The missing link: endocrine disrupting chemicals, epigenetics and breast cancer risk

A Thesis Submitted for the Degree of Doctor of Philosophy

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Declaration

I hereby declare that the work presented in this thesis has been carried out solely by myself, except where explicitly stated otherwise within the text. Work contained herein is original and has not been submitted, in whole or in part, for any previous degree or professional qualification.

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Abstract

Evidence suggests that 26.8% of new breast cancer cases can be attributed to extrinsic influences, such as lifestyle and environmental factors. Whilst we have increased knowledge surrounding factors like alcohol and obesity, little is known regarding environmental pollutants, such as endocrine disrupting chemicals (EDCs), which are defined by their ability to interfere with the regulation of an individual's endocrine system. Research has so far proven inconclusive, with effects only observed at concentrations considerably higher than those present in human tissues and in unrepresentative assay systems.

Utilising 3D *in vitro* assays that recapitulate characteristics of the human mammary gland, the relationship between low-dose EDC exposures (comparable to concentrations found in human tissues) and breast carcinogenesis was investigated. This work showed, both in primary cells and in the ER-positive MCF-12A cell line, that EDCs can affect acini development, gene expression and DNA methylation. Changes were indicative of neoplastic transformations, including increases to acini size and loss of circularity. Genetic and epigenetic modifications to genes associated with breast tumourigenesis, such as cell cycle regulators and tumour suppressors, were observed at concentrations relevant to human exposures. Similar changes were seen in predisposed individuals with a *BRCA1* mutation, translating into a significant impact on absolute risk of breast cancer. The ability of chemical mixtures to increase breast cancer risk was also considered. When combined at concentrations in human tissues, four EDCs (dichloro-diphenyl-trichloroethane, benzophenone 3, bisphenol A and propylparaben) acted together to produce a significant effect, by disrupting acini formation and gene expression.

These findings demonstrate the capacity of EDC exposures to contribute to breast cancer risk. Increasing our understanding of chemical contributions to cancer development provides opportunities for cancer prevention and more comprehensive risk model development. Results demonstrate a need for further research in this area and act as a foundation for future studies.

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Abbreviations

AF-1	Activation function 1
AF-2	Activation function 2
AKT	Protein kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
BBP	Benzyl phthalate
Bcl-2	B-cell lymphoma-2
BMI	Body mass index
bp	Base pair
BP-3	Benzophenone 3
BPA	Bisphenol A
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CA	Concentration addition
CAF	Cancer-associated fibroblasts
CDK	Cyclin dependent kinase
cDNA	Copied DNA
CF	Curve fit
CHL	Chlordane compound
CNA	Copy number abnormality
CpG	Cytosine guanine dinucleotide
Ct	Cycle threshold
CXADR	Ig-like cell adhesion molecule
-d(RFU) / dT	Rate of change in relative fluorescence with time
DAPI	4', 6-diamidino-2-phenylindole
DAVID	Database for annotation, visualisation and integrated discovery
DBD	DNA binding domain
DES	Diethylstilbestrol
DMEM	Dulbecco's Modified Eagle Medium
DMP	Differentially methylated position
DNA	Deoxyribonucleic acid
DNAme	DNA methylation
DNAse	Deoxyribonuclease

DNMT	DNA methyltransferases
E1	Oestrone
E2	17β-oestradiol
E3	Oestriol
ECM	Extracellular matrix
EDC	Endocrine disrupting chemical
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Oestrogen receptor
ERK1/2	Extracellular signal regulated kinase 1/2
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FADD	Fas-associated protein with death domain
FBS	Foetal bovine serum
FDA	US Food and Drug Administration
FDR	False discovery rate
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FPKM	Fragments per kilo base per million mapped reads
GFR	Growth factor reduced
GO	Gene ontology
GPER	G protein-coupled receptor 1
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2/3	Human epidermal growth factor receptor 2/3
HGF	hepatocyte growth factor
HGH	Human growth hormone
HPLC	High-performance liquid chromatography
HRT	Hormonal replacement therapy
IA	Independent action

IBCERCC	Interagency Breast Cancer and Environmental Research Coordinating Committee
JNK	c-Jun N-terminal
Kow	Octanol/water partition coefficient
LBD	Ligand-binding domain
LogFC	Log2 (fold change) over control
MAPK	Mitogen activated protein kinase
MOAP1	Modulator of apoptosis1
mRNA	Messenger RNA
miRNA	MicroRNA
MTOR	Mechanistic target of rapamycin
***o,p*'-DDT**	Dichloro-diphenyl-trichloroethane
PBS	Phosphate buffered saline
PCA	Principle component analysis
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PCT	Polycyclohexylenedimethylene terephthalate
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinases
POC	persistent organochlorines
POP	Persistent organic pollutants
Rb	Retinoblastoma
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
RNase	Ribonuclease
RNA-seq	RNA sequencing
rt-PCR	Real-time PCR
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphism
STAT3	Signal transducer and activator of transcription 3
STR	Short tandem repeat
SVD	Singular value decomposition
TCDD	2,3,7,8-tetrachloridibenzo-p-dioxin
TDLU	Terminal ductal lobuloalveolar units
TGF-β1	Transforming growth factor-β1
Tm	Melting temperature

TNF	Tumour necrosis factor
Tor	Protein kinase target of rapamycin
TRAIL	TNF-related apoptosis-inducing ligand
UNEP	United Nations Environment Programme
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated calcium channel
VGPC	Voltage-gated potassium channel
WHO	World Health Organization
2D	Two dimensional
3D	Three dimensional

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Chapter One: Chemicals, the epigenome and breast carcinogenesis

1 Chemicals, the epigenome and breast carcinogenesis

1.1. Introduction

Breast cancer is the most common malignant tumour within women in developed countries (Siegel, Miller and Jemal, 2015, 2016; Torre *et al.*, 2015). Only 5-15% of breast cancer incidents are attributed to hereditary factors, leaving a substantial proportion that could be related to extrinsic causes (Jenkins *et al.*, 2012). It is believed that cancers are caused by both intrinsic and extrinsic factors, with an estimated 26.8% of new breast cancer cases being related to external elements, such as lifestyle and environmental exposures (Howell *et al.*, 2014). One such external factor could be the exposure to so-called endocrine disrupting chemicals (EDCs). EDCs are chemicals that have the ability to interfere with the endocrine system and can often mimic endogenous hormones, such as oestrogen. Whilst we have increased knowledge surrounding factors such as alcohol, smoking, obesity and stress in relation to breast cancer, little is known in regard to the role of EDCs. Moreover, no single established risk factor has been shown to substantially contribute to the development of breast cancer, suggesting, instead, that a more multifactorial view must be taken (Wu *et al.*, 2016). Without a complete understanding of all the potential risk factors, including chemical exposures, and how they may interact to contribute to the overall risk of carcinogenesis, it is likely that we are significantly underestimating individual's true risk of breast carcinogenesis. The possibility that extrinsic factors play a significant role in breast cancer risk provides opportunity for prevention strategies in the future, through improving public awareness of risk factors and regulating harmful chemical exposures (Forman *et al.*, 2015). It will also allow for a more effective risk model development framework, aiding in identifying individuals at high risk for screening programmes, through the inclusion of factors that are currently not represented (Howell *et al.*, 2014; Gail, 2015).

A report entitled *Breast Cancer and the Environment: Prioritizing Prevention* (IBCERCC, 2013), called for further research to understand how environmental influences could lead to breast cancer, in order to mitigate these risks and move towards a more bespoke preventative strategy for carcinogenesis. Chemical exposures were named as a potential risk factor, yet their

association with breast cancer development is not as clear as factors like smoking and alcohol consumption (Forman *et al.*, 2015). Although substantial evidence exists supporting the association between chemical exposure and breast cancer risk, historically, much of this has been circumstantial population trends or studies with single chemicals at concentrations much higher than observed population exposures (WHO/UNEP, 2013). One reason for this is that we are yet to fully comprehend the mechanisms linking chemical exposure to breast cancer risk. Originally, EDCs were though to exert actions through nuclear hormone receptors, including oestrogen receptors (ERs) and progesterone receptors; however now research demonstrates that EDC mechanisms are much broader than this (Knower *et al.*, 2014). There is laboratory and human evidence supporting a role for chemical exposures in breast cancer through genotoxic action, alteration to hormone responsiveness in the breast and hormonal tumour promotion, however these do not occur with all chemicals at concentrations relevant to human exposures (Rodgers *et al.*, 2018). Thus, researchers have proposed the epigenome as an additional target for EDCs.

Increasing numbers of reports suggest that the epigenome plays a significant role in breast cancer development (Sarkar *et al.*, 2013; Widschwendter, Jones and Teschendorff, 2013; Byler *et al.*, 2014; Basse and Arock, 2015; Martín-Subero and Esteller, 2017; Widschwendter *et al.*, 2018), as it can act as the interface between our external environment and our genome, meaning that various external factors can influence cell behaviour and be linked to diseases like breast cancer (Jirtle and Skinner, 2007; Bollati and Baccarelli, 2010). Epigenetic alterations caused by chemical exposures represent a non-ER dependent mechanism that could provide insight into the links observed between some chemicals and breast cancer development. Substantial work has been undertaken to define the role of the epigenome in cancer biology and areas where epigenetics could be used in prevention, early detection and novel treatment options have already been identified, (Sarkar *et al.*, 2013). Also, it has been recognised that external and lifestyle factors have the ability to interact with the epigenome, resulting in an increased susceptibility to various diseases, including cancers (Feil and Fraga, 2012). With this in mind,

the epigenome is being studied to determine whether exposure to EDCs can alter the epigenetic profile of tissues and lead to carcinogenesis, providing insight into a possible mechanistic link (Knower *et al.*, 2014). Research undertaken in relation to smoking (e.g. Lee *et al.*, 2015; Maccani and Maccani, 2015; Shenker *et al.,* 2013; Teschendorff *et al.*, 2015) suggests that there is a strong possibility widespread chemicals have the potential to alter the epigenetic profile of an individual (Bromer *et al.,* 2010; Fernandez *et al.,* 2012; Vardi *et al.,* 2010). However, most EDC studies have been conducted in relation to general cancer risk or for other specific health effects, rather than breast cancer. It is important to note that epigenetic modifications are tissue specific (Minard, Jain and Barton, 2009), meaning that we must consider studies that focus specifically on the breast, given that the same environmental contaminant may illicit different epigenetic effects within other tissues. With the high rates of breast cancer incidents within industrialised countries, it is imperative that we fully understand the role of chemicals, alone and in combination, and their potential mechanism of action in cancer development. This chapter will critically review the current knowledge surrounding chemical exposures, epigenetics and breast cancer development to identify knowledge gaps and set the context for following experiments.

1.2. Mammary gland development

During postnatal development, the mammary epithelium has two main stages of development. The first is during puberty where a ductal elongation phase establishes a network of ducts spreading from the nipple. In response to an increase in endogenous levels of oestrogen, this stage is driven by specialised growth structures at the tips of ducts called terminal end buds. These buds consist of two distinct cell types, luminal epithelium and myoepithelium, which make up luminal and basal cell layers, respectively. From these networks, terminal ductal lobuloalveolar units (TDLUs) are developed, which can produce milk in the future (Figure 1.1). Whilst the specific mechanism that triggers breast development remains elusive, it coincides with the activation of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal

axis, which results in an increase of oestrogen, inducing cellular proliferation (Rosenfield, Cooke and Radovick, 2014).

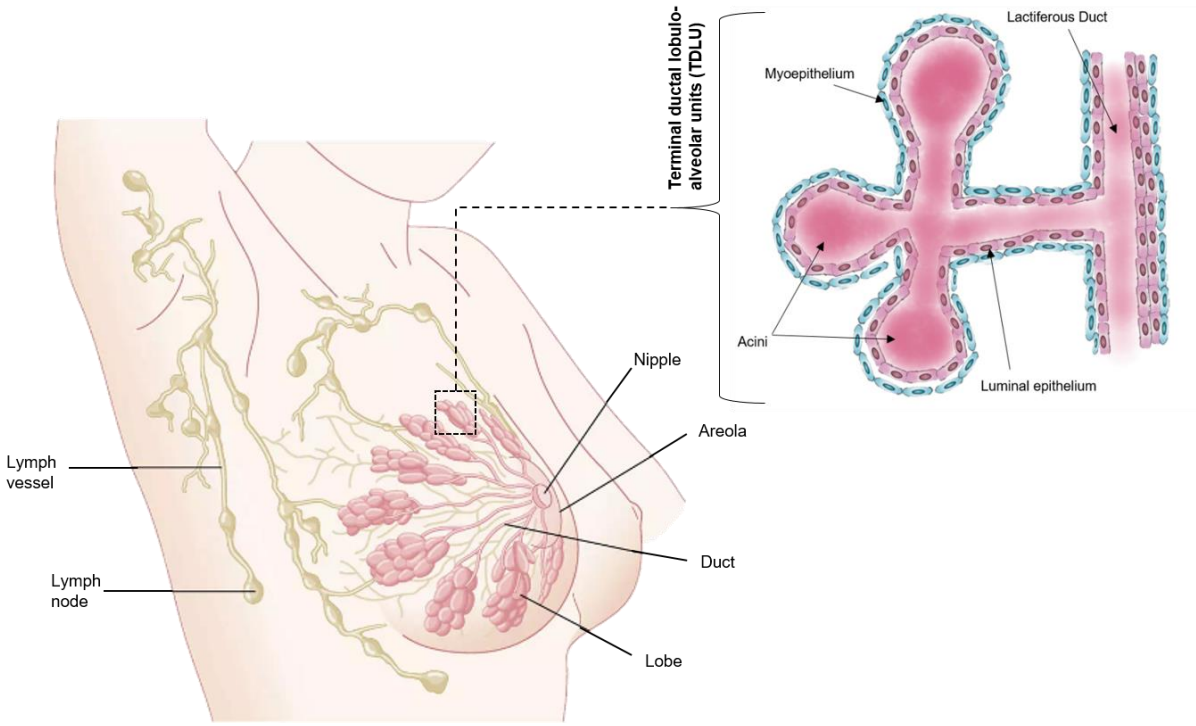

Figure 1.1. Overview of mammary gland morphology. Graphic depicting the structure of the female breast, highlighting terminal ductal lobuloalveolar units (TDLU). TDLUs are comprised of the acini structures made up of luminal and myo- epithelium which lead to ducts. Partly adapted from Breast Cancer Now, 2016.

In humans, TDLUs become more elaborate during pregnancy, which is the second phase of postnatal breast development. The main feature of this second phase of development is the extensive proliferation of epithelial cells and differentiation of TDLUs, induced by hormonal exposure. During lactation, luminal cells form differentiated milk-secreting cells and the basal layer (predominantly myoepithelial cells) will contract in response to oxytocin to force milk through the ducts to the nipple. Following pregnancy and breast feeding, the TDLUs regress through a process referred to as involution, which is largely regulated through apoptosis of the epithelial structures (Britt, Ashworth and Smalley, 2007; Kobayashi *et al.*, 2012). After this process, the gland will resemble a pre-pregnancy state, although some of the expansion will remain, namely the number of TDLUs and the degree of side branching will be higher (Kelly *et al.*, 2002).

1.3. Breast cancer

Breast cancer is the most common malignancy amongst women, with almost 50,000 women and 350 men diagnosed each year in the UK (Figure 1.2; Torre *et al.*, 2015; Breast Cancer Now, 2016; Desantis *et al.*, 2017; Siegel, Miller and Jemal, 2018). The number of cases has been increasing steadily in the western world since the mid-seventies, and this trend is now beginning to be observed in developing countries (Agarwal *et al.*, 2009; Torre *et al.*, 2016), posing major health challenges in the coming years. There is no single answer that can explain the observed increase, although several reasons have been proposed. Common themes include alterations to lifestyle factors (i.e. increases in obesity and reductions in physical exercise), people living longer and women choosing to have children later (discussed further in section 1.4). Breast cancer refers to a collection of breast related diseases that have diverse genetic, epigenetic and histopathological profiles (Vargo-Gogola and Rosen, 2007). Each type causes cells to change and grow out of control, no longer being bound by the regulatory and behavioural processes of normal cells. Despite significant improvements in detection, prevention and treatment, breast cancer remains the most frequent cancer type in women with around 1,000 women dying from the disease each month in the UK (Breast Cancer Now, 2016).

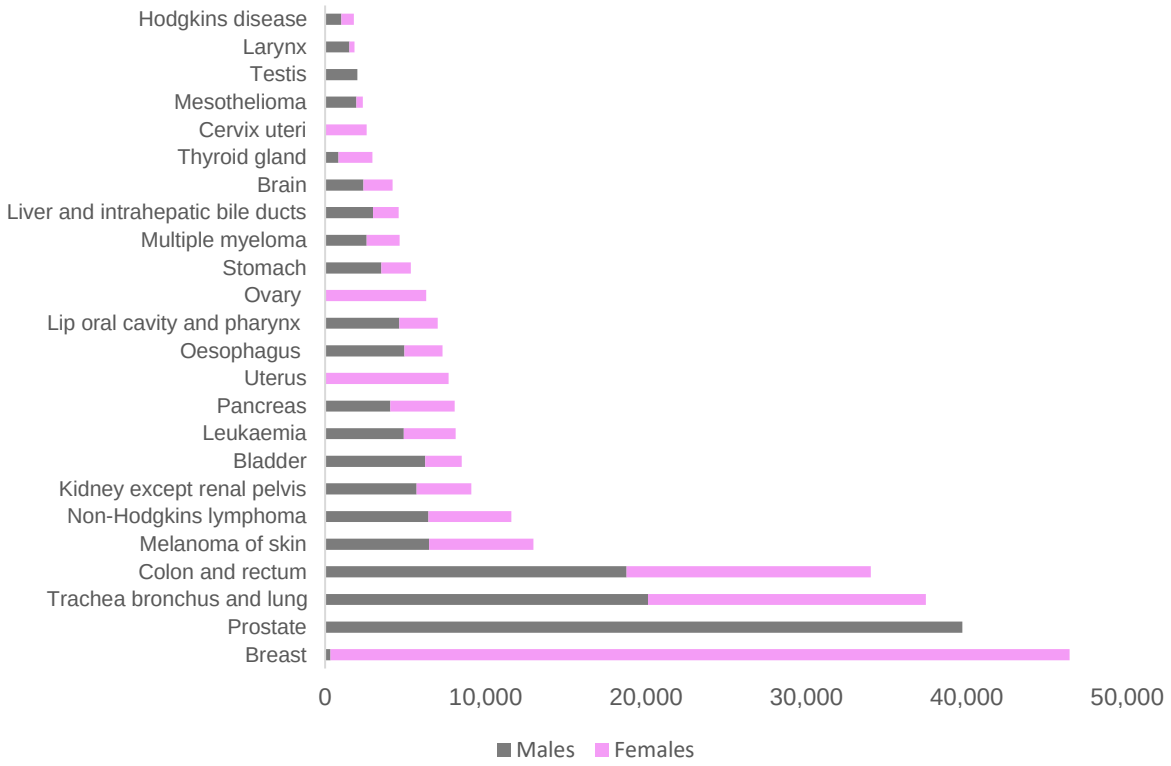

Figure 1.2. Number of cancer cases by site registered in the United Kingdom during 2014. All ages are combined and non-melanoma skin cancers were excluded. Data obtained from Office for National Statistics (2016).

1.3.1. Development and progression

Hanahan and Weinberg (2000) outlined six biological capabilities cells acquire though the development of tumours, referred to as the Hallmarks of Cancer. These included the resistance of cell death, angiogenesis induction, enabling replicative immortality, activating invasion and metastasis, evading growth suppressors and sustaining proliferative signalling. We now understand many of the mechanisms that underpin each of these hallmarks and the proposed framework has since been updated to reflect these advances (Hanahan and Weinberg, 2011). Within their paper, two additional hallmarks are speculated, including the deregulation of cellular energetics and the avoidance of immune destruction (Figure 1.3). Hallmarks are acquired via two enabling characteristics - the generation of random mutations and tumour promoting inflammation through genomic instability (Hanahan and Weinberg, 2011).

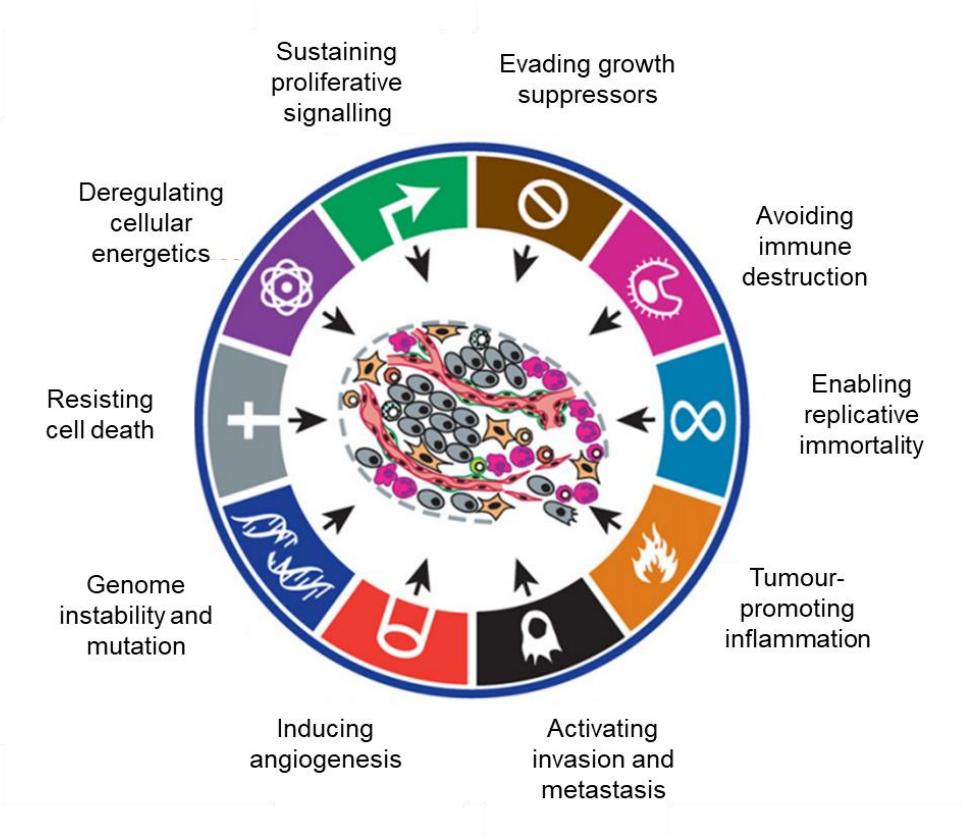

Figure 1.3. Characteristics acquired by cancer cells known as the hallmarks of carcinogenesis. Adapted from Hanahan and Weinberg (2011).

The development of breast cancer is a complex and multistage process whereby genetic and epigenetic changes can alter cell behaviour. These alterations result in dysregulated cell growth and proliferation, eventually breaking out from lobules or ducts to invade surrounding tissues (Gross *et al.,* 2016). Within the mammary gland, ducts are formed by luminal epithelial cells which are surrounded by a layer of myoepithelial cells. The majority of breast tumours begin in the epithelial tissue, most commonly in TDLUs, which contain the milk ducts. Uncontrolled proliferation of luminal epithelial cells occurs during the development of breast cancer. This can result in luminal filling and distortion of the ductal space and surrounding tissues. If left unchecked, proliferating cells can spread into lymph nodes and spread throughout the body.

1.4. Risk factors

Multiple risk factors have been identified that can influence the risk of breast cancer development, ranging from genetic predispositions and age, to lifestyle factors, such as diet and exercise (Table 1.1). Only 5-15% of breast cancer incidents can be attributed to familial genetic factors, with the remaining cases potentially relating to extrinsic influences (Anand *et al.*, 2008). However, much of the existing research effort has centred around intrinsic risks, leaving numerous environmental factors, including chemical exposures, comparatively less understood. In order to develop more effective preventative strategies, it is important that we further our understanding of extrinsic risks and the mechanisms that underpin their association with breast cancer development.

Table 1.1. Overview of established breast cancer risk factors and their respective lifetime risk. Adapted from McPherson, Steel and Dixon (2000).

Risk Factor	Relative Risk	High risk group
Age	>10	Elderly
Geographical location	5	Developed country
Age at menarche	3	Menarche before 11
Age at menopause	2	Menopause after 54
Age at first full pregnancy	3	First child in early 40s
Family history	>2	Breast cancer in first degree relative when young
Previous benign disease	4-5	Atypical hyperplasia
Cancer in other breast	>4	
Socio-economic group	2	Groups I and II
Diet	1.5	High intake of saturated fat
Body weight -		
Premenopausal	0.7	Body mass index >35
Postmenopausal	1.5	Body mass index >35
Alcohol intake	1.3	Excessive intake
Exposure to ionising radiation	3	Abnormal exposure in young females after the age of 10
Exposure to exogenous hormones -		
Oral contraceptives	1.24	Current use
Hormone replacement therapy	1.35	Use for >10 years
Diethylstilbestrol	2	Use during pregnancy

1.4.1. Genetic predispositions

Many genetic factors are known to be associated with breast cancer risk. There are three classes of genetic predispositions, grouped by their breast cancer risk; high-, intermediate- and low-penetrance genes (Turnbull and Rahman, 2008). Within the high penetrance class is the germline mutation of the breast cancer gene 1 (*BRCA1*). *BRCA1* falls into the tumour suppressor category, involved in DNA repair and ensuring the stability of the cell's genetic material. As a result, when *BRCA1* is mutated or altered, there is a risk of DNA damage not being repaired, meaning that cells are more likely to accumulate genetic mistakes and develop cancer (Wu, Lu and Yu, 2010). Tumours in patients with *BRCA1* mutations are often high-grade, oestrgen receptor negative invasive carcinomas, associated with poor clinical outcomes.

Breast cancer gene 2 (*BRCA2*) mutations are also connected with breast cancer risk through similar pathways. Together, mutations in *BRCA1* and *BRCA2* account for 40% of familial breast cancers and 10% of all breast cancers (Apostolou and Fostira, 2013). In most cases, mutations of *BRCA1* or *BRCA2* genes result in early protein truncation and deleterious mutations and deletions affecting splice sites (Mehrgou and Akouchekian, 2016). Both genes are associated with greater than a tenfold relative risk of breast cancer resulting in significant interest by researchers and the general public. Other candidate genes within this class include *TP53* (tumour protein p53), *PTEN* (phosphatase and tensin homolog)*, STK11* (serine/threonine kinase 11) and *CDH1* (cadherin-1).

Within the intermediate category, five genes have been identified that together account for around 2.3% of hereditary cases (Rahman *et al.*, 2007). The ataxia-telangiectasia mutated gene (*ATM*) plays a key role in the cellular response to DNA double-strand breaks, Heterozygous carriers of *ATM* mutations have an increased risk of breast cancer, equating to 6.02% cumulative risk by the age of 50 (Marabelli, Cheng and Parmigiani, 2016). Further genes in this cohort include *CHEK2* (checkpoint kinase 2), which is also involved in the repair of double-strand DNA breaks (Zhang *et al.*, 2004), *BRIP1* (BRCA1 interacting protein C-terminal helicase 1), *PALB2* (partner and localiser of BRCA2) and *RAD50* (RAD50 double strand break repair protein), all acting on similar pathways to *BRCA1* and *BRCA2*, yet in a much lower frequency (Turnbull and Rahman, 2008).

Large genome-wide studies have been conducted to identify areas of the genome that could be associated with breast cancer risk. This final category of low-penetrance alleles consists of mainly single-nucleotide polymorphisms (SNPs) in a limited number of genes, which are able to alter the expression of associated genes (Li *et al.*, 2018). Over 150 SNPs have now been linked to breast cancer susceptibility, and this number continues to rise (Michailidou *et al*., 2017 ;Ziv *et al*., 2017; van Veen *et al*., 2018), however their contribution to breast cancer risk is debated (Rae *et al.*, 2008; Skol, Sasaki and Onel, 2016). It has been suggested that individuals carrying many of these alleles may hold a lifetime risk of breast cancer development of up to

50% (Houlston and Peto, 2004), yet more recent studies claim they are unlikely to be a major contributor to breast cancer heritability (Shiovitz and Korde, 2015; Li *et al.*, 2018). Overall, heritable cases account for a very small proportion of breast cancer incidents and whilst their understanding has led to an increase in genetic screening and cancer prevention in many individuals, additional factors must be considered to account for the sizable number of sporadic breast cancer incidents.

1.4.2. Lifestyle risk factors

1.4.2.1. Diet

Diet can influence an individual's risk of developing breast cancer, which has been proposed to partially explain the high variation in geographic incident rates (Garland *et al.*, 1990; Grant, 2010; Torre *et al.*, 2015; Grosso *et al.*, 2017). There have been few clinical studies conducted, however case studies have found associations between certain foods and an increase, or decrease, in cancer risk. For instance, fat intake has been reported to increase endogenous oestrogen levels, however early cohort studies found only modest associations to support a link to breast cancer development (Kushi *et al.*, 1992). In contrast, dietary fibre intake has been associated with a significant reduction in breast cancer risk, with adolescent and young adult women observing lower breast cancer risk with higher intakes of soluble and insoluble fibre (Aune *et al.*, 2012; Farvid *et al.*, 2016). A recent study determined that high fibre intake may actually reduce the breast cancer risk related with alcohol consumption (Romieu *et al.*, 2017). Authors observed that alcohol was significantly associated with breast cancer risk amongst those with low fibre diets, yet this link was not observed in individuals with a high fibre intake. Although this is very much still a developing area of research, so far studies have shown mixed results (Hagmar and Törnqvist, 2003; Abbas *et al.*, 2013; Petridou, Georgakis and Antonopoulos, 2018; Taha and Eltom, 2018). The World Cancer Research Fund categorised the evidence supporting a link between diet and breast cancer risk as limited, stating that no

conclusions could be drawn based on the current information, suggesting that maintaining a healthy diet and weight should be the main focus (World Cancer Research Fund, 2014).

1.4.2.2. Weight

The 2012 Annual Report to the Nation on the status of Cancer, determined that women who were overweight or obese had a significantly higher risk of postmenopausal breast cancer (1.05 (1.03-1.07 95% CI) per 2 kg/m^2 increase in BMI). The high numbers of overweight individuals not only contributed to an increase in the cancer rate, but also led to a worsened prognosis (Eheman *et al.*, 2012; Crispo *et al.*, 2015). A recent study observed overweight women had a higher risk of invasive breast cancer when compared with healthy weight individuals and associated weight with tumour size and mortality (Neuhouser *et al.*, 2015). Recent evidence has suggested high insulin levels of overweight individuals may be one explanation for this relationship. Work by Gunter and colleagues (2015), found that metabolic health played a role in breast cancer risk and that metabolically unhealthy women had a higher breast cancer risk, regardless of whether they were normal weight or overweight. Similarly, they found women who were overweight, but metabolically healthy, did not have a significantly increased risk of breast cancer. As a result of this research, metabolic health has been suggested to have higher relevance to breast cancer risk than adiposity.

1.4.2.3. Physical activity

Although levels of physical activity are known to be associated with weight, independent from body mass index (BMI), an increase in physical activity is believed to decrease the risk of breast cancer development. One meta-analysis determined that two hours of recreational activity a week could reduce the risk of breast cancer by 5% (Wu, Zhang and Kang, 2013). Kyu and colleagues (2016) believed that just a small amount of additional exercise could see decreases in risk by 3%, with higher activity levels reducing risk by a considerable 14%. Whilst it is commonly agreed that physical activity reduces the risk of breast cancer and several other diseases, the exact contribution to risk reduction and how physical activity may influence other established risk factors remains elusive.

1.4.2.4. Alcohol consumption

High levels of alcohol intake have been associated with an elevated risk of several diseases, including breast cancer. A study of almost 22,000 women in Denmark observed that postmenopausal women who increased their alcohol consumption by just two drinks a day had an increased breast cancer risk of 30% (Dam *et al.*, 2016). Work conducted by Bagnardi *et al.,* (2015), observed a dose-response relationship between alcohol consumption and breast cancer risk, with a relative risk estimate of 1.16(1.33-1.94). Within the UK, alcohol is believed to account for 11% of breast cancer cases in women (Allen and Beral, 2009), with an International Agency for Research on Cancer (IARC) working group determining ethanol to be a carcinogen (Group 1; WHO International Agency for Research on Cancer, 2010). As with many of the other risk factors, the mechanisms that link alcohol to breast cancer are not well defined. However, it has been speculated that ethanol consumption in women causes an increase in endogenous oestrogen levels which increases breast cancer risk (Scoccianti *et al.*, 2014).

1.4.2.5. Smoking

The role of smoking in breast cancer has been heavily studied with multiple publications debating the contribution of tobacco to breast tumourigenesis. Whilst breast cancer is not generally thought to be a tobacco related disease, tobacco smoke contains numerous chemicals known to be carcinogens, that could have a role in breast cancer risk, notably several polycyclic aromatic hydrocarbons (Reynolds, 2013). There is evidence of such compounds being observed in the breast (Hecht, 2002), meaning it is plausible for these chemicals to contribute to mammary carcinogenesis. Much of the research has focused on active smoking with strong evidence now supporting the link to breast cancer (Connor *et al.*, 2017; Jones *et al.*, 2017; Pirie *et al.,* 2013). This association is particularly prominent in women who have smoked for long periods of time before their first pregnancy (Catsburg, Miller and Rohan, 2015). Less research has been undertaken in relation to the risk of passive exposure, however some

evidence exists to support the idea that passive smokers are also at risk (Macacu *et al.*, 2015; Regev-Avraham *et al.*, 2018).

1.4.2.6. Chemical exposures

The contribution of environmental chemical exposures to breast cancer risk is heavily debated. Considerable research has been undertaken, however much of the literature is inconclusive, with many population-based studies finding little association between environmental compounds and breast cancer development (Brody and Rudel, 2003). It must also be considered, that the thousands of compounds we are exposed to on a daily basis adds complexity to the understanding of this risk factors. We have a very limited evidence of how these compounds may interact with each other and indeed, interact with other established lifestyle and genetic risk factors. Furthermore, as with several of the other risk factors, the mechanistic link between exposure and carcinogenesis remains unclear.

1.5. Environmental chemicals and human health

Between 1957 and 2003, the American Chemical Society registered in excess of 15 million new chemicals being released into the environment, with over 1,000 being added every day (McKinney, Schoch and Yonavjak, 2007). Many of these compounds are able to persist within individuals for long periods of time, often spanning generations, even when the chemical is no longer in circulation (Botkin and Keller, 2010). As an example, the insecticide dichloro-diphenyl-trichloroethane (DDT) was banned within the United States in 1972, however as it takes 10-20 years for traces of DDT to disappear from the body after exposure, due to having a reported half-life of 2-15 years (Roy and Nath, 2016), low levels of DDT and its metabolites remain present in human tissues (Ellsworth *et al.*, 2018).

Cases of hormonal cancers, such as endometrium, prostate, thyroid and breast, along with other diseases are rising at an alarming rate within developed countries (Siegel, Miller and Jemal, 2016). Intrinsic factors can only account for a small proportion of cancer cases, leaving

researchers looking to external influences to explain the rate of increase (Wu *et al.*, 2016). Along with factors such as diet, alcohol, stress and body mass index, it has been hypothesised that chemical exposures could contribute to cancer risk (Bergman, Heindel, Jobling, *et al.*, 2013; Forman *et al.*, 2015). Cancer rates have been shown to increase with the levels of air pollution (Hystad *et al.*, 2015; Janitz *et al.*, 2016; Raaschou-Nielsen *et al.*, 2016).

Incident rates of prostate cancer have risen globally (Siegel, Miller and Jemal, 2016; Zhou, Check and Lortet-Tieulent, 2016). Epidemiological studies have suggested that pesticide application in agricultural practices, such as methyl bromide, phorate and butylate, could be associated with this growth (Lewis-Mikhael and Bueno-Cavanillas, 2016). Exposure to arsenic also has strong associations with the development of prostate cancer (Yaqub, Anetor and Olapade-Olaopa, 2014). Whilst associations with prostate cancer may not be directly applicable to the breast, being a hormonal cancer, similar links may be present in the breast providing justifications for similar research for breast cancer. Due to the critical role oestrogen plays in relation to breast development, maintenance and carcinogenesis, the chemicals that can mimic the endogenous hormone are of particular interest to the research community.

1.6. Hormonal carcinogenesis

Hormonal cancers, including breast, ovary, prostate, thyroid and endometrium can be associated with a common mechanism of carcinogenesis. Simply, endogenous and exogenous hormones are able to drive cell proliferation, resulting in a higher number of cell divisions and thereby increasing the opportunity for cells to accumulate random genetic errors, leading to tumorigenesis (Henderson and Feigelson, 2000). Oestrogens have been shown to interfere with various growth factor signalling pathways, which could explain some of the observed proliferation (Jia, Dahlman-Wright and Gustafsson, 2015). Disruption of key signalling pathways has been observed in breast cancer and can lead to the increased proliferation and survival of tumour cells. Studies have demonstrated oestrogen is able to interact with phosphoinositide 3

kinase (PI3K)/ protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) signalling cascades (Hall, Couse and Korach, 2001; Driggers and Segars, 2002; Ivanova *et al.*, 2002; Björnström and Sjöberg, 2005) altering the regulation of apoptosis, proliferation, cellular adhesion, metabolism and cell survival. In addition, oestrogen exposures have been suggested to cause genotoxicity by forming DNA adducts (Roy and Liehr, 1999), loss of heterozygosity (Russo and Russo, 2006) and single strand DNA breaks (Roy and Liehr, 1999; Rajapakse, Butterworth and Kortenkamp, 2005). A significant body of evidence now supports the role of oestrogens in promoting mammary tumorigenesis though this theory of hormonal carcinogenesis, with a cumulative exposure to oestrogen being recognised as one of the most significant risk factors (Travis and Key, 2003). Early menarche and late menopause have been identified as predictors of breast cancer risk, predominantly because they maximise the exposure time to endogenous oestrogens, with individuals experiencing an increased number of ovulation cycles (Collaborative Group on Hormonal Factors in Breast Cancer, 2012).

Women who have their first child before the age of 20 have a 50% reduction in lifetime risk compared to childless women (Britt, Ashworth and Smalley, 2007). The observed protective effect of early age at first birth has also been linked to oestrogen exposure. Whilst during the first trimester endogenous oestrogen levels rise rapidly, as the pregnancy develops these levels lower and sex hormone-binding globulin levels rise, resulting in an overall reduction in oestrogen profile (Kobayashi *et al.*, 2012). In addition, pregnancy induces pre-malignancy cells to terminally differentiate, losing future malignant potential (Russo *et al.*, 2005).

Population studies have shown an association between higher oestrogen levels, namely oestradiol, and breast cancer risk. For example, Bernstein *et al.,* (1990) found levels of oestradiol in pre-menopausal women in the US were 20% higher compared to China. Likewise, post-menopausal women in the US had oestradiol levels 36% higher than Japanese women (Shimizu *et al.*, 1990). Compared to China and Japan, women in the US are more likely to develop breast cancer in their lifetime and levels of oestrogen exposure has been put forward as one explanation of this (Torre *et al.*, 2016, 2017).

Exposure to exogenous oestrogen is linked to breast carcinogenesis as it adds to an individual's oestrogenic load (Hilakivi-Clarke, Assis and Warri, 2013). Observations from the Million Women Study showed that women currently using hormonal replacement therapy (HRT) had an increased risk of breast cancer development. Women using oestrogen-only forms of HRT experienced higher rates of breast cancer incidence, with risk increasing with the duration of HRT use. This risk was increased further for women using oestrogen-progesterone HRT (Beral and Million Women Study Collaborators, 2003). The ability for exposure to oestrogenic compounds to contribute to breast cancer risk has prompted substantial research to elucidate whether exposure to oestrogen-mimicking EDCs can lead to an increase in breast cancer risk via similar mechanisms.

Bisphenol A (BPA) is one of the highest volume chemicals produced worldwide, with more than $6x10^9$ lb/year (Gao *et al.*, 2015), and has been used in a variety of consumer products since 1957. BPA contaminates water supplies, dust and air with levels of up to 43 parts per billion ($4.3x10^{-8}$ M) being recorded in European waters and comparable concentrations have been observed in the United States (Klečka *et al.*, 2009). BPA can bind to oestrogen receptor alpha (ERα) and beta (ERβ), mimicking the endogenous hormone, even at very low doses (Gao *et al.*, 2015). However, BPA displays 1,000- to 2,000-fold less affinity to the ERs in comparison to 17β-oestradiol, and is therefore referred to as a weak oestrogen (Acconcia, Pallottini and Marino, 2015). As oestrogen levels have been shown to influence breast cancer risk (Clemons and Goss, 2001; Travis and Key, 2003; Petridou, Georgakis and Antonopoulos, 2018), BPA's ability to interact with these processes has resulted in the chemical being extensively studied, both *in vivo* and *in vitro*. Whilst BPA has not been shown to induce genomic instability, it has been demonstrated to be mitogenic, causing an increase in proliferation and increasing the risk of random genetic mutations, with this activity being the most likely mechanisms of carcinogenesis for BPA. A study in rats demonstrated that *in utero* exposure to BPA changed normal cell proliferation and apoptosis, and altered the timing of breast development (Wang, Jenkins and Lamartiniere, 2014). Further animal studies have shown that even low levels of BPA can alter

the development of mammary glands, inducing increased proliferation (Dhimolea *et al.*, 2014; Gao *et al.*, 2015; Wang, Liu and Liu, 2016; Perrot-Applanat *et al.*, 2018). Such changes may continue into puberty and adult life stages, affecting differentiated breast epithelial cells and increasing the risk of breast cancer (Paulose *et al.*, 2015; Mandrup *et al.*, 2016). *In vitro*, researchers have identified link between BPA exposure and breast cancer risk due to disruptions in cell signalling (Jenkins *et al.*, 2012). As with other oestrogenic compounds, BPA has been reported to interfere with ERα-mediated extracellular regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphatidyl-inositol-3-kinase/AKT (PI3K/AKT) pathways, resulting in ERK/MAPK and AKT phosphorylation (Acconcia, Pallottini and Marino, 2015; Villar-Pazos *et al.*, 2017).

Although compounds such as BPA have been demonstrated to act through genomic and non-genomic mechanisms and potentially increase the risk of breast cancer, the risk associated with exposure to EDCs is still heavily debated. Many oestrogenic effects are not observed at levels comparable to human tissue observations (Vandenberg *et al.*, 2012). In addition, it is argued that exposure to EDCs has an insignificant effect on the oestrogenic load, compared to the magnitude of effect induced by endogenous hormones (Kortenkamp, 2006) . Consequently, researchers have attempted to isolate further mechanisms that could explain the association observed between EDC exposure and breast cancer risk.

1.7. The epigenome

In an attempt to improve our understanding of the potential link between chemicals and cancer, an emphasis has been placed on isolating the specific mechanisms or targets that might lead to carcinogenesis. The epigenome, which has been referred to as the *'interphase between the genome and environment'* (Widschwendter, Jones and Teschendorff, 2013), could act as a target for EDCs, and may therefore provide a new insight into breast cancer risk. The term epigenetics refers to cellular processes that are able to influence gene expression and function, without altering the genome itself (Feinberg and Tycko, 2004; Goldberg, Allis and Bernstein,

2007). It is through epigenetic mechanisms that cells with identical genotypes are able to diversify into distinct cell types with unique gene expression and functions (Goldberg, Allis and Bernstein, 2007).

There are three main mechanism involved in epigenetic modification, all of which can alter gene activity without affecting the DNA sequence itself. These include DNA methylation, histone modification, and microRNAs. It is important to note that these mechanisms do not act alone and there is a significant amount of interaction amongst the different components. It is the integration of all mechanisms that maintain the natural state of the epigenome (Sharma, Kelly and Jones, 2010). We review each of these mechanisms briefly below, however more comprehensive reviews of the link between the epigenome and carcinogenesis have been compiled previously (e.g. Feinberg and Tycko, 2004).

1.7.1. DNA methylation

In vertebrate genomes, DNA methylation (DNAme) mainly occurs at cytosine guanine dinucleotide sites (CpGs) within the DNA (Veeck and Esteller, 2010), where a methyl group will bind to a cytosine (Figure 1.4). This process limits the access of transcription factors to the gene resulting in gene inactivation (Atalay, 2013). DNAme is facilitated by DNA methyltransferases (DNMTs) DNMT1, DNMT2, DNMT3a and DNMT3b, of which DNMT1 is the most abundant in mammalian cells (Subramaniam *et al.*, 2014).

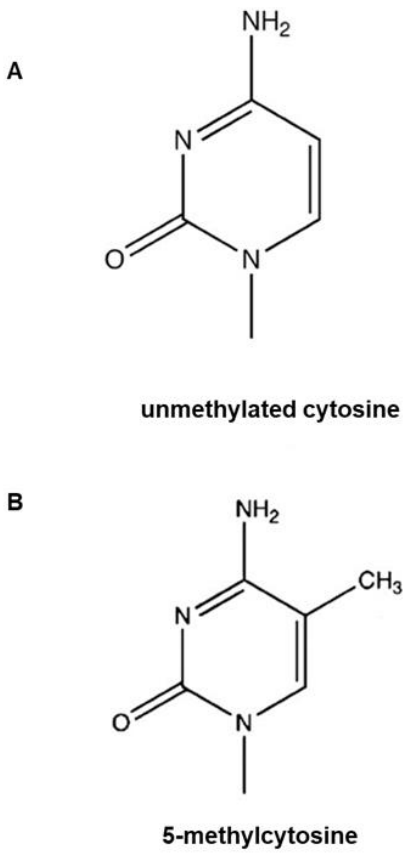

Figure 1.4. DNA methylation. Cytosine is one of the four main bases found in DNA and RNA (A), through the addition of a methyl group (B) in promotor regions, genes can become silenced.

Both global hypo- and hypermethylation have been associated with genomic instability and an increase in mutational events (Sandoval and Esteller, 2012). Therefore, any external interaction with the epigenome that results in large scale changes has the potential to impact on an individual's health. Further studies have shown that global hypomethylation with regional hypermethylation is observable in breast cancer tumours (Vo and Millis, 2012) stressing that methylation is not only restricted to CpG islands in cancer, but impacts multiple loci (Yan *et al.*, 2001).

1.7.2. Histone modification

Histones can be modified through a covalent post-translational modification to the histone proteins (Bowman and Poirier, 2015; Nadal *et al.*, 2018). Mechanisms involved include methylation, phosphorylation, acetylation, ubiquitylation and sumoylation (Zentner and Henikoff, 2013). These changes can alter the chromatin structure which impacts an individual's gene expression. Modification to histone proteins may also regulate the binding of effector molecules (Bannister and Kouzarides, 2011). Global histone modifications are an important event in breast cancer development (Zhao *et al.*, 2016) and have been found to correlate with tumour types and patient outcome in breast cancer cases (Elsheikh *et al.*, 2009). Whilst this mechanism has previously not received as much attention as DNAme, recent years have seen a sharp uptake in research surrounding histone modifications and cancer (Elsheikh *et al.*, 2009; Waldmann and Schneider, 2013; Hessler and Martin, 2016; Messier *et al.*, 2016; Zhao *et al.*, 2016), potentially attributed to equipment becoming more cost effective and attainable.

1.7.3. MicroRNAs

MicroRNAs (miRNAs) are small, non-coding RNAs around 20-24 nucleotides in length and are transcribed from DNA, but not translated into proteins. They have the ability to regulate gene expression at the post-transcriptional level by binding to 3'-untranslated regions of messenger RNAs (mRNAs) and silencing specific genes (Singh *et al.*, 2008). Depending on their target mRNA, miRNAs can act as either tumour suppressors or target oncogenes, and have been shown to play a role in breast carcinogenesis (Nelson and Weiss, 2008). A study by Lorio *et al.,* (2005) found that breast cancer cells could be differentiated from normal cells by the levels of miRNA deregulation, yet they could not make conclusions regarding the biological impact of these findings due to the limited understanding of the function of miRNA deregulation. Whilst knowledge has grown over the past decade, this mechanism still remains comparatively understudied.

1.8. Chemical exposure, epigenetics and breast cancer

The majority of studies investigating the impacts of chemicals present within the breast have used breast milk as a surrogate for breast tissue. These studies have identified an array of compounds, including BPA (Mendonca *et al.*, 2014), organochlorine pesticides (Rojas-Squella *et al.*, 2013; Pirsaheb *et al.*, 2015), parabens (Darbre *et al.*, 2004; Barr *et al.*, 2012) and even flame retardants (Kim *et al.*, 2014). However, we still lack information on whether these chemicals can impact breast cancer initiation or progression. The mechanism underlying carcinogenic effects remains elusive, as most of the chemicals are not considered classical genotoxicants or carcinogens (Del Pup *et al.*, 2015). Some of these compounds may be acting through alternative mechanisms, such as inducing mitogenicity or epigenetic effects (Smith *et al.*, 2016). The relationship between chemicals and the epigenome is beginning to receive interest from researchers, yet it is still unclear what the exact state of the science is in this field and what areas remain absent from our understanding. In the following sections we review the studies that have investigated breast cancer risk or breast carcinogenesis in response to chemical exposures via the epigenome.

1.8.1. Diethylstilbestrol (DES)

The story of Diethylstilbestrol (DES) marks an important point in medical history, highlighting the dangers of prescribing poorly understood chemicals on a large scale. The compound was frequently given to pregnant women between 1940 to 1971 to reduce the risk of miscarriage or prematurity. However, it became apparent that the drug carried sever health implications after it was noted that rare cases of cervicovaginal clear cell adenocarcinomas were observed in young women, whom had been exposed to DES *in utero* (Herbst, Ulfelder and Poskanzer, 1971). Although this discovery led to the banning of the substance during the 1970s, it became clear that these effects were passing across generations, which at the time was a new concept in medical research. However, the true impact of DES was still being uncovered, with second generation women showing increased breast cancer risk, and a high frequency of hypospadias

(unusual placement of the urinary opening) displayed even in third generations men (Fénichel, Brucker-Davis and Chevalier, 2015). Whilst mutagenic effects have been seen in women after high doses of DES were prescribed, rodent studies mimicking conditions provided early evidence that favoured foetal programming of adult diseases through transgenerational transmission of epigenetic modifications (Fénichel, Brucker-Davis and Chevalier, 2015). This concept is now considerably more understood as the transgenerational inheritance of exposure effects. Whereby the effects of exposure are passed from F0 to F3 generations, despite no direct exposure of the F3 generations occurring (Xin, Susiarjo and Bartolomei, 2015). DES can therefore be considered a model compound to study the relationship between chemicals, epigenetics and breast cancer risk. Yet, to our knowledge, only one study has investigated DES in terms of its epigenetic link to breast cancer. Doherty *et al.*, (2010) concluded that DES increased *EZH2* (enhancer of zeste 2 polycomb repressive complex 2 subunit) expression within the mammary gland, leading to epigenetic alterations and breast carcinogenesis. Whilst this presents compelling evidence when combined with years of previous research, more studies are required to confidently implicate the involvement of the epigenome in linking DES with breast cancer risk.

1.8.2. 2,3,7,8-tetrachloridibenzo-p-dioxin (TCDD)

A further chemical that has received significant interest is 2,3,7,8-tetrachloridibenzo-p-dioxin (TCDD). This organochlorine compound is produced as a by-product of the combustion and manufacture of chemicals. TCDD was weaponised by the US army during the Vietnam War, meaning that many military personnel and civilians were exposed to the compound. Traces of TCDD have since been detected in herbicides, including Agent Orange (Scialli, Watkins and Ginevan, 2015). Whilst levels of TCDD in human tissues are believed to be decreasing (Pelclová *et al.*, 2006), the compound has a long half-life (between six and nine years) enabling it to accumulate in human tissues. TCDD exposure has been linked with adverse health impacts,

including acute intoxication, hepatotoxicity, carcinogenicity and central neurotoxicity (Ogura, Masunaga and Nakanishi, 2004).

A handful of experiments have been undertaken to investigate whether TCDD can contribute to breast cancer risk through the epigenome. One study indicated that exposure to $1x10^{-7}$ M TCDD can alter BRCA1 protein and transcription levels through increasing the association of DNMT1 with methyl-binding-domain protein 2 (Papoutsis, Lamore and Wondrak, 2010). Further investigations by the same group reported that *in utero* exposures to TCDD reduced the expression of the *BRCA1* gene within the mammary tissue of offspring, which resulted from the *BRCA1* promotor being occupied by DNMT1 (Papoutsis *et al.*, 2015). Despite the small number of studies, evidence indicates that TCDD can be potentially be linked to breast cancer by inducing epigenetic modifications.

1.8.3. Dichlorodiphenyltrichloroethane (DDT)

Dichlorodiphenyltrichloroethane (DDT) was first synthesised in 1874, yet its ability to act as an effective insecticide was not clear until 1939 (Beard and Australian Rural Health Research Collaboration, 2006). From 1945 it was released commercially and widely used for its insecticidal properties on agricultural crops (Beard and Australian Rural Health Research Collaboration, 2006). Significant health effects to non-target wildlife became apparent in the 1950s (Berry-Caban, 2011). Notably, impacts were reported on the reproductive success of species, including brown pelicans (Blus, 1982; Fry, 1995) and peregrine falcons (Porter and Wiemeyer, 1969; Olsen *et al.*, 1992). These effects were most substantial in species at higher trophic levels, which raised questions about the compounds impact on human health. Studies have observed associations with exposure to DDT and an increased risk of reproductive disorders and cancers, including breast cancer (Beard and Australian Rural Health Research Collaboration, 2006). Although the compound has been banned in many countries since the 1970s, it is still in use in some geographical regions (Faroon *et al.*, 2002) and with the ability of DDT to remain in the

body for a long period of time, the pesticide can still be observed in human tissues (Roy and Nath, 2016).

Evidence supporting DDT's ability to impact breast cancer development through the epigenome is limited. One study has described the ability of DDT to alter miRNA expression in the breast (Tilghman *et al.*, 2012). However, cells in this experiment were only exposed to $1x10^{-5}$ M DDT, significantly higher than those observed in human tissues. Exposures were also only carried out for a period of 18 hours, meaning that it was not possible to capture the impact of long term, low dose exposures. In addition, this study was undertaken using the MCF-7 breast cancer cell line, and therefore may not be representative of the effects that would be observed in normal human tissues. Although conclusions cannot be drawn from a single study, considering the widespread historic exposure of populations to DDT the findings presented certainly justify additional work to reveal whether the compound impacts other epigenetic mechanisms.

1.8.4. Phytoestrogens

Phytoestrogens are plant-derived xenoestrogens, also known as dietary oestrogens. Due to their similarity to oestrogens at a molecular level, they are believed to mimic the endogenous hormone. Phytoestrogens are present in foods such as grains and some vegetables. Historically there has been much controversy surrounding this chemical group and their potential adverse health effects. For example, genistein, found in plants including soybeans, has been reported to both reduce and increase the risk of developing breast cancer (Bouker and Hilakivi-Clarke, 2000).

Our understanding of how phytoestrogens interact with the epigenome and whether they may lead to breast cancer is, again, relatively limited. In 2012, it was observed that genistein and daidzein (found in soy beans and other legumes) were able to reverse DNA hypermethylation of the tumour suppressor genes *BRCA1* and *BRCA2,* contributing to the regulation of these genes and restoring expression (Bosviel *et al.*, 2012). Although authors admitted further investigation

is required, this finding would suggest phytoestrogens reduce the risk of breast cancer development. Furthermore, Hsieh *et al., (2014)* demonstrated that arctigenin (lignin found in plants) may reduce ER-negative breast cancer risk by upregulating histone H3K9 trimethylation in the AP-1 binding region of the *BCL2* gene promoter, inducing apoptosis in cancer cells. This indicates a reduction in breast cancer risk. However, these studies represent a small subset of the phytoestrogen family and therefore their results cannot be extrapolated to all xenoestrogens. Larger studies that examine a variety of phytoestrogens are required whilst taking into consideration the possible mixture effects that may occur when these chemicals are combined with others within the body.

1.8.5. Bisphenol A (BPA)

BPA is manufactured on a commercial scale and is one of the highest volume chemicals produced globally (Rubin, 2011). Its main use is to harden plastics and epoxy resins and therefore it is used in a wide range of everyday consumer products. Its common use results in widespread human exposure, with BPA being recorded in the urine of over 90% of tested Americans between 2003-2004 (Calafat *et al.*, 2008). The main route of exposure to BPA is through ingestion of contaminated food and drink, where the chemical has leached from containers. BPA has been shown to interact with hormone signalling pathways leading to numerous adverse health effects; including diabetes, cardiovascular disease and several cancers (Bergman, Heindel, Jobling, *et al.*, 2013).

It is therefore not surprising that BPA is the most rigorously studied chemical in relation to epigenetics and breast cancer development. Experiments *in vitro* using immortalised breast epithelial cells have shown that exposure to BPA can alter the gene expression of a number of genes through hyper- and hypomethylation, including numerous genes associated with breast cancer, such as *BRCA1* and *BRCA2* (Fernandez *et al.*, 2012). Further research suggests that exposure to BPA *in utero* can alter the methylation profile of mice and that this profile persists after birth and through the individual's development (Bromer *et al.*, 2010). The chemical also has

the potential to interact with histones, with observations of BPA increasing levels of histone H3 and significantly increasing the expression of *EZH3* (a known epigenetic modifier in breast cancer) within MCF-7 cells (Doherty *et al.*, 2010). Multiple studies suggest that BPA has the potential to contribute towards breast cancer risk through epigenetic mechanisms (Ho *et al.*, 2006; Dolinoy, Huang and Jirtle, 2007; Prins *et al.*, 2008). The majority of these studies however, have relied on animal models or immortalised cell lines. The strength of the evidence presented could therefore be questioned in terms of its extrapolation to humans. Furthermore, the concentrations of BPA utilised within these experiments are often considerably higher than what is present within human tissues. Nonetheless, we have a very strong indication that BPA exposure can interact with the epigenome and lead to breast carcinogenesis.

1.9. Priorities for research

1.9.1. Additional chemicals

Whilst numerous compounds have been investigated in relation to their effects on the epigenome, there are several ubiquitous EDCs that have not been considered. For example, reports have suggested chemicals utilised in cosmetics could be linked to breast cancer development (Darbre, 2001; Darbre and Harvey, 2014). One such group of compounds are the cosmetic preservatives, parabens (4-hydroxybenzoic acid esters). Like the compounds discussed previously, parabens have been shown to possess oestrogenic properties in yeast and animal models (Byford *et al.*, 2002; Harvey and Darbre, 2004), however significant effects linked to carcinogenesis are often not observed at tissue relevant concentrations. To date, the potential impact of parabens on the epigenome has not been investigated. In addition, compounds such as Benzophenone-3 (BP-3; a UV filter used in sunscreens and other cosmetics) which are becoming increasingly widespread in the environment (Kerdivel *et al.*, 2013), have not been adequately examined. By determining whether these compounds have the ability to interact with the epigenome, it may be possible to determine additional mechanisms of action and strengthen the evidence linking EDC exposure to breast cancer risk.

1.9.2. Mixture effects

Individuals are never exposed to single chemicals in isolation. Instead, they are surrounded by hundreds of compounds that have the potential to act in combination to exert significant toxicity. For instance, traces of chemicals, including DDTs, hexachlorocyclohexanes, chlordane compounds, hexachlorobenzene, and polychlorinated biphenyls, were observed in mammary gland tissues collected from biopsies of women in China between 2000–2001 (Nakata *et al.*, 2002), all of which may interact with each other and amplify or reduce the possible overall toxicity. Understanding how chemicals in combination can impact individuals is essential to establishing a realistic picture of the potential detrimental effects of chemical exposure. Studies have shown that when chemicals, such as environmental oestrogens, are combined at low, ineffective concentrations, they can act together to produce significant effects (Silva, Rajapakse and Kortenkamp, 2002). These 'mixture effects' were first seen in simple yeast experiments (Rajapakse, Silva and Kortenkamp, 2002), however such observations have since been replicated in a number of *in vitro* (Silva, Rajapakse and Kortenkamp, 2002; Silva *et al.*, 2011; Orton *et al.*, 2014) and *in vivo* (Brian *et al.*, 2005; Hass *et al.*, 2007) systems with a large number of compounds, demonstrating that even though contaminants in isolation might not have significant toxic effect, toxicity may be exacerbated if they are found in combination, due to mixture effects (Carpenter, Arcaro and Spink, 2002; Goodson *et al.*, 2015).

From the literature we can see that although strong links may never be found between a single chemical exposure and breast cancer initiation (often the most common way of assessing chemical impacts on health), by investigating chemical mixtures, relationships may become more transparent and representative of the true risk posed to breast carcinogenesis. It is apparent that there is still a lack of research in terms of chemical mixtures and their impact on the epigenome, leaving a significant gap in our understanding of their potential to impact breast cancer risk. Experiments that examine the relationships between EDC mixtures and breast cancer in representative assays will make a considerable contribution to the current knowledgebase and the direction of future research.

1.9.3. Risk factor interaction

Additional uncertainty surrounds how chemical exposures may interact with other risk factors, such as genetic predispositions, alcohol consumption, diet, stress and obesity. In the case of genetic predispositions, whilst it is already known that individuals with mutated *BRCA1* or *BRCA2* function have an increased risk of breast cancer, the risk associated with these mutated genes appears to be changing over time. A study conducted by King *et al.,* (2003) observed an increase in breast cancer risk for *BRCA1* and *BRCA2* mutation carriers whom were born after 1940s. In addition, the age at which these individuals developed breast cancer was noticeably different, with those individuals born after 1940 being more likely to have breast cancer at an earlier age. They suggested that genetic risk factors were not responsible for these changes and believed instead that environmental factors including reduced exercise and higher obesity rates may be drivers (King *et al.*, 2003). It is plausible that chemical exposures may also contribute to this load. Furthermore, we are aware that in women with impaired *BRCA1* function, the epigenome differs significantly from the general population (Shukla *et al.*, 2010), as they exhibit global DNA hypomethylation and loss of genomic imprinting. One study has suggested that *BRCA1* mutation carriers may be more susceptible to the effects of oestrogenic effects of BPA (Fernandez *et al.*, 2012), however no subsequent studies have examined this. To our knowledge, no research has been conducted to look specifically at how environmental factors may interact with the altered epigenome of individuals with *BRCA1* or *BRCA2* mutations, specifically how chemical exposures may alter breast cancer risk in an already predisposed population. However, genetic predispositions are only one risk factor, and investigations into how chemical exposure may impact on the risks associated with other factors, such as stress or alcohol consumption could begin to provide insights into how these lifestyle factors interact with each other.

Risk models have been developed with the aim of predicting individual women's risk of developing breast cancer within their lifetime and identify those with a 'high risk' that may benefit from some form of intervention (Gail, 2015). Yet a recent article hypothesised that certain risk factors may interact in a synergistic way (Giovannucci, 2016), something that is not currently

ingrained in risk models. Without having a holistic view of each factor, their individual contribution to risk and the interaction between other risk factors, we are unable establish comprehensive risk models or design effective prevention strategies. In the absence of this knowledge we could be misinterpreting how likely individuals are to develop breast cancer. This in turn hinders the effective application of prevention strategies. In order to better understand breast cancer risk, we must move away from considering factors in isolation and begin to design multi-factorial studies that encapsulate all of the risk factors individuals are exposed to. Findings then need to be integrated into a more bespoke risk model which is capable of reflecting the cumulative risk of multiple risk factors.

1.10. Thesis aims and objectives

Breast cancer incidence is occurring at an alarming frequency and we must now look to external exposures to develop a more holistic understanding of breast cancer risk. Factors including obesity, genetic predispositions and stress have received significant interest from the research community, yet the impact of oestrogen-mimicking chemical exposures and how they fit into breast cancer risk, is less explicit. Although studies to date indicate that a relationship exists between chemicals, the epigenome and breast carcinogenesis, there are vast knowledge gaps, that are outlined above, which need to be addressed. Within the scope of this research we begin to address some of these knowledge gaps.

The overarching aim of this project is to investigate whether EDCs induce modifications to the epigenetic profile of human cells and if, in turn, these changes can be linked to breast cancer risk. This aim will be addressed within this thesis under the following objectives.

i.) Investigate the effect of four prevalent xenoestrogens, alone and in combination (DDT, BP-3, BPA and propylparaben) on the morphology of ER competent human mammary breast epithelial cells.

ii.) Identify whether observed morphological changes can be associated with genetic and epigenetic changes.

iii.) Examine the morphological, genetic and epigenetic impacts of environmental chemicals between different *in vitro* experimental systems of varying complexity.

iv.) Determine whether the presence of *BRCA1* mutations alters the susceptibility of individuals to the effects of EDCs.

Chapter Two: Characterisation of the non-tumorigenic mammary derived cell line MCF-12A

2 Characterisation of the non-tumorigenic mammary derived cell line MCF-12A

2.1. Introduction

The development and maintenance of the normal human mammary gland is heavily regulated by endogenous hormones, such as oestrogen (Pike *et al.*, 1993; Javed and Lteif, 2013). As discussed in Chapter 1, oestrogen also plays a critical role in breast carcinogenesis. Our aim within this thesis, is to study the effects of EDCs in a model that is representative of the normal breast. Therefore, we require a cell line that allows us to mimic characteristics of the normal, hormonally responsive, epithelium within the human mammary gland. Within this chapter, we intend to characterise the MCF-12A cell line in a control situation and in response to EDCs. This will assess if they express the oestrogen receptors and determine whether they respond to BPA and propylparaben exposure. These two ubiquitous compounds are used frequently in the following chapters.

2.1.1. MCF-12A cell line

The MCF-12A cells are immortalised, non-tumourigenic human breast epithelial cells. This cell line was first established from tissue taken during a reduction mammoplasty from a patient with fibrocystic breast disease and then derived from adherent cells within the population. MCF-12As typically show luminal epithelial morphology, and importantly, can be grown in three-dimension to form acini-like structures (Marchese and Silva, 2012). This cell line has been utilised in many studies investigating breast carcinogenesis (Tseng and Scott-Ramsay, 2004; Marchese and Silva, 2012; Cello, Flowers and Li, 2013; Gelfand, Vernet and Bruhn, 2016), including those considering epigenetic alterations (Wendt, Cooper and Dwinell, 2008).

Within the literature there are conflicting reports in regards to the oestrogen receptor status of the MCF-12A cell line (Soule *et al.*, 1990; Marchese and Silva, 2012). Initial studies concluded that the cell line was ER-negative (Paine *et al.*, 1992), with subsequent literature supporting the lack of ER expression (Subik *et al.*, 2010; Sweeney, Sonnenschein and Soto, 2018). However,

the cell line has also been identified as ER-positive (Eisen and Brown, 2002; Mitropoulou *et al.*, 2003; Dai *et al.*, 2008; Al-Souhibani *et al.*, 2010; Engel *et al.*, 2011; Wu *et al.*, 2011; Gelfand, Vernet and Bruhn, 2016). The responsiveness of the MCF-12A cell line to oestrogen exposure has also been reported as positive (Marchese and Silva, 2012) and negative (Sweeney, Sonnenschein and Soto, 2018).

Cell line variation between batches and laboratory groups has been reported to result in inconsistent and irreproducible findings (Freedman *et al.*, 2015; Neimark, 2015). Payne *et al.,* (2000) found that MCF-7 breast cancer cells were being frequently relied upon as a cell proliferation assay (E-SCREEN) to study the effects of weakly oestrogenic compounds, yet there were extensive differences in results between laboratories. Comparative genomic hybridisation, used to detect DNA sequence copy number changes on a genome-wide scale, found that differences in cytogenetic changes could be observed between various MCF-7 batches. This, in turn, altered the cell line's oestrogen responsiveness, proliferation rate and susceptibility to apoptosis. These differences could be attributed to differing culture conditions between groups, such as variations in seeding densities and medium supplements. Freedman *et al.,* (2015), stressed that cell lines were highly sensitive to changes in experimental and growth conditions. Long term culturing between lab groups can result in genomic, proteomic and phenotypic differences occurring in cell lines, resulting in different experimental outcomes. Based on this, it is imperative that we confirm the receptor status and responsiveness of our specific batch of MCF-12A cells to EDCs, at the tested passage number, to determine the suitability of the cell line for our study aims.

2.1.2. Bisphenol A exposure and breast cancer risk

To characterise the MCF-12A cell line, it is important that we assess the receptor status under control conditions and in the presence of EDCs. BPA is a highly produced plasticiser and plastic monomer, with over six billion pounds manufactured each year (Burridge, 2003). BPA is used as a coating for many products including containers for food and drink. Individuals are primarily

exposed to BPA through use of consumer products that contain the chemical, including polycarbonate plastics, food and drink containers, dental sealants and coated papers, such as till receipts (L. Vandenberg *et al.*, 2007). Due to its chemical structure (Figure 2.1) and its lipophilic qualities, it is believed that BPA has the ability to accumulate within the body (Fernandez *et al.*, 2012). Levels of BPA have been identified in amniotic fluid (1.1-8.3 ng/ml), placental tissue (11.2-104.9 ng/g), breast milk (0.28-3.41 ng/ml) and human serum (0.2-20 ng/ml; Vandenberg *et al.*, 2007a). From September 2018, new European regulations will restrict the use of BPA in food contact materials, particularly for the interior of food cans, and limit the amount of BPA that can be released into food (specific migration limit) to 0.05 mg/kg (European Commission, 2018). This regulation may reduce average tissue concentrations, however individuals will still be exposed to low levels of BPA, which may contribute to breast cancer risk. Furthermore, no such restrictions have been implemented in other regions, including the United States, meaning populations will remain highly exposed.

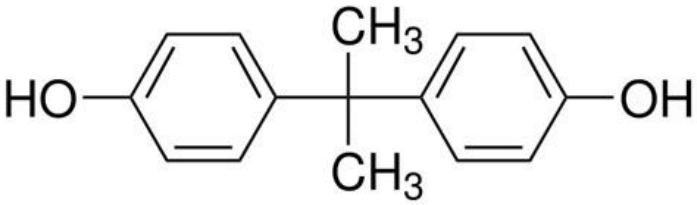

Figure 2.1. The chemical structure of the widely manufactured plasticiser bisphenol A.

The synthetic compound has a wide variety of toxic effects and is known to interfere with the androgen receptor, thyroid hormone signalling and the natural levels of oestrogen in a variety of organisms (Richter *et al.*, 2007; Wetherill *et al.*, 2007). There is some evidence between levels of BPA exposure and the likelihood of breast cancer development (Wetherill *et al.*, 2007), however clear links have still not been established. One population study found that an increase in breast density could be observed with an increase in serum BPA levels after adjusting for age, BMI and other potential confounding factors (Sprague *et al.*, 2013). This indicated that BPA exposure could be positively associated with an increase in breast cancer risk, as women that have mammographically-dense breasts, defined by a high concentration of epithelial and

stromal cells, have an elevated risk of developing breast cancer (Peres, 2012; Pettersson and Tamimi, 2014). For example, individuals with a mammographic density of >75% are associated with a 4.6 fold increase in breast cancer risk, in comparison to individuals with a density of <5% (McCormack and dos Santos Silva, 2006). It is possible that exposure to BPA could be causing this increase in breast density. Work undertaken using hormone therapy treatment has demonstrated that oestrogen-based therapy users had a delayed density decline, that may explain some of their increased breast cancer risk (van Duijnhoven *et al.,* 2007). The ability of BPA to mimic oestrogen suggests that a comparable relationship between exposure and breast density may be present. However, to date, no study has directly examined this possibility. Foetal exposure to BPA has been associated with morphological changes to mammary stroma and epithelium, including more advanced epithelial development in rhesus monkeys (Tharp *et al.*, 2012) and delayed ductal formation in rats (L. N. Vandenberg *et al.*, 2007), which has been linked to a greater risk of neoplasia later in life (Markey *et al.*, 2001; Munoz-de-Toro *et al.*, 2005; Timms *et al.*, 2005; Weber Lozada and Keri, 2011; Acevedo *et al.*, 2013).

BPA also plays a role in cancer progression. *In vitro*, chronic exposure to BPA elicits tumour proliferation and metastasis in breast cancer cell lines MDA-MB-231 and BT-549 (Zhang *et al.*, 2016). Direct BPA exposure can also affect multiple oncogenic pathways (Gao *et al.*, 2015), including the hypermethylation of DNA repair pathways, impacting the cell's ability to detect and fix abnormalities (Fernandez *et al.*, 2012), ERK1/2 activation through GPER (Dong, Terasaka and Kiyama, 2011) and increasing STAT3 signalling (Tan *et al.*, 2011). BPA can also stimulate cancer progression by inducing GPER target gene expression through the GPER/EGFR/ERK transduction pathway (Pupo *et al.*, 2012).

2.1.3. The role of parabens in breast cancer

The alkyl esters of p-hydroxybenzoic acid, more commonly referred to as parabens, are a group of chemically complex compounds thought to possess oestrogenic properties. Of this group, methyl-, butyl-, propyl-, ethyl- and isobutylparaben (Figure 2.2) are the most common. Parabens

are used in a variety of products including cosmetics, shampoos and body creams where they prolong shelf life and prevent microbial growth (Guo and Kannan, 2013; Hiatt and Brody, 2018). Levels of paraben have been identified in urine of over 96% of individuals in one study (Ye *et al.*, 2006), as well as in breast milk (Fisher *et al.*, 2017) and the breast itself (Barr *et al.*, 2012). Barr and colleagues (2012), showed that propylparaben had the highest concentration within the breast (16.8 ng/g), followed closely by methylparaben (16.6 ng/g). Their presence in human tissues can be attributed to their widespread use in consumer products that are used daily, exposing individuals via ingestion, skin absorption and inhalation (Anderson, 2008). In comparison, butylparaben (5.8 ng/g), ethylparaben (3.4 ng/g) and isobutylparaben (2.1 ng/g) had much lower concentrations in the breast (Barr *et al.*, 2012).

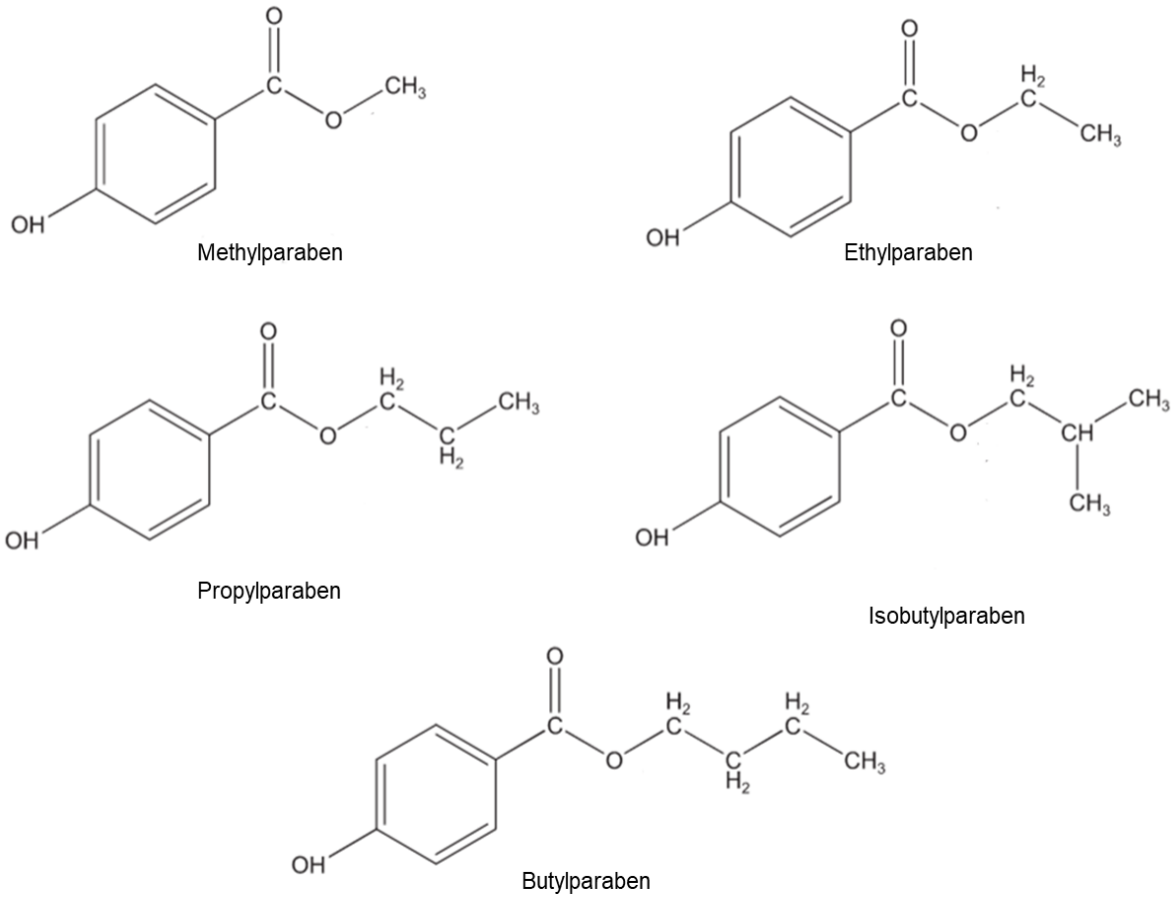

Figure 2.2. Chemical structure of five alkyl esters of p-hydroxybenzoic acid (parabens). Esters are commonly utilised in consumer products, specifically in cosmetics.

Like BPA, parabens have the ability to act as a weak oestrogens by binding to the oestrogen receptors (Hiatt and Brody, 2018). The oestrogenic activity of parabens has been demonstrated in an array of assays, including yeast assays (Routledge *et al.*, 1998), MCF-7 breast cancer cell line (Okubo *et al.*, 2001; Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003) and mice (Darbre *et al.*, 2002, 2003). Research has shown that parabens can significantly increase the proliferation of immortalised human mammary epithelial cells MCF-7 and MCF-10A (non-tumorigenic cell line) at concentrations as low as $2x10^{-10}$ M (Wróbel and Gregoraszczuk, 2013). Further research indicated that parabens can also down-regulate genes involved in cell cycle regulation, including *CDK6* (cell division protein kinase 6), *E2F3* (E2F transcription factor 3) and *ATM* (ATM serine/threonine kinase) in MCF-10A cells (Wróbel and Gregoraszczuk, 2014b). One study reported the increased migratory and invasive activity of breast cancer cells MCF-7, T-47-D and ZR-75-1 after 20 weeks of exposure to methyl-, propyl- and butylparaben (Khanna, Dash and Darbre, 2014), however the tested concentration of $1x10^{-5}$ M was considerably higher than environmental exposures. Nevertheless, concentrations of propylparaben and butylparaben previously reported in human breast tissue (Barr *et al.*, 2012) were observed to be oestrogenic and were seen to stimulate breast cancer cell proliferation, indicating a potential relevance of parabens to breast cancer progression (Pan *et al.*, 2016). Propylparaben has also been seen to up-regulate extrinsic and intrinsic apoptotic pathways, comparably to 17β-oestradiol (Wróbel and Gregoraszczuk, 2014b). Based on this, authors hypothesise propylparaben could possess carcinogenic properties and increase breast cancer risk.

Despite some evidence indicating a relationship between exposure to parabens and breast carcinogenesis, chemical regulators still allow their use in cosmetics. The European Union concluded that concentrations of up to 0.4%, when used individually, or 0.8% as a mixture of esters could be safely used within cosmetic formulations (Scientific Committee on Consumer Safety, 2013). In the US, the Food and Drug Administration (FDA) state they currently have no strong evidence that that use of parabens in cosmetics have any effect on human heath (US Food and Drug Administration, 2018). Within this thesis we examine propylparaben, one of the

predominant parabens identified in cosmetic products (Shen *et al.*, 2007), on account of the high levels observed in human tissues (Barr *et al.*, 2012).

2.1.4. Genes of interest

To address the aims of this chapter we identified genes associated with breast carcinogenesis that we believed could be disrupted by EDC exposure. Whilst there are hundreds of genes that are known to be linked to cancer development, due to the known mechanisms of the two test compounds, they are more likely to impact certain pathways more than others. We also identified genes that are not only thought to be altered by oestrogenic exposure, but also reported to be epigenetically regulated.

2.1.4.1. Oestrogen receptors

Natural oestrogen levels play an integral role in the regulation of growth and development of the mammary gland (E Swedenborg *et al.*, 2009; Javed and Lteif, 2013). These levels are predominantly regulated by oestrogen receptors (ERs), namely, oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ). Both receptors are encoded by distinctive target genes on different chromosomes. In humans, *ESR1* is on chromosome 6, whilst *ESR2* is located on chromosome 14. Both receptors are considered similar to the larger family of nuclear receptors in architecture, composed of independent, yet interacting functional domains (Figure 2.3; Gustafsson and Warner, 2000). Within these domains, activation function 1 (AF-1), located in the N-terminal A/B domain, is involved with protein interactions and the transcriptional activation of gene expression. The DNA binding domain (DBD) plays a role in receptor binding dimerisation. Finally, the ligand-binding domain (LBD) mediates ligand binding, receptor dimerisation, nuclear translocation, and transactivation of gene expression. Within this domain, activation function 2 (AF-2), which is ligand-dependant and undergoes a conformational change in the presence of ligands, determines binding of coactivators or corepressors (Shao and Brown, 2004).

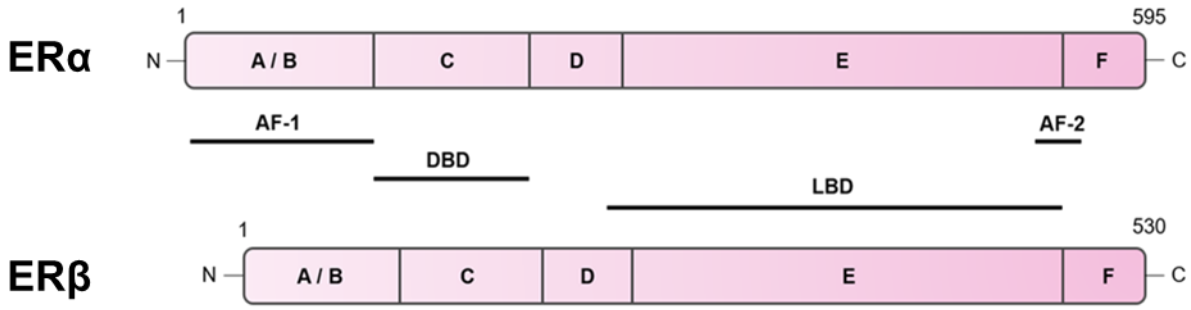

Figure 2.3. Functional domains of ERα and ERβ. Oestrogen receptor genes consisting of functional domains including the DNA-binding domain (DBD), the ligand-binding domain (LBD), the ligand-independent activation function (AF-1), and the ligand-dependent activation function (AF-2) Adapted from Shao and Brown (2004).

Both ERs have been studied in relation to breast carcinogenesis for some years and their roles within tumour development and progression are relatively well understood. Around 80% of tumours express ERα, meaning that oestrogen may be promoting tumour growth (Glass, Lacey and Carreon, 2007). High levels of the receptor found in benign breast epithelium, causing increased oestrogen sensitivity and breast cancer risk, also support the role of ERα in breast cancer initiation (Khan *et al.*, 1998; Ali and Coombes, 2000; Williams and Lin, 2013). *ESR1* mutations are also observed in breast cancer. *ESR1* mutations have been identified as a key mechanism in resistance to hormonal therapies such as aromatase inhibitors, that act by inhibiting aromatase enzymes to reduce oestrogen production (Clatot, Augusto and Di Fiore, 2017). Determining the ER status in breast cancer is also key to identifying treatment options. Hormone dependent tumours are often associated with good clinical outcomes and treated with antiestrogenic drugs, like tamoxifen, which block the effects of oestrogen on the ERs (Davies *et al.*, 2011).

ERβ has been detected frequently in breast tumours and is believed to relate to hormonal sensitivity and tumour resistance (Fuqua *et al.*, 2003). Since the discovery of ERβ in 1996, research has aimed to better understand ERβ expression and the relationship between aberrant *ESR2* expression and breast carcinogenesis to provide insights into treatment and patient outlook. Typically, ERβ expression declines during breast tumorigenesis (Leygue and Murphy, 2013), and high ERβ levels have been associated with good prognostic markers and more

positive clinical outcomes (Esslimani-Sahla *et al.*, 2004; Myers *et al.*, 2004; Gruvberger-Saal *et al.*, 2007). Most studies conducted conclude that ERβ has antiproliferative and pro-apoptotic properties (Leygue and Murphy, 2013). To date, there is no evidence that *ESR2* is a genetic determinant of breast cancer risk, however scientists have called for the further exploration of this genes' role in breast carcinogenesis (Haldosén, Zhao and Dahlman-Wright, 2014). For the purpose of the present study, we require a cell line that is ER competent and therefore it is necessary that we establish whether the genes are expressed in the MCF-12A cell line.

2.1.4.2. G protein-coupled receptor 1

In addition to traditional ERs, it has been shown that G protein-coupled receptor 1 (GPER1, previously GPR30) also binds to oestrogen. GPER1 is a seven-transmembrane domain protein, identified as distinctively different from ERα and ERβ (Maggiolini, Vivacqua and Fasanella, 2004), yet has been shown to be a genuine ER (Revankar *et al.*, 2005). G protein-coupled receptors are heptahelical transmembrane proteins and the largest class of signalling molecules within the human genome (Ji, Grossmann and Ji, 1998). *GPER1* is widely expressed in most breast cancer cell lines and primary breast tumours (Filardo, Quinn and Bland, 2000; Pandey *et al.*, 2009; Lappano, Pisano and Maggiolini, 2014). In 1997, a study revealed a significant positive correlation between ER and *GPER1* expression, suggesting the receptor was involved in physiologic responses to oestrogen in the breast and other hormonally sensitive tissues (Carmeci *et al.*, 1997). Controversies still surround the exact function of *GPER1*, however it has been speculated that the gene may act as a pro-apoptotic mediator and has the potential to interfere with the progression of breast cancer (Lappano, Pisano and Maggiolini, 2014). For instance, *GPER1* inhibited breast cancer cell proliferation via the induction of cell cycle arrest in the M-phase of MCF-7 and SK-BR-3 cells, with authors suggesting *GPER1* as a potential tumour suppressor gene (Weißenborn, Ignatov, Poehlmann, *et al.*, 2014). This action was also shown in the triple-negative breast cancer cell lines MDA-MB-231 and MDA-MB-468, which was seen to be epigenetically regulated via hypermethylation of promotor regions, further supporting the theory of *GPER1* having a protective role in breast cancer (Weißenborn, Ignatov, Ochel, *et al.*,

2014). Finally, *GPER1* expression can influence the responsiveness of ER-negative cancer cells to oestrogen, by activating MAPKs, Erk-1/-2, independent of ERα or ERβ (Filardo, Quinn and Bland, 2000). Again, it is important to establish the presence of *GPER1* in the MCF-12A cell line for subsequent experiments.

2.1.4.3. Cyclin D1

Cyclin D1 belongs to a family of D-type cyclins that are primarily responsible for the regulation of cyclin-dependent kinase (CDK4 and CDK6) activity. Cyclin D1 controls the progression of cells from G1 to S phase and is therefore essential for the maintenance of cell cycle regulation (Gillett *et al.*, 1994; Elsheikh *et al.*, 2008). Cyclin D1 functions to activate CDK4 and CDK6, which leads to the phosphorylation of the retinoblastoma tumour suppressor protein (Sutherland and Musgrove, 2004). Perturbations in cyclin D1 can result in uncontrolled cell proliferation, and as such, has been referred to as an oncogenic event (Tobin and Bergh, 2012). Cyclin D1 overexpression is reported in 40-90% of invasive breast carcinomas, resulting in the protein being amongst the most frequently overexpressed proteins in breast cancer (Zhang *et al.*, 1994; Mohammadizadeh *et al.*, 2013). The oncogene, *CCND1*, is located on chromosome 11q13 and encodes the cyclin D1 protein. Up-regulation of *CCND1* expression has been observed in 5-20% of breast tumours (Gillett *et al.*, 1994; Li *et al.*, 2016). This *CCND1* amplification has been reported to predict poor response to tamoxifen treatment in ER-positive tumours and has been associated with a poor clinical prognosis (Aaltonen *et al.*, 2009; Tobin and Bergh, 2012). Cyclin D1 expression is directly regulated by ER. When bound to oestrogen, ER has been shown to stimulate cyclin D1 protein and mRNA expression, resulting in an elevated level in ER-positive breast cancers (Musgrove *et al.*, 1993; Tobin and Bergh, 2012). However, additional pathways are also able to interfere with cyclin D1 expression, including MAP kinase, independently of ER activity (Modi *et al.*, 2012). More recently, studies have identified additional roles for *CCND1* outside of its traditional cell-cycle regulation function, such as invasion and metastasis (Fusté *et al.*, 2016). Few studies have investigated the impact of EDC exposures on *CCND1* expression. Mlynarcikova and colleagues (2013) observed a significant increase in *CCND1* expression after

24 hours of BPA exposure in MCF-7 cells, even at low, environmentally relevant concentrations ($1x10^{-12}$ M). Whilst our understanding of the role of *CCND1* expression is still developing, evidence strongly supports the ability of oestrogen to interfere with *CCND1* expression. and is therefore used in this chapter.

2.1.4.4. Cyclin D2

Cyclin D2 has a critical role in cell cycle regulation (Sherr and Roberts, 2004), yet its role in breast cancer has been less thoroughly investigated. In contrast to cyclin D1, cyclin D2 expression is frequently lost in breast cancers. One study reported loss of cyclin D2 expression in 25% of sporadic breast carcinomas and 54% of familial breast cancers (Fischer *et al.*, 2002). The reduction in expression at gene level for *CCND2* (the gene encoding cyclin D2) has also been documented in primary breast cancers (Oyama *et al.*, 1998). The reduction of *CCND2* expression may indicate a tumour suppressor function in normal breast cells, suggesting that the loss may be related to tumorigenesis (Oyama *et al.*, 1998). Several studies have now identified promotor region hypermethylation as a mechanism for this down-regulation in tumour tissues, with hypermethylation of *CCND2* being observed in 11-52% of breast malignancies (Fackler *et al.*, 2003, 2004; Li, Rong and Iacopetta, 2006; Sharma *et al.*, 2007; Li *et al.*, 2015; White *et al.*, 2015). However, little research has been undertaken to elucidate whether EDCs could induce hypermethylation of *CCND2*. One study did observe a significant down regulation of *CCND2* gene expression in pregnant OF-1 mice in response to a daily dose of $1x10^{-5}$ g/kg BPA, however authors did not investigate whether this alteration could be linked to epigenetic changes (Alonso-Magdalena *et al.*, 2015). To the best of our knowledge, no studies have investigated the impact of propylparaben on *CCND2* gene expression.

2.1.4.5. Breast cancer susceptibility gene 1

BRCA1 (breast cancer susceptibility gene 1) is a tumour suppressor gene located at chromosome 17q12-21, which plays a critical role in DNA repair and is also involved in controlling the cell cycle (Wu, Lu and Yu, 2010; Mehrgou and Akouchekian, 2016). Numerous studies have demonstrated the involvement of *BRCA1* in the repair of double-strand breaks and

initiation of homologous recombination (Scully *et al.*, 1997; Chen *et al.*, 1998; Liu and West, 2002; Zhang and Powell, 2005). *BRCA1 also* regulates S-phase and G2/M-phase checkpoints (Rosen *et al.*, 2003) and research has shown BRCA proteins to bind and interact with several other regulatory proteins (Table 2.1).

Table 2.1. Regulatory proteins known to interact with *BRCA1*. Adapted from Yoshida and Miki (2004).

Process	Protein symbol
DNA repair	ATM, CHK2, ATR, BRCA2, RAD51, RAD50 / MRE11/NBS1, BASC, PCNA, H2AX, c-Abl
Transcription	RNA polymerase II holoenzyme (RNA helicase A, RPB2, RPB10α), HDAC1, HDAC2, E2F, CBP/ p300, SWI/SNF complex, CtIP, p53, androgen receptor, ATF1, STAT1, ERα, c-Myc, ZBRK1
Cell cycle	RB, CDK2, p21, p27, BARD1
Other	BAP1, BIP1, BRAP2, importin α

Inherited mutations are associated with a predisposition to cancer in hormone responsive tissues, accounting for 40-45% of hereditary breast cancers (Lux, Fasching and Beckmann, 2006). Women born with mutations in the *BRCA1* gene have a significantly higher risk of developing breast cancer (>80% lifetime risk), along with a 40-65% lifetime risk of developing ovarian cancer (King *et al.*, 2003). Somatic deleterious mutations in *BRCA1* are extremely rare in sporadic breast cancers (Futreal *et al.*, 1994; Turner, Tutt and Ashworth, 2004), however 30-40% of sporadic breast cancers show reduced *BRCA1* gene expression, especially in high grade tumours (Wilson *et al.*, 1999; Alkam *et al.*, 2013). One mechanism for this down-regulation could be linked to hypermethylation in *BRCA1* promotor regions, which has been reported in 5-65% of sporadic breast cancers (Baldwin *et al.*, 2000; Esteller *et al.*, 2000; Chan *et al.*, 2002; Buyru *et al.*, 2009; Bal *et al.*, 2012; Hasan *et al.*, 2013; Ignatov *et al.*, 2013).

BRCA1 can influence oestrogen receptor signalling, either by inhibiting ERα directly, or by inhibiting downstream effectors of ER (Wang and Di, 2014). When cells proliferate due to the mitogenic effects of oestrogen, this interaction between *BRCA1* and oestrogenic signalling ensures replicated DNA is of high quality. When *BRCA1* expression is absent or reduced, cells begin to accumulate genomic mutations, contributing to breast cancer initiation (Li *et al.*, 2007;

Nishi *et al.*, 2014). This decreased expression has also been linked with an increased sensitivity to the effects of oestrogenic compounds. For example, Jones *et al.,* (2010) knocked down *BRCA1* in MCF-7 cells and treated cells with $1x10^{-8}$ M to $1x10^{-7}$ M BPA. After 72 hours significantly higher cell proliferation was observed in cells without *BRCA1*, compared with cells expressing *BRCA1* (Jones *et al.*, 2010). Moreover, 17β-oestradiol ($8x10^{-8}$ M), the insecticide β-hexachlorocyclohexane ($1x10^{-8}$ M) and *o,p'*-DDT ($1x10^{-5}$ M) have been shown to significantly increase *BRCA1* expression in MCF-7 cells (Silva, Kabil and Kortenkamp, 2010).

2.1.4.6. Ras associated domain family 1 isoform A

Ras associated domain family 1 isoform A (*RASSF1A*) is a tumour suppressor gene implicated in the development of several human cancers, including breast cancer. *RASSF1A* belongs to a family of six proteins. With the exception of *RASSF3*, all family members have now been categorised as tumour suppressors. *RASSF1A* holds a critical role within the G1/S checkpoint, through the inhibition of native cyclin D1 accumulation (Shivakumar *et al.*, 2002). The gene has also been implicated in the modulation of apoptotic processes. One such example is the direct binding of *RASSF1A* to MOAP1 (modulator of apoptosis 1), which is a bax-binding protein. As a member of the Bcl-2 family, bax is involved with key apoptotic pathways. The binding of *RASSF1A* to MOAP1 has been shown to regulate bax function, supporting the potential for *RASSF1A* to co-ordinate cell apoptosis (Baksh *et al.*, 2005; Vos *et al.*, 2006). Overactivation of *RASSF1A* has been observed to promote cell cycle arrest, apoptosis and reduces tumorigenicity of cancer cell lines (Agathanggelou, Cooper and Latif, 2005). Likewise, downregulation of the gene results in a loss of cell cycle regulation, increased genetic instability and enhanced cell mobility (Agathanggelou, Cooper and Latif, 2005; Dallol *et al.*, 2005; Song *et al.*, 2005; Vos *et al.*, 2006). DNA hypermethylation has been identified as the most likely mechanism behind the loss of *RASSF1A* expression in breast cancers, with genomic mutations being extremely rare (Agathanggelou, Cooper and Latif, 2005). Whilst methylation of *RASSF1A* seldom occurs in normal tissues, hypermethylation of the promoter region has been observed in 80-95% of primary breast tumours, resulting in gene silencing (Agathanggelou, Cooper and Latif, 2005;

Shinozaki *et al.*, 2005; Yeo *et al.*, 2005; Yadav *et al.*, 2016). This suggests that *RASSF1A* silencing is pivotal to breast carcinogenesis.

Little is known about the interaction between *RASSF1A* and oestrogen in comparison to the other genes of interest. Studies suggest *RASSF1A* could act as a tumour suppressor through the regulation of ERα. Thaler *et al.,* (2012) observed that the reconstitution of *RASSF1A* in MCF-7 cells resulted in a decrease in ERα expression. Additionally, the expression of *RASSF1A* led to a decrease in oestrogen-responsive genes *c-Myc* and *BCL2* and increased oestrogen sensitivity. Researchers are yet to specifically investigate the impact of EDCs on *RASSF1A* expression or methylation. Given the crucial role *RASSF1A* appears to have in breast cancer development, along with its association with ERs, elucidating whether ubiquitous EDCs can alter *RASSF1A* expression may provide insights into whether additional mechanisms, such as epigenetic modification, may link EDC exposure to breast cancer risk.

2.1.5. Chapter Scope

The primary aim of this chapter was to determine the suitability of the MCF-12A line for further experiments. This aim was addressed through the following questions:

1) Does the MCF-12A cell line express *ESR1, ESR2* and *GPER1* receptor genes in the presence and absence of endocrine disrupting compound, BPA and propylparaben?
2) Under control conditions, does the MCF-12A cell line express other genes associated with breast carcinogenesis that have previously been reported to be impacted by oestrogen?
3) Do the genes identified in Q2 respond to BPA and propylparaben exposure?

2.2. Methodology

2.2.1. Routine cell culture

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Dorset, UK). MCF-12A cells were obtained from the American Type Culture Collection and grown in monolayer within 75 cm^2 canter-neck tissue culture flasks. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM: F12; Invitrogen, Paisley, UK), supplemented with 5% horse serum (Invitrogen, UK), 0.02% epidermal growth factor, 0.01% cholera toxin, 0.1% insulin, 0.05% hydrocortisone and 1% pen/strep. Medium was replaced every four days and cells were kept in a humidified incubator at 37˚C and 5% CO_2. Cells were passaged at 70% confluence with 0.25% trypsin-ethylene diamine tetraacetic acid (trypsin-EDTA).

2.2.2. Chemical exposures

Stocks of BPA and propylparaben were prepared in 100% HPLC-grade ethanol as $1x10^{-3}$ M stocks. All chemical stocks and subsequent dilutions were stored at -20 °C in critically clean glass containers. Two concentrations of each compound were used: $1x10^{-7}$ M and $1x10^{-5}$ M. These concentrations were chosen based on their known ability to induce an effect in this model (Marchese and Silva, 2012). Cultures were treated with either BPA or propylparaben every three days throughout the experiment. RNA was isolated at 24, 168 and 336 hour timepoints after chemical exposure (Figure 2.4). In all cases, the final concentration of solvent did not exceed 0.5% to avoid ethanol toxicity. A total of three independent experiments were carried out.

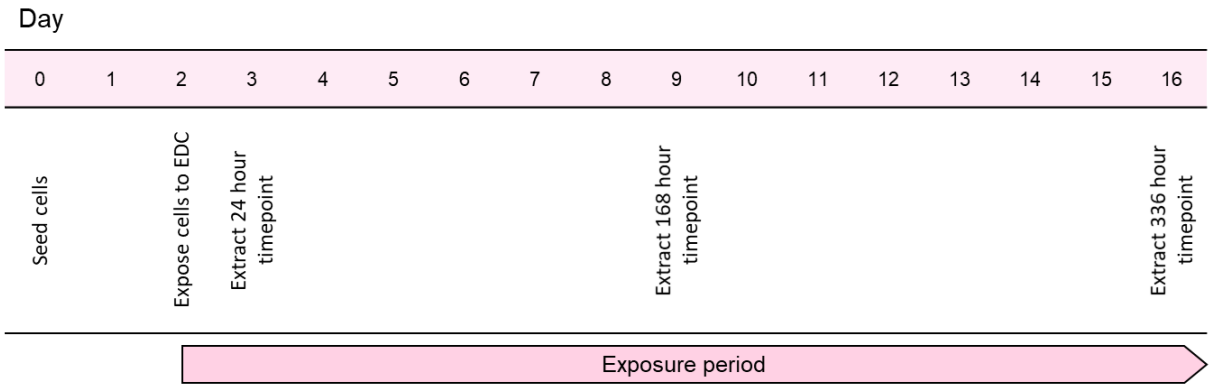

Figure 2.4. Experimental timeline for chemical exposures. MCF-12A cells were seeded on day 0 and left to attach before chemical treatment. RNA was isolated from treated samples after 24, 168 and 336 hours of chemical exposure.

2.2.3. RNA extraction

After the treatment period, cells were coated with trypsin-EDTA and incubated for five minutes at 37˚C and 5% CO_2. Once the cells were no longer attached to the flask, they were suspended in supplemented DMEM: F12 medium and used for RNA extraction. RNA was isolated from treated cells using RNeasy Mini Kit (Qiagen, Manchester, UK), following the manufacturer's instructions. Purity of the final RNA sample was confirmed using 260/280 and 260/230 ratios with the Nanodrop One (Thermo Scientific, Loughborough, UK).

2.2.4. Real-time PCR

A two-step approach was used for gene amplification, with the initial step being reverse transcription of the isolated RNA. Approximately 2.5 µg of RNA was reverse transcribed into cDNA using a previously described protocol (Marchese and Silva, 2012). This process allowed double stranded DNA molecules to be made from single stranded RNA, ready for gene amplification. Briefly, 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) 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). Next, samples

were heated to 65˚C for 10 minutes and cooled on ice for 2 minutes prior to 42˚C incubation with 2 µl Reverse Transcriptase (Promega) for 90 minutes. cDNA samples were stored at -80˚C until use in real-time PCR (rt-PCR).

rt-PCR is a powerful and sensitive tool that can be used for a broad range of applications. This method of gene expression analysis measures gene amplification as it occurs after each cycle, providing accurate and quantitative amplification data. Unlike traditional PCR, which is based purely on end-point detection of the amplified product, rt-PCR monitors the accumulation of PCR product making it possible to determine starting concentrations of nucleic acid in the sample. rt-PCR has also been cited as easy to use and highly reproducible with no post-PCR handling and no use of radioactivity (Radonić *et al.*, 2004). It is also possible to detect much smaller fold changes in gene expression, which may be invaluable when working with low-doses of endocrine disrupting chemicals.

In the present experiment, rt-PCR was performed using SYBR Green Supermix (PrimerDesign, Southampton, UK) in the BioRad rt-PCR iCycler system. SYBR Green is used to detect differences in target gene expression by incorporating itself into the minor grove of the cDNA. Once it binds to the DNA, SYBR Green emits fluorescence, whereas unbound SYBR Green does not. During the amplification process, the amount of DNA increases and therefore more SYBR Green binding occurs, resulting in proportionally increasing fluorescence being emitted and subsequently detected. The amplification curve produced after using real-time PCR depicts the number of cycles required before the level of emitted fluorescence crosses the cycle threshold (Ct). To calculate the Ct value, the baseline is adjusted to the point where the increase in PCR product becomes exponential (Figure 2.5). The baseline remains the same for each primer pair to ensure the Ct value can be accurately determined, allowing gene expression to be compared across samples. Alterations to the expression of each target gene is observed by a shift in the Ct value (Figure 2.5).

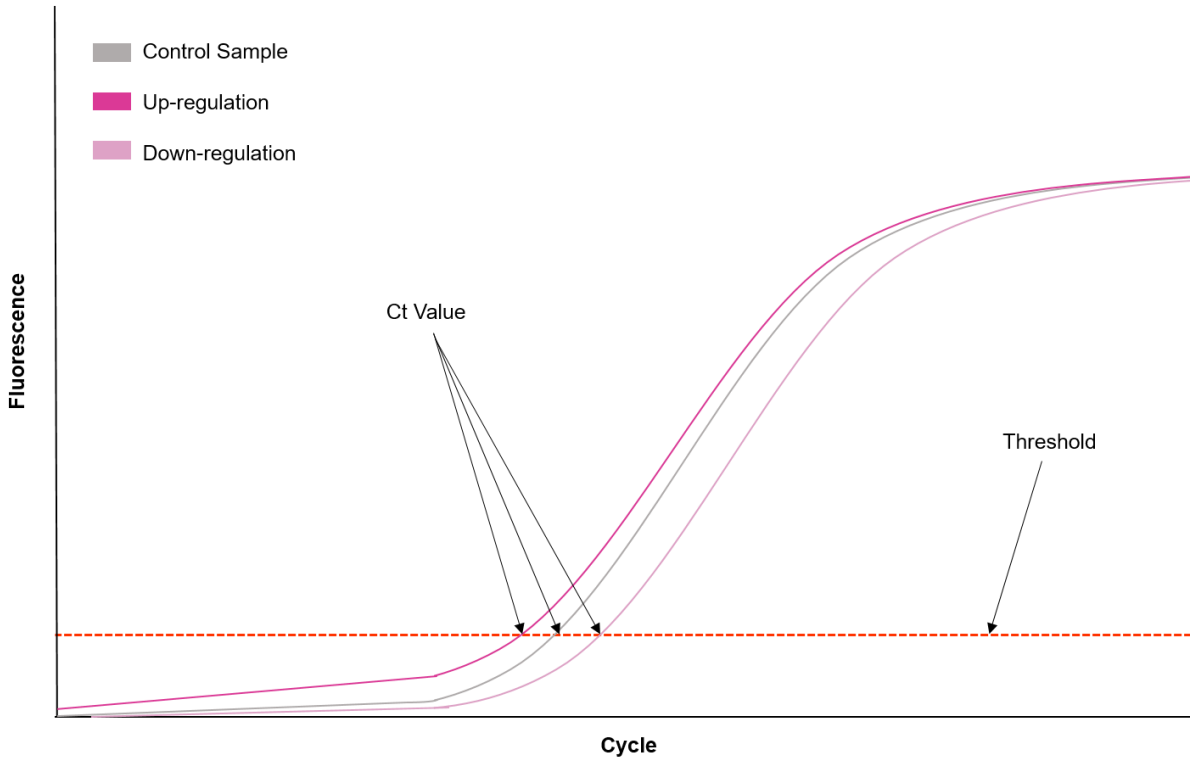

Figure 2.5. Hypothetical amplification curve produced following real-time PCR analysis. The cycle number is plotted against the arbitrary fluorescence. The threshold (red) is the point at which the fluorescence produced by SYBR Green becomes exponential. The cycle number at which the sample crosses the threshold is denoted as the Ct value. If the expression of the target gene is higher it will have a lower Ct, whereas a gene expressed at lower levels will have a higher Ct value.

2.2.4.1. Primer Selection and optimisation

Primer pairs were purchased as highly purified oligos. All primer pairs were optimised and the final concentrations are described in Table 2.2.

Table 2.2. Target gene primer sequences. Forward and reverse primer sequences are shown with accession numbers and final concentrations for use in real-time PCR analysis.

Gene	Accession number	Primer Sequence (5'->3')		Prod. Size	Conc. (M)
ESR1	NM_000125	Forward	GCCCTCCCTCCCTGAAC	146	$2.5x10^{-7}$
		Reverse	TCAACTACCATTTACCCTCATC		$2.5x10^{-7}$
ESR2	NM_001437	Forward	TCCTCCCAGCAGCAATCC	139	$2x10^{-7}$
		Reverse	CCAGCAGCAGGTCATACAC		$2x10^{-7}$
GPR30	NM_001505	Forward	GTTCCTCTCGTGCCTCTAC	132	$2x10^{-7}$
		Reverse	ACCGCCAGGTTGATGAAG		$2x10^{-7}$
BRCA1	NM_007294	Forward	ACATACCATCTTCAACCTCTG	122	$2.5x10^{-7}$
		Reverse	CGATGGTATTAGGATAGAAG		$2.5x10^{-7}$
CCND1	NM_053056	Forward	TGGAATGGTTTGGGAATAT	146	$2x10^{-7}$
		Reverse	CCTGGCAATGTGAGAATG		$2x10^{-7}$
CCND2	NM_001759	Forward	TGGGACAATGGGTGGTGAA	94	$3x10^{-7}$
		Reverse	GCAAAGCTGGCTCTTGAGAA		$3x10^{-7}$
RASSF1A	NM_007182	Forward	GCTAAGGGTGGGTGCTCAG	85	$3x10^{-7}$
		Reverse	TCAGGGTGTGTGAGGAGTTG		$3x10^{-7}$

Melt curves were produced to ensure the specificity of the primers and that no genomic contamination had occurred. The melt curve indicates the temperature against the relative fluorescence units over time (dFRU/dT), where peaks correspond to the amplified PCR product (Tm). Melt curves should contain one single peak (Figure 2.6) representing a single amplicon with a single melting temperature. Melt curves can depict the occurrence of contamination within genomic DNA, with the presence of a second curve, usually at a higher melting temperature of the target PCR product. Additional peaks at lower melting temperatures may represent primer dimers or mis-priming. The presence of additional peaks, indicating that additional products have been amplified, (Figure 2.6) would suggest issues with the primers and inaccurate PCR results.

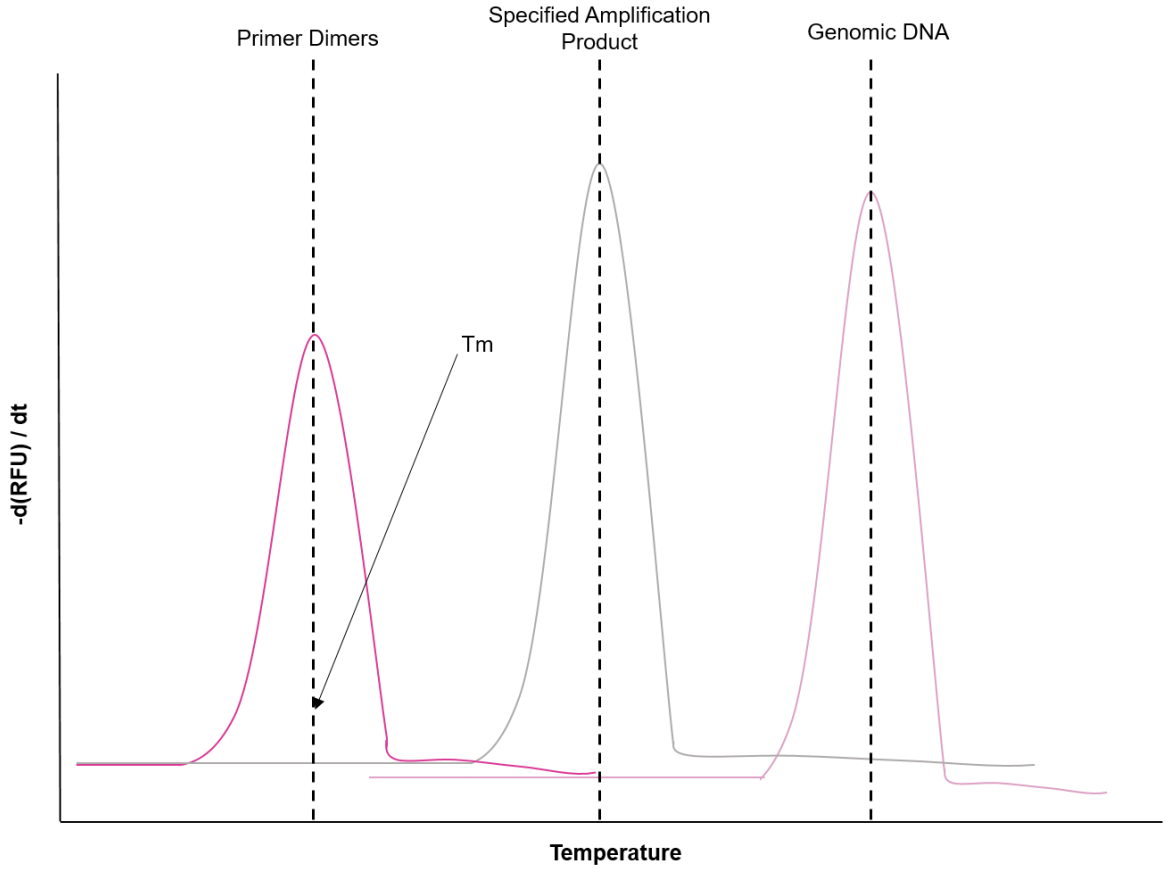

Figure 2.6. Hypothetical melt curve following real-time PCR analysis. Peaks represent the melting temperatures (Tm) of amplified products. The grey peak shows the melting temperature of the sample. Additional peaks within the melt curve indicate possible problems with the amplification process. To the left of the sample, primer dimers produce peaks at lower temperatures where the primers have bound to each other, whereas contamination with genomic DNA will be shown at a higher melting temperature, right of the sample.

2.2.4.2. Reference gene selection

Data were normalised against a reference gene to quantify changes to gene expression. This normalisation allowed for PCR and reverse transcription variation, along with sample loading errors, without impacting the final results. To be considered as a suitable reference gene, the target gene must be present in the cell type and its expression must not be impacted by chemical treatments. Any variation in Ct value would indicate that the gene expression had been altered by chemical treatment and therefore would be unsuitable as a reference gene.

To allow for the selection of the most suitable reference gene, several potential genes were identified based on their use in the literature and their presence in most cell types. It has been

argued that 'traditional' referencing genes, such as β-Actin (*ACTB*), are not always suitable as they can be influenced by treatments in some cell types, and therefore alternative genes were considered before deciding on the most robust choice (Radonić *et al.*, 2004). *ACTB, HPRT1, TBP, RPL13A, RPS18* and *UBC* (Table 2.3) were tested against $1x10^{-5}$ M BPA to ensure that chemical exposure did not disrupt the expression of candidate genes.

Table 2.3. Candidate reference gene primer sequences. Forward and reverse primer sequences are shown with accession numbers and final concentrations for use in real-time PCR analysis.

Gene	Accession number	Primer Sequence (5'->3')		Prod. size	Conc. (M)
ACTB	NM_001101	Forward	TCAGCAAGCAGGAGTATG	97	$3x10^{-7}$
		Reverse	GTCAAGAAAGGGTGTAACG		$3x10^{-7}$
HPRT1	NM_000194	Forward	CCTTGGTCAGGCAGTATAATCC	135	$3x10^{-7}$
		Reverse	GGGCATATCCTACAACAAACTTG		$3x10^{-7}$
TBP	NM_003194	Forward	TTGGACTTCAAGATTCAG	133	$3x10^{-7}$
		Reverse	AATAACTCTGGCTCATAC		$3x10^{-7}$
RPL13A	NM_012423	Forward	GAAAGGGTCTTAGTCA	134	$3x10^{-7}$
		Reverse	CTCCAATCAGTCTTCT		$3x10^{-7}$
RPS18	NM_022551	Forward	TACTCAACACCAACATC	97	$3x10^{-7}$
		Reverse	TTCCTCAACACCACAT		$3x10^{-7}$
UBC	NM_021009	Forward	TGACACCATTGAGAAT	128	$3x10^{-7}$
		Reverse	TCTGGATGTTGTAGTC		$3x10^{-7}$

2.2.5. Relative gene expression statistical analysis

The two most common methods for analysing PCR data are absolute quantification and relative quantification. Absolute quantification can be used to establish the input copy number of the target gene and can be determined through relating PCR signals to a standard curve (Livak and Schmittgen, 2001). However, this can be time consuming and not always necessary. In this case, having a relative quantification was more appropriate to identify whether chemical exposure has increased or decreased gene expression. Relative quantification can be quantified through the implementation of the $2^{-\Delta\Delta Ct}$ method, as described by Livak and Schmittgen (2001). This analysis assumes that the amplification efficiency of target and reference genes is approximately equal. We ensured these requirements were met during primer optimisation and only primers eliciting

the same level of PCR efficiency (between 98-100%) were used. In addition, a reliable reference gene must be validated to ensure it is not affected by experimental treatments. The analysis was undertaken here using the following calculation:

$$\Delta Ct = Ct\,(target\ gene) - Ct\,(reference\ gene)$$

$$\Delta\Delta Ct = \Delta Ct\,(treatment\ group) - mean\ \Delta Ct\,(control\ group) \quad \textbf{(Equation 2.1)}$$

$$2 - \Delta\Delta Ct$$

Results are reported as log2 (fold change), denoted as LogFC, with values obtained in controls standardised to 0. A LogFC-value close to 0 indicates that no change in gene expression has occurred between control and treated samples. Negative values indicate a decrease in gene expression, whilst positive values depict an increase. LogFC was utilised to ensure normal distribution of the data, which was required for the analysis, and visualisation of fold changes. After relative expression was calculated, normal distribution was confirmed and a parametric test was deemed appropriate. A two-way Analysis of Variance (ANOVA) test was undertaken to determine the significance of changes in gene expression between treated and control groups. To identify specific treatments that induced a significant differential gene expression, a post-hoc test was required. Here we used Bonferroni's multiple comparison test. All statistical analysis was carried out in R (version 3.1.2) and data visualisation undertaken in Prism (version 7.03).

2.3. Results

2.3.1. Selection of a suitable reference gene

Six genes were utilised to identify the most suitable candidate for the subsequent analysis of relative gene expression, including *ACTB* (β-actin), *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *TBP* (TATA-box binding protein), *RPL13A* (ribosomal protein L13a), *RPS18* (ribosomal protein S18) and *UBC* (ubiquitin C). Amplification curves showed shifts in expression after treatment in four of the six candidate genes (Figure 2.7). A decrease in

HPRT1 and *UBC* gene expression was observed after 24 hours of 1x10^{-5} M BPA exposure. *TBP* and *RPL13A* demonstrated an increase in expression, making them unsuitable as reference genes in the present study. No BPA-induced shifts could be observed in *ACTB* and *RPS18* indicating the suitability of both genes. However, *ACTB* displayed a lower Ct value, suggesting a higher expression level in MCF-12A cells that is easier to detect and prone to lower variability. Therefore, *ACTB* was used in subsequent relative gene expression analysis as the reference gene.

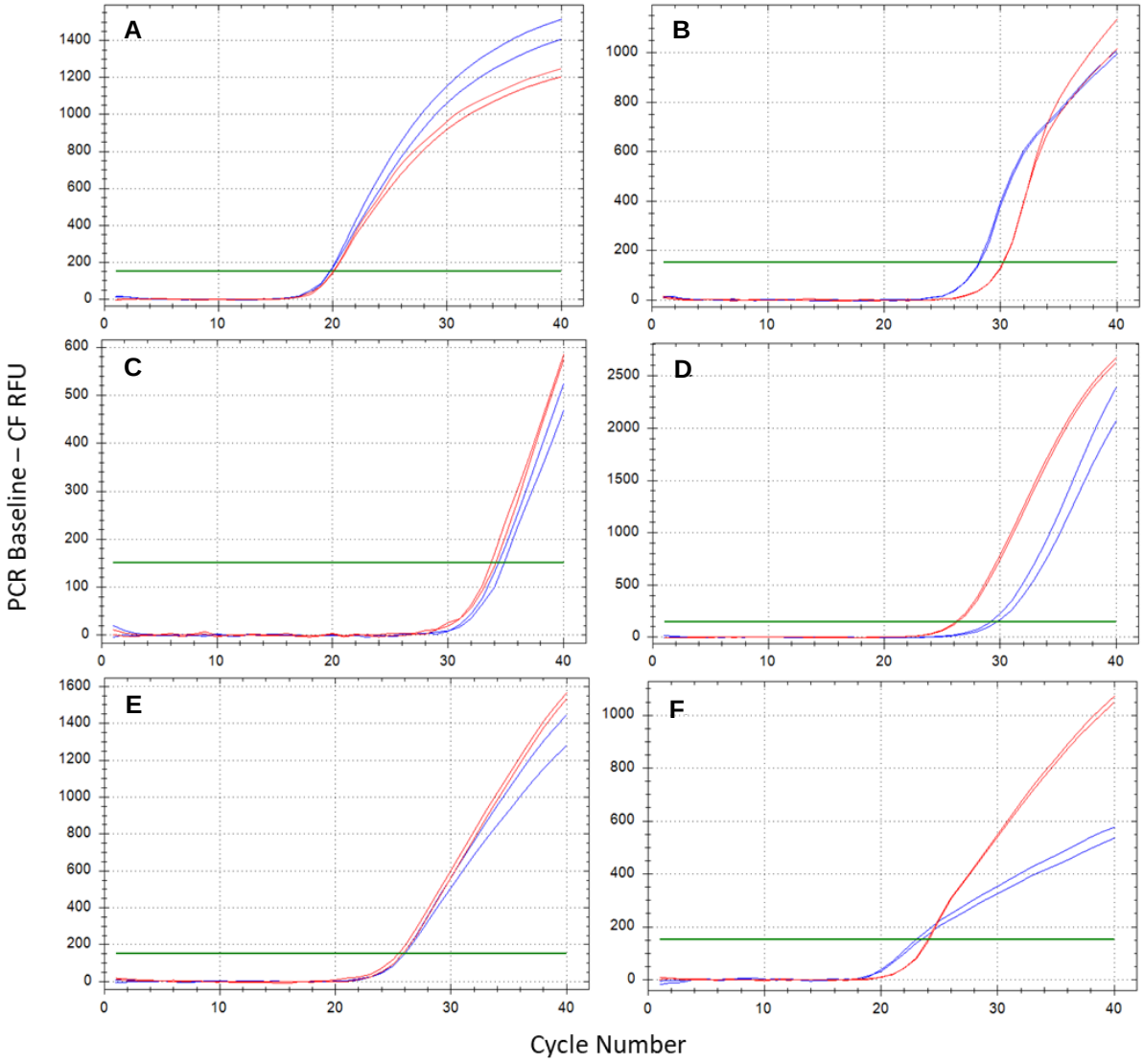

Figure 2.7. **Reference gene real-time PCR amplification curves for MCF-12A cell line.** Control samples in blue and bisphenol A (1x10^{-5} M) exposed samples in red. Graphs demonstrate (A) stable *ACTB* expression (B) decrease of *HPRT1* (C) increase of *TBP* (D) increase of *RPL13A* (E) stable *RPS18* expression (F) and reduction of *UBC*.

2.3.2. MCF-12A cell line expressed *ESR1, ESR2* and *GPER1*

Despite published confirmation of the ER status of MCF-12A cells, there are still conflicting reports in regards to the receptor status with MCF-12A cell line (Soule *et al.*, 1990; Liu *et al.*, 2007; Marchese and Silva, 2012; Sweeney, Sonnenschein and Soto, 2018). As the aims of this thesis are to investigate the effects of EDCs in a hormonally responsive assay, representative of the normal mammary epithelium, it is important to ensure we are using a cell line that is both ER and GPER competent. Consequently, we tested for *ESR1*, *ESR2* and *GPER1* expression at gene level at passage numbers that will be utilised in further experiments.

Real-time PCR analysis confirmed that the MCF-12A cell line expressed *ESR1*, *ESR2* and *GPER1* in untreated samples (Figure 2.8). Expression continued after 24 hours of $1x10^{-5}$ M of BPA exposure, however all receptors showed a higher Ct value in treated samples, suggesting a decrease in gene expression. Melt curves confirmed the specificity of the primers and the purity of the sample, demonstrated by the presence of a single peak for all samples (Figure 2.9).

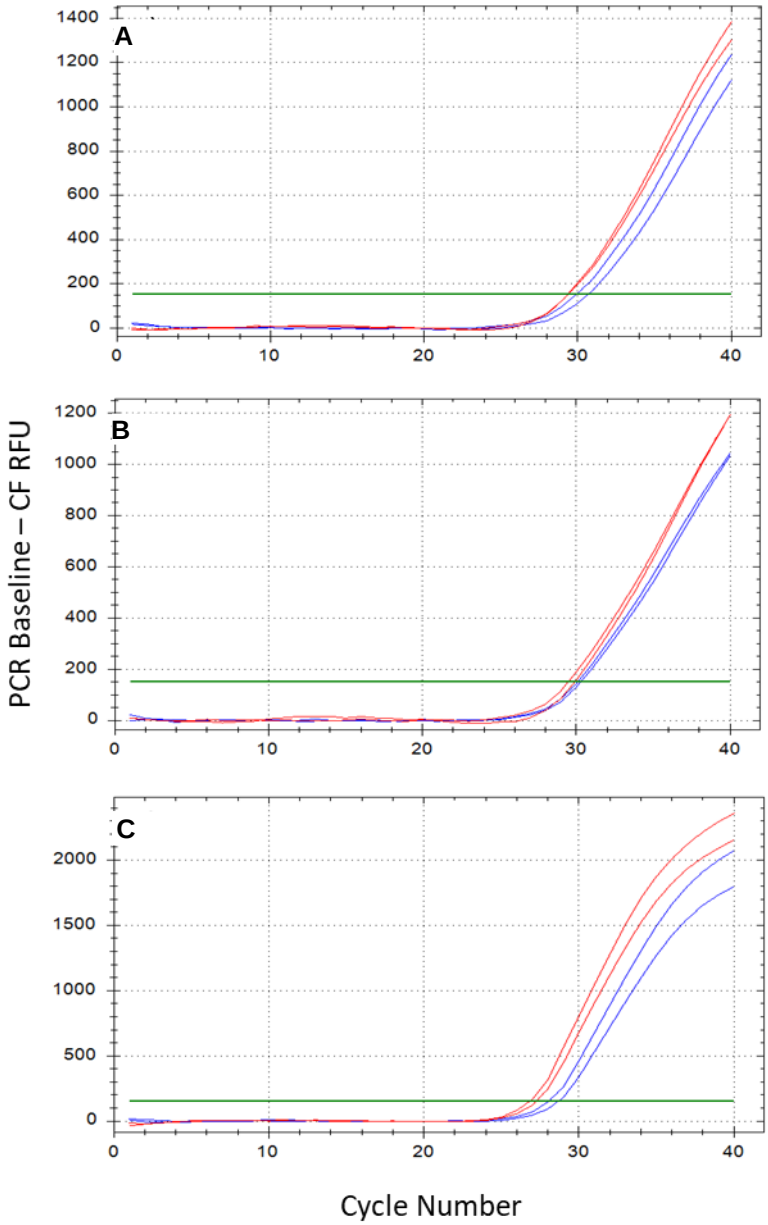

Figure 2.8. ER amplification curves for MCF-12A cell line. Amplification carried out in replicate, showing (A) presence of *ESR1* in the presence of pure ethanol (red) and 1x10^{-5} M bisphenol A (blue). *ESR2* (B) is also expressed in control and treatment samples, as is (C) *GPER1*. Curves are representative of three independent experiments.

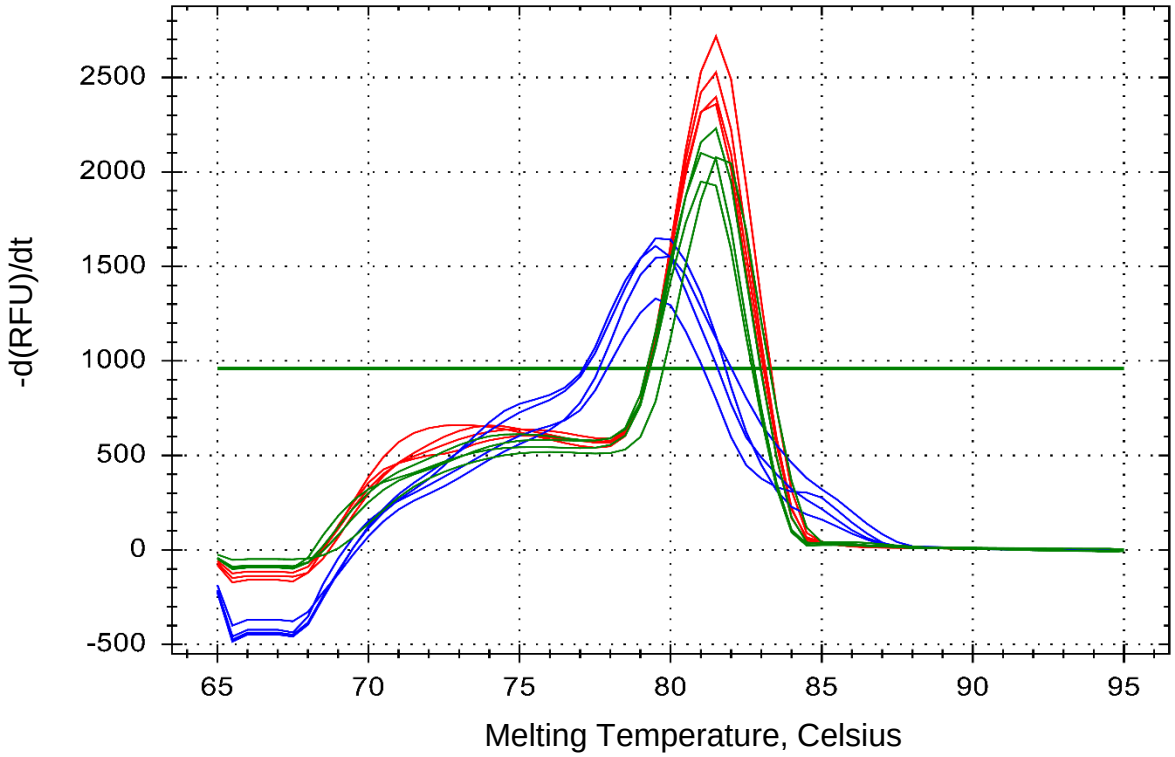

Figure 2.9. **Melt curve for *ESR1*, *ESR2* and *GPER1* gene amplification.** Amplified PCR product in MCF-12A cells for *ESR1* (red), *ESR2* (blue) and *GPER1* (green) after real-time PCR. The presence of a single peak for all target genes confirms the specificity of the real-time PCR amplification, with no primer dimers or genomic DNA contamination, and the reliability of results.

2.3.3. BPA exposure resulted in the significant down-regulation of oestrogen receptor genes

Once we established that MCF-12A cells were ER and GPER competent, we wanted to elucidate how their expression changed in the presence of xenoestrogens. To identify changes in gene expression, real-time PCR data were normalised to the reference gene *ACTB*. *ESR1, ESR2* and *GPER1* saw a significant down-regulation in gene expression after 24 hours of BPA exposure. The decrease in expression was most prominent in *ESR1*, with both tested concentrations instigating more than a 4-fold reduction in gene expression ($1x10^{-7}$ M; -4.33±0.51 LogFC, p <0.001, $1x10^{-5}$ M; -5.63±0.72 LogFC, p <0.001). After 168 hours the down-regulation of *ESR1* was no longer statistically significant in either concentration, with expression returning to levels indifferent to control samples by 336 hours (Figure 2.10A). *ESR2* was also significantly down-

regulated at 24 hours ($1x10^{-7}$ M; -2.99±0.33 LogFC, p <0.001, $1x10^{-5}$ M; -2.83±0.56, p <0.001), as was *GPER1* ($1x10^{-7}$ M; -1.76±0.37 LogFC, p = 0.002, $1x10^{-5}$ M; -1.71±0.49 LogFC, p = 0.002). Comparably to *ESR1*, neither *ESR2* or *GPER1* observed significant changes to gene expression at the 168 or 336 hour timepoints (Figure 2.10B,C).

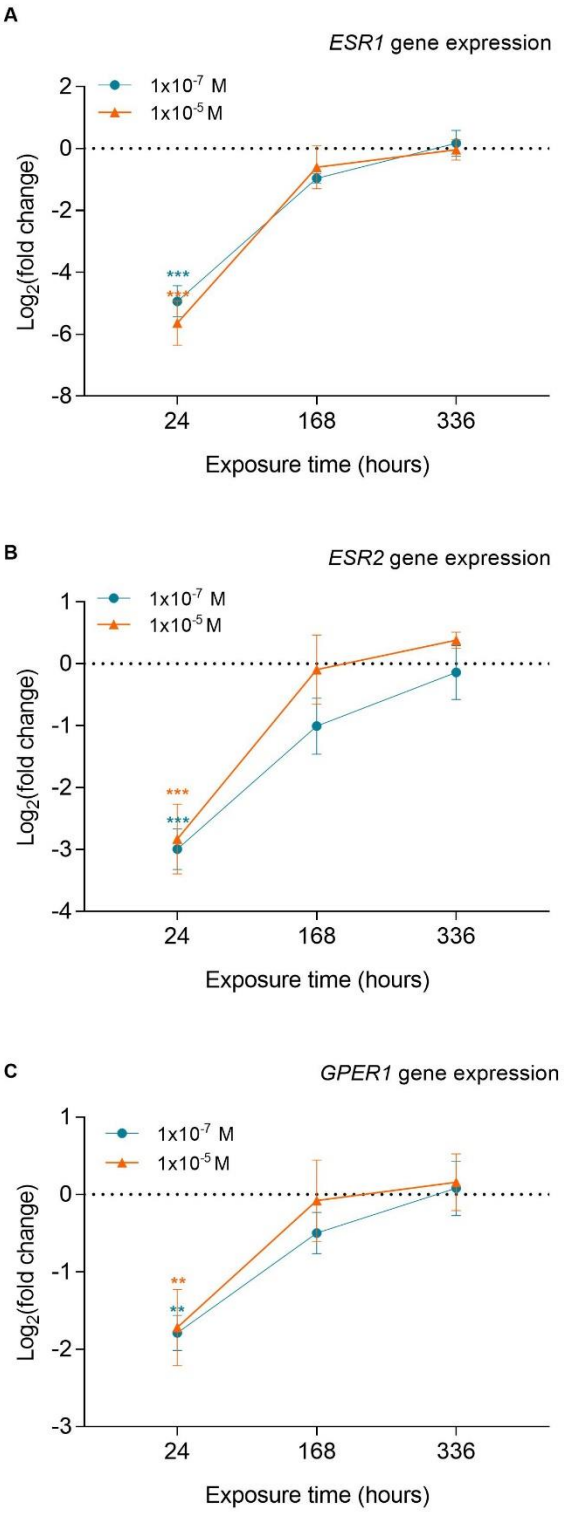

Figure 2.10. BPA-induced log2 (fold change) in *ESR1, ESR2* and *GPER1.* A decrease in gene expression is observed in *ESR1* (A) and *ESR2* (B). *GPER1* is also decreased in expression (C). Error bars indicate the standard error of the mean (SEM) for each treatment. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by two-way ANOVA with Bonferroni correction.

2.3.4. BPA exposure resulted in significant alterations to *CCND1, CCND2, BRCA1* and *RASSF1A* gene expression

Next, we assessed whether additional genes relevant to breast cancer and previously reported to be affected by oestrogen exposure, could be altered by BPA. We investigated *CCND1*, which is directly regulated by the ER. We also tested *CCND2* and *RASSF1A* that are both known to be epigenetically regulated, and we believed could be altered in response to EDC exposure. Finally, we included *BRCA1*, a gene that is associated with ER regulation, but also reported to be epigenetically regulated.

CCND1 showed a significant increase in gene expression after 24 hours of exposure ($1x10^{-7}$ M; 3.09±0.22 LogFC, p <0.001, $1x10^{-5}$ M; 2.47±0.55 LogFC, p <0.001), before returning to control levels by 168 hours. After 24 hours of $1x10^{-5}$ M exposure, *CCND2* showed little difference in gene expression, but a significant decrease was observed after 168 hours of continual exposure (-2.91±0.19 LogFC, p <0.001). Following $1x10^{-7}$ M exposure, *CCND2* saw a small increase at the 24 hour timepoint (1.51±0.11 LogFC, p =0.03), followed by a mildly significant decrease at 168 hours (-1.36±0.44 LogFC, p =0.03). Interestingly, *BRCA1* was significantly up-regulated after 24 hours of continual $1x10^{-7}$ M BPA exposure (3.86±0.38 LogFC, p <0.001), yet significantly down-regulated after exposure to the higher $1x10^{-5}$ M concentration (-4.38±0.37 LogFC, p <0.001), before returning to levels comparable to controls after 168 hours. *RASSF1A* expression was seen to decreased over time. At 24 hours there was no significant change in gene expression, but a significant down-regulation was observed after 168 hours of exposure to the highest concentration ($1x10^{-5}$ M; -4.47±0.63 LogFC, p <0.001). By the 336 hour timepoint, both concentrations of BPA elicited a highly significant reduction in expression ($1x10^{-7}$ M; -2.01±0.42 LogFC, p <0.001, $1x10^{-5}$ M; -3.15±0.56 LogFC, p <0.001). In summary, BPA induced significant expression changes to all of the genes tested at a minimum of one timepoint.

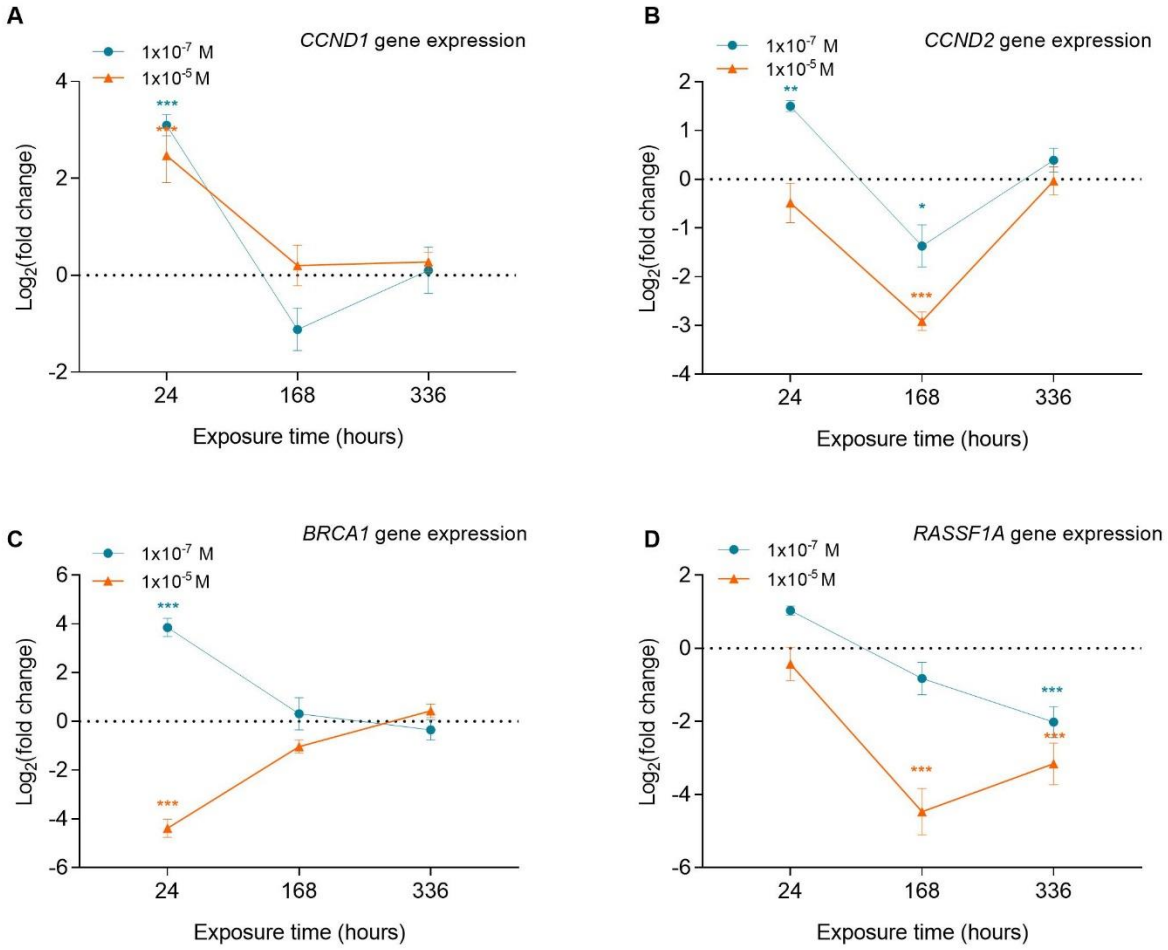

Figure 2.11. Alterations to *CCND1, CCND2, BRCA1* and *RASSF1A* gene expression after BPA exposure. Results are displayed as log2 (fold change) after exposure to BPA. Changes are observed in *CCND1* (A), *CCND2* (B), *BRCA1* (C) and *RASSF1A* (D). Error bars indicate the standard error of the mean (SEM) for each treatment. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by two-way ANOVA with Bonferroni correction.

2.3.5. Propylparaben exposure induced down-regulation of *ESR1, ESR2* and *GPER1*

We then proceeded to repeat the experiments described above with propylparaben, to elucidate whether similar patterns of gene alterations could be observed. We found that *ESR1* gene expression was significantly down-regulated after a 24 hour exposure period (Figure 2.12), with $1x10^{-5}$ M producing the most substantial effect ($1x10^{-7}$ M; -1.85±0.25 LogFC, p = 0.002, $1x10^{-5}$ M; -4.27±0.79 LogFC, p <0.001). Expression then returned to control levels by the 168 hour timepoint at the lowest concentration, however continued to be significantly down-regulated with

exposure to 1x10^{-5} M (-2.66±0.29 LogFC, p = 0.002). Both concentrations returned to control levels after 336 hours. Only 1x10^{-5} M propylparaben exposure induced a significant down-regulation in *ESR2* after 24 hours (-2.05±0.28 LogFC, p <0.001), with alterations at all other timepoints and concentrations being deemed insignificant. A slight decrease in *GPER1* gene expression could be observed after 24 and 168 hours of BPA exposure (1x10^{-7} M; -0.58±0.27 LogFC, p = 0.04, 1x10^{-5} M; -1.37±0.54 LogFC, p = 0.05), followed by a return to control levels, however this was not deemed to be significant.

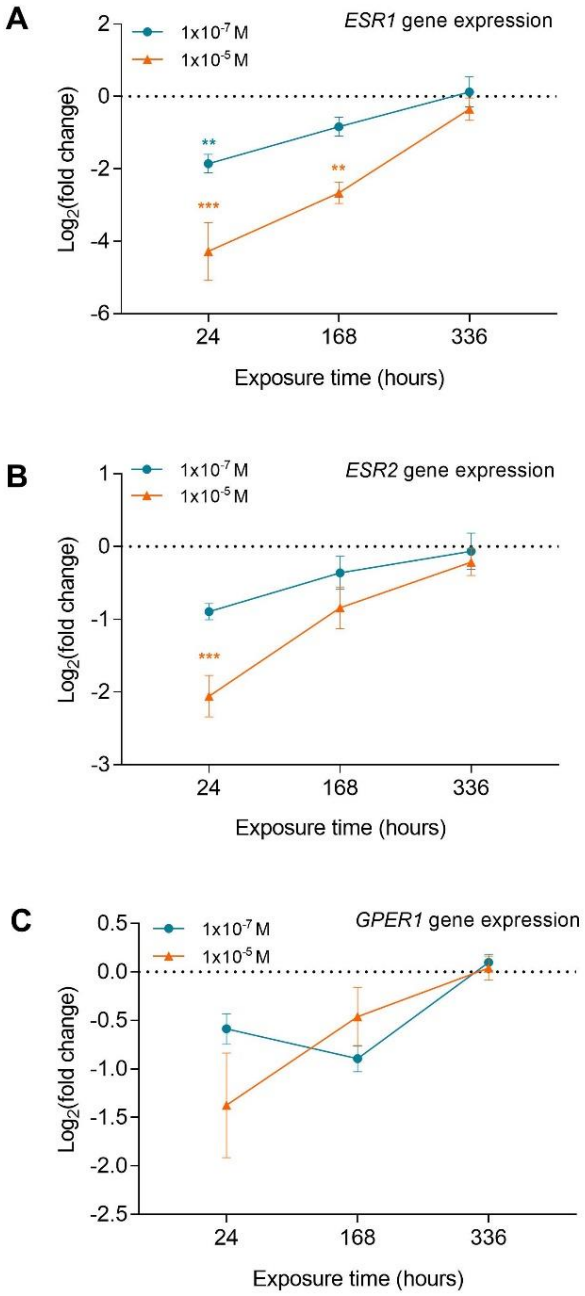

Figure 2.12. Log2 (fold change) observed in *ESR1, ESR2* and *GPER1* in response to propylparaben. Graphs depict alterations in *ESR1* (A) *ESR2* (B) and *GPER1* (C) expression. Error bars indicate the standard error of the mean (SEM) for each treatment. Significance of fold change in comparison to control is denoted by ***<0.001 ** 0.002 * 0.03.

2.3.6. Propylparaben exposure resulted in changes to *CCND1, CCND2, BRCA1* and *RASSF1A* gene expression.

As in the case of BPA, we tested the response of additional genes to propylparaben. *CCND1* and *CCND2* showed little difference between control and treated samples, with neither tested concentration resulting in significant changes to gene expression at any timepoint (Figure 2.13 A,B). After $1x10^{-7}$ M exposure, *BRCA1* showed a decrease in expression after 24 and 168 hours (-2.62±0.41 LogFC, p <0.001, -1.16±0.23 LogFC, p = 0.03 respectively), before returning to control levels by 336 hours (Figure 2.13C). With exposure to $1x10^{-5}$ M propylparaben, an increasing effect on *BRCA1* gene expression was observed over time, with the most significant effect seen after 336 hours (-2.26±0.25 LogFC, p <0.001). We observed little change to *RASSF1A* expression after exposure to $1x10^{-5}$ M propylparaben, with no significant difference between controls and treatments identified. A significant decrease in *RASSF1A* expression occurred at the 336 hour timepoint after exposure to $1x10^{-7}$ M propylparaben (-3.14±0.55 LogFC, p <0.001), however earlier timepoints showed no significant alteration to gene expression (Figure 2.13D).

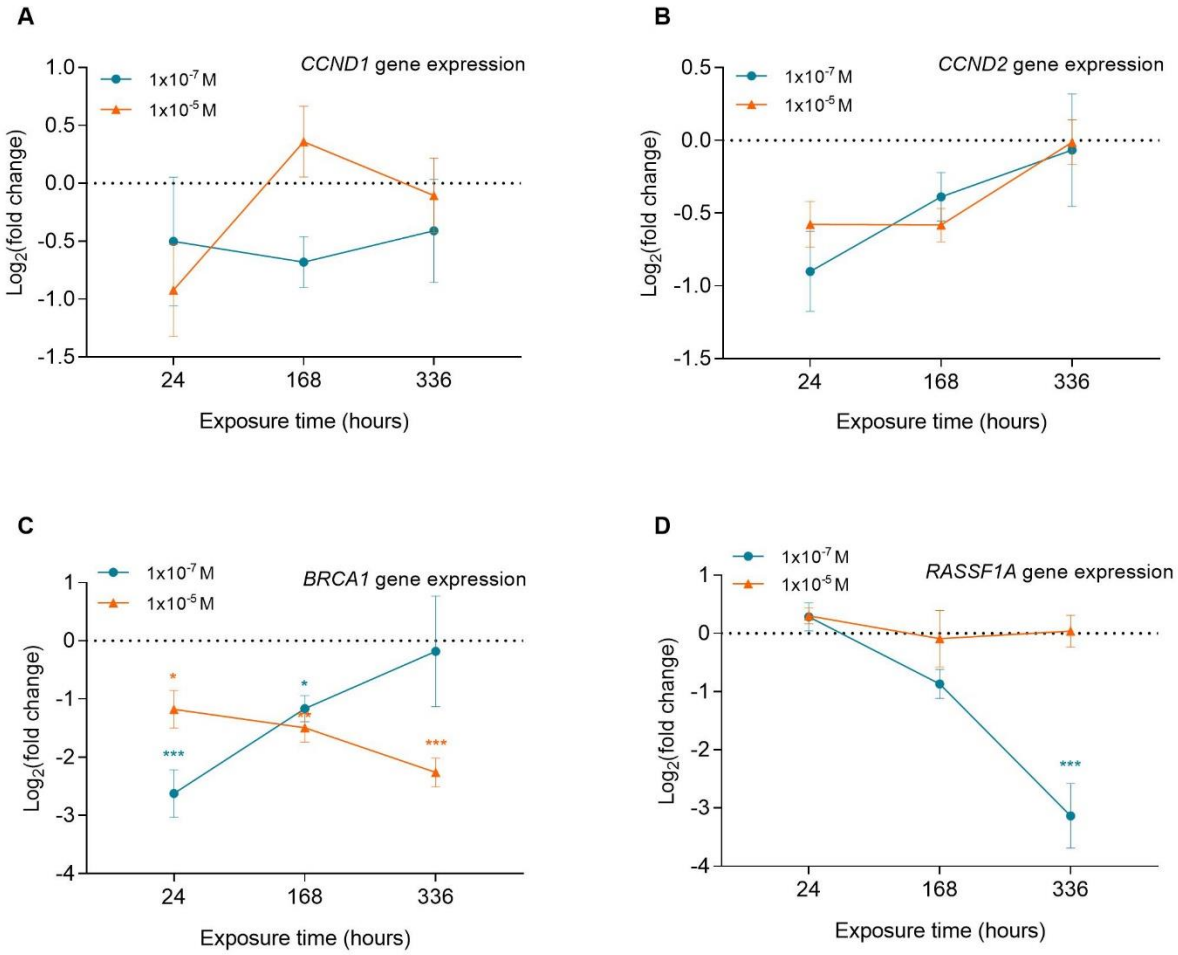

Figure 2.13. Effect of propylparaben exposure on *CCND1, CCND2, BRCA1* and *RASSF1A* gene expression. Results are depicted as log2 (fold change) after exposure propylparaben, with controls set to 0. Graphs depict *CCND1* (A), *CCND2* (B), *BRCA1* (C) and *RASSF1A* (D) expression in response to $1x10^{-7}$ M and $1x10^{-5}$ M propylparaben exposure. Error bars represent the standard error of the mean (SEM) for each treatment. Significance of fold change in comparison to control is denoted by ***<0.001 ** 0.002 * 0.03.

2.4. Discussion

Previous research has reported the MCF-12A cell line as both positive (Eisen and Brown, 2002; Marchese and Silva, 2012; Gelfand, Vernet and Bruhn, 2016) and negative (Subik *et al.*, 2010; Sweeney, Sonnenschein and Soto, 2018) for ER status. It was therefore important to establish whether our specific batch of MCF-12A cells were ER and GPER competent and whether they responded to compounds utilised in the following chapters. At the tested passage, it was shown that MCF-12A cells expressed *ESR1* and *ESR2* at measurable levels. This aligns with findings by Marchese (2013), who had similar requirements for a suitable experimental cell line. Marchese demonstrated a reduction in *ESR1* and *ESR2* expression after exposure to $1x10^{-9}$ M E2. It was speculated that this down-regulation could lead to a decrease in oestrogen responsiveness and could be attributed to a negative feedback mechanism, that would control the cellular response to oestrogen. Our results indicate that exposure to $1x10^{-5}$ M BPA could be inducing a similar effect. Data presented here demonstrate that BPA induced down-regulation of *ESR1* and *ESR2* expression was significant after 24 hours of exposure. The observed down-regulation of *ESR1* has been described in previous experiments at gene and also protein level. In MCF-7 cells, Jensen and colleagues showed that $1x10^{-9}$ M E2 induced a significant decrease in *ESR1* expression after 15 minutes (Jensen *et al.*, 1999). Authors also tested the ICI 182780-resistant (an oestrogen receptor antagonist) MCF-7.182^{R}-6 cell line. Although E2 exposure initially induced a decrease in expression, transcription levels began to increase again after 24 hours of continuous exposure, reaching control levels by 48 hours. Whilst we did not specifically test a 48 hour exposure timepoint, we observed a similar pattern of expression in MCF-12A cells. Jensen suggested that this reestablishment of ER control may be due to the induction of other mitogenic signals within the cell to compensate for blocked ER pathways. Additional studies that also reported a decrease in *ESR1* expression, attributed the alteration to a negative feedback mechanism, that prevents further transcription of the gene (Saceda *et al.*, 1989; Santagati *et al.*, 1997; Castellano *et al.*, 2009). The down-regulation in *ESR1* and *ESR2* presented here may be attributed to this mechanism. Propylparaben was also able to induce a comparable down-

regulation in *ESR1* and *ESR2* expression, however the effect was not as strong as observed with BPA. Propylparaben is generally considered to possess a weaker affinity to the classical ERs in comparison to BPA (Bolger *et al.*, 1998; Bouskine *et al.*, 2009; Pan *et al.*, 2016) and therefore this different in effect magnitude is expected.

Expression of *GPER1* was confirmed in untreated MCF-12A cells and showed a decrease in expression after exposure to $1x10^{-5}$ M BPA, demonstrating a response to oestrogenic compounds. A significant reduction in gene expression was recorded after 24 hours of $1x10^{-7}$ M and $1x10^{-5}$ M BPA exposure. As with the ERs, expression of *GPER1* returned to control levels by the 168 hour timepoint. This observation may indicate the regulation of cell responses to oestrogen exposure over time, comparable to what has been reported in *ESR1* and *ESR2*. Whilst *GPER1* showed a mild decrease in gene expression in response to propylparaben exposure, a significant effect could not be demonstrated. Again, this is likely due to propylparaben being a weaker oestrogenic compound in comparison to BPA, rather than a limitation of the cell line response. Wrobel and Gregoraszczuk (2014a) identified a significant increase in *GPER1* expression after $2x10^{-8}$ M propylparaben exposure in MCF-7 cells. The concentrations used here were substantially higher, yet could not induce a significant change in gene expression. It must be noted that the MCF-7 cell line has significantly higher levels of ERs in comparison to the MCF-12A cell line used in the present study, which would most likely explain the discrepancy. To the best of our knowledge, no comparable study has demonstrated a significant change in ER gene expression after exposure to propylparaben in MCF-12A cells.

To further ensure the appropriateness of using MCF-12A cells to study EDCs in this thesis we identified several other genes of interest that were reported in the literature to respond to oestrogen exposure. Furthermore, we selected a number of genes that were known to be regulated by epigenetic mechanisms. We demonstrated that untreated MCF-12A cells expressed these genes at measurable levels under control conditions and that both propylparaben and BPA could induce gene expression changes. Firstly, we saw a significant up-regulation of the cell cycle regulator *CCND1* after 24 hours of BPA exposure. Along with showing a BPA-induced response, up-regulation of *CCND1* could be associated with an increase in cell-

proliferation and an inhibition of apoptosis. Concentrations of $1x10^{-8}$ M to $1x10^{-7}$ M BPA have induced such effects through the increased *CCND1* expression in ER-positive MCF-7 cells (Pfeifer, Chung and Hu, 2015). An increase in *CCND1* expression has also been shown in response to other EDCs, including $1x10^{-6}$ M triclosan (an anti-fungal agent) and octylphenol (used in production of phenol/formaldehyde resins) exposure in MCF-7 cells after 24 hours, stimulating cell proliferation (H.-R. Lee *et al.*, 2014). As with ER encoding genes, we see *CCND1* regulating towards control levels at 168 and 336 hour timepoints. The regulation of *CNND1* by the ERs may account for this similarity.

Interestingly, *CCND2* shows a slight increase in expression after 24 hours of $1x10^{-7}$ M BPA exposure, yet after 168 hours a significant down-regulation was induced by both concentrations of BPA. *CCND2* expression is frequently silenced in breast cancer tissues (Oyama *et al.*, 1998; Fischer *et al.*, 2002). As reviewed previously, *CCND2* has been reported to be regulated epigenetic mechanisms, with hypermethylation of *CCND2* promotor regions observed in breast cancers (Fackler *et al.*, 2003, 2004; Li, Rong and Iacopetta, 2006; Sharma *et al.*, 2007; Li *et al.*, 2015; White *et al.*, 2015). Therefore, it may be possible that alterations to the methylation profile is responsible for the gene expression shifts presented here, however this would need to be confirmed with subsequent methylation analysis. Propylparaben did elicit a mild decrease in gene expression of the cyclin gene, however this was not deemed significant. Again, this is likely associated with the potency of the compound in comparison to BPA.

Next, we observed that BPA exposure elicited an effect on *BRCA1* expression that was concentration dependent. The lower $1x10^{-7}$ M exposure resulted in a significant increase in *BRCA1* expression of almost 4-fold. In contrast, $1x10^{-5}$ M, caused a down-regulation of the same magnitude. BPA-induced down-regulation of *BRCA1* has been reported previously by Singleton *et al.,* (2006), who showed a 12.8-fold decrease after three hours of $1x10^{-6}$ M BPA exposure in ERα-HA breast cancer cells (engineered to overexpress HA-tagged ERα). The observed concentration dependent difference is likely a matter of threshold. Other studies have also reported differential expression that is concentration dependent. Qin *et al.,* (2012) reported that cyclin E was both down- and up-regulated by BPA, depending on the exposure concentration.

For instance, after $1x10^{-8}$ M BPA exposure, cyclin E saw a significant decrease in expression, whilst after $1x10^{-7}$ M cyclin E was significantly enriched. Qin and colleagues suggested this difference in expression may be attributed to a concentration threshold required to elicit a deleterious effect. By 168 hours, neither concentration continued to significantly alter gene expression. Propylparaben exposure also instigated varying results that appeared to be concentration dependent. An increasing effect was observed with $1x10^{-5}$ M exposure, with the most substantial down-regulation occurring after 336 hours, whereas $1x10^{-7}$ M induced a decrease in gene expression after 24 hours, that gradually returned to control levels over time. Nevertheless, these data supported the ability of EDCs to induce changes to *BRCA1* expression in MCF-12A cells.

Finally, a decrease in *RASSF1A* could be induced by both EDCs. As with the other genes tested, this change was most significant after BPA exposure. Both BPA and propylparaben appeared to increasingly reduce gene expression over time. Unlike other genes tested here, *RASSF1A* is not regulated by ER activity, but is primarily modified by epigenetic mechanisms, which may explain the pattern of gene regulation we seen from this data (Agathanggelou, Cooper and Latif, 2005; Donninger, Vos and Clark, 2007). *RASSF1A* is one of the most commonly inactivated proteins identified in human cancers, with *RASSF1A* hypermethylation acknowledged as a key biomarker of early breast carcinogenesis, resulting in loss of expression (Fackler *et al.*, 2003; Shinozaki *et al.*, 2005; Donninger, Vos and Clark, 2007; Buyru *et al.*, 2009; Yadav *et al.*, 2016). *RASSF1A* has been reported to possess tumour suppressor properties (Donninger, Vos and Clark, 2007), inhibiting tumour cell growth via the regulation of the cell cycle, apoptosis and genomic instability (Agathanggelou, Cooper and Latif, 2005; Dallol *et al.*, 2005; Vos *et al.*, 2006). Whilst this significant decrease in *RASSF1A* expression is not immediate, it demonstrates the responsiveness of the gene within the MCF-12A cell line.

The aim of this chapter was to characterise the MCF-12A cell line in terms of ER status and response to the endocrine disrupting chemicals, BPA and propylparaben. Taken together, data presented in this chapter evidence the expression of *ESR1, ESR2* and *GPER1* in the MCF-12A cell line, contradicting some published reports (Subik *et al.*, 2010; Sweeney, Sonnenschein and

Soto, 2018). The controversy surrounding the ER status of MCF-12A cells has previously been attributed to the potential differences between passages (Paine *et al.*, 1992), which may account for some of the reported variation. It has also been speculated that cell lines may vary in hormone receptor status between labs (Burdall *et al.*, 2003) . Such differences have been shown in the MCF-7 (Osborne, Hobbs and Trent, 1987; Bahia *et al.*, 2002) and MDA-MB-231 (Watson *et al.*, 2004) breast cancer cell lines. It is plausible that similar differences may be present in the MCF-12A cell line, explaining the contradicting literature. Furthermore, we show that ER encoding genes are responsive to BPA and propylparaben exposure, making it a suitable cell line for further studies within this thesis. The controversy surrounding the suitability of MCF-12A cells to study the effects of oestrogenic compounds is most likely a result of spontaneous ER loss and differing culture conditions between research groups, which can have a significant impact on the cell line's response to EDCs (Payne *et al.*, 2000). Findings in this chapter have consequently established the importance of characterising cell lines at the tested passage and culture conditions to ensure the presence of ER receptors and response to chemical exposures. In the subsequent chapters we will utilise the MCF-12A cell line to investigate the impact of EDC exposure in *in vitro* assays more representative of the human breast.

Chapter Three: Mixture effects and breast carcinogenesis: the impact on morphology and gene expression of MCF-12A cells

3 Mixture effects and breast carcinogenesis: the impact on morphology and gene expression of MCF-12A cells

3.1. Introduction

Scientific research linking endocrine disruptors to cancer is currently inconclusive, frequently yielding contradictory results. Experimental work has demonstrated that EDCs, namely those which mimic the action of endogenous oestrogens, induce effects that are relevant to breast cancer initiation and progression (Diamanti-Kandarakis *et al.*, 2009; Doherty *et al.*, 2010; Forman *et al.*, 2015). Examples include the increase in mammary tumour incidence following prenatal exposure to bisphenol A *in vivo* and the induction of cancer cell proliferation and malignant invasion *in vitro* by phthalates, parabens and ultraviolet (UV) filters (Jenkins *et al.*, 2012; Darbre and Harvey, 2014). However, many of these effects have only been demonstrated at concentrations of the chemicals that are much higher than those found in human tissues and experiments are often undertaken in assays that are not representative of the human breast. Numerous epidemiological studies that have examined whether EDCs have a role in breast cancer have identified no significant correlations between exposures and cancer risk (Rudel *et al.*, 2007; Verloop *et al.*, 2010; Acheampong *et al.*, 2018; Rodgers *et al.*, 2018). Epidemiology studies have predominately focused on linking single compound exposures to breast cancer incidence rates, however, the concentrations of these chemicals in tissues are low and there a various confounding factors, such as occupation, diet and exercise, meaning that it is unlikely a correlation between a single compound and breast cancer development would be observed (Kortenkamp, 2006). Moreover, the putative effects of EDCs are often assessed in the context of endogenous hormones E1 (oestrone), E2 (oestradiol) and E3 (oestriol). The oestrogenic effects of EDCs are considerably weaker than the potent internal sex hormones. Thus, when considering the effects of EDCs in isolation, it is often concluded that they pose no significant health concern (Kortenkamp, 2006). Taken together, these observations often lead to the assumption that the concerns associated with the role of endocrine disruptors in breast cancer are unfounded, as their levels in tissues are not high enough to increase breast cancer risk

(Kortenkamp, 2006; Macon and Fenton, 2013). The problem with this assumption, is that humans are exposed to low levels of large numbers of chemicals that could act together, contributing to an individual's internal 'oestrogenic load' and increase breast cancer risk (Ibarluzea Jm *et al.*, 2004; Kortenkamp, 2006; Pastor-Barriuso *et al.*, 2016). However, to date, this possibility has not been sufficiently investigated, with most epidemiological and toxicological studies investigating individual compound effects, potentially underestimating the true impact of EDCs on breast cancer risk (Ekenga, Parks and Sandler, 2015; Gray *et al.*, 2017). Consequently, experiments to investigate the role of EDC mixtures in an assay that recapitulates some of the characteristics of the human breast, are required to have a comprehensive understanding of the relationship between exposures and breast carcinogenesis.

3.1.1. Three-dimensional cell culture

Over the past two decades, three-dimensional (3D) culture systems have become more frequently utilised to investigate processes in breast morphogenesis and carcinogenesis. In breast cancer, use of 3D systems has enabled the understanding of processes such as the formation and maintenance of the hollow lumen (Debnath *et al.*, 2002; Mailleux, Overholtzer and Brugge, 2008; Nguyen and Shively, 2016), tumour invasion (Berens *et al.*, 2015; Lv *et al.*, 2017; Ranftl and Calvo, 2017) and the regulation of acini polarity (Bryant and Mostov, 2008; Roignot, Peng and Mostov, 2013; Bidarra *et al.*, 2016). Despite being recognised as an essential tool in cancer biology, the vast majority of toxicological literature still remains based on data obtained from unrepresentative two-dimensional (2D) *in vitro* models (Kalvelyte *et al.*, 2017). Although these 2D studies have provided valuable insights into how EDCs may contribute towards breast cancer risk, they fail to capture the complex 3D structural architecture of epithelial cells within the breast. Also, when grown in 2D, normal epithelial cells are highly plastic and display many tumour cell characteristics (Bissell, 1981; Petersen *et al.*, 1992; Kim, Stein and O'Hare, 2004), meaning the responses to EDCs observed may not be representative of the normal breast. As an example, previous studies have shown the human epidermal growth factor receptor 2 (HER2)

forms heterodimers with human epidermal growth factor receptor 3 (HER3) resulting in PI3K signalling in 2D cultures, whereas in 3D cultures, HER2 forms homodimers that results in MAPK signalling (Pickl and Ries, 2009). Given the importance of HER2 and MAPK signalling in breast cancer development and the ability of EDCs to influence these pathways, it must be recognised that 2D cultures may not be accurately portraying the effects of EDCs on the breast. Monolayer cultures also lack the ability to recapitulate the microenvironment of the human breast, including stromal cells, such as adipocytes, fibroblasts, immune and endothelial cells, and the extracellular matrix (ECM). It is now understood that microenvironment plays an integral role in the development of the mammary gland and the initiation and progression of breast tumorigenesis (discussed further in Chapter 4). The use of 3D models of immortalised mammary cell lines begins to overcome such limitations by restoring some of the microenvironment properties.

3D cell culture models create artificial environments that have been shown to more accurately imitate *in vivo* cellular responses in comparison to single layer cells (Antoni *et al.*, 2015). The ability of 3D cultures to mimic events within the human breast make them an attractive tool to bridge the gap between traditional monolayer *in vitro* assays and the alternative animal models (Pampaloni, Reynaud and Stelzer, 2007). A collection of 3D cell culture methods have been developed, proving successful for the maintenance of various cell types (reviewed in Kim, Stein and O'Hare, 2004; Pampaloni, Reynaud and Stelzer, 2007; Hebner, Weaver and Debnath, 2008; Verjans *et al.*, 2018). Briefly, common methods for 3D cell culture can be divided into scaffold techniques and scaffold-free techniques. Scaffold techniques require either synthetic or biological polymers. Biological polymers are most commonly used for breast epithelial cell culture, with Matrigel being one of the most widely utilised (Hoarau-Véchot *et al.*, 2018). Matrigel is a commercial extra-cellular matrix (ECM), which is extracted from Engelbreth-Holm-Swarm mouse tumour cells and contains basement membrane proteins (Kleinman and Martin, 2005). These include collagen IV, entactin, laminin perlecan, matrix metalloproteinase-2, and growth factors, which are required for cellular processes such as polarisation, regulation of cell proliferation and adhesion (Barcellos-Hoff *et al.*, 1989; Aggeler *et al.*, 1991). Matrigel provides a stable matrix that allows mammary epithelial cells (MCF-10A and MCF-12A) to develop into

acini-like spheroids, comparable to the architecture of the human breast (Figure 3.1; Debnath, Muthuswamy and Brugge, 2003; Debnath and Brugge, 2005; Marchese and Silva, 2012).

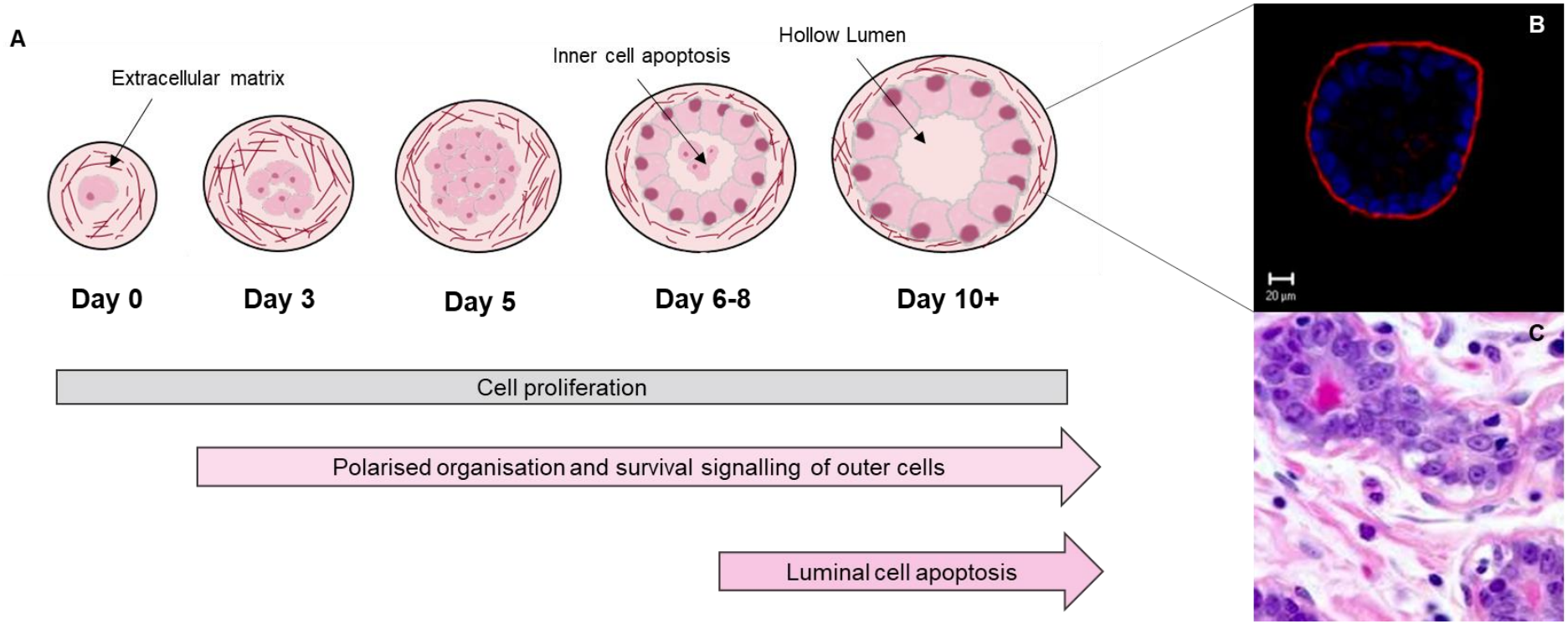

Figure 3.1. Mammary epithelial cells form acini-like structure when cultured on Matrigel. (A) An ordered sequence of events occurs during the development of human mammary epithelial cells grown on Matrigel, including proliferation, polarisation and apoptosis. Initially, cells proliferate to begin acini formation. By day 5-8, two distinct populations of cells form, with an outer layer, directly connected to the extracellular matrix, that remains polarised throughout acini development, and an inner layer. From around day 8, inner cells begin to die through apoptosis, allowing the formation of a hollow lumen by around day 10. (B) Confocal imaging demonstrates by day 10 normal epithelial cells have developed into acini-like structures with a single layer of epithelial cells and a hollow lumen. Acini show controlled proliferation and appear relatively circular. Acini structures cultured in Matrigel are comparable to the human breast aetiology (C). Adapted from Debnath, Muthuswamy and Brugge, (2003); Debnath and Brugge, (2005); Marchese and Silva, (2012).

Synthetic scaffolds are comprised of synthetic polymers, such as polyesters, polyanhydrides and polyethylene glycol (El-Sherbiny and Yacoub, 2013; BaoLin and Ma, 2014). For instance, systems have been developed, like the NanoCulture Plate, where a plastic film mimics the normal ECM. When cultured in the NanoCulture Plate, the epithelial cells cannot adhere strongly to the synthetic ECM structure and thus, start to reorganise themselves and form 3D structures that recapitulate some of the characteristics of *in vivo* tissue architecture (Yoshii *et al.*, 2011; Aritomi *et al.*, 2014; Verjans *et al.*, 2018). Although synthetic scaffolds have been proposed to overcome the constraints of expense and lot-to-lot variability of biological polymers, at the start of this project there was limited literature to support the use of such models in non-tumourigenic breast epithelial cells. Although there are now reports of synthetic assays that successfully maintain the growth of mammary acini-like spheroids (Rijal and Li, 2017; Rijal, Bathula and Li, 2017), these methods remain in their infancies. Scaffold-free methods include hanging drop, magnetic levitation, gyratory shaker and spinner tubes have also been utilised, however their use is not as common as biological ECMs including Matrigel (Figure 3.2).

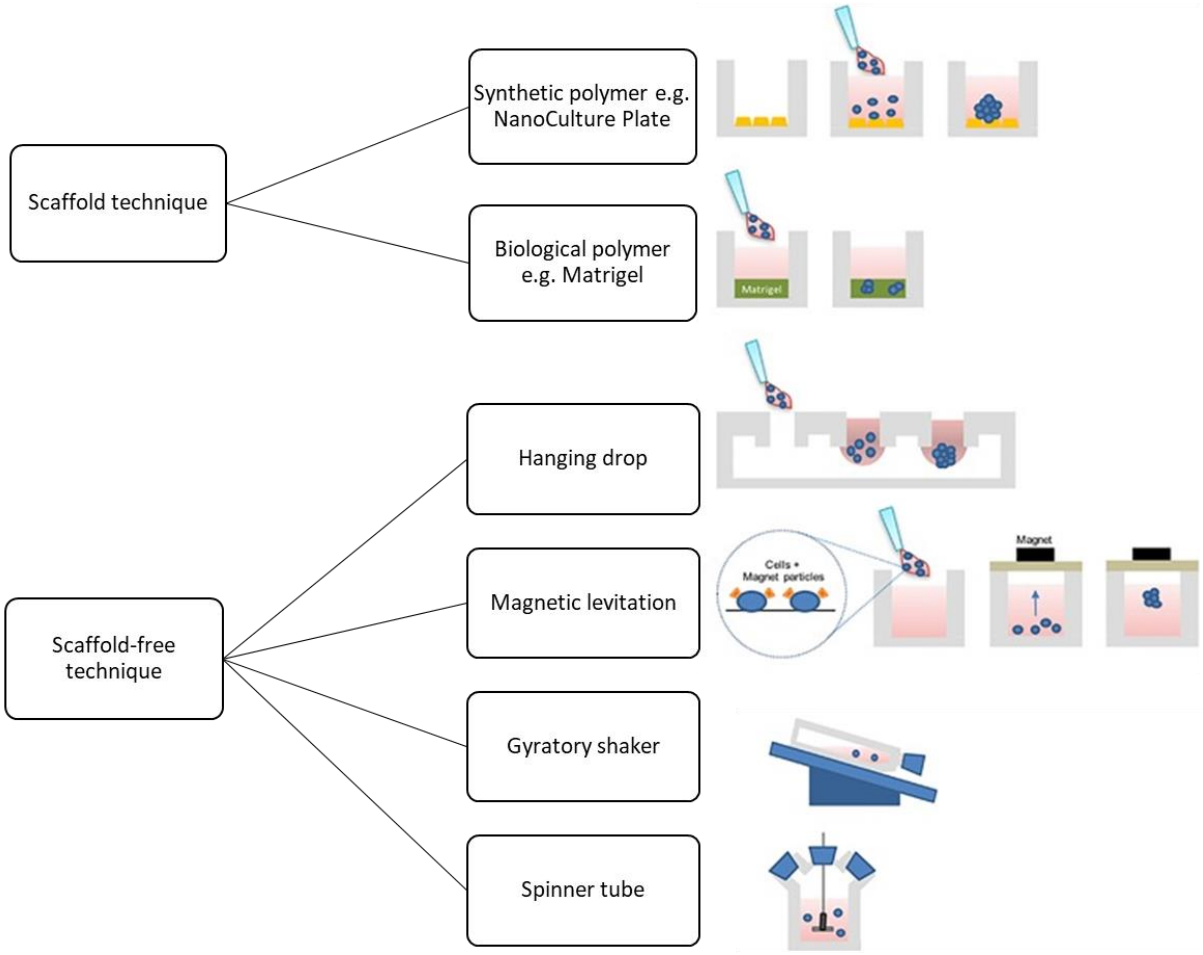

Figure 3.2. Schematic overview of commonly used three-dimensional (3D) cell culture techniques. Various 3D culture models have been developed to address the different requirements of cell types and assays. The most commonly used 3D cell culture methods can be divided into scaffold techniques (synthetic or biological scaffolds) and scaffold-free techniques. Adapted from Verjans *et al.,* (2018).

As outlined above, non-tumourigenic breast epithelial cells, such as MCF-12As, cultured using Matrigel, develop into polarised spheroids that resemble *in vivo* acini structures (Marchese and Silva, 2012). The spheroids consist of a single layer of epithelial cells surrounding a hollow lumen (Debnath *et al.*, 2002; Debnath, Muthuswamy and Brugge, 2003; Underwood *et al.*, 2006; Imbalzano *et al.*, 2009; Marchese and Silva, 2012). The spheroids are attached to a basement membrane containing laminin, collagen IV, entactin, proteoglycans and heparin sulphate (Debnath, Muthuswamy and Brugge, 2003) that affords structural support to the acini. Key hallmarks of early breast cancer include uncontrolled proliferation, loss of polarity and luminal

filling (Brennan *et al.*, 2010; Jiang *et al.*, 2015; Halaoui *et al.*, 2017). These characteristics can be recapitulated and studied within 3D cultures of breast epithelial cells (Inman and Bissell, 2010; Vidi, Bissell and Lelièvre, 2013; Asghar *et al.*, 2015; Singh *et al.*, 2016; Carter *et al.*, 2017). Thus, 3D cultures offer an opportunity to investigate the role of EDCs in these processes, within a physiologically relevant context.

3.1.1.1. Cell proliferation

The cell cycle starts with the mitotic phase, the process of cell division, which consists of mitosis and cytokinesis, producing two daughter cells. The mitotic phase accounts for only a small portion of the cell cycle, lasting typically no more than an hour in mammalian cells. The remainder of the cell cycle consists of various interphase stages (Figure 3.3). Cellular contents are synthesised throughout this interphase period, resulting in cell mass increasing as it prepares for division. Interphase is divided into three sub-phases: G1 (‘first gap’), S (DNA synthesis) and G2 (‘second gap’). During each of these phases the cell grows, however DNA replication only occurs during S phase. Non-dividing cells remain in a state of cell cycle arrest, referred to as G0.

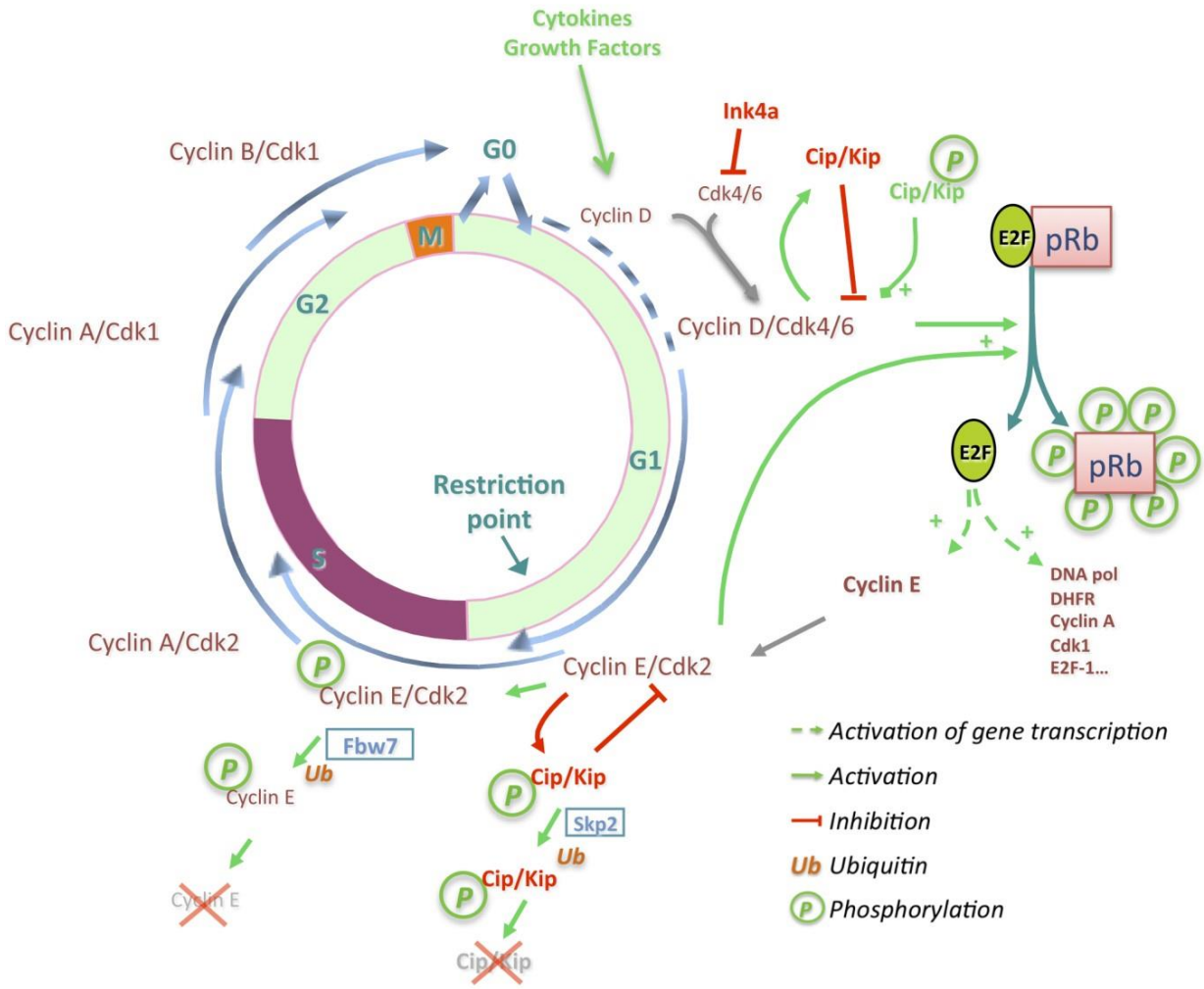

Figure 3.3. The eukaryotic cell cycle regulated by Cdk/cyclin complexes. Normal cells pass through the cell cycle phases, including mitosis (the process of cell division), two 'gap' phases G1 and G2, and an S phase for DNA replication. Non-dividing cells remain in a state of rest at G0. Ink4 inhibitors regulate cyclin D expression to bind and activate Cdk4 in G1 phase. Cdk4/cyclin D complexes are stabilised by Cip/Kip proteins and phosphorylate p21. pRb phosphorylation is initiated by Cdk4/cyclin D complexes, causing the release from E2F1 transcription factors. Cyclin E associates with Cdk2 enhancing pRb phosphorylation and phosphorylates Cip/Kip proteins leading to ubiquitination and degradation. Successive association of Cdk2 with cyclin A, after the ubiquitination and degradation of cyclin E by Fbw7, leads to S phase completion and entry into G2 phase. Association of Cdk1 with cyclin A and cyclin B1 enables G2/M transition, mitosis, and cytokinesis (Bisteau *et al.,* 2014).

Normal cells do not pass through the cell cycle phases and divide continually. Initiation and inhibition of cell division can be triggered by external factors, for example, in oestrogen responsive organs, such as the breast, the release of growth-promoting hormones like human growth hormone (HGH) or oestrogen can signal a cell to enter interphase. From this point, the

process is regulated at three internal cell cycle checkpoints G1, G2 and M. The G1 checkpoint controls for cell size, nutrient and growth factor content and DNA damage. G2 regulates cell size and DNA replication and finally the M checkpoint ensures chromosomal attachment to the spindle. Cyclins and cyclin dependent kinase (Cdk) proteins are responsible for progressing the cell through each of the checkpoints, known as positive regulators (Johnson and Walker, 1999; Kastan and Bartek, 2004; Hydbring, Malumbres and Sicinski, 2016). Levels of cyclins fluctuate through the cell cycle phases and regulate the cell cycle when bound to Cdks. The different cyclins and Cdks bind at the various checkpoints, regulating cell progression. For example, the G1 checkpoint is regulated by cyclin D, which binds to cyclin dependent kinases, CDK4 and CDK6 (Malumbres *et al.*, 2004).

Alongside positive regulators, there are also regulatory molecules that halt the cell cycle, referred to as negative regulators. The most understood negative regulators include the retinoblastoma protein (Rb), p53 and p21. Indeed, much of the understanding of how these proteins are involved in the cell cycle has originated from observing cells that have lost proliferative control, where all three of these proteins have been found to be impaired (Bartek, Bartkova and Lukas, 1997; Giacinti and Giordano, 2006; Chen, 2016; Karimian, Ahmadi and Yousefi, 2016). Rb, p53 and p21 are primarily involved in the G1 checkpoint. Whilst a cell is progressing through a checkpoint, a properly functioning p53 protein will halt the cell cycle progression in the presence of damaged DNA and recruit enzymes to repair the damage before progressing onto the next phase. In the case that DNA cannot be repaired, p53 can trigger cell death to prevent the damaged cell from being duplicated. p21 works alongside p53, where high levels of p53 trigger the production of p21 (Karimian, Ahmadi and Yousefi, 2016). This enforces the cell cycle to halt by binding to Cdk/cyclin complexes and inhibiting their activity. Rb primarily controls for cell size. The protein binds to transcription factor E2F. When Rb is bound to E2F, the proteins required for progression to S phase are inhibited. As the cell increases in size, the Rb protein becomes inactive and releases E2F allowing protein production (Giacinti and Giordano, 2006). Generally, in non-tumour cells, positive regulators (cyclins and Cdks) are active and negative regulators (Rb, p53 and p21) are inactive.

Abnormal functioning of positive or negative cell cycle regulators can allow for uncontrolled cell proliferation. Even minor changes to regulatory proteins can allow cells to pass through the cell cycle with errors, increasing the speed of the cell cycle. This increase in speed can further reduce the effectiveness of cell cycle checkpoints, resulting in the unchecked growth of mutated cells, ultimately contributing to a higher cancer risk (Kastan and Bartek, 2004; Massagué, 2004). Aberrant function of cell cycle regulators is commonly observed in cancer with uncontrolled proliferation of cells being a key hallmark of early carcinogenesis (Malumbres and Barbacid, 2009).

In 3D cultures of mammary epithelial cell lines, studies show that during acini formation, cell proliferation is initially high, but is then inhibited as the acini reaches full development (around day 10). This is evidenced by the gradual increase in expression of the Cdk inhibitor, $p27^{kip1}$ throughout morphogenesis. In addition, during early acini development, the Ki-67 proliferative marker is highly expressed. However, this expression declines at the later stages, following growth arrest (Coppock *et al.*, 2007). It has also been shown that 3D cultures of mammary epithelial cells proliferate excessively when cyclin D1 is overexpressed or Rb is inactivated (Muthuswamy *et al.*, 2001; Debnath, Walker and Brugge, 2003), demonstrating the ability to study early carcinogenic events within 3D *in vitro* assays.

3.1.1.2. Luminal filling

Within normal mammary tissue, lumen formation is essential to build functional networks of epithelial tubes required for the delivery of milk when breast feeding (Mailleux, Overholtzer and Brugge, 2008). The clearance of inner cell populations by apoptosis and autophagy is necessary to create this luminal space (Affolter *et al.*, 2003). Filling of the lumen is a prominent characteristic of early pre-invasive breast tumourigeneis (Debnath and Brugge, 2005; Patil *et al.*, 2015). A study in 2002, showed that an increase in cell proliferation alone was not sufficient to result in luminal filling (Debnath *et al.*, 2002). Instead, authors proposed that abnormalities in other processes, such as apoptosis, were required. As discussed above, the clearing of the acini lumen in 3D cultures occurs through a combination of processes, including apoptosis and autophagy (Debnath *et al.*, 2002; Mills *et al.*, 2004; Fung *et al.*, 2008). If just a single process

fails, the lumen formation is delayed, but not inhibited. Mills *et al.,* (2004) demonstrated that it was necessary for both apoptosis and autophagy to fail for luminal filling to occur. It has been evidenced that luminal filling through these mechanisms is a change indicative of early carcinogenesis (Debnath *et al.*, 2002; Russo *et al.*, 2010), therefore the study of how these events can be altered by extrinsic and intrinsic factors can provide valuable insights into breast cancer risk.

Apoptosis, also known as programmed cell death, can be triggered via internal and external signalling. In mammary epithelial cells, extrinsic apoptosis is regulated by the activation of receptors on the cell membrane via pro-apoptotic ligands, commonly Fas ligand (FasL), tumour necrosis factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL). The recruitment of proteins TNF receptor type 1-associated death domain protein (TRADD) and Fas-associated protein with death domain (FADD), then leads to the activation of caspase 8, followed by caspase 3 (Elmore, 2007). Intrinsic, or mitochondrial apoptosis, occurs under stress conditions, including hypoxia and exposure to radiation or toxins. Intrinsic apoptosis is regulated primarily by caspases and the B-cell lymphoma (Bcl-2) family (Cory and Adams, 2002), although numerous other proteins have been found to play a role (Table 3.1). A total of 25 genes have been identified within the Bcl-2 family, with some holding pro-apoptotic properties and others being anti-apoptotic. Whether the cell commits to apoptosis or not is dependent on the ratio, localisation and phosphorylation status of these proteins. The main mechanism of Bcl-2 family proteins is regulating the cytochrome c release from the mitochondria by altering the permeability of the mitochondrial membrane (Kale, Osterlund and Andrews, 2018). The release of cytochrome c activates caspase 9, which then results in caspase 3 activation. As with the extrinsic pathway, the activation of caspase 3 causes cell shrinkage, chromatin condensation and the formation of apoptotic bodies (Elmore, 2007). Finally, phagocytosis of the apoptotic bodies occurs. In 3D cultures of mammary epithelial cells, apoptosis can be observed by the presence of caspase 3 in the inner cells from around day 8, allowing for the hollowing of the acini lumen (Figure 3.4).

Table 3.1. Proteins involved in extrinsic and intrinsic apoptotic pathways. Protein abbreviations with the full protein name. Adapted from Elmore,(2007).

Apoptotic Pathway	Abbreviation	Protein Name	Pro- or anti- apoptotic
Extrinsic pathway related proteins	TNF-α	Tumour necrosis factor alpha	Pro
	TNFR1	Tumour necrosis factor receptor 1	Pro
	FasL	Fatty acid synthetase ligand	
	FasR	Fatty acid synthetase receptor	
	Apo3L	Apo3 ligand	
	DR3	Death receptor 3	
	Apo2L	Apo2 ligand	
	DR4	Death receptor 4	
	DR5	Death receptor 5	
	FADD	Fas-associated death domain	Anti
	TRADD	TNF receptor-associated death domain	pro
	RIP	Receptor-interacting protein	pro
	caspase-8	Cysteinyl aspartic acid-protease 8	Pro
	c-FLIP	FLICE-inhibitory protein	Anti
	Smac/DIABLO	Second mitochondrial activator of caspases/direct IAP binding protein with low PI	Pro
	HtrA2/Omi	High-temperature requirement	Pro
	IAP	Inhibitor of Apoptosis Proteins	Anti
	Apaf-1	Apoptotic protease activating factor	Pro
	Caspase-9	Cysteinyl aspartic acid-protease-9	Pro
	AIF	Apoptosis Inducing Factor	Pro
	CAD	Caspase-Activated DNAse	Pro
Intrinsic pathway related proteins	Bcl-2	B-cell lymphoma protein 2	Anti
	Bcl-x	BCL2 like 1	Pro
	Bcl-XL	BCL2 related protein, long isoform	Anti
	Bcl-XS	BCL2 related protein, short isoform	Anti
	Bcl-w	BCL2 like 2 protein	Anti
	BAG	BCL2 associated athanogene	Anti
	Bcl-10	B-cell lymphoma protein 10	Pro
	BAX	BCL2 associated X protein	Pro
	BAK	BCL2 antagonist killer 1	Pro
	BID	BH3 interacting domain death agonist	Pro
	BAD	BCL2 antagonist of cell death	Pro
	BIM	BCL2 interacting protein BIM	Pro
	BIK	BCL2 interacting killer	Pro
	Blk	Bik-like killer protein	Pro
	Puma	BCL2 binding component 3	Pro
	Noxa	Phorbol-12-myristate-13-acetate-induced protein 1	Pro
	Aven	Cell death regulator Aven	Anti
	Myc (c-myc)	Oncogene Myc	Pro

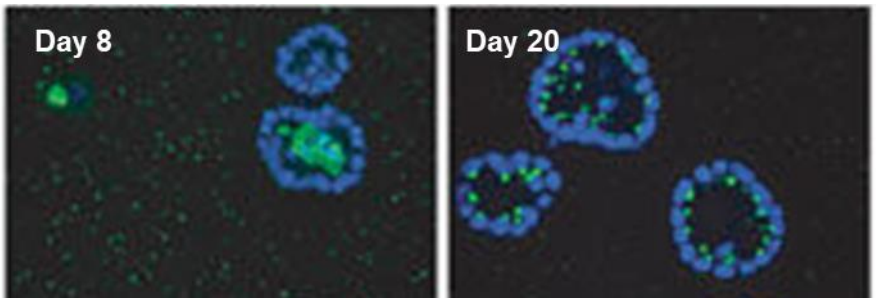

Figure 3.4. Evidence of apoptosis in 3D cultured MCF-10A cells. Immunofluorescent microscopy demonstrates apoptosis by the presence of caspase 3 (green) in the inner cells of the acini by day 8 of culture. This allows for the formation of a hollow lumen by day 20. Images taken from (Debnath and Brugge, 2005).

Autophagy is also involved in establishing and maintaining a hollow lumen (Mills *et al.*, 2004; Fung *et al.*, 2008). Autophagy, a non-apoptotic form of cell death, is a complex self-degradative process where cellular components degrade cytosolic material for reuse by lysosome action (Glick, Barth and Macleod, 2010; Solitro and MacKeigan, 2016). Autophagy is typically induced by intracellular stimuli. One such stimuli is the protein kinase target of rapamycin (Tor). Once initiated, a membrane surrounds the cellular organelles to form a vesicle (autophagosome), which then fuses with the lysosome. The lysosome contains acidic lysosomal hydrolases that then begin to degrade the cell organelles within the vesicle. In 3D mammary cell cultures, autophagic vacuoles have been observed in the inner cells of developing spheroids (Debnath *et al.*, 2002; Fung *et al.*, 2008; Bristol *et al.*, 2012; Jogalekar and Serrano, 2018). Moreover, Debnath *et al.,* (2002) reported that autophagy was observed in acini overexpressing Bcl-2. Accordingly, authors concluded that autophagy was occurring independently of apoptosis.

3.1.1.3. Loss of polarity

Aberrant cell polarity is an established hallmark of many cancers. Defined as the asymmetrical distribution of proteins in cellular domains, the proliferation, migration, survival and functioning of epithelial tissues is dependent on cell polarity. In the breast, normal epithelial cells display apical-basal polarity and the failure to maintain polarisation is associated with acini dysregulation, uncontrolled proliferation and the development of tumours (Wodarz and Näthke, 2007; Rejon, Al-Masri and McCaffrey, 2016). Polarity is maintained by tight junctions, as well as three major polarity complexes: the Crumbs complex, the Par complex (situated at the tight

junction) and the basolateral Scrib complex. The Crumbs complex is located at the apical membrane and is made up of Crumbs3 adaptor proteins Pals1 and Patj (pals1-associated tight junction protein). The Par complex consists of Par3, Par6, the small GTPase cdc42 and protein kinase C. The Scrib complex, contains the Scrib protein, discs large, and lethal giant larvae. Together these complexes are essential in preserving apical-basal polarity in breast cells (Rodriguez-Boulan and Macara, 2014).

Polarity is demonstrated by cell-cell tight junctions at the apical pole and hemidesmosomes at the cell basement membrane junctions within the acini structure. This is evidenced by the presence of polarity markers α6/β4 integrin (hemidesmosomes) and ZO-1 (tight junctions) evidences the apical-basal polarity in 3D cultures, comparable to the human breast tissue (Figure 3.5A). The loss of polarity can also be observed in 3D cultures, resulting in disorganised heterogeneous nodules (Figure 3.5B,C; Vidi, Bissell and Lelièvre, 2013)

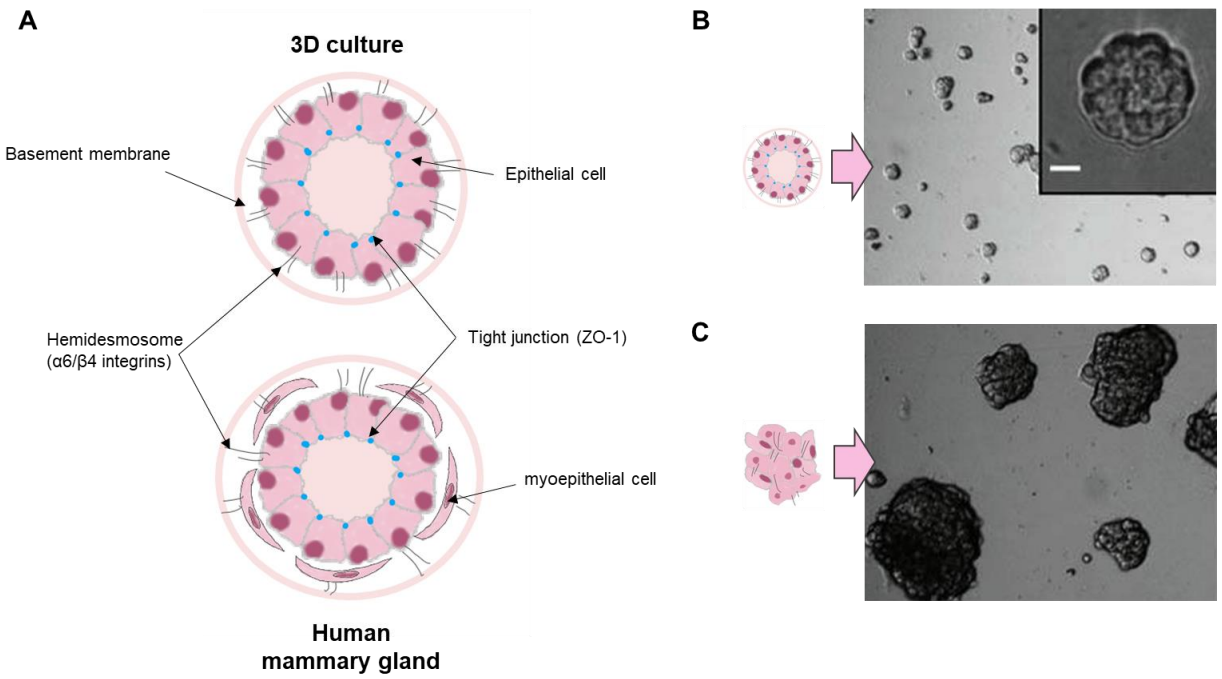

Figure 3.5. Apical-basal polarity in 3D cultures of mammary epithelial cells. (A) Cross section of acini in in 3D *in vitro* culture (top) shows tight junctions (blue dots) and hemidesmosomes (grey lines) comparably to the human mammary gland (bottom) demonstrating the ability of 3D cultures to display apical-basal polarity. Bright field images of non-neoplastic mammary cells form polarised spheres that are comparable to structures within the breast (B), whereas malignant cells lack polarity and develop into disorganised nodules (c). Adapted from Vidi, Bissell and Lelievre (2013).

3.1.1.4. Studying the effects of EDC exposures in 3D cultures

The mechanisms outlined above, in some cases, can be influenced by oestrogenic action. It has been long established that oestrogens can increase cell proliferation (Soto and Sonnenschein, 1985; Pike *et al.*, 1993; Ciocca and Fanelli, 1997; Russo and Russo, 2006; Tian *et al.*, 2018). For instance, studies using 2D cell culture methods, have shown that when MCF-7 cells are exposed to physiological concentrations of E2 ($1x10^{-10}$ M) an increase in proliferation and a decrease in apoptosis can be observed (F.-P. Chen *et al.*, 2013). More recently, Tian and colleagues (2018), showed that exposure to $1x10^{-10}$ M E2, promoted cell proliferation via the up-regulation of cyclin G1. Such oestrogenic effects have also been demonstrated in a 3D physiologically relevant context. After two weeks of exposure to $1x10^{-9}$ M E2, MCF-10A cells exhibited a significant loss of polarity when cultured in 3D (Wang and Kaplan, 2012). Concentrations of E2 between $1x10^{-13}$ to $1x10^{-9}$ M were also shown to disrupt MFC-7 cells cultured in 3D, displaying a decrease in lumen formation after 7 days of exposure (Vantangoli *et al.*, 2016).

Whilst much of the research surrounding the relationship between EDC exposure and breast cancer risk has been undertaken in monolayer cultures, 3D cultures have the ability to model oestrogen-induced changes indicative of early breast carcinogenesis. This provides an opportunity to study how oestrogen-mimicking EDCs may contribute towards breast cancer risk and the mechanisms that underpin this relationship. Morphological changes to acini development have been observed in Matrigel-based 3D cultures, in response to several EDCs, including propylparaben and BPA. MCF-12A acini have been observed to display an increase in size, along with luminal filling and a disorganised structure, comparable to changes observed in the human breast after EDC exposure (Marchese and Silva, 2012). Similar studies with BPA in ER-negative MCF-10A cells evidenced that concentrations of $1x10^{-7}$ M induced cell proliferation and significantly increased acini size (Pfeifer, Chung and Hu, 2015). Fernandez and Russo (2009), investigated the relevance of BPA and benzyl phthalate (BBP) had to breast cancer initiation, by exposing MCF-10F cells grown in 3D to concentrations of between $1x10^{-3}$ and $1x10^{-}$

^{6} M. Authors described that although concentrations higher than $1x10^{-4}$ M were toxic, evidence of an increase in proliferation could be observed at $1x10^{-5}$ M BPA and BBP.

Whilst these studies have validated the ability of EDCs to induce changes in 3D cultures, which could be considered indicative of neoplastic transformations, the concentrations tested are generally significantly higher than what is known to be present in human tissues. Therefore, it could be argued that they are not a true representation of the effects induced in populations. For instance, Marchese and Silva (2012) assessed the effects of $1x10^{-5}$ M BPA and propylparaben, which demonstrated the two compounds could induce significant changes to acini formation. However, the tested concentration was considerably higher than what has been observed in tissues, meaning these effects may not be representative of population exposures. Pfeifer and colleagues (2015), investigated the effects of $1x10^{-7}$ M BPA. Although authors categorised this concentration as 'low dose', it remains significantly higher than typical tissue concentrations of the compound. Furthermore, we lack a clear understanding of the mechanisms that underpin the observed morphological changes.

Often when low concentrations are tested, significant effects are not observed, which can lead to the belief that exposure to EDCs at low levels does not increase breast cancer risk (Bergman, Heindel, Kasten, *et al.*, 2013). However, as discussed previously, measuring the effects of single compounds in isolation, is not representative of real world exposures. Instead we are exposed to large numbers of chemicals at low levels in combination. To date, this has not been considered when testing EDCs in 3D models, highlighting a significant gap in our understanding.

3.1.2. Chemical mixtures

As discussed in Chapter 1, individuals are not exposed to single compounds and are in fact simultaneously exposed to a multitude of chemicals in combination. A clear EDC-mixture relationship has not yet been established in humans and, therefore, regulatory bodies have not been able to accurately assess the real impacts of exposures on human populations (Liu *et al.*, 2016; Gao *et al.*, 2018). However, the ability of these chemicals to act in combination in

experimental *in vitro* and *in vivo* assays has been reported by scientists for over two decades. As an example, the so-called “something from nothing” phenomenon has demonstrated that whilst individually, compounds at low levels are unable to elicit a measurable effect, in combination, this effect becomes more significant and easily detectable (Silva, Rajapakse and Kortenkamp, 2002; Jin *et al.*, 2014; Cobbina *et al.*, 2015; Seeger *et al.*, 2016).

Research into exposures and tissue concentrations has evidenced the myriad of compounds individuals are exposed to. Early studies showed that concentrations of persistent organic pollutants, including *o,p'*-DDT, hexachlorocyclohexanes (HCHs), chlordane compounds (CHLs), hexachlorobenzene (HCB), and polychlorinated biphenyls (PCBs), could be detected in human breast tissue (Nakata *et al.*, 2002). Although samples generally showed low amounts of the contaminants, they reflected what was found in the environment, which, combined, may result in significant health risks. Other studies have supported the presence of numerous EDCs in the breast. One overview highlighted low levels of *o,p'*-DDT, PCBs, PCTs, dioxins, lead, aldrin and dieldrin, amongst others, in breast milk, and authors again suggested that, when combined, these compounds may have negative impacts on health (Sonawane, 1995). More recently, Wang et al. (2015) showed 19 environmental phenolic and xenobiotic heterocyclic aromatic compounds accumulated in human adipose tissue. Wang and colleagues found that, out of 20 tissue samples, BPA was present in 18 at a concentration of up to 20.9 ng/g in US donor tissues. Benzophenone 3 (BP-3), a UV-filter used in sunscreens, cosmetics and some foodstuffs (Liao and Kannan, 2014), was shown to be present in all of the tested samples, with concentrations ranging from 3.76 to 4940 ng/g, suggesting a widespread exposure to the compound. Triclosan (an antibacterial/antifungal agent used in cosmetics and detergents) was also identified in all tissue samples tested, with concentrations ranging from 2.2 – 23.2 ng/g. They also observed levels of six parabens, with propylparaben observed in 50% of tissue samples (average of 0.49 ng/g), as well as levels of benzotriazoles and benzothiazoles. Further studies have reported traces of BP-3 (0-26 ng/g^{-1}), octylmethoxycinnamate (0-58.7 ng/g^{-1}) and 4-methylbenzilidenecamphor (0-25.6 ng/g^{-1}) in human breast tissue (Barr, Alamer and Darbre, 2018).

It must be noted that significant differences in chemical levels between tissue types are reported in the literature. For example, Wang et al (2015), described relatively low propylparaben concentrations in adipose tissue, whereas studies looking specifically at the breast have demonstrated much higher tissue concentrations (16.8 ng/g; Barr *et al.*, 2012). Further, there is substantial variation between geographic location. Studies comparing paraben exposures in women in the United States and China found that the sum of parabens in urine of women in the United states was 10-30 times lower than women in China (Ye *et al.*, 2006; Calafat *et al.*, 2010; Wang *et al.*, 2013). Nonetheless, a growing body of literature has shown the magnitude of chemicals individuals are exposed to on a daily basis, all of which may have the ability to work in combination, adding to an individual's 'oestrogenic load' and increasing breast cancer risk (Ibarluzea Jm *et al.*, 2004; Kortenkamp, 2006; Pastor-Barriuso *et al.*, 2016).

3.1.2.1. Assessing mixture effects

The number of chemicals individuals are consistently exposed to is substantial and the potential for these compounds to interact, increasing the overall toxic effect, has been established (Kortenkamp, 2008, 2014; Kjaerstad *et al.*, 2010; Ribeiro, Ladeira and Viegas, 2017). The importance of evaluating the effects of chemical mixtures has been long recognised and practiced within the field of toxicology (Groten, Feron and Sühnel, 2001; Carpenter, Arcaro and Spink, 2002; Rajapakse, Silva and Kortenkamp, 2002; Silva, Rajapakse and Kortenkamp, 2002; Silva *et al.*, 2011), however mixture studies remain uncommon when assessing the contribution of EDC exposure to breast cancer risk, despite being outlined as a priority (Kortenkamp, 2006; Engström *et al.*, 2015; Hu *et al.*, 2015).

Understanding how chemical mixtures interact is imperative to determine the true contribution EDC exposures may have to breast cancer risk. There are three main ways chemicals can interact in a mixture setting. Combinations can be defined as showing additive, synergistic or antagonistic relationships. In chemicals that elicit sigmoidal dose response curves, the term addivity is used to describe mixtures where compounds neither enhance or diminish each other's action (Kortenkamp, 2007). In additive mixtures, chemicals are not interacting with each other, but instead, they are inducing the same effects they would have individually. However, because

there are more compounds present the effect is increased. In cases of additivity, concentrations of the individual components can be added to predict what the overall effect would be, based on the dose response curves of the individual compounds, as if they were dilutions of each other. The two other types of chemical interactions cannot be predicted, yet they can be inferred when the observed mixture effect deviates from the additive prediction. If the observed effect is lower than predicted for a given concentration, an antagonistic interaction could be suggested. Antagonism occurs when substances work against each other and either completely cancel out or reduce the effects on the individual. For example, cadmium, a highly toxic metal, can cause anaemia and nephrogenic hypertension. When mixed with calcium, zinc and selenium, the negative impacts of cadmium are seen to be decreased (Yu, Tsunoda and Tsunoda, 2011). Zhang and Xiao, (1998) examined this relationship using root cells. Here authors described that cadmium exposure ($1x10^{-3}$ to $1x10^{-5}$ M) induced chromosomal aberrations and reduced the fidelity of DNA and RNA synthesis. However, when cadmium exposure was combined with $1x10^{-2}$ to $1x10^{-6}$ M of calcium, zinc and selenium, the frequency of chromosomal aberrations decreased. In contrast, if the observed mixture effect is higher than predicted, it would suggest a synergistic chemical interaction was occurring (Cedergreen, 2014). Synergism is often the result of one test compound interacting with the target of another. For instance, one compound may increase the expression of a gene encoding for a receptor. The effects of the second compound, which acts on this receptor, is then enhanced. Synergism creates significant challenges for chemical risk assessments of mixtures, however for environmental compounds such relationships are rare. Reviews have reported that in pesticide mixtures, approximately 5% of tested mixtures induce synergistic reactions (Belden, Gilliom and Lydy, 2007; Boobis *et al.*, 2011). Nevertheless, scientists have often been motivated to identify synergistic combination effects in an effort to explain the potential health risks induced by the low concentrations of compounds observed in tissues (Kortenkamp, 2007). A widely cited study by Arnold *et al.*, (1996) claimed to observe spectacular synergistic activity between oestrogenic pesticides. Yet the article was later retracted after several research groups failed to reproduce Arnold's findings (e.g. Ashby *et al.*, 1997; Ramamoorthy *et al.*, 1997). It has since been argued, that the

unrelenting search for EDC mixture synergisms is unhelpful. Silva (2003), stated that placing such importance on synergistic interactions may lead researchers to presume mere additive effects between EDCs hold no relevance to adverse human health effects. However, due to the volume of oestrogenic compounds we are now exposed to, this is not necessarily the case.

3.1.3. Chapter scope

As highlighted above, our understanding of how EDC mixtures can contribute to breast cancer risk is limited. Within this chapter, we aimed to determine whether four ubiquitous xenoestrogens; BPA, propylparaben, *o,p'*-DDT (oestrogenic mimicking pesticide) and benzophenone-3 (oestrogen-mimicking UV filter found in sunscreen and other cosmetics) could induce effects that may indicate an increase in breast cancer risk, both individually and in combination. Using a 3D culture model, more representative of the human breast than traditional 2D assays, we addressed this aim by investigating the following questions:

1) Do endocrine disrupting chemicals act together at tissue relevant concentrations to produce significant effects on acini morphology indicative of carcinogenesis?
2) Can the effects of the chemical combination be predicted, based on our knowledge of the effects elicited by individual mixture components?
3) Does exposure to individual compounds or mixtures elicit significant alterations to any of the genes described in Chapter 2 and can these alterations be associated with a possible increase in breast cancer risk?

3.2. Methodology

3.2.1. Chemical handling

All solutions of BPA, propylparaben, *o,p'*-DDT, and BP-3 were of analytical standard (>95% purity) and purchased from Sigma-Aldrich (Dorset, UK). Compounds were prepared at $1x10^{-3}$ M stock in 100% HPLC-grade ethanol in glass vials and stored at -20 °C. The molecular formulas and physicochemical properties of the EDCs are shown in Table 3.2. Serial dilutions were also prepared in 100% HPLC-grade ethanol, with six tested concentrations between $1x10^{-9}$ M to $1x10^{-5}$ M.

Table 3.2. Physiochemical properties of tested compounds.

Compound	Systematic name	Molecular Formula	Molecular weight	Water solubility[1]	Log K_{ow}[2]	CAS
BPA	4,4'-(2,2-Propanediyl)diphenol	$C_{15}H_{16}O_2$	228.29	173	3.64	80-05-7
Propylparaben	Propyl 4-hydroxybenzoate	$C_{10}H_{12}O_3$	180.08	529	2.98	94-13-3
o,p'-DDT	1,1'-(2,2,2-Trichloro-1,1-ethanediyl)bis(4-chlorobenzene)	$C_{14}H_9Cl_5$	354.49	0.007	6.79	50-29-3
BP-3	(2-Hydroxy-4-methoxyphenyl)(phenyl) methanone	$C_{14}H_{12}O_3$	228.24	69	3.52	131-57-7

[1]Data at 25 °C, mg/L obtained from chemspider.com, estimated from Log K_{ow} (WSKOW v1.41). [2]Predicted data cited from chemspider.com, generated using US Environmental Protection Agency's EPISuite, (KOWWIN v1.67 estimate).

3.2.2. Routine cell culture

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Dorset, UK). MCF-12A cells, were obtained from the American Type Culture Collection and maintained in monolayer in 75 cm^2 canter-neck tissue culture flasks. Cells were provided with Dulbecco's Modified Eagle Medium (DMEM: F12; Invitrogen, Paisley, UK), supplemented with 5% horse serum (Invitrogen), 0.02% epidermal growth factor, 0.01% cholera toxin, 0.1% insulin, 0.05% hydrocortisone and 1% pen/strep (growth medium). Medium was replaced every four days and cultures were maintained in a humidified incubator at 37˚C and 5% CO_2. Cells were passaged at 70% confluence with 0.25% trypsin-EDTA.

3.2.3. Three-dimensional Matrigel cultures

MCF12A cells were cultured on a Matrigel bed following a previously established protocol (Debnath, Muthuswamy and Brugge, 2003; Debnath and Brugge, 2005; Marchese and Silva, 2012). Briefly, 8-well chamber slides (MerckMillipore, Watford, UK) were coated with 70 µl 100% growth factor reduced (GFR) Matrigel (>10 mg/ml protein and <2 EU/ml endotoxin concentration; Corning, Wiesbadan, Germany) and allowed to polymerise at 37°C and 5% CO_2 for 15 minutes. Confluent cells were trypsinised and a single cell suspension was prepared in growth medium ($2x10^4$ cells/ml). Cell suspension was then added in a 1:1 ratio to assay medium (MCF-12A growth medium +4% GFR Matrigel). At this point, chemical treatment, dissolved in 100% EtOH, was added to the cell suspension solution, with the final concentration of solvent not exceeding 0.5%. As in Chapter 2, negative control samples were exposed to EtOH. Next, 400 µl per well cell suspension solution was overlaid on the polymerised Matrigel (Figure 3.6). Cultures were maintained in a humidified incubator at 37˚C and 5% CO_2. Growth medium was replenished every four days, containing 2% Matrigel. Cultures were incubated for a total of 20 days before being used for morphological or gene expression analysis (described below).

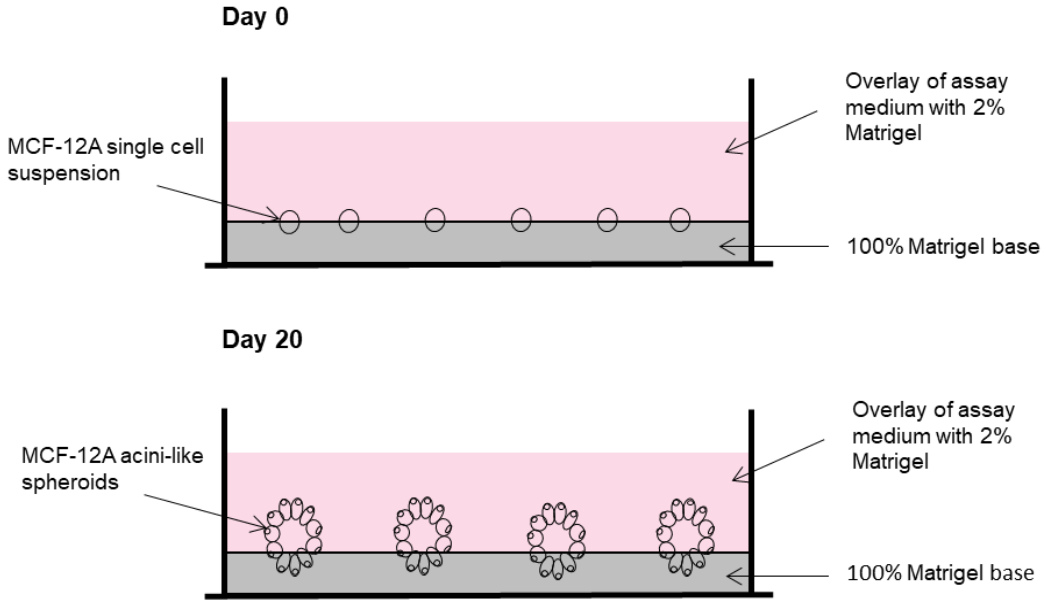

Figure 3.6. Set up of MCF-12A three-dimensional assay. A 100% GFR Matrigel bed is polymerised and overlaid with MFC-12A cells suspended in growth medium containing 2% Matrigel. By the 20 day incubation period, single cells develop into acini-like spheroids, comparable to structures observed in the human breast.

3.2.4. Morphological Analysis

Following the incubation period, medium was removed and slides were immediately fixed with a freshly prepared methanol and acetone solution (1:1 ratio) for 12 minutes at -20°C. Cell nuclei were counterstained with $2.5x10^{-6}$ M 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Thermo Fisher Scientific, Hemel Hempstead, UK) in PBS for 15 mins, mounted with freshly prepared Prolong Antifade Reagent (Molecular Probes) and allowed to dry overnight at room temperature. Immunofluorescence microscopy analysis was performed using an Olympus BX41 fluorescence microscope with IMSTAR Pathfinder™ software (IMSTAR, Paris, France). Analysis is representative of three independent experiments undertaken in duplicate.

To measure acini disruption, a minimum of 10 randomly selected acini from each duplicate were analysed per treatment for each independent experiment. Disruption was quantified by measuring the acini area and circularity (Carey, Martin and Reinhart-King, 2017; Corda *et al.*, 2017). Circularity was assessed using the following calculation:

$$Circularity = \frac{4\pi \text{ acini area}}{acini\ perimeter^2} \qquad \textbf{(Equation 3.1)}$$

A circularity value of 1 represents a perfect circle. As the value approaches 0, this indicates an increasingly elongated and irregular shape. Measurements were carried out using Image J (www.imagej.net) and statistical analysis was undertaken in Prism (version 7.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Data were normalised to negative controls and a Kruskal-Wallis test, followed by Dunn's multiple comparison, was performed to determine whether differences between treatments and negative controls were statistically significant.

3.2.4.1. Dose response curves

Based on the acini area analysis from single chemical exposures, a regression model was fitted to produce full dose response curves for each of the individual compounds. In total, four regression models were fitted; logit, hill three parameter and generalised logit I and II. Each

model describes monotonic sigmoidal dose response relationships and were fitted independently of each other to each compound's dataset. The final regression model was selected based on a statistical goodness of fit criterion (i.e. the distance between the model estimation and the observed data; Scholze, *et al.*, 2001; Slob, 2002).

3.2.4.2. Testing mixture effects

Morphological dose response curves were used to calculate a mixture effect prediction. The assessment of combination effects in relation to synergism, antagonism or additivity required accurate prediction of the combination effects expected from the mixture. Various models have been proposed to calculate additive expectations, and appropriate model selection is based on the type of mixture being investigated, the individual compounds within the mixture, their mechanism of action, and also the test system. Two of the most commonly utilised models, that are relied upon in current regulatory approaches, are independent action and dose addition (Baas *et al.*, 2007; Nagai and De Schamphelaere, 2016).

Independent action (IA) was originally developed by Bliss (1939). It is most commonly applied to mixtures containing dissimilar mechanisms of action (Jonker *et al.*, 2005; Baas *et al.*, 2007) and assumes that mixture effects are the result of interactions of individual mixture elements, meaning the effects of individual compounds are independent of each other.

Independent action is defined using the following formula:

$$1 - \prod [1 - E(c_i)] \qquad \textbf{(Equation 3.2)}$$

where ci is the concentration of chemical i in a mixture that produces an effect E of known magnitude. EC_i is the concentration of chemical i required to produce effect E on its own. IA is a probabilistic model where $E(c_i)$ is a fraction of the maximal possible effect, that cannot exceed 1.

Dose addition (DA), also referred to as concentration or response addition, works on the assumption that all the chemicals in the mixture act in a similar way, for example by having the same mechanism of action. In DA, each of the components behave as a dilution of each other and it is assumed that each component of the mixture can be replaced by an equi-effective concentration of another, without changing the overall mixture effect. Also, as with IA, it assumes that neither pharmacokinetic or pharmacodynamic interactions are present in the mixture (Kortenkamp, Backhaus and Faust, 2009). Belden *et al.* (2007) concluded that after reviewing 303 studies of aquatic pesticides, DA was the most frequently used prediction model for the majority of literature (207 experiments). DA has been reported to overestimate the mixture effect in comparison to IA (Tanaka and Tada, 2017). In contrast, IA has a tendency to underestimate the mixture effect and this must be considered when interpreting results (Orton *et al.*, 2014). Overall, DA has been successful at predicting the effects of a wide range of chemicals in a variety of test systems, both *in vivo* (Altenburger *et al.*, 2000; Brian *et al.*, 2005; Brion *et al.*, 2012; Gao *et al.*, 2018) and *in vitro* (Rajapakse, Silva and Kortenkamp, 2002; Ermler, Scholze and Kortenkamp, 2011; Hadrup *et al.*, 2013; Orton *et al.*, 2014). Thus, DA is often cited as a default approach to predicting mixture effects (Kortenkamp, Backhaus and Faust, 2009).

DA can be defined using the following formula:

$$\sum \frac{c_i}{EC_i} = 1 \qquad \textbf{(Equation 3.3)}$$

where ci is the concentration of chemical i in a mixture that produces an effect E of known magnitude. EC_i is the concentration of chemical i required to produce effect E on its own.

Model selection predominantly depends on the known mechanism of actions for each chemical in the mixture (Figure 3.7). In the present study, each of the compounds has been reported to be an ER agonist (Jaga, 2000; Okubo *et al.*, 2001; Kerdivel *et al.*, 2013; Lee *et al.*, 2013; Acconcia, Pallottini and Marino, 2015; Engeli *et al.*, 2017; Shafei *et al.*, 2018; Wnuk *et al.*, 2018).

Thus, DA was deemed the most appropriate model based on previous research outlining the mode of action for each of the target chemicals and relying on single compound dose response curves (Gennings and Carter, 1995; Groten, Feron and Sühnel, 2001; Kortenkamp, Backhaus and Faust, 2009).

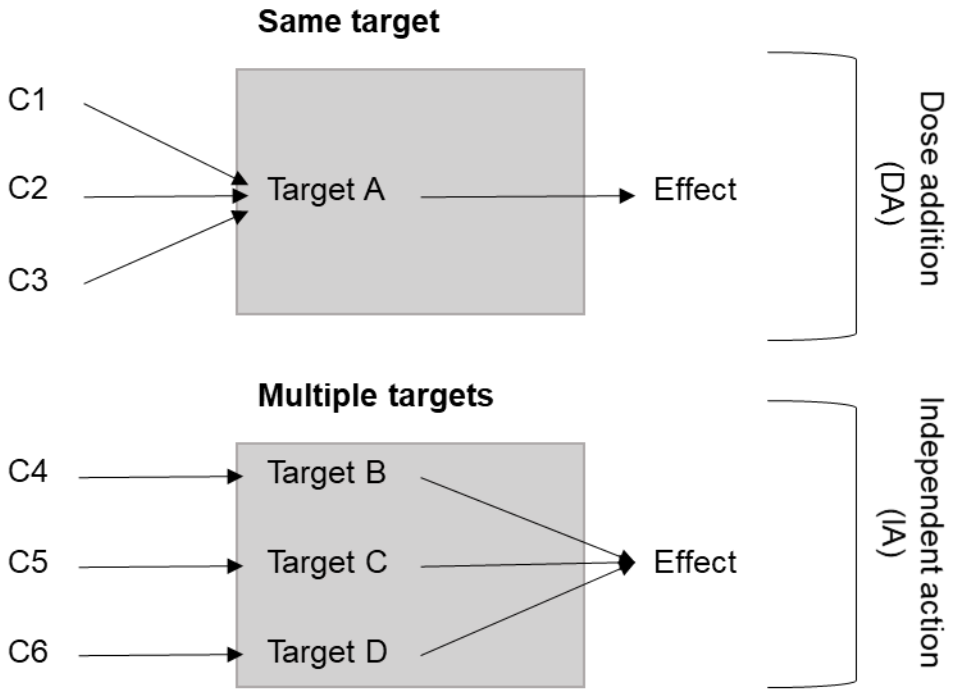

Figure 3.7. Mixture prediction model selection. Schematic represents hypothetical chemical mixtures, their mode of action and the most suitable prediction model. In the first mixture (C1-3), all compounds share the same mechanism of action and therefore dose addition is the most appropriate model choice. In the second mixture (C3-6), all the test compounds have a different mode of action that each contribute to the overall mixture effect, making independent action a suitable predictive model.

Here, the mixture experiments were designed according to a fixed-ratio mixture design. This is where the compounds are combined at a specific ratio (in this case, proportional to the concentrations present in tissues) and then a dilution series is prepared where this ratio is maintained (Evans, Scholze and Kortenkamp, 2012; Scholze, Silva and Kortenkamp, 2014; Runnalls *et al.*, 2015). Given the wide range of levels found in human tissues, the concentrations selected were based on the lower levels reported in the literature as follows: benzophenone-3: 8.09×10^{-10} M; bisphenol A: 8.76×10^{-9} M; *o,p'*-DDT: 1.9×10^{-10} M and propylparaben: 2.3×10^{-11} M (Kortenkamp, Scholze and Ermler, 2014). Due to the variations in compound levels between tissue types, concentration measurements were based on measurements taken from serum, as

a common tissue where data were available for all four compounds. Actual mixture effect observations were then compared to the DA prediction. Combination effects were considered additive if the 95% confidence intervals overlapped the mixture prediction (Orton *et al.*, 2014; Watt, Webster and Schlezinger, 2016; Thrupp *et al.*, 2018).

3.2.5. Gene Expression Analysis

To isolate acini from the Matrigel bed, assay medium was removed and wells were washed three times with PBS. Then, 200 µl/well Cell Recovery Solution (Corning, New York, USA) was added and Matrigel/ Cell Recovery Solution was removed and placed into an Eppendorf tube. A further 200 µl Cell Recovery Solution was used to rinse the wells. Matrigel/ Cell Recovery Solution was then incubated for 1 hour on ice and centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and the remaining pellet was washed once with PBS.

Following this, RNA was isolated and reverse transcribed before gene expression analysis using real-time PCR (performed as described in Chapter 2). The $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression in relation to negative controls with *ACTB* as the reference gene. Results are presented here as $\log_2$ (fold change), with control samples set to 0. Statistical significance was determined using analysis of variance (two-way ANOVA) with Bonferroni correction in R (version 3.4.3). A total of two mixture concentrations were analysed using real-time PCR, along with the corresponding concentrations for individual chemicals.

3.3. Results

3.3.1. Exposure to EDCs induced changes to acini morphology

To determine whether combination effects could be predicted, it was necessary to test six concentrations of each of the mixture compounds individually, to understand their effects on acini formation. Based on literature, normal acini grown in 3D on a Matrigel bed should form organised spheroids, with a single layer of cells and a hollow lumen (Debnath, Muthuswamy and

Brugge, 2003; Debnath and Brugge, 2005; Marchese and Silva, 2012). In the present study, after 20 days of incubation, negative control (0.5% EtOH) acini matched the expected morphology, with the majority of acini displaying a single layer of epithelial cells surrounding a hollow lumen. On average, negative control acini had an area of 3339±2744 μm^2 and a circularity of 0.94±0.01. When exposed to EDCs, we observed acini to be larger in area and circularity to be lost, which would suggest an increase in proliferation (Figure 3.8).

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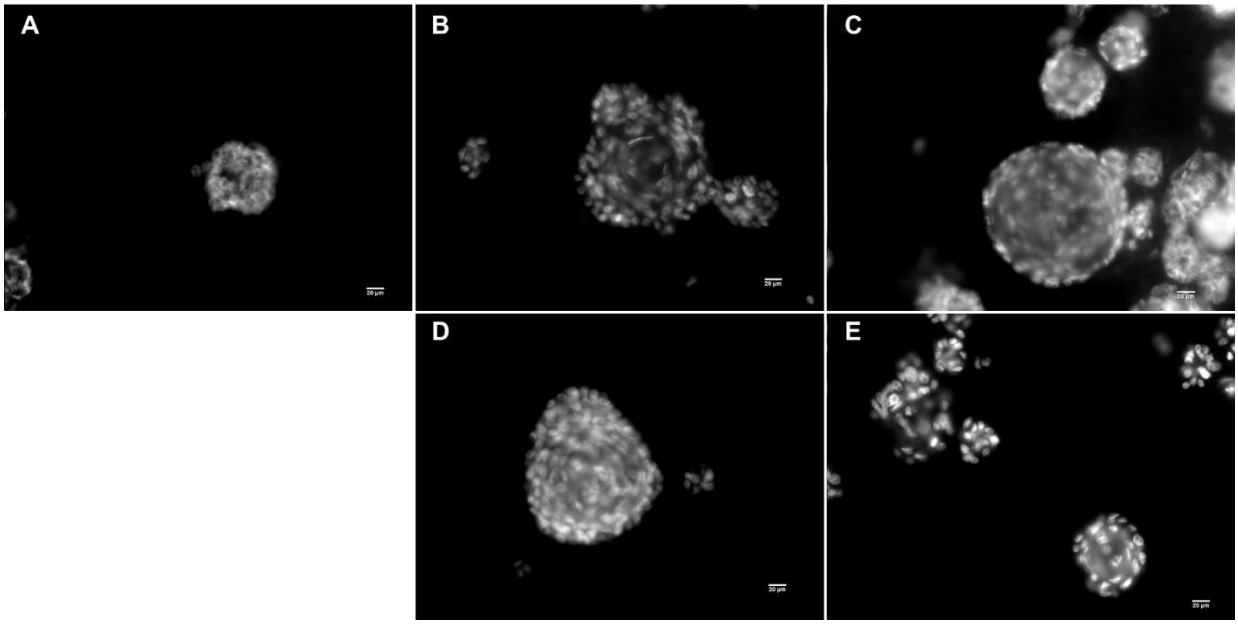

Figure 3. 8. Immunofluorescent images **of 3D cultures of mammary epithelial cells MCF-12A grown on a Matrigel bed.** Structures are depicted after treatment with 100% ethanol (A), $1x10^{-5}$ BP-3 (B), $1x10^{-5}$ propylparaben (c), $1x10^{-5}$ BPA (D) and $1x10^{-5}$ o,p'-DDT (E). Nuclei visualised with DAPI.

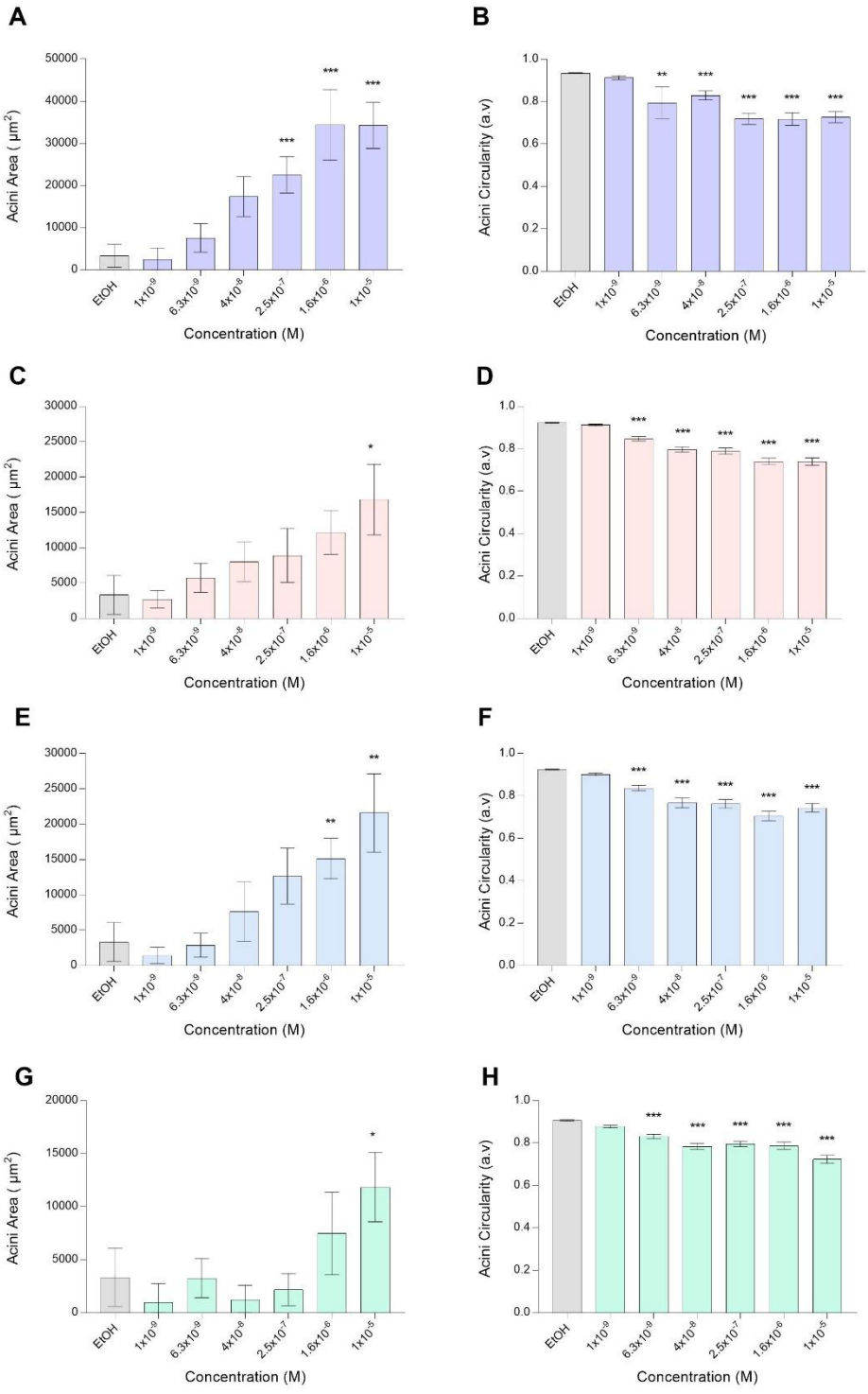

Figure 3.9. Quantification of acini area and circularity in response to EDC exposure. Acini area was measured using immunofluorescent microscopy images after 20 days incubation with EDC and showed an increase in acini size that was concentration dependent. Acini circularity decreased with an increase in EDC concentration. Graphs depict changes after exposure to BP-3 (A,B), propylparaben (C,D), BPA (E,F) and *o,p*'-DDT (G,H). Data are representative of three independent experiments run in duplicate with values corresponding to sample mean and SEM. Significance is denoted by *** <0.001 ** 0.002 * 0.03 as determined by Kruskal-Wallis test with Dunn's multiple comparison.

Out of the four compounds tested, BP-3 elicited the most substantial effect on acini morphology (Figure 3.9A,B). An increase in area could be seen at concentrations as low as $6.3x10^{-9}$ M (7615±3406 μm^2), however changes were only deemed significant at concentrations above $2.5x10^{-7}$ M (p <0.001). A loss of circularity was also induced by BP-3, with a significant decrease occurring after $6.3x10^{-9}$ M exposure (circularity 0.79±0.07, p = 0.002). This dysregulation of acini circularity continued in a dose dependent manner, with the highest concentration possessing a circularity value of 0.73±0.02 (p <0.001).

Propylparaben and BPA exposure also resulted in changes to acini circularity and size (Figure 3.9C,D and 3.9E,F respectively). Significant changes to acini size in BPA-treated samples were only observed in the highest two concentrations ($1.6x10^{-6}$ M, 15157±2860 μm^2, p <0.001; $1x10^{-5}$ M, 21604±5546 μm^2, p = 0.002). Acini exposed to propylparaben only saw a mildly significant alteration to area at the highest concentration ($1x10^{-5}$ M, 16813±4992 μm^2, p = 0.01). Highly significant changes in circularity were recorded in response to propylparaben, with concentrations as low as $6.3x10^{-9}$ M causing a substantial decrease in acini circularity (0.85±0.01, p <0.001). This was also the case for acini exposed to BPA ($6.3x10^{-9}$ M, circularity 0.84±0.01, p <0.001).

Finally, *o,p'*-DDT only elicited a significant effect on acini size after $1x10^{-5}$ M exposure (11847±3274 μm^2, p = 0.03 ;Figure 3.9G,H). As with the other tested EDCs, a significant decrease in acini circularity could be seen from $6.3x10^{-9}$ M (circularity 0.83±0.01, p <0.001).

3.3.2. Chemical mixture exposures resulted in a predictable additive effect

We then proceeded to use the data generated from individual compounds to calculate dose response curves. It was decided that acini area would be the endpoint used for dose response curves and mixture predictions, as it is the more widely used parameter within the literature (e.g. Shaw, Wrobel and Brugge, 2004; Marchese and Silva, 2012; Vidi, Bissell and Lelièvre, 2013; Sweeney, Sonnenschein and Soto, 2018), allowing us to compare the study results more effectively to existing research. As described in the methodology, for each compound, four

regression models were tested and the final presented model was identified based on goodness of fit criterion (Figure 3.10). After assessing each fitted regression model, it was decided Logit was the most appropriate for each of the four individual compounds, based on the similarity between the model and the observed data. Each of the compounds exhibited dose response curves comparable in shape, however BP-3 showed the highest maximal effect, whereas *o,p'*-DDT was seen to elicit the weakest maximum effect of the four EDCs.

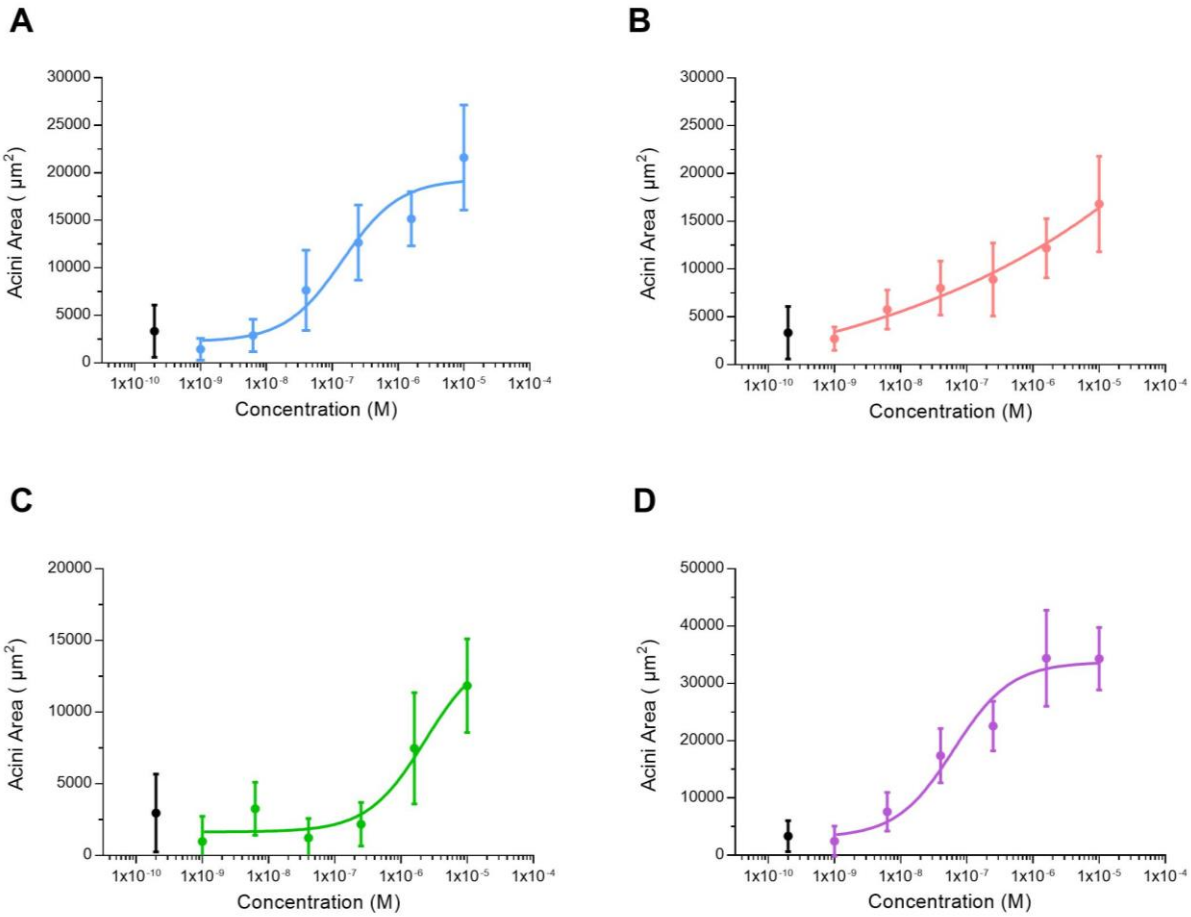

Figure 3.10. Dose response curves for four oestrogenic chemicals with regression lines derived from the best fit models for acini area. Individual compound dose response curves for (A) BPA, (B) propylparaben, (C) *o,p'*-DDT and (D) BP-3 exposure on MCF-12A cells. Data shown represent observed chemical effects on acini area (coloured circles) and controls (black circles) with corresponding SEM values from three independent experiments run in duplicate.

Next, we wanted to use data obtained from the individual exposure dose response curves to calculate a mixture effect prediction using the DA concept. It was only possible to predict the mixture effect up to 2.32×10^{-6} M, as this was the concentration that elicited the maximum effect

from *o,p'*-DDT (Figure 3.11). Due to the formula behind DA (Equation 3.3), where the mixture effect is predicted based on the sum of the individual concentrations, it is only possible to predict the effects as high as the lowest maximum effect of the individual chemicals, which in this case was *o,p'*-DDT.

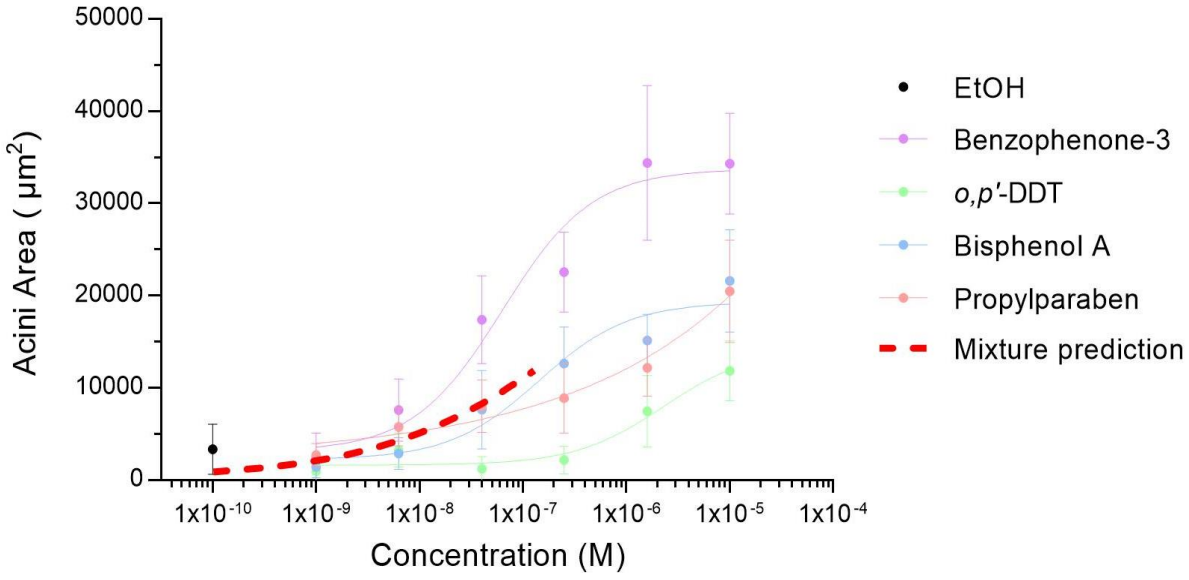

Figure 3.11. Predicted mixture effect of four oestrogenic compounds based on dose addition (DA). Individual compound dose response curves are shown in solid lines with observations (coloured circles) and SEM. Negative control acini area is represented in black, with the DA mixture prediction shown as a broken red line.

Following this, the mixture was tested experimentally, based on a fixed-ratio model. As with the individual compounds, a dose response curve for the mixture effects was fitted to a regression model (Figure 3.12). Again, the most appropriate model was selected based on the goodness of fit criterion. In this case, Logit was identified as the best fitting model for the observed mixture effects. As described in other publications (Orton *et al.*, 2014; Watt, Webster and Schlezinger, 2016; Thrupp *et al.*, 2018), an overlap of the mixture prediction and the 95% confidence interval for the mixture indicates no deviation from additivity. This was the case in the present study, with the majority of the dose response curve overlapping with the DA mixture prediction. Notably, we saw that the mixture concentration representative of tissue levels ($4x10^{-8}$ M), elicited a larger

effect on acini area than the individual EDC exposures. However, as with the single compounds, the mixture observations showed considerable experimental variation within this model.

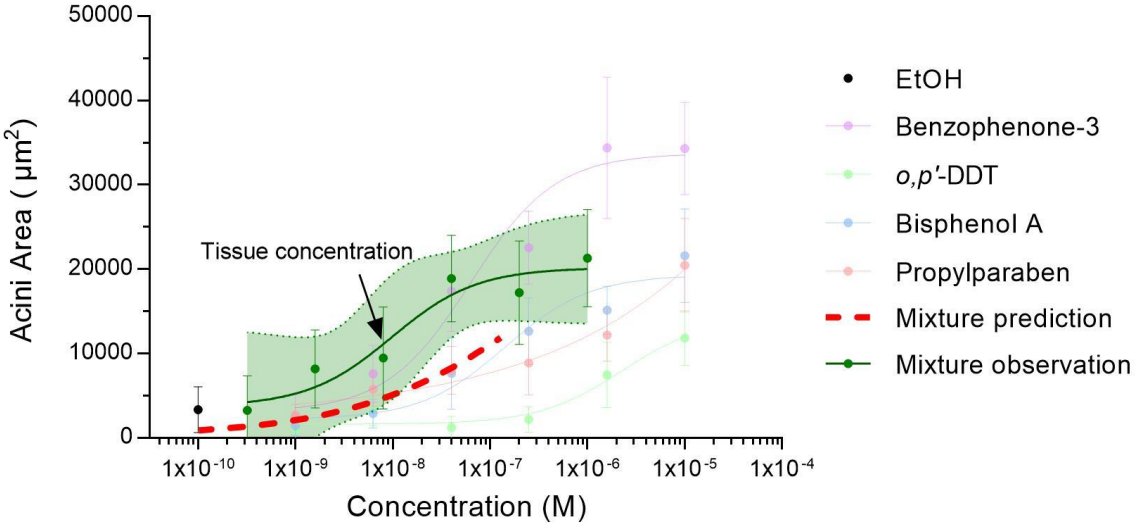

Figure 3.12. Observed changes to acini area in response to the tested mixture of four oestrogenic compounds. The observed increase in acini size in response to the mixture (dark green circles) with SEM and the mixture prediction (red broken line). Dotted green lines indicate 95% confidence intervals of the fitted regression curve (solid dark green line). The third highest concentration ($4x10^{-8}$ M) corresponds to levels found in human tissues and is highlighted by the arrow. Individual compound dose response curves are shown in solid lines with observations (coloured circles) and SEM. Negative control acini area and corresponding SEM is depicted in black.

Table 3.3. Concentration-response curve parameters of individual compounds and mixture.

Compound	Regression model	EC_{50} (M)[1]	Min[2]	Max[2]	Proportion in mixture[3]
BP-3	Logit	$6.33x10^{-6}$	3109	33724	0.8955
BPA	Logit	$1.4x10^{-7}$	2230	19337	0.0023
Propylparaben	Logit	$6.77x10^{-7}$	3223	16912	0.0194
o,p'-DDT	Logit	$2.32x10^{-6}$	1638	14395	0.0827
Mixture	*Logit*	*$9.8x10^{-9}$*	*3738*	*20173*	-

[1]EC50 refers to the concentration required to elicit a response half way between the min and max values of the regression model. [2]Min and max refer to the top and bottom plateaus of the dose response curve (µm²). [3]The proportion of each individual compound was determined by concentrations observed in human tissues (described in methodology).

3.3.3. Exposure to individual EDCs resulted in significant gene expression change

After seeing an additive mixture effect in morphological changes, we wanted to determine whether combination effects were also observable at gene expression level in this system. Using the genes identified in Chapter 2, the change in gene expression was measured at six

concentrations of each individual compound. After, two mixture concentrations were tested and compared with the effect of the individual compound concentration that was present in the mixture. One of the mixture concentrations ($4x10^{-8}$ M), resembled the levels observed in human tissues. The second concentration tested ($2x10^{-7}$ M) was higher and chosen for hazard characterisation to determine whether effects were possible. Variation in gene expression data was much more substantial than morphological changes, and clear dose response relationships could not always be observed. Indeed, many of the genes saw both increases and decreases in expression levels in response to the same compound. This occurred most consistently in samples exposed to BPA (Figure 3.13). Lower concentration exposures were seen to elicit an increase in gene expression in ER mediated genes. This up-regulation was most significant after exposure to $6.3x10^{-9}$ M BPA (6.26±0.93 LogFC, p <0.001). The same genes were then seen to decrease in gene expression at higher concentrations. In both *ESR1* and *ESR2* this down-regulation was determined to be moderately significant ($1.6x10^{-6}$ M, -2.92±0.47 LogFC, p = 0.01; -2.58±0.64 LogFC, p = 0.03 respectively).

Genes known to be mediated by other mechanisms outside of ER regulation (*CCND2, BRCA1* and *RASSF1A*) did not follow the same pattern. *CCND2* showed little change at lower concentrations and a significant change in gene expression was only observed at the highest tested concentration ($1x10^{-5}$ M, -3.55±0.56 LogFC, p <0.001). Expression of *RASSF1A* was also very significantly decreased at concentrations above $2.5x10^{-7}$ M (-3.63±0.74 LogFC, p <0.001). In the case of *BRCA1* expression, we observed a significant increase in gene expression ($2.5x10^{-7}$ M, 2.64±0.18 LogFC, p = 0.02; $1.6x10^{-6}$ M, 3.39±0.47 LogFC, p = 0.002), however, at the highest concentration no significant change could be seen, with expression appearing to return to levels comparable to controls (0.78±0.45 LogFC, p >0.05). Generally, BPA induced significant changes in gene expression at low, tissue relevant concentrations. In some cases, changes were more significant at low levels compared to high concentration exposures, suggesting the possible presence of non-monotonic activity in response to BPA at gene level. This effect was not observed in the morphological data presented previously.

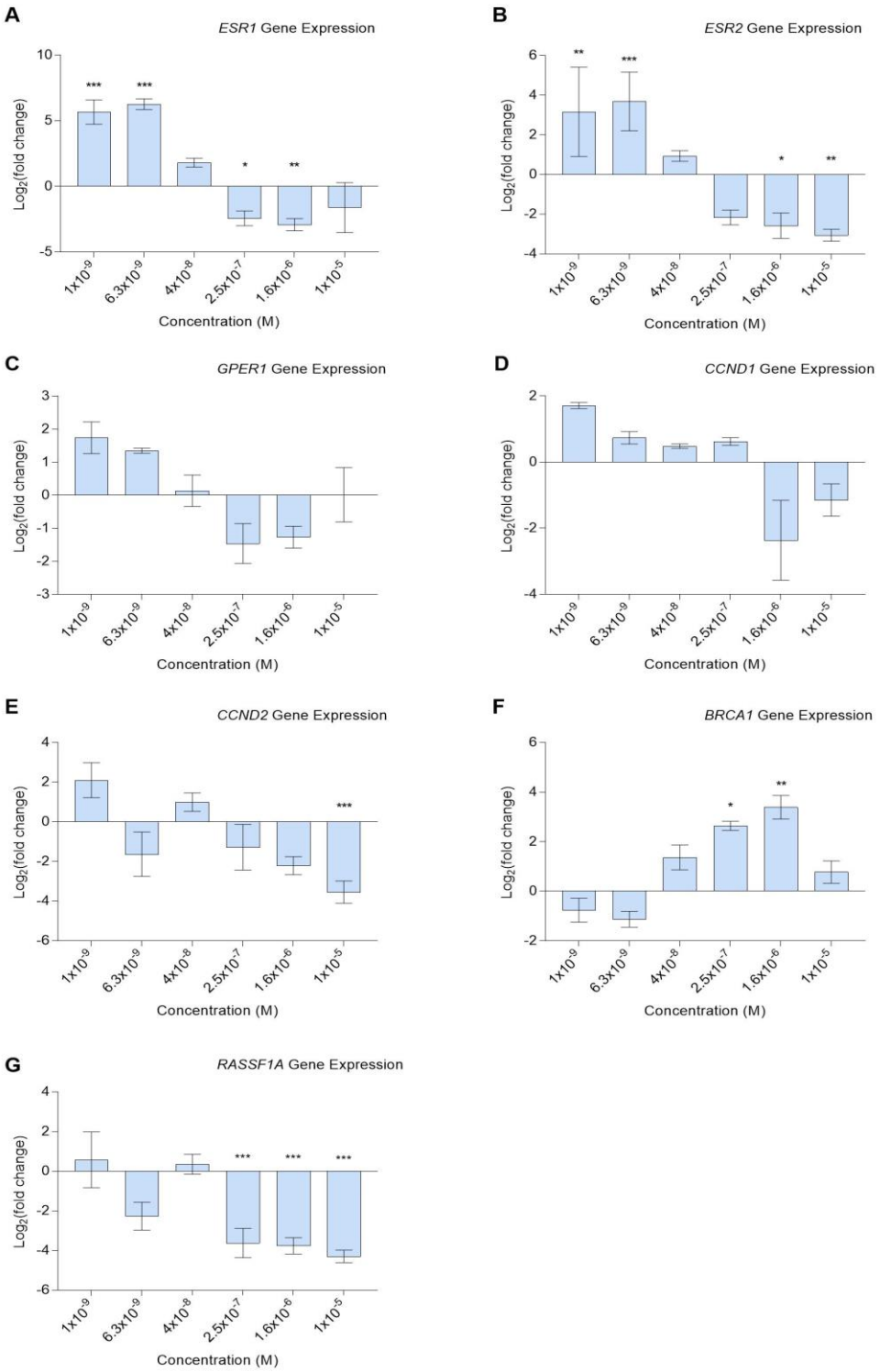

Figure 3.13. Differential gene expression observed in 3D cultured MCF-12A cells in response to BPA exposure. Log_2 fold change in response to six tested concentrations of BPA for (A) *ESR1*, (B) *ESR2*, (C) *GPER1*, (D) *CCND1*, (E) *CCND2*, (F) *BRCA1* and (G) *RASSF1A*. Data are representative of three independent experiments run in duplicate, with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

Unlike BPA, propylparaben consistently induced a decrease in gene expression (Figure 3.14). However, again, a clear dose response relationship was not clear, which may have been a result of the substantial experimental variation. The majority of significant changes were to *ESR1,* where significant alterations to expression were induced in all concentrations, with the exception of $1x10^{-9}$ M and $4x10^{-8}$ M. Out of the tested concentrations, $2.5x10^{-7}$ M elicited the most significant decrease in *ESR1* expression (-2.58±0.61 LogFC, p <0.001). Mildly significant changes could be seen in *GPER1* ($2.5x10^{-7}$ M, -2.11±0.25 LogFC, p = 0.003; $1.6x10^{-6}$ M, -1.63±0.49 LogFC, p = 0.03), *CCND1* ($1x10^{-5}$ M, -1.72±0.18 LogFC, p = 0.02) and *BRCA1* (-1.58±0.56 LogFC, p = 0.04). Decreases to *RASSF1A* expression showed a general concentration dependent response relationship, however this change was only significant at the highest tested concentration of propylparaben (-2.05±0.29 LogFC, p = 0.004).

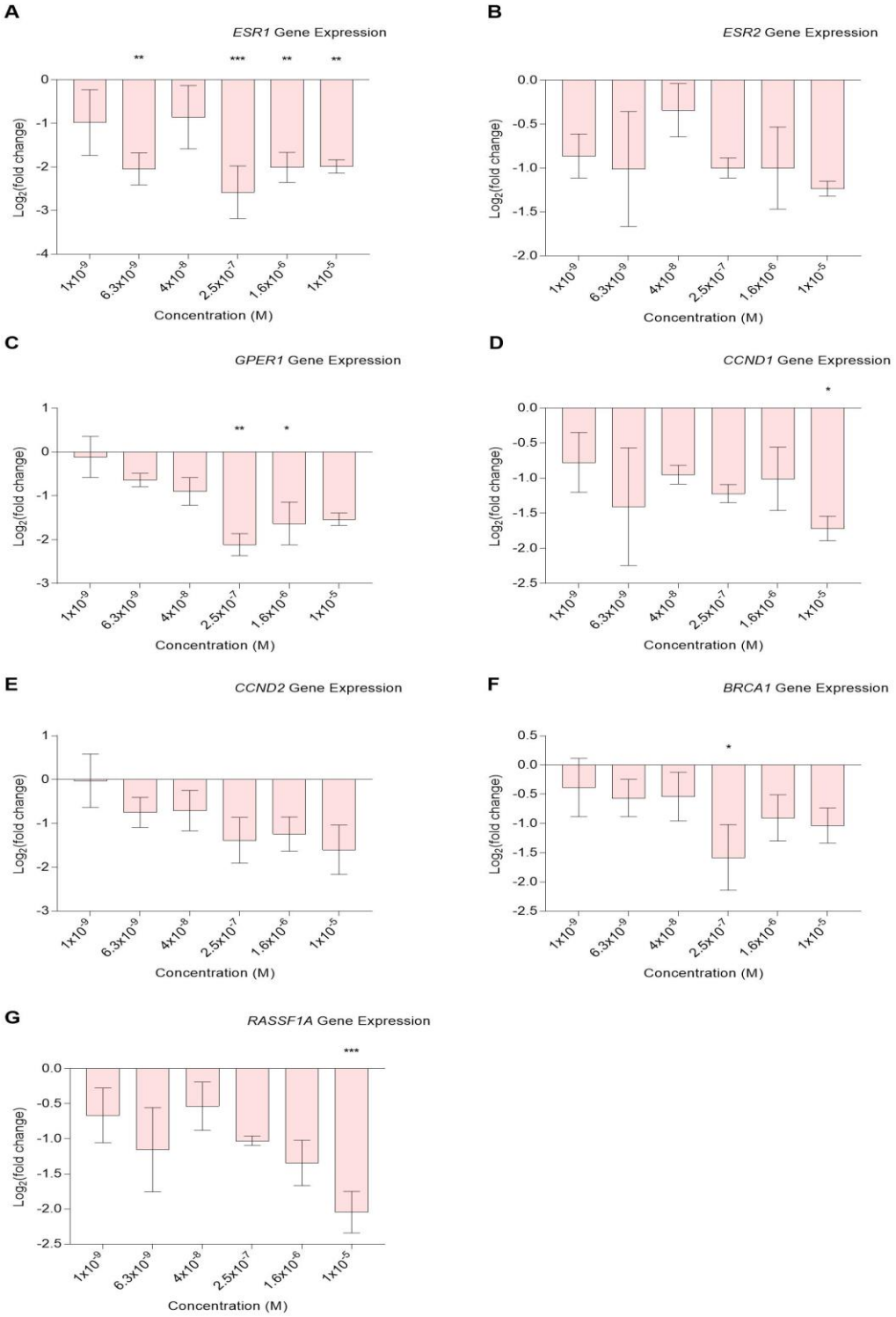

Figure 3.14. Differential gene expression observed in 3D cultured MCF-12A cells in response to propylparaben exposure. Log_2 fold change in response to six tested concentrations of propylparaben for (A) *ESR1*, (B) *ESR2*, (C) *GPER1*, (D) *CCND1*, (E) *CCND2*, (F) *BRCA1* and (G) *RASSF1A*. Data presented are representative of three independent experiments run in duplicate, with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

Samples exposed to *o,p'*-DDT appeared to demonstrate a clearer dose response relationship overall, in comparison to the other test compounds (Figure 3.15). *ESR1*, *ESR2*, *GPER1* and *BRCA1* all showed similar alterations with an increase in gene expression observed in a concentration dependent manner. As seen in response to BPA and propylparaben, the largest gene expression change was observed in *ESR1*, with a moderate significance identified at the highest exposure concentration ($1x10^{-5}$ M, 1.91±0.43 LogFC, p = 0.003). *CCND1* and *RASSF1A* expression decreased with concentration, however only the highest concentration elicited a significant effect to *CCND1* (-3.53±1.08 LogFC, p = 0.01). Likewise, only samples exposed to the highest two concentrations induced a significant decrease in *RASSF1A* ($1.6x10^{-5}$ M, -1.47±0.63 LogFC, p = 0.03; $1x10^{-5}$ M, -2.04±0.84 LogFC, p = 0.001). In the case of *CCND2*, the lowest three concentrations appeared to induce both increases and decreases in gene expression, however when the sample variation was considered, it was probable very little change was actually occurring. Also, whilst a down-regulation was shown after exposure to the higher concentrations, the experimental variation was so large it was not possible to say whether a true decrease in expression is occurring and no changes were deemed significant.

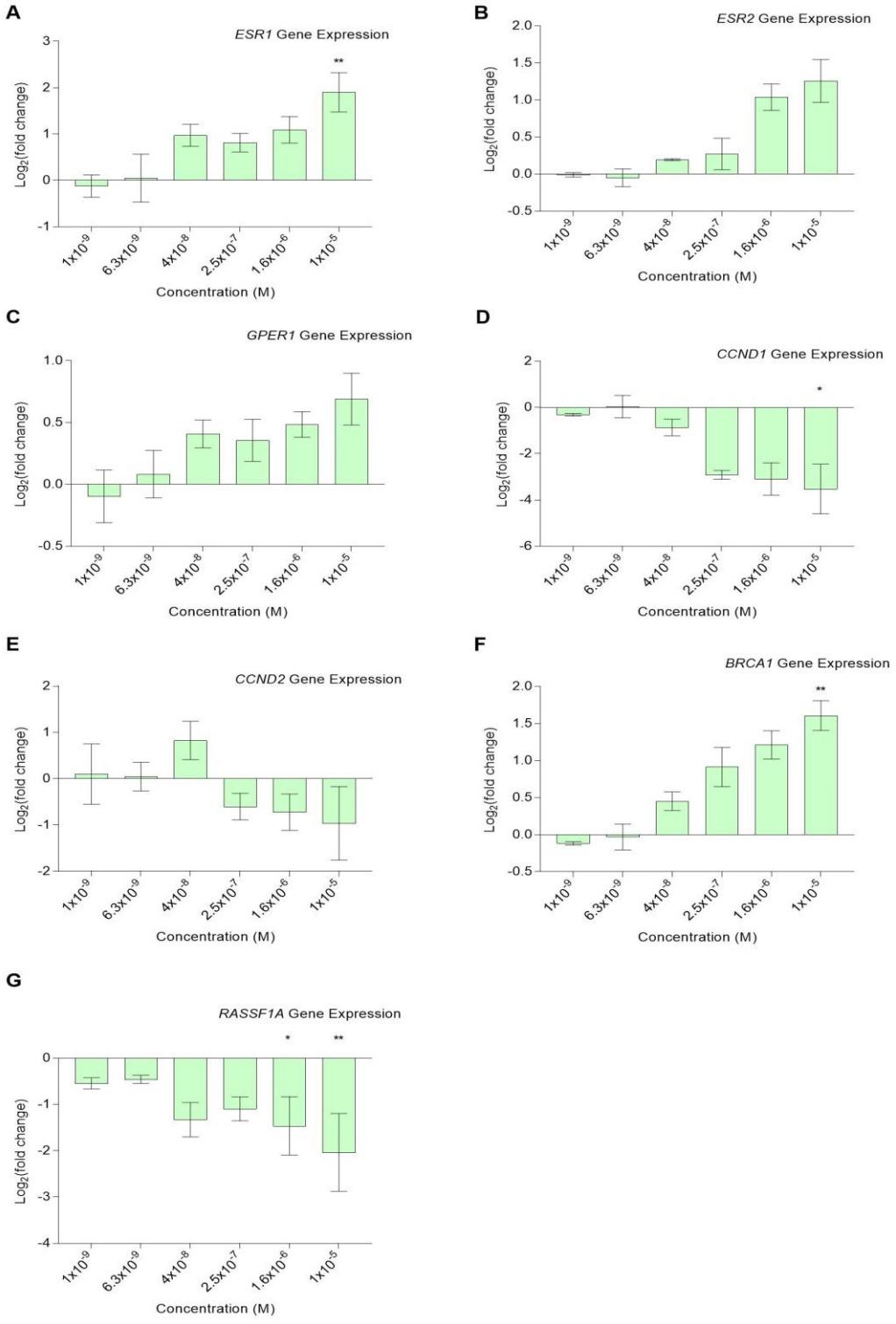

Figure 3.15. Differential gene expression observed in 3D cultured MCF-12A cells in response to *o,p'*-DDT exposure. Log_2 fold change in response to six tested concentrations of *o,p'*-DDT for (A) *ESR1*, (B) *ESR2*, (C) *GPER1*, (D) *CCND1*, (E) *CCND2*, (F) *BRCA1* and (G) *RASSF1A*. Data presented are representative of three independent experiments run in duplicate with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

Finally, we tested gene expression change in response to BP-3 exposure. BP-3 treated cells showed a similar pattern to BPA in some of the tested genes (Figure 3.16). With the exception of *ESR2*, all genes displayed an up-regulation in response to low concentrations of BP-3 exposure, before a down-regulation was seen at higher concentrations. *ESR1*, *CCND1* and *BRCA1* were significantly changed at some exposure concentrations, with no other genes demonstrating a significant alteration. The largest increase induced by BP-3 was to *CCND1* expression ($1x10^{-9}$ M, 2.81±0.36 LogFC, p = 0.005; $6.3x10^{-9}$ M, 2.43±0.68 LogFC, p = 0.02), however higher concentration exposures failed to elicit significant changes. This was followed by increases to *BRCA1* expression after exposure to $4x10^{-8}$ M (2.75±1.19 LogFC, p = 0.006), followed very closely by *ESR1* ($4x10^{-8}$ M, 2.64±0.31 LogFC, p = 0.009).

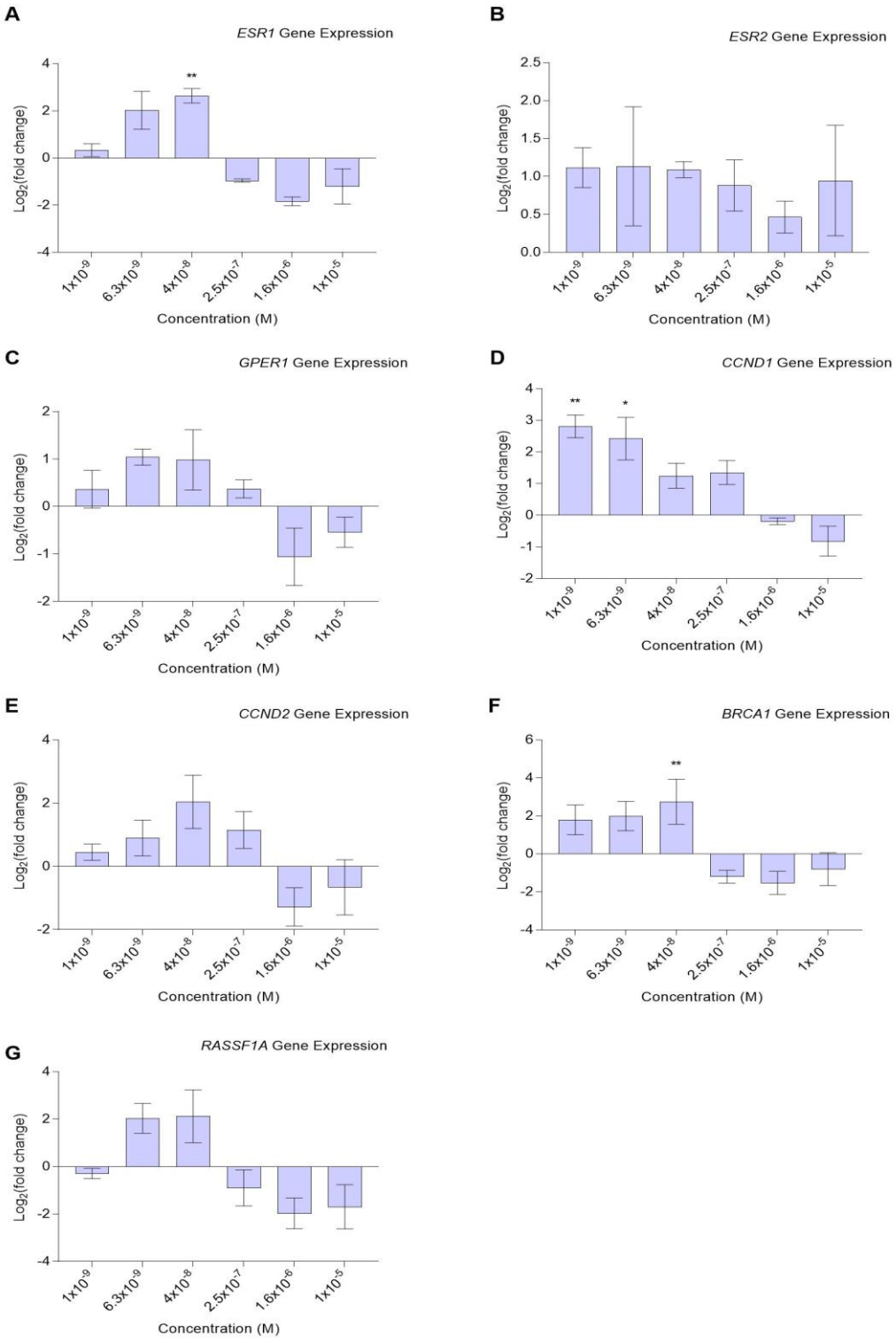

Figure 3.16. Differential gene expression observed in 3D cultured MCF-12A cells in response to BP-3 exposure. Log_2 fold change in response to six tested concentrations BP-3 for (A) *ESR1*, (B) *ESR2*, (C) *GPER1*, (D) *CCND1*, (E) *CCND2*, (F) *BRCA1* and (G) *RASSF1A*. Data presented are representative of three independent experiments run in duplicate, with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

3.3.4. Exposure to chemical mixtures elicited significant changes to gene expression

Due to a lack of concentration dependent effects observed in gene expression in response to individual chemical exposures, it was not possible to generate a mixture prediction for this endpoint using currently established methods. Consequently, we compared the mixture concentration with corresponding concentrations of individual compounds present in the mixture to see if an increase in the magnitude of effect could be seen in response to the mixture exposure.

The results observed were again very gene specific, with no general trend seen across the tested genes (Figure 3.17). In *ERS1*, *ESR2* and *GPER1* the effect of the lowest mixture concentration was lower than effects induced by exposure to BPA in isolation. Both *ESR1* and *ESR2* saw a much more significant alteration to gene expression when exposure to BPA individually, rather than the mixture ($8x10^{-9}$ M Mixture 2.06±0.38 LogFC, p = 0.03; $6.3x10^{-9}$ M BPA, 6.26±0.41 LogFC, p <0.001). In *GPER1* this was also apparent, however no significance was recorded for any of the tested individual exposures or the mixture ($8x10^{-9}$ M Mixture, 0.31±0.12 LogFC, p >0.05; $6.3x10^{-9}$ M BPA, 1.35±0.08 LogFC, p >0.05). When assessing the $2x10^{-7}$ M concentration of the mixture, there was an increased magnitude of effect in response to the mixture, with a significant down-regulation observed in both *ESR1* (-3.79±0.49 LogFC, p <0.001) and *ESR2* (-2.16±0.17 LogFC, p = 0.02), greater than any of the compounds when tested individually. This could not be observed in *GPER1*, with BPA continuing to induce the most substantial change. Analogous to *ESR1* and *ESR2*, *CCND1* effects showed no evidence of an increased response to the lower mixture concentration, however when samples were exposed to the highest $2x10^{-7}$ M mixture concentration, a clear increase in effect could be observed in response to the mixture that was considerably higher than the individual compounds (Figure 3.18; -5.82±0.46 LogFC, p <0.001). Finally, no evidence could be seen that combination exposures increased the effect on *CCND2* and *RASSF1A* expression, with neither mixture concentration inducing a significant response. However, in *BRCA1*, both mixture concentrations resulted in a more significant change to expression levels in comparison to the individual chemicals ($8x10^{-9}$ M

Mixture -3.19±0.66 LogFC, p <0.001, 2x10^{-7-} M Mixture, -4.46±0.46 LogFC, p <0.001). An increase in effect magnitude in response to both mixture concentrations was unique to *BRCA1* expression.

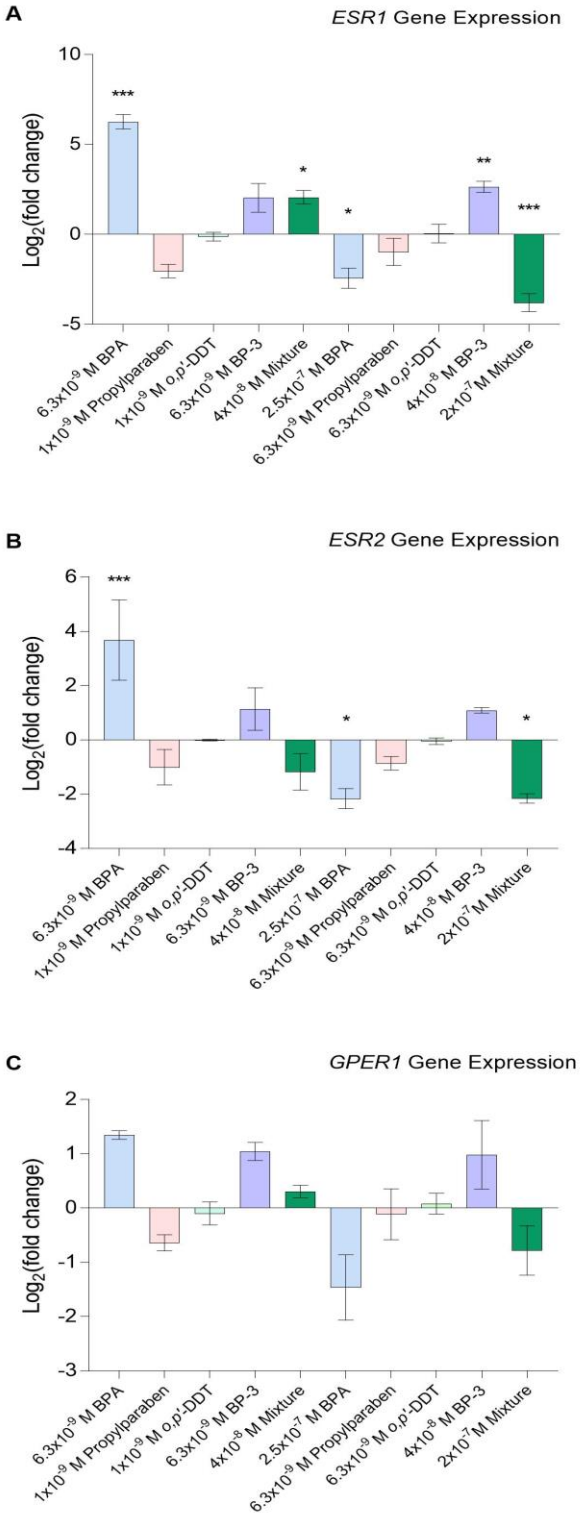

Figure 3.17. Comparison of change in ER gene expression elicited by individual and combination exposures in 3D cultured MCF-12A cells. Log_2 fold change in response to individual compounds and mixtures for (A) *ESR1*, (B) *ESR2* and (C) *GPER1*. Data presented are representative of three independent experiments run in duplicate, with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

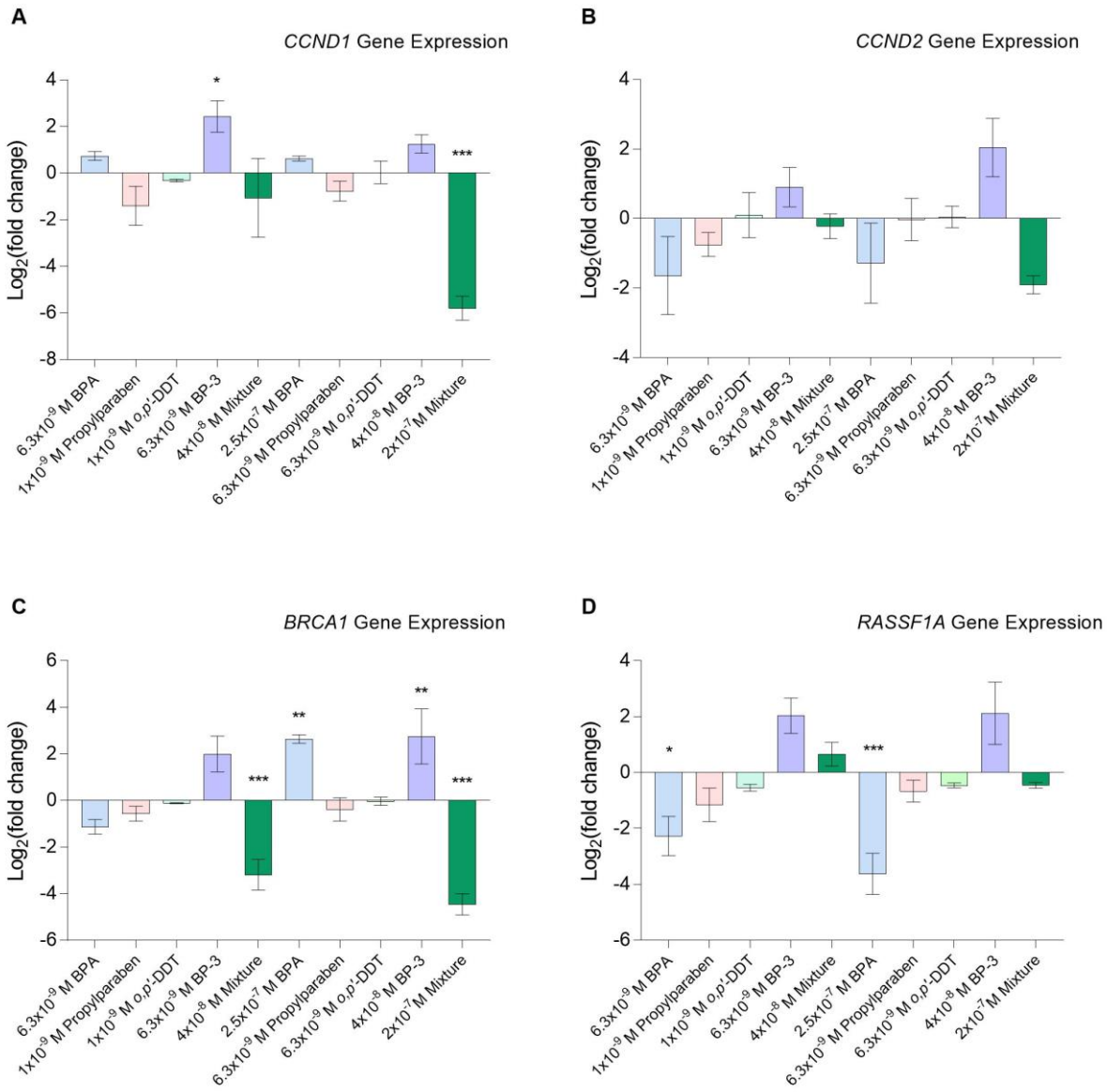

Figure 3.18. Comparison of change in expression of genes associated with breast cancer risk elicited by individual and combination exposures in 3D cultured MCF-12A cells. Log_2 fold change in response to individual compounds and mixtures for (A) *CCND1*, (B) *CCND2*, (C) *BRCA1* and (D) *RASSF1A*, Data presented are representative of three independent experiments run in duplicate, with values corresponding to sample mean and SEM. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by ANOVA with Bonferroni correction.

3.4. Discussion

Previous studies surrounding the ability of oestrogen-mimicking compounds to increase breast cancer risk have proven inconclusive, with deleterious effects observed mostly at concentrations

higher than human exposures (Rodgers *et al.*, 2018). This observation has often led to the assumption EDCs cannot contribute towards breast cancer development. However, this assumption does not consider that humans are exposed to complex mixtures of chemicals that have the potential to act in combination, adding to an individual's oestrogenic load (Ibarluzea *et al.*, 2004; Kortenkamp, 2006; Pastor-Barriuso *et al.*, 2016), that could contribute to a carcinogenic effect. In this study, we present the first evidence of combination effects occurring in an ERα, ERβ and GPER competent 3D cell culture model, which recapitulates some of the characteristics of the human mammary gland.

First, analysis of negative control acini morphology demonstrated that the 3D model utilised was fit for purpose, being comparable to published literature (Debnath, Muthuswamy and Brugge, 2003; Debnath and Brugge, 2005; Marchese and Silva, 2012). We then showed MCF-12A acini were sensitive to oestrogenic exposures, with treated samples displaying changes to acini area and circularity, indicative of the early stages of neoplastic transformation (Debnath *et al.*, 2002; Russo *et al.*, 2010). All the four compounds were able to elicit significant decreases in acini circularity, even at tissue comparable concentrations. This observation of acini disorganisation could potentially be attributed to an increase in proliferation and a loss of cellular apico-basal polarity, which is essential for maintaining acini structure (Shi *et al.*, 2014; Carey, Martin and Reinhart-King, 2017). In 3D cultured MCF-10A cells, loss of polarity has been linked to aberrant activation of PI3K/Akt cascade (Liu *et al.*, 2004). Exposure to $1x10^{-6}$ M E2 has been shown to induce hyperactivation of the cascade after binding to ERα in rat uterus cells (Kazi, Molitoris and Koos, 2009) and MCF-7 cells (Pesiri *et al.*, 2014). EDCs, including BPA and propylparaben, have also been reported to activate the PI3K/Akt signalling pathway in breast epithelial cell lines, such as MCF-7 and MCF-10A (Ptak and Gregoraszczuk, 2012; Gao *et al.*, 2015; Wróbel and Gregoraszczuk, 2015). Results observed in the present study may be induced by similar pathway activation. Overall, few studies have looked specifically at alterations to acini circularity, however data presented here suggests, along with area, circularity could be a valid endpoint that may be indicative of neoplastic transformations.

Despite seeing significant decreases in circularity at tissue relevant levels, no such significant alterations were present to acini area in response to single compound exposures. We speculate that this may be attributed to circularity being a more sensitive endpoint to detect uncontrolled proliferation or loss of polarity. However, changes to acini area is a much more commonly used measurement (e.g. Lee *et al.*, 2007; Marchese and Silva, 2012; Vidi, Bissell and Lelièvre, 2013; Feigin *et al.*, 2014; Abu-Tayeh *et al.*, 2016), but there is a lack of research demonstrating significant increases in size after exposure to individual EDCs at tissue relevant concentrations. Thus far, studies have primarily focused on changes induced by high, unrealistic concentrations of EDCs (e.g. $1x10^{-5}$ M BPA; Fernandez and Russo, 2010; Marchese and Silva, 2012). Fernandez and Russo (2010) demonstrated that exposure to E2 ($7x10^{-8}$ M), BPA or benzyl phthalate (BBP; concentrations between $1x10^{-6}$ M and $1x10^{-5}$ M) resulted in the neoplastic transformation of 3D cultured MCF-10F cells. The collagen grown MCF-10F cells were reported to form a high percentage of solid masses and were seen to have significantly less duct-like structures in treated samples compared to controls. The authors concluded that whilst BPA and BBP have a much lower affinity to ERα and ERβ than E2, the EDCs were able to induce effects comparable to $7x10^{-8}$ M E2 exposures. Marchese and Silva (2012), investigated the effects of $1x10^{-5}$ M BPA and propylparaben on MCF-12A acini formation. They described that after 8 days of exposure to EDCs, dramatic impacts could be observed on acini morphogenesis, including the presence of a filled lumen, lack of a regular spheroid shape and an increase in acini size. Within this chapter we were not able to observe significant increases in acini area in response to individual compounds at low, tissue relevant exposures. To the best of our knowledge, no study has shown the compounds tested here have the ability to elicit significant changes to acini size in ER-positive cell lines at tissue relevant concentrations. However, we see here that at higher concentrations ($1x10^{-5}$ M), tested EDCs were seen to induce large and misshapen acini. The observed changes resemble events that occur during early breast carcinogenesis (Debnath and Brugge, 2005; Hebner, Weaver and Debnath, 2008), which have been suggested to be indicative of neoplastic transformations (Marchese and Silva, 2012). This finding supports our

argument that we need to start looking towards combinations of chemicals to gain a more realistic understanding of whether 'low-dose' chemical exposures pose a risk to human health. We then carried out a mixture prediction using DA to determine whether the combination of EDCs had an additive, synergistic or antagonistic relationship, with the hope of obtaining a better understanding of the mechanisms behind the mixture toxicity and the risks posed when individuals are exposed to these compounds in combination. Data presented here largely supported the presence of an additive effect, with the mixture observation generally agreeing with the DA prediction. Comparable conclusions of additivity have been drawn previously in the literature (Orton *et al.*, 2014; Watt, Webster and Schlezinger, 2016; Thrupp *et al.*, 2018). At higher concentrations, there was some difference between the 95% confidence intervals and the prediction. This effect appeared to be slightly synergistic, with the observed effect being higher than the DA prediction. However, as previously discussed within the literature, synergistic effects between mixtures of EDCs are extremely rare. Often apparent synergisms in EDCs can be explained by alternative factors, such as experimental variability, which is possibly the case here. To confirm this was the case, studies to elucidate the mechanism of interaction or experiments using different mixture ratios would be required. For instance, in this study we tested a mixture ratio that was based on reported tissue concentrations to understand the effects of a mixture relevant to human exposures. This may mean that stronger compounds that represent a higher proportion of the mixture, such as BPA or BP-3, are masking the effects of other tested chemicals. It is more common for studies to develop combination experiments using equi-effective mixture ratios, based on EC50 concentrations (Kunz and Fent, 2006; Larsson, Giesy and Engwall, 2014; Gu *et al.*, 2015; Rossier *et al.*, 2016). This design ensures all the individual compounds are contributing to the mixture equally. Future work may benefit from testing an equi-effective mixture to compare to effects reported here and further our understanding of the mixture effects occurring in response to these compounds. Nonetheless, given it is the tissue relevant concentrations we are most interested in here, it can be seen that DA was a useful prediction method for the prediction of EDC mixture effects, where compounds are known to act predominantly via ER activation. This agreement has been reported consistently within the

literature in other study systems, first in yeast experiments (Rajapakse, Silva and Kortenkamp, 2002), and since in numerous *in vitro* (Payne, Scholze and Kortenkamp, 2001; Silva, Rajapakse and Kortenkamp, 2002; Orton *et al.*, 2014; Watt, Webster and Schlezinger, 2016) and *in vivo* (Brian *et al.*, 2005; Correia *et al.*, 2007; Christiansen *et al.*, 2009; Brion *et al.*, 2012) models.

The results from this experiment show that widespread EDCs can work in combination to produce measurable effects when present at concentrations that individually were not able to elicit significant effects. It has previously been argued that EDCs do not have the ability to increase breast cancer risk at concentrations individuals are exposed to, leading to the assumption that these compounds pose no threat to human health. In this proof of principle study, we have demonstrated that just four compounds can work in combination and induce a measurable effect indicative of early breast carcinogenesis, in a 3D culture model representative of the human breast. Previously, combinations of low dose isobutyl- ($1x10^{-7}$ M), butyl- ($2x10^{-7}$ M), propyl- ($2x10^{-7}$ M), ethyl- ($8x10^{-7}$ M) and methylparaben ($2x10^{-5}$ M) have been shown to work together and stimulate the proliferation of 2D cultured MCF-7 cells (Charles and Darbre, 2013). Work with more complex mixtures has also evidenced such combination effects. For example, Orton and colleagues (2014) tested a combination of 24 androgen receptor antagonistic compounds at concentrations representative of human exposures. They found the compounds acted in combination to produce an impact much more substantial than the individual chemical exposures, using the immortalised breast cancer cell line, MDA-kb2. Whilst these findings support our results, they were carried out using simplistic monolayer assays, unrepresentative of breast architecture. To the best of our knowledge, the present study is the first experiment to show mixture effects in such a physiologically relevant *in vitro* assay. This presents a more realistic representation of combination effects than more 2D assays used in earlier mixture assessments. Overall, our work confirms that the DA prediction is also appropriate for 3D mammary cell culture models, evidencing that 3D models are suitable to test the effect of EDCs and predict the outcome of mixture experiments.

As well as morphological changes, alterations to the genome can provide a valuable insight into the ability of these compounds to contribute towards breast carcinogenesis and highlight

mechanisms of action that may underpin this relationship. We examined the gene expression of seven genes, identified in Chapter 2. These genes were known to play a role in breast carcinogenesis, however could also be used to characterise the responses of cells to EDC exposures, for instance, whether the compounds were acting by ER-dependent mechanisms. Overall, the effect on gene expression after individual and combination chemical exposures was difficult to interpret. Unlike morphological changes, with the exception of *o,p'*-DDT, clear dose response relationships could not be observed in response to single compound exposures. Gene expression response to *o,p'*-DDT has previously been confirmed to be regulated by classical ER binding activity and our results agree with reports that the compound acts in a generally concentration dependent manner (Silva, Kabil and Kortenkamp, 2010). Other EDCs have been described to be much more varied in their mechanism of actions, despite resulting in comparable outcomes to acini morphology (Rubin, 2011; Vandenberg *et al.*, 2012; Lee *et al.*, 2013; Acconcia, Pallottini and Marino, 2015; Sharma, Schuhmacher and Kumar, 2016; Ribeiro, Ladeira and Viegas, 2017). In light of this, we suggest that the substantial experimental variation and appearance of 'random' transcriptional changes observed in response to the other tested compounds are due to the multitude of intra-cellular effects the other compounds have the ability to interfere with, rather than an issue with the test system itself. For instance, BPA has been reported to act and an ER agonist, however it can also affect other processes, such as the signalling of the androgen receptor, oestrogen-related receptors and the thyroid hormone receptor (Acconcia, Pallottini and Marino, 2015; Shafei *et al.*, 2018).

In response to individual compound exposures, significant effects could be observed in some genes at low, tissue relevant concentrations. This was especially clear in the case of BPA where the most substantial changes to ER-related gene expression was after exposure to low concentrations. This observation may potentially be linked to previous reports of BPA displaying a non-monotonic dose response curve (Angle *et al.*, 2013; Vandenberg, 2014). A recent article discussed the non-monotonic effects of BPA on *ESR1* in male and female peripheral blood samples (Awada *et al.*, 2018). Through bisulfite sequencing, authors found this was associated with BPA-induced *ESR1* promoter methylation and shorter telomere lengths. Other studies have

also linked BPA to a dysregulation of epigenetic patterns in a non-monotonic manner, suggesting a common mechanism that may be responsible for inducing significant effects at low concentrations (Faulk *et al.*, 2015; Santangeli *et al.*, 2016). The biological phenomena of non-monotonic dose response relationships, characterised by a slope that changes direction within the range of tested doses, has been suggested in the literature for many years (Kohn and Melnick, 2002), however is only now becoming affirmed in toxicology. Although more studies are being designed to address complications surrounding non-monotonicity, we still know very little about the mechanisms behind this and it presents a challenge not only for mixture predictions, but for wider toxicology and risk assessments (Zoeller and Vandenberg, 2015; Villar-Pazos *et al.*, 2017). Studies so far have reported non-monotonic dose response curves most frequently for BPA, however other environmental compounds, including genistein, PCBs, DES and various pesticides have also been reported to display non-monotonic relationships under some conditions (reviewed in Lagarde *et al.*, 2015). It must be noted however, that at the morphological level, BPA appeared to induce a monotonic dose response curve. The difference in dose response relationships on varying endpoints has been discussed previously, with researchers noting significant endpoint variation in response to BPA (Vandenberg *et al.*, 2012; Beausoleil *et al.*, 2013; Lagarde *et al.*, 2015). This observation has previously led researchers to conclude non-monotonic relationships are not established or reproducible (Melnick *et al.*, 2002), however after re-examination, it has come to be accepted that these relationships are more complex than initially thought and that disagreement between endpoints does not mean the possibility of low-dose effects should be ignored (Vandenberg *et al.*, 2012).

The impact of BPA inducing highly significant changes in gene expression at low concentrations may have also had an effect on our attempt to see whether chemical combinations elicited a more significant effect than individual exposures. The absence of dose response relationships meant it was not possible to calculate dose response curves and carry out mixture predictions. Instead, it was decided to consider the effect magnitude to determine if EDC mixtures could add to the oestrogenic load of an individual when acting in combination. However, we were not able to show mixture combinations could elicit a greater effect. For genes regulated by ER activation,

the effect induced by BPA overshadowed effects by the chemical mixture. We saw that BP-3 had the most significant effect on acini formation when tested individually. Interestingly, BP-3 was not seen to have the most significant effect on gene expression. We did see a significant increase in *CCND1* expression at concentrations below $1.6x10^{-6}$ M, which may underpin some of the increases in proliferation observed in acini development by promoting cell cycle progression at low concentrations (Alao, 2007; Foster *et al.*, 2010), however few other significant alterations could be observed in the other tested genes. This indicated that for BP-3, mechanisms inducing a change in morphology may be regulated at protein level or via genes not tested here. BP-3 exposure ($1x10^{-8}$ to $1x10^{-6}$ M) has previously been seen to induce MCF-7 proliferation via the C-X-C motif chemokine 12c (CXCL12) chemokine protein (Kerdivel *et al.*, 2013).

Interestingly, a clear increase of effect in response to chemical mixtures could only be observed in *BRCA1* expression, where both concentrations of the mixture elicited a decrease in gene expression much more substantial that any of the tested compounds individually. Very limited research has previously been undertaken to assess mixture effects on gene expression. One study did examine the impact of three environmental persistent organochlorines (POCs) in combination with E2 and saw that the addition of POCs had the ability to alter *BRCA1* expression by down-regulating promotor activity in both the ER-positive cell line, MCF-7(BUS), and the ER-positive cell line, MDA-MB-231, at a concentration of $1x10^{-5}$ M (Rattenborg, Gjermandsen and Bonefeld-Jørgensen, 2002). As discussed in Chapter 2, *BRCA1* is known to have a critical role in cell cycle control, DNA repair and maintaining genomic stability. Moreover, it is frequently down-regulated in breast cancer tumours (Rosen *et al.*, 2003). Expression of *BRCA1* has been reported to be down-regulated due to hypermethylation in response to oestrogen-mimicking EDCs (Qin *et al.*, 2012), resulting in irregular functioning of the gene and potentially leading to an increased risk of tumourigenesis. The results presented in this chapter suggest that, for *BRCA1* expression, combinations of EDCs may be increasing this reported promotor hypermethylation and down-regulation, potentially increasing breast cancer risk.

At this stage, it is not possible to confirm chemical combinations elicit an increased deleterious effect on gene expression due to results being so varied between each of the genes tested. This, combined with the lack of a clear concentration response relationship to individual compounds, made it very difficult to draw conclusions about the effect of mixtures on gene expression. Although the results shown here may be a true reflection that no mixture effects are occurring, the variability of this assay must also be considered when interpreting these results. Unlike monolayer systems, the 3D assay utilised here is comprised of a heterogeneous population of cells. Using fluorescence activated cell sorting (FACS), it was seen that 3D cultures MCF-12A cells formed acini comprising of two distinct populations of cells, referred to as 'inner' and 'outer' cells (Marchese, 2013). Inner cells underwent apoptosis during acini formation, whilst the outer cells, in contact with the basement membrane, did not undergo apoptosis. Marchese showed that within individual acini, inner and outer cells also had differing gene expression profiles. Specifically, it was reported the genes involved in apoptosis and proliferation, such as *BAX* (BCL2 associated apoptosis regulator) and *CCND1,* were differentially expressed between the populations. In control acini, expression of *CCND1* was lower in the inner population of cells in comparison to the outer cells, whereas *BAX* was more highly expressed in the inner population. Marchese found that after exposure to $1x10^{-9}$ M E2, inner and outer cells displayed significantly different expression of *BCL2*, *BAX* and *BAD* (BCL2 associated agonist of cell death). For example, in the case of *BAD*, the inner cells were seen to have an E2-induced down-regulation, whereas expression in the outer cells was enhanced. These findings indicate it may also be possible for the two cell populations to respond differently to exposures of oestrogen-mimicking EDCs. Although it was not possible to carry out cell sorting within the scope of this PhD, future work may benefit from dividing the inner and outer acini cell populations to see if clearer dose response curves could be observed in gene expression. This may then allow for mixture predictions to be calculated and for more apparent mixture effects to be seen.

The main aim of this chapter was to determine whether, in combination, low-dose EDC exposures could act together to increase the effects and potentially contribute towards breast cancer risk. It is clear at the morphological level that such mixture effects can be seen with the

four tested compounds acting in an additive manner; an effect that could be predicted using DA. Considering the number of compounds that individuals are exposed to, it is likely that moving forward, experiments will be required to include well defined mixtures, representative of real-life exposures, in order to possess a comprehensive understanding of how EDCs contribute to breast cancer risk. Our work clearly shows DA is an effective prediction tool to underpin such experiments. The observed additive effect confirms previous suggestions that EDCs, whilst individually may not be able to elicit measurable effects at concentrations observed in human tissues, can interact and add to the internal oestrogenic load, potentially increasing breast cancer risk (Ibarluzea Jm *et al.*, 2004; Kortenkamp, 2006; Pastor-Barriuso *et al.*, 2016).

In addition, we wanted to establish whether similar effects could be seen at the gene level, to investigate the mechanisms that may be underpinning phenotypic alterations. Although work presented here did not confirm the presence of a mixture effect on gene expression, the possibility of this occurring cannot be ruled out and is most likely gene specific. Our conclusions are also limited by testing a tissue specific mixture ratio, rather than an equi-effective mixture, meaning that some compound effect may be masked. Moreover, previous work has identified sub-populations of cells in 3D cultures with distinct gene expression profiles, which could be having an impact on the results presented in this chapter. Overall, despite the lack of mixture effects observed at gene level, the observation of combination effects on acini area warrant further investigation into the ability of chemical mixtures to contribute to breast cancer risk.

Unfortunately, results shown here highlight considerable variability in gene expression profiles, especially at higher concentrations, and it was not possible to adequately assess mixture effects at gene level. Originally it was anticipated that EDC mixtures would be assessed throughout this thesis, however in light of the findings presented in this chapter, including the significant variation, lack of clear dose response curves and the inability to separate acini populations, it was decided that further mixture experiments would not be feasible within the scope of this PhD. Accordingly, the experiments following this will be undertaken using single chemical exposures to allow a more in-depth understanding of how the genome and epigenome respond to EDC exposures. In this case, dose response curves are not necessarily required and investigating

changes to the acini as a whole can still provide useful insights into the mechanisms that underpin the association between EDC exposures and breast cancer risk.

Chapter Four: Investigating the impact of bisphenol A and propylparaben on three-dimensional co-cultures of MCF-12A cells

4 Investigating the impact of bisphenol A and propylparaben on three-dimensional co-cultures of MCF-12A cells

4.1. Introduction

In Chapter 3 we demonstrated the impact of EDCs in a 3D model that allowed for the culture of acini-like structures, which is more representative of the human breast than classic 2D cultures. However it did not account for stromal-epithelial interactions, that are known to play a significant role in tumorigenesis (McCuaig *et al.*, 2017). To fully understand how the breast responds to EDC exposures, it is important that these stromal interactions are represented in test systems. To date, studies have largely ignored the fact that epithelial cells are not isolated within the breast, potentially leaving a significant gap in our knowledge of how the EDC exposure contributes towards breast cancer risk. Studies have expressed the need for stromal representation in 3D cultures models and have shown they can be hormone responsive and more comparable to the human breast (Wang and Kaplan, 2012). Despite this, the stroma is rarely represented in *in vitro* assays, which may lead to inaccurate conclusions when quantifying the contribution EDCs have on breast cancer risk.

4.1.1. The role of the stroma within mammary carcinogenesis

The mammary gland is composed of various cell types that make up complex interaction networks. Such interactions are essential for the normal development of the breast, as well as playing key roles within tumorigenesis (McCuaig *et al.*, 2017). The stroma refers to the area surrounding the functional cells within tissues (Arendt *et al.*, 2010). In the mammary gland, the stroma consists of cellular components including fibroblasts, immune cells, endothelial cells, adipocytes, bone marrow-derived cells and the extracellular matrix. This accounts for approximately 80% of the tissue volume within the normal breast (Shekhar, Pauley and Heppner, 2003; Rønnov-Jessen and Bissell, 2009). Under normal conditions, the stroma acts as an essential barrier to epithelial transformation and is involved in maintaining cell polarity and

growth regulation (Quail and Joyce, 2013). DeCosse *et al.,* (1975) first showed the stroma had a role in mammary tumorigenesis over four decades ago, by showing that mammary mesenchyme tissues were able to induce cytodifferentiation in murine mammary tumour cells. Authors proposed the interaction between cell types resulted in a change in gene expression and that this was sufficient to cause a compaction of the tumour mass. Although research predominantly continues to focus on the epithelial cells, it is now recognised that the stroma has a central function in tumour initiation, progression and metastasis (Noël *et al.*, 1998; Saad *et al.*, 2000; Mao *et al.*, 2013). Thus, within this chapter we begin to capture these interactions by including two additional cell types, fibroblasts and endothelial cells, in the culture assay.

4.1.1.1. Fibroblasts

Often identified by their spindle-shaped morphology, fibroblasts form loose connective tissue that surrounds epithelial cells. The primary function of fibroblasts in the breast is to provide support to epithelial cell structures. Fibroblasts produce a scaffold of ECM proteins including collagen, fibronectin, collagen type I, II and IV and laminin (Yamazaki and Eyden, 1998). It has been documented that fibroblast growth factor (FGF) signalling plays an important role in the development and homeostasis of the breast. The FGF signalling pathway is comprised of 22 members and four single-pass membrane receptor tyrosine kinases, FGFR1-4 (Eswarakumar, Lax and Schlessinger, 2005). The importance of this signalling has been demonstrated in rodent models. In the absence of kinases, mice have been observed to have abnormal mammary development, including lack of mammary bud formation, increased apoptosis and loss of terminal end buds (Xu *et al.*, 1998; Dillon, Spencer-Dene and Dickson, 2004; Parsa *et al.*, 2008). It is apparent that breast cancer progression is strongly influenced by cross-talk between epithelial tumour cells and tumour associated fibroblasts. For instance, cancer-associated fibroblasts (CAFs), a distinct group of heterogenous cells that exhibit mesenchymal-like features, account for the largest component of tumour stroma (Kalluri and Zeisberg, 2006; Östman and Augsten, 2009; LeBleu and Kalluri, 2018). CAFs are known to produce tumour-promoting growth factors including transforming growth factor-β1 (TGF-β1) and HGF (hepatocyte growth factor;

Kalluri and Zeisberg, 2006). Moreover, CAFs have been shown to remove toxic metabolites and buffer cancer cell generated acidity, exerting a metabolic pro-tumour effect (Koukourakis *et al.*, 2006). There are a collection of theories regarding the origin of CAFs. Normal fibroblasts, stem cells, hematopoietic stem cells, adipocytes, epithelial and endothelial cells have all been suggested as potential predecessors of CAFs, however it is now generally accepted that CAFs are derived from several types of cells, resulting in a heterogenous population (Shiga *et al.,* 2015). Resident tissue fibroblasts are thought to be reprogrammed by tumour cells to become CAFs via miRNAs (Mitra *et al.*, 2012). Wen and colleagues reported that bone marrow derived mesenchymal stem cells have also been shown to promote the conversion of resident tissue fibroblasts into CAFs by altering the secreted TGF-β1 (Wen *et al.,* 2015). CAFs have also been proposed to be derived from epithelial cells through epithelial mesenchymal transition (EMT). EMT is a process where epithelial cells possessing tight junctions convert to mesenchymal cells with loose cell-cell contacts, obtaining mesenchymal properties (Greenburg and Hay, 1982). This process is believed to be regulated by TGF-β1. Watanabe-Takano *et al.,* (2015) demonstrated that the alveolar epithelial type 2 cell line, RLE-6TN, can convert into CAFs via epithelial mesenchymal transition through the Ras-ERK pathway, when treated with TGF-β1. Further studies have speculated that the conversion of normal fibroblasts to CAFs occurs in early initiation stages of breast carcinogenesis and that each can have a significant impact on tumour development (Qiao *et al.*, 2016).

Normal fibroblasts have also been implicated in the initiation of breast cancer. Over the past few decades, studies have shown the involvement of fibroblasts and suggested aberrant fibroblast behaviour is directly related to breast cancer initiation. For example, one study showed that fibroblasts overexpressing TGF-β and HGF was associated with increased incidents of breast tumours (Kuperwasser *et al.*, 2004). A recent study by Chen and colleagues (2017) found that the tumour suppressor, p85α (a regulatory subunit of PI3K), which is often found to be lost in human carcinomas, played a critical function in breast cancer initiation and metastasis. Interestingly, they found that loss of p85α expression in stromal fibroblasts regulated tumourigenesis and progression by interfering with stromal-epithelial crosstalk through the

paracrine Wnt/β-catenin pathway. Using MCF-10A and preneoplastic mammary EIII8 cell, Shekhar and colleagues (2001) emphasised the critical role of CAFs and normal fibroblasts in cancer initiation. The presence of normal fibroblasts inhibited the morphological conversion and uncontrolled proliferation of MCF-10A and EIII8 cells in the presence of E2 ($1x10^{-8}$ M). Indeed, normal fibroblasts were able to supress the oestrogen-induced growth of EIII8 cells, indicating a protective role of the stroma. In contrast, the presence of CAFs supported and maintained the oestrogen-responsiveness of EIII8s and promoted the morphological conversion to a neoplastic phenotype. Authors suggest that this observation may be a result of cell-cell interactions causing aberrant genetic functioning. They believed this may be particularly important in EIII8 cells that have already undergone genetic alterations akin with early epithelial neoplasms. Combined, these studies evidence the importance of both fibroblasts and CAFs in the initiation phases of breast cancer.

Although there is currently no literature examining whether the presence of normal fibroblasts impacts the epithelial response to EDC exposure, one study has shown EDCs can directly affect tumour associated fibroblasts. It was shown that $1x10^{-7}$ to $1x10^{-6}$ M BPA could induce the proliferation of CAFs via the GPER/EGFR/ERK transduction pathway, which could promote tumour growth (Pupo *et al.*, 2012). Overall, the role of fibroblasts with in the breast and in cancer initiation and progression means it is imperative to ensure they are represented in exposure studies.

4.1.1.2. Endothelial Cells

Endothelial cells have been reported to be associated with tumour growth and invasion (Cameron *et al.*, 2005; Serratì *et al.*, 2008; Franses *et al.*, 2011; Buchanan *et al.*, 2012; E. Lee *et al.*, 2014). The primary function of endothelial cells is to form a barrier between blood and other tissues, whilst being selectively permeable for certain molecules to pass. They are also involved in angiogenesis, a process of forming and maintain new blood vessels, which is vital for tumour development and survival. Tumours have a very restricted growth capacity without the presence of vascular support. As a result, the formation of blood vessels is an essential step,

required to sustain the delivery of nutrients to the tumour (Longatto Filho, Lopes and Schmitt, 2010). One of the most prominent molecules in regulating angiogenesis is the vascular endothelial growth factor (VEGF). Angiogenesis is promoted by VEGF secretion that binds to receptors VEGFR1 and VEGFR2 (Shibuya, 2011). The first step of angiogenesis is initiated by VEGF, which signals activated endothelial cells to migrate and proliferate in the direction of the signalling (Claesson-Welsh and Welsh, 2013). Whilst tumour cells are an important source of VEGF, the receptor is also expressed in endothelial cells themselves (Heloterä and Alitalo, 2007). Any increase in the production of VEGF can promote angiogenesis and tumour growth (Longatto Filho, Lopes and Schmitt, 2010).

Endothelial cells were originally believed to hold a passive role, yet literature now suggests they may directly influence epithelial cell behaviour. For example, research by Shekhar and colleagues suggests that endothelial and premalignant breast epithelial cell interaction is required to allow epithelial proliferation and induce branching ductal-alveolar morphogenesis (Shekhar, Werdell and Tait, 2000; Shekhar *et al.*, 2001). In addition, Ingthorsson *et al.,* (2010) demonstrated that the inclusion of breast endothelial cells (BRENCs) in 3D *in vitro* systems could induce proliferation of the MCF-10A and MDA-MB-231 cell lines. Authors suggested that the proliferative effect of endothelial cells was due to the delivery of soluble factors. Although Ingthorsson and colleagues could not identify the specific factors responsible, previous work saw endothelial cells enhanced migration of epithelial cells through STAT3/Akt/ERK signalling (Neiva *et al.*, 2009). These studies further support the notion that paracrine interactions between the stroma and epithelial cells are important for the maintenance of the breast.

In relation to EDC exposure, again, few studies have been conducted and very little is known about the ability of EDCs to directly impact endothelial cells. A recent study examined the effects of BPA on swine endothelial cell function (Basini *et al.*, 2017). Authors utilised a 3D *in vitro* angiogenesis assay to evaluate the effect of BPA on the production of VEGF. They saw that BPA could elicit a stimulatory effect on endothelial cells, causing them to increase VEGF production. Although this study did not specifically look at breast cancer, the observation suggests BPA could be associated with carcinogenesis by interacting with endothelial cell

regulation, potentially encouraging uncontrolled neovascularisation. It is not yet known whether other xenoestrogens can have similar effects. Nevertheless, due to their importance in vessel formation and apparent impact on epithelial cell behaviour, endothelial cells were included here to provide a more realistic representation of the mammary gland *in vitro*.

4.1.2. The epigenome and breast cancer initiation

Thus far, much of the literature surrounding the ability of EDCs to contribute to breast cancer development has been unconvincing, with many studies failing to demonstrate deleterious responses at concentrations relevant to human exposures. It has been suggested that this lack of clarity may be addressed by considering alternative endpoints, such as the epigenome, which authors believe may provide the link between EDC exposures and breast cancer risk (Feil and Fraga, 2012; Kim *et al.*, 2012; Knower *et al.*, 2014). Consequently, the work described in this chapter attempts to elucidate whether exposure to ubiquitous compounds BPA and propylparaben can be associated with epigenetic modifications that are indicative of early breast carcinogenesis.

As discussed in Chapter 1, there are three major epigenetic mechanisms that have the ability to interfere with gene expression and contribute to the development of breast cancer. These are DNA methylation, the modification of histones and miRNAs. Amongst these mechanisms, DNA methylation is by far the most extensively researched and understood modification in relation to breast cancer. Methylation is mediated by four DNA-modifying enzymes, referred to as DNA methyltransferases (DNMTs): DNMT1, DNMT2, DNMT3a and DNMTb. The activity of DNMTs is highly regulated, owing to a myriad of complex processes (Lyko, 2017). These processes can be grouped into four main categories, including molecular interactions, post-translational modifications (e.g. phosphorylation), alternative splicing, and gene loss and duplication. For instance, DNMT1 is the most abundant DNMT in human cells, is generally considered to be the main maintenance DNMT, and is highly expressed during the S-phase of the cell cycle (Mirza *et al.*, 2013). It has been shown that DNMT1 can bind to non-coding RNA arising from CCAAT enhancer binding protein α (*CEBPA)*, preventing the methylation of *CEBPA* (Di Ruscio *et al.*,

2013). DNMT3, which is responsible for establishing methylation profiles during embryonic development (Basse and Arock, 2015), has been reported to be modified by alterations to the gene copy number, enabling activity to be adapted to species-specific requirements. This is demonstrated by the increase and loss of DNMT3 throughout evolution (Lyko, 2017). Whilst, DNMT2 has been shown to be a tRNA methyltransferase (Jeltsch *et al.,* 2017).

In breast cancer patients, the expression of both DNMT1 and DNMT3 is enriched, evidencing their role in breast carcinogenesis (Girault *et al.*, 2003; Shin, Lee and Koo, 2016). Interestingly, the overexpression of DNMTs is similar between tumour types. DNMT3b is enriched in approximately 30% of breast cancer patients, with up to an 81.8 fold increase in expression (Girault *et al.*, 2003). DNMT1 and DNMT3a also display an increase in expression, however this is only reported in 3-5% of patients (Subramaniam *et al.*, 2014). Also, the fold increase of DNMT1 and DNMT3a is much lower, with a maximum of 16.6 and 14 fold increases respectively (Roll *et al.*, 2008), indicating that DNMT3b may play a more prominent function in breast tumourigenesis.

Literature has cited DNMT-inhibitors as an attractive treatment option, with the aim of lowering DNMT expression (Subramaniam *et al.*, 2014). Notably, some environmental compounds have also been shown to induce a down-regulation of DNMTs in breast cancer patients. Mirza and colleagues (2013), found that genistein, a phytoestrogen reported to have both pro- and anti-cancer effects (Bouker and Hilakivi-Clarke, 2000), reduced the expression of DNMT1, DNMT3a and DNMT3b in MCF-7 ($1.5x10^{-5}$ M genistein) and MDA-MB-231 cells ($1x10^{-5}$ M genistein).

DNA methylation is an essential process in normal development, aging and gene regulation throughout life (Nagy and Turecki, 2012). Whilst an array of studies have demonstrated the environment can influence an individual's methylation profile at all stages of development, there are a number of sensitive periods where these changes are more likely to occur, such as embryogenesis and early life. During these critical periods, factors including diet (total caloric intake), chemical exposure (toxins, EDCs and pharmaceuticals), social environment and stress have all been shown to influence DNA methylation (Faulk and Dolinoy, 2011). For example,

individuals abused in early life have been shown to have increased methylation levels at the NR3C1 glucocorticoid receptor promoter in the hippocampus (McGowan *et al.*, 2009). Also, individuals that were subject to famine in childhood show aberrant methylation at the insulin-like growth factor 2 locus (Heijmans *et al.*, 2008). The reasons behind why these timepoints are so susceptible to methylation modification are still being revealed. During embryogenesis, the epigenome is highly vulnerable to modification because of the high DNA synthesis rate. The complex methylation pattern needed for tissue development is also being established at this stage, meaning that any irregular methylation can have serious consequences in later life. For instance, exposure to BPA *in utero* has been associated with multiple development, metabolic and behavioural disorders (Bernal and Jirtle, 2010). These windows of vulnerability can also be modulated in later life stages, such as puberty and pregnancy (Walker, 2016).

Epigenetic dysregulation is believed to be one of the earliest events in cancer development, with the literature suggesting that, once modifications have occurred in premalignant tissues, they will continue to accumulate as the cancer progresses (Leu *et al.*, 2004; Dworkin, Huang and Toland, 2009). Global hypomethylation was one of the first epigenetic characteristics observed in cancer cells and is considered a hallmark of carcinogenesis (Gama-Sosa *et al.*, 1983; Narayan *et al.*, 1998). In mice possessing a hypomorphic DNMT1 allele (which reduces DNMT1 expression by 10%, resulting in genome-wide hypomethylation), global hypomethylation has been shown to trigger carcinogenesis by promoting genomic instability (Gaudet *et al.*, 2003). Hypermethylation and inactivation of transposable genetic elements is essential for the maintenance of regular cell function (Daskalos *et al.*, 2009). Global hypomethylation has been shown to reactivate silenced elements, resulting in genomic instability (Locke and Clark, 2012). In addition, hypomethylation at the pericentric regions can result in the mis-segregation of chromosomes during cell division and lead to aneuploidy (Narayan *et al.*, 1998; Prada *et al.*, 2012). This hypomethylation-induced genomic instability results in a higher frequency of genomic rearrangements, causing gene fusions and abnormal gene regulation (Locke and Clark, 2012).

Despite displaying an overall decrease in methylation, cancer cells contain hypermethylation of specific regions, including tumour suppressor genes (Baylin *et al.*, 2001). This hypermethylation, particularly when located in promoter regions, can lead to inappropriate gene silencing (Jones and Baylin, 2002). Amongst the genes commonly identified as hypermethylated and silenced within breast cancer are proapoptotic genes (e.g. homobox A5 and target of methylation-induced silencing 1), cell cycle regulators (*CCND2, p16, p15, RASSF1A*) and genes involved in DNA repair, such as *BRCA1* and O-6-methylguanine-DNA methyltransferase (*MGMT*; Esteller, 2002; Lustberg and Ramaswamy, 2011; Radpour *et al.,* 2011). Interestingly, the hypermethylation of *BRCA1* is associated with the same gene expression pattern as *BRCA1*-mutated breast cancers (Hedenfalk *et al.*, 2001). In addition, significant hypermethylation of genes, including the tumour suppressor, runt related transcription factor 3 (*RUNX3*) and *ESR1*, have all been identified in breast cancer, contributing to pro-tumorigenic processes (Widschwendter *et al.*, 2004; Gaudet *et al.*, 2009; Huang *et al.*, 2011; Chen, 2012; Stefansson and Esteller, 2013; Martínez-Galán *et al.*, 2014).

Regional hypomethylation of specific genes can also occur in breast cancer and contribute to tumourigenesis. *TET1* (tet methylcytosine dioxygenase 1) hypomethylation was observed to activate oncogenic signalling pathways in triple negative breast cancer, including the hyperactivation of PI3K/AKT (Good *et al.*, 2017). Earlier literature also reported the hypomethylation of oncogenes was common in breast cancer (Ehrlich, 2009). For example Parris *et al.,* (2014) found the oncogene *SQLE* (squalene epoxidase) was differentially hypomethylated and up-regulated in breast carcinoma samples. *SQLE* expression is associated with aggressive breast cancers and is currently being considered as a target for therapy (D. N. Brown *et al.*, 2016). Despite receiving less attention than regional hypermethylation, evidence indicates regional hypomethylation does play a role in breast cancer.

With epigenetic modification now recognised as a key mechanism contributing to carcinogenesis, studies have turned to the epigenome as a potential biomarker for detection of breast cancer (Lo and Sukumar, 2008; Dworkin, Huang and Toland, 2009; Parrella, 2010).

Research by Hocque and colleagues (2006) has focused on identifying methylation changes unique to tumours. They showed that the aberrant hypermethylation of tumour suppressor genes, adenomatous polyposis coli (*APC*), glutathione s-transferase P1 (*GSTP1*), *RASSF1A* and retinoic acid receptor β2 (*RARβ2*) in the serum of breast cancer patients could be detected, allowing for the identification of 33% of early-stage tumours (Hoque *et al.*, 2006). Authors went on to show aberrant methylation of *APC*, cadherin 1 and catenin β1 were directly implicated in the early development of breast cancer (Hoque *et al.*, 2009), demonstrating that alterations to the methylation profile could be utilised for the early detection of breast carcinogenesis. Other studies have also supported the ability to use epigenetic modifications as a biomarker for breast cancer. Takahashi *et al.,* (2005), suggested the epigenetic modification of large tumour suppressor kinase 1 (*LATS1*) and large tumour suppressor kinase 2 (*LATS2*) as a biomarker, due to hypermethylation being associated with large ER-negative tumours that were likely to metastasise. In addition, hypermethylation of Wnt agonist, secreted frizzled-related protein 1 (*SFRP1*) has been put forward as a biomarker for poor survival (Veeck *et al.*, 2006). Recently it was found the extent of *BRCA1* promotor methylation could be correlated with clinicopathological features in breast cancer, including tumour grade and metastasis, with researchers believing the gene to be a highly reliable biomarker (Sun *et al.*, 2018).

Although studies looking at changes to single genes have previously dominated the literature, due to technological advancements there is movement towards an epigenome-wide approach, allowing the identification of many genes that are involved in cancer initiation and progression. The adoption of epigenome-wide approaches has provided new insights into breast cancer diagnosis, prognosis and therapy (Davalos, Martinez-Cardus and Esteller, 2017), however few studies have utilised these tools to investigate the impact of EDCs on the epigenome. Thus, in the present study we use an epigenome-wide approach to explore whether exposures to xenoestrogens can elicit alterations to the methylation profile of cells and whether these changes could be associated with breast cancer risk.

4.1.3. Chapter scope

Within this chapter we built on work undertaken in Chapter 3, by improving the relevance of the 3D MCF-12A assay to the human breast. The main aim of the present study was to investigate the impact of BPA and propylparaben exposure in a more realistic assay, that accounts for the interaction between the epithelium and the surrounding stroma. We then used this model to further our understanding of the mechanism of action of these compounds and whether they can induce changes indicative of early breast carcinogenesis, by addressing the following questions:

1) Can exposure to BPA or propylparaben interfere with acini development in a representative 3D co-culture? Specifically, can we observe changes that occur in early stages of breast carcinogenesis like increases in acini area and cell number?
2) Using an epigenome-wide approach, can differentially methylated genes be observed in response to BPA and propylparaben exposure and are these genes associated with tumourigenesis?
3) Are any of the observed epigenetically modified genes also differentially expressed?
4) Finally, can any of the genes identified in Q2 be associated with functional processes relevant to breast carcinogenesis?

4.2. Methodology

4.2.1. Concentration selection and chemical handling

Solutions of BPA, propylparaben and 17β-oestradiol (E2) were of analytical grade (>95% purity) and all concentrations were prepared as $1x10^{-3}$ M stock in 100% HPLC-grade ethanol. Stocks and subsequent dilutions were stored at -20°C. The concentrations tested in Chapter 3 were based on the levels measured in human serum. This decision was based on the availability of a common tissue for each of the four compounds. However, both BPA and propylparaben have been measured in the human breast and therefore we were able to utilise tissue specific concentrations here.

For BPA, a total of three concentrations were tested, including a high concentration ($1x10^{-5}$ M) and a lower concentration ($1x10^{-7}$ M). A concentration comparable to that observed in the human breast (Fernandez *et al.*, 2007; Zimmers *et al.*, 2014) was utilised to elucidate the effects of the chemicals at concentrations realistically present in average human populations ($5x10^{-9}$ M). Two concentrations of propylparaben were also tested within this experiment, including a low concentration ($1x10^{-7}$ M) and a concentration recorded in the mammary gland ($1x10^{-8}$ M) based on previous research (Barr *et al.*, 2012). Finally, cells were exposed to $1x10^{-8}$ M of 17β-oestradiol (E2) as a comparison compound. E2 is known to elicit oestrogenic effects and acini alterations within 3D *in vitro* systems at this concentration, without inducing toxicity (Marchese and Silva, 2012). As in previous experiments, 100% EtOH was used for negative control samples. Solvents with chemical treatments were added to the co-culture system and cells were exposed to treatments for the entirety of their 14-day incubation period. Solvents did not exceed 0.5% of the co-culture medium to avoid ethanol toxicity.

4.2.2. Routine cell culture

Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (Dorset, UK). MCF-12A cells were obtained from the American Type Culture Collection and grown in monolayer within a 75 cm^2 canter-neck tissue culture flasks. Cells were provided with DMEM: F12 (Invitrogen, Paisley, UK), supplemented with 5% horse serum (Invitrogen), 0.02% epidermal growth factor, 0.01% cholera toxin, 0.1% insulin, 0.05% hydrocortisone and 1% pen/strep. Medium was replaced every four days and cells kept in a humidified incubator at 37˚C and 5% CO_2. Cells were passaged at 70% confluence with 0.25% trypsin-EDTA.

Primary human mammary fibroblasts (HMFs) were obtained from ScienCell (Buckingham, UK) and maintained in accordance to supplier's instructions in 75 cm^2 canter-neck tissue culture flasks pre-coated with 0.15% poly-l-lysine in PBS. Coating was removed and replaced with Fibroblast Medium supplemented with 2% FBS, 1% Fibroblast Growth Supplement and 1% pen/strep solution (ScienCell). HMF cells were seeded at $5x10^3$ cells/cm^2. Cells were kept in a

humidified incubator at 37˚C and 5% CO_2. Medium was replaced every three days up to 70% confluence and then every other day up to 90% confluence where they were passaged with 0.25% trypsin-EDTA.

Primary human endothelial cells (HMMECs) were sourced from ScienCell and cultured in 75 cm^2 canter-neck tissue culture flasks pre-coated with 3% fibronectin in PBS. Cultures were maintained in Endothelial Cell Medium, supplemented with 5% FBS, 1% Endothelial Growth Supplement and 1% pen/strep solution (ScienCell). HMMECs were seeded at a density of $5x10^3$ cells/cm^2 and fresh medium was added every three days up to 70% confluence and every other day to 90% confluence, where cells were passaged using 0.25% trypsin-EDTA. HMMECs were maintained in a humidified incubator at 37˚C and 5% CO_2. All cell types were passaged at least once in monolayer after resurrection to ensure optimal cell viability before being utilised within the 3D cell culture system.

4.2.3. Three-dimensional co-cultures

In the present study it was decided that collagen gels would be used, in place of Matrigel, for 3D cultures. This decision was based on several factors, however was mainly due to Matrigel batch variation, which was having a significant impact on acini growth. For instance, despite containing comparable protein and endotoxin levels, the use of some lots led to disorganised acini structures in control samples that varied considerably in size and lumen formation (data not shown). Due to a shortage of high quality Matrigel batches, along with the significant cost associated with them, Rat Tail Collagen I was used instead. Experiments using so called ‘floating collagen gels’ have been published, evidencing their reliability as a scaffold to grow 3D breast epithelial cultures that resemble TDLUs (Carter *et al.*, 2017; Linnemann *et al.*, 2017).

Here, MCF-12A cells were trypsinised at optimal confluence (70%) and centrifuged at 1000 rpm for five minutes. Supernatant was removed and the cell pellet was resuspended in 3 ml DMEM: F12. Cells were counted and the volume of medium was adjusted to achieve a final concentration of $2x10^5$ cells/ml. The cell suspension was combined at a 1:1 ratio with 4 mg/ml Rat Tail Collagen

I (Scientific Laboratory Supplies, Nottingham, UK), stabilised with 1 M NaOH and 1 M HEPES, achieving a final collagen concentration of 2 mg/ml. Following this, 1.5 ml of collagen/cell suspension solution was added into a tissue culture insert (0.4 µm pore Ø, $2x10^6$ pore/cm^2 density; Sarstedt, Leicester, UK), placed in a 6-well plate and allowed to set at 37˚C and 5% CO_2 for 1 hour.

Next, $5x10^4$ HMMEC and $3.5x10^4$ HMF cells (comparable ratios to Li and Lu, 2011; Buchanan *et al.*, 2012), were combined in 2 ml co-culture medium (MCF-12A growth medium with an additional 5% FBS). Next, 2 ml of the HMF/HMMEC cell solution was added to the 6-well plate. An additional 1 ml co-culture medium was added to the tissue culture insert to cover the collagen gel. Gels were then detached and allowed to 'float' (Figure 4.1; Burkel *et al.*, 2016; Carter *et al.,* 2017). Medium containing chemical treatment was replenished every three days for a total incubation period at 37˚C and 5% CO_2 of 14 days.

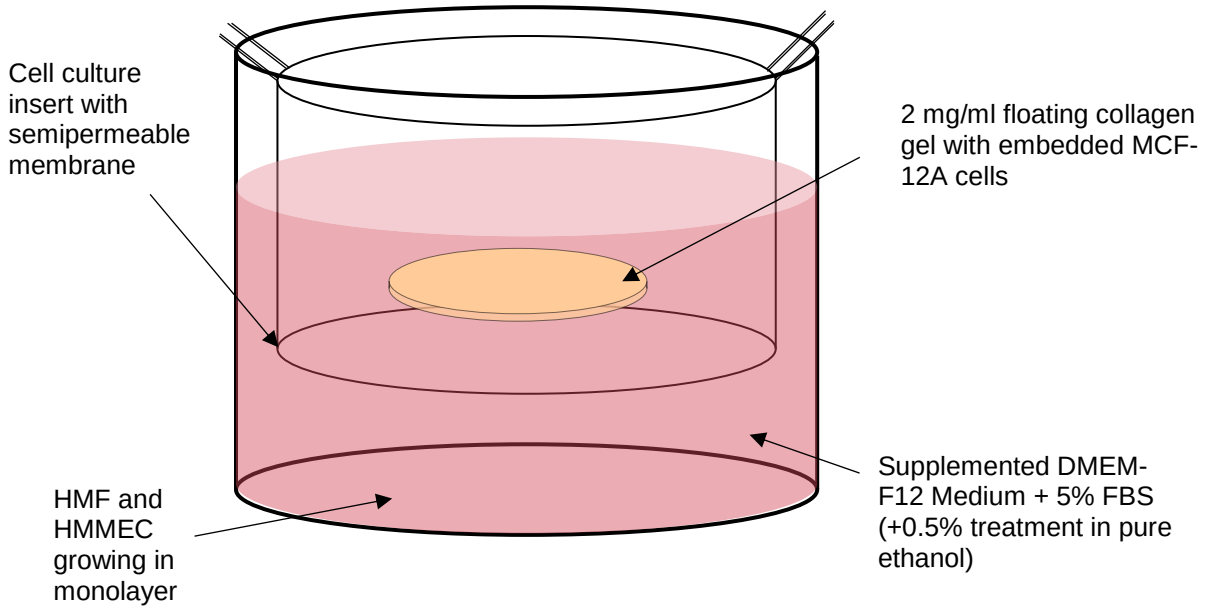

Figure 4.1. Three-dimensional co-culture system. MCF-12A cells are cultured within a floating collagen gel, separated from fibroblast and endothelial cells by a semipermeable tissue culture insert, allowing for paracrine interactions between cell types.

4.2.4. Morphological analysis

To visualise morphological changes that occured due to chemical exposures, collagen gels were partially digested with 1 mg/ml collagenase I in PBS for 10 minutes at 37˚C and 5% CO_2 (Carter *et al.,* 2017), prior to fixation with 4% PFA. Cells were then permeabilised with 0.5% Triton-X in PBS overnight at 4˚C, to avoid the disruption of protein tertiary structure. A block solution of 2% BSA and 5% FBS in PBS was placed on the gels for 1 hour, after which, primary antibodies for mouse anti-laminin V (ABcam, Bristol, UK) and rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Hertfordshire, UK) were diluted 1:200 in block solution and incubated for 48 hours at 4˚C. This was followed by a 2 hour incubation at room temperature with species-appropriate secondary antibodies (Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 555 goat anti-mouse; Molecular Probes, Invitrogen, Loughborough, UK) in block solution (1:200 dilution). Gels were incubated with 1 µg/ml DAPI before mounting with Prolong Antifade Reagent (Molecular probes) to label nuclei.

Acini structures were imaged using the Zeiss LSM 710 confocal microscope (Carl Zeiss Ltd, Hertfordshire, UK). Measurements of acini area and cell number were then taken. Cell number was calculated as the number of individual cells present at the most central point of the acini. Measurements were carried out using Image J (www.imagej.net) and statistical analysis was undertaken in Prism (version 7.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). Data were normalised to negative controls and a Kruskal-Wallis test, followed by Dunn's multiple comparison, was used to determine whether differences between treatments and negative controls was significant. Results presented are representative of three independent experiments, with a minimum of 10 acini randomly selected for each treatment.

4.2.5. Methylation analysis

4.2.5.1. Isolation of genomic DNA

Collagen gels were incubated with 1 mg/ml collagenase I for 2 hours at 37˚C and 5% CO_2 to isolate acini. The digested gel solution was then washed twice with PBS to ensure all collagen

was removed. DNA was isolated from epithelial cells using Quick-DNA Universal kit (Zymo Research Corp., CA, USA), per manufacturer's instructions. Eluted DNA was measured and assessed for purity by determining 260/280 and 260/230 ratios with the Nanodrop One (Thermo Scientific, Loughborough, UK).

4.2.5.2. Bisulfite conversion

Prior to sequencing, 0.1 µg DNA was bisulfite converted using EZ DNA Methylation kit (Zymo Research Corp., CA, USA). During this process, methylated DNA was treated with sodium bisulphite, which converted unmethylated cytosines to uracils (Figure 4.2), leaving methylated cytosines unaffected (Frommer *et al.*, 1992; Clark *et al.*, 1994). This conversion allowed for the differentiation between methylated and unmethylated regions in downstream analysis. Following conversion, samples were stored at -80°C until further use.

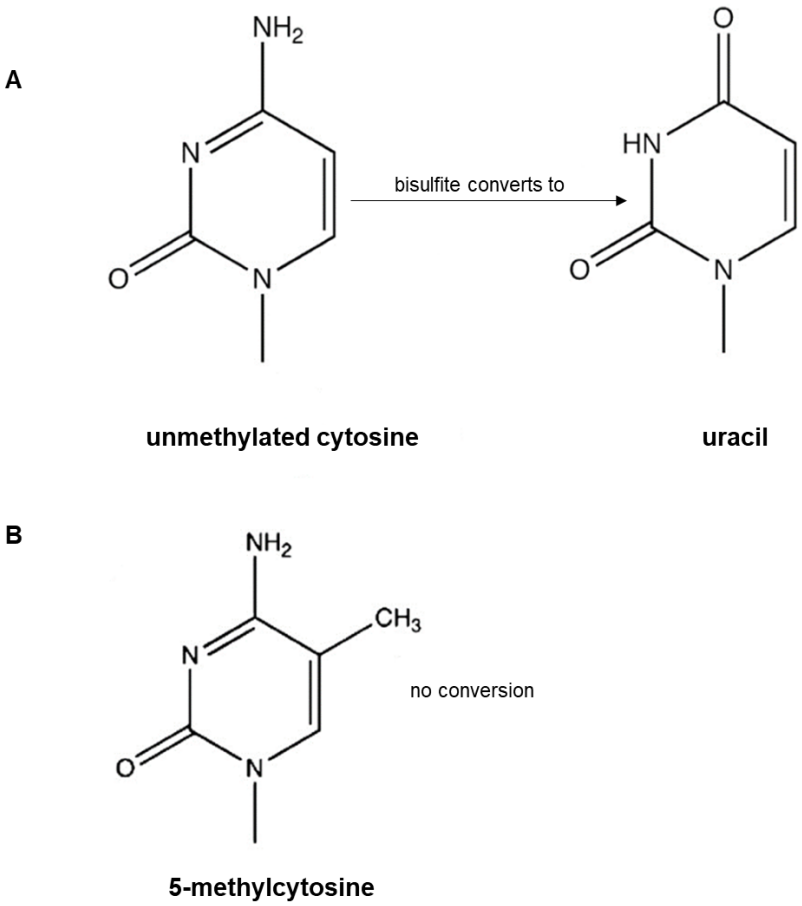

Figure 4.2. Bisulfite conversion treatment. (A) Unmethylated cytosines convert to uracil upon bisulfite treatment. (B) 5-methylcytosines (methylated cytosines) do no undergo conversion, remaining unaffected, allowing the detection of methylated sites in downstream analysis.

4.2.5.3. Methylation array

To detect whether methylation changes occurred in response to BPA or propylparaben exposure, a Infinium MethylationEPIC BeadChip microarray (Illumina, CA, USA) was used by UCL Genomics (Institute of Child Health, London). The recently developed Infinium MethylationEPIC BeadChip microarray, provides comprehensive genome-wide coverage and identifies over 850,000 CpG sites. Here, the assay was used to map regions that are differentially methylated/unmethylated in treated samples compared to controls.

Previous studies have confirmed that this method demonstrates high reproducibility and is a reliable tool for the analysis of DNA methylation profiles from the human genome (Moran, Arribas

and Esteller, 2016; Pidsley *et al.*, 2016). In addition, this assay allows for low sample input (as little as 250 ng DNA), which is a significant asset, due to the low cell numbers obtained from the 3D co-culture system. Moreover, the EPIC array covers a substantially higher amount of CpG sites in comparison to alternative techniques, including the Infinimum HumanMethylation450 BeadChip (Figure 4.3), providing and enhanced coverage.

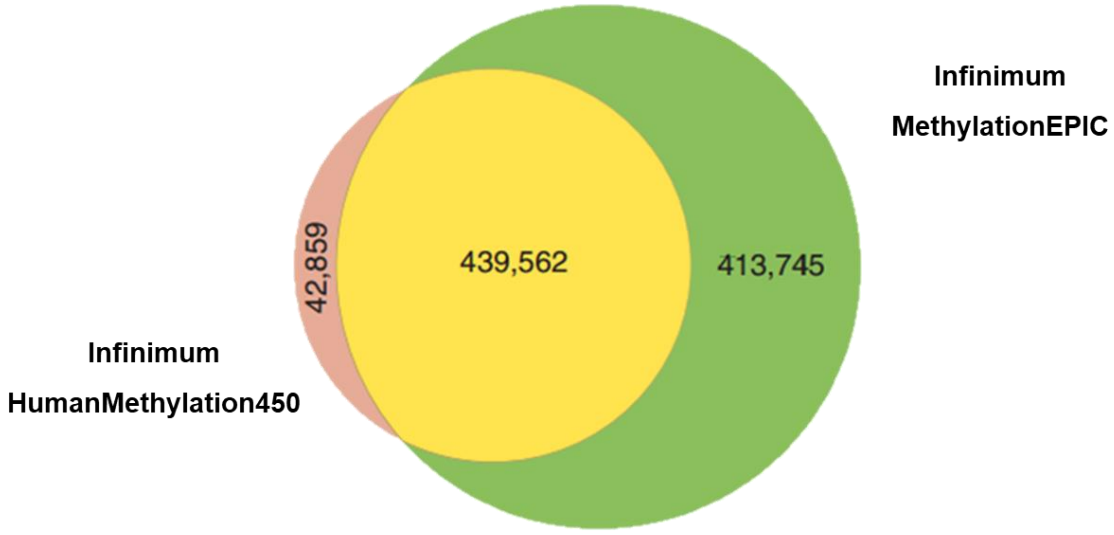

Figure 4.3. Comparison of 450k and 850k methylation arrays. Ven diagram identifying degree of CpG coverage and overlapping (yellow) between two frequently used methylation microarrays; Infinium HumanMethylation450 BeadChip (450K; red) and the MethylationEPIC BeadChip (850K), which is utilised in this chapter (green). Adapted from Moran *et al.,* (2016).

The EPIC array uses bead technology to achieve a highly multiplexed measurement of DNA methylation. Within the platform there are two unique array designs. Type I contains an individual bead for methylated and unmethylated sites (two probe sequences per CpG site; Figure 4.4A). Type II probes differ by only containing one bead per CpG site to measure methylation (Figure 4.4B). Regardless of the probe type, the array produces two measurements per CpG locus; the methylated intensity (M), and an unmethylated intensity (U). These values are used to estimate the proportion of methylation at each CpG site in downstream analysis.

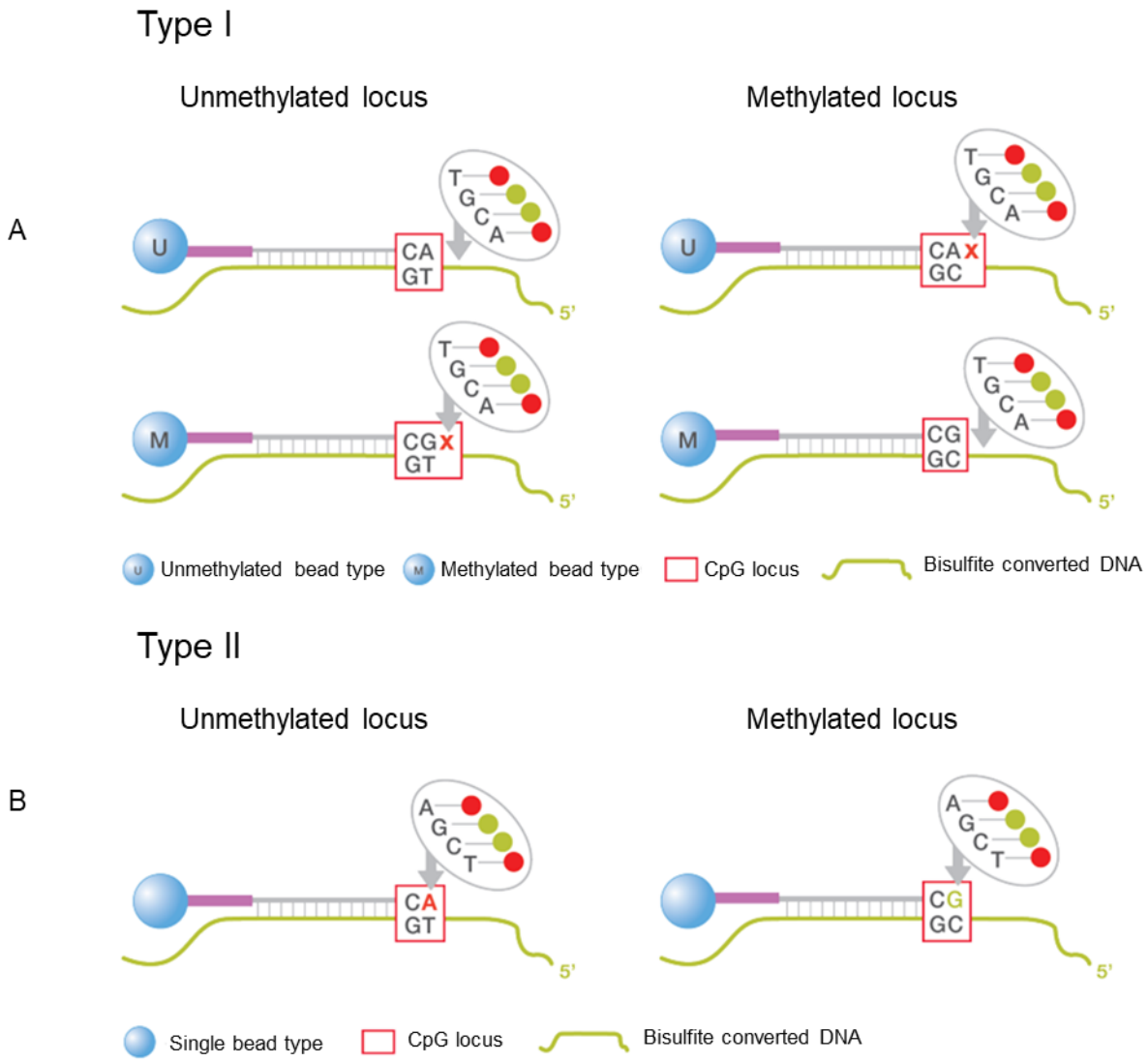

Figure 4.4. Infinium MethylationEPIC BeadChip type I and type II array designs. (A) Type I probe relies on two beads; one for methylated and one for unmethylated CpG locus. (B) Type II design contains a single bead (Illumina, 2017).

4.2.5.4. Bioinformatic analysis

Bioinformatic analysis was undertaken in R (Version 3.4.0.) using Minfi and Champ packages (Aryee *et al.,* 2014; Fortin *et al.,* 2016; Morris *et al.,* 2014) within Bioconductor. Data were mapped to the hg19 human genome reference sequence. An established processing pipeline was followed to include quality control, filtering and normalisation (Maksimovic *et al.,* 2016). Thus far, two methods have been proposed to quantify methylation status. The Beta-value, which ranges from 0 to 1, has been most commonly used to measure methylation and is the method

currently recommended by Illumina (Bibikova *et al.*, 2006; Bibikova and Fan, 2009). This method relies on the following equation:

$$Beta_i = \frac{\max(y_{i,methyl,}, 0)}{\max(y_{i,unmethyl,}, 0) + max(y_{i,methyl,}, 0) + \alpha} \quad \textbf{(Equation 4.2)}$$

where $y_{i,methyl}$ and $y_{i,unmethyl}$ are the intensities measured by the methylated and unmethylated probes, respectively. Illumina recommends the addition of a constant offset α (by default α = 100) to regularise Beta-values in the presence of low probe intensities. The second method, referred to as the M-value, is the log2 ratio of the intensities of the methylated probe verses the unmethylated probe (Irizarry *et al.*, 2008), shown in the following equation:

$$M_i = log_2 \left(\frac{\max(y_{i,methyl,}, 0) + \alpha}{\max(y_{i,unmethyl,}, 0) + \alpha} \right) \quad \textbf{(Equation 4.2)}$$

Here the offset value α is by default set to 1 (Du *et al.*, 2010), which prevents significant changes due to small intensity estimation errors, that can have a large impact on the M-value. Here, M-values close to 0 indicate a balance in intensities between the unmethylated and methylated probes, suggesting the CpG site is about 50% methylated. Values above 0 indicate more molecules are methylated than unmethylated, whilst a value below 0 suggests the opposite. M-values are commonly utilised for microarray analysis, however can be applied to methylation data. The Beta-value offers a direct biological interpretation, corresponding to an approximate level of methylation at a given CpG site. In contrast, the M-value has no biological meaning but has been cited as more statistically valid when used in more complex models. Based on the aims of this chapter it was decided that Beta-values were most suitable, due to its intuitive biological interpretation and widespread use in the literature. In the present study, all probe reads with p >0.01 were removed and Beta-values were extracted using the Illumina definition and

data were normalised using Beta Mixture Quantile dilation that corrected for probe design bias (Teschendorff *et al.*, 2013).

Next, confounding factors were identified by applying singular value decomposition (SVD; the factorisation of a matrix) and principle component analysis (PCA; a dimension-reduction tool). It was essential to adjust for confounders (so called 'batch effects') as they may affect between 80-90% of the variance observed, attributed to laboratory conditions, leading to significant bias in statistical significance and incorrect conclusions (Leek *et al.*, 2010). The application of SVD/PCA within pre-processing of methylation analysis to identify such confounders is now common practice (Zhuang, Widschwendter and Teschendorff, 2012; Teschendorff, Renard and Absil, 2014). SVD components were evaluated and removed using ComBat function. SVD/PCA was then rerun to confirm any batch effects were successfully removed.

Finally, differentially methylated positions (DMPs) were identified using the Delta Beta-value. The Delta Beta-value represents the mean DNA methylation difference between treated and control samples for each probe. For example, a Delta Beta-value of 0.1 would indicate a 10% difference between two samples, whilst a value of 0.5 would represent a 50% change. Here, we implemented the champ.DMP() function, which uses linear regression to calculate the p-value for differential methylation. EDC-treated samples were compared to EtOH-treated samples (negative control). DMPs were defined as probes with Delta Beta >0.1 and false discovery rate (FDR) adjusted p <0.05. This criteria was based on thresholds generally adhered to in the literature (Martino *et al.*, 2014; Nazarenko *et al.*, 2015; Maksimovic *et al.*, 2017). Results presented are representative of three independent experiments, with DNA from approximately 200 acini per treatment, from each experiment, being extracted.

4.2.6. Gene expression analysis

Total RNA was isolated from epithelial cells using the RNeasy Mini Kit (Qiagen, Manchester, UK), following manufacturer instructions and impurities were removed using DNase I treatment. Purity

of the final RNA sample was confirmed using 260/280 and 260/230 ratios with the Nanodrop One (Thermo Scientific, UK). Approximately 2.5 µg of RNA was reverse transcribed into cDNA and then stored at -80°C until use. Real-time PCR was performed on the CFX96 (Bio-Rad), using SYBR Green Master Mix (Primer Design, Southampton, UK). Primer pairs (Table 4.1) were purchased as highly purified oligos and *ACTB* was utilised as the reference gene. All primer pairs were optimised and used at a final concentration of $3x10^{-7}$ M. Relative gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Results are presented as log2 (fold change), with control levels set to 0. Statistical significance was determined using two-way ANOVA with Bonferroni correction.

Table 4.1. Primer sequences for real-time PCR amplifications. *ACTB* was utilised as the reference gene for fold change analysis.

Gene	Accession number	Primer Sequence (5'->3')		Prod. size
ACTB	NM_001101	Forward	TCAGCAAGCAGGAGTATG	97
		Reverse	GTCAAGAAAGGGTGTAACG	
FYN	NM_002037	Forward	GGAGAGGACCATGTGAGTGG	100
		Reverse	AGGCAGGACTGGTCTTTTTCC	
HCFC1	NM_005334	Forward	CATCCAACACATTTGTGGCCC	122
		Reverse	CAGGTCTGGCTTCACACCCA	
DCHS1	NM_003737	Forward	CTGAAACACGGTTGGTGCTG	102
		Reverse	GGCGATTGTCATTGGTGTCG	
CLMP	NM_024769	Forward	GGAGCCCTGCTGATTTTCCT	109
		Reverse	GAGCTTCAGCATCTTCTCGAAT	
NUAK1	NM_014840	Forward	TTACATCAGTGAGCGGCGAC	102
		Reverse	TGGACCACACCGTTCTTGTG	
FOXN3	NM_001085471	Forward	GCCCTTCTCCAAGATCCTGAC	80
		Reverse	AAACCTGCTTGTATCTCAGGGG	
ARHGAP10	NM_024605	Forward	ACTGAAACCCTGATTAAACC	168
		Reverse	ATCTGCCTCTTGTAAATGTG	
NAAA	NM_001042402	Forward	CCCAAGGAAGATGACCGGAG	104
		Reverse	TGGGTTCTTCTTGCTCACTGG	
USH1C	NM_020798	Forward	CAAGGAGGACTCGAACTCGG	132
		Reverse	ACATGGAGGTCCTTGATGGC	
CBFA2T2	NM_005093	Forward	AGCACAATCCTGACTGTACCTG	136
		Reverse	AGGACCAACTTTCCATTGCC	
FLI1	NM_002017	Forward	AGGCTGTAACCGGGTCAATG	91
		Reverse	CACCGACAGAGCCTCCTTAAT	
LIFR	NM_001127671	Forward	GCAGTGGCTGTCATTGTTGG	153

		Reverse	AAGAGCACTGCTTCCCTCAC	
JAM3	NM_032801	Forward	GTGACACGGAGAGACTCAGC	112
		Reverse	GGGTCACTGGCTTCACTTGC	
NRG1	NM_013959	Forward	TGTGCAAGTGCCCAAATGAG	127
		Reverse	GAAAGCAGCACCAACTGAGC	
PAK6	NM_001276717	Forward	GCAGACAGGGCTACCTGAGT	93
		Reverse	CCAGATAGGGTTTTGGGGTGG	
RUNX3	NM_001031680	Forward	GGATGGTACGGTGGTGACTG	147
		Reverse	GTGAAACTCTTCCCTCGCCC	
PRKX	NM_005044	Forward	AGGTGATGAGCATTCCCGAC	114
		Reverse	ACGTCCAGAACAGCCTGATG	
RPTOR	NM_020761	Forward	CCTACATGCCAGCTGAACACC	103
		Reverse	ATGAGGTTTCCCTGAAGGCAG	
CLASP2	NM_029633	Forward	GCTCACAAGAAAGTCTCAATCGTC	92
		Reverse	GCTGCCTCCCACAGATACTC	
ZFHX3	NM_006885	Forward	CTCCACGGACCCAGAAGAAG	154
		Reverse	GATGCGTTTGGAGGTTGCAG	
USP35	NM_020798	Forward	CAAGGAGGACTCGAACTCGG	132
		Reverse	ACATGGAGGTCCTTGATGGC	
BCL6	NM_001706	Forward	ACTGGGGTTCTTAGAAGTGGTG	102
		Reverse	GTCTTCACCAATGCCTTGCTTC	

4.2.7. Functional analysis

Functional annotation clustering analysis was performed using DAVID (the database for annotation, visualisation and integrated discovery) Bioinformatics Resources (version 6.8; https://david.ncifcrf.gov/). DAVID is an open access high-throughput data-mining environment that can analyse gene lists derived from arrays (Huang *et al.*, 2007; Sherman *et al.*, 2007). The integrated functional annotation clustering tool allowed for the investigation of how epigenetically altered genes were associated with biological functions in a network format.

Following an established protocol (Huang, Sherman and Lempicki, 2009), for each chemical exposure, two gene lists were defined: hypomethylated genes and hypermethylated genes. Each of these were analysed independently and a minimum of 100 genes were required to perform the clustering. To increase the number of genes to meet this requirement, DMPs were defined as probes with false discovery rate (FDR) adjusted p <0.05. This definition still met recommended statistical thresholds, however the magnitude of effect criteria was removed. For

each gene list, it was ensured that a minimum of 80% of genes were recognised by DAVID. The default medium strength classification stringency was utilised. Other stringencies were tested but were deemed either too tight or broad, reducing the result relevance to the study aims. Genes were then mapped to gene ontology (GO) terms divided between molecular function (molecular activities of gene products), cellular component (where the gene products are active) and biological process (pathways and larger processes made up of the activities of multiple gene products; Gene Ontology Consortium 2004). Further descriptions of these categories can be accessed via the Gene Ontology project (http://geneontology.org/). GO terms are well established and widely recognised within the biological community, being referred to as the 'common language for annotation' (Ashburner *et al.*, 2000).

Upon the application of the functional annotation clustering tool, two key statistics were generated including a p value for each individual term and an enrichment score for the cluster. The enrichment score ranks the overall enrichment of annotation term clusters. It is calculated as the geometric mean of the individual term's p value within the cluster. A high score indicates terms included within the cluster are playing a more important role in the given study. Huang (2009) recommends a statistical threshold of an enrichment score >1.3, but notes groups with slightly lower scores may also be of relevance and explored. For this reason, clusters with an enrichment score >1 were presented.

4.3. Results

4.3.1. Malformed acini occur in response to chemical exposures

To ensure the validity of the new system, acini response to chemical exposures were tested to determine whether they were comparable to more commonly used models, like the single-cell Matrigel overlays used in Chapter 3. In the co-culture collagen assay, epithelial cells behaved comparably to previously published work by our group in Matrigel (Marchese and Silva 2012) and observations reported in Chapter 3 of this thesis. Single cells began to form acini-like structures, with a spheroid shape and hollow lumen in control samples. Cell apoptosis could not

be measured, due to a lack of Caspase 3 (Figure 4.5). This was most likely due to the lack of apoptotic cells present at the 14 day timepoint. The lack of apoptotic markers at this timepoint has been reported previously, due to luminal clearing being completed at this stage (Marchese and Silva 2012).

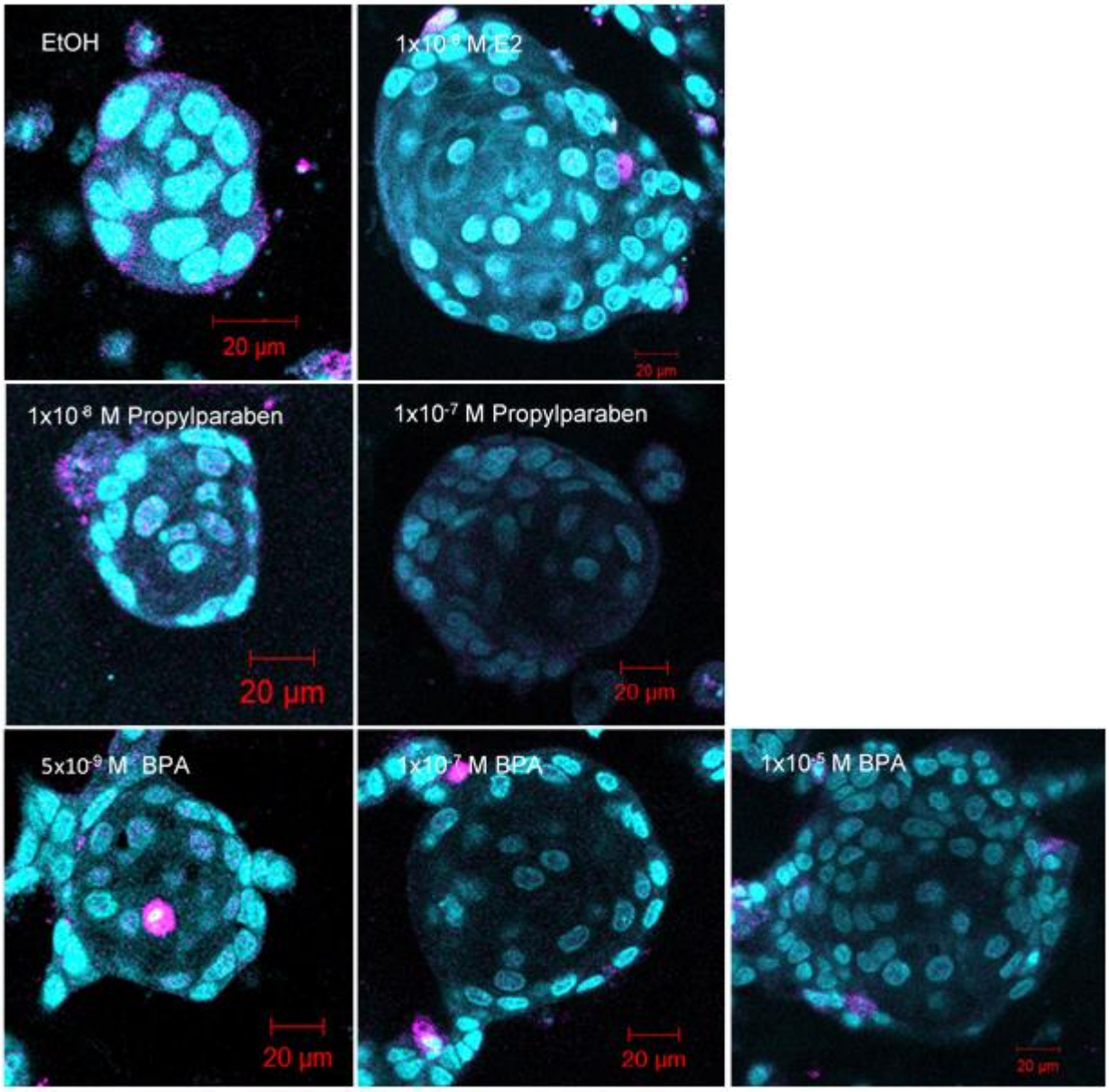

Figure 4.5. Confocal images of 3D cultures of mammary epithelial cells MCF-12A grown in co-culture with endothelial (HMMEC) and fibroblast (HMF) cells. Acini structures treated with antibodies against Integrin (magenta) and nuclei visualised with DAPI (cyan). No caspase-3 could be detected.

Cells exposed to 0.5% ethanol were, on average, 935.71±300.81 µm^2 in area and the central part of the acini comprised of 6.43±1.37 cells (Figure 4.6A). To establish whether these structures responded to chemical exposures, we tested 1x10^{-8} M E2, which resulted in a

significant increase in acini size (6338.71±334.89 μm^2, p <0.001). The cell number also significantly increased to 25.38±6.06 μm^2 after E2 exposure (p <0.001). The same experiment was then repeated with three concentrations of BPA ranging from 5x10^{-9} M to 1x10^{-5} M. All three concentrations elicited a significant increase in acini area. At the highest concentration, acini area was 6956.33±761.49 μm^2 (p <0.001), comparable to E2. A mildly significant increase in cell number was observed after 1x10^{-7} M exposure (18.13±4.06, p = 0.03), however a more substantial increase could be seen at 1x10^{-9} M (24.56±5.23, p <0.001; Figure 4.6B). Next, two concentrations of propylparaben were tested; 1x10^{-8} M and 1x10^{-7} M. Only the higher concentration caused a significant alteration to acini area (2841±238.18 μm^2, p = 0.002) and cell number (17.16±3.97, p = 0.03).

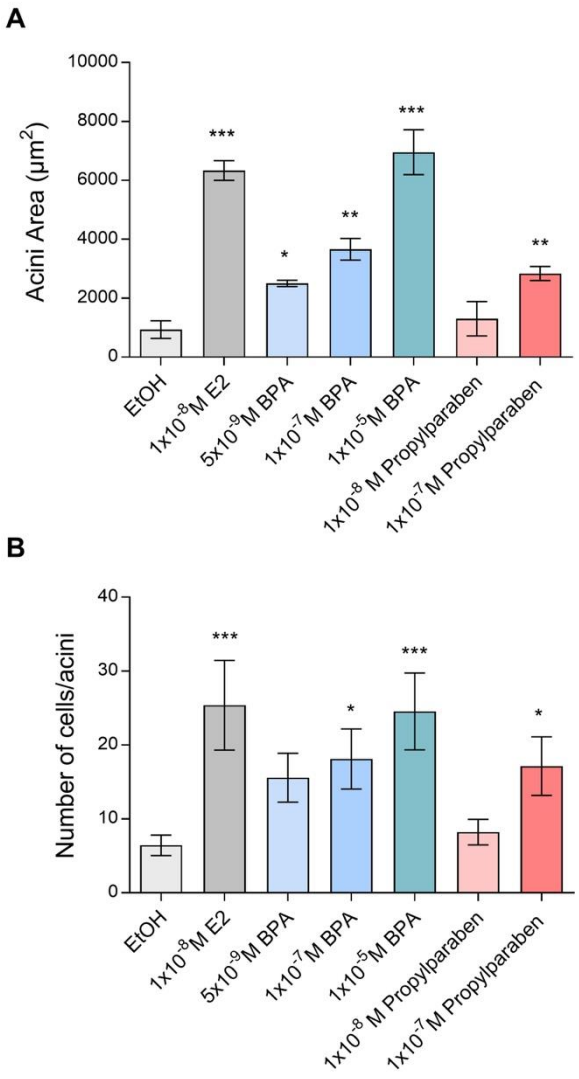

Figure 4.6. Quantification of acini area and cell number/acini in response to chemical exposure. Acini show significant increases in area (A) and cell number (B) after 14 days of EDC exposure. Significance is denoted by ***<0.001 ** 0.002 * 0.03 as determined by Kruskal-Wallis test, followed by Dunn's multiple comparison.

4.3.2. The methylation profile of mammary epithelial cells is significantly altered in response to chemical exposures

We then wanted to investigate whether concentrations that elicited clear morphological changes could also induce epigenetic changes that could be related to the observed phenotypic changes and cancer initiation. First, we performed initial quality control and removal of reads with p >0.01

leaving a total of 862,967 probes. Overall the quality of the samples and probes were high and no samples needed to be removed due to poor quality (Figure 4.7).

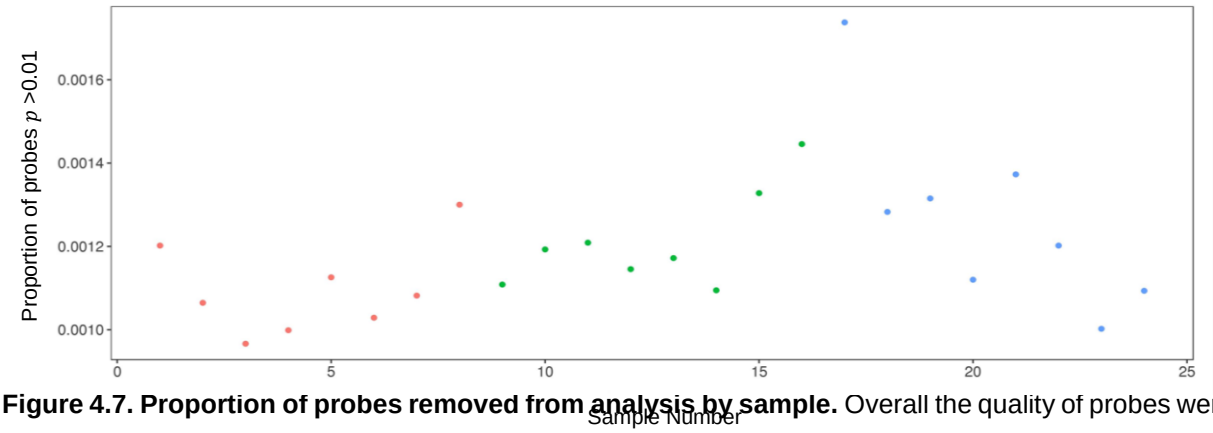

Figure 4.7. Proportion of probes removed from analysis by sample. Overall the quality of probes were high with <0.2% of probes being removed in any one sample. A total of 24 samples were analysed. Sample number was divided by independent experiment, depicted in red, green and blue.

The application of SVD/PCA determined whether any batch effects were present. The experiment number was identified as a significant confounding factor (p <0.001; Figure 4.8). After the application of ComBat the confounding effect was successfully removed (Figure 4.9).

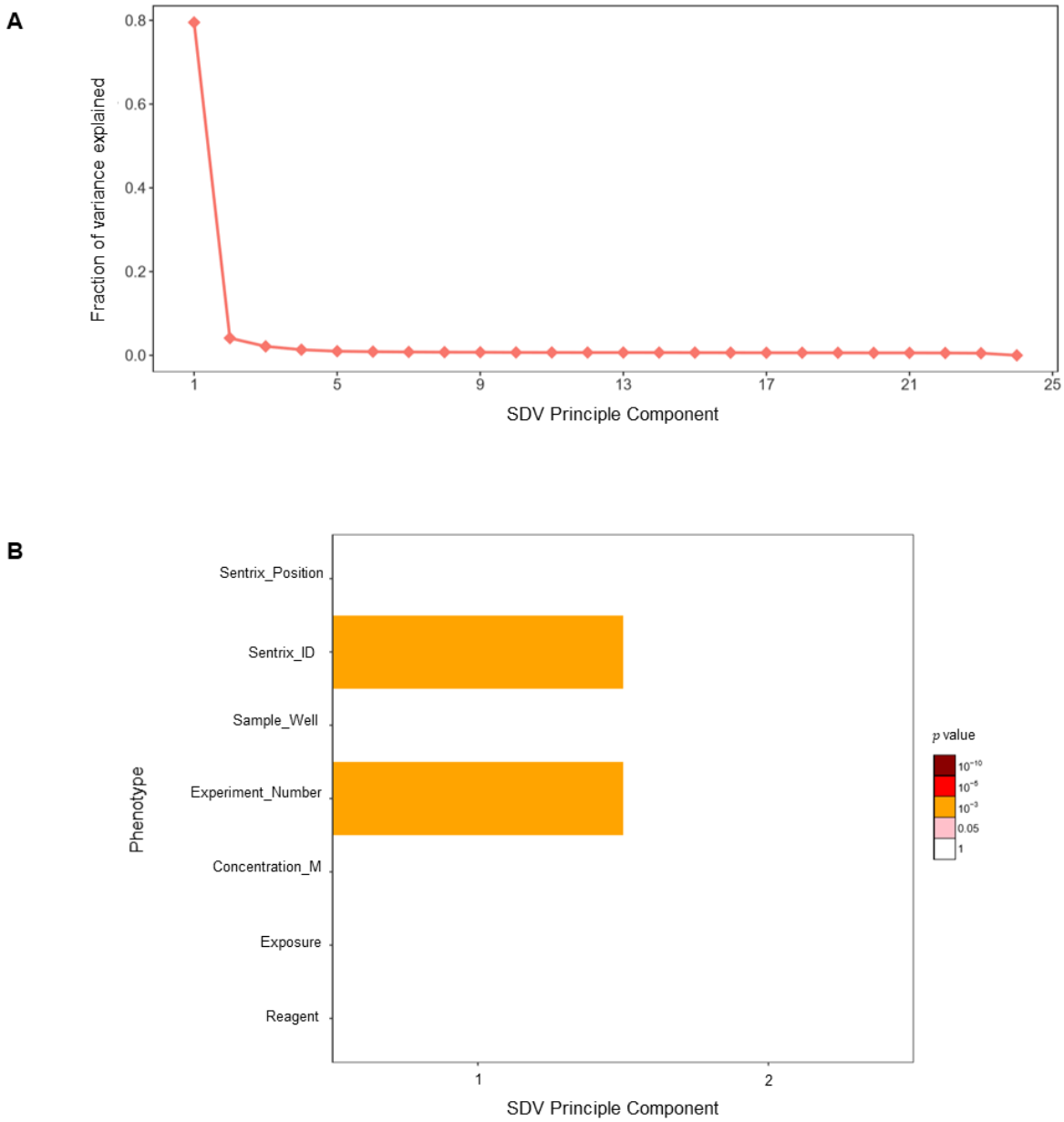

Figure 4.8. Significant confounder identified through singular value decomposition and principal component analysis. One principle component is found to explain a substantial fraction of the variance explained (A). Experiment number and sentrix ID are identified as significant variables responsible for confounding effects (B).

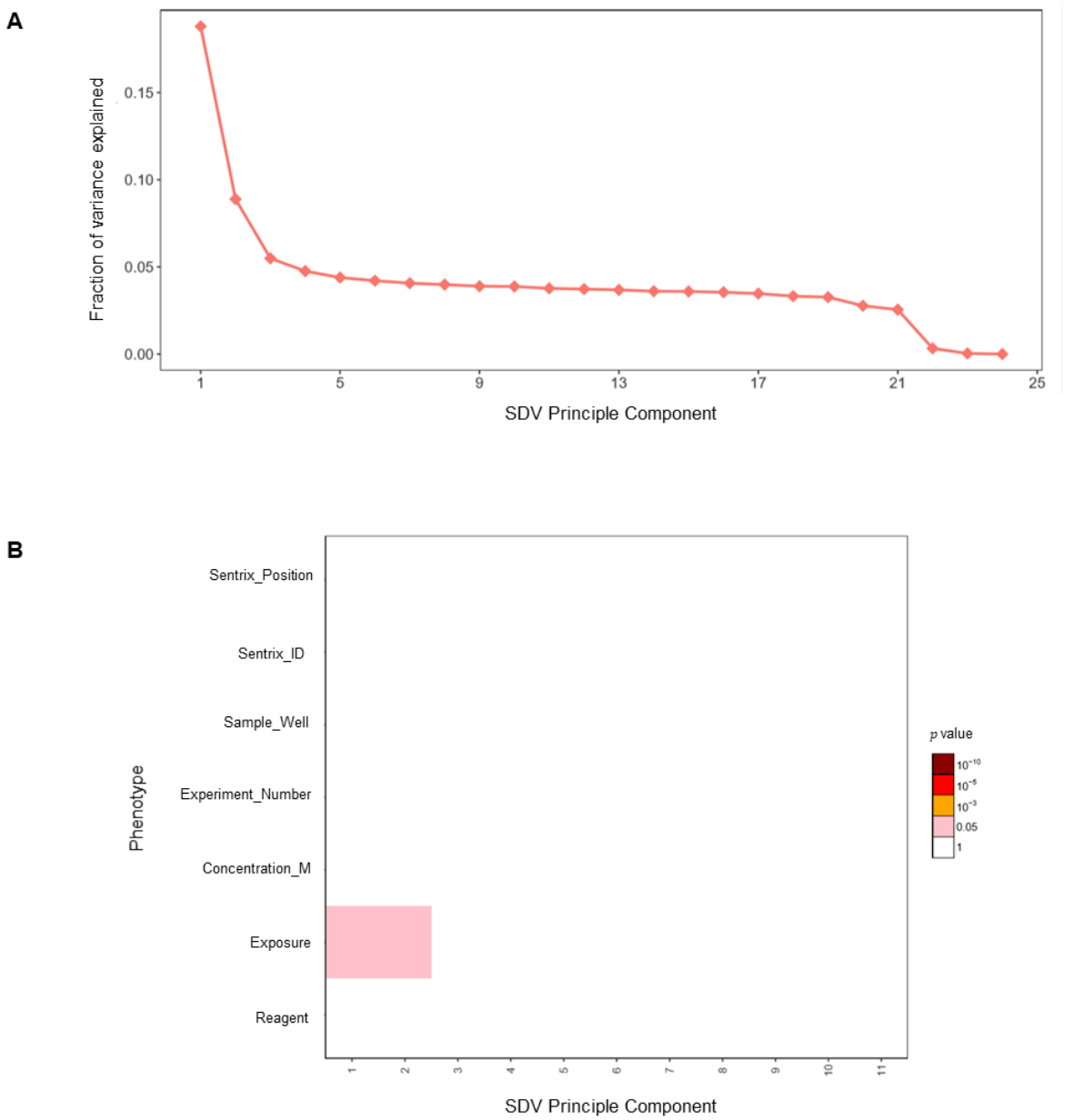

Figure 4.9. Successful removal of confounders achieved using ComBat. (A) No single principle component accounts for a significant fraction of the observed variation. (B) None of the phenotypes have a p value <0.05. The application of ComBat has successfully removed confounding factors that would have the ability to interfere with downstream analysis.

To investigate if any of the EDC exposures resulted in a global methylation shift, the global Beta-value distribution in each of the samples was examined. The sample medium for all tested samples was in the range of Beta-value 0.75, indicating that most molecules were approximately 75% methylated (Figure 4.10). From this we can see that no global shift in methylation profiles occurred in response to any of the compounds tested. We were not able to adjust for concentration within the statistical model, due to the limited statistical power.

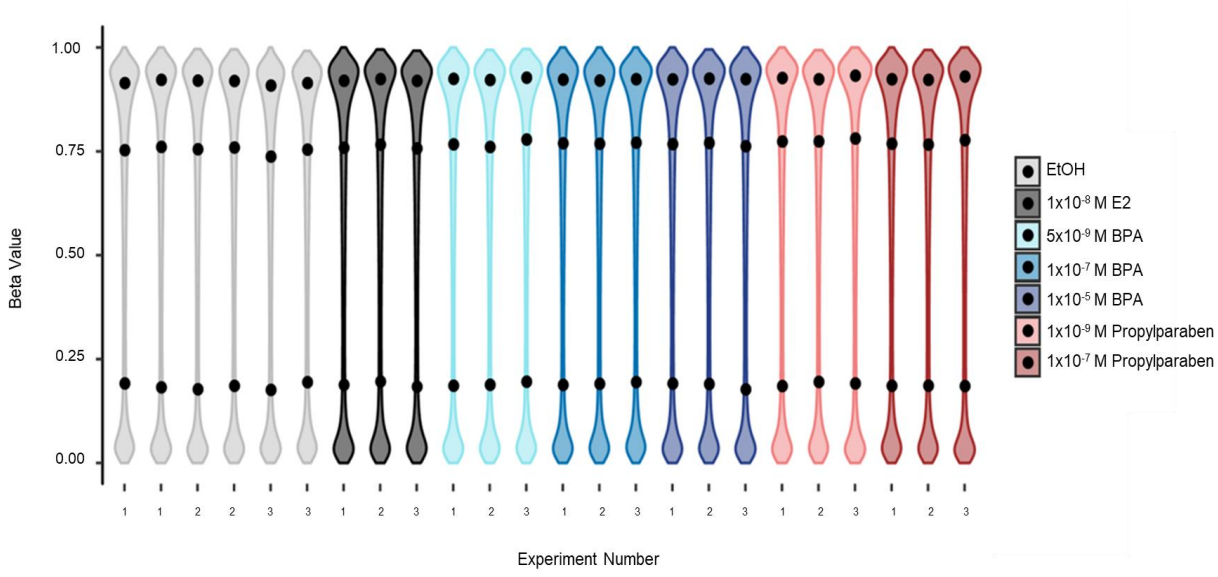

Figure 4.10. Global Beta-value distribution of MCF-12A cells exposed to EDCs. Plots separated by chemical treatment and experiment number (1-3) with violin plots representing the distribution of Beta-values within each sample. Dots depict the sample median, along with upper and lower quartile range.

Next, we wanted to see if regional differential methylation had occurred in response to EDC exposure. Due to only having data from three independent experiments, we had limited statistical power to perform this bioinformatic analysis. After further investigation, it was determined that no significant differences in methylation profiles could be observed between the different tested concentrations of BPA. The same was also observed with individual propylparaben concentrations. Based on this, it was decided that data resulting from the different chemical concentrations would be pooled within each compound to increase the statistical power of the following analysis. No DMPs could be identified after exposure to E2 at the threshold of Delta Beta-value >0.1 and p <0.05. Both BPA and propylparaben exposure elicited DMPs in the epigenome. Compared to 0.5% EtOH, BPA exposure induced a total of 52 DMPs. These DMPs could be mapped onto a total of 39 hypermethylated genes (Figure 4.11). There were no hypomethylated genes identified after BPA exposure. Propylparaben exposure instigated the highest number of DMPs, totalling at 144. Again, these were dominated by hypermethylations and could be mapped to 108 hypermethylated genes and a single hypomethylated gene (Figure 4.11). Out of the differentially methylated genes, 15 were common to both BPA and propylparaben.

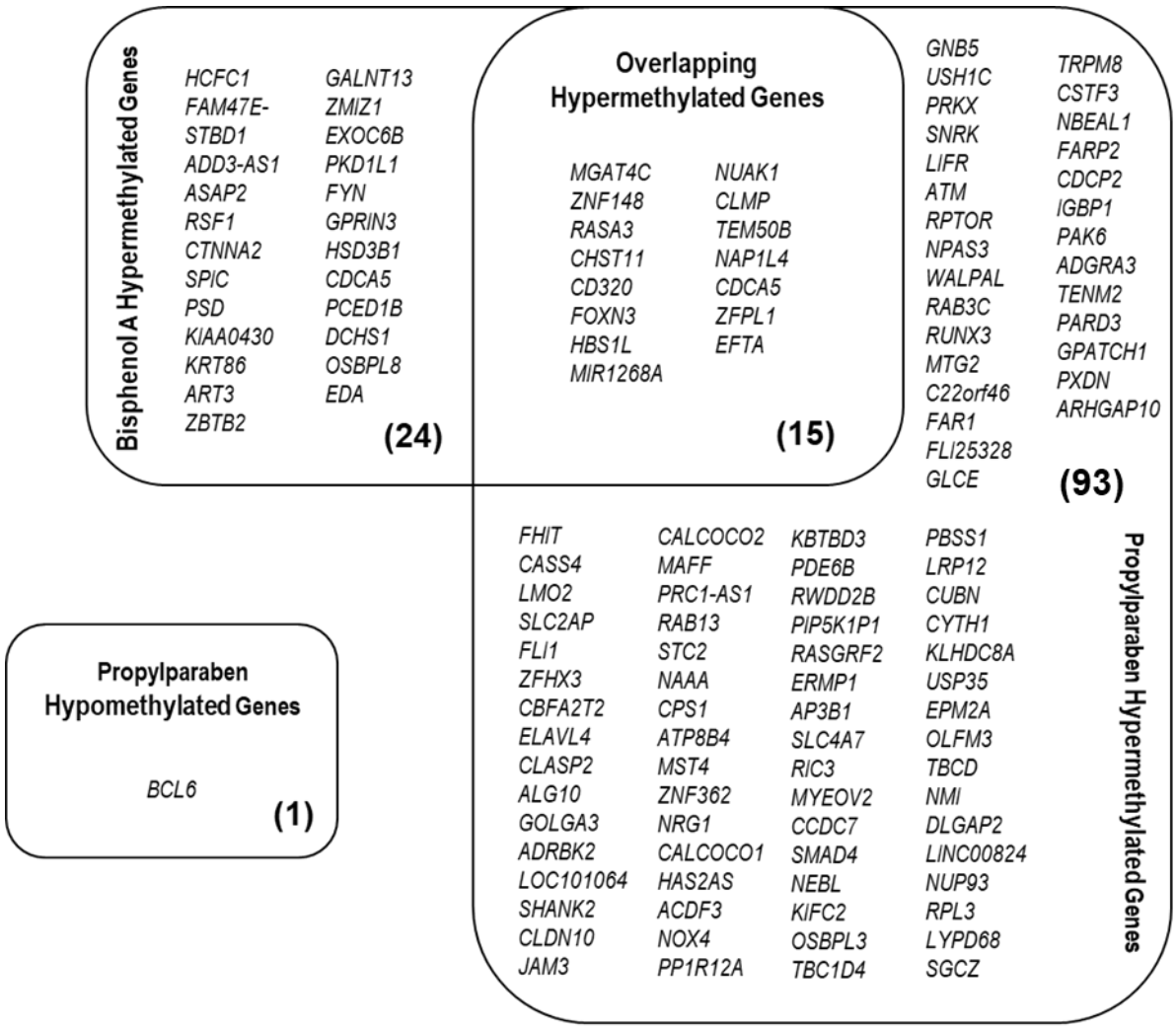

Figure 4.11. Genes significantly altered in methylation profile after exposure to bisphenol A or propylparaben. BPA exposure can be associated with 24 unique gene hypermethylations, whereas 93 genes displayed hypermethylation in the presence of propylparaben. An additional 15 gene hypermethylations were identified in both BPA and propylparaben exposures. Only one gene hypomethylation was deemed as significant and this was specific to propylparaben.

Overall, it could be seen that both EDCs elicited a measurable effect in genes associated with breast cancer (Figure 4.13). Common to both EDCs, the cell proliferation inhibitor *FOXN3* (forkhead box N4) was observed to be significantly hypermethylated in both BPA and propylparaben exposed samples (Delta Beta 0.11, p = 0.02 and Delta Beta -0.11, p = 0.03 respectively). A further regulator of cell proliferation, *NUAK1* (NUAK Family Kinase 1) was differentially hypermethylated, again in both BPA and propylparaben exposed cells (Delta Beta -0.13, p = 0.03 and Delta Beta -0.12, p = 0.04 respectively). Cell-cell adhesion molecule, CXADR

like membrane protein (*CLMP*) was also significantly hypermethylated in BPA and propylparaben exposed samples (Delta Beta -0.147, p = 0.02 and Delta Beta -0.149, p = 0.02 respectively).

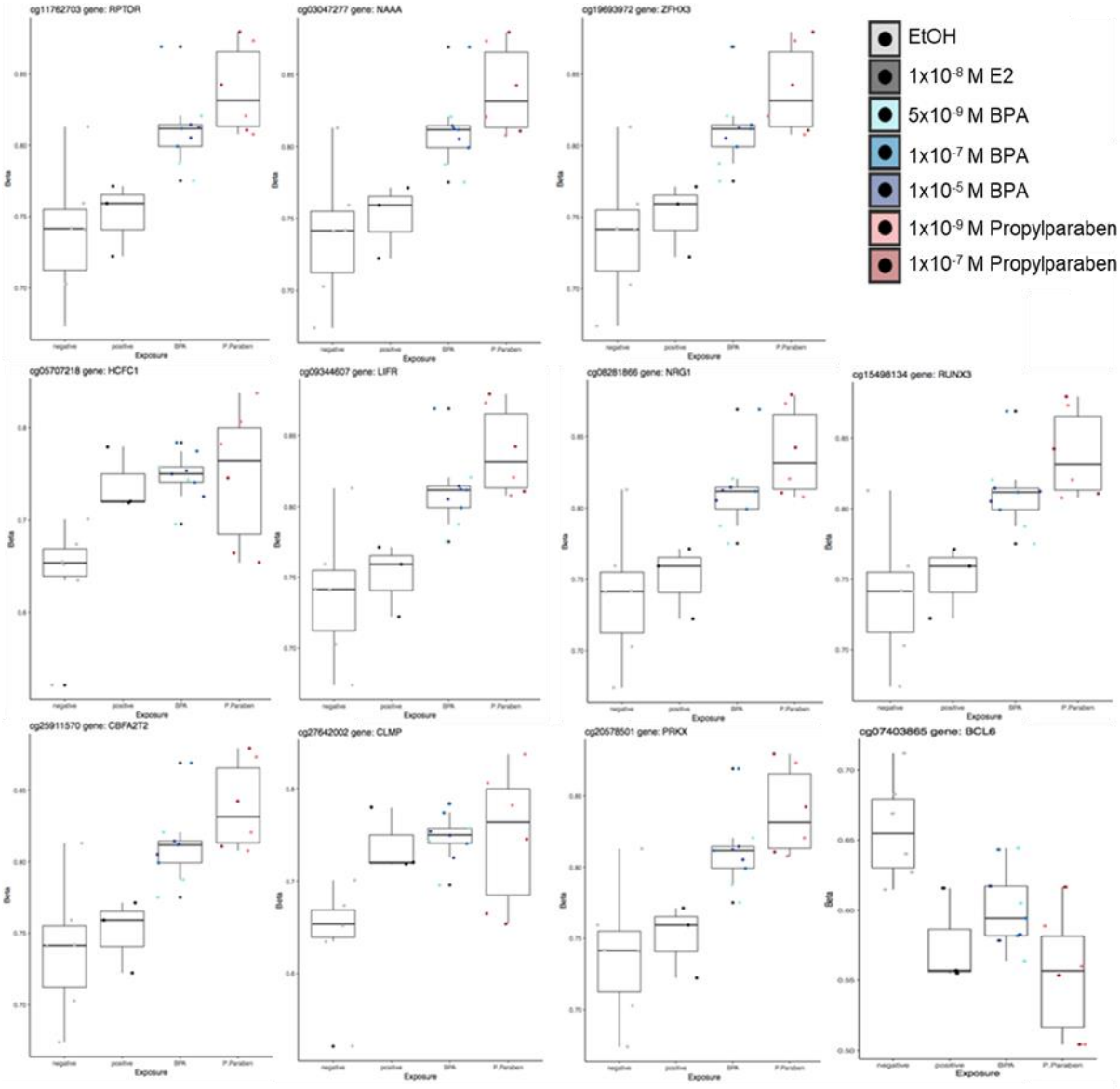

Figure 4.12. Significant alterations to methylation status observed in genes associated with breast cancer. Through the identification of differentially methylated positions (Delta Beta >0.1 and FDR adjusted p <0.05) numerous genes were identified as being significantly altered in methylation status in comparison to negative control samples and associated with breast cancer.

There were multiple genes of interest unique to BPA exposed cells. Dachsous1 (*DCHS1*) was significantly hypermethylated in BPA exposed cells (Delta Beta -0.12, p = 0.04), as was *HCFC1* (host cell factor C1; Delta Beta -0.11, p = 0.003). Interestingly, the proto-oncogene *FYN* was

also hypermethylated in BPA exposed cells (Delta Beta -0.13, p = 0.03). Unique to propylparaben, several tumour suppressor genes, including *RUNX3* (Delta Beta -0.11, p = 0.003), *LIFR* (LIF receptor alpha; Delta Beta -0.11, p = 0.002) and neuregulin 1 (*NRG1*; Delta Beta -0.13, p = 0.009) were hypermethylated. Included in propylparaben hypermethylated genes was *ARHGAP10* (Rho GTPase activating protein 10), which significantly increased in methylation (Delta Beta -0.12, p = 0.02). *RPTOR* (regulatory associated protein of MTOR complex 1) was also hypermethylated in response to propylparaben with a Delta Beta-value of -0.14 (p = 0.001). Propylparaben exposure resulted in the single significantly hypomethylated gene, the oncogene *BCL6* (B cell CLL/lymphoma 6; Delta Beta 0.11, p = 0.01).

4.3.3. Methylation profiles translate to gene expression changes in response to chemical exposure

To elucidate whether the changes seen in the epigenome could be correlated with a change in gene expression, real-time PCR was performed. A total of 22 genes were identified that were most significantly altered in methylation status and were also found to be associated with breast cancer after extensive literature searches (Figure 4.14; Table 4.2).

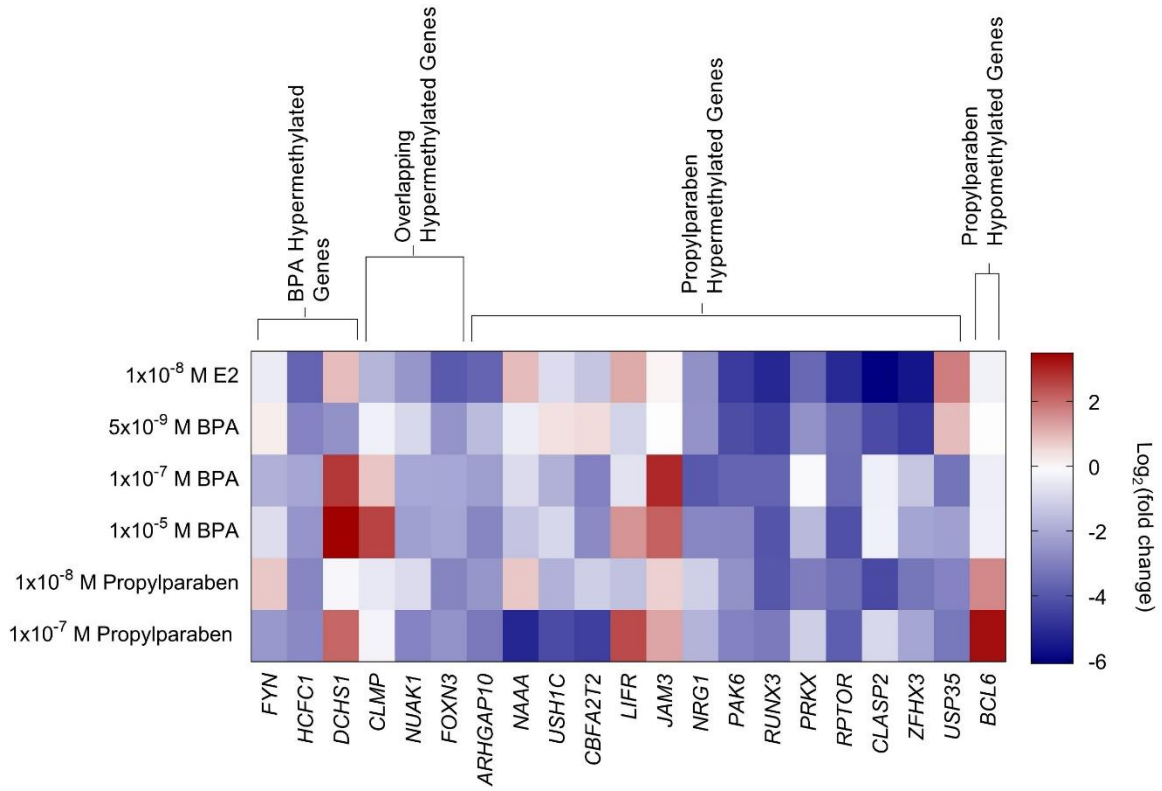

Figure 4.13. Change in gene expression observed in differentially methylated genes after EDC exposure. Summary of log2 (fold change) expression over negative controls in genes associated with breast carcinogenesis after exposure to E2, BPA or propylparaben. Intensity of cell colour indicates the level of change with red demonstrating an up-regulation and blue a down-regulation in gene expression. Fold change was calculated using $2^{-\Delta\Delta Ct}$. *ACTB* was utilised as the reference gene.

Table 4.2. Role of EDC-induced epigenetically modified genes in breast cancer.

Gene	Function
FYN	Proto-oncogene
HCFC1	Frequently hypermethylated in breast cancer
DCHS1	Part of Hippo pathway with deregulation associated with tumour development
CLMP	Role in cell-cell adhesion and suspected tumour suppressor
NUAK1	Promotes migration and invasion in breast cancer
FOXN3	Tumour suppressor
ARHGAP10	Suspected tumour suppressor
NAAA	Expression associated with aggressive tumours
USH1C	Encodes for scaffold protein
CBFA2T2	Tumour suppressor
LIFR	Tumour suppressor
JAM3	Involved in signalling cascades that promote endothelial angiogenesis
NRG1	Tumour suppressor
PAK6	Shown to inhibit oestrogen and androgen receptor activity
RUNX3	Tumour suppressor

PRKX	Regulates endothelial cell proliferation and migration. Overexpressed in triple negative breast cancers
RPTOR	Within mTOR pathway associated with breast cancer development
CLASP2	Involved in cellular movement and cytoskeletal organisation
ZFHX3	Tumour suppressor
USP35	Inhibits epithelial cell proliferation
BCL6	Expressed in breast cancer and prevents epithelial differentiation

Three hypermethylated genes unique to BPA were analysed (Figure 4.15). Neither *FYN* or *HCFC1* showed any significant change in gene expression at any of the tested BPA concentrations. *DCHS1* had a mildly significant increase in expression at 1x10^{-7} M (3.05±0.38 LogFC, p = 0.03) and 1x10^{-5} M (3.46±0.32 LogFC, p = 0.01) BPA concentrations.

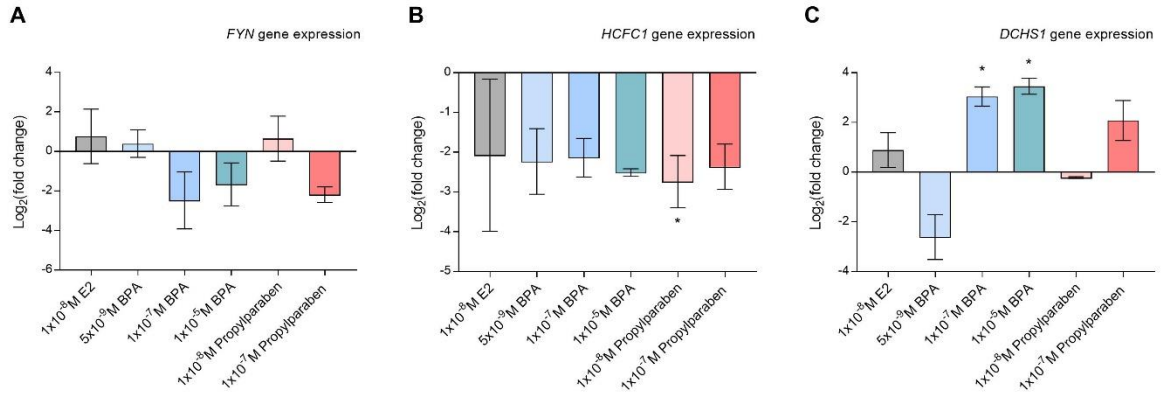

Figure 4.14. Change in gene expression seen in differentially methylated genes unique to BPA exposure. No changes were observed in *FYN* (A), however both *HCFC1* (B) and *DCHS1* (C) showed significant alterations in response to EDC exposures. Fold change was calculated against the negative control sample using $2^{-\Delta\Delta Ct}$. *ACTB* was utilised as the reference gene.

Of the genes tested common to BPA and propylparaben exposed cells, all showed a decreasing trend in gene expression after EDC exposure (Figure 4.16). None of the down-regulations were significant in *CLMP* or *FOXN3*. However, a mild significant decrease in gene expression was observed in *NUAK1*, after 1x10^{-5} M BPA (-3.25±1.22 LogFC, p = 0.02) and 1x10^{-7} M propylparaben exposure (-2.77±0.57 LogFC, p = 0.04).

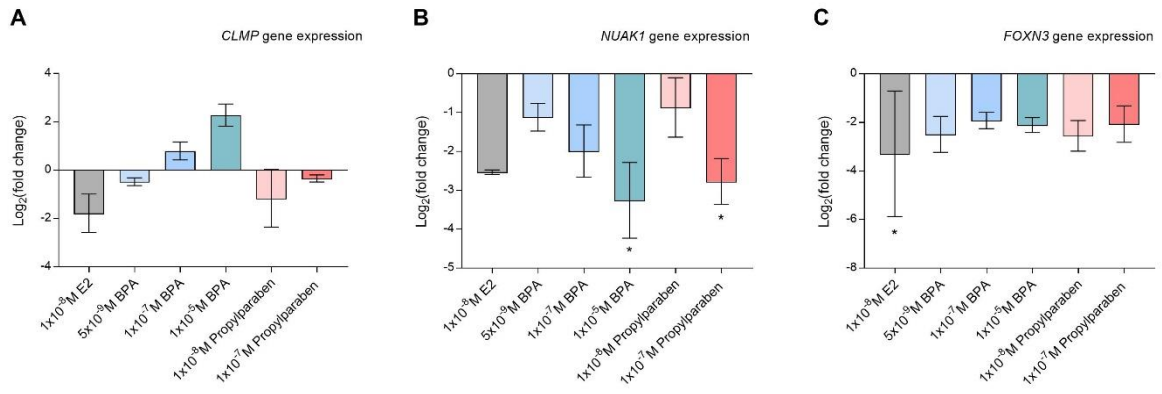

Figure 4.15. Change in gene expression seen in differentially methylated genes common to both BPA and propylparaben exposure. Depiction of log2 (fold change) in response to E2, BPA and propylparaben exposures in *CLMP* (A), *NUAK1* (B) and *FOXN3* (C). Fold change was calculated against the negative control sample using $2^{-\Delta\Delta Ct}$. *ACTB* was utilised as the reference gene.

Tested hypermethylated genes after propylparaben exposure showed a much more consistent trend in down-regulation. However, this decrease in gene expression was not limited to propylparaben exposure, with most genes also showing down-regulation after BPA and E2 exposure (Figure 4.17). *ARHGAP10* was down-regulated in both propylparaben concentrations ($1x10^{-8}$ M; -3.41±1.19 LogFC, p = 0.01, $1x10^{-7}$ M; -3.01±0.96 LogFC, p = 0.03). However, it was most significantly altered by E2 (-4.55±1.01 logFC, p <0.001). $1x10^{-5}$ M BPA was also able to decrease *ARHGAP10* expression by -2.91±0.72 LogFC (p = 0.03). *NAAA* (n-acylethanolamine acid amidase) was down-regulated by BPA ($1x10^{-5}$ M; -3.27±2.52 LogFC, p = 0.02) and propylparaben exposure ($1x10^{-7}$ M; -4.75±1.41 LogFC, p <0.001). *USH1C* (USH1 protein network component harmonin) was only significantly down-regulated in the highest concentration of propylparaben (-3.67±1.16 LogFC, p = 0.007). *NRG1* showed no change after propylparaben exposure, however it was significantly decreased in BPA exposed cells ($5x10^{-9}$ M; -2.89±0.63 LogFC, p = 0.03, $1x10^{-7}$ M; -3.98±0.96 LogFC, p = 0.009, $1x10^{-5}$ M; -3.49±1.29 LogFC, p = 0.01). A reduction in gene expression in *CLASP2* (cytoplasmic linker associated protein 2) and *ZFHX3* (zinc finger homeobox 3) was observed after exposure to $5x10^{-9}$ M BPA (-4.31±0.35 LogFC, p = 0.005; -3.92±1.51, p = 0.005 respectively) and $1x10^{-8}$ M propylparaben (-4.34±0.88 LogFC, p = 0.005; -2.69±1.52 LogFC, p = 0.05 respectively). *USP35* (ubiquitin

specific peptidase 35) showed significant decreases in gene expression after $1x10^{-7}$ M propylparaben (-3.16±0.87 LogFC, p = 0.02), as well as $1x10^{-7}$ M (-3.57±1.21 LogFC, p = 0.009) and $1x10^{-5}$ M (-3.17±1.52 LogFC, p = 0.02) BPA. *PAK6* (p21 activated kinase 6), *RUNX3* and *RPTOR* showed significant down-regulation after exposure to all compounds at all concentrations. No significant alterations were observed in *LIFR*, *JAM3* (junctional adhesion molecule 3) or *PRKX* (protein kinase, x-linked) expression. The single hypomethylated gene, *BCL6*, was also tested for changes to gene expression and an up-regulation was observed that was limited to propylparaben exposure. Only $1x10^{-7}$ M propylparaben was deemed significant (3.33±0.36 LogFC, p = 0.02).

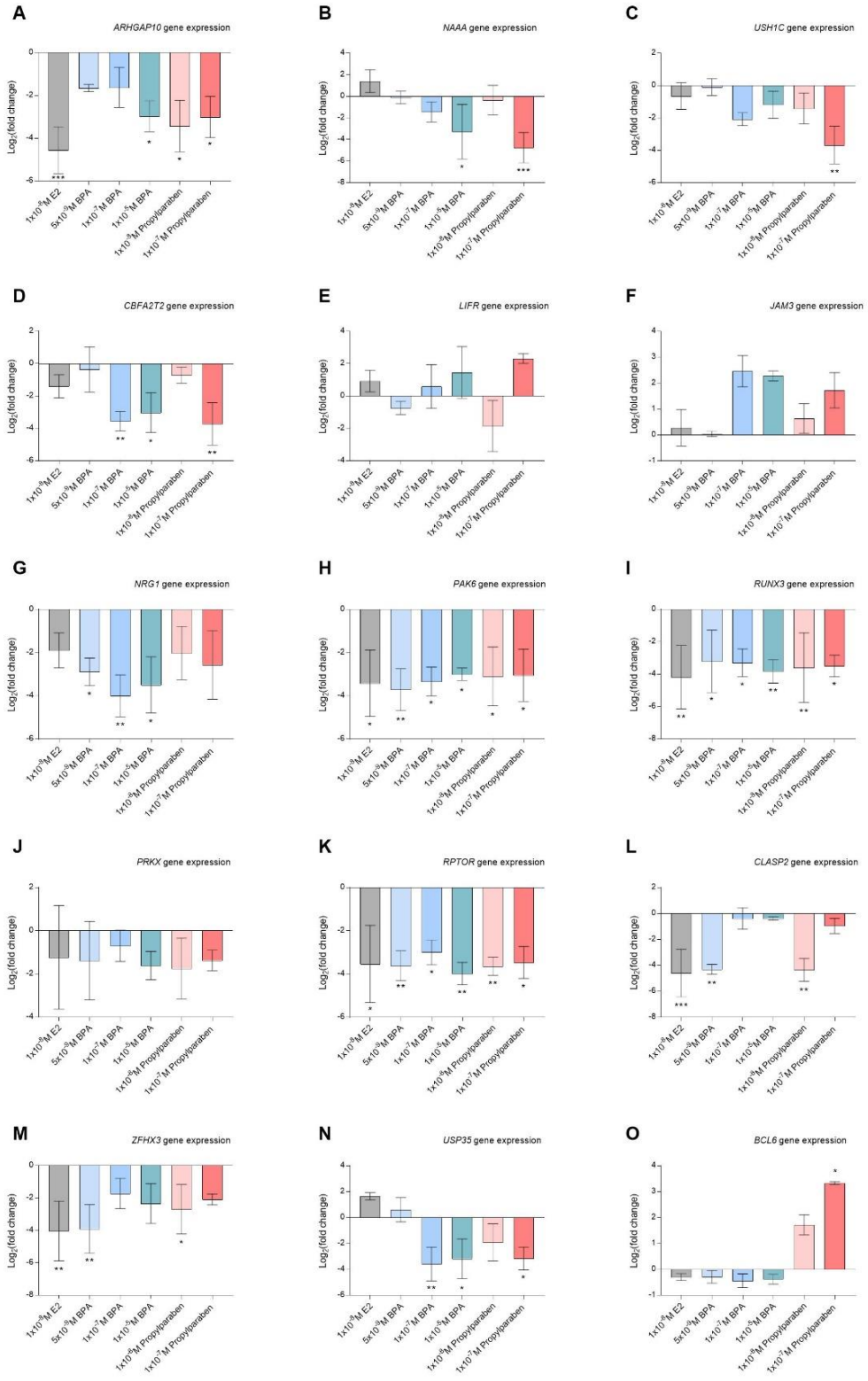

Figure 4.16. Change in gene expression seen in differentially methylated genes unique to propylparaben exposure. Expression of *ARHGAP10* (A), *NAAA* (B), *USH1C* (C), *CBFA2T2* (D), *LIFR* (E), *JAM3* (F), *NRG1* (G), *PAK6* (H), *RUNX3* (I), *PRKX* (J), *RPTOR (*K), *CLASP2* (L), *ZFHX3* (M), *USP35* (N) and *BCL6* (O). Fold change was calculated against the negative control sample using $2^{-\Delta\Delta Ct}$. *ACTB* was utilised as the reference gene.

4.3.4. EDC exposure induces alterations to functional gene annotations relevant to breast cancer risk

Finally, to explore whether the observed epigenetic changes could highlight pathways and processes that linked EDCs to breast cancer risk, a functional analysis was performed. First, DMP criteria were relaxed to allow additional epigenetically modified genes to be identified, as described in the methods section. A total of 2545 hypermethylated DMPs in response to BPA were found, 1698 of these could be mapped onto genes for use in the analysis. GO annotations associated with these genes were grouped into 39 functional clusters. Ten clusters had enrichment scores >1 (Table 4.3). The cluster that included GO terms associated with the phosphorylation of amino acids had the highest enrichment score (2.77). This cluster included genes that were most significantly altered in methylation, including *FYN*, *NUAK1* and *PAK6*. Other clusters included those associated with tyrosine phosphate activity (enrichment score 2.39), potassium ion transportation (enrichment score 2), glucose metabolism (enrichment score 1.57) and cell-cell adhesion (enrichment score 1.27). The regulation of Rho proteins was enriched with a score of 1.29. Genes involved in the regulation of cilia assembly and morphogenesis were also identified as enriched (enrichment score 1.11). Despite the altered criteria, only 44 hypomethylated DMPs, mapping to 35 genes could be identified after BPA exposure, meaning it was not possible to perform the cluster analysis.

Table 4.3. Functional annotation clustering of BPA-induced hypermethylated genes. Ten clusters with enrichment score >1 were produced from analysis of 1698 hypermethylated genes. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term. Clusters 1-5 met the enrichment score >1.3 threshold (shown in italics).

Cluster Rank		GO Annotation Term	Enrichment Score	Count	*p*
1	Biological Process	protein phosphorylation	*2.77*	56	<0.001
	Molecular Function	protein serine/threonine kinase activity		44	0.01
	Molecular Function	protein kinase activity		38	0.01
2	Biological Process	peptidyl-tyrosine dephosphorylation	*2.39*	18	0.001
	Molecular Function	protein tyrosine phosphatase activity		17	0.002
	Biological Process	protein dephosphorylation		16	0.04
3	Molecular Function	voltage-gated potassium channel activity	*2*	14	<0.001
	Biological Process	potassium ion transmembrane transport		20	<0.001
	Cellular Component	voltage-gated potassium channel complex		13	0.02
	Biological Process	potassium ion transport		11	0.07
	Molecular Function	delayed rectifier potassium channel activity		5	0.26
4	Biological Process	regulation of glucose metabolic process	*1.57*	8	0.01
	Molecular Function	pyruvate dehydrogenase (acetyl-transferring) kinase activity		3	0.02
	Biological Process	glucose metabolic process		9	0.12
	Biological Process	regulation of acetyl-CoA biosynthetic process from pyruvate		3	0.22
5	Biological Process	regulation of cardiac conduction	*1.38*	11	0.01
	Cellular Component	sarcoplasmic reticulum		8	0.01
	Cellular Component	junctional sarcoplasmic reticulum membrane		4	0.03
	Cellular Component	sarcoplasmic reticulum membrane		6	0.09
	Biological Process	cellular response to caffeine		3	0.11
	Cellular Component	calcium channel complex		4	0.26
6	Biological Process	regulation of Rho protein signal transduction	1.29	12	0.03
	Molecular Function	Rho guanyl-nucleotide exchange factor activity		11	0.04

	Molecular Function	guanyl-nucleotide exchange factor activity		14	0.08
	Cellular Component	cell-cell adherens junction		33	0.03
7	Biological Process	cell-cell adhesion	1.27	28	0.06
	Molecular Function	cadherin binding involved in cell-cell adhesion		29	0.06
	Molecular Function	ATPase activity, coupled to transmembrane movement of substances		9	0.01
8	Molecular Function	xenobiotic-transporting ATPase activity	1.18	3	0.04
	Biological Process	xenobiotic transport		3	0.08
	Biological Process	drug transmembrane transport		3	0.41
	Biological Process	peptidyl-tyrosine autophosphorylation		8	0.02
	Molecular Function	protein tyrosine kinase activity		17	0.02
9	Cellular Component	extrinsic component of cytoplasmic side of plasma membrane	1.13	10	0.05
	Molecular Function	non-membrane spanning protein tyrosine kinase activity		7	0.11
	Biological Process	adaptive immune response		12	0.53
	Biological Process	cilium assembly		15	0.07
10	Biological Process	cilium morphogenesis	1.11	16	0.07
	Cellular Component	ciliary basal body		12	0.08

Analysis was undertaken for propylparaben in both hypo- and hypermethylated genes. For the hypermethylated list, 8459 DMPs were identified containing 5640 genes. As the DAVID software is currently restricted to 3000 genes per analysis, the list was narrowed based on Delta Beta values, with the most substantially altered 3000 genes used in the analysis. Of the genes entered, 2658 were recognised by the software. A total of 52 clusters were generated that were associated with hypermethylated genes induced by propylparaben, 13 clusters held and enrichment score >1 (Table 4.4). Clusters 1-3 were most significantly enriched with a score >1.3, which was less than BPA despite a higher number of genes being entered into the analysis. The most enriched cluster (enrichment score 2.52) pertained to voltage-gated calcium channel activity. This was followed by terms associated with protein kinase activity (enrichment score 2). Several other clusters were of interest, including Rho protein signalling (enrichment score 1.26), potassium ion transport (enrichment score 1.23), amino acid transport (enrichment score 1.08) and microfilament activity (enrichment score 1.04). The overall function of some of the clusters was not as clear, however they contained several annotation terms of interest. For example, cluster 8 (enrichment score 1.12) comprised the GO term 'negative regulation of epithelial cell proliferation' (p = 0.2), whilst cluster 13 (enrichment score 1.03) included terms such as the 'regulation of MAPK cascade' (p = 0.27), 'cell development' (p = 0.12), 'positive regulation of pathway-restricted SMAD protein phosphorylation' (p = 0.01) and 'SMAD protein signal transduction' (p = 0.04).

Table 4.4. Functional annotation clustering of propylparaben-induced hypermethylated genes. Thirteen clusters with enrichment score >1 were produced from analysis of 2658 hypermethylated genes. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term. Clusters 1-3 met the enrichment score >1.3 threshold (shown in italics).

Cluster Rank		GO Annotation Term	Enrichment Score	Count	p
	Cellular Component	voltage-gated calcium channel complex		10	<0.001
1	Molecular Function	voltage-gated calcium channel activity	*2.52*	11	<0.001
	Molecular Function	high voltage-gated calcium channel activity		4	0.05
	Molecular Function	protein serine/threonine kinase activity		51	<0.001
2	Biological Process	protein phosphorylation	*2*	56	0.01
	Molecular Function	protein kinase activity		37	0.17
	Molecular Function	biotin carboxylase activity		4	0.01
3	Molecular Function	biotin binding	*1.48*	3	0.06
	Biological Process	biotin metabolic process		4	0.11
	Biological Process	regulation of Rho protein signal transduction		14	0.02
4	Molecular Function	Rho guanyl-nucleotide exchange factor activity	1.26	13	0.03
	Molecular Function	guanyl-nucleotide exchange factor activity		13	0.30
	Biological Process	potassium ion transmembrane transport		18	0.03
5	Cellular Component	voltage-gated potassium channel complex	1.23	13	0.06
	Molecular Function	voltage-gated potassium channel activity		9	0.12
	Cellular Component	extrinsic component of cytoplasmic side of plasma membrane		13	0.01
6	Biological Process	peptidyl-tyrosine autophosphorylation	1.19	8	0.05
	Biological Process	B cell receptor signalling pathway		8	0.18
	Molecular Function	non-membrane spanning protein tyrosine kinase activity		7	0.19
	Molecular Function	voltage-gated sodium channel activity		6	0.02
7	Biological Process	regulation of postsynaptic membrane potential	1.13	6	0.04
	Biological Process	sodium ion transmembrane transport		11	0.09
	Biological Process	membrane depolarization during cardiac muscle cell action potential		4	0.09

	Cellular Component	voltage-gated sodium channel complex		3	0.33
	Biological Process	SMAD protein import into nucleus		4	0.04
8	Molecular Function	transforming growth factor beta receptor binding	1.12	8	0.06
	Biological Process	negative regulation of epithelial cell proliferation		8	0.20
	Cellular Component	stereocilium		7	0.02
9	Biological Process	equilibrioception	1.09	3	0.09
	Cellular Component	photoreceptor outer segment		8	0.14
	Biological Process	sensory perception of light stimulus		3	0.18
	Molecular Function	basic amino acid transmembrane transporter activity		4	0.01
10	Biological Process	basic amino acid transmembrane transport	1.08	3	0.09
	Biological Process	cellular amino acid metabolic process		4	0.68
	Molecular Function	3',5'-cyclic-nucleotide phosphodiesterase activity		6	0.04
11	Molecular Function	3',5'-cyclic-AMP phosphodiesterase activity	1.05	4	0.13
	Biological Process	cAMP catabolic process		4	0.13
	Molecular Function	actin-dependent ATPase activity		5	0.02
	Molecular Function	microfilament motor activity		6	0.02
12	Biological Process	actin filament-based movement	1.04	4	0.17
	Cellular Component	myosin complex		7	0.23
	Molecular Function	motor activity		8	0.33
	Biological Process	positive regulation of pathway-restricted SMAD protein phosphorylation		11	0.01
	Biological Process	SMAD protein signal transduction		11	0.04
	Molecular Function	growth factor activity		21	0.06
	Molecular Function	transforming growth factor beta receptor binding		8	0.06
13	Biological Process	cell development	1.03	7	0.12
	Molecular Function	BMP receptor binding		3	0.17
	Biological Process	growth		5	0.22
	Biological Process	regulation of MAPK cascade		6	0.27
	Molecular Function	cytokine activity		16	0.53

Finally, 277 hypomethylated DMPs (242 annotated genes) induced by propylparaben exposure were analysed. Of the genes entered, 201 genes were registered by DAVID. A total of five clusters were identified, with just one having an enrichment score >1 (Table 4.5). This cluster (enrichment score 1.86) contained three terms including 'transcription factor activity, sequence-specific DNA binding' (p <0.001), 'DNA binding' (p = 0.04) and 'regulation of transcription, DNA-templated' (p = 0.14).

Table 4.5. Functional annotation clustering of propylparaben-induced hypomethylated genes. A single cluster with an enrichment score >1 was produced from the analysis of 201 hypomethylated genes. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term.

Cluster Rank		GO Annotation Term	Enrichment Score	Count	p
	Molecular Function	transcription factor activity, sequence-specific DNA binding		25	<0.001
1	Molecular Function	DNA binding	*1.86*	29	0.04
	Biological Process	regulation of transcription, DNA-templated		24	0.14

4.4. Discussion

In this chapter we aimed to determine whether BPA and propylparaben could affect epithelial cells cultured in an *in vitro* system that was more representative of the human mammary gland by allowing for epithelium-stromal interactions. Here, evidence is presented from three key endpoints known to be disrupted in early breast carcinogenesis: acini morphology, the methylation profile and gene expression.

Negative control acini formation and development within the collagen co-cultures were comparable to cultures using Matrigel and to single cell collagen cultures (Marchese and Silva, 2012; Carey, Martin and Reinhart-King, 2017; Carter *et al.*, 2017; Linnemann *et al.*, 2017; Wood *et al.*, 2018). The morphological analysis showed that acini exposed to oestrogenic compounds were larger and contained more cells in comparison to negative controls. This response demonstrated that despite differences in the culture set up, cells responded comparably to those presented in Chapter 3 and to previously published work with MCF-12A cells (Marchese and Silva, 2012). An increase in median acini area was observed after exposure to all concentrations of BPA, even at $5x10^{-9}$ M, which is the concentration reported in breast tissue (Fernandez *et al.*, 2007; Zimmers *et al.*, 2014). Whilst the increase in area after $5x10^{-9}$ M BPA exposure was only mildly significant, it not only demonstrates the sensitivity of the assay, but suggests that, even at low concentrations, BPA has the ability to induce changes to acini structure indicative of early breast carcinogenesis (Pal and Kleer, 2014). Similar observations were reported in 3D cultures of ER-negative MCF-10A cells, where 14 days of $1x10^{-7}$ M BPA exposure enhanced acini proliferation resulting in a higher median area (Pfeifer, Chung and Hu, 2015). Likewise, Marchese and Silva (2012) described a comparable increase in acini area and cell number in MCF-12A cells cultures on a Matrigel scaffold. Propylparaben also elicited an increase in acini area and cell number, however this was not deemed statistically significant in concentrations reported in the human breast ($1x10^{-8}$ M). After $1x10^{-7}$ M propylparaben exposure, the increases in both acini area and cell number were comparable to changes induced by BPA. Work

undertaken by Kahanna and Darbre (2011) tested the effect of propylparaben on MCF-10A suspension cultures, where significant increases in colony size at concentrations from $1x10^{-7}$ M. Unlike BPA, the tissue level concentration of propylparaben did not result in a significant change to acini area or cell number. The most likely explanation of this observation is that the two compounds have different potencies. This was demonstrated in Chapter 3, where only $1x10^{-5}$ M propylparaben was able to induce a significant change to acini area. It was also seen in Chapter 2, where propylparaben resulted in a smaller decrease in gene expression to *ESR1*, *ESR2* and *GPER1*. An alternative possibility is that propylparaben has a different mechanism of action to BPA. For example, work carried out by Marchese and Silva (2012) showed that when treated with a combination of ER and GPER inhibitors, BPA-exposed acini returned to a control-like state, exhibiting characteristics of untreated cells, including a hollow lumen and controlled growth. In contrast, propylparaben-treated cells continued to show a disrupted morphology in the presence of inhibitors, suggesting the alterations to acini were being regulated by other mechanisms, outside of traditional ER pathways. More recently, researchers identified c-Myc as an additional target of BPA (Pfeifer, Chung and Hu, 2015). BPA-exposed cells ($1x10^{-7}$ M) were found to have an increased expression of the c-Myc protein, associated with higher levels of DNA damage and cell proliferation. Pfeifer and colleagues established that BPA-induced cell proliferation in 3D cultures could be prevented by blocking the transcription of c-Myc. Alternative mechanisms linking propylparaben to cell proliferation have been suggested, including the up-regulation of the aromatase gene, *CYP19A1* (cytochrome p450 family 19 subfamily A member 1), allowing parabens to increase cell proliferation indirectly by increasing endogenous synthesis of oestradiol (Wróbel and Gregoraszczuk, 2013). It is plausible that the concentrations of chemicals needed to activate each of these effects varies depending on the target in question. If this was the case, it would result in different concentrations being required to induce cell proliferation, depending on the specific mechanism behind this effect. Nonetheless, this data shows that the morphological response to oestrogenic compound exposure is in line with previous literature and confirms the suitability of the co-culture system to study EDCs. In

addition, data presented here supports a growing body of evidence that BPA and propylparaben are indeed capable to eliciting morphological alterations indicative of neoplastic transformations. The exact mechanisms that underpin morphological changes in response to EDC exposure are still relatively unknown. In this chapter, data support the theory of the epigenome being one such mechanism that can link EDC exposure to breast cancer risk. It is well established that two types of DNA methylation changes occur in breast cancer; global hypomethylation (Narayan *et al.*, 1998) and regional hypo- and hypermethylation of specific genes (Baylin *et al.*, 2001). Whilst we did not observe any global shifts in methylation profile, numerous changes could be observed in regional methylation. The most significantly altered positions were overwhelmingly hypermethylated, with only a single hypomethylated gene identified at the highest threshold level. The higher frequency of hypermethylated genes has been previously seen in non-transformed ER-negative MCF-10F cells exposed to $1x10^{-5}$ M and $1x10^{-6}$ M BPA, resulting in 1,178 hypermethylated, 110 hypomethylated genes and 545 hypermethylated, 111 hypomethylated genes respectively (Fernandez *et al.*, 2012). No comparable study has looked at the impact of propylparaben exposure. Cultures exposed to propylparaben saw the highest number of DMPs in comparison to negative controls. The most significantly changed genes induced by propylparaben exposure included the hypermethylation of *RUNX3* which acts as a tumour suppressor gene known to be hypermethylated and frequently inactivated in breast cancer (Huang *et al.*, 2011; Chen, 2012; Chen *et al.*, 2014). Likewise, *NRG1* was significantly hypermethylated after propylparaben exposure. *NRG1* has been cited as having both tumour suppressor and oncogenic characteristics (Chua *et al.*, 2009), however Chua and colleagues showed that the gene was frequently silenced by methylation in breast cancer cell lines. They concluded *NRG1* may be a key tumour suppressor gene located on the short arm of chromosome 8, the loss of which was strongly associated with a poor prognosis in breast cancer. The observed hypermethylation of *ARHGAP10* could also be linked to breast cancer, being identified as one of the most frequently altered loci in 4,335 women diagnosed with invasive breast cancer (Azzato *et al.*, 2010). The exact role of *ARHGAP10* in breast cancer remains elusive, however, authors have speculated it may hold a tumour suppressor function (Teng *et*

al., 2017). *ARHGAP10* is commonly down-regulated in cancers, further indicating an essential function of the gene in tumorigenesis (Luo *et al.*, 2016; Teng *et al.*, 2017). Several other tumour suppressor genes known to be implicated in breast carcinogenesis were also hypermethylated by propylparaben exposure, including *ZFHX3* (Chen and Yang, 2014) and *LIFR* (Chen *et al.*, 2012). Interestingly, the hypermethylation of tumour suppressor *FOXN3* was common to both BPA and propylparaben exposure. Research has found the gene to play a vital role in the regulation of cell proliferation through the down-regulation of the reported oncogene E2F transcription factor 5 (Sun *et al.*, 2016). This observed regional hypermethylation of tumour suppressor genes is indicative of potential breast cancer initiation and progression (Szyf, Pakneshan and Rabbani, 2004). Moreover, the induction of tumour suppressor gene hypermethylation may suggest ER-independent mechanisms of action, previously unexplored in relation to EDC exposure. For example, it has been speculated that EDCs may reprogramme the epigenome by modulating s-adenosylmethionine levels and the activity of DNMTs (Walker, 2016), which can be translated into a change in DNA methylation (Kim, 2005; Crider *et al.*, 2012; Feil and Fraga, 2012).

In addition to tumour suppressors, alterations in multiple genes known to be hypermethylated frequently in breast cancer tissues were differentially methylated in response to EDC exposure. *RPTOR,* which is responsible for the regulation of cell proliferation and plays a pivotal in the cancer-associated mTOR pathway, was identified as significantly hypermethylated after propylparaben exposure. Studies analysing epigenome-wide DNA methylation have recently identified the increased methylation of *RPTOR* in breast cancer tissues, identifying this as a biomarker of early breast carcinogenesis (Wu *et al.*, 2017). Likewise, the observation of *HCFC1* hypermethylation has also been identified frequently in breast cancer tissues (Hu *et al.*, 2005). Taken together, the hypermethylation of the above genes supports a link between EDC exposure and changes indicative of early breast carcinogenesis through the epigenome.

Exposure to propylparaben resulted in the single hypomethylated gene *BCL6*. Studies have identified high expression levels of *BCL6* in breast cancer cell lines and found it to be commonly expressed in all three major classes of breast cancer (Walker *et al.*, 2015). Walker and

colleagues determined that the activation of *BCL6* played a key role in breast cancer cell survival. To our knowledge, no studies have looked specifically at the hypomethylation of *BCL6*, however, as the observed hypomethylation translated to an increase in expression levels, propylparaben may associated with breast cancer risk by increasing the expression of this oncogene.

Whilst many of the genes did suggest a clear link to breast cancer risk, EDC exposure also resulted in the hypermethylation of several potential oncogenes. *FYN* is reported to be over expressed in breast cancer and is linked to increased cell proliferation, migration and invasion (Xie *et al.*, 2016). Our results showed this gene was significantly hypermethylated after BPA exposure. Likewise, both propylparaben and BPA induced *NUAK1* hypermethylation. High *NUAK1* expression has been reported to promote migration and invasion in MDA-MB-231 cells (Liu *et al.,* 2013), and has been associated the a poor prognosis in lung (P. Chen *et al.*, 2013) and ovarian cancers (Phippen *et al.*, 2016).

It is clear from these findings that both BPA and propylparaben can induce changes to the methylation status of an array of genes, and we cannot be certain what biological implications all of these changes would result in. From our study, it is not possible to determine the relative contribution of each of these genes towards breast cancer risk and how much of an impact differential methylation of these genes will have at a higher level. What we can see from this data, however, is that EDC concentrations that result in changes to acini morphology, can also elicit relevant changes in the epigenome that could be associated with breast carcinogenesis.

Out of these differentially methylated genes, a subset were analysed using real-time PCR to elucidate whether the change in methylation could correlate with altered gene expression. The genes were chosen based on the most significant changes in methylation status and their association with breast cancer. Uniquely to BPA exposure, three hypermethylated genes were identified and their expression in response to chemical exposure was investigated; *FYN*, *HCFC1* and *DCHS1*. Of these genes, a significant change in gene expression could only be detected in *DCHS1*. However, the expression of the gene increased, which was not expected considering the observed hypermethylation. Whilst high levels of methylation have been correlated with low or no transcription (Lim and Maher, 2010), the location of the epigenetic mark is essential in

determining the outcome on gene expression. If the methylation is not located in the promotor region of the gene, it may have little impact on transcription levels (Suzuki and Bird, 2008). It may be possible that this was the case for these genes and therefore hypomethylation had no impact on gene expression in this study. Alternatively, other mechanisms may be having a more significant impact on transcription regulation of these genes. For example, we saw little change in *FYN* expression. *FYN* has been reported to be regulated by redox (Gao *et al.*, 2009). Ban and colleagues (2008) found that *FYN* expression in leukaemia was directly regulated by *BCR-ABL1* oncogene expression that is able to raise intracellular oxidant levels driving redox. Authors described that an up-regulation in *BCR-ABL1* resulted in an increase in *FYN* transcription. Whilst BPA has been seen to induce oxidative stress *in vivo* (Eid, Eissa and El-Ghor, 2015; Kaur, Saluja and Bansal, 2018) and *in vitro* (reviewed in Gassman 2017), the same effects may not be occurring in our model. In addition, *BCR-ABL1* is not present in breast cancers and this may explain why a change in *FYN* transcription is not seen in this study. Variations between models and endpoints has been noted previously to explain contradictory findings, along with the complex and pleiotropic mechanisms of BPA (Gassman, 2017).

Genes that were uniquely hypermethylated in response to propylparaben exposure were more consistently down-regulated. This decrease in gene expression was more statistically significant and we reported the silencing of several tumour suppressor genes known to be involved in breast cancer, including *ZFHX3*, *RUNX3*, *ARHGAP10* and *NRG1*. Given the role of such critical genes, it is plausible that exposure to propylparaben can have deleterious effects on the genome, leading to an increase in breast cancer risk. Evidence has indicated *RUNX3* is a key tumour suppressor gene and frequently inactivated in breast cancer due to hypermethylation of the promoter region (Chen, 2012). The inactivation of *RUNX3* has now been established as an early event in breast cancer progression (Subramaniam *et al.*, 2009). Loss of *RUNX3* led to the development of ductal carcinomas in over 20% of female mice (Huang *et al.*, 2011). Likewise, increasing *RUNX3* expression in breast cancer cells has been seen to reduce the tumorigenic potential (Lau *et al.*, 2006; Chen *et al.*, 2007; Huang *et al.*, 2011). One study observed the hypermethylation of *RUNX3* by oestrogen in primary mammary epithelial cells, with authors

suggesting that some methylation of *RUNX3* may be regulated via ER signalling (Cheng *et al.*, 2008). The exact mechanisms of how ER signalling induces *RUNX3* methylation still need to be determined, however this relationship may indicate how exposure to oestrogen-mimicking compounds could elicit hypermethylation of *RUNX3* and cause a reduction in the gene's expression. The hypomethylation and down-regulation of *LIFR*, which has been identified as a breast cancer metastasis suppressor (Chen *et al.*, 2012), suggests that the EDC exposure may also have the ability to play a role in breast cancer progression. One gene that did not match the expected decrease in gene expression after hypermethylation was *JAM3*, which is involved in the signalling cascades that promote endothelial angiogenesis. Such mechanisms have led researchers to speculate that JAM proteins may therefore be indirectly supporting cancer progression (Offiah, Brennan and Hopkins, 2011). Whilst tissue relevant concentrations of the compounds caused no change to *JAM3* expression, higher concentrations saw an increase in expression, suggesting that methylation was not the predominant mechanism impacting this gene.

As for the genes common to both BPA and propylparaben, *CLMP*, *NUAK1* and *FOXN3*, whilst a general trend in down-regulation could be seen, this was not always deemed significant. The higher concentration of propylparaben ($1x10^{-7}$ M) did elicit a mildly significant down-regulation in *NUAK1* expression. Only $1x10^{-5}$ M BPA could induce a significant change in *NUAK1* expression, which is a concentration much higher than seen in human tissues. Also, *FOXN3* was only significantly changed in response to E2 exposure. As this gene was not seen as differentially methylated in response to E2, it suggests that additional factors, such as classical ER signalling pathways, may be having a more dominant effect on influencing gene expression than methylation.

Generally, genes involved in tumour suppressor functions saw a more significant decrease in gene expression compared with genes possessing oncogenic properties. This observation supports the theory that EDCs can be linked to breast tumorigenesis through epigenetic mechanisms, however the lack of expression changes in some genes indicates EDC-induced methylations are not always occurring within promoter regions, or that there are additional

factors influencing expression that are not solely limited to methylation status. Interestingly, many of the genes where an increase in methylation was only observed in exposure to propylparaben, also saw a decrease in gene expression in response to BPA exposure. Also, several of the genes tested observed a significant decrease after exposure to E2 where no changes in methylation were detected. Some of the genes have been linked to other regulators, such as ER signalling, including *RUNX3* and *ZFHX3*. Both E2 and BPA have been reported to directly bind to ERs and impact ER signalling (Rubin, 2011; Acconcia, Pallottini and Marino, 2015; Yaşar *et al.*, 2017), which may be one explanation of how they are able to influence gene expression without interfering with the epigenetic profile of cells. The *RPTOR* gene is known to negatively regulate the mTOR kinase, which is responsible for the regulation of cell growth and survival. One study found that E2 and BPA exposure were able to activate the mTOR pathway in non-malignant primary breast cells, resulting in suppressed apoptosis (Goodson *et al.*, 2011). Goodson hypothesised that the phosphorylation of AKT (cellular homolog of murine thymoma virus akt8 oncogene), a key regulatory step in mTOR activation, could be a possible mechanism of action. Although research did not look specifically at the expression of *RPTOR* and whether this was associated with xenoestrogen-induced mTOR activation, it is possible that BPA and E2 may be influencing *RPTOR* gene expression via a similar mechanism. Also down-regulated was *PAK6*. This result was unexpected, as it is not in agreement with published literature on the levels of *PAK6* in breast cancer tissues. *PAK6* is a member of the p21-activated kinase family, implicated in in the regulation of cell motility, gene transcription, apoptotic signalling and is known to interact with the ligand binding domain of the androgen receptor (AR), where *PAK6* expression can inhibit AR-mediated transcription (Yang *et al.*, 2001; Lee *et al.*, 2002). *PAK6* has also been reported to bind to ERα, inhibiting transcriptional activities (Lee *et al.*, 2002). The expression of *PAK6* is generally enriched in breast tumours, however very little is known about its functional role in breast cancer and additional research is required to understand this further (Eswaran, Soundararajan and Knapp, 2009). Regulation of *PAK6* has previously been directly linked to MAP kinase activity, specifically by stimulation from MAPK kinase 6 and p38 MAP kinase (Kaur *et al.*, 2005). BPA has been shown to induce p38 MAPK activation in rat sertoli cells after

exposure to concentrations between $5x10^{-5}$ M to $7x10^{-5}$ M (Qi *et al.*, 2014), however if this was occurring here, an increase in *PAK6* transcription would be expected. It is possible this effect is concentration dependant, although further research would be required to elicit the specific mechanism responsible for the decrease in *PAK6* expression observed here.

The single hypomethylated gene, *BCL6*, also demonstrated a significant increase in gene expression. Unlike many of the hypermethylated targets, this down-regulation was limited to propylparaben, with no other exposures resulting in a change in gene transcription. This finding suggested the regulation of *BCL6* may be a mode of action unique to propylparaben, not associated with traditional ER-mediated pathways. *BCL6* expression is rare in normal mammary epithelium yet activated in breast cancer and has been shown to promote the survival of breast cancer cells, preventing apoptosis and epithelial differentiation (Logarajah *et al.*, 2003; Walker *et al.*, 2015).

Many of the genes identified as epigenetically altered due to EDC exposure within this chapter have not previously been investigated in relation to BPA or propylparaben exposures. A more in-depth study of these genes may significantly increase our understanding of the mechanisms of action of compounds and their potential link to breast cancer risk. Due to the number of differentially methylated positions, we grouped the genes based on the processes they are associated with, in order to see what functions may be altered in response to BPA and propylparaben exposure.

In the case of propylparaben, biological processes associated with both hyper- and hypomethylated genes could be identified, many of which were relevant to breast carcinogenesis. Interestingly, two common processes were associated with BPA- and propylparaben-induced hypermethylated genes. Firstly, we saw that many of the differentially methylated genes could be associated with protein phosphorylation, specifically related to serine and threonine kinase activity. One such gene was *PKD1*, which encodes the serine/threonine-protein kinase D1. The serine/threonine-protein kinase is known to phosphorylate the epidermal growth factor receptors, which results in the suppression of EGF-induced MAPK8/JNK1 activation and subsequent c-JUN phosphorylation. *PKD1* has also been implicated in various

functions within the cell including proliferation, apoptosis, adhesion and cell motility (Sundram, Chauhan and Jaggi, 2011). Aberrant expression of *PKD1* is linked to the activation of the MAPK signalling pathway through the phosphorylation of Ras effector proteins (Van Lint *et al.*, 2002; Jaggi *et al.*, 2007). Significant down-regulation of *PKD1* has been observed in 95% of tested breast cancer tissue samples (Eiseler *et al.*, 2009), as well as in breast cancer cell lines MD-MD-321 and MCF-7 (Qin *et al.*, 2015). This reduction in gene expression has been attributed predominantly to DNA hypermethylation (Eiseler *et al.*, 2009). The ability for EDCs to elicit effects on the epigenome, which, in turn, alter the function of genes associated with protein phosphorylation has not yet been investigated. Yet, the findings here indicate the epigenome may be playing a role in these processes and therefore justify further research.

A second function common to both propylparaben- and BPA-induced hypermethylated genes was Rho-protein signal transduction. Rho-proteins play a pivotal role in driving metastasis in breast cancer, although each of the proteins holds distinct functions (Ridley *et al.*, 1992; Nobes and Hall, 1995; Tang *et al.*, 2008; Okada *et al.*, 2014; Aleskandarany *et al.*, 2017). Typically, rho-proteins are over-expressed in breast cancer tissues (Lin and Van Golen, 2004). In one study, *RhoC* was reported to be enriched in over 90% of tested inflammatory breast cancer tumour samples (van Golen *et al.*, 1999). Some of the Rho-protein regulation was shown to be influenced via p38 MAPK pathways (van Golen *et al.*, 2002; Okada *et al.*, 2014), however the superfamily currently includes over 130 members that still require further research to understand the role each holds in breast cancer and how they are regulated (Lin and Van Golen, 2004). One Rho GTPase activating gene, *ARHGAP10,* has already been discussed and the demonstrated hypermethylation and down-regulation supports the hypothesis that EDCs can contribute to breast cancer risk, yet investigations of other genes affected in this complex functional group may not be as clear. Nevertheless, this is cluster was identified in as enriched after exposure to both tested EDCs and therefore warrants further investigation.

Several additional functional clusters were highlighted that may also provide insights into how EDC exposures could be linked to breast carcinogenesis via the epigenome. BPA hypermethylated genes were associated with glucose metabolism processes. During glycolysis,

energy is produced in the form of activated carrier molecules (adenosine triphosphate and nicotinamide adenine dinucleotide), providing a net gain in energy. Aberrant metabolism of glucose has been associated with a spectrum of diseases including breast cancer (Li *et al.*, 2011). Cancer cells can be characterised by their uncontrolled proliferation, which requires accelerated glucose metabolism to meet the energy requirement (Long, Li and Zhang, 2016). BPA exposure has been shown to affect glucose metabolism in mice, implicating the compound in numerous metabolic disorders (such as obesity and metabolic syndrome; Elin Swedenborg *et al.*, 2009; Alonso-Magdalena *et al.*, 2010; Angle *et al.*, 2013; J. Liu *et al.*, 2013; Mimoto, Nadal and Sargis, 2017), however there is limited research tying BPA-induced changes in glucose metabolism to breast cancer risk, resulting in a potential knowledge gap. Cell-cell adhesion and cilium assembly were also identified in functional clusters and are known to be associated with carcinogenesis (Moh and Shen, 2009; Menzl *et al.*, 2014), however they were not amongst the most significantly enriched clusters, with both being below the 1.3 enrichment score threshold, and therefore may not be considered as a priority to investigate.

Propylparaben hypermethylated genes could be associated with functions related to breast cancer risk. Although not as many clusters passed the 1.3 enrichment score threshold, several clusters contained processes of interest, which may provide insight into novel mechanisms linking EDC exposure to breast cancer risk. In comparison to BPA, comparatively little is known about the mechanisms of action of propylparaben, therefore these processes may provide a crucial starting point to further understand how the compound can be associated with breast tumorigenesis. The most significantly enriched cluster pertained to the hypermethylation of genes connected to voltage-gated calcium channel (VGCC) activity. Calcium ions play an essential role in cells and are involved in processes, such as cell motility, apoptosis, exocytosis and endocytosis (Buchanan and McCloskey, 2016). VGCCs are one mechanism responsible for regulating the level of calcium ions within the cell and, therefore, any abnormal behaviour could have deleterious consequences. Within cancer, VGCCs are widely expressed at gene and protein level, with a dramatic up-regulation observed in breast tumours (reviewed in Wang *et al.,* 2015). Wang and colleagues concluded that VGCCs are likely to play essential roles in most

cancer types and expressed a need for further research to determine the exact underlying carcinogenic mechanisms. Meijer *et al.,* (2014) identified VGCCs as a common mode of action within insecticides. The study demonstrated that the insecticides cypermethrin, α-cypermethrin, endosulfan, and chlorpyrifos, could inhibit VGCC activity and disturb calcium homeostasis in rat pheochromocytoma cells. Moreover, the insecticides were able to exert this effect at concentrations relevant to human exposures (as low as $7.8x10^{-8}$ M in the case of cypermethrin). Low-dose ($1x10^{-10}$ M to $1x10^{-6}$ M) BPA exposure has also been seen to interact with the regulation of VGCCs via ERα and ERβ in mouse pancreatic β-cells (Villar-Pazos *et al.*, 2017). This mode of action has not yet been explored in breast cells, nor have studies investigated whether propylparaben can have a comparable effect. Data presented here strongly suggest that propylparaben exposure may be influencing VGCC regulation, revealing a novel mechanism that could link exposure to breast cancer development.

As well as influencing VGCCs, propylparaben induced the hypermethylation of genes associated with voltage-gated potassium channel (VGPC) regulation, responsible for controlling the flow of potassium across cell membranes. Akin to VGCCs, VGPCs have been implicated in carcinogenesis, in addition to other disorders including epilepsy, cardiac arrhythmias and neuromuscular disorders (Huang and Jan, 2014). The aberrant regulation of key VGPC-regulating genes has been seen to enhance tumorigenic processes, such as cell proliferation, migration and metastasis (Bielanska *et al.*, 2009; Williams, Bateman and O'Kelly, 2013; Pardo and Stühmer, 2014). As with VGCCs, there is very limited research surrounding xenoestrogen exposure and VGPC activity. Soriano *et al.,* (2016) reviewed evidence surrounding the impacts of BPA on ion channels and concluded that whilst research indicated these molecules could be key targets for EDCs, they were poorly understood and required further study. Results shown here also support ion channels as a target for EDC exposure.

Although not within one of the most significantly enriched clusters, six genes associated with MAPK cascade regulation were significantly hypermethylated in response to propylparaben. Aberrant MAPK signalling plays a critical role in the development and progression of cancers, and is also related to therapy resistance (Dhillon *et al.*, 2007; Low and Zhang, 2016). Moreover,

the MAPK pathway is responsible for the regulation of several other enriched processes discussed previously. Hypermethylated genes associated with this annotation included *ATP6AP2* (ATPase H+ transporting accessory protein 2), *BMP6* (bone morphogenetic protein 6), *BMP8A* (bone morphogenetic protein 8a), *GDF3* (growth differentiation factor 3), *GDF5* (growth differentiation factor 5) and *INHBC* (inhibin beta C subunit). There is evidence linking *GDF5* and *BMP8A* expression to p38 MAPK functioning (Nakamura *et al.*, 1999; Wu, Chen and Li, 2016), yet how they function in the context of breast carcinogenesis is unknown. The impact of propylparaben on MAPK activation has been explored in MCF-7 and MCF-10A cells (Wróbel and Gregoraszczuk, 2015). Authors reported that exposure to $2x10^{-8}$ M propylparaben induced ERK1/2 activation via GPER1, inhibiting apoptosis, however it is likely this was due to an increase in protein phosphorylation, rather than changes in gene expression. There is currently very limited evidence of what biological implications hypermethylation of MAPK would have and controversy remains surrounding how each of the kinases are implicated in breast cancer. For example, whilst activation of p38 kinases and JNK have been associated with tumour suppression (Brancho *et al.*, 2003; Bulavin *et al.*, 2004; Timofeev, Lee and Bulavin, 2005; Cellurale *et al.*, 2011), biological outcomes are often dependent on the stimuli strength, duration and cell type (Ventura *et al.*, 2006). Nevertheless, data presented here supports previous evidence of parabens being able to influence MAPK signalling and we propose that changes to methylation may be another mechanism influencing this. Future studies should aim to develop our understanding of the biological effect of this, in assays representative of the human breast.

Finally, genes hypomethylated by propylparaben were associated with just a single functional cluster pertaining to DNA binding and transcription. Potentially not as insightful as the hypermethylated clusters, this function related to the frequency, rate or extent of DNA-templated transcription. Within these terms were genes including *BCL6*, SMAD family member 1, zinc finger protein 74 and *MTA1* (metastasis associated 1). Whilst these have been associated with breast cancer related processes, the cluster does not provide a useful insight into novel processes that may be influenced by paraben exposures. The reduced cluster number is most likely a product of the significantly lower number of genes inputted into the analysis. It may also

be due to the spread of the genes and the processes they are involved with, suggesting that this form of analysis may not be the most suitable in this case. Further research should instead focus on the specific genes that are most significantly altered, such as *BCL6*.

Overall, data presented in this chapter demonstrate the ability of EDC exposures to induce effects that could be associated with breast cancer initiation. Alterations to three endpoints indicative of the early stages of breast carcinogenesis have shown that even at tissue relevant concentrations, both BPA and propylparaben can elicit deleterious effects. Furthermore, the involvement of the epigenome has highlighted new pathways and functions that are influenced by EDC exposure, providing avenues for future research. Literature has often concluded that EDCs, like BPA, are unable to elicit carcinogenic effects at concentrations relevant to human exposures and, therefore, are considered safe. The majority of research has focused on the compound's oestrogenic properties where the ER binding affinity is considerably less than the endogenous hormones. Yet, here we have shown tissue relevant concentrations interact with a variety of ER-independent processes, altering the epigenetic and genetic profile of cells.

Although some of the genes were not differentially expressed at average concentrations seen in the human breast, it must be recognised that some subpopulations may be subject to higher exposure concentrations. With an increase in cosmetic use around the world, populations exposed to high concentrations of propylparaben will become more frequent, potentially magnifying the effects described in this chapter. Likewise, despite many products now advertising being BPA free, initial research on 'safer' replacement compounds indicates their effects may be comparable to BPA, if not worse (Eladak *et al.*, 2015; Mesnage *et al.*, 2017; Zhang *et al.*, 2018). Our work has highlighted the possibility that the current regulation of these compounds may be putting the population at risk and further work to ascertain their effects at relevant concentrations needs to be carried out. Furthermore, individuals are not exposed to these compounds in isolation. As we demonstrated in Chapter 3, EDCs can act together, contributing to the overall oestrogenic load, and this must always be considered when interpreting the results reported here.

Whilst we have highlighted new mechanisms that can possibly link EDCs to breast cancer, further research will be required to confirm these findings through more detailed and focused analysis, considering multiple endpoints including protein expression and biological implications. It must also be noted that results presented here are representative of just three independent experiments. Reproducing this experiment to include additional replicates would strengthen the reliability of conclusions made here and potentially highlight additional factors influenced by EDC exposure, due to an increase in statistical power. Despite using a superior cell culture model compared with the assay in Chapter 3, the reliability of cell lines has been criticised and their relevance to mammary tissues is continually debated. In addition, there are other risk factors that EDCs may interact with to add to the 'risk load' that have not been included here. Chapter 5 will begin to model these interactions with the inclusion of genetic risk factors in primary donor derived cells.

Chapter Five: Investigating the effect of endocrine disrupting chemicals on primary breast epithelial cells: a comparison between individuals with and without *BRCA1* mutations

5 Investigating the effect of endocrine disrupting chemicals on primary breast epithelial cells: a comparison between individuals with and without *BRCA1* mutations

5.1. Introduction

5.1.1. Advancing 3D co-cultures using primary donor cells

In Chapter 3, we moved away from traditional 'flat biology' methods and towards a model that more effectively recapitulates the human breast. Then, we advanced in Chapter 4 by including additional cell types, to further improve the relevance of the cell culture model. Whilst this is a considerable development on simplistic 2D models, it remains limited by a reliance on the MCF-12A cell line. Cells lines have historically been relied upon for studying cell biology and pathologies, such as cancer development progression and therapy. This can be attributed to their convenience, ease of use and relatively minimal cost (Kaur and Dufour, 2012; Srivastava *et al.*, 2018). However, the scientific community has raised concerns about their similarity to cells within the human breast, and consequently, about the relevance of results to human responses. Studies have compared the gene expression profiles of multiple cell lines with primary tissues and indicated that no single cell line was truly representative of *in situ* tumours (Ross *et al.*, 2000; Neve *et al.*, 2006). Using 51 different breast cancer cell lines, Neve *et al.,* (2006) identified numerous key differences between cancer cell lines and primary tumours. Notably, the group reported that primary luminal and basal sub-type tumours had a significantly different number of genome copy number abnormalities (CNAs). In contrast, luminal and basal cell lines contained analogous numbers of CNAs. This finding highlighted a pronounced difference between the heterogeneity of tumours and cell lines. Authors suggested this difference may have originated from long-term culture conditions selecting for specific genomic alterations and although the MCF-12A cell line is non-cancerous, it is plausible that similar selections have occurred. The divergent effects of long-term culture on cell lines has been reported previously, with impacts being seen in the morphology, development and gene expression of a variety of cell lines (Briske-Anderson, Finley and Newman, 1997; Chang-Liu and Woloschak, 1997; Esquenet *et al.*, 1997; Sambuy *et al.*, 2005; Mazzatti *et al.*, 2007; Riedl *et al.*, 2017).

As well as potentially being unrepresentative of human breast cells, cell lines have been frequently cross-contaminated or misidentified. One review estimated that between 1968 and 2007, 18-36% of cell lines may have been contaminated with other cell lines or misidentified (Hughes *et al.*, 2007). The high frequency of contamination with other cell lines has resulted in dissimilar genetic and epigenetic profiles in comparison to the original donor cells (Hughes *et al.*, 2007). For example, over 1000 studies have been published on the triple negative breast cancer cell line MDA-MB-435, yet it was found to have an identical short tandem repeat (STR) profile to a melanoma cell line, M14. This indicated that the samples were potentially identical and therefore the same cell line (Christgen and Lehmann, 2007; Chambers, 2009). This finding called into question the validity of studies relying on both cell lines and also the prevalence of misidentification across other cell lines (Lacroix, 2008). Whilst the requirement of cell line authentication has attempted to address these concerns, scientists have been criticised for not tackling this problem sufficiently (American Type Culture Collection Standards Development Organization Workgroup ASN-0002, 2010), with numerous studies still failing to confirm the authenticity of cell lines. Two articles highlighted this issue and questioned the value of cell lines studies when inconsistent results are observed between lab groups and batches, leading to irreproducible findings (Freedman *et al.*, 2015; Neimark, 2015).

A further limitation of cell lines is that individual cell lines originate from a single donor's tissue. The MCF-12A cell line was first established from cells taken from a single patient with fibrocystic breast disease (described in Chapter 2). Consequently, results obtained from experiments performed using MCF-12A cells will, at best, be representative of a single individual's response to EDC exposures. Yet it is likely no two individuals will have the same response to chemical exposures. Diamanti-Kandarakis *et al.,* (2009), speculated that susceptibility to EDCs may vary greatly due to metabolism, body composition and genetic polymorphisms. Research has shown individuals differ greatly in pharmacodynamics and pharmacokinetics, impacting their responses to drugs, which can be influenced by age, BMI, alcohol intake, internal hormone levels and genetic differences (Sellers and Holloway, 1978; Sweeney, 1983; Evans and Johnson, 2001; Wilson *et al.*, 2001; Lonergan *et al.*, 2017). As such, whilst several independent experiments

using cell lines can be used to support hypotheses of EDC effects, they will never fully capture the response variation within populations. Hughes *et al.* (2007) argued that when taken together, the selective pressures of genetic drift, over passaging and prevalence of misidentification bring into question the value of continuing to use cell lines in research.

The use of primary donor cells offers the opportunity to overcome some of these limitations, providing a more representative model for toxicological research (Kaur and Dufour, 2012; Kodack *et al.*, 2017). Unlike immortalised cell lines, primary cells have a limited period of viability and therefore maintain very similar characteristics to the originating donor tissues, thus, producing more biologically relevant results (Borrell, 2010; Edmondson *et al.*, 2014). Primary cells have been seen to mimic target tissue responses to anticancer drugs more effectively than cell lines during screening. Work by Jabs *et al.* (2017) found that results from patient-derived 3D cultures provided a much more realistic prediction of *in vivo* responses. Specifically, authors highlighted how primary cell results were more diverse in their responses to anticancer agents, representing individual variation. Jabs *et al.* also reported that cytostatic drugs tested in primary cultures were found to have a lower therapeutic potential in comparison to cell lines, which was more representative of actual patient responses. This was supported further by Vlachogiannis *et al.* (2018), who showed patient-derived 3D culture models maintained the molecular profiles of donors and reliably recapitulated patient responses to 51 anticancer agents, currently being trialled for gastrointestinal cancer treatment. Together, these findings suggest that using primary cells to study the effects of EDCs may significantly increase the relevance of findings to human responses.

Whilst cell lines are still heavily relied upon, researchers are recognising the added value primary cells can have in breast cancer research. To support an increasing demand, tissue and cell banks have been established, with the aim of improving the ease of access to primary cells. In the United Kingdom, the Breast Cancer Now Cell Bank is currently the only facility to offer donor-derived primary cells isolated from human breast tissue. At the time this research was conducted, the cell bank provided primary epithelial, myoepithelial and fibroblast cell stocks from non-tumorigenic breast tissue. After applying for access, we were informed two distinct patient

biotypes were available for research: wildtype individuals, where tissues were obtained due to cosmetic reduction mammoplasties, and *BRCA1* mutation carriers, where tissues were obtained through prophylactic mastectomies. The availability of these two biotypes provided a valuable opportunity to consider the contribution of additional risk factors on the responses to EDC exposure. Thus far, studies have generally focused on demonstrating how chemicals can contribute to carcinogenesis in isolation. However, in order to have a comprehensive understanding of the relationship between EDCs and breast cancer, we must consider that breast cancer risk can be increased by an accumulation of factors, not just single factors in isolation (Arthur *et al.*, 2018; Ellingjord-Dale *et al.*, 2018; Friedenreich and McTiernan, 2018; Heitz *et al.*, 2018). In Chapter 3 we started to address this by investigating the effect of mixtures of low-dose EDCs. Although this provided valuable results, we must also consider that individuals are subject to a myriad of other intrinsic and extrinsic risk factors (reviewed in Chapter 1), that when combined, may have a substantial effect on an individual's risk. The study of individuals with and without *BRCA1* mutations (a significant genetic risk factor), begins to incorporate this.

5.1.2. Multifactorial effects of risk factors

Individuals with germline mutations in the *BRCA1/2* genes carry a high risk of developing breast cancer. Indeed, mutations in either *BRCA1* or *BRCA2* account for the majority of hereditary breast cancers (Kuchenbaecker *et al.*, 2017), explaining 5-10% of all breast cancers diagnosed in women before the age of 40 (Mehrgou and Akouchekian, 2016; Milne and Antoniou, 2016). The *BRCA1* gene is located on chr17q, and *BRCA2* is found on chr13q. Both are well known tumour suppressor genes and any aberrant regulation or mutations in either gene is associated with an increased risk of developing breast and ovarian cancer in women (Brody and Biesecker, 1998; Rosen *et al.*, 2003; Eccles and Pichert, 2005; Kuchenbaecker *et al.*, 2017). Savage *et al.,* (2014) demonstrated that *BRCA1* regulates oestrogen metabolism metabolite-mediated DNA damage by suppressing the transcription of oestrogen-metabolising enzymes, such as CYP1A1,

which converts androgens into bioactive oestrogens. Authors suggested that *BRCA1*-dificient mammary cells de-regulated the metabolism of oestrogen, which resulted in increased levels of genotoxic metabolites and increased DNA damage, and when this was combined with *BRCA1*-induced defective DNA repair, would lead to genomic instability.

Studies have noted a significant variation of risk in women who have inherited *BRCA1/2* mutations in regards to age at diagnosis and site of cancer occurrence (Friebel, Domchek and Rebbeck, 2014), even between close relatives with the same mutation (Antoniou *et al.*, 2003; Begg *et al.*, 2008). Although the cause of this variation remains unclear, one influential study in 2003 investigated the occurrence of breast cancer in a community of Ashkenazi Jews, that were known to have relatively high penetrance of *BRCA1* mutations. King *et al.* (2003) emphasised that the cancer development risks associated with *BRCA1* mutations, within this population, appeared to have been increasing since the 1940s. They showed the lifetime risk associated with women born before the 1940s was 24%, whereas individuals born after 1940 had a 67% lifetime risk of developing breast cancer. Furthermore, the study showed that the age of first diagnosis was decreasing, with *BRCA1* mutated individuals developing breast cancer at a younger age. King and colleagues proposed that a reduction in physical exercise and higher obesity rates during adolescence could be the reason for this observed increase in lifetime risk. This paper highlighted that, even in populations with a high penetrance of *BRCA1* mutations, the timing and severity of the cancer incidents appeared to be influenced by additional extrinsic factors. Other groups have also found that breast cancer risk varies amongst *BRCA1/2* mutation carriers (Antoniou *et al.*, 2003; Simchoni *et al.*, 2006; Brohet *et al.*, 2014). It has been hypothesised that such observations could be attributed to environmental and lifestyle factors, such as reproductive history, oral contraception, obesity, physical activity and alcohol consumption, that can modify the breast cancer risk of mutation carriers (Milne and Antoniou, 2016).

Research has endeavoured to improve our understanding of such risk factor interactions, however many of the studies have been limited due to the ascertainment of reproductive history

and by recall and testing bias, which may have significantly impacted study results (Antoniou *et al.*, 2009). Studies have also been restricted by a lack of statistical power, due to small sample sizes (Milne and Antoniou, 2016). Moreover, very few consistent findings have been reported in the literature, causing significant confusion over how environmental and lifestyle factors can modify breast cancer risk in *BRCA1/2* mutation carriers (Friebel, Domchek and Rebbeck, 2014). For instance, the benefits of *BRCA1/2* carriers adopting a Mediterranean diet have been reported inconsistently (Pepe, Pensabene and Condello, 2012; Kiechle *et al.*, 2016; Pollan *et al.*, 2017; Bruno *et al.*, 2018; Turati *et al.*, 2018). Despite this, some relatively robust associations have been found. Smoking was found to be associated with a higher breast cancer risk in *BRCA2* mutation carriers (Friebel, Domchek and Rebbeck, 2014). However, in *BRCA1* carriers, results surrounding breast cancer risk and smoking are inconclusive and remain under investigation (Brunet *et al.*, 1998; Breast Cancer Family Registry, 2008; Ginsburg *et al.*, 2009; Lecarpentier *et al.*, 2011). Reproductive history has also been reported to be a significant modifier of risk in mutation carriers, with a higher age at first full-term pregnancy consistently demonstrating a decreased risk of breast cancer in *BRCA1* carriers (Andrieu *et al.*, 2006; Antoniou *et al.*, 2006; Lecarpentier *et al.*, 2012; Friebel, Domchek and Rebbeck, 2014; Pan *et al.*, 2014). This finding contrasts with what is known about the general population, implying that the presence of a *BRCA1* mutation may have a significant impact on how lifestyle factors influence risk. Authors have suggested the difference in between mutation carriers and the general population may be attributed to differing responses to hormonal influences, however stressed that further studies were required to fully understand what mechanisms underpin these discrepancies (Pan *et al.*, 2014). Although we have significantly improved our understanding of *BRCA1* modifiers of risk, a review by Milne and Antoniou (2016) concluded considerable work is still required to establish the relative risks for *BRCA1* and *BRCA2* mutation carriers, that are associated with external factors. To achieve this, Milne and Antoniou called for multidisciplinary collaborations to investigate the interaction of risk factors and incorporate these into bespoke prediction models. Notably for this study, no research has so far been conducted to examine the effect of EDC exposure on individuals with *BRCA1* mutations. Within this thesis we have discussed the role of

the *BRCA1* gene in the cell cycle and in DNA repair. Furthermore, in Chapters 2 and 3, we have shown that EDC exposures have the ability to elicit changes to *BRCA1* expression, comparably to what is presented in the wider literature (Singleton *et al.*, 2006; Fernandez *et al.*, 2012). Authors have previously hypothesised that individuals with a mutation in the *BRCA1* gene may have an increased susceptibility to the effects of oestrogen and oestrogen-mimicking compounds (Jones *et al.*, 2010; Fernandez *et al.*, 2012), yet no study has tested this theory in response to xenoestrogen exposure in primary mammary cells donated by *BRCA1* mutation carriers. The potential for lifestyle and environmental factors to increase the risk of *BRCA1* mutation carriers is of great relevance to risk prediction and management of this predisposed population. As *BRCA1* mutation carriers have such a high lifetime risk of developing breast cancer, any factor that adds to this risk can translate into significant impacts on the absolute risks of developing the disease (Antoniou *et al.*, 2008, 2010). This, in turn, has substantial implications for risk management and the ability to provide personalised care. Consequently, it is of paramount importance that we understand whether EDC exposures can contribute to the likelihood of breast cancer development in this high-risk population.

5.1.3. Chapter scope

Within this chapter, we aimed to understand the effect of BPA and propylparaben exposure on individuals with and without *BRCA1* mutations, in a representative primary 3D co-culture model. We addressed this aim by investigating the following research questions:

1) Can BPA or propylparaben induce differential gene expression in primary cells from patients with and without *BRCA1* mutations?
2) Does exposure to BPA or propylparaben alter the methylation profile of primary cells from patients with and without *BRCA1* mutations?
3) Do any observed EDC-induced epigenetic modifications correlate with gene expression changes?

4) Are EDC-induced genetic or epigenetic changes associated with processes associated with breast cancer risk and are enriched processes comparable between the general population and *BRCA1* mutation carriers?

5.2. Methodology

5.2.1. Concentration selection and chemical handling

Solutions of BPA and propylparaben were of analytical grade (>95% purity) and all concentrations were prepared as $1x10^{-3}$ M stock in 100% HPLC-grade ethanol in glass vials. Stocks and successive dilutions were stored at -20°C. Solvents did not exceed 0.5% of the co-culture medium to avoid ethanol toxicity. For each patient, samples were exposed to $1x10^{-7}$ M BPA or propylparaben. This concentration was selected based on the results observed in Chapter 4. The concentration of $1x10^{-7}$ M is closer to higher tissue levels reported in the literature (Barr *et al.*, 2012; Lan *et al.*, 2015) and was selected to increase the likelihood of detecting an effect. As in previous chapters, 100% EtOH was used as a negative control.

5.2.2. Primary cell isolation and routine culture

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Dorset, UK). Ductal organoids originated from reduction mammoplasty specimens or preventative mastectomy procedures. Briefly, organoids were digested with 0.05% trypsin-EDTA, 0.4 mg/ml DNAse solution at 37°C for 15 minutes, following an established protocol (Gomm *et al.*, 1995). Epithelial, myoepithelial and fibroblast cells were then isolated through fluorescence activated cell sorting. Cells were provided by the Breast Cancer Now Cell Bank after the above processing steps and after being passaged at least once (for an in-depth explanation of the pre-processing steps refer to Carter *et al*., 2017). Due to cell type availability at the Breast Cancer Now Cell Bank, it was not possible to obtain patient-derived endothelial cells. This meant we were unable to replicate the assay from Chapter 4 with the primary cells. However, the cell bank did offer patient-derived

myoepithelial cells. Whilst these were not incorporated into the culture model previously, we saw this as an opportunity to include an alternative cell type that also played an important role within the normal mammary gland.

As described in Chapter 1, the normal mammary epithelium is comprised of an inner layer of secretory cells (luminal epithelial cells) and an outer layer of basal lamina (myoepithelial cells). The basal cells form an essential selective barrier separating the epithelial cells from the ECM (Faraldo *et al.*, 2006; Pandey, Saidou and Watabe, 2010; Weigand *et al.*, 2016). Myoepithelial cells play a critical role in mammary gland morphogenesis within all stages of development, regulating the proliferation and differentiation of epithelial cells (Gudjonsson *et al.*, 2005; Makarenkova and Dartt, 2015). For instance, mapsin is expressed solely by the myoepithelial cells, but has been shown to be essential for the appropriate regulation of duct formation (Pandey, Saidou and Watabe, 2010; Sopel, 2010). During lactation, myoepithelial cells participate in milk ejection, where oxytocin initiates myoepithelial contractions around the breast ducts, leading to milk expulsion (Breton, Di Scala-Guenot and Zingg, 2001; Hawley *et al.*, 2018). Myoepithelial cells are also important in breast tumourigenesis. Myoepithelial cells act as cancer suppressors, due to their involvement in maintaining the normal breast function (e.g. maintaining polarity, regulating proliferation and filtering endocrine and paracrine signalling) and their ability to secrete tumour suppressor proteins like mapsin (Pandey, Saidou and Watabe, 2010; Sopel, 2010; Ingthorsson *et al.*, 2015). These functions have been evidenced to limit cancer progression and metastasis (Lakhani and O'Hare, 2001; Gudjonsson *et al.*, 2002; Jones *et al.*, 2003; Barsky and Karlin, 2005; Polyak and Hu, 2005; Duivenvoorden *et al.*, 2017). Hence, including patient derived myoepithelial cells in the co-culture model, represents a valuable opportunity include an important component of the mammary gland that contributes to the behaviour of epithelial cells. Patient derived myoepithelial cells have been included in collagen 3D mammary co-cultures before (Carter *et al.*, 2017), however such assays have not been utilised for the study of EDCs. In total, samples from six patients were kindly donated by the Breast Cancer Now Cell Bank; three samples from *BRCA1* mutation carriers and three from patients with no known family history of breast cancer (Table 5.1).

Table 5.1. Biotype, Patient ID, donor age and cell passage number of tested primary cells.

			Cell passage number		
Biotype	Patient ID	Patient Age	Epithelial	Myo-epithelial	Fibroblast
Wildtype	NP1	33	1	2	3
	NP2	25	1	2	3
	NP3	28	1	2	3
BRCA1 mutation carrier	BRCAP1	32	1	2	3
	BRCAP2	41	1	3	3
	BRCAP3	27	1	3	3

Following isolation, epithelial cells were cultured for one passage in 25 cm^2 canter-neck tissue culture flasks pre-coated rat tail collagen I solution (2.3% collagen in $2x10^{-2}$ M acetic acid). Cells were maintained in DMEM: F12 medium, supplemented with 10% FBS, 1% pen/strep, 1% fungizone (Invitrogen, Thermo Scientific, Loughborough, UK), 0.1% apo-ternsferrin, 0.1% human EGF and 0.1% insulin. Myoepithelial cells were also cultured in 25 cm^2 canter-neck tissue culture flasks pre-coated Rat Tail collagen I solution. Myoepithelial cells were kept in HuMEC medium (Invitrogen, Thermo Scientific, Loughborough, UK), supplemented with 10% pen/strep, 0.5% fungizone, 0.4% BPE, 0.1% hydrocortisone, 0.1% human EGF and 0.02% gentamicin. Finally, fibroblasts were cultured in 75 cm^2 canter-neck tissue culture flasks and maintained in DMEM: F12 medium supplemented with 1% pen/strep, 1% fungizone and 10% FBS. All cell types were maintained in an incubator at 37°C and 5% CO_2. At optimal confluence cells were trypsinised, as described in Chapter 4, ready for use in the 3D co-culture assay.

5.2.3. Primary 3D co-cultures

Three-dimensional co-cultures were performed as described in Chapter 4, with minor modifications to account for the use of primary cells. Epithelial cells were suspended in DMEM: F12 medium at a final concentration of $2x10^5$ cells/ml. This was then combined with a 1:1 ratio with 4 mg/ml Rat Tail Collagen I (Scientific Laboratory Supplies, Nottingham, UK), stabilised with 1 M NaOH and 1 M HEPES, achieving a final collagen concentration of 2 mg/ml. Next, 1 ml of collagen/cell suspension solution was added to a tissue culture Insert (0.4 µm pore Ø, 2 x 10^6

pore/cm^2 density; Sarstedt, Leicester, UK), before being placed in a 6-well plate and allowed to polymerise at 37˚C and 5% CO_2 for 1 hour. Myoepithelial cells (5x10^4 cells/ml) and fibroblasts (1.75x10^4 cells/ml) were then combined in epithelial cell assay medium. Cell ratios were comparable to published experiments carried out by Li and Lu (2011) and Carter *et al.,* (2017). The myoepithelial and fibroblast cell solution was then added to the 6-well plate (2 ml/well). As in Chapter 4, an additional 1 ml epithelial cell medium was added to the tissue culture inserts and the collagen gels were detached, allowing them to float (Figure 5.1). Cultures were maintained at 37˚C and 5% CO_2, with medium being replaced every three days for a total incubation time of 14 days. Solvents containing chemical treatments were added to the primary co-culture medium every three days to the entirety of the 14-day incubation.

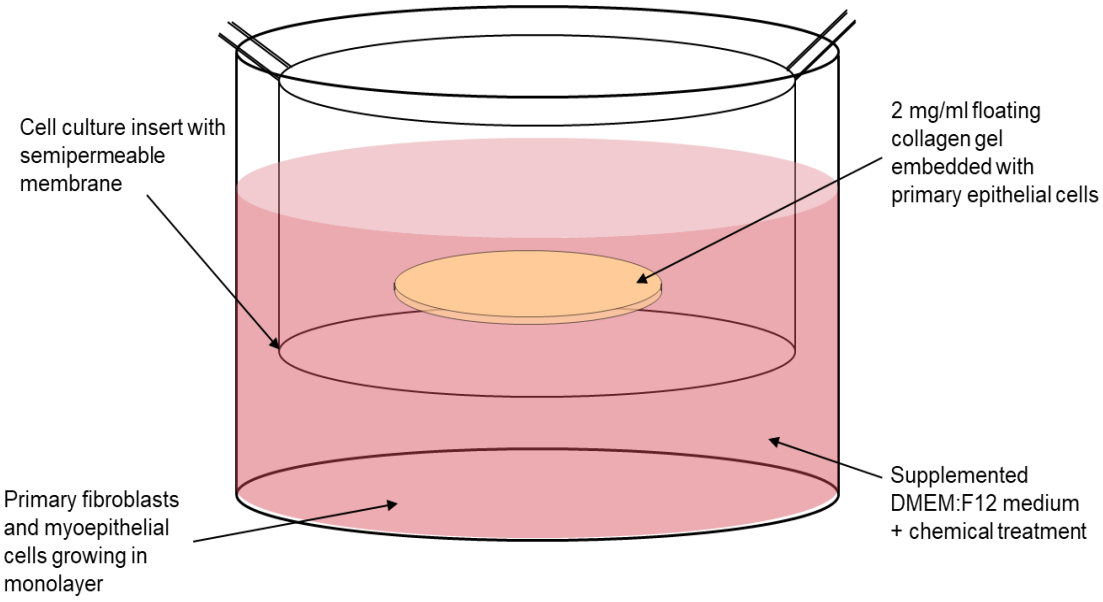

Figure 5.1. Three-dimensional primary co-culture system. Primary donor epithelial cells are cultured within a floating collagen gel, separated from fibroblast and myoepithelial cells by a semi-permeable membrane. This allowed for paracrine interactions between cell types without combining them in culture. Cells are maintained in DMEM: F12 medium for a total of 14 days.

5.2.4. Genomic analysis

5.2.4.1. RNA isolation

Total RNA was isolated from primary epithelial cells with the RNeasy Mini Kit (Qiagen. Manchester, UK), following manufacturer instructions. Impurities were removed using DNase I treatment and the final RNA purity was confirmed using 260/280 and 260/230 ratios. RNA samples were then stored at -80 °C before being shipped on dry ice for RNA sequencing.

5.2.4.2. RNA-sequencing

Quantifying gene expression using traditional PCR based techniques can be incredibly useful, however this method has a major limitation in terms of the number of genes that can be profiled simultaneously (van Hal *et al.*, 2000; Smith and Osborn, 2009). The development of sequencing technologies allows for the testing of thousands of genes, providing a fast, reliable and effective method for understanding gene expression profiles on a large scale (Buermans and den Dunnen, 2014; Reuter, Spacek and Snyder, 2015; Conesa *et al.*, 2016). RNA-seq is the first sequencing based tool to allow high-throughput quantification of gene expression (Wang, Gerstein and Snyder, 2009) and is not limited by previous knowledge of genomic sequences like PCR or microarray techniques. RNA-seq has been cited as highly reproducible and enables the detection of differentially expressed genes in the presence of very small changes (Wang, Gerstein and Snyder, 2009). During RNA sequencing, millions of short strings, commonly referred to as reads, are sequenced from random positions within the input RNA. After, these reads are mapped to a reference genome during bioinformatic analysis, revealing a transcriptional map. The number of reads aligned to each position on this map provides a measurement of gene expression, generally reported as fragments per kilobase per million mapped reads (FPKM; Finotello and Di Camillo, 2015).

The following processing steps were performed by Source Bioscience RNA-Seq Facility (Nottingham, UK). RNA was first reverse transcribed and adaptors were attached at each end of the molecule using the SMARTer Stranded Total RNA-Seq (Pico input mammalian, Clontech, Takara Bio Europe, Saint-Germain-en-Laye, France), according to manufacturer instructions to

create a library. This kit chosen based on its ability to process the low volume of RNA isolated from the primary 3D cultures. Next, the library was assessed for quality and sequenced using Illumina HiSeq 4000 whole-genome sequencing technology, which has been cited as cost effective and able to produce highly accurate readings in a short time period (Reuter, Spacek and Snyder, 2015). Single-end sequencing was chosen to address the aims of this chapter. Single-end sequencing refers to sequencing the cDNA from one end only. This is the simplest way to approach sequencing and is also considerably quicker, more economic and has low input DNA requirements (as little as 10 ng). In comparison, whilst paired-end sequencing has been reported to have a higher accuracy, it is considerably more expensive and time consuming. It was determined that for the aims of this study the level of accuracy from single-end sequencing was sufficient, as we were predominately interested in identifying differentially expressed genes. Appropriate RNA-seq read length is an area that has been consistently debated. Often it has been assumed that longer read lengths produce better results, however a study by Chhangawala et al. (2015) demonstrated that this was not the case. Within their paper, authors concluded that, with the exception of 25 base pair (bp) readings, there was very little difference in the detection of differentially expressed genes regardless of read length and that there was no benefit of increasing the read length past 50 bp, or performing paired-end sequencing, unless the aim of the study was to detect splice junctions. Consequently, to conserve substantial resources, single-end 50 bp sequences were suitable and selected for this study (sequencing information Appendix I).

4.1.1.1. Bioinformatic analysis of gene expression

RNA-sequencing data analysis was undertaken in collaboration with Dr. Cristina Sisu (Brunel University), following an established analysis workflow (Figure 5.2; Sisu *et al.,* 2014; Gerstein *et al.*, 2014). Briefly, fastq. read files were obtained from Source Bioscience after sequencing. We then mapped single-end reads to the hg19 human genome reference sequence using TOPHAT2 (version 2.1.1 with Bowtie 2.2.6.0) following standard conditions. Next, using the resulting alignment files, we identified mapped reads with a mapping quality score >30 for subsequent

analysis using Samtools (version 0.1.19). Using Cufflinks (version 2.2.1) and the human genome annotation from GENCODE (version 19), we quantified gene expression based on FPKM. To calculate differential expression, the Cuffdiff process from Cufflinks package was used under standard conditions. Genes were classed as significantly differentially expressed based on the criteria of log2 (fold change) >2 and p <0.00001. Genes that passed the threshold of log2 (fold change) >2 and p <0.05 were categorised as potentially differentially expressed.

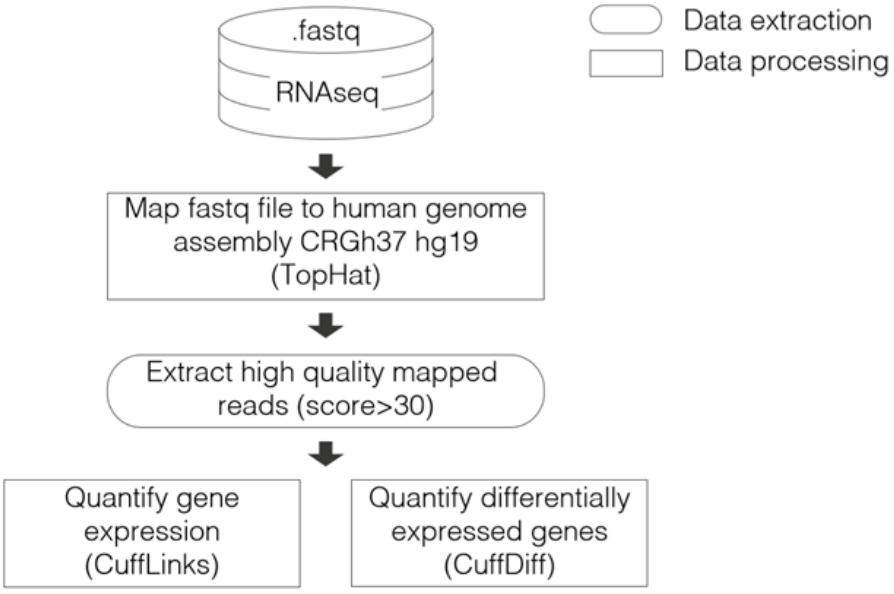

Figure 5.2. RNA-sequencing data analysis pipeline. Main RNA-sequencing analysis steps include mapping the data to a reference genome, extracting reads and quantifying gene expression and differentially expressed genes between treatments.

4.1.2. Epigenetic analysis

Isolation of genomic DNA and bisulfite conversion were performed as described in Chapter 4. Again, bisulfite converted samples were analysed using the Infinium MethylationEPIC BeadChip microarray at UCL Genomics (Institute of Child Health, London, UK).

4.1.2.1. Bioinformatic analysis of methylation profiles

Bioinformatic analysis was undertaken in R (Version 3.4.0.) using Minfi and Champ packages (Aryee *et al.*, 2014; Morris *et al.*, 2014; Fortin, Triche and Hansen, 2016) within Bioconductor. An established processing pipeline was followed to include quality control, filtering and

normalisation (Maksimovic *et al.*, 2017). Reads with p >0.01 were removed and Beta-values were extracted. The same process outlined in Chapter 4 was followed to identify confounders and adjust for batch effects.

EDC treated samples were compared to negative controls to identify DMPs. Analysis of *BRCA1* mutated and normal patients was carried out independently to ensure differences between the groups could be identified. Due to significant patient variation within cohorts, it was decided that DMP thresholds would be relaxed to provide an indication of epigenetic alteration (discussed further in results section).

4.1.3. Functional analysis

Genes that were found to be differentially expressed (p <0.05) were grouped based on their associated GO processes using the previously described DAVID software (Chapter 4). This process was undertaken separately for individuals with and without *BRCA1* mutations, with the aim of identifying unique and common cellular processes that may be altered in response to EDC exposure. Functional clusters with an enrichment score >1 were presented.

5.3. Results

Whilst the aim of this study was not to assess morphological changes induced by EDC exposures, it was important to ensure similar cell behaviour was occurring to demonstrate the assay was suitable for use with primary cells. Despite a slower rate of proliferation in comparison to the MCF-12A cell line, both biotypes formed acini-like spheroids that resembled the morphological structures reported in Chapter 4. In general, structures appeared less developed than previous reports of acini morphology in these cells (Carter *et al.*, 2017), however, this discrepancy may be due to epithelial and myoepithelial cells being separated by the semi-permeable membrane. This comparable development of the primary cells to previous chapters

within this thesis and the wider literature, indicated the assay was suitable to investigate the research questions of this chapter.

5.3.1. Significant genetic differences between individuals with and without *BRCA1* mutations

Before proceeding onto downstream analysis, we wanted to determine how the genome and epigenome differed between patients with and without a *BRCA1* mutation. To do this, we first carried out quality control steps on the RNA-seq data. When looking at the expression level of each patient, it was apparent that the majority of genes had a low expression of <50 FPKM. This pattern continued in all of the patients tested and across treatments, regardless of the biotype (Figures 5.3). Although each patient followed a similar pattern of gene expression, we wanted to further evaluate the variability between patients. Therefore, we directly compared gene FPKM values of patients between biotypes As the majority of genes were expressed a low-levels, we concentrated this analysis on FPKM values between 0-1000 to visualise and compare the transcription of these low expressed genes more effectively (Figures 5.4).

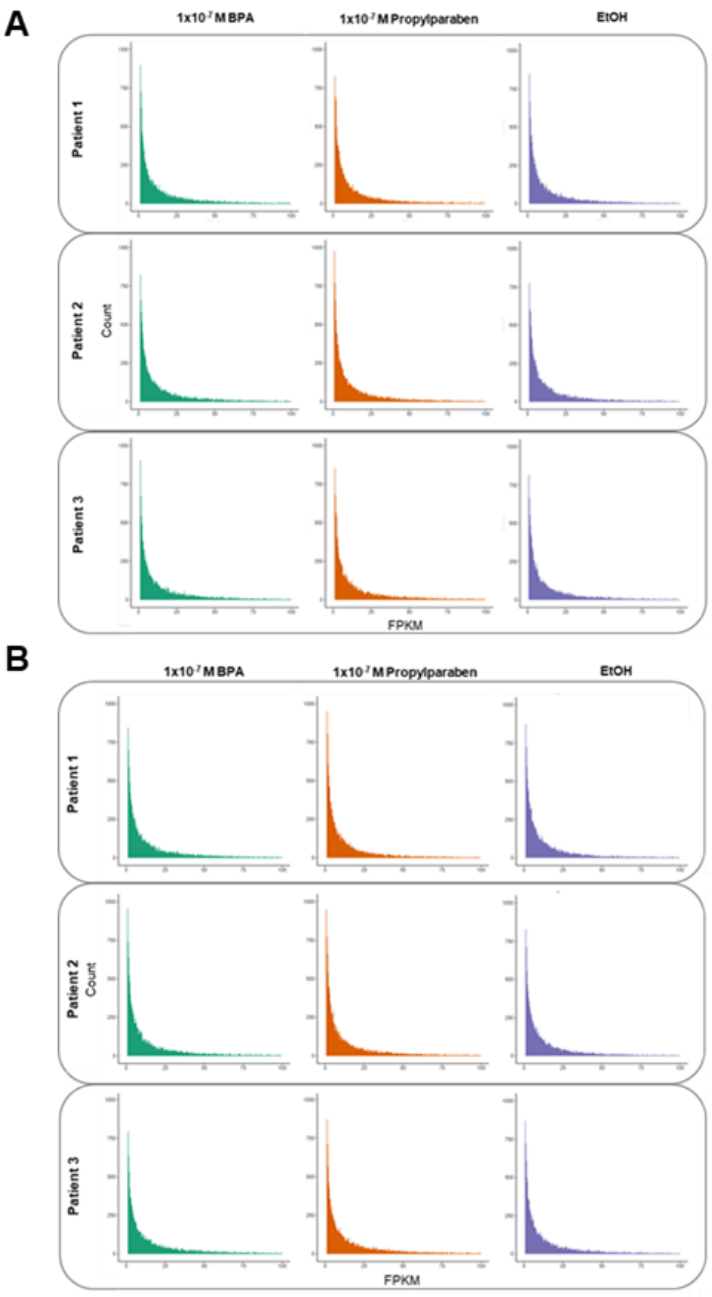

Figure 5.3. Distribution of fragments per kilobase of transcript per million mapped read (FPKM) values of patient cells. Depiction of number of genes (y-axis), sorted by their expression levels (FPKM value; x-axis) as determined by RNA-seq analysis for wildtype patients (A) and those with a *BRCA1* mutation (B). Across all patients and treatments, the majority of genes displayed a low level of expression (<50 FPKM).

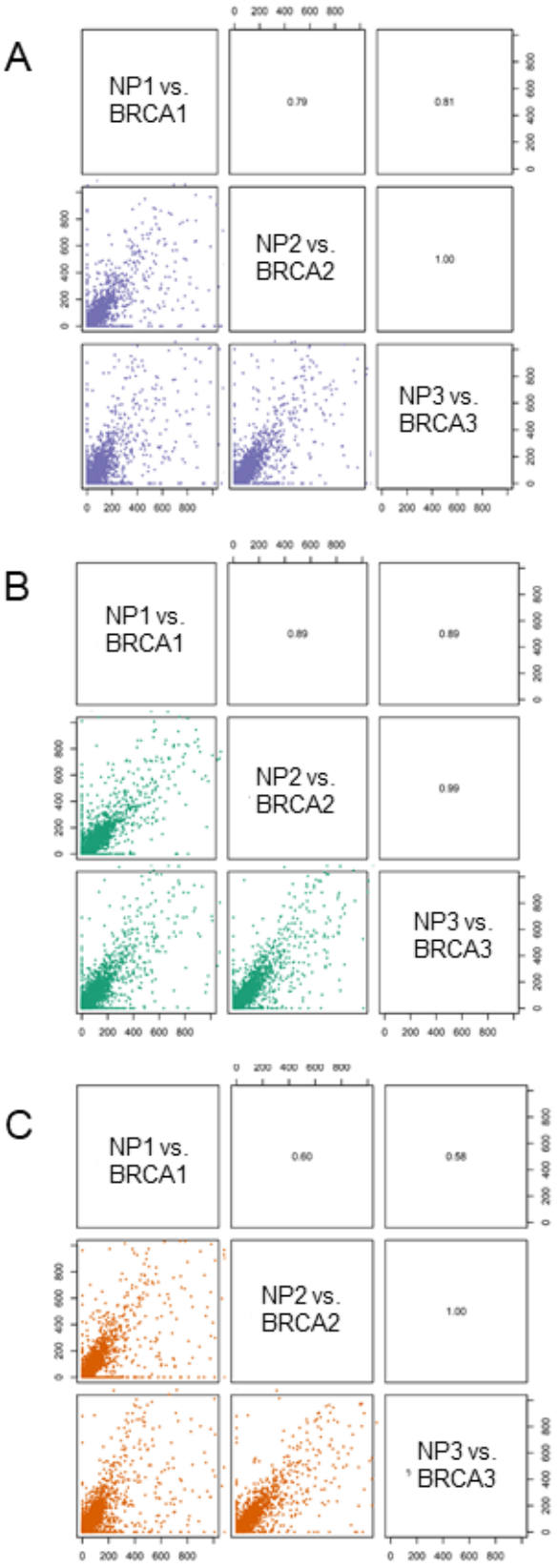

Figure 5.4. Comparison of fragments per kilobase of transcript per million mapped read (FPKM) values between 0-1000. Comparison of gene FPKM reads between 0-1000 for each patient, divided by treatment with EtOH (A), $1x10^{-7}$ BPA (B) and $1x10^{-7}$ propylparaben (C).

We then examined the genetic differences between individuals with and without *BRCA1* mutations. When the data from patients were pooled, we identified 50 highly significant differentially expressed genes between the two biotypes, which translated to 30 genes that were increased and 20 genes with a lower expression (Figure 5.5) in *BRCA1* mutation carriers. Notable genes that were seen to be differentially increased, included *CCND2* (6.25 LogFC, p <0.00001) and a marker of basal-like breast cancer *MMP-7* (2.02 LogFC, p <0.00001). We also saw a significant decrease in the expression of mammary stem cell marker *KRT6* (keratin 6; -3.74 LogFC, p <0.00001). When considering genes with p <0.05, an additional 770 genes (526 down-regulated and 244 up-regulated) were found to be differentially expressed in patients with *BRCA1* mutations.

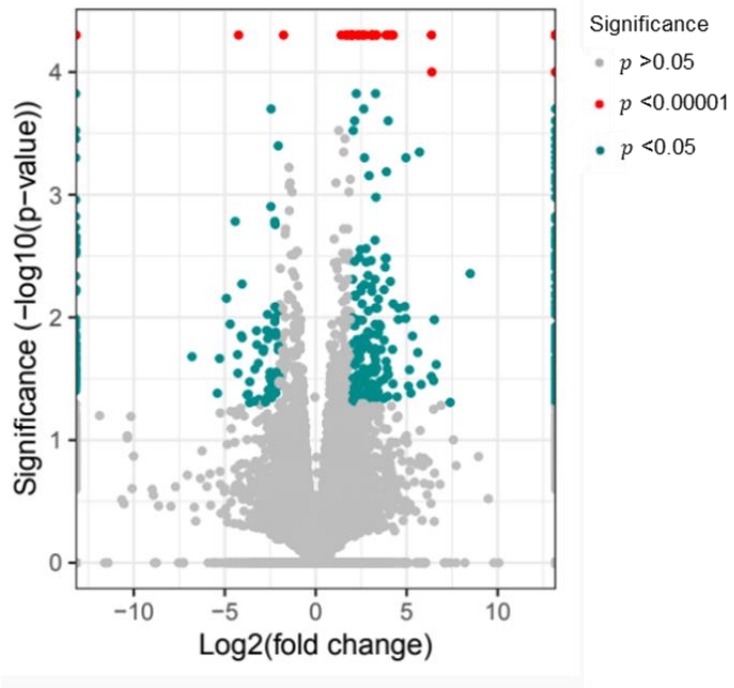

Figure 5.5. Differential expression analysis of individuals with and without *BRCA1* mutations. Differential expression is determined by log2 (fold change) >2 and p <0.05, with genes passing this threshold shown in blue. Genes significant to p <0.00001 are shown in orange. Data points at the edge of the x-axis correspond to + or – infinity. This occurs due to the comparison of two values where one value equals 0.

5.3.2. Differences in epigenetic profiles of individuals with and without *BRCA1* mutations

After observing genetic differences between individuals with and without *BRCA1* mutations, we wanted to elucidate whether differences were also present at the epigenetic level. Again, we carried out numerous pre-processing steps to ensure the quality of the data and subsequent analysis were of high standard. Sample quality was assessed based on the number of probes that failed to meet the threshold of p <0.01. None of the samples had more than 8% of probes that did not meet this threshold (Figure 5.6). Indeed, with the exception of a single *BRCA1* mutation carrier (BRCAP1), samples had less the 2% of probes removed, as a result of this criteria.

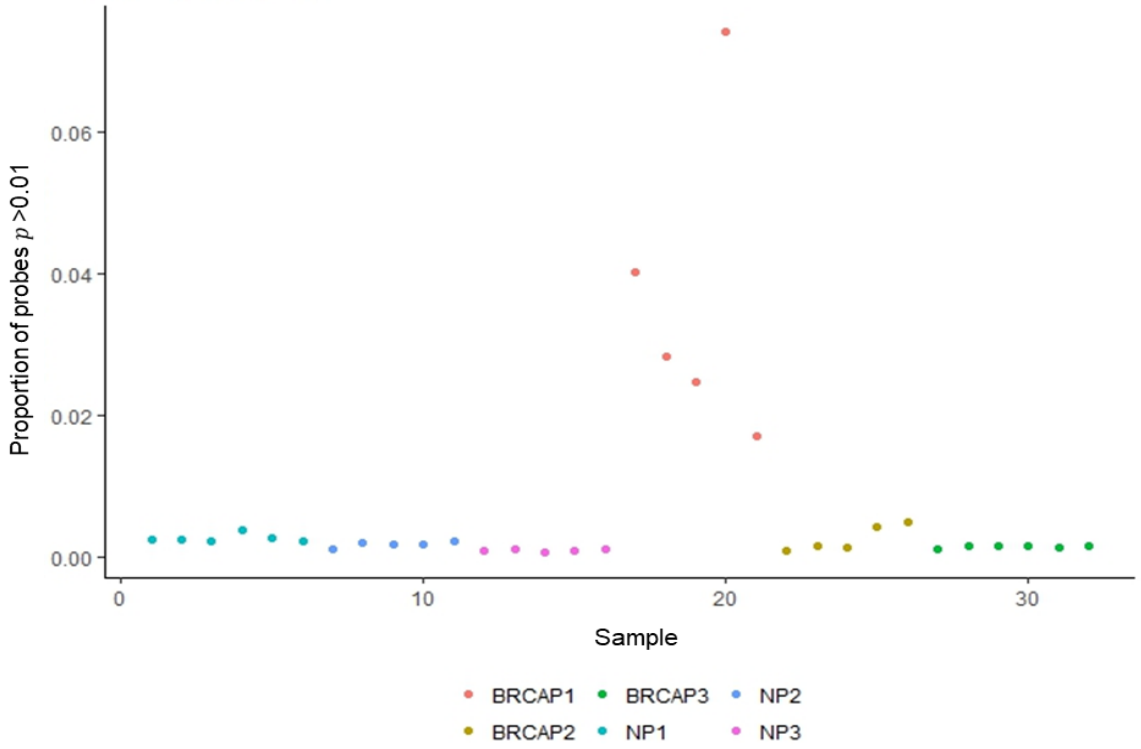

Figure 5.6. Proportion of probes removed from methylation analysis by sample and donor. The quality of the probes was generally high with no more than 8% of probes being removed from any given sample.

SVD/PCA analysis highlighted biotype, patient ID and slide as significant confounding factors (p <0.001; Figure 5.7), which had the potential to affect downstream analysis. We examined this relationship further by producing a multidimensional scaling plot that depicted the variability between patients. This predominantly visual analysis represented the distances or dissimilarities between patients. Where patients are shown close together this represented similarities in the epigenome. Likewise, patients depicted further apart implied significant differences in the epigenetic profile. As can be seen from Figure 5.8, the most substantial variation observed originated from patient ID (Figure 5.8A). Whilst changes could be seen between the different EDC treatments, the epigenetic profiles remained relatively close to the control samples. Furthermore, when considering individuals with and without *BRCA1* mutations, we saw a much larger patient variation in individuals with the *BRCA1* mutation (Figure 5.8B) than those that do not carry the mutation. Interestingly, NP2 and NP3 appeared to possess relatively similar epigenetic profiles, whereas NP1 was depicted as dissimilar. This observation may be due to age-induced epigenetic differences, with NP1 being slightly older than the other patients included in this category. However, it is important to note that there are a multitude of other factors that may be responsible for this difference, including lifestyle factors. Unfortunately, we were unable to obtain this information for the tested patients, so it is not possible to speculate further in the present study.

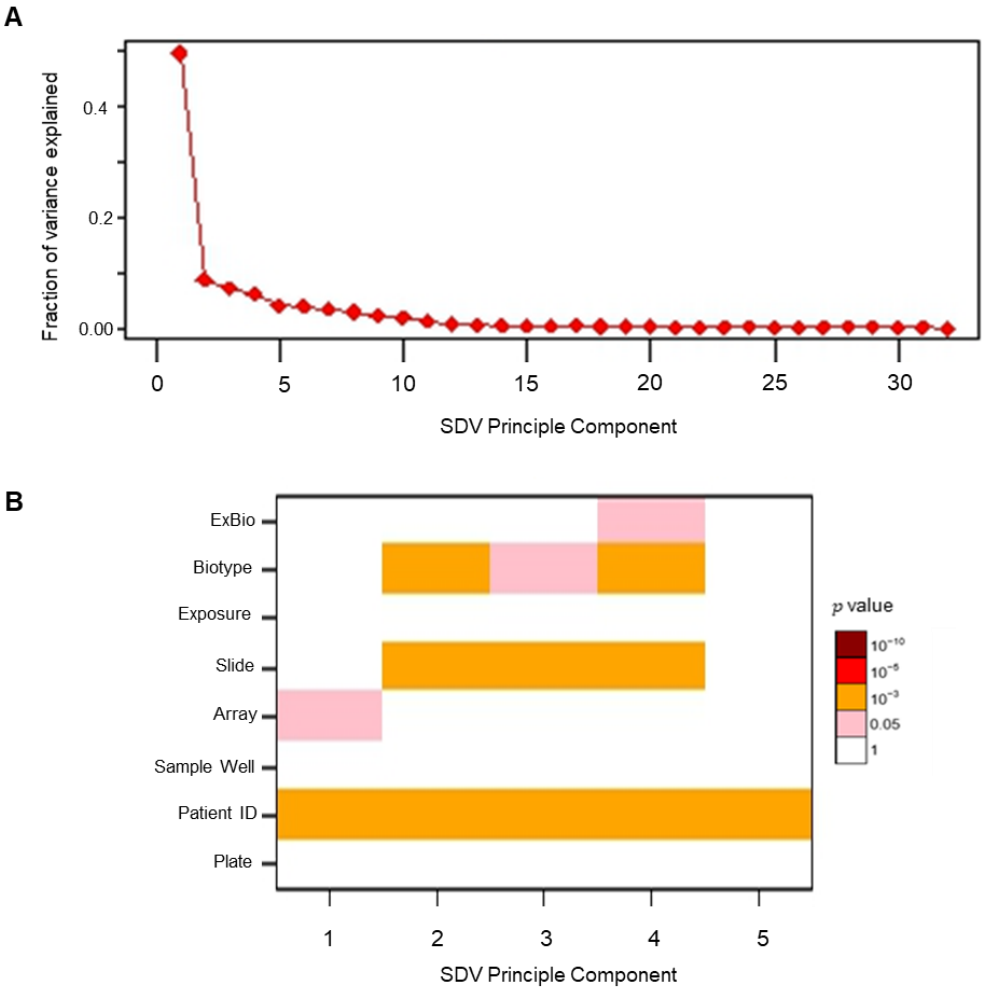

Figure 5.7. Significant confounders identified in primary cell data using singular value decomposition and principle component analysis. Several components were found to account for a significant amount of the variation observed (A). Patient ID, Biotype and Slide were identified as significant confounders, with p <0.05 (B).

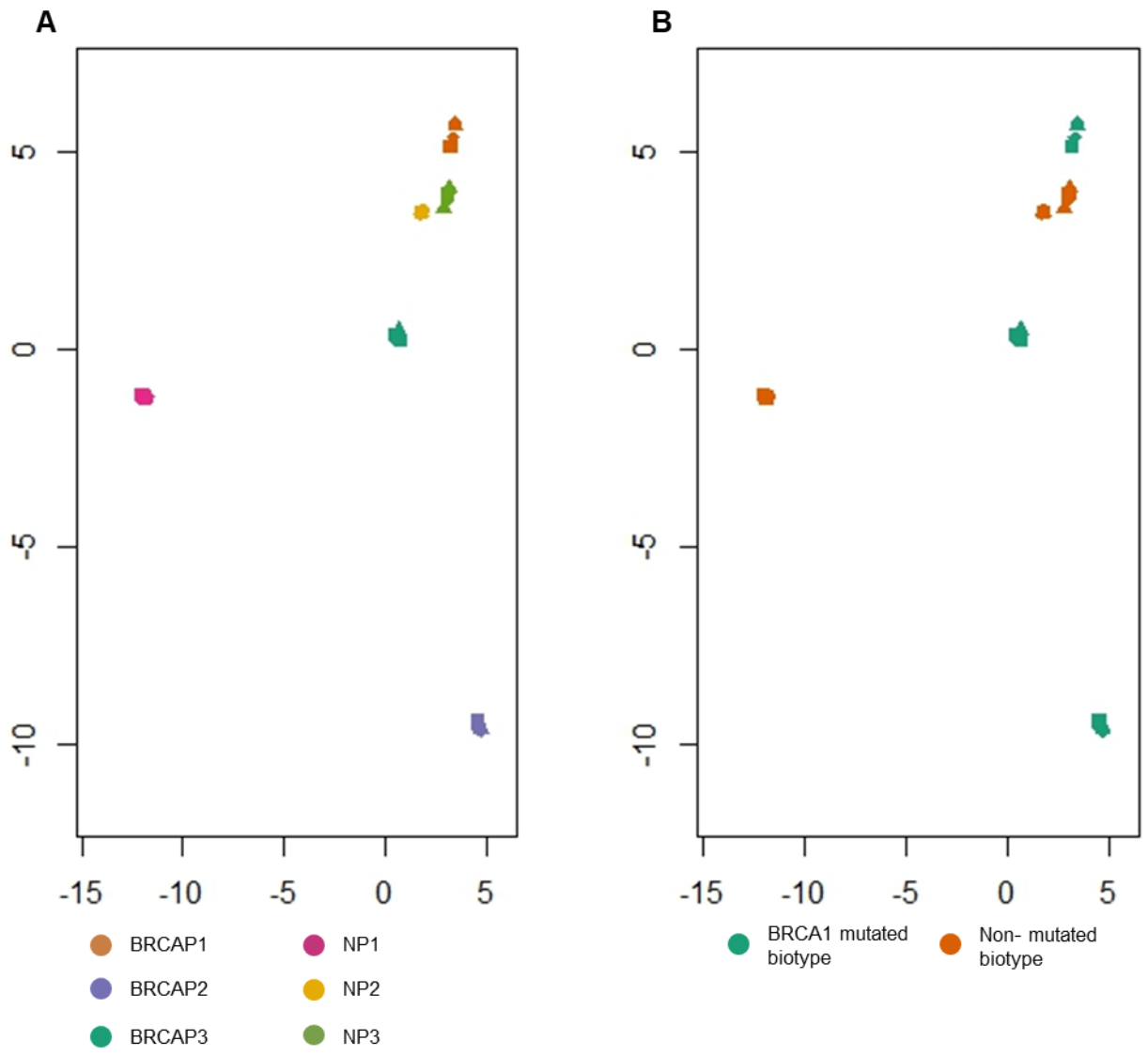

Figure 5.8. Multidimensional scaling analysis of patient samples by patient ID and biotype. Distance of samples indicates the similarity of the epigenetic profile. Closely plotted samples suggest similar epigenetic profiles, whereas samples located further apart demonstrate dissimilarity. (A) Patient ID caused a substantial amount of variation in how similar samples were. (B) Biotype also distinguished patients, with the largest differences seen in the *BRCA1* mutation biotype.

Such significant differences have the ability to impact downstream analysis, which may mask true EDC-induced DMPs. Consequently, we applied ComBat to reduce these effects, which successfully removed the presence of confounders (Appendix II). We then compared the differences between individuals with and without *BRCA1* mutations using Delta Beta-values to explore how the biotypes differed in terms of their epigenetic profile. Due to intra-biotype patient variability, no DMPs could be identified as significant to p <0.05 and it could be seen that the

majority of probes were not changed (Figure 5.9). However, we observed a considerable number of DMPs with a Delta Beta-value >0.1 (representing >10% difference in methylation), indicating the biotypes had considerable differences in some regions of the epigenome, despite not being deemed statistically significant. A total of 2827 DMPs were identified that mapped onto 1952 genes, with 801 genes seeing a lower methylation and 1151 genes having higher methylation in individuals with *BRCA1* mutations when compared with wildtype individuals. These data, combined with the observed genetic differences between individuals with and without *BRCA1* mutations, supported the presence of two distinct biotypes.

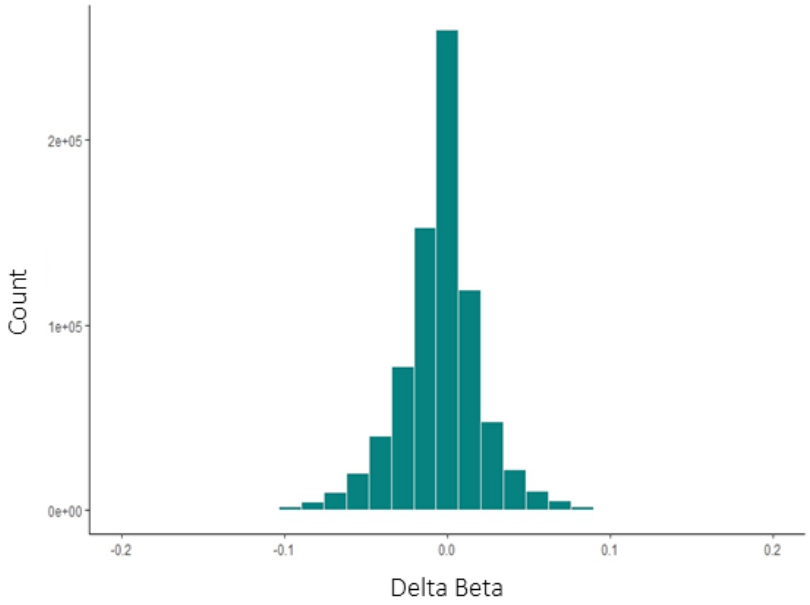

Figure 5.9. Distribution of Delta Beta-values between individuals with and without *BRCA1* mutations. Most probes have a Delta Beta-value of 0, indicating homogeneity between the two biotypes. Numerous probes have a Delta Beta-value of between 0.1 and 0, with a Delta Beta-value of 0.1 suggesting a 10% difference between the distinct biotypes.

5.3.3. Transcriptional alterations are induced by EDC exposure in individuals without *BRCA1* mutations

As the data strongly suggested there were differences between individuals with and without *BRCA1* mutations, both at the genetic and epigenetic level, we treated the biotypes as two distinct populations for further analysis. Firstly, we wanted to determine whether exposure to

BPA or propylparaben could alter the genome of wildtype individuals. We saw that both compounds were able to elicit effects on gene expression, however significant variation between patients was seen (Figure 5.10). For instance, neither exposure to BPA or propylparaben resulted in genes being highly significantly changed (p <0.00001) in NP1. In contrast, both NP2 and NP3 had numerous highly significant genes (p <0.00001).

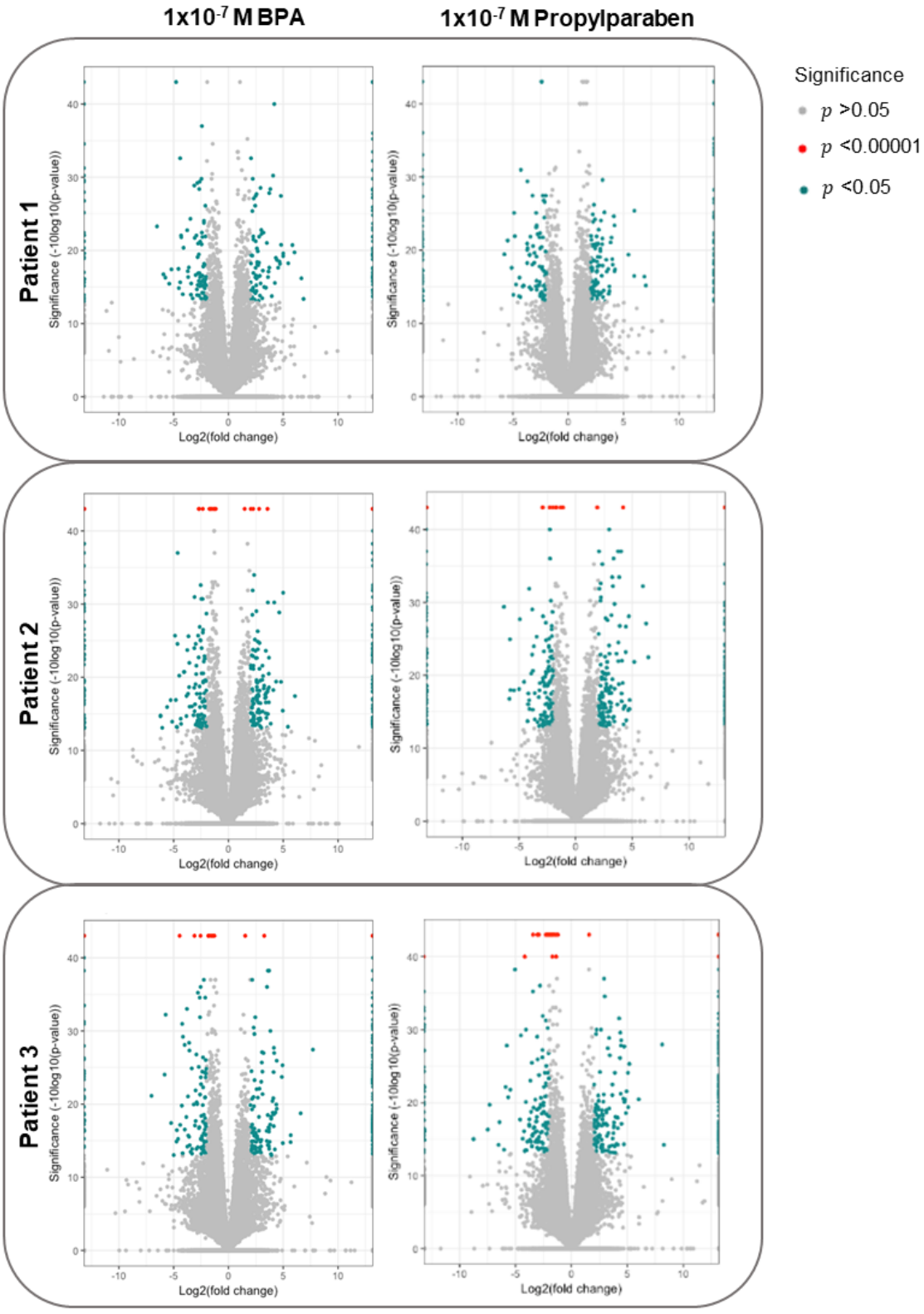

Figure 5.10. Differential gene expression of wildtype primary cells exposed to BPA and propylparaben. Differentially expressed genes identified by patient. Differential expression is determined by log2 (fold change) >2 and p <0.05, with genes passing this threshold shown in blue. Genes significantly differentiated to p <0.00001 are shown in orange.

We then pooled the patient data to see if a general trend in gene expression change could be found in response to the two compounds. A total of 32 genes were identified as highly significantly down-regulated after exposure to $1x10^{-7}$ M propylparaben (log fold change >2, p <0.00001). Eleven genes in this category were small nucleolar RNAs (snoRNAs). In addition, 25 genes were highly significantly up-regulated. This included genes such as Amphiregulin (*AREG;* 3.02 LogFC p <0.00001), Aurora-A (*AURKA;* 4.18 LogFC, p <0.00001) and regulator of G protein signalling 6 (*RGS6;* 1.85, p <0.00001), all of which have been previously associated with breast cancer risk (Couch *et al.*, 2007; Maity *et al.*, 2011, 2013; Peterson *et al.*, 2015; Feimeng Zheng *et al.*, 2016). When expanding the significance threshold to p <0.05, we recorded a further 484 down-regulated genes and 598 up-regulated genes in response to propylparaben exposure. This included an additional 13 down-regulated snoRNAs and 18 genes in the Zinc finger family with reduced expression. Interestingly, *TP53 (2.07* LogFC, p = 0.04*)* and *STAT4 (*signal transducer and activator of transcription 4*;* 2.89 LogFC, p = 0.01*)* were both seen to be up-regulated in this category.

Comparable results were seen in response to BPA exposure. We found 52 genes that were considered significantly differentially expressed (p <0.00001) after exposure to $1x10^{-7}$ M BPA. This translated into 26 down-regulated genes and 26 up-regulated genes. As with propylparaben exposure, this list included several down-regulated snoRNAs. Genes reported to be up-regulated, for instance, angio associated migratory cell protein (*AAMP;* 4.66 LogFC, p <0.00001) and polymeric immunoglobulin receptor (*PIGR;* 3.51 LogFC, p <0.00001), have previously been linked to breast cancer development (Harris, Caleb and South, 1975; Welinder *et al.*, 2013; Yin, Sanders and Jiang, 2013). Again, more genes were identified as altered in response to BPA when the significance threshold was expanded (p <0.05). A further 447 genes exhibited a decreased expression, whilst 585 genes were up-regulated. Genes known to be up-regulated in breast cancer tissue samples, including talin 2 (*TLN2;* 2.41 LogFC*,* p = 0.04) and ADP-ribosyltransferase 3 (*ART3;* 3.18 LogFC, p = 0.02), were here seen to be enriched after BPA exposure. As an example, RAD51 paralog D (*RAD51D*), classified as a moderate risk gene for

breast cancer (Lose *et al.*, 2006; Alshareeda *et al.*, 2016), was seen to be up-regulated (2.02 LogFC, p = 0.02) after exposure to BPA. Included in the list of down-regulated genes was the apoptosis regulator *BCL2* (-2.48 LogFC, p = 0.02), which represses apoptosis when expressed (Lam *et al.*, 1994; Kirkin, Joos and Zörnig, 2004; Williams and Cook, 2015).

5.3.4. Transcriptional alterations are induced by EDC exposure in *BRCA1* mutation carriers

We then looked at individuals with *BRCA1* mutations to see if comparable EDC-induced genomic changes were present. We first examined patients individually (Figure 5.11). After exposure to BPA, whilst there was individual variation, each of the patients displayed highly significant changes to gene expression (p <0.00001). However, although propylparaben exposure induced mildly significant changes in all patients (p <0.05), only Patient 2 was seen to have highly significant gene changes (p <0.00001). This result confirms individual response variation in individuals with *BRCA1* mutations, comparable to what was observed in individuals with no *BRCA1* mutation.

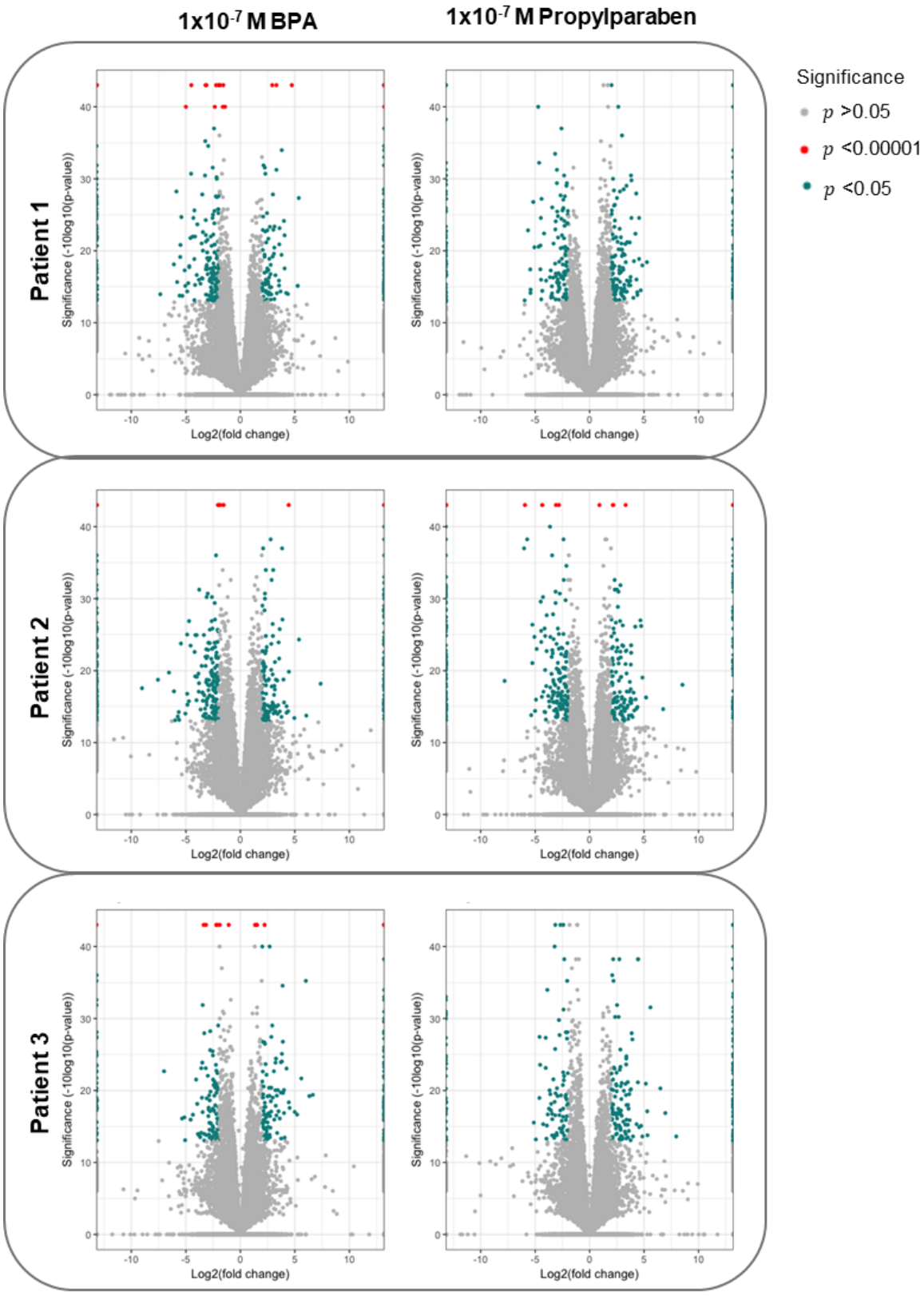

Figure 5.11. Differential gene expression of *BRCA1* mutated primary cells exposed to BPA and propylparaben. Differentially expressed genes identified by patient. Differential expression is determined by log2 (fold change) >2 and p <0.05, with genes passing this threshold shown in blue. Genes significant to p <0.00001 shown in orange.

When the patient data were pooled, we identified 15 highly significantly down-regulated genes and 12 up-regulated genes in response to propylparaben exposure. Notably, we recorded the down-regulation of tumour suppressor gene, *ST18* (suppression of tumorigenicity 18; -4.83 LogFC, p <0.00001). Further, *CDH5* (cadherin 5) expression was enhanced (2.46 LogFC, p <0.00001), which exhibits an increased expression in breast cancer tissues, promoting cell proliferation (Andrews, Kim and Hens, 2012; Fry *et al.*, 2016). When looking at the mildly significant gene changes induced by propylparaben exposure, we uncovered an additional 588 genes with decreased expression and 610 genes with increased expression. Included in this category was the down-regulation of suspected tumour suppressor and DNA-repair gene *RAD51* (-3.21 LogFC, p = 0.03) and *PRRX1* (paired related homobox 1; -4.79 LogFC, p = 0.02), where loss of function has been associated with tumour tissues (Zhu and Sun, 2017). We also saw an increase in *GALNT6* (polypeptide N-acetylgalactosaminyltransferase 6; 2.09 LogFC, p = 0.01) and *LPAR1* (Lysophosphatidic acid receptor 1; 2.51 LogFC, p = 0.004) expression, which is consistently reported in breast carcinomas (Potapenko *et al.*, 2010; Wang *et al.*, 2016).

Significant changes in response to BPA exposure were also seen. We observed the highly significant up-regulation of 25 genes, including amphiregulin (*AREG*; 3.06 LogFC, p <0.00001) that has been reported as enriched in ERα- and HER2 positive breast tumours (Peterson *et al.*, 2015; Schmucker *et al.*, 2018). In addition, 42 genes were highly significantly down-regulated, such as the discs large homolog 5 (*DLG5*; -4.56 LogFC, p <0.00001), which plays a central role in the maintenance of epithelial cell polarity (Liu *et al.*, 2014, 2017). When accounting for genes significant to p <0.05, we highlighted a further 668 down-regulated genes and 537 up-regulated genes.

5.3.5. EDC exposure results in changes to the epigenetic profile of individuals without and without *BRCA1* mutations.

After identifying changes in the genome, we wanted to understand how EDCs interacted with the epigenome of patient-derived cells. Specifically, we wanted to see if alterations could be

induced in either of the biotypes and whether any of the observed epigenetic changes could be correlated with the changes reported in the genome. We first explored the distribution of Delta Beta-values in individuals without *BRCA1* mutations. When assessing samples exposed to BPA or propylparaben, we saw that the majority of probes had a Delta Beta-value <0.1, with very few probes expressing more than a 10% change in response to EDC exposure (Figure 5.12A,B). A similar distribution was seen in individuals with *BRCA1* mutations (Figure 5.12C,D).

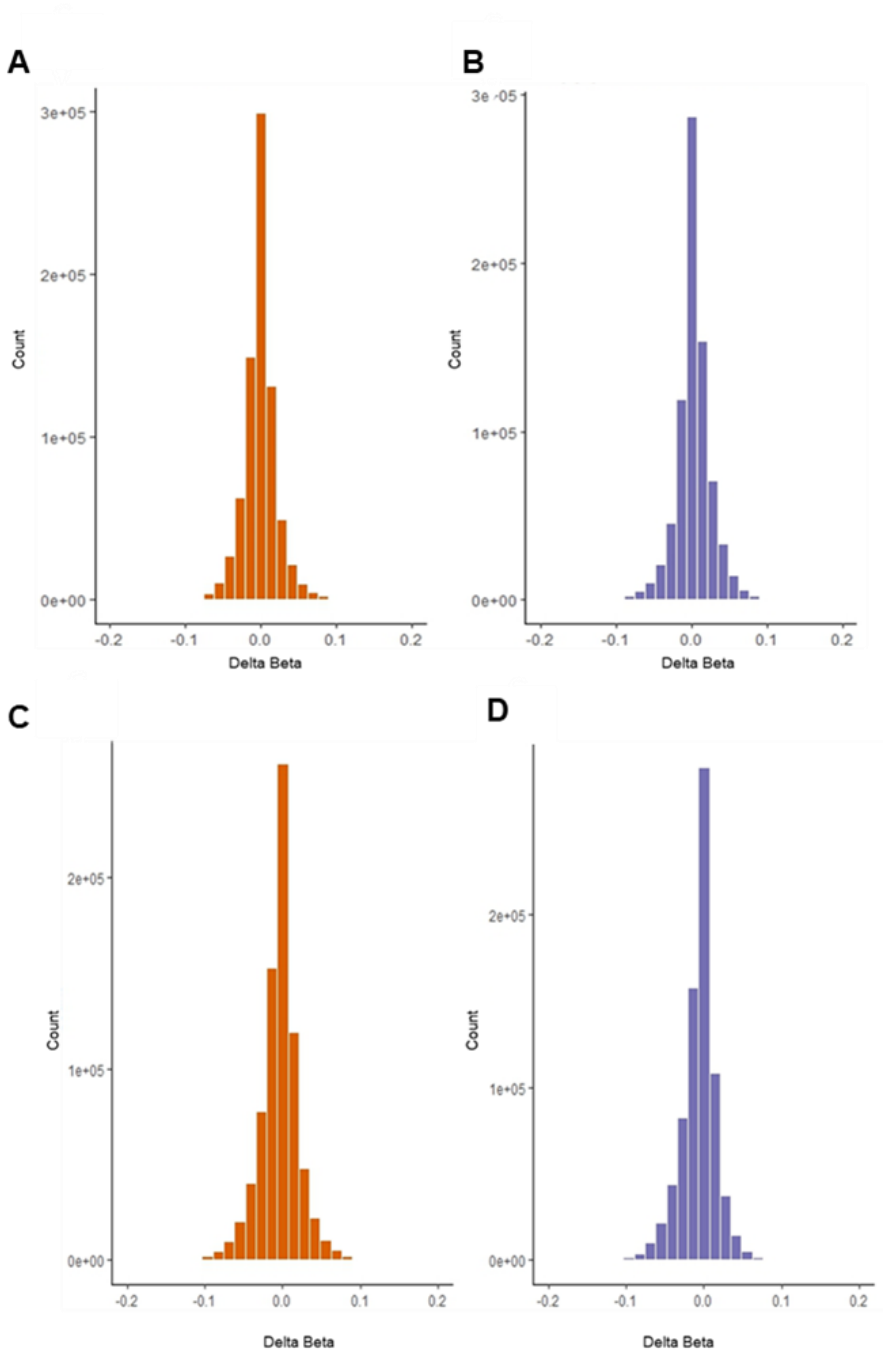

Figure 5.12. Change in methylation status of individuals with and without *BRCA1* mutations exposed to endocrine disrupting chemicals. Distribution of Delta Beta-values of wildtype individuals in response to bisphenol A (A) and propylparaben (B) and of individuals with BRCA1 mutations in response to bisphenol A (C) and propylparaben (D).

We then investigated whether any genes were significantly differentially methylated by identifying DMPs, based on the criteria of FDR adjusted p <0.05 and Delta Beta >0.1. We found that no DMPs could be identified using these criteria, regardless of biotype or exposure. Indeed, even when removing the requirement of Delta Beta >0.1, no DMPs were reported and we

determined that this result was due to the constraint of p <0.05. The variation between individuals was the most probable cause for this lack of statistical significance. Consequently, we explored the data for DMPs based on the criteria of purely Delta Beta-values, as performed by Xie *et al.* (2011). We grouped the DMPs based on Delta Beta >0.2 and >0.1 to highlight genes that had been most substantially changed in response to EDC exposure. Whilst these criteria did not account for the significance of the changes across patients, they provided an indication of how EDC exposure may be interacting with the epigenome and whether these changes can be linked to alterations in gene expression.

In the case of individuals without *BRCA1* mutations, we identified 1243 DMPs with Delta Beta >0.1 in response to BPA exposure. These DMPs mapped onto 893 genes, translating to 207 hypomethylated and 686 hypermethylated genes. We then examined DMPs with Delta Beta >0.2. This category contained 18 DMPs, mapping to 12 genes; 7 hypomethylated genes and 5 hypermethylated genes. Genes found to be differentially hypermethylated to Delta Beta >0.2 included *GPBP1* (Delta Beta 0.26, p = 0.23) and the tumour growth factor agonist, Decorin (*DCN;* Delta Beta 0.21, p = 0.63). Genes differentially hypomethylated to Delta Beta >0.2 included suspected oncogene, LIM-only protein 3 (*LMO3*; Delta Beta -0.22, .p = 0.82).

After exposure to propylparaben 1744 positions were observed to be differentially methylated (Delta Beta >0.1), mapping onto 870 hypomethylated genes and 358 hypermethylated genes. Of these, 12 DMPs were identified as having a Delta Beta-value >0.2, mapping to six hypermethylated genes and four hypermethylated genes. Analogous to BPA exposed samples, *GPBP1* was identified as hypermethylated (Delta Beta 0.22, p = 0.49), along with *LMO3* being hypomethylated (Delta Beta -0.21, p = 0.77).

Finally, we compared *BRCA1* mutation carriers, to examine whether there was a comparable change to the epigenome in response to EDC exposures. Exposure to BPA elicited 1060 DMPs, that mapped onto a total of 740 genes. This equated to 256 hypermethylated genes and 484 hypomethylated genes. Of these, 13 DMPs were identified with Delta Beta >0.2, mapping to eight genes (4 hypermethylated and 4 hypomethylated genes). We again found *LMO3* to be hypomethylated in response to BPA exposure (Delta Beta -0.21, p = 0.91). Ribosomal protein

L13 (*RPL13*) was found to be hypermethylated (Delta Beta 0.22, . p = 0.86). Reduced expression of this gene has previously been suggested as a biomarker for poor survival in pancreatic ductal adenocarcinoma (Goudarzi and Lindström, 2016). Similar results were seen in response to propylparaben exposure. We found 1493 DMPs (Delta Beta >0.1), accounting for 860 hypomethylated genes and 117 hypermethylated genes. A total of 18 positions were seen to be differentially methylated by Delta Beta >0.2. Again, *LMO3* was included in this category seeing a 22% decrease in methylation (Delta Beta -0.22, p = 0.82). We also noted the hypermethylation of *GPBP1* (Delta Beta 0.26, p = 0.23) and *DCN* (Delta Beta 0.21, p = 0.63).

Next, we wanted to understand whether these alterations to the epigenome correlated with any of the changes to gene expression. To do this, we overlaid genes with a change in methylation status (Delta Beta >0.1) and those with change in gene expression (p <0.05; Figure 5.13). Following propylparaben exposure, we found 11 genes that demonstrated a differential decrease in gene expression and hypermethylation in individuals without *BRCA1* mutations (Appendix III). For instance, *CUX2* (cut like homeobox 2), a gene involved in the repair of oxidative DNA damage (Pal *et al.*, 2015) and *DCN.* An additional 68 genes were identified as having an increase in expression and hypomethylation, including *SOX5* (SRY-related HMG-box 5), which is known to contribute to the regulation of breast cancer progression via binding to the promoter of *TWIST1* (Pei, Lv and Li, 2014). Next, 36 genes could be observed to be both down-regulated and hypermethylated in response to BPA (Appendix IV). These included *DCN* and the PI3K/Akt/p70S6K regulator, *FOXP1* (forkhead box P1). Fewer genes were seen to be enriched in expression and hypomethylated, with only 10 genes epigenetically and genetically modified, including *LGR4* (leucine-rich repeat-containing G-protein-coupled receptor 4), which has been reported to possess oncogenic properties (van Andel *et al.*, 2017). Combined, epigenetically modified genes overlapped with 5.62% of gene expression changes induced by BPA and propylparaben exposure.

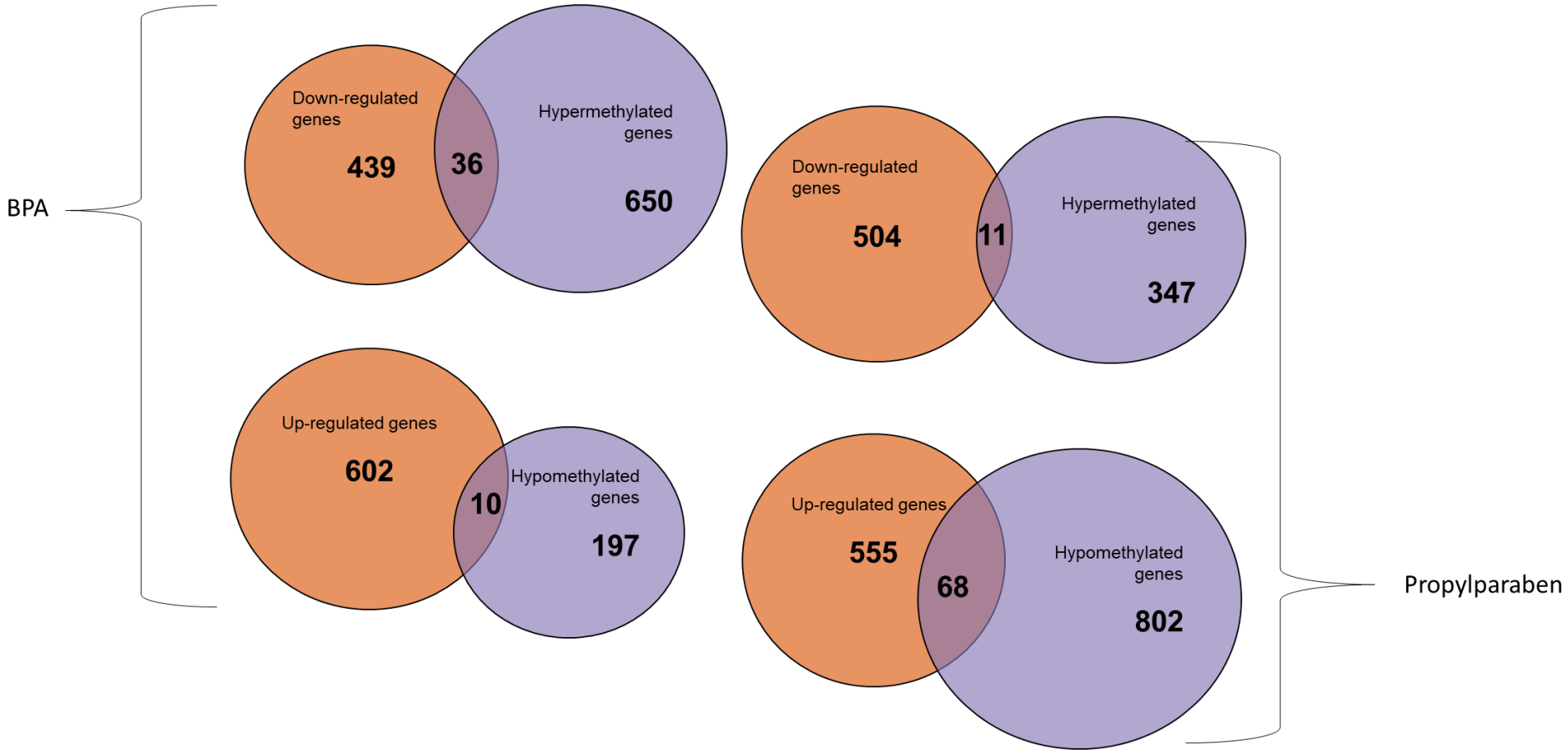

Figure 5.13. Ven diagram depicting genes altered at genetic and epigenetic level in individuals without *BRCA1* mutations.

We then assessed the genetic and epigenetic overlap in individuals with *BRCA1* mutations (Figure 5.14). We found comparable results in the case of propylparaben, with 8 genes identified as hypermethylated and having a decreased expression after exposure (Appendix V). This included *DUSP9* (dual-specificity phosphatase 9), where epigenetic silencing has been reported to induce cell proliferation by activating ERK and JNK kinases in the MAPK pathway (Wu *et al.*, 2015). In addition, 63 genes were found to be both hypomethylated and enriched in expression, including the oestrogen receptor *ESR1*. In BPA exposed samples, 14 genes were both hypermethylated and decreased in expression (Appendix VI), for example, the apoptosis inducing factor *BLCAP*. We also found 26 genes that were hypomethylated and increased in expression. Notably, *LARP4* (La-related RNA-binding protein 4), which induces the loss of polarity in 3D cultures of MDA-MB-231 breast cancer cells (Seetharaman *et al.*, 2016), was here seen to be hypomethylated and reduced in expression. In total 4.44% of differentially expressed genes induced by EDC exposure in individuals with *BRCA1* mutations were also epigenetically modified. However, it must be noted that some of this overlap would be expected due to chance (Table 5.2), and this should be considered when interpreting this data. For instance, for individuals without *BRCA1* mutations, the number of down-regulated and hypermethylated genes that would be expected to overlap by chance after propylparaben exposure would be 11.22. This indicates most genes seen to be overlapping in this group could be entirely by chance. However, if we consider genes up-regulated and hypomethylated after propylparaben, the number of genes we would expect to see by chance is 29.67. As we report 68 common genes, it is possible that this is more meaningful.

Figure 5.14. Ven diagram depicting genes altered at genetic and epigenetic level in individuals with *BRCA1* mutations.

Table 5.2. Number of genes expected to be altered genetically and epigenetically by chance. Calculation based on 15000 genes analysed.

Treatment		Genes expected by chance	Genes observed
BPA	Wildtype down-regulated and hypermethylated	19.02	36
	BRCA1 mutated down-regulated and hypermethylated	11.22	14
	Wildtype up-regulated and hypomethylated	7.9	10
	BRCA1 mutated up-regulated and hypomethylated	16.33	26
Propylparaben	Wildtype down-regulated and hypermethylated	11.65	11
	BRCA1 mutated down-regulated and hypermethylated	4.35	8
	Wildtype up-regulated and hypomethylated	29.67	68
	BRCA1 mutated up-regulated and hypomethylated	29.8	63

5.3.6. EDC exposure elicits alterations to functional gene clusters relevant to breast cancer risk in individuals without *BRCA1* mutations.

After determining that both BPA and propylparaben could induces changes to the genome and epigenome of individuals with and without *BRCA1* mutations, we wanted to see whether these alterations could be linked to pathways or processes associated with breast cancer risk. Due to epigenomic patient variability, we were not able to identify significant EDC-induced differentially methylated genes. Consequently, it was not possible to include epigenetically altered genes in the functional analysis. Therefore, the subsequent analysis was therefore performed using only genes identified as differentially expressed. First, we focused on wildtype patients and performed a functional cluster analysis on differentially modified genes based on the criteria of log2 (fold change) >2 and p <0.05. As in Chapter 4, clusters with enrichment scores >1 were reported. Down-regulated differentially expressed genes caused by propylparaben exposure could be grouped into seven functional clusters based on associated GO annotation terms. Two of these clusters possessed an enrichment score higher that 1 (Table 5.3). The most significantly enriched cluster pertained to DNA-binding processes, with between 38 and 22 genes mapping onto each term and an enrichment score of 1.29. The second cluster (enrichment score 1.04) included terms concerning the MAPK cascade and phosphatidylinositol signalling, suggesting these processes may be disrupted in propylparaben exposed cells. We then looked at clusters associated with genes that were up-regulated in response to propylparaben exposure. A total of

six clusters were generated. However, only two of these had an enrichment score >1 (Table 5.4). The most significantly enriched cluster, with an enrichment score of 1.28, included terms surrounding carbonate activity, such as carbonate dehydratase activity (p = 0.02), bicarbonate transport (p = 0.04) and one-carbon metabolic processes (p = 0.11). The final cluster included terms associated with the cell cycle (enrichment score 1.1). Specifically, genes associated with G2/M transition (p = 0.04) and cell division (p = 0.01) were seen to be enriched.

Table 5.3. Wildtype patient functional annotation clustering of propylparaben-induced differentially down-regulated genes. Two clusters with enrichment score >1 were identified. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term.

Cluster Rank		GO Annotation Term	Enrichment score	Count	p
1	Biological Process	regulation of transcription, DNA-templated	1.29	33	0.04
	Molecular Function	DNA binding		35	0.04
	Molecular Function	transcription factor activity, sequence-specific DNA binding		22	0.05
	Biological Process	transcription, DNA-templated		38	0.1
2	Biological Process	positive regulation of MAP kinase activity	1.04	5	0.01
	Biological Process	phosphatidylinositol phosphorylation		6	0.02
	Biological Process	phosphatidylinositol-mediated signalling		6	0.02
	Molecular Function	phosphatidylinositol-4,5-bisphosphate 3-kinase activity		4	0.06
	Molecular Function	Ras guanyl-nucleotide exchange factor activity		5	0.09
	Biological Process	regulation of phosphatidylinositol 3-kinase signalling		4	0.12
	Molecular Function	1-phosphatidylinositol-3-kinase activity		3	0.13
	Molecular Function	protein tyrosine kinase activity		5	0.14
	Biological Process	phosphatidylinositol-3-phosphate biosynthetic process		3	0.17
	Biological Process	positive regulation of GTPase activity		12	0.25
	Biological Process	MAPK cascade		6	0.37
	Biological Process	peptidyl-tyrosine phosphorylation		4	0.42

Table 5.4. Wildtype patient functional annotation clustering of propylparaben-induced differentially up-regulated genes. Two clusters with enrichment score >1 were identified. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term.

Cluster Rank		GO Annotation Term	Enrichment Score	Count	p
	Molecular Function	carbonate dehydratase activity		3	0.02
1	Biological Process	bicarbonate transport	1.28	4	0.04
	Biological Process	one-carbon metabolic process		3	0.11
	Biological Process	G2/M transition of mitotic cell cycle		7	0.04
2	Biological Process	mitotic nuclear division	1.1	9	0.09
	Biological Process	cell division		11	0.12

Differentially expressed genes in response to BPA exposure could be grouped into seven clusters associated with down-regulated genes and four clusters related to enriched genes. None of the identified clusters passed the criteria of enrichment score >1 and therefore were not considered significant. The lack of identified significantly enriched clusters is most likely due to the diversity of genes that were differentially expressed, with limited relatedness between them.

5.3.7. EDC exposure elicits alterations to functional gene clusters relevant to breast cancer risk in individuals with *BRCA1* mutations.

We continued by investigating whether comparable functions were enriched in *BRCA1* mutation carriers. Genes down-regulated by propylparaben exposure could be grouped into four functional clusters, however none of these clusters were found to be significant based on the criteria of enrichment score >1. Significant clusters were associated with genes significantly up-regulated by propylparaben. A total of 12 clusters were found, with three of these possessing an enrichment score >1 (Table 5.5). The most significantly enriched functional cluster contained terms pertaining to cilium morphogenesis (enrichment score 1.34). Two biological processes, cilium assembly (p 0.08) and cilium morphogenesis (p = 0.12) were enriched, along with the ciliary transition zone cellular component (p <0.01). This was then followed by a cluster associated with glycosylation, specifically sialylation (a glycosylation pattern associated with cancer progression). Sialytransferase activity was the most significantly enriched term in this cluster (p = 0.05), indicating genes associated with this process were being enhanced. The final cluster (enrichment score 1.07) surrounded the functioning of collagen, with the most enriched term being collagen fibril organisation (p = 0.01), signifying processes that determine the size and arrangement of collagen fibrils within the extracellular matrix.

Table 5.5. Functional annotation clustering of propylparaben-induced differentially up-regulated genes in individuals with a *BRCA1* mutation. Three clusters with enrichment score >1 were found. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term.

Cluster Rank		GO Annotation Term	Enrichment Score	Count	p
1	Cellular Component	ciliary transition zone	1.34	4	<0.01
	Biological Process	cilium assembly		6	0.08
	Biological Process	cilium morphogenesis		6	0.12
2	Molecular Function	sialyltransferase activity	1.09	3	0.05
	Biological Process	sialylation		3	0.05
	Cellular Component	integral component of Golgi membrane		4	0.09
	Biological Process	protein glycosylation		5	0.17
3	Cellular Component	endoplasmic reticulum lumen	1.07	9	0.02
	Biological Process	collagen fibril organization		3	0.01
	Cellular Component	collagen trimer		4	0.25
	Biological Process	collagen catabolic process		3	0.34

Genes up-regulated by BPA exposure could be mapped onto 11 functional clusters, with two clusters passing the criteria of enrichment score >1 (Table 5.6). The most enriched cluster pertained to processes surrounding cilium function (enrichment score 1.28). This result is comparable to what was observed after propylparaben in *BRCA1* mutation carriers. Further, cilium assembly (p = 0.16) and cilium morphogenesis (p = 0.03) terms appeared in both cluster results, indicating the presence of a potential common target between the two compounds. Following this, we observed the enrichment of genes associated with cell cycle regulation (enrichment score 1.1). Notably, the most significant term within the cluster was DNA-polymerase activity (p = 0.02), followed by telomere maintenance via recombination (p = 0.03). Finally, genes that were down-regulated in response to BPA exposure could be linked to seven functional clusters, yet none were considered significantly enriched.

Table 5.6. Functional annotation clustering of BPA-induced differentially up-regulated genes in individuals with a *BRCA1* mutation. Two clusters with enrichment score >1 were identified. Gene ontology (GO) annotation terms are grouped in clusters based on the association of genes within each term.

Cluster Rank		GO Annotation Term	Enrichment Score	Count	p
1	Cellular Component	primary cilium	1.28	6	0.01
	Biological Process	cilium morphogenesis		7	0.03
	Cellular Component	axoneme		4	0.15
	Biological Process	cilium assembly		5	0.16
2	Molecular Function	DNA-directed DNA polymerase activity	1.1	4	0.02
	Biological Process	telomere maintenance via recombination		4	0.03
	Biological Process	DNA replication initiation		3	0.15
	Biological Process	G1/S transition of mitotic cell cycle		4	0.37

5.4. Discussion

Research has suggested that endocrine disrupting chemicals have the ability to contribute towards breast cancer risk, yet evidence to support this claim is often inconsistent. Most literature aimed at investigating the links between EDC exposure and breast carcinogenesis has focused on end points, such as cell proliferation or changes to the morphology of acini (Okubo *et al.*, 2001; Byford *et al.*, 2002; Marchese and Silva, 2012; Wróbel and Gregoraszczuk, 2013; Pfeifer, Chung and Hu, 2015; Rodgers *et al.*, 2018), yet at lower concentrations, it is possible that alternative endpoints, such as methylation profiles, may need to be explored to observe EDC-induced effects. Alterations to the genome and epigenome are now recognised as some of the earliest changes to occur in breast cancer development (Dworkin, Huang and Toland, 2009; Stefansson and Esteller, 2013). Understanding how chemicals interact with the genome and epigenome of cells could provide vital insights into the relationship between EDC exposures and breast cancer risk. In Chapter 4, we demonstrated the ability of BPA and propylparaben to interact with the epigenome. Here, we investigated whether exposure could elicit changes to the genome of primary cells derived from individuals with and without *BRCA1* mutations and if such changes could be correlated with alterations to the epigenome. In addition, we explored whether such changes could be associated with processes involved in breast cancer risk.

The results presented in this chapter strongly indicate that EDCs have the ability to significantly affect the genomic landscape of primary cells, even when taking into account individual variation. However, it must be noted that the number of differentially expressed genes identified was considerably less than previous studies using cell lines. For instance, Fernandez *et al.,* (2012) showed $1x10^{-5}$ M and $1x10^{-6}$ M BPA could induce the down-regulation of 1675 and 1368 genes respectively and up-regulate 1939 and 1796 genes respectively in collagen grown MCF-10F cells. The group utilised a comparable significance criterion of log2 (fold change) >2 and FDR <5%. This disparity may originate from the use of primary cells in this chapter. Indeed, by relying on an immortalised, relatively homogenous cell line, Fernandez and colleagues did not account for the variation in response between individuals, which is a factor in the research presented

here. We demonstrated that individuals differ in their genetic profile even before treatment with EDCs and this must be taken into consideration when extrapolating results from cell lines to human populations. This finding was expected and not surprising, however it was something that had to be considered throughout the analysis and result interpretation of the present research. Jabs *et al.,* (2017) showed that patient-derived cells were significantly more representative of *in vivo* responses to exposures, producing more diverse results and indicating a lower therapeutic potential of drugs. Our work supports this notion by showing a relatively lower number of differentially expressed genes in response to BPA, in comparison to work published by Fernandez (2012), and demonstrating considerable patient variation in response to exposure. Thus far, no study has examined the effects of propylparaben using RNA-sequencing or comparable genome-wide technologies. The few articles that have been published on propylparaben induced changes have focused on specific genes. For example, Wróbel and Gregoraszczuk (2015) showed the modification of genes involved in cell cycle regulation in MFC-10A cells exposed to $2x10^{-7}$ M propylparaben relying on real-time PCR. GPR30 was seen to be the most significantly altered gene looked at, with an up-regulation observed after being exposed to propylparaben. Interestingly, GPR30 was not identified as a significantly altered gene within the present study. It is likely that this difference could be attributed to the significant differences between the model used by Wróbel and Gregoraszczuk and that used in the present study. For instance, here we used primary cells in a 3D co-culture assay, rather than a cell line in a 2D model. As discussed previously, these differences can result in significantly different results. Despite this, the vast number of genetically altered genes identified using RNA-sequencing suggests propylparaben has a much wider impact on a number of genes much higher than has previously been investigated.

As well as changes to the genome, we observed substantial changes to the profile of the epigenome in response to BPA and propylparaben exposure. Despite having to modify the threshold due to significant individual variation, BPA-induced differentially methylated genes in wildtype samples were comparable in number to work by Fernandez (2012), who reported 545 hypermethylated genes and 111 hypomethylated genes in response to $1x10^{-6}$ M BPA. However,

the number of DMPs is considerably inflated in comparison to results seen in Chapter 4 using MCF-12A cells, where only 39 genes were identified as hypermethylated and no genes were hypomethylated in response to BPA. This difference can most likely be attributed to the criterion employed within this chapter. When attempting to identify genes based on Delta Beta >0.1 and FDR adjusted p <0.05 (as performed in Chapter 4), no DMPs could be isolated using data obtained from primary cells. As noted above, this change was made to account for the patient variability and whilst it provided an indication of what genes may be epigenetically modified in response to EDC exposure, the results are not as statistically robust as work presented in Chapter 4. This difference can also be seen in response to propylparaben, where a much higher number of hypomethylated genes were observed in comparison to Chapter 4. To the best of our knowledge, no comparable study has been published with propylparaben, despite a long standing call for further research (Harvey and Darbre, 2004; Darbre and Harvey, 2014). Interestingly, no study has investigated the impact of propylparaben on the epigenome. Data presented here and in Chapter 4 suggest the epigenome may play a significant role in linking propylparaben exposure to breast cancer risk and justifies further examination. The lack of work undertaken to date highlights a significant gap in our understanding of how propylparaben contributes to breast cancer risk, leaving the potential for the compounds effects to be underestimated.

Both EDCs also elicited a higher number of differentially hypomethylated genes in *BRCA1* mutation carriers. Global hypomethylation was the initial epigenetic aberration recognised in human tumours, being first reported in 1983. Despite this, most research has focused on regional hypermethylation, specifically in tumour suppressor genes. However, global hypomethylation is known to contribute towards carcinogenesis (Ehrlich, 2009) and, therefore, the considerable amount of hypomethylated genes induced by EDCs here should not be overlooked. *BRCA1* mutation carriers are a particularly high-risk population, with a predisposition to breast and ovarian cancers (King *et al.*, 2003; Antoniou *et al.*, 2006; Kuchenbaecker *et al.*, 2017). Antoniou and colleagues (2008,2010) have previously stressed the importance of identifying risk factors that can add to the *BRCA1/2* mutation associated risk, stating that such factors can have a large

impact on an individual's absolute risk of developing breast or ovarian cancer. Whilst our results may not be considered statistically significant due largely to individual variation, they strongly suggest both BPA and propylparaben can elicit deleterious effects to *BRCA1* mutation carriers, potentially increasing the already heightened risk of these individuals.

We then wanted to understand whether there was a possibility changes to the epigenetic profile of exposed cells could be associated with alterations to the genome. By overlaying differentially expressed genes with differentially methylated genes we were able to identify several common targets and align this with published research. For instance, Fernandez reported that both *FOXP1* and *DCN* were significant down-regulated and hypermethylated after being treated with $1x10^{-5}$ M BPA. *FOXP1* has been demonstrated as an oncoprotein due to its ability to supress immune response and promote tumour cell survival in leukaemia and B-cell lymphoma (Flori *et al.*, 2016; P. J. Brown *et al.*, 2016). In contrast, when expressed in neuroblastomas and prostate cancer *FOXP1* can repress cell proliferation and reduce tumorigenicity (Ackermann *et al.*, 2014; Takayama *et al.*, 2014), indicating a tissue dependent role from *FOXP1*. Comparatively less is known about the role of *FOXP1* in breast cancer, however there is some evidence that a decrease in expression is correlated with relapses in breast cancer patients (Xiao *et al.*, 2016). *FOXP1* is also assumed to be a transcription factor for ERα signalling and serves as a predictive factor for acquiring endocrine resistance in breast cancer (Ijichi *et al.*, 2013). Studies using ERα-positive MCF-7 cells reported *FOXP1* to be up-regulated after 3 hours of $1x10^{-6}$ M E2 exposure, resulting in an increased growth rate (Shigekawa *et al.*, 2011), which is inconsistent with the findings here. This difference may be attributed to a different impact in breast cancer cell lines as opposed to non-cancerous primary breast cells, but also to the significantly higher affinity of E2 for the ERs, especially at such a high concentration. *DCN* has been reported to have antifibrotic, anti-inflammatory and anti-cancer effects (Gubbiotti *et al.*, 2016). Early studies found *DCN* expression inhibited tumour cell proliferation and metastasis (Yamaguchi and Ruoslahti, 1988; Ruoslahti and Yamaguchi, 1991). Clinically, loss of *DCN* expression is regarded as a strong biomarker of invasive and metastatic breast cancer (Goldoni and Iozzo, 2008). Following these findings, significant work has concentrated on targeting the gene for its therapeutic anti-

cancer potential (extensively reviewed by Järvinen and Prince, 2015). Together, the hypermethylation and down-regulation of genes like *FOXP1* and *DCN* strongly supports previous work demonstrating the ability of BPA to interact with the genome and epigenome to increase breast cancer risk. The hypermethylation and down-regulation of *DCN* was also observed after exposure to propylparaben. Interestingly, neither gene has received much attention regarding their expression in response to EDC exposure, indicating a potentially critical knowledge gap that should be addressed in future research. Overall, epigenetically modified genes could account for just under 5% of differentially expressed genes. Whilst this could be considered a relatively minimal proportion, further studies using demethylation techniques (Piccolo and Fisher, 2014; Xu *et al.*, 2016; Adli, 2018) are recommended to confirm if and how methylation is mechanistically regulating gene expression.

Fernandez (2012) predicted that individuals with *BRCA1* mutations would be more susceptible to the effects of xenoestrogens. Additional studies have also suggested that the loss of *BRCA1* function could be associated with an increased sensitivity to the impacts of oestrogen-mimicking EDCs. For instance, Jones and colleagues (2010) used mouse models to demonstrate that the loss of *BRCA1* expression enhanced BPA-induced cell proliferation effects by increasing the impact of BPA on ERα signalling. From our results it is not possible to determine whether either BPA or propylparaben have an enhanced effect on individuals with *BRCA1* mutations. Here, exposure induced a comparable number of differentially expressed genes in individuals with and without *BRCA1* mutations. Nevertheless, our data does indicate that the compounds may be affecting the two biotypes in different ways, potentially by impacting different genes. When comparing the list of genes affected by BPA and propylparaben across the two biotypes, very few common genes could be identified. This is further supported by the functional analysis, where we observed different enriched clusters between the two biotypes. Whilst both biotypes shared the up-regulation of genes involved in the mitotic cell cycle (via propylparaben exposure in wildtype and BPA in *BRCA1* mutation carriers), the other processes shared little similarity. For example, we identified significantly enriched clusters associated with up-regulated genes after propylparaben exposure in both wildtype and *BRCA1* mutation carriers. In individuals without

BRCA1 mutations, these clusters focused on functions surrounding carbon transport and cell cycle regulation. The one-carbon metabolism term included in the first cluster, referred to a web of biological reactions that serve a critical role in DNA methylation and synthesis, facilitating cross-talk between the genome and epigenome. Consequently, the role of this process in breast carcinogenesis has been investigated with the up-regulation of one-carbon metabolism being observed in mammary tumours (Shuvalov *et al.*, 2017). Thus far, many of the studies published have focused on the effect of diet on one-carbon metabolism regulation, with research indicating the adoption of low fat and carbohydrate diets that are rich in protein can reduce breast cancer progression (Willcox *et al.*, 2009; Blagosklonny, 2010; Ho *et al.*, 2011). Evidence was reviewed by Xu and Chen, with the authors concluding that whilst the relationship between one-carbon metabolism and breast cancer risk remained complex, there was sufficient literature to suggest the involvement of the pathway in breast cancer development by altering the epigenetic profile (Xu and Chen, 2009). Metabolic pathways are considered important targets of anticancer therapy, with metabolic inhibitors being used clinically for over 50 years (Shuvalov *et al.*, 2017) and we should not ignore the possible ability of EDCs to interfere with such central processes. BPA exposure has previously been shown to dysregulate metabolic pathways, such as glucose metabolism, resulting in disorders such as glucose intolerance and insulin resistance (Alonso-Magdalena, Rivera and Guerrero-Bosagna, 2016; Menale *et al.*, 2016; Heindel *et al.*, 2017), however research has not been conducted in the context of breast cancer risk. In contrast, clusters deemed most significantly impacted in *BRCA1* mutation carriers, involved genes associated with cilium assembly, sialylation and fibril organisation. Whilst these processes have previously been associated with the development of cancer, they are significantly different from what was reported in wildtype samples. Loss of primary cilia occurs early in breast cancer development, both in the tumour cells and the surrounding stroma (Menzl *et al.*, 2014). In addition, sialyation is an altered glycosylation pattern strongly associated with breast cancer progression and metastasis, with associated genes, such as *ST8SIA4* (ST8 alpha-n-acetyl-neuraminide alpha-2,8-sialyltransferase 4) and *ST3GAL6* (ST3 beta-galactoside alpha-2,3-sialyltransferase 6), being more enriched in the highly metastatic breast cancer cell line MDA-

MB-231 compared with MCF-7, which is less metastatic (Ma *et al.*, 2016). It must be noted however, that it was not possible to identify significant clusters for each of the compounds across biotypes and that individual variation may have a critical role in the observed differences, in addition to the presence of *BRCA1* mutations.

Previous work aimed at elucidating the clinical significance of *BRCA1/2* mutations in cancer and has shown that the presence of a mutation can influence the response to chemotherapy treatment in breast and ovarian cancers. Yang *et al.*, (2011) observed that individuals with a mutated *BRCA2* gene were more sensitive to the effects of platinum-based chemotherapy compared to individuals without a *BRCA1/2* gene mutation. Also, using the MDA-MB-231 (wildtype) and MDA-MB-436 (mutant) cell lines, Stefansson *et al.*, (2012) found that the epigenetic inactivation of *BRCA1* was a predictor of increased sensitivity to platinum-based chemotherapy. Although authors could not determine why this was the case, from our data we could speculate that similar response differences are occurring due to EDC exposure. Indeed, it may be possible that the previously hypothesised increased sensitivity to EDCs is a result of differing biological processes that are targeted between the two biotypes, however additional evidence would be needed to support this claim. It has also recently shown, that the variant of *BRCA1* mutation can have an impact on the way individuals respond to treatment (Anantha *et al.*, 2017). Within the scope of this study it was not possible to look at the response of individual mutation variants, however future experiments, that incorporate higher patient numbers, should consider this variable. At this stage, the potential for EDCs to induce a more significant effect on *BRCA1* mutation carriers should not be ruled out. The small sample size used here, when accounting for patient variability, means that it may not be possible to fully capture the exposure effects on this predisposed population.

A final interesting result to be taken from this experiment is the considerable variation between individuals, even within the same biotype. For experiments based on cell lines, three independent experiments are generally considered sufficient to address most objectives (Vaux, Fidler and Cumming, 2012). Results are, in most cases, consistent across experiments, allowing trends to be identified. Here three patients from each biotype were sampled, akin to three

independent experiments, yet the significant variation between individuals is not comparable to that of cell lines. In this case, experimental data from three patients were not sufficient to fully address the research questions of this chapter. Even before compounds were treated with EDCs, individuals possessed significantly different epigenetic and genetic profiles, consequently, it is not surprising that despite normalisation to controls, there was a considerable variation in responses. In turn, the statistical power required to identify trends at this resolution could not be achieved. This was most evident in the epigenetic data, where due to the considerable variation between individual responses, we were not able to identify any significant DMPs to p <0.05, despite the presence of numerous DMPs with high Delta Beta-values. From this it could be implied that more patient variation is present in the epigenome. Epigenetic heterogeneity may be attributed to environmental conditions and lifestyle factors of the individuals that have an impact on the epigenome, but not the genome. Whilst we were provided individual's age, gender and biotype, we were provided no further lifestyle information such as diet, whether they smoke, their level of exercise or their BMI, all of which can have an impact on an individual's epigenetic profile (Motta *et al.*, 2017). For example, DNA methylation signatures have been altered in response to various factors including smoking (Zeilinger *et al.*, 2013; Tsaprouni *et al.*, 2014), arsenic exposure (Argos *et al.*, 2014), BMI (Dick *et al.*, 2014; Aslibekyan *et al.*, 2015; Bell, 2017; Wahl *et al.*, 2017), exercise (Rönn *et al.*, 2013) and pregnancy (Barua and Junaid, 2015), as well as conditions such as rheumatoid arthritis (Y. Liu *et al.*, 2013), type 2 diabetes (Chowdhury *et al.*, 2014; Chambers *et al.*, 2015; Scott *et al.*, 2017), asthma (Arathimos *et al.*, 2017), schizophrenia (Montano *et al.*, 2016) and panic disorders (Shimada-Sugimoto *et al.*, 2017). If this information was known it may be possible to identify where some of the heterogeneity originated from, yet the sample size in this current study would still not be sufficient to tease out the relative contribution of EDC exposure to breast cancer risk. A recent review suggested that, based on previous work, 300 individuals per biotype should be tested to identify differentially methylated CpGs between individuals with and without cancer (Widschwendter *et al.*, 2018). Such a substantial sample size is 100 times larger than the study conducted here and this must be considered when interpreting the results. Although

Widschwendter and colleagues recognise that resources (both financial and temporal) limit the ability of researchers to conduct studies on this scale, future research should aim to include a larger sample size than the present study.

DNA methylation-based risk-prediction models have recently been suggested as a novel opportunity to deliver bespoke state-of-the-art breast cancer screening and prevention programmes (Widschwendter *et al.*, 2018). Authors emphasised the need for a multidisciplinary and collaborative approach to research in order to overcome the current scientific challenges currently preventing the development of such models, including understanding the contribution of lifestyle risk factors, discovering methylation markers and developing robust analytical methodologies to incorporate such complex relationships. Results presented here indicate that EDCs can interact with the epigenome, however the individual response variation is too large to definitively understand whether this can increase breast cancer risk, both for the general population and those considered high-risk due to *BRCA1* mutations. Yet, if we are to incorporate lifestyle and environmental factors into epigenome-based risk prediction models, it is imperative that we determine how EDCs interact with the epigenome. To achieve this, additional patients would be required to fully address the aims of this chapter, both from wildtype and *BRCA1* mutated biotypes. Further studies should also be undertaken that utilise primary, donor-derived cells that fully capture the variation in responses necessary to extrapolate findings to human populations. Finally, through the adoption of integrated analysis of omics based techniques, it may be possible to uncover the impact of EDCs more rapidly, providing the ability to reveal stronger and more direct contributions to breast cancer risk (Shenker *et al.*, 2013; Pineda *et al.*, 2015; van Veldhoven *et al.*, 2015; Sun and Hu, 2016; Bonder *et al.*, 2017; Johansson and Flanagan, 2017; Johnson *et al*., 2017; O'Brien *et al.*, 2018; Widschwendter *et al.*, 2018).

Despite the limitations imposed by patient variability, the results of this study indicate that EDCs can alter the genome and the epigenome of breast tissue. We also demonstrated effects in high-risk individuals, which could translate to a significant increase in absolute risk. Moreover, we observed differences in the responses of *BRCA1* mutation carriers compared with wildtype patients, suggesting the potential for unique targets between biotypes that should be explored

further. Notably we also saw common genes both genetically and epigenetically modified, supporting the hypothesis that the link between EDC exposure and breast carcinogenesis, could, at least in part, be mediated by epigenetic mechanisms.

Chapter Six: Final conclusions and future work

6 Conclusions and future work

6.1. Introduction

Cancer remains one of the leading causes of mortality in the world and represents a significant global health burden (Grosso *et al.*, 2017). Breast cancer is the most common malignancy diagnosed in women residing in western countries and, despite considerable research into identifying risk factors, early diagnosis strategies and treatments, it continues to be the second leading cause of female cancer deaths in the US (DeSantis *et al.*, 2017). As little as 5% of breast carcinomas can be attributed to genetic factors, with the remaining sporadic cases potentially being related to lifestyle and environmental factors (Anand *et al.*, 2008; Howell *et al.*, 2014; Hiatt and Brody, 2018). Although researches have highlighted numerous well-established lifestyle risk factors, such as smoking (Ginsburg *et al.*, 2009; Reynolds, 2013; Jones *et al.*, 2017), diet (Michels *et al.*, 2007; Harvie, Howell and Evans, 2015) and BMI (Eheman *et al.*, 2012; Crispo *et al.*, 2015), comparatively little is known about the contribution of EDC exposures to breast cancer risk.

There is a general consensus that the human population is exposed to a large number of oestrogen-mimicking compounds (Morgan *et al.*, 2016; Sifakis *et al.*, 2017; Zhou *et al.*, 2017). However only a limited number of EDCs have been extensively studied and the mode of action of many compounds is yet to be determined. Exposure to these chemicals has been associated with numerous diseases, including breast cancer (Rachoń, 2015; Holmes, 2017). However, even with the wealth of literature indicating a link between EDC exposure and breast cancer risk, few studies have been able to demonstrate this association at concentrations relevant to human exposures. Due to inconsistent study findings and reports of effects only occurring at high concentrations, it has often been assumed EDCs, at concentrations reported in human tissues, pose no threat to health (Acerini and Hughes, 2006; Dietrich *et al.*, 2013).

Traditionally, it was believed that the effects of EDCs were predominately mediated through classic ER signalling pathways (Rodgers *et al.*, 2018), yet this could only be induced at high, often irrelevant concentrations in terms of general population exposures (Vandenberg *et al.*,

2012; Lee *et al.*, 2013; Schug *et al.*, 2016). In recent years scientists have proposed that investigations into the role of the epigenome could shed light on how low-dose environmental exposures may be connected to breast tumourigenesis (Bernal and Jirtle, 2010; Shahidehnia, 2016; Walker, 2016). Epigenetic research has provided insights into established risk factors including smoking, diet, obesity and exercise. Yet, few studies have thoroughly examined the ability of EDCs to interact with the epigenetic profile, in test systems that recapitulate mammary gland characteristics and at concentrations relevant to those reported in human tissues. The aim of this thesis was to begin addressing this critical knowledge gap, to further our understanding of how exposure to xenoestrogens may contribute to breast cancer risk. Specifically, we wanted to determine whether exposure to environmental compounds could result in changes to the epigenome of mammary epithelial cells and if such changes could be correlated with genetic and morphological alterations that are indicative of breast cancer development. Within this final conclusion chapter, we highlight the main findings from the series of experiments presented in this thesis and make recommendations for future studies.

6.2. Main findings

This thesis has made a valuable contribution to the knowledge of how EDCs can contribute to breast cancer risk, in cell culture models representative of the human breast. We have demonstrated that environmental chemicals cannot only induce changes to acini development, but also elicit genetic and epigenetic changes, indicative of the early stages of breast cancer, at concentrations relevant to levels observed in human breast tissue.

6.2.1. EDCs can induce changes to mammary acini morphology

In Chapter 2, we demonstrated that the MCF-12A cell line was ER and GPER competent and represented a normal hormonally responsive mammary epithelial cell line, suitable for the aims of the present study. In Chapter 3 we then utilised MCF-12A cells in a 3D culture that allowed for the development of acini-like spheroids that resembled structures in the human breast. Based

on existing literature, which has demonstrated the ability of E2, BPA and propylparaben to alter acini development (Marchese and Silva, 2012), we hypothesised chemicals with similar a mode of action would induce comparable effects.

We tested four widespread xenoestrogens found in human tissues (*o,p'*-DDT, BP-3, BPA and propylparaben), at concentrations between $1x10^{-10}$ M and $1x10^{-4}$ M, and saw the development of acini could be influenced by exposure. Changes observed included increases in acini size and a decrease in circularity, which are characteristics indicative of the early stages of breast tumourigenesis. We proposed that these changes may be regulated by activation of the PI3K/Akt cascade, which has been reported after exposure to E2 by binding to ERα (Guo *et al.*, 2006; Kazi, Molitoris and Koos, 2009; Pesiri *et al.*, 2014). The ability for EDCs to elicit acini malformations was further evidenced in Chapter 4, where we improved the cell culture model by including stromal cells, allowing for cell-cell communication. Again, BPA and propylparaben were seen to result in alterations to acini morphology, including the development of larger acini, with a higher number of cells observed in the most central point of the acini. Moreover, in Chapter 4 we saw that morphological changes associated with BPA and propylparaben exposure were comparable to E2-induced effects. In addition, results presented in Chapters 3 and 4 demonstrated 3D assays using MCF-12A cells could be utilised as a valuable tool to study the effects of EDCs on acini morphology.

Overall these findings support existing literature indicating that EDC exposure can induce morphological changes relevant to breast cancer development (e.g. Marchese and Silva, 2012; Halsne *et al.,* 2016). The observation of EDCs eliciting changes that are indicative of neoplastic transformations supports claims that these compounds possess carcinogenic properties that could be associated with an increase in breast cancer risk. Despite not being able to observe morphological changes at all tested concentrations, significant effects at concentrations higher than the average tissue levels should not be overlooked. Populations with higher exposures exist and are subject to concentrations shown here to elicit changes indicative of breast carcinogenesis. For instance, Ruiz *et al.* (2018) reported that minority groups (including Latino, African American and low-income individuals) in the US appeared to have a significantly higher

exposure to EDCs like BPA, PCBs and phthalates. Here, authors highlighted low-income populations had substantially higher levels of BPA, with studies finding children that received emergency food assistance had a 54% higher concentration of BPA in tissues compared to individuals from more affluent families (Nelson *et al.*, 2012). Ruiz and colleagues believed this correlation could be attributed to a reliance on processed foods stored in plastic containers and cans. Moreover, we must also remember that individuals are not exposed to single compounds in isolation and each individual chemical could be contributing to an individual's oestrogenic load, which was examined further in Chapter 3.

6.2.2. EDCs can elicit relevant changes to the genome and epigenome

Elucidating whether the epigenome played a role in linking EDCs to breast cancer risk was an integral part of this thesis. Many EDCs are not considered classical genotoxicants or carcinogens. Thus, it has been speculated that some of these compounds may be acting through alternative mechanisms, such as inducing epigenetic effects (Smith *et al.*, 2016). Here, we investigated changes to DNA methylation, the most established epigenetic mechanism in relation to breast carcinogenesis. Aberrant methylation has been reported in breast tumours and is detectable at early, preneoplastic stages of the disease, serving as a useful biomarker (Buyru *et al.*, 2009; Veeck and Esteller, 2010; Atalay, 2013; Davalos, Martinez-Cardus and Esteller, 2017). Studying the effects of EDC exposures on the epigenome offers a novel opportunity to further our understanding of how these compounds work and whether measurable impacts can be induced by concentrations relevant to human exposures. Whilst there is evidence to support a relationship between EDC exposures, the epigenome and breast cancer (Anway and Skinner, 2006; Bromer *et al.*, 2010; Doherty *et al.*, 2010; Weng *et al.*, 2010; Fernandez *et al.*, 2012; Hsieh *et al.*, 2014), few studies have shown effects in assays representative of the human breast and at concentrations comparable to levels reported in tissues.

In Chapters 4 and 5 we used the Infinium MethylationEPIC BeadChip microarray to examine how BPA and propylparaben affected the methylome. In Chapter 4 we saw that whilst no global

methylation changes occurred, both BPA and propylparaben could elicit regional differential methylation. The majority of these alterations were increases in methylation, often to well-known tumour suppressor genes such as *RUNX3* and *NRG1*, which have been cited as epigenetic biomarkers in early breast cancer (Chua *et al.*, 2009; Huang *et al.*, 2011; Chen, 2012). Then, using functional analysis we reported that many of the differentially methylated genes were associated with processes currently unexplored in relation to EDC exposures. For example, several genes seen to be hypermethylated in response to BPA could be associated with metabolic processes, such as glucose metabolism. Such processes are essential in regulating tumour initiation and progression (Li *et al.*, 2011), yet have not been investigated in relation to EDC exposures. In addition, propylparaben exposure induced the hypermethylation of genes that were connected to voltage-gated calcium channel activity. Whilst limited research has examined the relationship with insecticides and VGCC activity (Meijer *et al.*, 2014), there is no literature to suggest propylparaben could be interfering with such processes. Taken in isolation, the effects on the epigenome are not proof that these EDCs cause breast cancer and additional work would be required to explore this possibility further. However, we did show that concentrations which resulted in epigenetic changes also induced morphological effects. This finding strengthens the hypothesis that the two may be correlated.

Next, we wanted to see if similar mechanisms could be observed in primary cells. In Chapter 5 we began to assess this, however trends were not as clear as with MCF-12A cells. There was significant variation between the three wildtype patients analysed, which meant EDC-induced modifications were difficult to identify. Despite this, with just three patients we were able to provide an indication that changes to the epigenome could be observed in primary cells. Again, numerous hypermethylations of tumour suppressor genes were highlighted, suggesting the epigenome could link EDC exposure to an increase in breast cancer risk. Although the results appeared to indicate a higher frequency of EDC-induced hypomethylations in comparison to MCF-12As, the thresholds used in Chapter 5 were not as stringent due to individual variation, so this would need to be confirmed with additional patients. Nevertheless, findings presented in Chapter 5 suggest that, even taking into account the expected variation in response and lower

effects due to the use of primary cells (Jabs *et al.*, 2017), alterations to patient methylomes may still be occurring in response to EDCs.

We then wanted to see if changes in the epigenome could be associated with changes in gene expression. In Chapter 4 we investigated this using rt-PCR on genes identified as significantly differentially methylated. Overall, we saw a trend in changes to gene expression, yet, interestingly, a change in gene expression could be seen after exposure to compounds that induced no change in methylation. For example, no E2-induced DMPs could be identified, yet exposure to $1x10^{-8}$ M E2 elicited a significant change in gene expression. This led us to hypothesise that additional mechanisms may also be influencing expression, such as direct binding to ERs and impacting classical signalling pathways.

In Chapter 5 we took a genome-wide approach by utilising RNA-sequencing technology to quantify gene expression changes. Several hundred differentially expressed genes were identified in response to both BPA and propylparaben, many of which could be related to breast cancer risk. Whilst just 5% were also differentially methylated, this finding indicated that, at least for some genes, methylation may be playing a role in regulating gene expression. If, after investigating additional patients, similar trends were found to be significant, it would support the hypothesis that the epigenome may link EDC exposures and breast cancer risk, potentially by influencing gene expression.

Alterations to key genes were also reported in Chapter 3. Whilst in this chapter we did not examine whether epigenetic changes were also present, these data evidenced the ability of EDCs to interfere with cellular functioning. Alterations to ERs, as well as genes involved in cell cycle regulation were all seen to be differentially expressed after exposure to *o,p'*-DDT, BP-3, BPA and propylparaben. Interestingly however, many of the changes were not seen to be significant. Furthermore, of all the genes tested in Chapter 3, only *ESR1* was seen to be differentially methylated in either Chapter 4 or 5. This suggests that despite a wealth of literature suggesting the ability of these genes to be impacted by EDC exposure (e.g. Silva, Kabil and Kortenkamp, 2010; Mlynarcikova, Macho and Fickova, 2013; Alonso-Magdalena *et al.*, 2015), in this case, it may not be methylation driving such a change. Thus, whilst these experiments

suggest it is possible the epigenome can link EDC exposure and breast cancer risk, methylation changes do not necessarily regulate traditionally investigated pathways and genes. Instead, it is possible the epigenome may be regulating novel pathways that are associated with breast cancer risk, but have not yet been considered in relation to xenoestrogen exposures. Consequently, in the future, research designed to examine these pathways closer to improve our understanding of the ability of EDCs to alter their regulation and contribute to breast carcinogenesis will be necessary.

6.2.3. The use of primary cells offers a novel opportunity to investigate effects in a more representative assay

In Chapter 5, we discussed the need to progress away from cell line dependent studies and towards primary cells. Primary cells are generally deemed more representative of human responses to exposures and overcome several limitations of cell lines including lack of heterogeneity and batch variations (Neimark, 2015). Findings described in Chapter 5 support this need. We found that whilst epigenetic and genetic changes could still be seen in primary cells, the changes appeared to be different in comparison to the MCF-12A cell line. For instance, there was little overlap in regional methylation sites between Chapters 4 and 5. This difference may be attributed to the patient variation seen in primary cells. All tested patients possessed significantly different genetic and epigenetic profiles, even before cells were exposed to compounds. Whilst we may begin to see shared pathways between cell lines and primary cells with the incorporation of additional patients, our findings call into question the relevance of conclusions drawn from cell lines to human population responses. If we are to truly understand how EDCs could be associated with breast cancer risk in human populations, it is essential that future studies recognise the value primary cells can bring to research. With the increasing availability of primary cells from cell banks such as the Breast Cancer Now facility, researchers have the opportunity to utilise these resources and dramatically improve the reliability of study extrapolations.

An additional strength of work within this thesis was that the medium used was not stripped, meaning there was background levels of oestrogen present in the model. Furthermore, the EDCs were diluted in 100% ethanol. Both factors add an additional layer of complexity to the model, with both being considered risk factors to breast cancer. However, this is a further demonstration of how the model was representative of conditions in the human breast.

6.2.4. Mixture effects can be observed with as little as four EDCs

Numerous papers are now calling for studies that begin to mimic the large number of compounds individuals are exposed to in the environment. It has been suggested that whilst effects at low concentrations may not be observed with individual chemical exposures, compounds may act in combination to add to an overall oestrogenic load. Taken together, such mixtures could make a significant contribution to breast carcinogenesis. The so called 'something from nothing effect' has been seen in a variety of test systems (Silva, Rajapakse and Kortenkamp, 2002; Jin *et al.*, 2014; Cobbina *et al.*, 2015; Seeger *et al.*, 2016), however to date has not been explored in an *in vitro* system that recapitulates the mammary gland. One aim within this thesis was to not only investigate the effects of individual compounds, but also examine how they acted in combination. Based on evidence from other test systems we believed such combinations could elicit more significant effects.

In Chapter 3 we used a morphological endpoint (acini area) to produce individual compound dose-response curves and used these to predict the effect of a mixture of four widespread EDCs (*o,p'*-DDT, benzophenone 3, BPA and propylparaben). After testing the mixture in a 3D culture of mammary epithelial cells we showed that by combining just four common compounds, EDCs could be working in combination, resulting in an additive mixture effect. Interestingly, despite the lack of significant effects in acini development induced by with single compounds at tissue relevant concentrations, the chemical mixture did elicit such effects. This finding evidences the hypothesis that EDCs can act in combination to induce a more significant effect in comparison to individual exposures. This led us to speculate that if more complex mixtures were tested with

additional chemicals, we would start to see how EDC exposure could make a significant contribution to breast cancer risk.

We also tried to see if similar mixture effects could be inferred by changes to gene expression. We were not able to produce dose response curves based on log2 (fold change) results, on account of the variation and apparent lack of a concentration dependent effect in many of the target genes. Consequently, we tried to determine if a difference in effect magnitude could be observed, comparatively to the 'something from nothing effects' (Silva, Rajapakse and Kortenkamp, 2002). In some genes, such as *BRCA1*, an increased effect in response to the mixture could be seen. However, potentially due to the assay sensitivity, other genes showed little evidence of an increased mixture response. We concluded that this absence of any clear trend may be attributed to distinct populations of cells within the analysed acini, that have previously been shown to possess differing genetic profiles (Marchese, 2013). In the present study we were not able to split these cell populations, however suggest that if subsequent experiments could isolate inner and outer acini cells, clearer mixture effects may be observed. Finally, it is plausible that such mixture effects may be gene specific. By adopting more advanced genome-wide analyses like RNA-sequencing used in Chapter 5, key genes displaying clear mixture effects may be identified.

Despite a lack of clear results at gene level, findings in Chapter 3 support the argument that the array of chemicals individuals are exposed to on a daily basis may be acting together and producing a more substantial effect. These chemicals could then make a contribution to an individual's oestrogenic load and this in turn could be associated with an increase in breast cancer risk. We therefore recommend that we continue to develop our understanding of how mixtures of EDCs could be associated with breast carcinogenesis, with the aim of incorporating this knowledge into comprehensive risk models and accurate patient awareness campaigns.

6.2.5. EDCs may be affecting populations predisposed to breast cancer development

Whilst investigating mixture effects is a considerable step forward, it is important to note that individuals are also subject to a range of other risk factors that are all adding to and individual's breast cancer risk. Arguably the most understood genetic risk factor is the presence of a germline *BRCA1* mutation. Individuals are reported to be predisposed to an increased risk of developing breast and ovarian cancers within their lifetime (Milne and Antoniou, 2016). Thus, any factors that can impact this high-risk population can translate into a dramatic increase in absolute risk for individuals (Antoniou *et al.*, 2008, 2010). Literature has previously suggested that the presence of a *BRCA1* mutation could increase the susceptibility of individuals to exposure to xenoestrogens (Jones *et al.*, 2010; Fernandez *et al.*, 2012), however no study has extensively investigated this theory. Consequently, in Chapter 5 we wanted to determine whether this was the case using primary cells from individuals with a confirmed *BRCA1* mutation.

Unfortunately, due to patient variability we were not able to conclude whether the *BRCA1* mutated biotype was more susceptible to the impacts of EDC exposures. What could be drawn from Chapter 5, however, is that both BPA and propylparaben may be inducing changes to the genome and epigenome of this predisposed population, which could have a significant impact on individual risk. Such changes included the hypermethylation of several tumour suppressor genes like *LMO3* and the biomarker *RPL13*. We also reported the genetic and epigenetic alteration of *DCN*, which has been associated with breast cancer (Goldoni and Iozzo, 2008; Gubbiotti *et al.*, 2016). Due to the epigenetic response variation, we did not perform functional analysis on genes epigenetically modified, however differentially expressed genes were found to be associated with processes including cilium assembly and cell cycle regulation, which have been associated with breast tumourigenesis. The investigation of additional individuals with a *BRCA1* mutation would be required in order to speculate whether this population would be considered more susceptible to the impacts of EDCs. Nevertheless, any factors that can be associated with breast cancer risk in an already vulnerable population should not be overlooked.

6.3. Future work

Despite making a contribution to the current knowledge surrounding EDC exposures and breast cancer risk, this series of experiments has highlighted multiple avenues for future work. Such research would not only improve the relevance of the findings outlined here, but also provide additional evidence to strengthen our understanding of how these compounds work and how they are associated with breast carcinogenesis. This knowledge could then be used in the development of bespoke risk models and prevention strategies, ultimately improving patient care.

6.3.1. Strengthen present conclusions

In Chapter 5 we began to see that the effects of EDC exposures recorded in cell lines could also be observed in primary cells. Regrettably, due to the funding and time constraints of the present study, we were unable to test more than three patients per biotype. Most likely attributed to the variation between patients, this number was not sufficient to identify significant changes to the methylome and therefore thresholds had to be reduced. This meant we could only obtain an indication of what epigenetic changes may be occurring, however no clear definitive alterations could be identified at this time. Furthermore, this meant we could not state whether predisposed populations were more or less susceptible to the impacts of EDCs. By adding additional patients to this study, we believe firmer conclusions could be reported. These findings could then make a significant impact to the wider literature.

6.3.2. Epigenetics and mixtures

In Chapter 3 we saw that mixtures of xenoestrogens can elicit additive mixture effects at the morphological level. Whilst changes were also induced to gene expression it was not possible to demonstrate a mixture effect due to the lack of clear dose response curves. Interdisciplinary research groups would benefit from the development of mixture prediction models in cases where compounds do not elicit traditional concentration dependent effects. In addition,

investigating whether mixture effects can be seen at the epigenetic level would be a valuable step forward and shed light on whether the large number of EDCs individuals are exposed to can have a significant impact on an individual's epigenetic profile. Initially, simple measures of global methylation would be suitable to address this. Looking further into the future, employing array-based technologies, such as the 850k methylation array used in Chapters 4 and 5, could provide a more detailed and insightful result. Mixture studies on this scale may become more accessible once array-based technologies become more affordable.

6.3.3. Improving the co-culture assay

Throughout this thesis, significant enhancements have been made to the relevance of the cell culture assay. From studies relying on unrepresentative 2D models, we have utilised more sophisticated methods that begin to recapitulate structures within the human breast. However, there are still numerous improvements that could be made to the models used here that would further improve how well these systems represent the human mammary gland. There is a wealth of literature that demonstrates cells respond differently depending on the culture conditions (Payne *et al.*, 2000; Dhiman, Ray and Panda, 2005; Horning *et al.*, 2008; Pickl and Ries, 2009; Marchese, 2013). Therefore, it is of paramount importance that experiments are conducted in assays that are as representative of the breast as possible.

In Chapters 4 and 5 we added two additional stromal cell types, which allowed for cellular interactions. Yet the breast consists of more than three cell types and having additional cell types represented in an *in vitro* assay would improve the relevance of exposure responses. For example, it is now known that adipocytes play a critical role in breast development, maintenance and tumourigenesis (Dirat *et al.*, 2011; Wang *et al.*, 2017; Zwick *et al.*, 2018).

A further limitation of the culture systems used in this thesis is that they remain static. One tool that may address this constraint is the organ-on-a-chip (OOCs). OOCs are multi-channel 3D microfluidic cell culture models that simulate organ properties (Aziz *et al.*, 2017). The approach has already been utilised to predict human pharmacokinetic and pharmacodynamic responses to drugs and are being widely hailed as an alternative to extensive animal testing (Han *et al.*,

2012; Baker *et al.*, 2013; Fuyin Zheng *et al.*, 2016). The microfluidic cell culture devices can be used to simulate tissue physiology by recapitulating cellular interactions and mechanical properties of a functioning tissue. OOCs can also reproduce normal mechanical cues, which have been shown to influence organ development and function, such as fluid shear stress, cyclic strain and mechanical compression (Bhatia and Ingber, 2014). Breast-on-a-chip models were first successfully developed in 2011 and were shown to mimic the mammary ductal system and allow for paracrine interactions (Grafton *et al.*, 2011). To date these assays have not been used to investigate the effects of EDCs, but represent a critical step towards representing the breast and its responses *in vitro* (Nawroth *et al.*, 2018).

6.3.4. Impact of EDCs on the stroma

Whilst including stromal cells is important, this thesis has not examined the effects of EDCs on these various cell types. Within the literature there is a distinct lack of research in this area, meaning we have very little understanding of how EDCs interact with the stroma. As it is now recognised the stroma is involved in tumour development and progression, understanding whether xenoestrogens directly impact different cell types within the breast could be considered fundamental when speculating the contribution of exposure to breast cancer risk. Thus, future research should investigate how EDCs interact with stromal cells genetically, epigenetically and morphologically.

6.3.5. Alternative epigenetic mechanisms

In this thesis we have investigated the effects of EDC exposures on the epigenome of epithelial cells by examining the DNA methylation profiles. In addition to methylation, two other epigenetic mechanisms have been well evidenced in their role in breast cancer. Both histone modification and miRNAs have been shown to be implicated in breast cancer initiation and progression (Nelson and Weiss, 2008; Elsheikh *et al.*, 2009; Zhao *et al.*, 2016). It must be noted that none of these mechanisms act in isolation. Interactions between each of these mechanisms is required to maintain the natural state of the epigenome (Sharma, Kelly and Jones, 2010).

Consequently, we must understand how EDCs affect each of these processes to achieve a comprehensive understanding of how the epigenome can link exposure to breast cancer risk. Although literature has predominantly focused on DNA methylation, it is important that other mechanisms are not overlooked, as they should be considered equally important. Limited evidence does exist to suggest EDCs could interfere with these alternative mechanisms (e.g. Kumar and Thakur, 2017; Senyildiz *et al.,* 2017), however our understanding is not as developed as methylation.

6.3.6. Multifactorial effects

A recent article by Friedenreich and McTiernan (2018) stressed the importance of combining risk variables to create a realistic and inclusive assessment of an individual's breast cancer risk. By doing so, both intrinsic and extrinsic risks can be jointly considered when developing cancer prevention guidelines. To date, the majority of research has focused on understanding individual risk factors and their relation to breast cancer, yet no single risk factor has been identified that can exclusively account for and individual's risk. Friedenreich and McTiernan believe future investigations should examine combinations of risk factors using randomised clinical trials, however it is possible to begin some of this work *in vitro*. Although additional investigations are required to further our understanding of chemical exposures, it would be of great interest and benefit to understand how EDCs interact with other risk factors such as stress, alcohol, smoking and diet. Many such risk factors have been represented in *in vitro* assays, yet we do not understand how they might work in combination to influence breast cancer risk. Additional authors have also expressed the necessity to consider combined associations of risk factors (Garcia-Closas, Gunsoy and Chatterjee, 2014; Wu *et al.*, 2016; Arthur *et al.*, 2018; Heitz *et al.*, 2018). Therefore, studies that consider these multifactorial processes may make a significant impact to breast cancer prevention.

6.4. Conclusion

This thesis examined each of the study objectives outlined in Chapter 1 and made a significant contribution to the current knowledge surrounding how EDCs may induce changes indicative of early breast cancer development. We have demonstrated, at multiple endpoints, the ability of widespread oestrogen-mimicking compounds to alter normal cellular functioning, individually and in combination, that could potentially be associated with breast cancer development. In addition, this work supports a wealth of emerging literature evidencing that EDCs can interact with the epigenome, linking exposures to breast carcinogenesis through a novel mode of action. Here we showed this with two ubiquitous compounds, both in the MCF-12A cell line and in primary cells derived from individuals with and without *BRCA1* mutations. Together these findings provide a strong evidence base demonstrating the ability of EDCs to induce alterations inactive of neoplastic transformations. Such observations support the presence of a relationship between EDC exposures and breast cancer risk and justify further research into this field.

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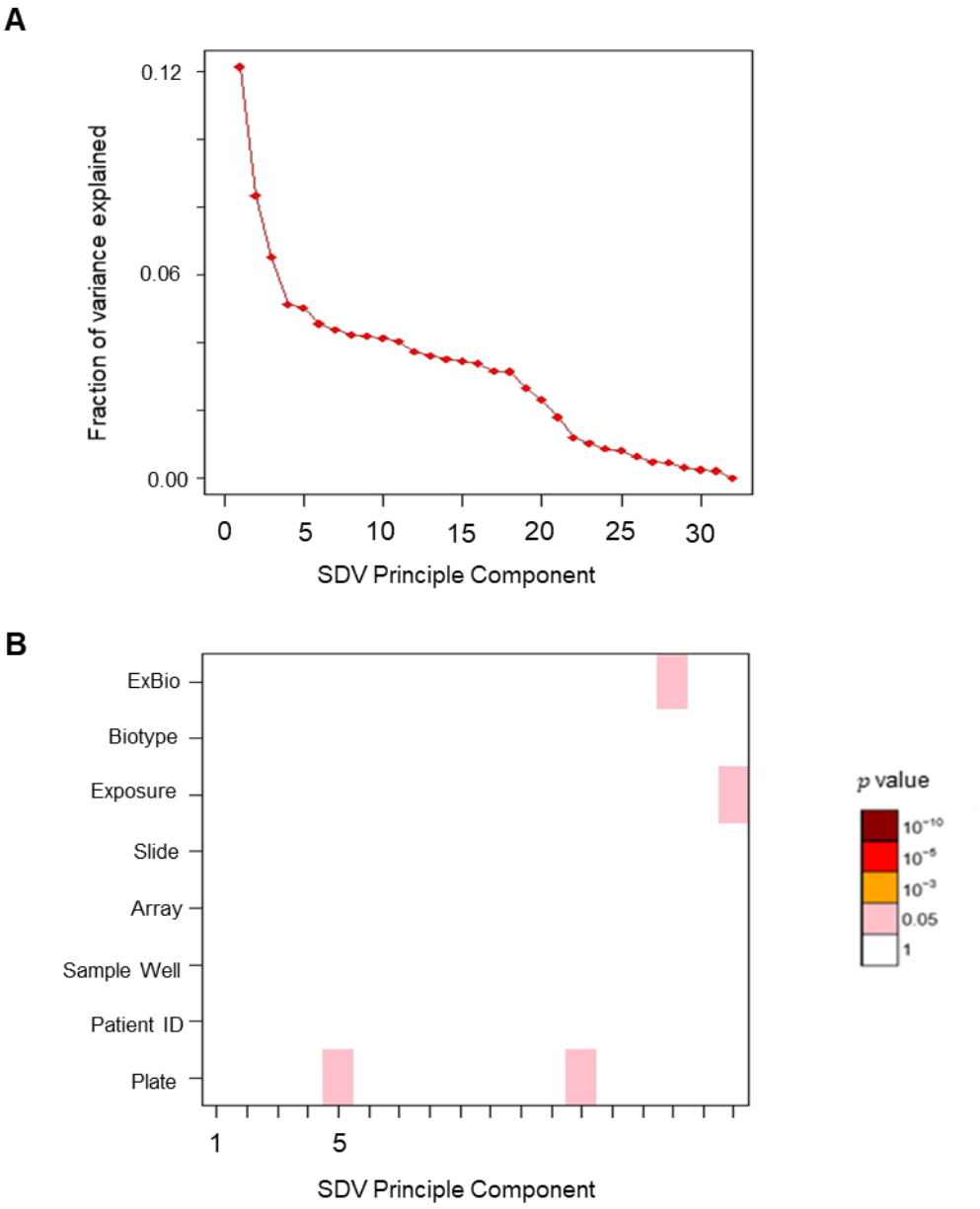
Appendices

Appendix. I. Sequencing information for single-end 50 bp sequences RNA-Seq analysis.

Patient	Treatment	Barcode	
NP1	EtOH	TATAGCCT	ATTACTCG
	1x10-7 BPA	TATAGCCT	TCCGGAGA
	1x10-7 Propylparaben	TATAGCCT	CGCTCATT
NP2	EtOH	TATAGCCT	GAGATTCC
	1x10-7 BPA	TATAGCCT	ATTCAGAA
	1x10-7 Propylparaben	TATAGCCT	GAATTCGT
NP3	EtOH	TATAGCCT	CTGAAGCT
	1x10-7 BPA	TATAGCCT	TAATGCGC
	1x10-7 Propylparaben	TATAGCCT	CGGCTATG
BRCA1	EtOH	TATAGCCT	TCCGCGAA
	1x10-7 BPA	TATAGCCT	TCTCGCGC
	1x10-7 Propylparaben	TATAGCCT	AGCGATAG
BRCA2	EtOH	ATAGAGGC	ATTACTCG
	1x10-7 BPA	ATAGAGGC	TCCGGAGA
	1x10-7 Propylparaben	ATAGAGGC	CGCTCATT
BRCA3	EtOH	ATAGAGGC	GAGATTCC
	1x10-7 BPA	ATAGAGGC	ATTCAGAA
	1x10-7 Propylparaben	ATAGAGGC	GAATTCGT



Appendix. II. Successful removal of cofounders in primary cells achieved using ComBat. (A) no single principle component accounted for a significant fraction of the observed variation in primary cell methylation alterations. (B) After applying ComBat, neither patient ID or Biotype remain as significant cofounders.

Appendix. III. Genes differentially altered genetically and epigenetically in primary cells without *BRCA1* mutations after exposure to propylparaben. Delta Beta and LogFC in gene expression values are shown, along with respective statistical significance denoted by p.

Gene	Delta Beta	p	LogFC	p
ADD3	-0.15	0.36	3.92	0.01
AHI1	-0.14	0.49	4.21	0.04
AHRR	-0.10	0.84	2.64	0.04
ANKRD45	-0.11	0.91	4.74	0.03
ANO4	-0.17	0.38	3.54	0.03
BASP1	-0.12	0.42	5.65	0.03
BBS2	-0.13	0.46	2.99	0.03
BCL9	-0.17	0.17	3.04	0.02
BTAF1	-0.12	0.30	4.48	0.04
C4orf47	-0.16	0.24	3.39	0.03
C8orf37-AS1	-0.17	0.31	4.55	0.02
CAMK2B	-0.12	0.41	3.22	0.03
CAMK2D	-0.13	0.57	3.27	0.03
CCDC129	-0.12	0.36	2.78	0.03
CCDC15	-0.13	0.35	5.09	0.03
CCDC39	-0.12	0.40	3.11	0.04
CCND3	-0.12	0.58	3.75	0.03
CDK17	-0.13	0.20	3.33	0.03
CHCHD3	-0.11	0.92	3.04	0.04
CUX2	0.12	0.26	-4.93	0.03
DAAM1	-0.14	0.20	3.17	0.03
DCN	0.12	0.34	-4.76	0.03
DNAH5	-0.18	0.29	3.70	0.03
DNAH7	-0.17	0.62	2.95	0.03
DNM3	-0.12	0.30	2.78	0.03
DROSHA	-0.13	0.38	4.69	0.04
ERC1	-0.13	0.52	3.90	0.03
EREG	-0.15	0.56	2.63	0.03
FAM133B	-0.12	0.51	2.85	0.03
FLJ43860	-0.10	0.38	5.45	0.05
GLS2	-0.11	0.56	3.61	0.03
GNAQ	-0.14	0.41	3.59	0.02
GPHN	-0.13	0.43	5.17	0.03
GRID2	-0.13	0.38	3.52	0.03
HLTF	-0.17	0.29	2.83	0.04
MAGI2	-0.12	0.43	5.70	0.04
MCTP1	-0.11	0.75	2.58	0.03
NCKAP1	-0.13	0.19	4.44	0.02
NR3C1	-0.12	0.52	2.72	0.03
PCLO	-0.14	0.32	4.68	0.02
PCYT1B	0.15	0.46	-2.64	0.03

PDE11A	-0.15	0.38	2.82	0.02
PDE3A	-0.18	0.46	2.71	0.03
PDE4D	-0.15	0.29	2.79	0.03
PDGFD	-0.11	0.40	4.44	0.04
PEG3	-0.13	0.55	2.96	0.03
PIWIL1	-0.13	0.41	3.93	0.03
PLN	-0.13	0.71	3.94	0.04
PNPLA7	-0.17	0.85	4.33	0.03
PPAP2B	-0.17	0.31	3.03	0.02
PRDM16	0.10	0.26	-5.61	0.03
PRKCH	-0.10	0.78	3.44	0.03
PSD3	-0.14	0.79	2.86	0.03
PTPRN2	0.14	0.46	-3.47	0.03
RABGAP1L	-0.13	0.39	3.42	0.04
RANBP9	-0.15	0.52	2.73	0.04
ROR1	-0.16	0.23	3.15	0.03
RUFY3	-0.16	0.72	2.67	0.01
RWDD4	-0.16	0.18	3.64	0.03
SDC3	-0.12	0.87	3.25	0.02
SDR39U1	0.14	0.19	-5.18	0.03
SFXN5	0.13	0.51	-2.82	0.03
SHPRH	-0.13	0.83	2.64	0.03
SLC7A8	0.10	0.67	-2.92	0.03
SOX5	-0.16	0.23	3.50	0.03
SPEF2	-0.14	0.35	3.78	0.03
STK3	-0.13	0.24	3.15	0.03
TBC1D5	-0.12	0.32	3.98	0.03
TBL1XR1	-0.13	0.49	5.61	0.02
TCERG1L	-0.14	0.35	2.98	0.03
TLN2	-0.13	0.29	3.67	0.04
TRIP13	0.11	0.34	-5.73	0.03
UBR5	-0.13	0.38	2.94	0.03
UNC119B	-0.16	0.25	2.86	0.03
VWA8	-0.13	0.95	5.54	0.03
WDR70	-0.11	0.33	3.15	0.02
YTHDC2	-0.13	0.49	2.84	0.04
ZNF630	0.13	0.24	-3.24	0.03
ZNF639	0.10	0.85	-2.91	0.04

Appendix. IV. Genes differentially altered genetically and epigenetically in primary cells without *BRCA1* mutations after exposure to BPA. Delta Beta and LogFC values are shown, along with respective statistical significance denoted by p.

Gene	Delta Beta	p	LogFC	p
ABCG1	0.11	0.83	-6.34	0.01
ATP8B4	0.57	0.69	-3.07	0.01
BBS2	0.10	0.64	-3.10	0.02
C3orf58	-0.11	0.76	3.00	0.02
CAMK2D	0.14	0.85	-3.92	0.02
CCDC146	0.11	0.88	-2.67	0.02
CCDC170	0.11	0.79	-4.33	0.01
CCDC88A	0.12	0.82	-3.59	0.04
CCNY	0.16	0.27	-2.80	0.02
CDKAL1	0.13	0.92	-3.28	0.02
CHEK1	-0.11	0.66	3.42	0.02
DCN	0.21	0.63	-5.35	0.01
DHRS9	0.11	0.85	-3.38	<0.01
DOCK2	0.36	0.91	-6.09	0.03
DTNBP1	0.12	0.61	-3.09	0.04
ESRRG	-0.11	0.59	3.49	0.04
FOXP1	0.21	0.81	-3.59	0.05
FUBP1	-0.16	0.67	3.47	0.01
GPHN	0.11	0.83	-2.74	0.01
LGR4	0.11	0.67	-3.26	<0.01
LMO3	-0.22	0.82	6.73	0.01
lrg4	-0.11	0.56	5.00	0.04
METTL8	0.15	0.89	-2.67	0.02
MYO1D	0.11	0.82	-5.13	0.02
NCKAP1	0.10	0.80	-2.77	0.04
NR6A1	-0.20	0.67	2.62	0.04
PCDHB1	-0.12	0.30	5.01	0.01
PDE11A	0.11	0.93	-3.70	0.02
PDE4D	0.50	0.75	-3.15	0.01
PEX7	0.10	0.80	-2.98	0.04
PNCK	0.10	0.77	-5.22	0.02
ROBO1	-0.15	0.90	3.23	0.02
SDC2	0.40	0.85	-3.38	0.02
SLC15A1	0.12	0.93	-3.94	0.02
SORBS1	0.11	0.43	-5.42	0.02
STAG3	-0.18	0.49	3.70	0.02
TCF4	0.14	0.72	-3.29	0.02
THSD7A	0.70	0.67	-3.66	0.04
UBE4B	0.15	0.82	-3.16	0.02
UBR5	0.14	0.77	-6.63	0.02

XKR6	0.13	0.94	-2.90	0.04
ZCCHC11	0.11	0.94	-2.98	0.04
ZFY	0.12	0.33	-3.61	0.04
ZNF177	0.12	0.96	-2.75	0.05
ZNF596	0.16	0.84	-4.37	0.01
ZWILCH	0.11	0.91	-2.77	<0.01

Appendix. V. Genes differentially altered genetically and epigenetically in primary cells with *BRCA1* mutations after exposure to propylparaben. Delta Beta and LogFC values are shown, along with respective statistical significance denoted by p.

Gene	Delta Beta	p	LogFC	p
ABL1	-0.10	0.86	3.97	0.03
ACP2	-0.10	0.85	3.08	0.03
ADARB2	-0.10	0.96	5.47	0.02
ADGRG1	-0.10	0.84	2.92	0.03
AHCTF1P1	0.12	0.78	-6.59	0.01
AMMECR1	0.12	0.70	-3.22	0.04
ARHGAP6	-0.10	0.83	3.53	0.01
BAZ2B	-0.10	0.82	3.07	0.04
BRSK2	-0.10	0.97	3.70	0.03
C6orf10	-0.24	0.79	2.85	0.03
CACNA1C	0.12	0.64	-5.36	0.04
CCDC50	-0.23	0.90	3.11	0.05
CLVS1	-0.11	0.86	2.78	0.01
CNGB1	-0.12	0.92	4.48	0.08
CNR2	-0.17	0.86	3.22	0.01
DLG2	-0.17	0.87	5.65	<0.01
DNMT3L	-0.17	0.78	2.72	0.05
DUSP9	0.12	0.80	-2.59	0.03
ESR1	-0.17	0.85	2.01	<0.01
ETS1	-0.16	0.87	3.78	<0.01
FAM47E	-0.16	0.97	5.35	0.03
FCHO1	-0.16	0.79	3.05	<0.01
FGF5	-0.16	0.64	4.49	0.04
FGGY	-0.15	0.94	2.76	0.04
GABBR2	-0.15	0.96	2.07	0.01
GPD2	-0.15	0.70	2.31	0.05
GRIA3	-0.15	0.96	3.34	0.01
GRIK4	-0.15	0.94	3.75	0.03
GRIN1	-0.15	0.79	7.35	0.03
GRIN2A	-0.15	0.75	3.85	0.03
HIST1H2AE	-0.15	0.87	3.82	0.05
HNMT	-0.14	0.62	2.87	0.01
INADL	-0.14	0.90	3.56	0.04

KAT2B	-0.14	0.82	3.09	0.01
KCNMB2	-0.14	0.96	3.21	0.04
LACC1	-0.14	0.81	3.06	0.01
LCE3D	-0.14	0.78	3.90	0.03
LDB2	-0.14	0.70	2.66	0.05
MACROD2	-0.14	0.79	2.64	0.03
MAST4	0.12	0.60	-2.74	0.01
MCFD2	-0.14	0.73	3.46	0.03
MCTP1	-0.14	0.92	2.49	0.03
MEF2C	-0.14	0.89	2.83	0.03
MFSD4	-0.13	0.98	2.95	<0.01
MYF5	-0.13	0.60	2.89	0.04
NELL1	-0.13	0.76	2.19	0.05
NHS	-0.12	0.79	3.97	0.01
NHSL2	-0.12	0.61	4.36	0.03
PCDHB1	-0.12	0.96	3.20	0.03
PRDM16	-0.12	0.90	4.82	0.03
PTPRN2	-0.12	0.85	2.21	0.01
RAPSN	-0.12	0.83	3.06	0.01
RBFOX1	-0.12	0.86	3.01	0.02
RIMS3	-0.12	0.62	2.01	0.03
SDK2	-0.12	0.84	2.83	0.03
SH3KBP1	-0.12	0.93	2.14	0.04
SIM1	-0.12	0.63	2.94	0.03
SLC25A21	-0.12	0.98	5.64	0.03
SLIT2	-0.12	0.73	3.23	0.01
SLIT3	0.12	0.89	-3.92	0.04
SMS	0.12	0.90	-2.83	0.03
SORCS2	-0.12	0.80	2.33	0.03
SRPX	-0.12	0.65	3.76	0.03
TEX41	-0.12	0.98	3.41	0.03
TMEM187	0.12	0.74	-3.02	<0.01
TMEM91	-0.12	0.79	2.11	0.03
TSPAN7	-0.12	0.88	3.35	0.05
UBQLN1	-0.12	0.95	4.84	0.03
UGGT2	-0.12	0.96	1.43	<0.01
WDR66	-0.12	0.35	2.06	0.05
ZNF555	-0.18	0.74	2.26	0.05

Appendix. VI. Genes differentially altered genetically and epigenetically in primary cells with *BRCA1* mutations after exposure to BPA. Delta Beta and LogFC values are shown, along with respective statistical significance denoted by p.

Gene	Delta Beta	p	LogFC	p
ADGRG1	0.11	0.50	2.43	0.03
ANKS1B	0.18	0.89	-2.01	0.05
APBA2	0.16	0.66	-3.87	0.06
ATAD2B	0.26	0.79	3.41	0.03
BCAR3	0.15	0.59	2.18	0.03
BCLAP	0.13	0.98	-2.36	0.03
C21orf2	-0.21	0.88	2.75	0.01
CADPS	-0.21	0.79	3.93	0.06
CAMK2B	0.15	0.74	-2.28	0.01
DDAH2	-0.21	0.79	2.96	0.01
DIP2C	-0.21	0.72	3.56	<0.01
DLG2	-0.21	0.94	4.51	<0.01
EGFL8	-0.20	0.86	2.90	0.01
ESYT3	0.14	0.75	-2.05	0.01
FAM199X	0.14	0.93	-2.65	0.04
FBXL16	-0.20	0.91	1.33	0.04
FCHO1	-0.20	0.65	2.34	0.03
GAB3	0.14	0.96	-2.92	0.04
GIPR	0.14	0.90	-3.13	0.04
IZUMO1	-0.20	0.77	3.42	0.04
LACC1	-0.19	0.80	6.24	0.04
LARP4	-0.42	0.61	3.51	0.04
LY6D	-0.19	0.86	4.70	0.01
MAP3K15	0.14	0.90	-6.14	0.03
MFSD4	-0.19	0.91	2.79	0.01
NLGN4Y	-0.19	0.46	6.53	0.01
OTUD5	0.13	0.88	-3.59	0.01
PPARG	-0.19	0.56	2.53	0.04
PPP1R3F	0.13	0.88	-3.83	<0.01
PRRT1	-0.19	0.86	3.90	0.01
PTPRN2	-0.19	0.95	2.98	0.03
RBM20	0.13	0.76	-3.22	0.01
RFTN1	-0.19	0.87	2.80	0.02
RGN	-0.19	0.90	4.60	0.01
RP2	-0.18	0.48	3.70	0.04
SAMD11	0.12	0.90	-4.63	0.01
SORCS2	-0.15	0.65	5.55	0.01
TSPYL2	-0.15	0.69	3.20	0.01
TTTY14	-0.15	0.89	5.07	<0.01
WWC3	-0.15	0.77	3.64	0.01