al*, 2002; Lee *et al*, 2005). Following the identification of GPER-1, it has also been demonstrated that E2 can induce the activation of these pathways through this receptor (Filardo *et al*, 2002; Dong *et al*, 2011). To investigate the putative role of GPER-1 in mediating the non-genomic effects of E2 in the MCF-10A, MCF-12A, MCF-7 and MDA-MB-231 cell lines, cells were treated with E2 or G-1, either alone or in combination with the GPER-1 antagonist, G-15. The primary aim of this was to investigate the non-genomic responses to estrogens in the MCF-12A cell line, which to date, has not been characterized. The remaining cell lines served as a means of comparison of ER competent and ER negative, tumourigenic and non-tumourigenic cell lines.

In the MCF-12A cell line, a basal level of P-AKT is present in controls and G-15 treated samples. However, it is worth noting that the level of total-AKT is also higher than the other samples for the G-15 well. The growth factor, EGF (used here as a positive control for comparison with estrogen treatment), causes the phosphorylation of AKT and this is not inhibited by co-incubation of EGF with G-15.

Treatment with E2 or G-1 individually, also increased the amount of P-AKT detected relative to controls and this was not inhibited by co-incubation with G-15 in either E2 or G-1 treated samples (**Figure 6.13**).

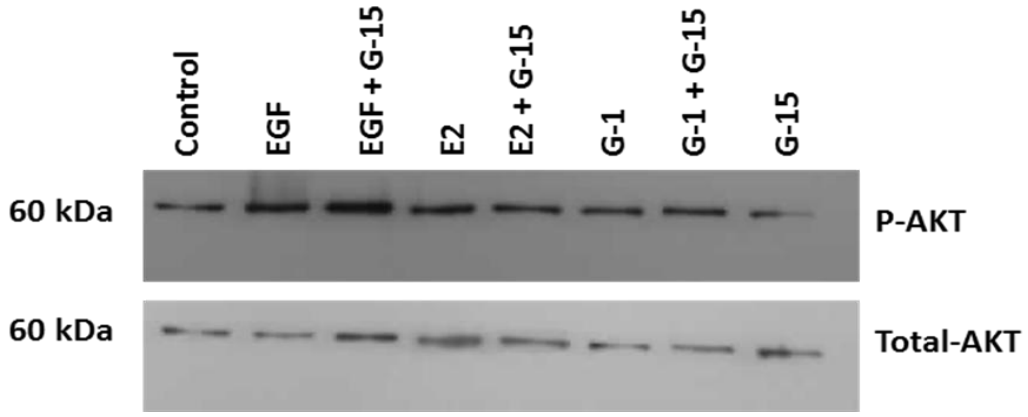

Figure 6.13: Activation of AKT by phosphorylation in MCF-12A cells in response to E2 and G-1. Immunoblot results for MCF-12A cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-AKT, stripped and probed again for antibodies against Total-AKT.

Control and G-15 treated MCF-12A cells have a basal level of ERK1/2 phosphorylation. Treatment with EGF, E2 or G-1 induced the phosphorylation of ERK1/2. The phosphorylation of ERK1/2 induced by EGF was not inhibited upon co-incubation with G-15. Co-incubation of E2 with G-15, however, did slightly decrease the level of P-ERK1/2 detected. Finally, co-incubation of G-15 with G-1 completely abrogated P-ERK1/2 (**Figure 6.14**).

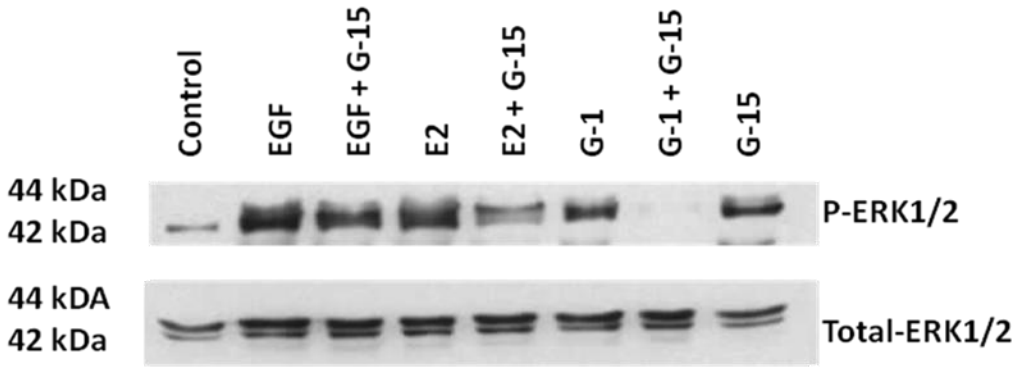

Figure 6.14: Activation of ERK1/2 by phosphorylation in MCF-12A cells in response to E2 and G-1. Immunoblot results for MCF-12A cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-ERK1/2, stripped and probed again for antibodies against Total-ERK1/2.

In the ER negative, GPER-1 competent MCF-10A cell line, EGF induced the activation of the PI3K pathway, as indicated by the increase of P-AKT levels, which results from AKT activation by PI3K. This was inhibited upon co-incubation with G-15. E2 treatment did not, however, induce measurable AKT activation. Treatment with G-1 induced AKT activation, although not to the extent observed with EGF. As with EGF treatment, co-incubation of G-1 with G-15 prevented the activation of AKT (**Figure 6.15**).

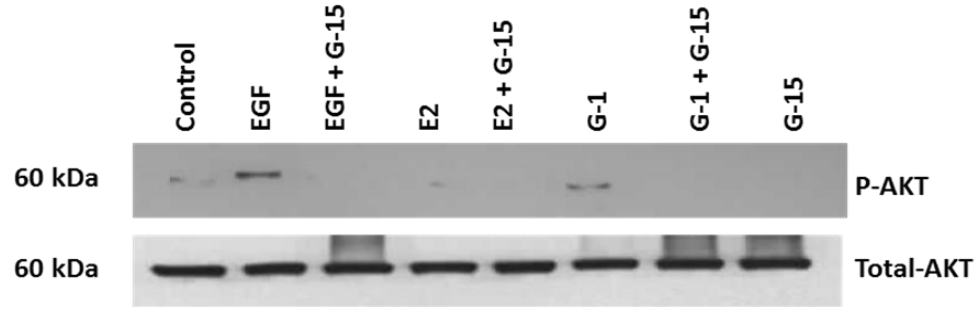

Figure 6.15: Activation of AKT by phosphorylation in MCF-10A cells in response to E2 and G-1. Immunoblot results for MCF-10A cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-AKT, stripped and probed again for antibodies against Total-AKT.

A basal level of MAPK pathway activation was observed in untreated MCF-10A controls (no EGF) as shown by the detection of P-ERK1/2. EGF treatment caused a pronounced activation of ERK1/2 and this was not abrogated upon co-incubation with G-15, although the effect was clearly reduced. E2 treatment resulted in a level of P-ERK1/2 that was similar to that observed in controls and upon co-incubation with G-15, the level of P-ERK1/2 was reduced even further. It is important to note, however, that the total level of ERK1/2 (T-ERK1/2) in G-15 treated samples is lower than seen in controls. As a decrease in both total and phosphorylated forms of ERK1/2 were observed, it is not possible to make conclusions in relation to the phosphorylation status of ERK1/2 in this sample. It is likely that the decrease in both forms of ERK1/2 was due to a lower protein loading onto the gel during western blot analysis.

Activation of GPER-1 using G-1 resulted in increased P-ERK1/2 relative to controls. Contrary to what was expected, this was not reversed upon co-incubation with G-15. G-15 alone resulted in a level of P-ERK1/2 similar to that observed in control cells (**Figure 6.16**).

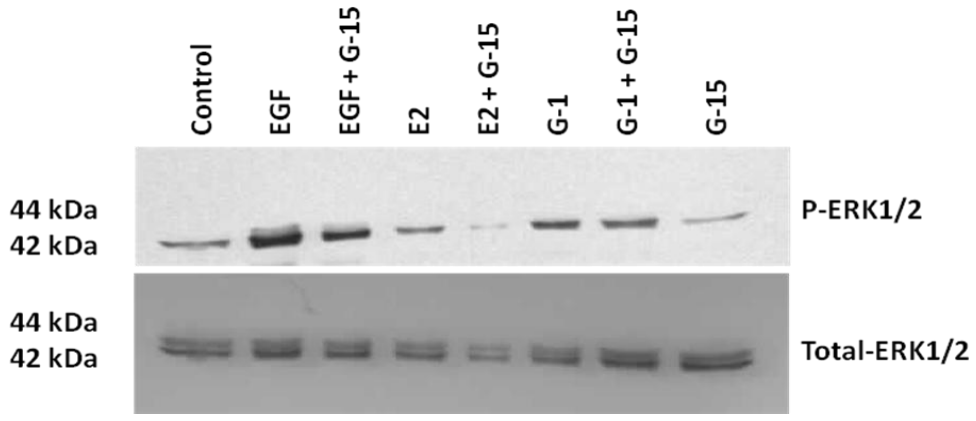

Figure 6.16: Activation of ERK1/2 by phosphorylation in MCF-10A cells in response to E2 and G-1. Immunoblot results for MCF-10A cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-ERK1/2, stripped and probed again for antibodies against Total-ERK1/2.

In the MCF-7 cell line, a basal level of P-AKT was present in untreated controls and G-15 treated cells. EGF and E2 treatment resulted in activation of AKT and in neither case did co-incubation with G-15 inhibit this effect. G-1 treatment, on the other hand, did not appear to activate the kinase. Strangely,

G-1 in combination with G-15 induced a slight increase in the intensity of the P-AKT band, but this could be attributable to a small increase in the total-AKT loaded protein (**Figure 6.17**).

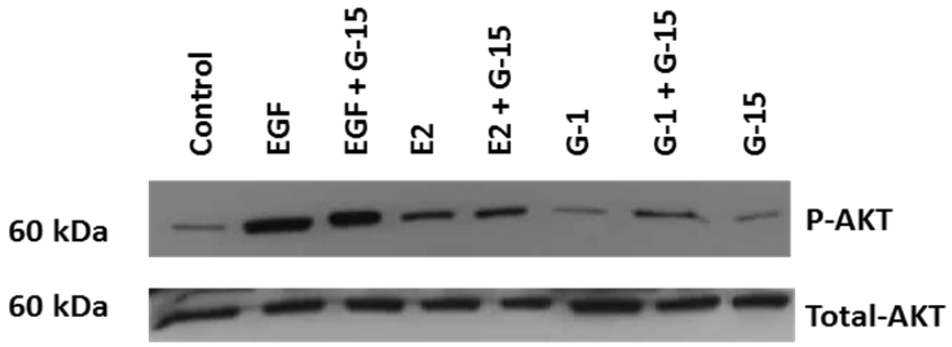

Figure 6.17: Activation of AKT by phosphorylation in MCF-7 cells in response to E2 and G-1. Immunoblot results for MCF-7 cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-AKT, stripped and probed again for antibodies against Total-AKT.

MCF-7 cells had a basal level of P-ERK1/2 in controls. G-15 treatment of these cells inhibited this background level of P-ERK1/2. As expected, EGF treatment induced significant activation of P-ERK1/2 and this was inhibited in the presence of G-15. In E2 treated cells, there was a small detection of P-ERK1/2, however, this was lower than in control samples. This very weak activation of the MAPK signalling pathway was, however, abrogated upon co-incubation of E2 with G-15. Finally, G-1 treatment induced the activation of P-ERK1/2 and this was completely inhibited by co-incubation of G-1 with G-15 (**Figure 6.18**).

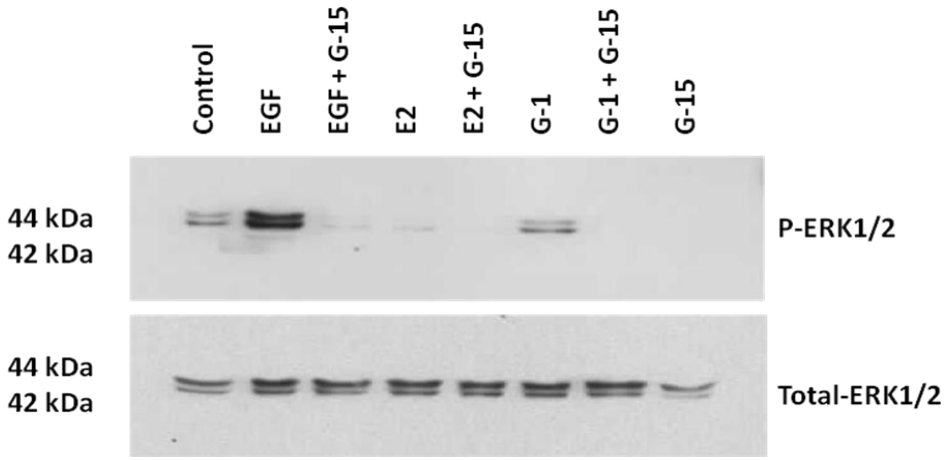

Figure 6.18: Activation of ERK1/2 by phosphorylation in MCF-7 cells in response to E2 and G-1. Immunoblot results for MCF-7 cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-ERK1/2, stripped and probed again for antibodies against Total-ERK1/2.

In the MDA-MB-231 cell line, P-AKT was only detected in cells treated with EGF, either alone or in combination with G-15. No activation of the PI3K pathway was observed in controls or following G-15, E2 or G-1 treatment, in any combination (**Figure 6.19**).

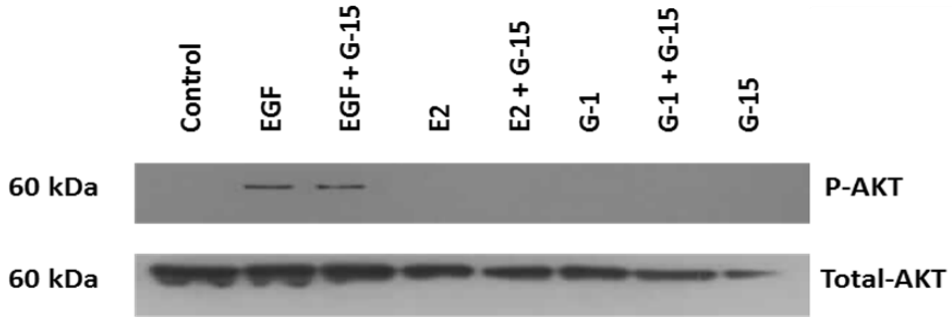

Figure 6.19: Activation of AKT by phosphorylation in MDA-MB-231 cells in response to E2 and G-1. Immunoblot results for MDA-MB-231 cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-AKT, stripped and probed again for antibodies against Total-AKT.

In the MDA-MB-231 cell line, both in control conditions and G-15 treatment P-ERK1/2 was still detected. EGF treatment caused the levels of ERK1/2 activation to increase compared to controls and this was not

inhibited upon co-incubation with G-15. The level of P-ERK1/2 detected in response to E2 treatment was similar to that observed in controls, suggesting that E2 does not cause activation of the MAPK signalling cascade in this cell line. Co-incubation of E2 with G-15, however, appeared to increase the level of P-ERK1/2 relative to controls. As with E2, G-1 treatment did not elevate the level of P-ERK1/2 beyond the levels observed in controls, but co-incubation of G-1 and G-15 did (**Figure 6.20**).

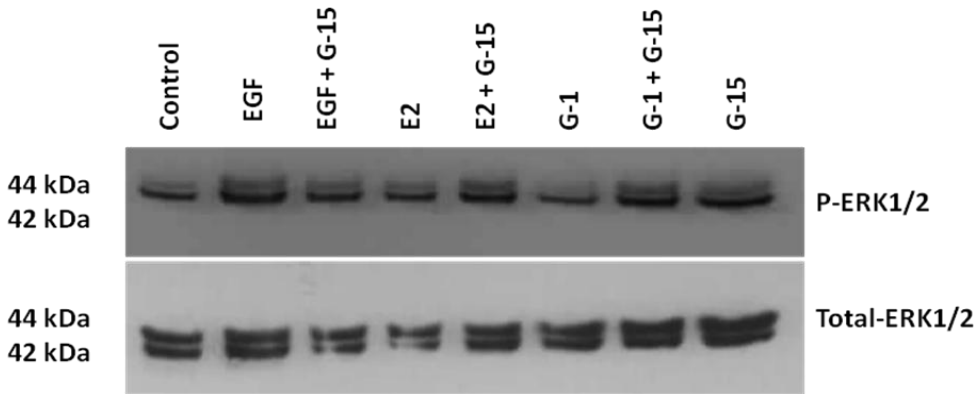

Figure 6.20: Activation of ERK1/2 by phosphorylation in MDA-MB-231 cells in response to E2 and G-1. Immunoblot results for MDA-MB-231 cells treated with E2 or G-1 with or without co-incubation with G-15. Blots were probed with antibodies against P-ERK1/2, stripped and probed again for antibodies against Total-ERK1/2.

6.6 Discussion

In **Chapters IV** and **V**, we showed that estrogens disrupt MCF-12A acini formation. As described previously, estrogens bind to estrogen receptors in order to regulate the transcription of estrogen-responsive genes. In the context of the breast, this regulation is required for the growth and differentiation of TDLUs during breast development (Bocchinfuso *et al*, 2000; Feng *et al*, 2007). We aimed to investigate the effects of E2 on the ERα, ERβ and GPER-1 competent MCF-12A cell line in terms of apoptotic factor expression and the activation of signalling cascades in monolayer. The data presented in this chapter not only provides novel information on the responses of this non-tumourigenic cell line to estrogen treatment, but will also allow us to compare the effects of estrogens in monolayer and 3D cultures of MCF-12A cells, and, therefore, assess whether outcomes observed in monolayer are predicative of those observed in 3D cultures. We achieved our aims to characterise the genomic and non-genomic responses to estrogens in MCF-12A cells. Furthermore, by testing an agonist and

antagonist of GPER-1, we have developed some understanding of this receptor, and its actions, in MCF-12A cells. We also repeated the experiments with three further cell lines for means of comparison with tumourigenic and ER negative cell lines.

6.6.1 Genomic Responses to the Antagonists, ICI 182,780 and G-15

Contrary to expectations, the ER antagonist, ICI 182,780, significantly down-regulated *BAX* expression in both ER negative cell lines: MCF-10A and MDA-MB-231. Further to this, ICI 182,780 also down-regulated *BCL-2* and *BCL-XL* expression in MDA-MB-231 cells. If these observations were due to the presence of residual estrogens in the CDS serum, the addition of ICI 182,780, would counteract the effects of any residual estrogens, resulting in the down-regulation of the aforementioned genes (Rea & Parker, 1996; Silva *et al*, 2010). The problem with this theory, is that these cells are ERα negative. Furthermore, if this were the case, the effects of ICI 182,780 would oppose those observed with E2 and this was not seen in MDA-MB-231 cells. It is therefore unlikely, that residual estrogens were accountable for the effects of ICI 182,780 alone.

Another possible explanation for these effects is linked to the presence of GPER-1 in these cell lines. It has previously been demonstrated that ER antagonists, such as ICI 182,780, can act as agonists for GPER-1, causing up-regulations in the expression of target genes such, as *BCL-2* (Filardo *et al*, 2000; Somaï *et al*, 2003; Thomas *et al*, 2005). This may account for why ICI 182,780 alone, in some instances, induced small changes in gene expression. It may also account for why, in some cases, G-1 + ICI 182,780 (both agonists for GPER-1), caused a greater change in gene regulation than G-1 alone. In addition to having agonistic activities towards GPER-1, ICI 182,780 can also activate signalling pathways via the ER splice variant, ERα36 (Wang *et al*, 2006). It is, therefore, plausible that some of the effects of ICI 182,780 resulted from non-genomic signalling following the activation of ERα36. It is, however, important to note here that the lack of AF-1 and AF-2 in ERα36 has been demonstrated to render ERα36 incapable of inducing transcriptional responses directly and, therefore, any changes in gene expression would be as a consequence of signalling pathway activation (Wang *et al*, 2006; Chaudhri *et al*, 2012). The reported presence of ERα36 has been shown in MCF-7 and MDA-MB-231 cells, but it is unknown whether MCF-10A or MCF-12A cells express this receptor (Zhang *et al*, 2012). Therefore, we cannot conclusively attribute the observations described above to ERα36 without further experiments.

In the case of the treatment with the GPER-1 antagonist, G-15, an up-regulation of *BCL-2* expression was observed in MCF-10A and MCF-12A cells, and a down-regulation of *BAX* expression was

seen in MCF-7 cells following treatment. This could mean that residual estrogens are still interacting with GPER-1 to induce changes in the transcription of apoptotic genes. This idea remains unlikely, as if this were the case, the effects of G-15 would be the opposite to those observed with E2, and in the instance of MCF-7 cells, both G-15 and E2 induced a down-regulation in *BAX* gene expression. Recently, it has been demonstrated that antagonists of GPER-1 are capable of inducing ER-dependent transcriptional responses (Dennis *et al*, 2011). This could account for the up-regulation of genes in response to G-15 treatment or following co-incubation of E2 with G-15.

6.6.2 Effects of E2 on Apoptotic Factor Expression in MCF-12A Cells and A Comparison with Other Cell Lines

The effects of estrogens on the expression profiles of apoptotic genes were studied in three other cell lines (MCF-7, MDA-MB-231 and MCF-10A), in addition to the cell line of interest: MCF-12A. These cell lines, which vary in terms of their ER and tumourigenic status, were used primarily for comparison with MCF-12A cells and to help draw conclusions, where possible, as to the role of the ER and GPER-1, and the impact of E2 on apoptotic events.

In the ERα, ERβ and GPER-1 competent MCF-12A cell line, E2 induced a significant up-regulation in the expression of *BCL-2*, *BCL-XL* and *BAX*. Although the up-regulation of *BCL-2* and *BCL-XL* in response to E2 would make sense, as E2 promotes cell survival, the up-regulation of *BAX* expression was unexpected. Despite this, E2 decreased the Bax/Bcl-2 ratio, which would, overall, promote survival, rather than cell death.

The up-regulation of *BCL-2* expression in MCF-12A cells following E2 treatment was not observed in ERα negative MCF-10A cells or MDA-MB-231 cells, but was seen in ER-competent MCF-7 cells, and has been reported previously in the same cell line (Wang & Phang, 1995).

In MCF-12A cells, ICI 182,780 reverted the E2-induced up-regulation of *BCL-2* and *BCL-XL* expression, even though this was not statistically significant. On the other hand, it appears that GPER-1 does not mediate the genomic responses of the *BCL-2* and *BCL-XL* genes, as G-15 failed to decrease the actions of E2. These observations suggest that, although weakly, the nuclear ER, and not GPER-1, is involved in regulating the expression of the apoptotic genes in this cell line. It is worth noting, however, that there was a degree of variability in the results obtained for the *BCL-2* data set. Therefore, a role for GPER-1 in mediating *BCL-2* transcription in response to E2 cannot be conclusively ruled out. Interestingly, in addition to ICI 182,780, G-15 also inhibited the E2-induced up-regulation of *BCL-2*

expression in MCF-7 cells, which was not observed in MCF-12A cells, and this could mean, that in some instances, GPER-1 is involved in the transcriptional response of *BCL-2* to estrogens.

To date, a correlation between GPER-1 expression and *BCL-2* expression has not been documented in breast cancer patients, whilst a positive correlation between Bcl-2 and ER expression has (Rehman *et al*, 2000; Sjöström *et al*, 2002). This, in combination with the data presented here, suggests that the ER is involved in the transcriptional regulation of the proto-oncogene, *BCL-2*. Given its overexpression in 40-80% of breast cancer samples, and an involvement of the ER in its expression, it may mean that one of the contributing factors of ER involvement in breast cancer progression, is increased *BCL-2* expression (Nahta & Esteva, 2003; Planas-Silva *et al*, 2007). It is worth noting that the *BCL-2* gene does not contain a perfect ERE within its promoter region, which raises the question as to how the ER may control *BCL-2* expression. There are two possibilities that may answer this: the first is the presence of imperfect ERE sequences in the coding region of *BCL-2*; the second is the presence of potential Sp-1 binding sites in the promoter region (Seto *et al*, 1988; Dong *et al*, 1999; Perillo *et al*, 2000). More recently, it has been shown that E2 mediates a decrease in the expression of mi-R21, a miRNA which targets *BCL-2* (Wickramasinghe *et al*, 2009). This down-regulation could, therefore, result in less *BCL-2* degradation and increased *BCL-2* transcript level, hence increased anti-apoptotic Bcl-2.

The E2 induced up-regulation of *BCL-XL* expression seen in the MCF-12A cell line was not observed in MCF-10A, MCF-7 or MDA-MB-231 cells. This raises the question to whether *BCL-XL* expression is affected by E2 in cell lines other than MCF-12A, although the gene does contain a putative ERE (Gollapudi & Oblinger, 1999; Koski *et al*, 2004). Indeed, immunohistological studies have demonstrated that unlike Bcl-2 expression, Bcl-xl expression does not correlate with ER expression in breast cancer and in MCF-12A cells ICI 182,780 did not inhibit the E2-indced up-regulation of *BCL-XL* expression (Sjöström *et al*, 2002). Unexpectedly, co-incubation of E2 with G-15 and ICI 182,780 together caused a significant down-regulation in *BCL-XL* expression in the tumourigenic MCF-7 and MDA-MB-231 cell lines. An up-regulation in *BCL-XL* expression was also observed in the MCF-7 cell line upon co-incubation of E2 with G-15. These observations may be due to off-target effects of G-15 or ICI 182,780 with ERα or GPER-1, respectively. In addition to these off-target interactions, it has also recently been demonstrated that ICI 182,780 can activate signalling pathways via ERα36 and activation of this pathway may have later consequences for gene transcription (Zhang *et al*, 2012). This ER isoform has been confirmed in MCF-7 and MDA-MB-231 cells and therefore the changes in *BCL-XL* expression may be due to interactions of ICI 182,780 with ERα36. This would, however, be counterintuitive as a decrease in Bcl-

xl would confer a pro-apoptotic influence. The exact reason for the down-regulation of *BCL-XL* in response to E2 and ICI 182,780 in MCF-7 and MDA-MB-231 cells, therefore, remains unknown.

The E2-induced up-regulation of *BAX* expression was significantly reduced upon co-incubation with G-15, relative to *BAX* expression in E2-treated MCF-12A cells. This seems to indicate GPER-1 is involved in mediating the transcriptional regulation of *BAX* in response to E2 treatment in this cell line. In addition to this, ICI 182,780 also negated the effect of E2 on *BAX* expression (although this effect was not significant), and therefore it appears that the transcriptional regulation of *BAX*, by estrogens, can be mediated by either of these receptors. Given that in patients with ductal adenocarcinoma of the breast, the level of *BAX* expression does not correlate with ER status, it may mean that GPER-1 is involved in the transcriptional regulation of *BAX* (Yang *et al*, 1999; Rehman *et al*, 2000; Sjöström *et al*, 2002).

The GPER-1 and ER-mediated up-regulation of *BAX* expression that was observed in response to E2 in MCF-12A cells was not observed in MCF-10A cells or MDA-MB-231 cells. A change in expression was observed in MCF-7 cells, but this was a down-regulation in gene expression, which has previously been documented (Teixeira *et al*, 1995; Lucas *et al*, 2010). It is worth noting at this point that E2, in MCF-7 cells, has also been shown to have no impact upon *BAX* expression or even elevate it (Wang & Phang, 1995; Gadd *et al*, 2002; Lewis *et al*, 2005). Given that only MCF-12A and MCF-7 cells express ERα, the data would suggest that, in the case of *BAX*, ERα is necessary for the transcriptional response to estrogens. However, given that ICI 182,780 was unable to inhibit the effect in MCF-7 cells, but G-15 was, GPER-1 also plays a role. This supports some previous findings where the ER was required for GPER-1 mediated responses to estrogens in ER negative SKBR-3 cells (Pedram *et al*, 2006). As to how estrogens may illicit their effects through the ER, no ERE has been identified within the *BAX* gene, thus far. The *BAX* gene does, however, contain Sp-1 sites and therefore E2 may regulate *BAX* expression through interactions with Sp-1 (Thornborrow & Manfredi, 2001).

E2 did not impact upon *BAD* gene expression in MCF-12A cells. However, in the MCF-10A and MCF-7 cell lines, E2 down-regulated *BAD* expression. A down regulation in *BAD* following E2 treatment has previously been documented in neuronal cells, which, supports these findings (Gollapudi & Oblinger, 1999). In the case of MCF-10A cells, this down-regulation was reversed by both ICI 182,780 and G-15. This was not anticipated, as MCF-10A cells lack the ER and, hence, ICI 182,780 should not have been able to reverse this effect. A possible explanation, other than experimental error, is the off-target effects of ICI 182,780 (Filardo *et al*, 2002). As G-1 did not induce an up-regulation in *BAD* expression, it is unlikely that the effects of ICI 182,780 were mediated by GPER-1. It, therefore, remains unclear as to why and

how ICI 182,780 was able to reverse the effect of E2. Regardless, it would appear that GPER-1 is implicated in the E2-induced *BAD* transcriptional response. This idea is supported by the data for the MCF-7 cells, where only G-15, and not ICI 182,780 was able to abrogate some of the effects of E2 upon *BAD* expression. To date, no ERE, Ap-1 or Sp-1 sites have been identified within the *BAD* gene suggesting that the genomic responses observed are not mediated by the ER and this supports the data presented above concerning a role for GPER-1 in regulating *BAD* expression in response to E2. Indeed, it has previously been reported that the level of *BAD* expression does not correlate with ER expression (Yu *et al*, 2010). Estrogens can induce CREB phosphorylation in an ERα dependent fashion via activation of the MAPK pathway (Szego *et al*, 2006). The transcription factor, CREB can then regulate the transcription of genes such as *Bad* (Finegan *et al*, 2009) This could mean that estrogens are capable of inducing transcriptional responses in cells following the induction of non-genomic signalling pathways such, as the MAPK or PI3K pathways.

Perhaps more importantly than the gene analysis results for the effect of E2 upon the expression of individual apoptotic genes, is the impact of E2 upon the Bax/Bcl-2 ratio. We found that E2 treatment in the ERα positive MCF-12A and MCF-7 cells decreased the Bax/Bcl-2 ratio. A similar, but much smaller, decrease in the Bax/Bcl-2 ratio was also observed in ERα negative, but ERβ competent MDA-MB-231 cells. This may mean that in ER competent cell lines, E2 has an inhibitory effect upon apoptosis. In **Chapter II**, however, we observed no significant change in cell number following E2 treatment in these cell lines which may mean that other mechanisms are in place in monolayer cultures of mammary epithelial cells that circumvent the anti-apoptotic effect of E2.

The E2-induced decrease in the Bax/Bcl-2 ratio was not greatly reversed by co-incubation with ICI 182,780 or G-15, either alone or combination, in the MCF-12A cell line. This was unexpected, as in this cell line, ICI 182,780 abrogated the E2-induced up-regulation of *BCL-2*. However, ICI 182,780 also inhibited the E2-induced up-regulation of *BAX*. This observation could therefore be due to ICI 182,780 reversing both effects of E2, resulting in a lack of change in the overall Bax/Bcl-2 ratio. In MCF-10A cells, E2 increased the Bax/Bcl-2 ratio, which is probably due to the E2-induced increase in *BAX* expression, and this was reversed by co-incubation with ICI 182,780 or G-15, suggesting that both of the receptors are responsible for this overall pro-apoptotic effect. In the case of ICI 182,780, this reversal is probably due to the up-regulation of *BCL-2* in response to E2 + ICI 182,780. However, in the case of co-incubation with G-15, the effect may be attributable to the down-regulation of *BAX* expression in response to E2 + G-15. A more marked reversion of the E2-induced reduced Bax/Bcl-2 ratio by ICI 182,780 or G-15 was

observed in the MCF-7 cell line, and this was more pronounced when the antagonists were used in combination. This, like the relative expression data, suggests that both the ER and GPER-1 are implicated in apoptotic responses to estrogens, in respect to gene expression. In MDA-MB-231 cells, the Bax/Bcl-2 ratio was slightly reduced by E2 treatment, and the presence of G-15, reversed this, implying that in MDA-MB-231 cells GPER-1 may be implicated in regulating the anti-apoptotic response to E2, in terms of gene expression. Taken together, the data suggests that both the ER and GPER-1 may be involved in the anti-apoptotic effect that estrogens can exert on cells, in respect to gene expression.

6.6.3 The Non-Genomic Effects of E2

The protein analysis data showed that, in control conditions lacking EGF, activation of the MAPK and AKT pathways still occurred, as inferred by the detection of P-ERK1/2 and P-AKT. This could be due to the presence of residual growth factors or estrogens in the assay media or CDS serum that were able to induce MAPK signalling (Rea & Parker, 1996; Silva *et al*, 2010). It has been demonstrated that in the MCF-7 cell line, the PI3K pathway is constitutively activated (Sivko & DeWille, 2004). However, in the MCF-10A and MCF-12A cell lines, this does not occur (Vasudevan *et al*, 2010). The constitutive activation of components of the MAPK pathway have also been reported in MCF-10A, MCF-7 and MDA-MB-231 cells (Krueger *et al*, 2001; Yue *et al*, 2002; Normanno *et al*, 2006). In spite of the observed residual activation of the PI3K and MAPK signalling cascades, these effects were very small, and probably are not significant.

In MCF-12A cells, E2 caused the phosphorylation, and, hence, activation of AKT and ERK1/2. This suggests that estrogens are capable of activating PI3K and MAPK signalling in this cell line. GPER-1 does not appear to play a role in mediating the induction of the PI3K pathway following E2 treatment, suggesting that other estrogen-responsive receptors may mediate this non-genomic effect of estrogens. Conversely, GPER-1 does appear to, at least partially, mediate the activation of the MAPK pathway in this cell line.

We described above how the presence of E2 resulted in the phosphorylation of both AKT and ERK1/2, which is indicative of the activation of the PI3K and MAPK signalling cascades, and these effects were mediated partially by GPER-1. As the effects were only partially inhibited by GPER-1 antagonism, another receptor is probably involved, potentially the ER, or a truncated form of it. In MCF-7 cells, E2 treatment resulted in the activation of AKT, but not ERK1/2, although previous work has shown that estrogens induce both PI3K and MAPK signalling (Improta-Brears *et al*, 1999; Pedram *et al*, 2006). The

data also indicates that this was mediated by another factor, rather than GPER-1, although other groups have demonstrated that GPER-1 mediates the activation of MAPK signalling in response to E2 (Filardo *et al*, 2000). However, these pathways did not appear to be activated by E2 treatment in ER negative MCF-10A or MDA-MB-231 cells. This may indicate that the presence of the ER is required for E2 to activate the PI3K and MAPK signalling pathways. Indeed, it has been demonstrated that the presence of the ER is a requisite for E2-induced activation of signalling pathways, via GPER-1 in SKBR-3 cells (Pedram *et al*, 2006). Contradictory evidence, however, has also been documented in the SKBR3 breast cancer cell line where estrogens activated MAPK signalling via the GPER-1 independently of ER expression (Filardo *et al*, 2000).

G-15 did not always abrogate the effects of E2 and this indicates other estrogen-responsive receptors may be involved in mediating the non-genomic effects of estrogens. These non-genomic effects may be mediated by the classical ER, although there are also membrane-associated ER's that have been hypothesised to mediate these effects, such as ERα36 and ERα46 (Flouriot *et al*, 2000; Wang *et al*, 2006).

6.6.4 A Function for GPER-1

To ascertain the effect of GPER-1 activation upon apoptotic gene expression, cells were treated with the GPER-1 agonist, G-1. We observed that G-1 up-regulated the expression of *BCL-2* in MCF-10A, MCF-12A cells and MDA-MB-231 cells significantly and induced a small increase in expression in MCF-7 cells. G-15 was not always able, however, of inhibiting this effect, suggesting off-target effects of G-1 or G-15 (Kang *et al*, 2010; Dennis *et al*, 2011). A down-regulation in *BCL-XL* expression following G-1 treatment was observed in MDA-MB-231 cells, and this was inhibited by G-15, suggesting that GPER-1 may mediate the transcription of *BCL-XL*. G-1 also affected *BAX* expression, although the observed effect was not the same in each cell line: in MCF-10A cells, G-1 up-regulated *BAX*, whereas, in MCF-12A and MCF-7 cells, *BAX* was down-regulated. Given that in MCF-7 cells the E2-induced down-regulation of *BAX* expression was inhibited by G-15 and that treatment with G-1 produced similar effects to that seen with E2, it is plausible that GPER-1 may be mediating the effects of E2 upon *BAX*. Similar observations have also been made in sertoli cells and this supports the findings presented above (Lucas *et al*, 2010). In MCF-10A cells, he G-1 induced up-regulation of *BAX* expression and subsequent down-regulation following co-incubation with G-15 suggests that GPER-1 is capable of regulating the transcription of *BAX,* but, E2 does not utilise this pathway in this ER negative cell line. Strangely, in MCF-10A cells, G-1 in

combination with ICI 82,780 also caused a down-regulation in *BAX* expression relative to controls and E2 treated samples. As ICI 182,780 alone also induced a down-regulation of *BAX* expression in this cell line, this observation may be attributable to the presence of ICI 182,780. In a similar fashion to *BAX*, *BAD* expression was also affected by G-1 differently in the four cell lines (down-regulated in MCF-10A cells and up-regulated in MCF-7 and MDA-MB-231 cells). The reason for these observations remains unclear as it does not appear that a cells tumourigenic state or ER status corresponds to genomic response to G-1. In MCF-10A cells, co-incubation of G-1 with G-15 inhibited the effect of G-1 confirming the effect seen was due to GPER-1. ICI 182,780 in combination with G-1 however further decreased *Bad* expression which may be due to the agonistic properties of ICI 182,780 towards GPER-1 (Filardo *et al*, 2000). In the case of MDA-MB-231 cells, E2 treatment did not induce the same effect as with G-1. This suggests that although GPER-1 activation can impact upon *BAD* expression, E2, although a ligand for GPER-1, cannot induce this effect. Furthermore, in the MCF-7 cell line, the effect induced by G-1 was the opposite of that induced by E2 which raises the question as to whether the effects induced by E2 were mediated by GPER-1. It has been demonstrated by some that activation of GPER-1 can have opposing effects to ERα (Oprea *et al*, 2010). Although GPER-1 activation did have an impact apoptotic factor expression, it is important to note that the response of an individual gene may not alone have an impact on a cell's apoptotic potential. It is more likely that the cumulative effect of changes in the expression multiple genes has a greater impact upon whether a cell enters apoptosis, or not.

In respect to the non-genomic response to GPER-1 activation, G-1 resulted in the increased presence of P-AKT in MCF-10A cells and P-ERK in MCF-10A and MCF-7 cells. As with the real-time PCR data, G-15 was not always capable of inhibiting the effects of G-1 upon the phosphorylation status of AKT or ERK1/2. Nevertheless, AKT activation appeared to be mediated by GPER-1 to some extent at least, suggesting that the activation of GPER-1 can result in MAPK or PI3K signalling. These results, when compared to the activation status of AKT and ERK1/2 following E2 treatment, did not always correlate. This suggests that although E2 is capable of activating signalling pathways via GPER-1, it does not always do so, and therefore other factors may be implicated.

Although the reason why G-15 did not always inhibit the effects of G-1, it possible to suggest that GPER-1 activation can induce transcriptional responses and activation of the PI3K and MAPK signalling pathways. Hence, the activation this receptor may have an impact on apoptosis through the transcriptional regulation of key apoptotic genes, and proliferation through the activation of the PI3K pathway. Furthermore, the presence of GPER-1, regardless of ER expression, did not always result in

AKT or ERK1/2 activation following treatment with G-1. This further implies that the response of GPER-1 to stimulation involves factors other than whether cells express GPER-1, or indeed the ER.

6.6.5 Possible Off-Target Effects

We observed that the data for G-1 and E2 + G-15 did not always agree, or that in some instances ICI 182,780 was able to inhibit the effects of G-1. A plausible explanation could be off-target interactions of ICI 182,780, G-1 or G-15. Indeed, it has been demonstrated recently that G-1 can bind to and activate ERα36 (Filardo *et al*, 2002; Kang *et al*, 2010). The ERα36 status of the MCF-12A cell line is not currently reported in the literature, and therefore, G-1 interactions with this receptor cannot be ruled out and this could present a reason for the gene analysis discrepancies. At high enough concentrations, G-15 can cause activation of ERα, although the concentrations used in this work were not high enough to produce this effect (Dennis *et al*, 2011).

As detailed above, G-15 did not always abrogate the effects of G-1. Recently G-15 has been shown to possess a low affinity for ERα. Hence a second GPER-1 antagonist with improved selectivity has recently been synthesised. This new GPER-1 antagonist has been called G-36 and displays a lower degree of ERα binding and activation (Dennis *et al*, 2011). As this data was not available at the time when experiments were being conducted, it would be of significance to repeat the G-15 co-incubations with G-36 and investigate whether the effects elicited by G-1 or indeed E2 can be abrogated with this new GPER-1 antagonist. Even more recently, the compound, ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1Hindol-3-yl] but-2-enoate (MIBE), that has the ability to bind to and inhibit both ERα and GPER-1 in breast cancer cells has been identified (Lappano *et al*, 2012). This dual antagonist could provide a promising treatment for breast cancer patients as it would ablate the effects of E2 mediated by both the ER and GPER-1 simultaneously. MIBE has another advantage in relation to other ER antagonists as it does not display agonistic properties towards GPER-1, which both tamoxifen and ICI 182,780 do (Filardo *et al*, 2000; Filardo *et al*, 2002). MIBE's antagonistic properties for GPER-1, are also greater than those of G-15, making it the better choice for antagonism of this receptor in particular (Lappano *et al*, 2012). Given the dual-agonistic properties towards both ERα and GPER-1, it would be of future interest to investigate whether MIBE was capable of completely reverting the effect of E2 and G-1 upon gene expression in the cell lines studied.

We also observed that ICI 182,780, when in combination with E2, was able to reverse the effects of E2 in ER negative cell lines. It is worth considering that ICI 182,780, although an ER antagonists, has

agonistic properties towards other receptors. Indeed, it has been demonstrated that ICI 182,780 acts as an agonist for GPER-1 (Filardo *et al*, 2000; Filardo *et al*, 2002; Thomas *et al*, 2005).

6.6.6 Conclusions

In this chapter, we have shown for the first time, that in ER, GPER-1 competent non-tumourigenic MCF-12A cells, E2's intracellular effects promote proliferation, through activation of signalling pathways and suppression of apoptosis, by lowering of the Bax/Bcl-2 ratio. However, as shown in **Chapter II**, E2 treatment had very little impact upon cell number, and if apoptosis were being inhibited, even in the lack of increased proliferation, an increase in cell number would be expected. Therefore, other mechanisms may be in place in this cell line that control the overall cell number. One possibility is that other factors, not included in this study, such as MDM2, Bim or PUMA, were regulated by estrogens, and these counteracted the decreased Bax/Bcl-2 ratio or activation of MAPK and PI3K signalling. Also, it is worth noting that an increase in the expression of a gene does not always correspond to an increase in protein or activity levels, due to post-translational modifications of various apoptotic factors. For instance, Bcl-2, Bcl-xl and Bad can all be phosphorylated, and this impacts upon their ability to form dimers, and hence their activity: phosphorylation of Bcl-2, Bcl-xl or Bad has an inhibitory effect on their activity (Munger & Roizman, 2001; Delivora-Papadopoulos & Mishra, 2010). The phosphorylation of Bad, in particular, can be achieved through activated AKT (Lewis-Wambi & Jordan, 2009). Given that activated AKT (P-AKT) was detected, in response to E2, in MCF-12A and MCF-7 cells, this may further impact on the overall apoptotic status of these cells. Furthermore, ubiquitylation of Bax targets it for degradation and hence lowers the protein level of this pro-apoptotic factor (Amsel et al, 2008). In the future, it would be of interest to extend the panel of apoptotic genes studied and to perform protein analysis in addition to gene analysis.

The next step in our work is to perform gene and pathway activation analysis in 3D cultures of MCF-12A cells treated with E2. As discussed in the next chapter, we assessed the impact of E2 upon apoptotic gene expression in these 3D cultures. Unfortunately, due to time and technical limitations, the signalling cascades following E2 treatment of MCF-12A cells grown in 3D could not be investigated. Obviously, in the future, this would be of interest to study.

Chapter VII: Separating the Two Cell Populations in Acini

In **Chapter III**, **IV** and **V**, we showed that in 3D *in vitro* cultures of MCF-12A cells, the seeded cells organise themselves into acini, comprising of two populations: the inner and outer cells. We observed that it was the inner cells that underwent apoptosis, as inferred by the expression of activated caspase-3, and were then cleared. The outer cells, on the other hand, were in contact with the basement membrane and did not undergo apoptosis. Other differences between these populations have been described in the literature. For example, in 3D cultures of MCF-10A cells, the outer cells are polarised, whereas inner cells do not display this level of polarisation (Debnath *et al*, 2002). Furthermore, activated AKT is mainly expressed by the outer cells and only during the later stages of MCF-10A acini morphogenesis (Debnath *et al*, 2002[b]), when the inner cells are undergoing clearance. 3D cultures of cell lines derived from other tissues have also shown that the inner and outer cells of 3D structures have different characteristics. For example, in 3D cultures of colonic HCT116 cells, following 4 days incubation, the proliferative marker, ki-67, is only detected in the outer cell population (Tsunoda *et al*, 2010).

Given that the two populations within acini display distinct characteristics, it may be the case that these cells respond differently to treatments or stimuli. However, currently, it is not fully understood what factors determine which cells are to survive and which are to be cleared by apoptosis. It is unclear whether the central cells of acini receive, and respond to, death signals; or whether the outer cells receive, and respond, to proliferative stimuli. Given that it is the cells lacking contact with the basement membrane that undergo cell death, it is plausible that these cells die through the lack of signalling pathway activation, which is achieved through basement membrane interaction. Indeed, evidence for this specialised form of apoptosis, termed anoikis, has been documented. For example, in 3D cultures of MCF-10A cells, the pro-apoptotic factor Bim, is induced in cells following detachment from the basement membrane (Reginato *et al*, 2003; 2005). Furthermore, when ECM-derived signalling is disrupted in CID-9 mammary epithelial cells, increased indicators of cell death are observed in cells in contact with the basement membrane (Boudreau *et al*, 1995). This observation suggests that apoptotic suppression is one way in which cells in contact with the basement membrane escape clearance. Regardless of this, the precise reasons for the final fate of the two acini cell populations remains unclear.

It is important to better understand the processes involved in acini formation, i.e. apoptosis and proliferation, and how these processes differ between the outer and central cells of acini. This would be

likely to provide valuable information on how the disruption of these normally highly regulated processes can result in cancers.

In vivo, homeostasis of mammary tissue is achieved through a controlled balance between proliferation and apoptosis. Whilst proliferation is necessary for the growth of the breast, the apoptotic process is equally important for the normal development of the mammary gland. This is because the process of apoptosis leads to hollow duct formation and involution, the process by which the lobules of the breast reduce in size, once they are no longer functionally necessary. The importance of apoptotic and proliferative control is exemplified by luminal filling, which appears to occur when both processes are dysregulated, and is often observed *in vivo* and in breast cancer patients (Harris *et al*, 1995; Humphreys *et al*, 1996).

One way in which these processes can be studied is by assessing the expression profile of genes involved in apoptosis and proliferation, such as *BCL-2*, *BAX* and *CCND1* (the gene encoding cyclin D1). The development of the mammary gland *in vivo*, involves extensive changes in gene and protein expression levels of various apoptotic factors. The expression of *BAX* and *BCL-XL* appears to remain relatively constant during mammary gland development, but both are down-regulated during lactation and then up-regulated during the onset of mammary gland involution. *BAD* expression, however, increases significantly during the late pregnancy and lactation phases of breast maturation, until involution has been achieved (Heermeier *et al*, 1996; Schorr *et al*, 1999). The anti-apoptotic factor, Bcl-2, also undergoes dynamic changes in expression during the morphogenic process. In non-pregnant females and during the early stages of pregnancy, *BCL-2* is expressed, but during lactation, or in the early stages of mammary gland involution, its levels become undetectable. *CCND1* expression, on the other hand, is associated with cell cycle progression and thus, proliferation. Cyclin D1 is involved in the regulation of cell cycle progression during the G1 phase. The overexpression of *CCND1* is a commonality in malignancies of the breast and is amplified in approximately 20% of mammary tumours (Zhou *et al*, 2009; Lari & Kuerer, 2011). The correlation between ER and *CCND1* expression in breast cancer patients remains unclear, with some studies reporting a positive correlation (Oh *et al*,2001) and other studies finding no association between the two (Lebeau *et al*, 2003; Millar *et al*, 2007).

By conducting gene analysis of key apoptotic genes and *CCND1* in the inner and outer cells of MCF-12A acini, we may shed some more light onto the differences between the two cell populations. These genes have already been demonstrated to be involved in the formation of the hollow lumen during acini morphogenesis, as demonstrated by delayed lumen formation following ectopic expression

of Bcl-2, Bcl-xl or cyclin D1 (Debnath *et al*, 2002; 2003). As it is more likely that a combination of factors contribute to the dysregulation of acini formation, rather than a single factor, it was decided to see if estrogens affected the gene expression of these factors. Additionally, Bax, a key factor in the apoptotic process, and Bad, an inhibitor of Bcl-2 and Bcl-xl function, were also investigated. This investigation can also provide some information on whether these populations respond differently to external signals, in this instance, the hormone E2. Given that it is the inner cells that undergo apoptosis and are cleared, one would expect that this population of cells expressed higher levels of *BAX* and *BAD*, and lower levels of *BCL-2*, *BCL-XL* and *CCND1*, relative to the outer cells. This would favour apoptosis. However, following E2 treatment, we would expect the inner cells of E2-treated acini to express higher levels of *BCL-2*, *BCL-XL* and *CCND1*, and lower levels of *BAX* and *BAD,* in comparison to the inner cells of control acini. This would, instead, favour survival of the inner cells of acini, and would correspond to the increased luminal filling observed in 3D cultures of MCF-12A cells described as in previous chapters.

The overall increased proliferation, as inferred by increased acinar size and irregular shape, in response to estrogens, did not appear to be restricted to the inner cells exclusively, indicating that the outer cells are also affected. In this instance, it would be expected that *CCND1* expression, an indicator of proliferation, would increase in both populations following treatment with estrogen. By defining the impact of estrogenic action on target genes, we could further clarify the processes involved in hormone-mediated acinar malformations, and potentially speculate how each population impacts on hormonal carcinogenesis.

7.1 Studying Gene Expression Profiles in Cells from 3D Cultures

The study of gene expression profiles in 3D cultures has been met with difficulty in the past: firstly, the low number of cells present in cultures, although this can be counteracted through increasing the seeding density and pooling of wells, has proven to be a limitation. Secondly, cells grown in a 3D matrix are embedded within the Matrigel that must be removed prior to analysis, which can now be achieved using cell recovery solutions, but was not always a straightforward task. More recent solutions permit the depolymerisation of Matrigel without enzyme action and high temperatures, processes that can cause biochemical changes, and, thus, maintain the integrity of RNA.

Studying gene expression in monolayer may obviate some of these problems, but, monolayer cultures themselves present their own problems, when used to study gene expression in response to a stimulus, such as E2. This can be demonstrated by the work conducted in **Chapter VI**, where E2 did not

show a great impact upon the expression of apoptotic genes in the MCF-12A cell line. However, it was clearly demonstrated in previous chapters that in 3D cultures, apoptosis is suppressed by estrogens, as inferred by the reduction in the levels of activated caspase-3 in acini, following E2 treatment. Given that cells *in vivo* or cultured in 3D matrices behave differently from those grown in monolayer, it is plausible that E2 also impacts on apoptotic gene expression differently in different settings (Horning *et al*, 2008; Pickl & Ries CH, 2009; Yang *et al*, 2009). There are limited publications regarding the gene expression profiling of 3D cultures of mammary epithelial cells. The most thorough work has focused on comparing the gene expression profiles of different breast cell lines cultured in 3D to identify differences in their gene expression signatures, using Affymetrix high-density oligonucleotide arrays. It was observed that the gene expression profile of mammary epithelial cells cultured in 3D reflects the morphology they adopt in a 3D matrix (Kenny *et al*, 2007). However, to our knowledge, there has been no work conducted on comparing the gene expression of the inner cells with the outer cells, within acini.

In our work, initially, whole acini were extracted from Matrigel for gene expression analysis. However, as mentioned before in this chapter and presented in **Chapters III**, **IV** and **V**, as well as in the reported literature (Debnath *et al*, 2002; 2003), two populations of cells exist within acini cultured in Matrigel: the outer cells attached to the basement membrane and the inner cells that undergo apoptosis and are progressively cleared, leaving a hollow lumen. As these two distinct populations of cells within the acini of 3D cultures seem to have specific functions and fates, it is likely that the gene expression profile of these cells will also differ, as described previously. Based on this, another potential limitation associated with studying the expression of genes in whole acini becomes clear, as it is possible that the differences in gene expression in one population might mask the changes in gene expression of the other, and lead to results that are not representative of what actually occurs in the acini. In order to evaluate the differences between the two populations and study how gene expression profiles change in each one, in response to E2, it would be necessary to first separate the two populations of acini. This, however, is no easy task and, to our knowledge, has not been carried out previously. Consequently, we devised, developed and tested a novel method by which the inner and outer cells of acini can be separated.

One way in which cells of a mixed population can be separated into their component populations is by fluorescence activated cell sorting (FACS). FACS works by separating cells by fluorescent markers that are uniquely present in the desired population that you wish to separate. Through the use of lasers, these fluorophores can be excited, the emitted light detected and the cells

separated into a population of cells expressing the marker and a second population of cells lacking the marker.

7.2 The Principles of FACS

FACS is a form of flow cytometry that enables a heterogeneous mixture of cells to be separated into distinct cell populations based on fluorescent and light scattering properties of individual cells. Antibodies against specific markers and bound to specific fluorophores are used to label individual cell populations within this mixture. These markers are used to distinguish the different populations during the FACS procedure. Different fluorophores have characteristic excitation and emission wavelength spectra. This means that multiple fluorophores can be used within one session and that multiple populations within a mixture can be obtained, providing the emission wavelengths of the fluorophores do not overlap.

In terms of the sorting methodology, the cell mixture passes through a vibrating pressurized liquid stream whereby clumps of cells are separated into single droplets. Next, the single-file cell stream passes through a series of lasers and detectors. The various laser wavelengths excite the fluorescent molecules and these, in return, emit light of a particular wavelength, which is detected by a photomultiplier tube. Depending on the fluorescence emission wavelength, cells are differentially charged. The charged cells can then be separated according to this charge by electrodes resulting in the separation of a cell mixture into its component populations (**Figure 7.1**). As all these procedures can be done under sterile conditions, cells can then be re-cultured or undergo further analysis via methodologies, such as real-time PCR.

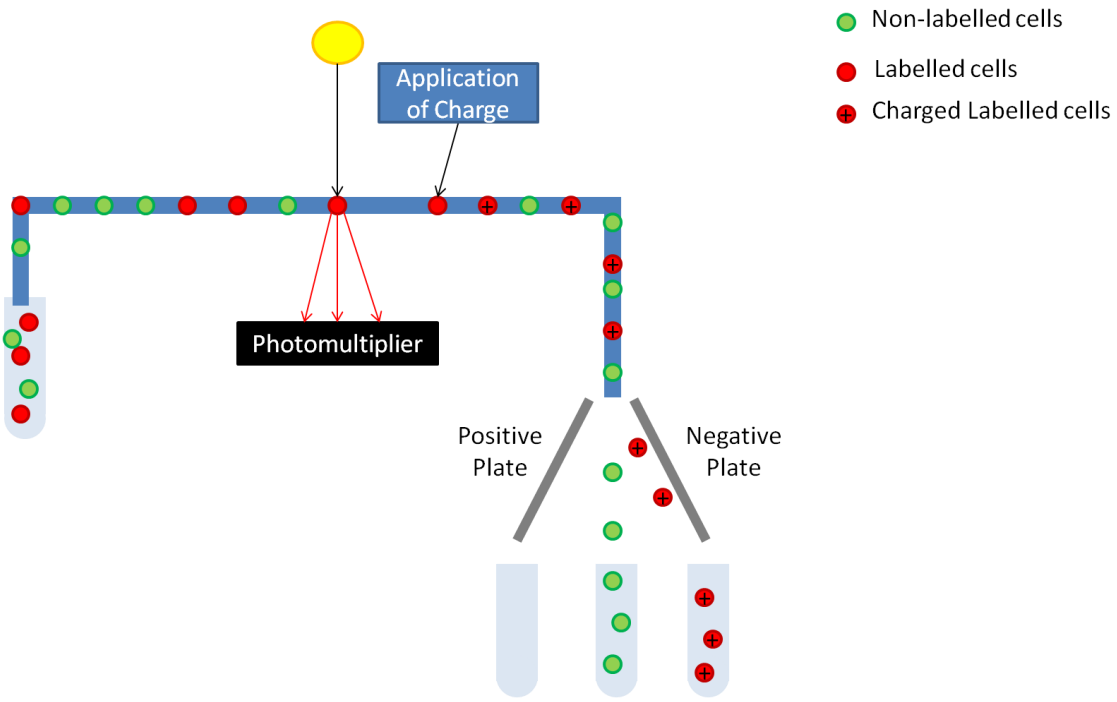

Figure 7.1: The Process of FACS. Fluorescently labelled cells (red) become charged upon excitation by lasers and are separated according to this positive charge. Non-labelled cells (green) are not excited by the laser and hence produce no emission spectra. These cells remain uncharged and are separated from the mixed population accordingly.

The data from FACS can provide information on the total number of cells present within each population and their characteristics, in various formats. Firstly, an intensity plot depicting fluorescence (x-axis) against frequency (y-axis) depicts the number of cells present with each emission wavelength (**Figure 7.2A**). Alternatively, a dot plot depicting the fluorescence of one dye (y-axis) against another (x-axis) can show the number of cells with no staining, staining with one fluorophores, or both fluorophores (**Figure 7.2B**).

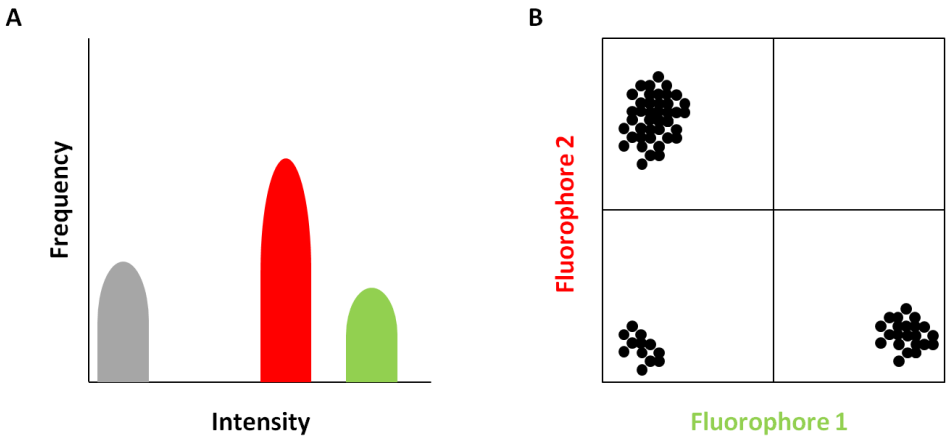

Figure 7.2: Schematic Representations of Data Following FACS Analysis. A) Cell number is plotted against fluorescence wavelength. Three populations are shown: Grey = unstained cells, Green = Fluorophore 1, Red = Fluorophore 2 **B)** Non-labelled cells are shown in the bottom left quadrant. Red and green labelled cells are shown in the upper left and bottom right quadrants respectively. Cells with both markers would be shown in the upper right quadrant. Each dot represents a single event, i.e. a single cell.

In order to perform FACS and obtain two separate populations of cells from acini, it is first necessary to find a specific marker for one, or both cell populations. From our work with 3D cultures, and published literature, laminin, which composes the basement membrane, was observed to be localised to the outer edge of acini and in contact with the outer cells (Debath *et al*, 2002; 2003). Moreover, this protein binds to transmembrane receptors, namely, integrins which, in turn, have been shown to locate specifically to the outer cells of the spheroid structures (Debnath *et al*, 2003). For this reason, integrin β4 was considered a possible good candidate for using in the separation of the two populations within acini, as it would act as a marker for the outer cells.

7.3 Integrin

In the context of the breast, interactions between cells and the ECM *in vivo* are involved in the branching of the mammary ducts at the embryonic stage (Silberstein *et al*, 1992) and during mammary gland proliferation, differentiation, lactation and involution (Talhouk *et al*, 1992; Sympson *et al*, 1994). This is achieved through signalling from the ECM, via receptors, such as the integrins. Reciprocally, cellular aspects, such as shape, internal cell architecture and expression of particular genes can impact upon the integration of the signals originating from the ECM and even impact upon the architecture of the ECM itself (Roskelley *et al*, 1994; Ingber *et al*, 1995).

Integrins comprise a large family of transmembrane proteins that are involved in cellular adhesion to the ECM and link the components of the extracellular environment, such as laminin, collagen, fibronectin and vitronectin, to the intracellular actin cytoskeleton. Upon integrin binding to the ECM, integrin clustering occurs. This adhesion can, in turn, engage various signalling pathways, such as the MAPK and PI3K cascades, that mediate survival, proliferation, migration and invasion of cells (Chen *et al*, 1994) (**Fig 7.3**)

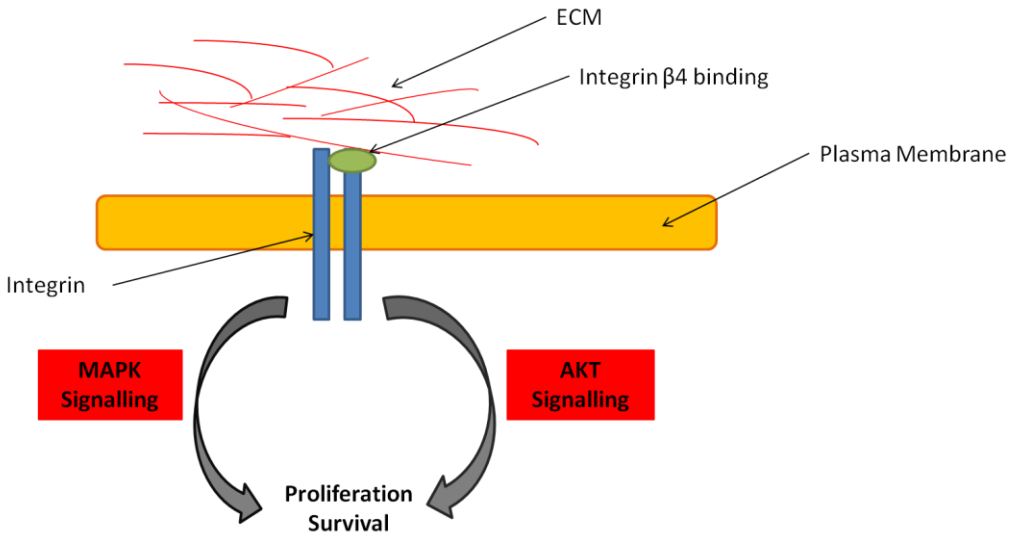

Figure 7.3: Integrin signalling. Activation of the AKT and MAPK signalling pathways cumulating in increased proliferation and survival in response to the binding of integrins to components of the ECM. The antibody against integrin β4 (green) binding to integrin β4 (blue) is also shown.

Previous work has identified integrins, such as integrin α6, as markers of basal polarity in 3D cultures of MCF-10A cells, and this localization has been implicated in mediating particular functions, such as establishment of polarity and activation of survival pathways (Debnath *et al*, 2003; Liu *et al*, 2004). Integrin β4 forms complexes with integrin α6 in epithelial cells and acts as a receptor for laminin, making it an ideal candidate marker for future work. An antibody against this specific integrin was selected due to its availability and compatibility with our previously established protocols.

7.4 Other Considerations

When developing this new approach, another important point to consider was the selection of an appropriate time point at which to extract acini from Matrigel and separate the two populations. The

ideal time point to do this would be just before cells undergo clearance from the centre of the acini, but while these are entering the apoptotic programme. This is because we want to analyse the gene expression of the inner cells before they are cleared from the lumen, so that they are viable for RNA extraction and gene analysis. However, we also had to ensure that the fates of the two cell populations had been determined and that the acini were comprised of two distinct cell populations. The time-course analysis in **Chapter III**, showed that following approximately 8 days incubation, the central cells of MCF-12A acini were starting to express activated-caspase 3, an indicator of apoptosis, but had not yet been cleared. It was therefore decided to perform experiments following 7 days incubation in Matrigel to ensure that cells would be present in each population, but would still be preparing for their determined fate.

7.5 Materials and Methods

The same protocol was used to grow MCF-12A acini for whole and separated acini in preparation for extraction and gene analysis. MCF-12A cells were seeded onto Matrigel, as previously described, at a seeding density of 100,000 cells per ml to ensure a significant number of cells would be recovered for FACS analysis and subsequent real-time PCR. Cells were treated with either 0.5% ethanol or 1 nM E2 in 0.5% ethanol for 7 days with media changes performed every 3 days. At the end of the incubation period, cells were pooled from 8 chambers of an 8 chamber slide to ensure an adequate cell number was obtained for further analysis.

7.5.1 Cell Recovery

After maintenance in 3D culture for 7 days, cells were recovered from the Matrigel using MatriSperse Cell Recovery solution (BD Biosciences, Bedford, MA USA). For that, medium was removed and wells washed 3 times with cold PBS. 200 µl Matrisperse cell recovery solution (BD Biosciences, Bedford, MA USA) was added to each well and a P1000 pipette tip was used to gently dislodge the Matrigel and transfer it to 1.5 ml centrifuge tubes that were kept on ice. Wells were washed twice with 100 µl cell recovery solution and samples left on ice for 90 minutes with gentle mixing at 30 minute intervals. Samples were centrifuged at 6000 rpm for 3 minutes at 4^{o}C and washed twice with cold PBS. They were centrifuged again at 6000 rpm for 3 minutes at 4^{o}C and the PBS aspirated, so only the pellet remained.

7.5.2 FACS Staining and FACS

Cells were fixed by re-suspension in freshly prepared 4% PFA for 20 minutes at room temperature, centrifuged at 4000 rpm for 5 minutes and washed with PBS. Cells were then permeabilised with 0.5% Triton-X for 10 minutes at 4^{o}C, centrifuged at 4000 rpm for 5 minutes and washed with PBS. Buffer (130 mM NaCl, 7.7 mM NaN_3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) containing 10% goat serum was applied to the pellet to prevent unspecific antibody binding and cells were centrifuged for 10 minutes at 4000 rpm. Cells were then re-suspended in 100 µl antibody against human integrin β4 (mouse monoclonal antibody) at a 1:100 dilution in buffer containing 10% goat serum and incubated at 4^{o}C overnight. 1 ml PBS was added and the cells were centrifuged for 10 minutes at 4000 rpm, then washed in PBS, before being re-suspended in 100 µl anti-mouse Alexafluor 555 (secondary antibody comprised of an anti-mouse antibody conjugated to a fluorophore with a similar excitation and emission spectra to Cy3, but with increased photostability and brighter resultant protein staining) at a 1:100 dilution and incubated at 4^{o}C for 45 minutes. 1 ml PBS was added and the cells were centrifuged for 10 minutes at 4000 rpm, then washed in PBS three times. The final pellet was re-suspended in 1 ml PBS and transferred into 12 x 75 mm microtubes (BD Biosciences, Bedford, MA USA). FACS was performed using the BD FACS Aria II cytometer (BD Biosciences, Bedford, MA USA) to separate integrin and non-integrin labelled cells.

Cells were separated into three populations: integrin-labelled, non-integrin-labelled and a mixed population. The mixed population was discarded, as it was only necessary to ensure thorough sorting of the labelled and non-labelled cells. Data analysis was performed using BD FACS Diva (Biosciences, Bedford, MA USA).

7.5.3 Confirmation that Integrin β4 is Unique to One Population

Prior to FACS, it was necessary to confirm that integrin β4 was differentially expressed by the two cell populations of acini, and that cell liberation from Matrigel did not affect integrin β4 detection (for example, loss of integrin β4 due to internalisation and degradation). In order to do this, firstly, cells were grown in 3D culture for 8 days, as at this point acini remain whole with no luminal clearing evident, and whole acini were stained with antibodies against integrin β4 and TOPRO-3 used as a nuclear counterstain, as described previously.

In a second experiment, following 8 days incubation, MCF-12A acini were extracted from the Matrigel, as described above, and a sample of cell suspension prior to FACS was dropped onto glass

slides into a defined area. After drying, cells were fixed and stained using antibodies against integrin β4 and the nuclear counterstain, 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) as described in the immunocytochemistry protocol detailed in previous chapters, with a few adjustments to the protocol. As the desired areas to be stained were marked by a PAP pen, a volume of 500 µl of 2% PFA, 0.5% Triton X and primary and secondary antibody dilutions were required to sufficiently cover the desired area. Washing steps were performed in petri dishes with gentle rocking in volumes of 5 ml to sufficiently wash away excess antibodies. In order to counterstain the nucleus using DAPI, the immunocytochemistry was followed until the washing steps following incubation with the secondary antibodies. After this, 500 µl DAPI (300 nM) prepared in dH_20 was applied to the marked areas and samples were incubated for 5 min at room temperature. Samples were then washed in PBS for 5 min with gentle rocking. The PAP pen was removed and the samples mounted as described previously.

Both samples were analysed using the Zeiss LSM 710 confocal microscope (Carl Zeiss Ltd).

7.5.4 Gene Analysis Procedures

RT real-time PCR was used to analyse the difference in gene expression between inner and outer cell populations in EtOH controls and 1 nM E2-treated samples. As before, *β-ACTIN* was used as the reference gene.

7.5.5 RNA Extraction

RNA extraction was performed on separated samples using the Nucleospin® RNA XS kit (Machery-Nagel, Düren, Germany), which is specifically designed for RNA extraction from small cell numbers. Samples were lysed using 100 µl RA1 lysis buffer and 2µl tris 2-carboxyethylphosphine (TCEP) and vortexing vigorously. 5 µl 4ng/µl carrier RNA solution was added and samples were again vortexed and spun down. 100 µl 70% ethanol was added to optimise binding conditions and samples were mixed by pipetting up and down before being loaded onto NucleoSpin® XS columns. Samples were centrifuged for 30 seconds at 11,000 x g. Desalting of the silica membrane was achieved by the addition of 100 µl membrane desalting buffer (MDB) and centrifugation at 11,000 x g for 30 seconds. 25 µl rDNase was applied directly to the membrane of the columns and samples were incubated at room temperature for 15 minutes. 100 µl RA2 buffer was added to the columns and samples were incubated for 2 minutes at room temperature before centrifugation for 30 seconds at 11,000 x g. 400 µ RA3 buffer was then added and samples were centrifuged at 11,000 x g for 30 seconds. A final wash with 200 µl RA3 buffer and

centrifugation for 2 minutes at 11,000 x g was performed before RNA was eluted in 10 µl RNase-free water at 11,000 x g, for 1 minute.

7.5.6 Reverse Transcription Real-Time PCR

Reverse transcription was performed as described previously. Real-Time PCR was performed as described previously, using primers for *BAX*, *BAD*, *BCL-2*, *BCL-XL* and *CCND1* (**Table 7.1**). *β-ACTIN* was used as a reference gene. Finally, gene expression analysis and determination of the Bax/Bcl-2 ratio was performed as described previously. Statistics was performed using the pair wise fixed reallocation randomisation test (REST, Pfaffl *et al,* 2002) and the unpaired t-test for gene expression and Bax/Bcl-2 ratios, respectively. A *p-value* of 0.05 was considered statistically significant.

Table 7.1: Primers used for real-time PCR analysis of 3D cultures of MCF-12A cells. Primers were designed using Beacon Designer 5 and purchased from Eurogentec.

cDNA	Genebank Ascension Number	Primer Sense	Primer Sequence (5'-3')	Concentration (nM)	Target Size (bp)
β-ACTIN	X00351	Forward	TGCTATCCAGGCTGTGCTAT	300	97
		Reverse	GATGGAGTTGAAGGTAGGTT	300	
BCL-2	EU287875	Forward	CCTGGTGGACAACATC	100	160
		Reverse	GAGCAGAGTCTTCAGAG	100	
BCL-XL	BT007208	Forward	TGAACAGGTAGTGAATGAAC	100	105
		Reverse	TCCTTGTCTACGCTTTCC	100	
BAX	BC014175	Forward	CCTCCTCTCCTACTTTGG	150	103
		Reverse	GCCTCAGCCCATCTTC	150	
BAD	BT006678	Forward	GATGAGTGACGAGTTTGTG	150	133
		Reverse	GCCCAAGTTCCGATCC	150	
CCND1	AF212040	Forward	TGGAATGGTTTGGGAATAT	200	146
		Reverse	CCTGGCAATGTGAGAATG	200	

7.6 Results

7.6.1 Gene Expression of Whole Acini

Firstly, we investigated the expression of key apoptotic and proliferative genes in whole acini following 7 days incubation in Matrigel. Following gene expression analysis by real-time PCR, the relative expression ratio, as calculated using the Pfaffl equation (Pfaffl *et al*, 2002), were plotted for each gene. We observed that the only significant changes in gene expression in response to E2 were an up-regulation of *CCND1* and a down-regulation of *BAX* ($P < 0.05$) (**Figure 7.4**).

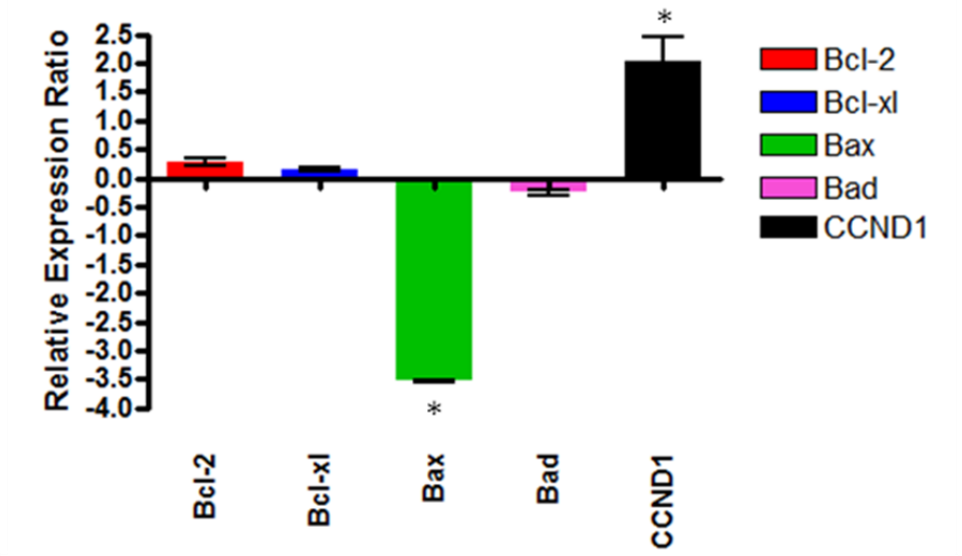

Figure 7.4: Effect of E2 on gene expression in MCF-12A acini. Negative controls (etOH) are set to 0. Bars show relative changes in gene expression following 1 nM E2 stimulation during the incubation period in 3D culture. Positive values indicate a gene up-regulation and negative values indicate gene down-regulation. Error bars represent +/- standard error of the mean (n = 6). *P*-values are shown for each data set. A *P-value* of 0.05 was considered statistically significant and is donated by a *.

7.6.2 Bax/Bcl-2 Ratio of Whole Acini

The Bax/Bcl-2 ratio was calculated as previously described. Although the up-regulation in *BCL-2* expression in response to E2 treatment was not statistically significant (**Figure 7.4**), E2 treatment did decrease the Bax/Bcl-2 ratio relative to etOH controls (**Figure 7.5**). This potentially means that E2 can have a negative impact upon apoptosis and, thus, promote cellular survival in 3D cultures of MCF-12A cells.

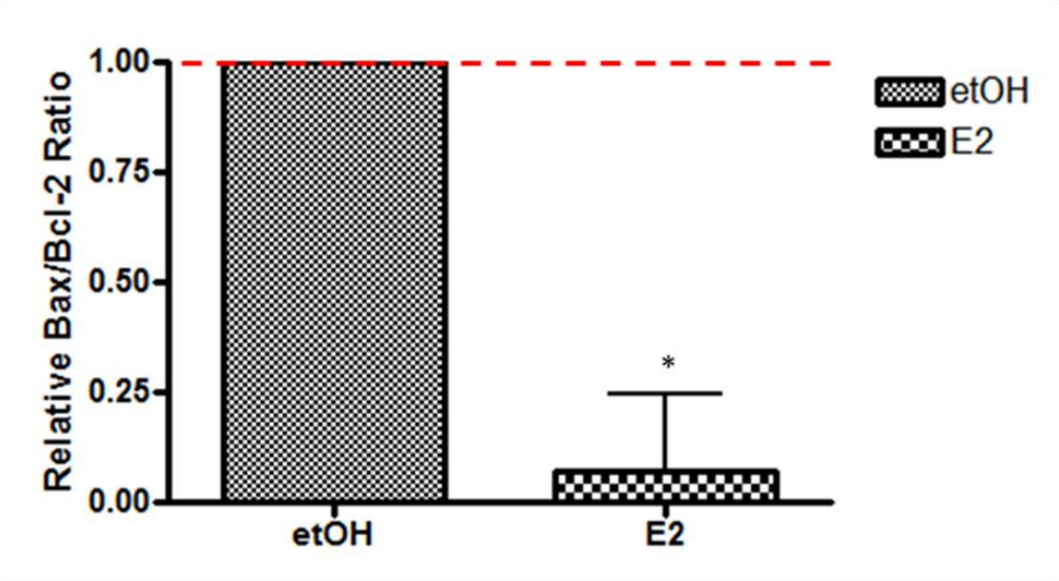

Figure 7.5: Relative Bax/Bcl-2 ratio for MCF-12A cells cultured in Matrigel for 7 days and treated with 1 nM E2. A value of 1 was set for the Bax/Bcl-2 ratio for etOH controls and treatment ratios are relative to this. Data is normalised to β-actin. Error bars represent standard error of the mean (n = 6). *P-values* < 0.05 were considered statistically significant and are denoted by an *.

As can be seen in **Figure 7.4**, the expression of pro-apoptotic *BAD*, as well as the anti-apoptotic factors, *BCL-2* and *BCL-XL*, was not significant. This observation partially contradicts the observations in monolayer, where cultures of MCF-12A cells showed an increase in *BCL-2* expression following E2 treatment (**Figure 6.5**). Although this may have been due to differences in cell responses determined by whether they were cultured in monolayer or 3D, another reason may be that the outer and inner cell populations of E2 respond differently to E2, in terms of gene expression. If this is the case, it is plausible that the response to E2 in one population may mask that of the other. The need to test this hypothesis emphasises the need to separate the two populations of cells (which has been discussed previously), and analyse gene expression in both of them, in response to E2 treatment. This requirement led to the development FACS separation of MCF-12A acini into inner and outer cell populations for gene expression analysis.

7.6.3 Confirmation of Integrin β4-Specific Localisation to Outer Acinar Cells

In order to ensure the correct separation of both inner and outer cell populations from acini, it was necessary to confirm that only one cell population expressed integrin β4, so that cell sorting could be

performed reliably. Following immunocytochemistry and microscopic analysis of MCF-12A cells grown in 3D cultures with an antibody against integrin β4 and TOPRO-3 nuclear counterstain, integrin β4 was seen to localise to the basal side of the outer acinar cells. There was no staining for integrin in the inner cells of acini (**Figure 7.6**). This observation indicates that integrin β4 can potentially be used as a marker to separate the outer from the inner populations of MCF-12A acini by FACS, as its expression is limited to the outer cells of the spheroid structures.

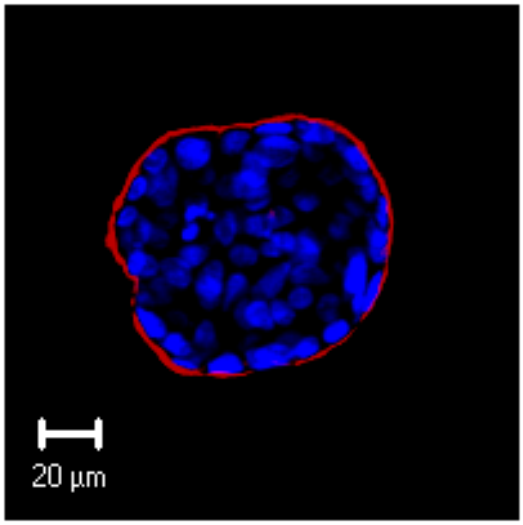

Figure 7.6: LCM image of MCF-12A acinus after 8 days incubation in Matrigel. Acini were fixed and stained with antibodies against integrin β4 (red) and then counterstained with TOPRO-3 (blue) as a nuclear marker.

7.6.4 Proof That Integrin β4 is not Lost During Sample Preparation

To confirm that acini recovery from Matrigel did not result in the loss of integrin β4 (as a result of receptor internalisation, ubiquitylation or degradation), the expression of this marker was analysed by immunocytochemistry and LCM, following liberation from the Matrigel. We observed that there appeared to be two different populations: one positive for DAPI only, and another that expressed both DAPI and integrin β4 (**Figure 7.7**). Assumedly, these populations correlate with the inner and the outer cells of MCF-12A acini, respectively. Taking this together with the staining of whole acini, these observations seem to imply that the expression of integrin β4 is exclusive to cells of acini that are in contact with the basement membrane. Furthermore, we can now be confident that the process of acini extraction from Matrigel does not result in the loss of integrin β4 and consequently, integrin β4 can be used as a marker to separate the inner cells of acini from the outer.

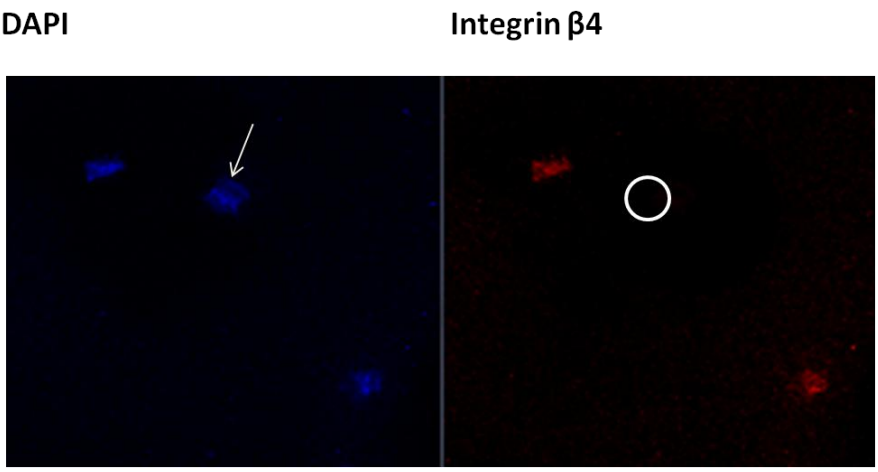

Figure 7.7: DAPI and integrin β4 staining of cells after acini extraction from Matrigel. Following acini isolation from Matrigel after 8 days incubation, cells were stained for integrin β4 (red) and counterstained with DAPI (blue). Despite all cells being positive for the nuclear counterstain DAPI (blue), only some cells expressed integrin β4 (red) which confirmed that not all cells expressed integrin β4. Arrow indicates cell where only nuclear staining is present as inferred by positive DAPI staining but lack of integrin β4. Circle indicates where cell would be if it had stained positive for integrin β4.

7.6.5 Selection of a Time-Point for Acini Separation

The final step in optimisation of conditions for the separation of the inner and outer cell populations of MCF-12A acini was to select an appropriate time point at which to perform FACS. In **Chapter III**, we performed a time-course analysis for the morphogenesis of MCF-12A acini (**Figure 7.8**).

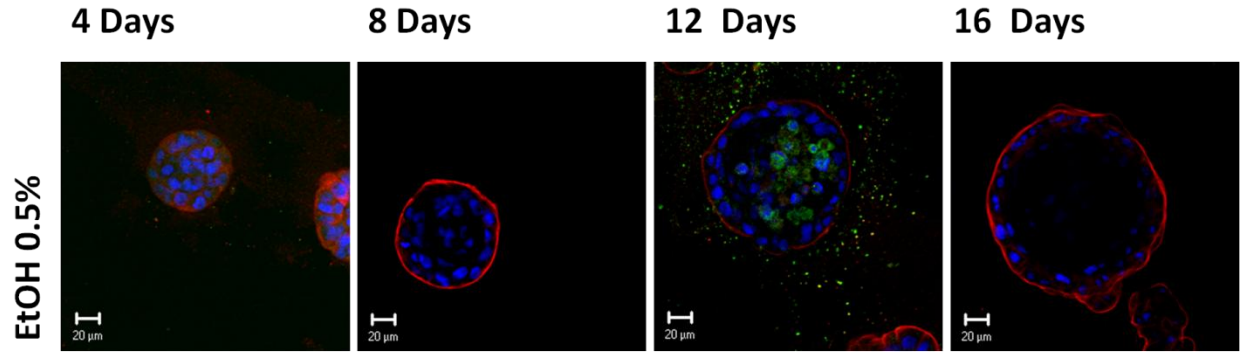

Figure 7.8: Time-course of MCF-12A acini over 16 days. Acini were stained with antibodies against integrin β4 (red) and activated caspase-3 (green) to identify basement membrane and apoptotic cells, respectively. Cells were counterstained with TOPRO-3 (blue) as a nuclear stain.

At 4 days, acini were small in size and because we required the maximum cell number possible, this time point was not optimum for future gene analysis. Also, at this point, the staining for integrin β4 was diffuse and disperse throughout the acini structure, probably indicating that, at this early time-point, the two distinct populations of cells were not yet completely differentiated and expression of integrin β4 was not significant. Following 8 days incubation in Matrigel, MCF-12A acini were larger and showed no sign of apoptosis, as indicated by the lack of activated caspase-3. However, at 12 days, luminal clearing and extensive activated caspase-3 was evident. This means that time-points after 12 days are not candidates for the comparison of outer and inner cells, as some cells had already cleared from the central area. Furthermore, as the remaining inner cells were clearly marked an on the path to cell death, it is likely they would not be functionally active anymore and the expression of apoptotic genes no longer observable. Therefore, a time-point at around 8 days, where two clear populations of viable, live cells were obtained, would be the optimum time-point. 7 days was selected to perform FACS, as this time-point was adequate for our needs and was convenient for the collaborative effort with the FACS operator.

7.6.6 FACS

Following staining of the extracted acini with antibodies against integrin β4, cells were separated into three populations: P1 (integrin β4 labelled), P2 (a mixed population) and P3 (unlabelled). The P2 cells were disregarded as contained a small fraction of both tagged and untagged cells. The P1 and P3 populations, however, were prepared for gene analysis by real-time PCR. The majority of cells were untagged, which is probably due to more cells being contained within the volume of a sphere than those present on the surface (**Figure 7.9; 7.10; 7.11**).

etOH

Population	#Events
All Events	10,000
P1	247
P2	659
P3	9,032

E2

Population	#Events
All Events	10,000
P1	308
P2	625
P3	8,981

Figure 7.9: FACS Raw Data: The number of events, or cells, in each population. FACS was performed until a total of 10,000 events had occurred. Red (P1) = Integrin β4-labelled cells, Pink (P2) = mixed population of labelled and unlabelled cells, and Blue = unlabelled cells.

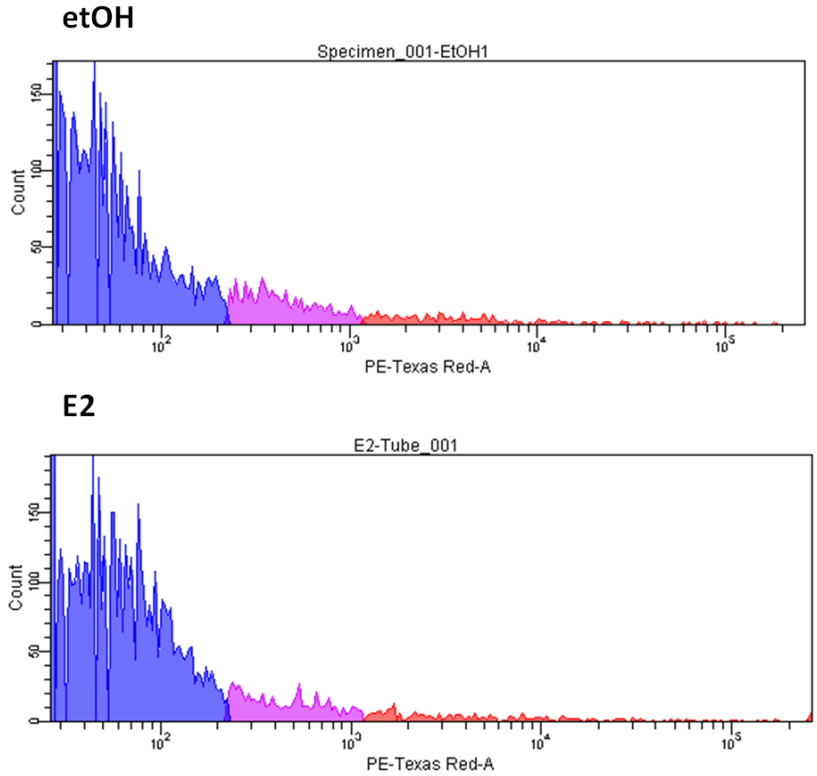

Figure 7.10: Intensity plot for FACS-separated cells for etOH controls and E2-treated samples. Fluorescence is plotted along the x axis against frequency (y-axis). The three populations are shown: Red (P1) = Integrin β4-labelled cells, Pink (P2) = mixed population of labelled and unlabelled cells, and Blue = unlabelled cells.

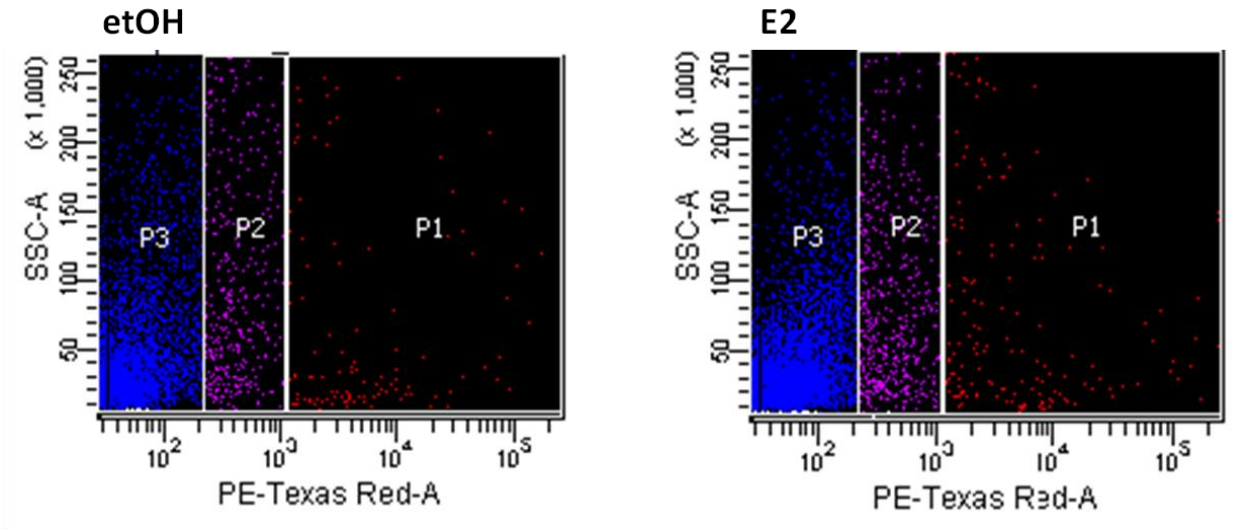

Figure 7.11: Dot plot diagrams for FACS-separated etOH and E2-treated samples. Fluorescence in the range of Texas Red is plotted against side scatter (SSC-A). Each dot corresponds to a single event. Red (P1) = Integrin β4-labelled cells, Pink (P2) = mixed population of labelled and unlabelled cells, and Blue = unlabelled cells.

7.6.7 The Two Populations of Acini Have Different Expression Profiles

After the inner and outer populations of etOH control and E2 treated acini were separated by FACS and real-time PCR was performed, changes in gene expression were observed in apoptotic genes that were not previously observed, following gene expression analysis of whole acini. As can be seen in **Figure 7.12,** in etOH controls *BCL-XL* expression was lower in the inner than the outer cells of acini (outer cell expression is set to 0, so positive values indicate an up-regulation of a gene in the inner cells relative to the outer cells, and a negative expression value indicates a down-regulation in the inner cells). Following E2 treatment, however, *BCL-XL* expression increased in inner cells relative to the outer cells ($p < 0.05$). It is likely that this E2-induced up-regulation of an anti-apoptotic factor would contribute towards an anti-apoptotic influence and, hence, promote the survival of the inner population of cells. Although not statistically significant, the inner cells of etOH controls expressed higher levels of *BAX* and *BAD* compared to the outer population. This may have a pro-apoptotic influence on these inner cells. When acini were treated with E2, however, the inner cells expressed less *BAX* ($p < 0.05$) and *BAD* than the outer cells and, thus, would be more likely to survive and evade apoptosis.

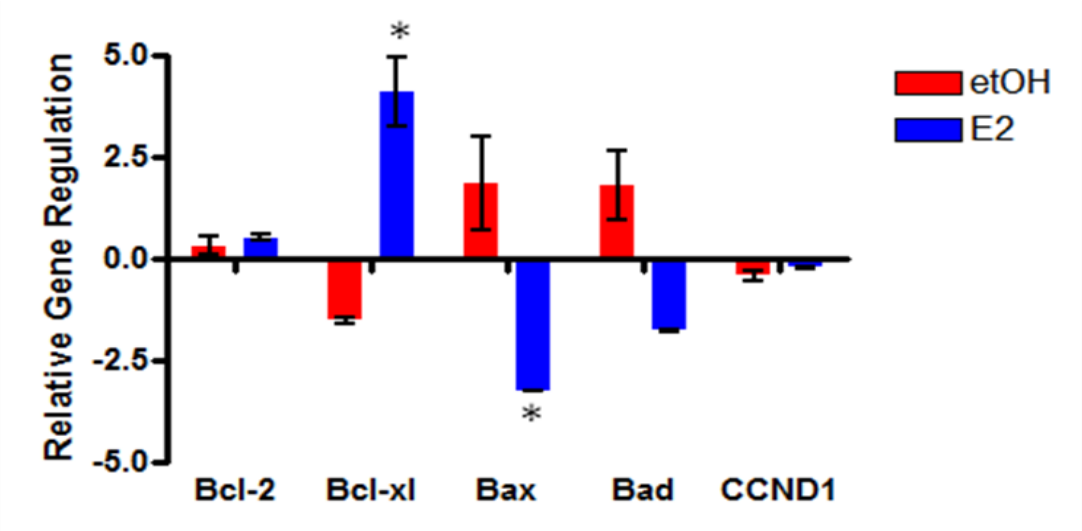

Figure 7.12: Relative gene expression of inner cells compared to outer cells. Red bars are controls and blue bars are E2-treated cells. A positive value is indicative of an up-regulation in gene expression and a negative value is indicative of a gene down-regulation. Data is normalised to β-actin. Error bars represent the standard error of the mean (n = 6). A *p-value* of 0.05 was considered statistically significant and is donated by an *.

7.6.8 Gene Expression of FACS-Separated Acini in Response to E2

We then sought to see if E2 treatment caused changes in gene expression in both inner and outer populations, relative to controls (controls are set to 0). *BCL-2* expression was up-regulated by E2 in the both the inner and outer cells of MCF-12A acini, but was only significant in the outer cells ($p < 0.05$). Interestingly, E2 significantly down-regulated the expression of *BCL*-XL which was unexpected as this would contribute to a pro-apoptotic effect. As expected, *BAX* expression was down-regulated by E2 in both the inner and outer cell populations ($P < 0.05$), and this was more marked in the inner cells. This may present one part of the mechanism by which estrogens act to induce luminal filling in MCF-12A acini, as decreased *BAX* would have an anti-apoptotic effect. In addition to this, E2 also down-regulated *BAD* expression relative to controls in the inner cells of acini ($P < 0.05$). However, unexpectedly, BAD expression was up-regulated in the outer cells of acini following E2 treatment ($P < 0.05$). Although not statistically significant, E2 induced a small up-regulation in *CCND1* expression in the outer and inner cells (**Figure 7.13**). This may contribute to an increase in proliferative ability of both populations, and may account for the increased acinar size that was observed when 3D cultures of MCF-12A cells were treated with estrogens.

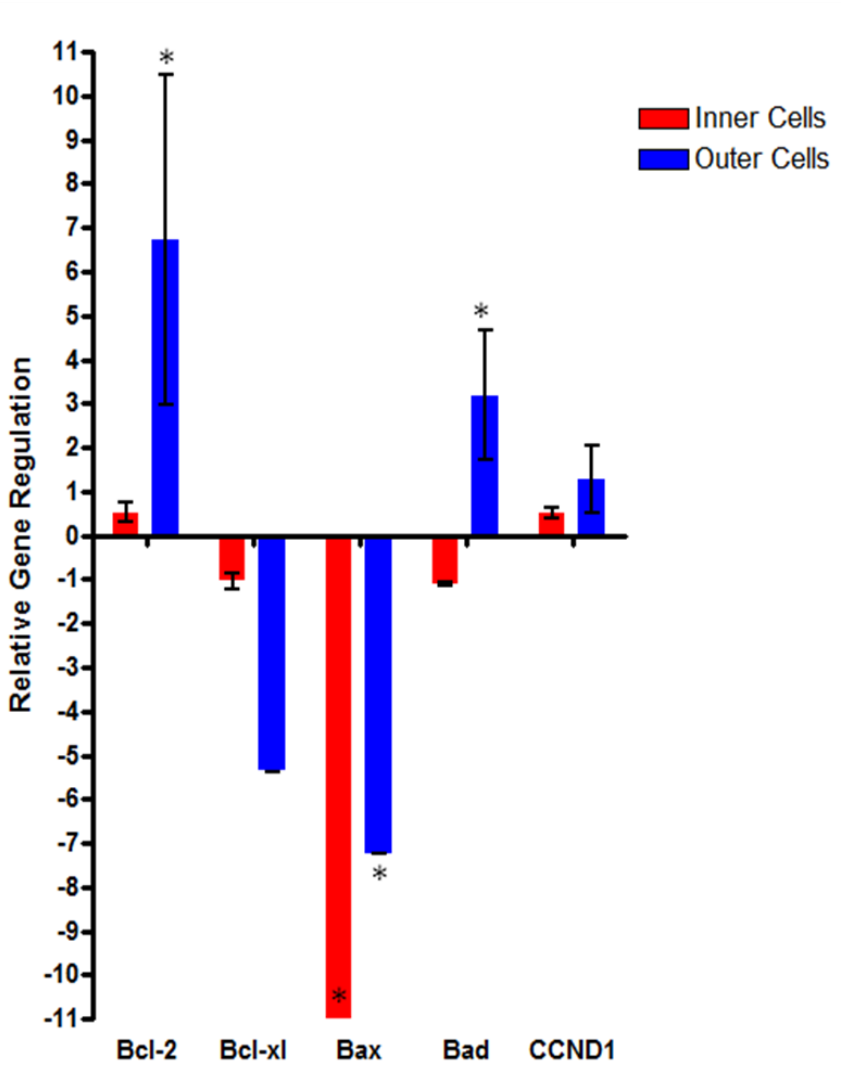

Figure 7.13: Relative gene regulation of E2-treated inner and outer cells compared to controls (x-axis). Red bars are from inner cells and blue bars are from outer cells. A positive value is indicative of an up-regulation in gene expression and a negative value is indicative of a gene down-regulation relative to controls. Data is normalised to β-actin. Error bars represent the standard error of the mean (n = 6). A *p-value* of 0.05 was considered statistically significant and is donated by an *.

7.6.9 Bax/Bcl-2 Ratio

The Bax/Bcl-2 ratio was calculated by dividing the relative expression ratio of *BAX* by that of *BCL-2* obtained from the Pfaffl equation (Pfaffl *et al*, 2002). The lower the Bax/Bcl-2 ratio, the more likely a cell is to undergo apoptosis. The Bax/Bcl-2 ratio for solvent controls was lower in the inner population of MCF-12A acini compared to outer cells, which would indicate that the inner cells are more likely to

undergo apoptosis. In both the inner and outer populations, E2 treatment reduced the Bax/Bcl-2 ratio (**Figure 7.14**). This would likely contribute to an anti-apoptotic effect and hence may explain why E2-treated acini underwent less apoptosis, and displayed luminal filling, as observed by marked reductions in activated caspase-3 expression in **Chapter IV**.

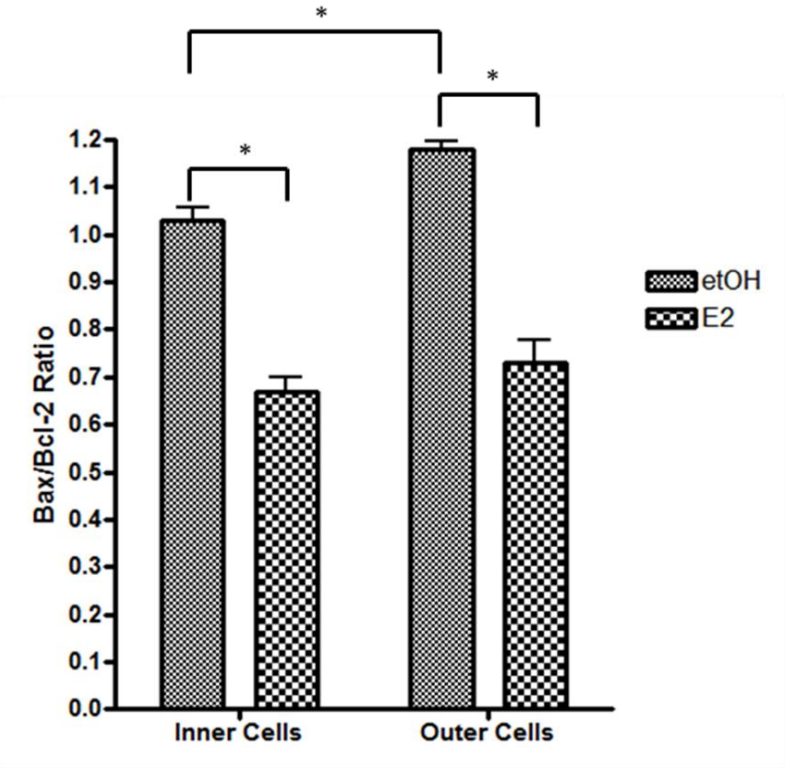

Figure 7.14: Bax/Bcl-2 ratio for FACS-separated MCF-12A cells cultured in Matrigel and treated with 1 nM E2. Lower Bax/Bcl-2 ratios correlate with an increased pro-apoptotic influence. Error bars represent standard error of the mean (n = 6). Data is normalised to β-actin. A *p-value* < 0.05 was considered statistically significant and is denoted by an *.

7.7 Discussion

7.7.1 Analysis of Whole MCF-12A Acini Cultured in Matrigel

In **Chapter VI**, it was demonstrated that E2 induced up-regulation of *BCL-2*, *BCL-XL* and *BAX* expression in monolayer cultures of MCF-12A cells. Given that cells behave differently in monolayer from *in vivo* or in 3D, it was important to investigate if the same gene regulation was seen in our 3D cultures (Horning *et al*, 2008; Pickl & Ries CH, 2009; Yang *et al*, 2009). By studying the expression profile of genes involved in cell proliferation and apoptosis in 3D cultures, we hoped to provide a further understanding of the

mechanisms that regulate acini formation, as well as those involved in estrogen-induced acini disruption.

Firstly, gene expression analysis of specific apoptotic and proliferative genes was carried out in whole acini, recovered from 7 days Matrigel cell cultures. As described in the results section, when real-time PCR was performed on E2-treated whole acini extracts, *BAX* expression was down-regulated and no significant changes were seen in the expression of *BCL-2* and *BCL-XL*. The effect on these apoptotic genes was accompanied by an increase in *CCND1* levels. In contrast to this, E2-treated monolayer cultures of MCF-12A cells resulted in the up-regulation of *BCL-2* and *BCL-XL*, in addition to *BAX* (**Chapter VI**). The differences in gene expression of cells cultured in 3D and in monolayer have been described previously. For example: following gene analysis of prostate cells, cultured in monolayer and in Matrigel, approximately 3400 mRNA's were expressed differently (Härmä *et al*, 2010). This study identified that prostate cells cultured in Matrigel, without treatments, differed from monolayer cultures, in terms of the expression of genes associated with metabolism, proliferation and epigenetic modifications. The differences in gene expression were hypothesised to account for further observations that prostate cultures grown in Matrigel displayed reduced growth and proliferation than monolayer cultures. If the same pattern holds true for mammary epithelial cells, in particular for MCF-12A cells, the difference in gene expression profiles may account for why MCF-12A cells grown in monolayer or Matrigel responded differently to E2, in respect to apoptotic factor expression. Indeed, it has been shown that the gene expression profile of bovine mammary epithelial cells cultured in Matrigel differs from that of cells grown in monolayer (Kozlowski *et al*, 2009). These changes in expression between monolayer and 3D cultures involved genes associated with cell signalling, apoptosis and proliferation.

An overall increase in the expression of *CCND1* of E2-treated MCF-12A acini may account for the increased proliferation, as inferred by the increased acinar size and deviation from a uniform spherical shape, and the ability of estrogen-treated acini to escape proliferative suppression, observed in 3D cultures of these cells in **Chapter IV**. Likewise, the down-regulation in *BAX* expression may be a contributing factor to the decreased levels of apoptosis in E2-treated acini. These observations are supported, at least in respect to the role of cyclin D1, by previous findings in which ectopic expression of *CCND1* caused cells to evade proliferative suppression (Debnath *et al*, 2002). Following gene expression analysis, the Bax/Bcl-2 ratio was also calculated. We did not observe a significant change in expression of *BCL-2* following E2 treatment. However, the Bax/Bcl-2 was reduced, and this would favour survival,

rather than apoptosis. This change in the Bax/Bcl-2 ratio may therefore account for the reduction in apoptosis observed in estrogen-treated acini in **Chapter IV**.

7.7.2 Optimisation of Separation of Acini Cell Populations by FACS

As mentioned earlier, it is worth noting that there are two cell populations present within the acini (the inner and the outer cells). It is likely, given that these two populations undergo different fates, that the gene expression profile of the inner and outer cells differs. We observed an overall increase in *CCND1* and a decrease in *BAX* expression following gene analysis of whole MCF-12A acini. However, the methodology did not allow us to ascertain the specific impact of E2 upon each individual population found in acini. For this reason, we then proceeded to separate the inner cells from the outer cells of acini by FACS. In order to achieve this, a protocol for the fluorescent tagging of the two populations had to first be developed and optimised, as, to our knowledge; there are no published reports of the use of FACS technique to separate cells within 3D cultures of immortalised cells. We utilised this technique to develop a novel way to investigate the difference between the two populations of MCF-12A acini, and the impact of E2 upon each. For the first time, we achieved the separation of MCF-12A cells cultured in Matrigel into the inner and outer populations for gene analysis investigation.

In this chapter, integrin β4 was identified as a suitable marker to separate acinar cells, as our work demonstrated that this receptor is only expressed by the outer cells of acini in contact with the basement membrane, in a similar fashion to integrin α6 (Debnath *et al*, 2003; Liu *et al*, 2004). Following the successful separation of the cells of acini by FACS, the RNA from these samples had to be extracted and real-time PCR conducted. These processes had to be adapted from our standard protocols to accommodate for the fact that the cell number available was markedly lower. Nevertheless, using RNA extraction kits designed for small quantities of cells and careful adaptation of the protocol, sufficient RNA was extracted in order for real-time PCR to be conducted. Despite the good quality data obtained for the separated acinar cells, it should be noted that the protocol is in its infant stages and still requires optimization, which will be discussed briefly in the following paragraphs. Having said this, we have, nevertheless, demonstrated for the first time that cells grown in 3D culture can be separated based on integrin β4 expression and used for further analysis by techniques, such as real-time PCR.

Following the separation of MCF-12A cells grown in 3D culture for 7 days, the data from the FACS software was analysed for both etOH controls and E2 treated samples. It was observed that only a small proportion of the cell visualised were labelled for Integrin β4. One possible explanation for this

was that the labelling of integrin β4 was insufficient. Various factors can contribute to this, such as insufficient antibody concentration and incubation periods, or the use of an inappropriate buffer. Furthermore, loss of integrin β4 expression, through internalisation and degradation, could contribute to the low abundance of cells in the labelled population (Kelly & Owen, 2011). We demonstrated by immunocytochemistry, that some cells still retained their integrin β4 expression following extraction from Matrigel. However, this approach yielded only qualitative data, and it is possible that some level of integrin expression was lost. An alternative possibility for these results could be due to a larger quantity of cells occupying the volume of a sphere compared to that present on the surface. This means that the majority of cells would not be labelled (as they would be inner cells) and would account for the lower proportion of integrin β4 labelled cells. Given the confirmation of integrin β4 presence following acini extraction from Matrigel and various optimisation steps to ensure sufficient labelling of integrin β4, one of the most likely possibilities for the low number of cells was the difference in cell number between the two populations.

Although our data still demonstrated clearly that there are differences in the gene expression of cells obtained from each population, more work will have to be performed in the future to further improve and validate this novel technique. In future, it may be of interest to attempt the procedure with a higher concentration of integrin β4 antibody, or to try and increase overall cell number, possibly through the pooling of additional wells.

Cell sorting with other markers for each population may also further validate our findings. Such markers include antibodies against tight junctions, for example, zonula occludens protein 1 (ZO-1) antibodies. This would allow separation as ZO-1 is reported to be expressed exclusively by the outer cells of mammary epithelial cells cultured in Matrigel (Plachot *et al*, 2009).

7.7.3 Effects of Estrogen Treatment on Inner and Outer Cells of MCF-12A Acini

Firstly, one of the important points for discussion is the differences in gene expression between the outer and inner cell populations of control acini. In control acini, the expression of *BCL-XL* and *CCND1* was lower in the inner population of cells compared to the outer population. In addition to this, the expression of *BAX* and *BAD* was higher in the inner population. A decrease in the expression of *BCL-XL* and *CCND1* and an increased expression of *BAX* and *BAD* in the inner population would have a pro-apoptotic and anti-proliferative influence. This observation was expected, as this inner population is composed of cells that are destined to be cleared during the formation of the hollow lumen. Having

analysed the difference in gene expression between the inner and outer cells within MCF-12A acini under control conditions, we can now potentially further understand the mechanisms behind acini morphogenesis. Our data appear to indicate that the inner cells of acini, under control conditions, express lower levels of anti-apoptotic factors (*BCL-XL*) and a higher abundance of pro-apoptotic factors (*BAX* and *BAD*), suggesting that the regulation of the balance of apoptotic factors in implicated in the hollowing of the central lumen by apoptosis. Furthermore, decreased *CCND1* in the inner cells, would imply that these inner cells have a lower proliferative ability than the outer cells, and this may account for why they undergo proliferative suppression.

Having classified the differences in gene expression between the inner and outer populations found within acini under control acini, we could then investigate the impact of estrogens upon this, in the hope that it may provide us with some information on the mechanisms involved in the estrogen-induced acinar malformations.

E2 induced an increase in *BCL-2* expression in the outer population of cells (and a small increase in the inner cells) within MCF-12A acini. *In vivo*, Bcl-2 overexpression has been found to partially suppress apoptosis during the formation of the TEBs of the breast, however, this induced over-expression of *BCL-2* does not prevent the mammary gland from developing *in vivo* and does not result in luminal filling *in vitro* (Humphreys *et al*, 1996; Debnath *et al*, 2002; Underwood *et al*, 2006). Despite this, a role for Bcl-2 in the initiation and progression of breast cancer is suggested as the anti-apoptotic factor is overexpressed in approximately 80% of breast tumours (Zhang et al, 1999). We observed that E2 did not greatly impact upon the expression of *BCL-2* in the inner cells of MCF-12A acini, suggesting that luminal filling was not due to increased *BCL-2*. This observation is supported by previous studies whereby ectopic *BCL-2* expression in 3D cultures of MCF-10A cells resulted in larger acini with delayed onset on hollow lumen formation, but where complete luminal filling did not occur (Debnath *et al*, 2003; Karantza-Wadsworth *et al*, 2007).

E2 induced a down-regulation of *BCL-XL* expression in comparison to untreated controls in both the inner and outer cell populations, but this was only statistically significant in the outer population. As Bcl-xl is an anti-apoptotic factor, its down-regulation would contribute to a pro-apoptotic influence. Our observations were therefore, unexpected, as in **Chapter IV**, we demonstrated that estrogens induced acinar malformations that appeared to be associated with an increase in cell proliferation and a decrease in cell death. Furthermore, gene expression analysis of whole acini showed that E2 up-regulated *BCL-XL* expression. The reason for the discrepancies in the data may be down to experimental

error or it could be that E2 did in fact down-regulate *BCL-XL* expression. In this instance, given the reduced levels of apoptosis observed in estrogen-treated acini, it would appear that Bcl-xl alone does not play a major role in the formation and maintenance of the hollow lumen. Indeed, it has been shown that ectopic expression of *BCL-XL* did not inhibit lumen formation, but did delay its formation (Debnath *et al*, 2002; Mills *et al*, 2004). However, a down-regulation in *BCL-XL* expression in response to estrogens has not previously been reported in the literature and in the future will have to be confirmed. The gene expression data from FACS-separated acini agrees with previous observations that E2, in monolayer cultures of MCF-12A cells, up-regulated *BCL-2*. However, the remaining gene data is not in agreement with the monolayer data, and although this may be due to experimental error, it is well known that cells cultured in 3D behave differently to those cultured in monolayer in response to a stimulus (Horning *et al*, 2008; Pickl & Ries CH, 2009; Yang *et al*, 2009).

E2 treatment of MCF-12A acini induced a significant down-regulation of *BAX* expression in both the inner and outer cell populations within MCF-12A acini. This effect seemed to indicate that E2 treatment has an anti-apoptotic influence in both populations, which was corroborated by the dramatic decrease in the Bax/Bcl-2 ratio induced by E2 in both inner and outer cells. Decreased Bax expression and a role for Bax in tumour progression have been postulated in patients with breast cancer (Krajewski *et al*, 1995; Sturm *et al*, 2000). In combination with the data presented here, this may mean that Bax is involved in the estrogen-induced acinar malformations, in regards to luminal filling, and that this may be one contributing factor that leads to a cancerous phenotype. To date, there is little literature concerning Bax expression in 3D cultures of mammary epithelial cells. In one of the few reports concerning this gene, acini formed from primary breast cancer cells treated with PI3K pathway inhibitors, displayed increased Bax at the protein level. The authors concluded that activation of the PI3K pathway was necessary for tumour growth in a 3D setting, and acted in a way that suppressed the apoptotic process (Polo *et al*, 2010). In **Chapter VI**, we demonstrated that in monolayer cultures of MCF-12A cells, E2 was capable of inducing PI3K, as inferred by the increased detection of activated AKT. Furthermore, in **Chapter V**, we observed that inhibition of PI3K signalling by the AKT inhibitor, LY 294002, resulted in increased signs of apoptosis. Although the activation of AKT in 3D cultures of MCF-12A cells is yet to be investigated, it may be plausible that through the activation of PI3K signalling, *BAX* expression is decreased, and, hence, less caspase-mediated apoptosis is observed.

To date, no ERE has been confirmed in the promoter region of the *BAX* gene, which raises the question as to how E2 was able to impact upon its gene expression. The presence of Sp-1 or Ap-1

binding sites in the *BAX* gene have not yet been disproved and, therefore, the ER may be co-operating with Sp-1 or Ap-1 in order to induce transcriptional responses. Alternatively, it is possible that the E2-induced activation of the MAPK and PI3K signalling cascades, culminating in a transcriptional response. Indeed, as mentioned above, E2 resulted in the activation of both PI3K and MAPK signalling in MCF-12A cells, and in other cell lines. Given that c-Myc activity is increased upon MAPK pathway activation and p53 activity is repressed upon PI3K pathway activation; and that p53 and c-Myc regulate *BAX* transcription, it is plausible that estrogens impact upon the expression of *BAX* via this mechanism (Miyashita & Reed, 1995; Mitchell *et al*, 2000).

In **Chapter VI**, no significant changes were observed in *BAD* expression in response to E2 in the monolayer cultures of MCF-12A cells. When gene expression analysis for whole MCF-12A acini was performed, again, no significant changes in *BAD* expression were observed in response to E2 either. However, when E2-treated FACS-separated acini were analysed, it was observed that E2 induced a down-regulation of *BAD* expression in the inner cell population in relation to control samples. However, an up-regulation of the gene was observed in the outer cells. Given that E2 had opposing effects on *BAD* expression in each population, this may explain why, in whole acini, no effect was observed. As described previously, Bad is a pro-apoptotic factor; consequently, a down-regulation of this gene would result in an anti-apoptotic influence over the central cells of MCF-12A acini promoting increased cell survival, which would not occur under control conditions. Taken together with the E2-induced reduction in Bax/Bcl-2 ratio, our data suggests that E2 has an anti-apoptotic effect upon both populations of acini, although compared to etOH controls, this is more pronounced in the inner population. Interestingly, this observation is in agreement with the observations presented in **Chapter IV**, where estrogens disrupted acini formation. Although BPA and n-propylparaben appeared to impact on acini as a whole (i.e. both the inner and outer populations), the disruption induced by E2 appeared to concentrate on the inner cells within acini. This was inferred from the observations that BPA and n-propylparaben resulted in much larger acinar with greater malformation in regards to their shape, in comparison with E2-treated acini. We speculate that, if the endogenous hormone, had a strong impact upon the proliferation of the outer cells, the resultant acini would be more deformed and larger (as seen with the other treatments). Taking into account the gene expression data presented in this chapter, it is possible that E2 targets the inner cells more than outer cells, and therefore has a greater impact on lumen formation, as opposed to acini size and morphology.

In addition to investigating the effects of E2 upon the expression of key apoptotic factors during acini formation, the expression of the cell cycle progression marker, *CCND1*, was also studied. Cyclin D1 has previously been suggested to mediate the proliferation of cells treated with steroids, such as estrogen, and studies have shown that increased ER expression in breast cancer cells correlates with increased levels of the *CCND1* gene (Buckley *et al*, 1993; Sicinski *et al*, 1995). Under control and E2 conditions, the inner cells of acini expressed less *CCND1* compared to the outer cells, although this was not statistically significant. This would appear to suggest that the outer population of cells within MCF-12A acini have a greater level of proliferative ability. It is important to note at this point, that the proliferative ability of cells within acini differs depending on incubation time in Matrigel. We demonstrated in **Chapter III**, when we defined the morphogenesis of MCF-12A acini, that cells undergo proliferative arrest, as inferred by a cessation in the increase of acinar size, following approximately 16 days in 3D culture. Similar findings have been reported in the literature (Debnath *et al*, 2003). At 7 days, acini are still growing, whilst hollow lumen formation simultaneously occurs, and therefore it would be expected to observe active proliferative genes in the outer cells. However, if cells were recovered following 20 days incubation in Matrigel, when growth-arrest of acini has occurred, it is likely that genes associated with proliferation would not be expressed at such levels. It may be of interest in the future, to perform gene analysis on FACS-separated acini at various time-points in the morphogenic process to identify how the expression profile of both the inner and outer populations changes over time.

Following whole extraction of E2-treated acini from Matrigel and gene analysis on the whole acini population, *CCND1* was significantly up-regulated. In the FACS-separated MCF-12A 3D cultures, E2 treatment increased the expression of *CCND1* in both the inner and outer cell populations. However, this was only a small effect and was not significant. Regardless, it would appear E2 up-regulates the expression of *CCND1* in MCF-12A cells grown in 3D, and this may confer a stimulatory effect on proliferation, and account for the lack of proliferative suppression in E2-treated samples. A role for cyclin D1 in mediating acinar malformations in 3D cultures of MCF-10A, as discussed previously, has already been alluded to, and our data supports these findings as increased *CCND1* expression correlated with distortion of MCF-12A acini (Debnath *et al*, 2003).

In this chapter we have demonstrated for the first time that estrogen treatment of ERα, ERβ, GPER-1 competent non-tumourigenic MCF-12A cells cultured in 3D impacts upon the gene expression of apoptotic factors. These changes in gene expression impact upon the Bax/Bcl-2 ratio and may be

involved in the malformations induced by estrogens, described in **Chapter IV**. Previous studies utilising 3D cultures to study the impact of apoptotic factors upon acini formation concentrate on knock-out or ectopic expression of single genes, and has shown that although implicated in luminal clearing, many factors are not solely responsible for the correct formation of acini. For example, the disruption of *BAD* or *CCND1* expression alone in 3D culture has previously been found not to induce luminal filling, but did correlate with increased levels of DNA fragmentation and partial apoptotic resistance after apoptotic stimulus, or delayed luminal clearance (Debnath *et al*, 2003; Schmelzle *et al*, 2007). These previous observations have led to the hypothesis that in order to induce luminal filling, there must be increased proliferative signalling in addition to decreased apoptosis (Humphreys *et al*, 1996; Debnath *et al* 2002; Shaw *et al*, 2004). The data presented in this chapter partially supports this theory, in that changes in *BCL-XL*, *BAX*, *BAD* and *CCND1* expression were observed in E2-treated samples, rather than a change in the expression of a single gene. Thus, our data appears to indicate that changes in the expression of multiple genes are responsible for the malformed MCF-12A acini observed, when these cells are treated with estrogens, and the impact of estrogens on gene expression differs between the inner and outer populations of MCF-12A acini. It should be noted, though, that apoptotic genes may undergo post-transcriptional modifications which affect their ability to perform their functions, as described previously. It may, therefore, be of interest in the future to study the presence of activated protein forms by immunocytochemistry of acini, as performed in **Chapter III**, **IV** and **V**, or protein analysis of FACS-separated acini.

In conclusion, we have developed a technique by which acini can be separated into the inner and outer populations and have shown that these two populations differ in their gene expression profiles. The protocol is, however, in its infant stages and requires further optimisation and validation. Following this, we would be able to investigate of a wider selection of genes which would enable us to further understand the mechanism by which estrogens impact upon acini formation. Furthermore, it would also be of interest to conduct protein analysis of the two populations within acini, with targets such as P-AKT and P-ERK1/2, as was performed in monolayer cultures. Nevertheless, it appears that E2 does impact upon gene expression in a way that would contribute to an inhibitory effect upon apoptosis and this may, therefore, account for the E2-induced acinar malformations. Given that attachment of normal mammary epithelial cells to the basement membrane is essential for cell growth and survival, the reduction in the Bax/Bcl-2 ratio and Bad expression within the inner cell population may play a role in enabling these cells to evade apoptosis (Weaver *et al*, 1997).

Chapter VIII: Final Discussion and Conclusions

As detailed in **Chapter I**, estrogens are essential for the normal development of the mammary gland (Korach *et al*, 1996). Traditionally, it was thought that the actions of these endogenous hormones were mediated solely by a nuclear ER, in the form of ERα and ERβ. More recently, it has been proposed that estrogens are capable inducing extra-nuclear effects by interacting with estrogen responsive receptors associated with the cell membrane. One such transmembrane receptor is the G-protein-coupled receptor: GPER-1 (Filardo *et al*, 2002), and hence, we also investigated the role of this receptor in cellular responses to endogenous estrogens and xenoestrogens, in addition to the traditional ER.

Despite the essential role of estrogens in breast development, they are, paradoxically, also linked to the development and progression of breast cancer. It is now widely accepted that some of the most important risks factors for breast cancer are, in fact, linked to the cumulative lifetime exposure to estrogens. Although the intracellular mechanisms of estrogenic action and the role of estrogens in neoplastic transformation have been extensively studied, little is known about how estrogenic effects lead to changes in the structure of the mammary epithelium, which are characteristic of breast cancer. In addition to this, even though a large number of environmental contaminants have been shown to have estrogenic activity at different physiological levels, evidence that these might also be responsible for cancer development is still missing. We, therefore, selected the endogenous estrogen, and a small panel of xenoestrogens, to investigate the impact of estrogenic compounds on acini development in an *in vitro* model.

8.1 Evaluating the Effects of Estrogens in Monolayer

The aim of the work conducted in this thesis was to utilise a 3D model to investigate the impact of estrogens on acini morphogenesis, using an ER competent non-tumourigenic mammary cell line. Therefore, our first task was, to identify a cell line in which the ER was expressed. We identified the MCF-12A cell line expressed ERα and ERβ, and, for the first time, showed that this cell line was also GPER-1 competent. Based on this, MCF-12A cells were selected as an example of an ER positive non-tumourigenic cell line for future 3D studies. In conjunction with this, the ERα, ERβ and GPER-1 status of the MCF-10A, MCF-7 and MDA-MB-231 cell lines was also confirmed. This was particularly necessary for the MCF-10A cell line, which was to be used as an example of an ER negative non-tumourigenic mammary cell line for comparison with MCF-12A cells grown in Matrigel.

We next sought to investigate whether E2 stimulated cellular proliferation of normal MCF-12A cells, and whether this was mediated by the ER or GPER-1. Unsurprisingly, E2 did not impact upon the proliferation of the ERα negative MCF-10A and MDA-MB-231 cell lines. However, due the presence of ERα, we expected that E2 treatment would increase the proliferation of MCF-12A and MCF-7 cells. This was only observed in MCF-7 cells, where E2 induced a small increase in proliferation, agreeing with previously published data (Spink *et al*, 2006; Wang *et al*, 2008[b]). As discussed previously, there were a few reasons why we did not observe a proliferative response to E2 in MCF-12A cells, and such a small increase in MCF-7 proliferation. Briefly, it may be that ER-competent cells do not proliferate in response to estrogens and instead secrete growth factors via a paracrine fashion to ER negative cells in close proximity and it is these ER negative cells that then proliferate (Russo *et al*, 1999; Mallepell *et al*, 2006; Gupta *et al*, 2007). Another possibility is that the expression of ERβ, negated the proliferative effect of E2 mediated by ERα (Sotoca *et al*, 2008). However. it may be that non-tumourigenic cell lines do not proliferate in response to E2 (Haslam, 1986). Regardless of the reason, we observed that monolayer cultures of MCF-12A cells do not proliferate in response to E2 and these unexpected observations led us to wonder if the effects of estrogen treatment in a 3D culture model would substantiate those observed in monolayer culture.

8.2 The Complexities of 3D Culture

Prior to studying the impact of estrogens in a 3D culture model of MCF-12A cells, it was first necessary to adapt the methodology used by Debnath *et al* (2002), for use with this cell line. In **Chapter III**, we successfully adapted the 3D methodology (Debnath *et al*, 2002) to the MCF-12A cell line. The most challenging aspect of this was selecting the correct composition of Matrigel. As Matrigel is produced in batches, the availability of a particular specification is not always a possibility, which forced us to screen several batches of Matrigel with different endotoxin and protein concentrations to identify the most appropriate compositions available to us. We found that MCF-12A cells grown in Matrigel only formed spherical acini with hollow lumen in one batch of Matrigel tested. This batch had a protein composition of 10.3 mg/ml and an endotoxin level of less than 1.5 EU/ml. In batches where the protein composition was less than 9.5 mg/ml, or the endotoxin level greater than 2 EU/ml, MCF-12A, acini did form, but lacked completely hollow lumen. It remains unclear whether the determining factor concerning the suitability of a Matrigel batch was the protein concentration or the endotoxin level, although it is known that endotoxins can impact upon cellular and physiological responses, both *in vitro* and *in vivo* (Kendrick

et al, 2008). Regardless, these observations highlight the importance of selecting the appropriate Matrigel for individual cell lines, prior to commencing experiments. Importantly, it appears that a low endotoxin level and high protein concentration is necessary for the formation of acini, and this was also reported with 3D cultures of MCF-10A cells (Debnath *et al*, 2003).

Using our optimised 3D methodology, we proceeded to establish a time-course for the morphogenesis of MCF-12A acini, in order to understand when some of the key events in the morphogenic programme occurred. Basement membrane deposition was an early event in the morphological programme and is present following just four days incubation. Acini increased in diameter up until approximately day 12 and then underwent growth arrest. Apoptotic onset, as indicated by the presence of activated caspase-3 and luminal clearing, was evident following 12 days incubation and after 16 days, the lumen were near hollow. The final acini consisted of a spherical single layer of cells, in contact with a basement membrane, surrounding a hollow, or near-hollow, lumen. These MCF-12A acini were very similar to those formed in the 3D culture of MCF-10A cells, both in ours and in other laboratories, in respect to their morphological programme and final structure (Debnath *et al*, 2002; 2003). This similarity acted as validation to our 3D model of ER competent mammary epithelial cells, which allowed us to proceed to the next stage of investigating the impact of estrogenic compounds upon MCF-12A acini formation with confidence in the reliability of the chosen assay.

8.3 Estrogens in a 3D Culture Model Disrupt Acini Formation

In **Chapter IV**, we began to investigate the impact of estrogens on the formation of MCF-12A acini. Firstly, we conducted a dose response for MCF-12A acini with E2 concentrations ranging from 1 pM to 10 nM. We observed that disruption of the MCF-12A acini structures occurred in a dose-dependent fashion. This disruption presented as increased acinar size, luminal filling, and, at concentrations exceeding 1 nM, loss of spherical shape. From these results, it was decided to use 1 nM E2 in subsequent experiments, as it physiologically relevant, commonly used in the literature to study estrogenic effects in *in vitro* studies and induced clearly identifiable disruption in 3D cultures of MCF-12A acini.

Four estrogenic compounds were then selected, in addition to the endogenous hormone, to investigate the impact of estrogenic compounds on MCF-12A acini, following 16 days incubation: *o,p'*-DDT, β-HCH, BPA and n-propylparaben. All of the test compounds induced acinar malformations to some extent, such as luminal filling, irregular shape, increased acinar size and decreased apoptosis. BPA

and n-propylparaben were selected, in addition to E2, to be used in further experiments, due to their current interest in the literature and the media (in regards to their endocrine disrupting properties and everyday exposure), their estrogenic qualities and their clear effects on MCF-12A acini.

In time-course analysis experiments, basement membrane deposition was not affected by the test compounds. However, increased size of treated samples, compared to controls, was observed following just four days and continued throughout the incubation period. An additional indicator of uncontrolled proliferation presented as misshapen acini, as opposed to the spherical acini observed in controls throughout the time-course.

In addition to allowing acini to escape proliferative control, estrogen treatment also had a suppressive impact on apoptosis, as shown by the reduction in activated caspase-3. Furthermore, there was no evidence of hollow lumen formation with samples treated with the test compounds. This adds to the evidence presented in this thesis, that estrogens induce luminal filling of MCF-12A acini and do so, at least to some extent, through suppression of caspase-mediated apoptotic pathways. One interesting observation was that acini aberrations were more pronounced upon treatment with BPA and n-propylparaben than with the endogenous hormone, E2. This suggests that although these xenoestrogens are considered less potent than E2, they cause greater acinar deformations in a 3D culture model. This raises the question as to whether the effects of these xenoestrogens are mediated by the same receptors or pathways as endogenous E2, or whether they target additional processes, that have not been covered in this thesis. Indeed, the metabolites of BPA, have been shown as having the ability to form DNA adducts, a process which may lead to genotoxicity (Atkinson & Roy, 1995). It is worth noting at this point that the BPA, in addition to the metabolites of E2, are also capable of transforming MCF-10F cells, a normal breast epithelial cell line that is ERα negative but ERβ competent (Russo *et al*, 2002; Fernandez & Russo, 2010) and that these effects have been attributed to genotoxicity. Another hypothesis is linked to the genomic effects of BPA, as the gene expression profile of cells, both *in vitro* and *in vivo*, is affected differently by E2 and BPA, and this may account for some of the differences in the acinar malformations between the two estrogenic compounds (Shioda *et al*, 2006; Natsoulis *et al*, 2008).

On the other hand, it has been shown, that BPA, even at low doses, is capable of activating non-genomic signalling pathways, mediated by a membrane-associated estrogen receptor, in a different manner to E2 (Bouskine *et al*, 2009). Similar observations, relating to different activation of pathways have been made with other environmental estrogens, such as endosulfan, nonylphenol and β-HCH,

(Bulayeva and Watson, 2004; Silva *et al*, 2010). Overall, this may mean that xenoestrogen evaluation needs to be based, not only on the affinity to the nuclear ER, but also on the affinity to other membrane-associated receptors, as well as activation of extranuclear cascades. It is important that the non-genomic effects are taken into consideration when studying the full estrogenic profile of xenoestrogens, as these may have physiological effects *in vivo* and in humans that are not directly linked to the ER.

If the perturbations in MCF-12A acini in response to estrogens are indicative of neoplastic transformation, the question arises as to whether BPA and n-propylparaben are more carcinogenic than endogenous E2. The question to whether BPA does act as a carcinogen remains of some debate. A study in rats and mice showed that BPA had carcinogenic properties in rats, but not in mice, and concluded from this that BPA was not considered a carcinogen (NTP, 1982). However, more recently, a link between neonatal exposure to BPA and breast cancer in rats has been demonstrated (Durando *et al*, 2007). Furthermore, it is proposed that increased exposure of developing foetuses to endogenous and exogenous estrogens (including BPA and n-propylparaben) sensitize tissues, such as the prostate or the breast, via epigenetic mechanisms (i.e. methylation). With exposure in later life, estrogens act upon these "sensitized" tissues to promote cancer initiation and progression (McLachlan *et al*, 2001). However, it remains to be seen whether the carcinogenic properties of BPA, n-propylparaben, or indeed other xenoestrogens are stronger than endogenous E2 in humans, and although our 3D model clearly shows that the effects elicited by BPA and n-propylparaben were greater than those induced by E2, this *in vitro* assay does not provide us with the full picture as to the mechanisms and carcinogenic properties of the test compounds.

Regardless as to the mechanism through which xenoestrogens appeared to induce greater acinar malformations that endogenous E2, it is clear that these compounds induce perturbations in MCF-12A acini. Both BPA and parabens have been identified in human breast tissue samples, suggesting that they may accumulate in the body, leading to continuous long-term exposure (Darbre *et al*, 2004; Fernandez *et al*, 2007). These compounds are also widely used: BPA in food packaging and parabens in cosmetics and as food preservatives, and hence, exposure to them occurs on a daily basis. Therefore, if these compounds, were indeed found to induce neoplastic lesion formation, it could have dramatic consequences for an individual's cancer risk.

In **Chapter IV**, we observed that both the ER and GPER-1 are involved in estrogen-induced disruption of MCF-12A acini. The antagonists of these receptors did not, however, completely revert all

of the effects of the estrogens tested and this may suggest that other receptors or factors are involved. Alternatively, it may be that the estrogens or their metabolites are exerting genotoxic effects, independently of any receptor. Indeed, it has been demonstrated that estrogen metabolites are capable of forming DNA adducts, and this can lead to mutations (Ashburn *et al*, 1993). We also observed that small perturbations in MCF-10A acini (ER negative, GPER-1 positive) were present following treatment with the test compounds. It is unclear at this point whether this effect was mediated by GPER-1, or indeed another receptor, or was due to genotoxicity, which has been demonstrated in 3D cultures of MCF-10F cells in response to E2 (Russo *et al*, 2002; Lu *et al*, 2007).

In **Chapter V**, we demonstrated that both the PI3K and MAPK pathways are involved in the estrogen-induced disruption of MCF-12A acini. Inhibition of these pathways resulted in smaller spherical acini, indicating that these pathways may be disrupted by estrogens, resulting in the loss of proliferative control. The impact of pathway inhibition upon estrogen-induced apoptotic suppression and luminal filling was less significant, although there was a small increase in luminal clearing and activated caspase-3 following incubation with PI3K or MAPK inhibitors, in combination with the test compounds.

The data presented in **Chapters IV and V** supports previous *in vivo* evidence that estrogens can disrupt the processes involved in mammary gland development. For example: *in utero*, estrogen exposure results in less apoptosis in the TEBs of rat mammary glands; and E2 causes over-proliferation in rat mammary glands (Hilakivi-Clarke *et al*, 2006; Murray *et al*, 2007; Starlard-Davenport *et al*, 2010).

Although it is clear that estrogens induce acinar disruptions in 3D cultures of MCF-12A cells and that these perturbations resemble those seen in the early stages of breast cancer *in vivo* and in humans, it cannot be claimed that the structures are of a malignant phenotype. Genotyping of acini would allow us to see if genes involved in cancer progression, such as *TP53* (the gene that encodes p53), have been affected by estrogen treatments. Furthermore, and more importantly, these findings would need to be reproduced *in vivo*, to validate the hypothesis that estrogens impact on the architecture of the breast. One such way in which a transformed phenotype of estrogen-treated acini could be confirmed, is through the implantation of treated acini into mice, and seeing if tumour formation occurs.

Regardless of whether estrogens elicit their effects via genomic, non-genomic mechanisms or through genotoxicity, it is apparent that the test compounds impact on both proliferation and apoptosis. Previous studies have shown that ectopic expression of single genes does not impact upon lumen formation. However, when apoptosis is suppressed and proliferation stimulated simultaneously,

luminal filling is observed (Debnath *et al*, 2002; Reginato *et al*, 2005). This would suggest that the disruption of acini requires a combination of uncontrolled hyper-proliferation and apoptotic suppression, both of which were observed in the estrogen-treated 3D cultures of MCF-12A cells. In light of work carried out by Russo and colleagues (2002; 2003; 2006), a possible role for estrogen-induced genotoxicity may also contribute to the malformations of MCF-12A acini in response to the presence of estrogenic compounds. Importantly, the work presented in this thesis, has demonstrated that estrogens and xenoestrogens impact on the formation of mammary acini in a 3D *in vitro* model. If the same is true *in vivo*, or in humans, these estrogenic compounds, particularly BPA and n-propylparaben, may have serious detrimental consequences for the formation and maintenance of the mammary gland in humans and, hence, exposure to them may contribute to an individual's risk of developing breast cancer. Given that many xenoestrogens are highly lipophilic and are consequently stored in breast and adipose tissues, and the prevalence of BPA and parabens in everyday products, exposure is common place and the effect of these compounds, and their impact on human health, needs to be better understood.

8.4 The Effects of Estrogens on in Monolayer and a Role for GPER-1

In **Chapter VI**, we investigated the impact of E2 on the gene expression of key apoptotic factors in monolayer cultures, with the aim to extend this to 3D cultures of MCF-12A cells. We observed that estrogens did impact on apoptotic factor gene expression in monolayer cultures of breast cells. ER antagonism did not always abrogate the genomic effects of E2, implying that although the ER does have a role in the transcriptional responses of apoptotic genes to estrogens, another estrogen-responsive receptor is also implicated. We identified a potential role for GPER-1 in mediating some of the genomic responses to endogenous E2, in both ER negative and ER positive mammary epithelial cell lines. Correlations between ER and Bcl-2 expression have been reported in breast cancer patients, thus supporting a role for this receptor in regulating the expression of *BCL-2* (Rehman *et al*, 2000; Sjöström *et al*, 2002). However, to our knowledge, there is no data concerning the correlation between GPER-1 and apoptotic factor expression. It remains to be seen if these observations in monolayer are replicated in 3D models, or indeed *in vivo*. However, it is clear that E2 can impact upon the expression of genes involved in apoptosis and, thus, act to suppress cell death.

The Bax/Bcl-2 ratio was calculated to see if the changes in gene expression would result in an overall apoptotic or anti-apoptotic influence. We observed that in ERα cells, the Bax/Bcl-2 ratio was decreased by E2, which would contribute towards an anti-apoptotic effect. This may imply that the

presence of ERα has an impact on responses of cells following estrogen treatment, regardless of the expression of other estrogen-responsive receptors, such as GPER-1. In the future, investigating the effects of E2 on a wider panel of breast cell lines or isogenic cell line studies (for example, where the genomic effects of E2 are analysed in two MCF-12A populations: one expressing ERα, and the other where ERα expression has been abrogated) may shed some further light onto this hypothesis.

In **Chapter VI**, we also investigated the impact of E2 upon PI3K and MAPK signalling, concentrating on whether this was mediated by GPER-1. It was our hope to continue this investigation with 3D cultures of MCF-12A cells; however, time, technical and financial constraints ultimately prevented this. We found that E2 activated MAPK signalling in MCF-10A and MCF-12A cells, via GPER-1. This supports previous findings that E2 is capable of inducing non-genomic responses in the ER negative SK-BR-3 breast cancer cell line, possibly through activation of GPER-1 (Filardo *et al*, 2000). E2-induced activation of the PI3K was also observed in MCF-12A, MCF-7 and MDA-MB-231 cells, although this was not GPER-1 mediated. This may implicate the ER, as opposed to GPER-1, in mediating these effects, as has been demonstrated in MCF-7 cells (Lee *et al*, 2005; Pedram *et al*, 2006). In combination, this protein analysis data suggests that E2 can activate the PI3K and MAPK signalling cascades, and that GPER-1 mediates some of these non-genomic responses, even though it is clearly not the sole player in these events. It is unclear whether the ER is mediates these responses in the cells studied, but given that MDA-MB-231 cells do not express ERα, it may be that ERβ or another estrogen-responsive receptor is responsible. Indeed, it has been demonstrated that in non-small cell lung cancer (NSCLC) cells, ERβ can mediate the activation of the MAPK and PI3K signalling pathways (Zhang *et al*, 2009). Unfortunately, there is not a wealth of data concerning the non-genomic responses to estrogens in the cell lines studied (with the exception of the MCF-7 cell line). This prevents us from comparing some of the results with pre-existing data. It does however for the first time demonstrate that in ER negative MCF-10A, as well as ER positive MCF-12A cells, estrogens are capable of inducing non-genomic responses.

Given that both in monolayer and in 3D, estrogens exerted some of their effects via GPER-1, it may be that GPER-1 is a potential target for breast cancer therapy and that, to negate the effects of estrogens on breast cancer cells in humans, both the ER and GPER-1 need to be targeted. This theory will need further investigation in animal models, but may be a possibility in overcoming some of the effects of endocrine-therapy resistant tumours or targeting ER negative breast cancers.

8.5 Acini can be Separated into Inner and Outer Cell Populations

From the LCM images in **Chapter III**, **IV** and **V**, it was clear that within MCF-12A acini, two cell populations exist: an inner and an outer population. The outer cells organise themselves into a single layer surrounding a hollow lumen, do not undergo apoptosis and have contact with the basement membrane. The inner cell population, on the other hand, are the centrally located cells that undergo apoptosis and are cleared, resulting in the formation of a hollow lumen. Given that these two populations have two very different fates, it was hypothesised that their gene expression profiles would be different. In order to investigate this, however, it was first necessary to find a method that was capable of separating the two populations. In **Chapter VII**, we developed a method through which we could separate the inner from the outer cells of MCF-12A acini for gene analysis studies, which to our knowledge, has not been previously reported. The yields were low, but sufficient sample was obtained to proceed with RNA extraction and real-time PCR analysis of the expression of the selected target genes. This technique provides us the opportunity to further understand the molecular mechanisms involved in the morphological programme of acini, and how compounds, such as estrogens, can act on these structures to cause a phenotype that resemble breast cancer *in vivo* and in humans.

Analysis of whole MCF-12A acini showed that E2 increased the expression of *CCND1*. This would aide in cell cycle progression and, therefore, may also be one of the mechanisms through which the estrogens in **Chapter IV** increased cellular proliferation, and consequently led to the formation of larger, disorganised acini. We then proceeded to investigate the differences in gene expression between the inner and outer cell populations in solvent control acini. We observed that the inner cells expressed less *BCL-XL* compared to the outer cells and increased levels of *BAD* and *BAX*. This means that the inner cells of acini are more likely to undergo apoptosis compared to the outer cells under normal conditions.

We next compared the gene expression of target genes in the outer and inner populations in E2-treated samples, relative to controls. *CCND1* expression was up-regulated in response to E2 in both populations, indicating increased proliferative ability or the ability to overcome proliferative suppression. We also found that E2 increased the expression of the anti-apoptotic factor, *BCL-2* in both populations. Unexpectedly, E2 also down-regulated the expression of *BCL-XL* in both populations, and the exact reason for this remains unclear. The most pronounced observation was that E2 down-regulated *BAX* expression in both inner and outer cells, which would confer an anti-apoptotic influence. *BAD* was down-regulated in the inner population also, although was up-regulated in outer cells. When the Bax/Bcl-2 ratio was calculated, E2 decreased the Bax/Bcl-2 ratio in both the inner and outer cell

populations, which implies an anti-apoptotic effect. Overall the data appears to suggest that E2 impacts upon the gene expression of the inner and outer populations differently. The data appears to indicate that E2 has a more profound effect upon apoptotic genes in the inner cells, which is hardly surprising considering that these cells undergo apoptosis normally, and that the outer cells are already primed to survive.

8.6 Estrogens Induce Different Effects Depending on Culture Conditions

In **Chapter II**, we observed that E2 does not increase the proliferation of MCF-12A cells in monolayer culture. Conversely, when the same cell line was cultured in 3D and treated with estrogenic compounds, including the endogenous hormone, the resulting acini were larger than control structures (**Chapter IV**), an indication of increased proliferation and uncontrolled growth. On the other hand, the work conducted in **Chapter VI** showed that E2 treatment of monolayer MCF-12A cultures resulted in a significant increase in the gene expression of *BCL-2*, *BCL-XL* and *BAX*, whereas in 3D cultures, *BAX* and *BAD* expression were down-regulated in the inner cell population and *BCL-XL* expression was down-regulated in the outer population after estrogen treatment.

The discrepancies between monolayer and 3D culture of MCF-12A cell line is supported by previous studies in which cells grown in monolayer respond differently to cells cultured in 3D or *in vivo* (Horning *et al*, 2008; Pickl & Ries CH, 2009; Yang *et al*, 2009). Importantly, primary cultures of mammary epithelial cells have shown that in monolayer, ER's functionality is compromised, whereas it is regained in 3D cultures of the same cells, allowing estrogens to elicit their effects (Novaro *et al*, 2003). Furthermore, cells cultured in 3D systems express higher levels of ERα compared to those cultured in monolayer (Yang *et al*, 2000). Combined with the literature findings, our observations demonstrate the limitations of monolayer cultures, particularly in relation to the effects of estrogens upon proliferation. It also highlights the necessity to use more physiologically relevant *in vitro* models to investigate the impact of estrogenic compounds, or indeed any compound, upon cells behaviour and function. Unmistakeably, it cannot be denied that monolayer cultures have their benefits: they are cost-effective, easy to set-up and maintain, and are extensively used. They do, however, lack stroma and structural architecture, hindering the study of physiological processes. 3D cultures overcome some of these problems and have the advantage of being more physiologically relevant than traditional monolayer cultures. These 3D cultures permit a further understanding of cell organisation, cell-ECM interactions and function, and the effect of treatments on these processes. They are more complex and costly than

monolayer cultures and still not perfectly recapitulate the microenvironment of tissues, such as the breast, or take into account interactions between different cell and tissue types, or indeed, the interaction of different organ systems. More recently, 3D platforms that allow multiple cell types to be co-cultured have been developed, although *in vivo* models still provide the most physiologically relevant model we have available to us. *In vivo* models are however, more expensive, require special expertise and approval, and are subject to variability. Furthermore, there is now general pressure to encourage scientist to adopt experimental designs that move away from animal testing in a bid to reduce the amount of *in vivo* work (http://www.nc3rs.org.uk/landing.asp?id=2). For these reasons, developments in *in vitro* techniques, such as 3D cultures, are needed to replace or reduce the number of animals used in experimental research, whilst still maintaining a higher level of physiological relevance in comparison with traditional monolayer methodologies. Although work is still ongoing to improve 3D methodologies, these advances are a step in the right direction and present us with the most relevant way of studying physiological events, such as breast morphogenesis and the impact of estrogens on this process, without the need of *in vivo* studies.

8.7 Future Work

Although we observed various perturbations of MCF-12A acini following estrogen treatment, similar to those observed in the early stages of breast cancer *in vivo* (i.e. luminal filling and increased proliferation), the results do not yet tell us whether estrogens induced a neoplastic phenotype. Genetic analysis for markers of carcinogenesis, such as LOH and chromosome amplifications would need to be performed to confirm this. Furthermore, it would be interesting to extend this study into *in vivo* investigations and see if acini grown in the presence of E2 and implanted into the mammary fat pads of mice or rats, go on to form tumours.

Although more physiologically relevant than traditional monolayer cultures, 3D Matrigel still does not fully recapitulate the environment of the mammary gland. For instance, the mammary gland is not just comprised of epithelial cells; adipocytes, myoepithelial and fibroblasts are also present. As Matrigel cultures do not allow the interactions between the epithelia and stroma to be investigated, we are left with a lack of data concerning how these factors contribute to acini morphogenesis under normal and test conditions. This leads to failing in understanding the origin of tumour formation. Co-cultures of mammary epithelial cells with fibroblasts, a cellular component within the breast stroma, may have recently been developed in a bid to further understand how the interactions with the

epithelial cells and stroma impact upon mammary gland development (Sadlonova *et al*, 2005). More recently, co-cultures of epithelial cells with adipocytes has been achieved. This has involved culturing pre-adipocytes in a collagen system, to form a synthetic fat pad that recapitulates the mammary stroma, and then seeding epithelial cells on top of it. The result is the formation of branched structures with hollow lumen, similar to the ducts of the breast (Campbell *et al*, 2011). This may shed even further light into how the interactions between the various cell types that compose the breast stroma and epithelial cells converge to result in the development of the mammary gland. This co-culture methodology is not without its drawbacks, as different cell types require different growth conditions, leading to technical complexity. Nevertheless, developing such a system to test estrogen treatments, would allow us to more thoroughly investigate the effects of estrogens on the environment of the mammary gland and the consequences for the formation of the mammary ducts.

As stated in **Chapter VII**, time, financial and technical constraints, meant that we were unable to study the impact of estrogens on MAPK and PI3K signalling in 3D cultures of MCF-12A cells. It would be of great interest in the future to investigate whether these pathways are activated differently in the inner and outer populations of MCF-12A acini. This may provide further information relating to the reasons why only the inner cells undergo apoptosis and clearing, whereas, the outer cells survive. Further to this, it would be interesting to see if E2 impacts upon the activation of signalling pathways in each population, as this may allow us to understand more fully why estrogen treatment induced luminal filling and increased proliferation. As activation of the MAPK and PI3K signalling cascades are implicated in proliferation and survival, E2 is capable of activating these pathways in monolayer cultures of MCF-12A cells, and specific inhibitors reverted some of the effects of estrogens on 3D acini, it is plausible that some of the estrogen-induced acinar malformations are, in fact, attributable to the activation of these pathways. Further, direct confirmation of this possibility is required which could be achieved by experiments involving the study of activated AKT and ERK1/2, by immunoblotting, in separated MCF-12A acini.

Although we observed changes in gene expression in FACS-separated MCF-12A acini, following treatment with E2, it would be interesting to see if the same changes are observed in response to BPA and n-propylparaben. As these estrogenic compounds induced greater malformations of MCF-12A acini than E2, it may be that these have a greater impact on apoptotic factors or *CCND1* expression in a 3D setting. Until we further understand how xenoestrogens, such as these, impact upon gene expression or signalling pathways in a more physiologically relevant 3D setting, it remains hard to evaluate the impact

these compounds potentially have for the mammary gland development, breast carcinogenesis and cancer progression.

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