Molecular Mechanisms of Chemotherapy Resistance in Oestrogen Receptor Positive Breast Cancer

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Published work is presented in Chapter 6 of this thesis (BAXTER, D. E., KIM, B., HANBY, A. M., VERGHESE, E. T., SIMS, A. H. & HUGHES, T. A. 2018. Neoadjuvant Endocrine Therapy in Breast Cancer Upregulates the Cytotoxic Drug Pump ABCG2/BCRP, and May Lead to Resistance to Subsequent Chemotherapy. *Clinical Breast Cancer,* 18**,** 481-488.). All work presented in this chapter was performed by the candidate except the work presented in Figure 6.2.1 where BCRP expression was investigated in tumour samples of breast cancer.

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Abstract

Resistance to chemotherapy is a major obstacle to the successful treatment of breast cancer. In order to optimise treatment regimens and identify novel drug targets, it is important first to understand the molecular mechanisms that can lead to chemoresistance. In this thesis, I have investigated mRNA and miRNA expression profiles of tumour samples taken from patients with oestrogen receptor positive primary breast cancers treated with neoadjuvant chemotherapy (NAC) who displayed only a partial response. Samples were analysed before and after chemotherapy treatment. Gene expression profiles post-NAC suggested that the MAPK and PI3K-AKT pathways were activated. MiRNA expression profiles demonstrated three miRNAs that were consistently deregulated post-NAC. Further *in vitro* studies revealed that the increased expression of miR-26b and miR-195 contributed to significant increases in chemoresistance ($p<0.05$).

Pulldown assays using mimics of miR-26b and miR-195 as bait, together with RNA-Seq, led to the identification of possible mRNA targets of these two miRNAs. Further *in vitro* studies confirmed *REEP4* and *SEMA6D* as targets of miR-26b and miR-195 respectively. As targets of these miRNAs, decreased expression of these mRNAs would be expected to contribute to chemoresistance. Chemosensitivity assays suggested a consistent but not significant increase in resistance when *REEP4* was silenced, and a significant increase in resistance when *SEMA6D* was silenced ($p<0.05$). Investigations were performed to determine whether the expression of either of the corresponding proteins had any prognostic value. Results suggested that REEP4 expression was significantly related to disease free survival, although the precise relationship was unclear.

The effect of increased expression of the xenobiotic drug pump BCRP induced by endocrine therapy on chemoresistance was also investigated. Results suggested that increased BCRP expression led to significant increases in chemoresistance ($p<0.05$), thus suggesting that a treatment regimen of endocrine therapy followed by chemotherapy may not be beneficial.

I have identified in this thesis several molecular changes that are induced by chemotherapy or endocrine therapy that contribute to chemoresistance, including changes in mRNA, miRNA and xenobiotic drug pump expression.

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Abbreviations

AAV	Adeno-Associated Viral
ABC	ATP-Binding Cassette
Ago	Argonaute
AI	Aromatase Inhibitor
AMO	Anti-MiRNA Oligonucleotides
BCRP	Breast Cancer Resistance Protein
BCS	Breast Conserving Surgery
BRCA	Breast Cancer susceptibility gene
CAF	Cyclophosphamide, Doxorubicin, Fluorouracil
CDK	Cyclin Dependent Kinase
CI	Confidence Interval
CTCL	Cutaneous T Cell Lymphoma
DAB	3,3'-diaminobenzidine
DCIS	Ductal Carcinoma In Situ
DFS	Disease Free Survival
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
DTT	Dithiothetriol
EC	Epirubicin and Cyclophosphamide
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial to Mesenchymal Transition
EnR	Endoplasmic Reticulum
ER	Oestrogen Receptor
ERE	Oestrogen Response Element
FCS	Foetal Calf Serum
FFPE	Formalin-Fixed Paraffin Embedded
GPCR	G-Protein Coupled Receptor
H&E	Haematoxylin and Eosin
HER2	Human Epidermal Growth Factor Receptor 2
HNSCC	Head and Neck Squamous Cell Carcinoma
IHC	Immunohistochemistry

ISH	In Situ Hybridsation
LCM	Laser Capture Microdissection
LNA	Locked Nucleic Acid
MACS2	Model-based Analysis of ChIP-Seq 2
MAPK	Mitogen Activated Protein Kinases
MDR	Multi-Drug Resistance
miRNA	MicroRNA
mRNA	messenger RNA
MTE	Multiplexed Target Enrichment
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	Neoadjuvant Chemotherapy
NAET	Neoadjuvant Endocrine Therapy
NF	Nuclease Free
OS	Overall Survival
PARP	Poly(ADP-Ribose) Polymerase
PBS	Phosphate Buffered Saline
pCR	pathologic Complete Response
PCR	Polymerase Chain Reaction
PFS	Progression Free Survival
Pgp	P-glycoprotein
PI	Propidium Iodide
PMSF	phenylmethane sulfonyl fluoride
PPARE	Peroxisome Proliferator-Activated Receptor Response Element
PR	Progesterone Receptor
PRE	Progesterone Response Element
Pri-miRNA	Primary miRNA
PSI	Plexin-Sema-Integrin
PVDF	Polyvinylidene Fluoride
R	Resistant
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcription
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate

SEM	Standard Error of the Mean
SERD	Selective Oestrogen Receptor Degrader
SERM	Selective Oestrogen Receptor Mediator
SNP	Single Nucleotide Polymorphism
STAR	Spliced Transcripts Alignment to a Reference
TAM	4-hydroxy-tamoxifen
TBST	Tris Buffered Saline and Tween-20
TE	Tris and EDTA
TG	Thioglycerol
TGF-Beta	Transforming Growth Factor - Beta
TMA	Tissue Microarray
UTR	Untranslated Region
WT	Wild Type

Chapter 1: Introduction

1.1 Introduction to breast cancer

1.1.1 Incidence of breast cancer

Breast cancer is the second-leading cause of cancer death in women worldwide after lung cancer, and accounted for a quarter of all cancers diagnosed in women in 2012 worldwide (Ferlay et al., 2015). It is currently estimated that one in seven women in the UK will be diagnosed with breast cancer sometime in their lifetime, amounting to approximately 50 000 diagnoses per year (CRUK.Available:https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer/risk-factors#heading-Zero). However, with ongoing research into novel treatments and the advancement of technologies that allow earlier detection of cancer, a greater proportion of patients are surviving longer post-diagnosis. This is reflected in the fact that female breast cancer death rates (European age-standardised) have fallen by 40% in the UK since the mid-1980s (CRUK. Available: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/breast-cancer/risk-factors#heading-Zero).

1.1.2 Classifying breast tumours

Breast tumours are classified using a variety of systems in order to assess the extent to which they have spread, their relative growth rates, their aggressive potential, and to some extent the likelihood of them responding to specific therapies.

The extent to which the cancer has progressed or spread is commonly classified using the TNM staging system (Classification of Malignant Tumours) (Gospodarowicz et al., 2017). The ‘T’ indicates the size of the primary tumour and the extent of invasion of surrounding tissues (if any). The ‘N’ indicates if and how many lymph nodes are involved and the ‘M’ indicates whether any distant metastasis has occurred and if so, to what extent. Each classification and their stages are shown in Table 1.1.1.

Table 1.1.1: Parameters and classifications used to classify breast tumours (Senkus et al., 2015, Giuliano et al., 2017)

Parameter	Classification	Description
T	Tis	Carcinoma in situ
	T1, T2, T3, T4	Size of primary tumour (T4 is the largest)
N	N0	No evidence of regional lymph node metastasis
	N1-3	Extent/location of metastases present in lymph nodes (N3 is the largest number of nodes, or more distant nodes)
M	M0	No distant metastasis
	M1	Metastasis to distant organs

Depending on their TNM classification, tumours are subsequently divided into stages. These categories give a summarised overview of the extent of the disease and are shown in Table 1.1.2.

Table 1.1.2: Stages of breast tumours (Senkus et al., 2015)

Stage	TNM classifications		
0	Tis	N0	M0
IA	T1	N0	M0
IB	T0/1	N1	M0
IIA	T0/1	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0/1/2	N2	M0
	T3	N1/2	M0
IIIB	T4	N0/1/2	M0
IIIC	T0/1/2/3/4	N3	M0
IV	T0/1/2/3/4	N1/2/3	M1

Tumours are also classified according to their grade. This is based on the appearance of the tumour cells. Tumours are given grades of 1, 2 or 3, with ‘1’ designated to tumours where cells appear to be relatively normal, ‘2’ designated to tumours where cells appear to be slightly bigger than normal and vary more in shape, and ‘3’ designated to tumours where cells are very different to normal cells due to poor differentiation (Bloom and Richardson, 1957). It has been noted that tumours with lower grades have better outcomes

than higher grade tumours, which tend to recur and metastasise much earlier after initial diagnosis (Rakha et al., 2010).

Growing evidence has shown that earlier detection of cancer when tumours are of lower stages leads to better outcomes than late diagnoses (Hiom, 2015). This is likely due to fewer incidences of metastasis and involvement of lymph nodes at earlier stages of the disease (Saadatmand et al., 2015).

1.1.2.1 Molecular subtypes of breast cancer and associated survival statistics

Breast cancer is a heterogeneous disease that can be further classified into several different molecular subtypes. The subtypes that are used clinically were initially defined using hierarchical clustering of the gene expression profiles of breast tumours (Perou et al., 2000, Sorlie et al., 2001), but in clinical practice this classification is made using surrogate markers, based around three receptors (Onitilo et al., 2008). The expression of these receptors is determined at diagnosis and, importantly, partially determines what treatment regimens would be the most effective (see section 1.2). The three receptors used by pathologists to determine subtype are the oestrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). The presence and absence of the various combinations of expression of these receptors largely determines into which subtype of breast cancer the tumour is defined (Brenton et al., 2005, Voduc et al., 2010), although the proliferation marker Ki67 is also sometimes used (Hugh et al., 2009, Keam et al., 2011).

The four main subtypes of breast cancer used clinically in the UK are luminal A, luminal B, HER2 and triple negative, sometimes referred to as basal, although it should be noted that the terms actually describe different groups of tumours (Brenton et al., 2005, Onitilo et al., 2008, Voduc et al., 2010). A summary of marker expression for each subtype is shown in Table 1.1.3. In brief, as the name suggests triple negative breast tumours do not express any of the three receptors, HER2 subtype breast tumours only express the HER2 receptor, luminal B breast tumours express ER and may express PR and HER2 receptors, and luminal A breast tumours express ER and PR but not the HER2 receptor.

Table 1.1.3: Molecular subtype classifications of breast cancers showing receptor status (Feeley et al., 2014, Yersal and Barutca, 2014, Prat et al., 2015)

MOLECULAR SUBTYPE	RECEPTOR STATUS			Ki67
	ER	PR	HER2	
Luminal A	+	+/-	-	Low
Luminal B	+	+/-	+/-	High
Triple Negative	-	-	-	High
HER2	-	-	+	High

The prevalence of each subtype of breast cancer is largely weighted towards those expressing the oestrogen receptor, therefore luminal A and luminal B subtypes, with 65-75% of all breast cancers being diagnosed expressing this receptor (Voduc et al., 2010, Ignatiadis and Sotiriou, 2013). Breast cancers that express the HER2 receptor (both the HER2 subtype and HER2 positive luminal B subtypes) account for roughly 15% of all breast cancers (Loibl and Gianni, 2017), and triple negative breast cancers account for the remaining proportion. Long-term survival and risk of metastasis are just some of the factors that differ between these subtypes. Response to therapies also differs between subtypes (Rouzier et al., 2005).

Different molecular subtypes of breast cancer are associated with different prognoses. Despite the fact that luminal A and luminal B subtypes both largely express the ER, the former generally has a better prognosis associated with it than the latter. Luminal A breast cancers have a ten-year survival rate of roughly 85% whereas luminal B breast cancers have a ten-year survival rate of just over 65% (Haque et al., 2012). However, luminal A breast cancers have been noted to have a tendency to lay dormant for many years and can recur beyond a decade after initial diagnosis (Di Leo et al., 2012, Haque et al., 2012). It is therefore important for this particular subgroup of patients to be carefully monitored for any recurrences well beyond the first five years post-initial diagnosis. Luminal A breast tumours generally have higher expression of ER related genes whereas luminal B tumours tend to have higher expression of genes involved in cell proliferation (Brenton et al., 2005).

Basal-like breast cancers are associated with an initial poor prognosis as they typically have an aggressive nature and suffer relapses rapidly (Toft and Cryns, 2010). However, survival rates ten years post-initial diagnosis tend to stabilise at roughly 75% in this subtype with later recurrences being relatively rare

(Haque et al., 2012). HER2 positive breast cancers are similar to triple negative breast cancers in that they are also associated with an initial poor diagnosis and aggressive nature (Voduc et al., 2010). Over-expression of this receptor correlates with numerous poor prognostic features including large tumour size and high nuclear grade (Nielsen et al., 2009). Ten-year survival rates of HER2-positive breast cancer are just below 65%. However, unlike with basal-like breast cancer, the survival probability of the HER2 molecular subtype continues to decrease beyond ten years post-initial diagnosis (Haque et al., 2012). The poor prognoses associated with ER negative tumour subtypes has been hypothesised to be linked to their resistance to the ER-targeted treatments that are routinely used with ER positive tumours (Dai et al., 2015).

1.2 Therapies in the treatment of Breast Cancer

1.2.1 Local and systemic therapies are available for the treatment of breast cancer

There are multiple treatment options available for primary breast cancer. However, viable treatment options for individual patients depend on a number of factors. The majority of treatment strategies include surgery to remove the primary tumour (Miller et al., 2016). This involves either breast conserving surgery (BCS), where a minimal amount of breast tissue is removed including the tumour mass and a margin of normal tissue (Park et al., 2000), or a mastectomy in which all of the breast tissue is removed (Shimkin et al., 1961). However, unfortunately, not all patients are eligible for surgery for a number of reasons. Some of these reasons include frailty, particularly in elderly patients who would not be able to tolerate the stress of undergoing a major procedure, and comorbidities that may cause an increased risk to the procedure (Chou et al., 2016, Ethun et al., 2017).

Another available treatment option, almost always used in conjunction with surgery is radiotherapy. This involves the use of accelerated particles, usually photons, to create therapeutic, high energy beams. The energy and the focus of these beams are calculated to target the site of the tumour mass, whilst minimising the reach of these beams to surrounding normal tissue (Pereira et

al., 2014). The aim of post-surgery radiotherapy is to target any tumour cells remaining within the breast or local tissues that were outside the main tumour mass that was resected. The resultant ionising radiation causes clusters of DNA damage sites, in particular double strand breaks. These clusters refer to multiple sites of damage within one or two helical turns of DNA. Sites of DNA damage clusters are more difficult to repair than naturally occurring DNA damage sites (Lomax et al., 2013). Successful radiotherapy causes clusters of DNA damage to such an extent that cells are unable to repair the damage and apoptosis is induced (Roos and Kaina, 2013). Less successful radiotherapy causes clusters of DNA damage such that cell cycle arrest is induced in order to allow the sites of DNA damage to be repaired (Wang et al., 2000). A disadvantage of using radiotherapy in the treatment of breast cancer is the associated increased risk of toxicities that sometimes do not appear until over 15 years post-treatment (Poortmans, 2013). The most concerning toxicity that does not appear until many years later is cardiac toxicity. Studies have shown that ionising radiation used in the treatment of breast cancer also exposes the heart to this radiation. This exposure increases the rate of subsequent ischemic heart disease. It was also shown that this increased rate was proportional to the mean dose of ionising radiation to which the heart was exposed (Darby et al., 2013).

A third treatment option is the use of systemic therapies, also often used in conjunction with surgery. The advantage of this treatment type is that it is not targeted to a specific region of the body and is therefore able to target cancer cells that have left the local environs of the primary tumour as well, in particular sub-clinical micro-metastases. Targeting these cells, if present, is critical since it is the full metastatic recurrences that grow from these cells that lead to patient deaths in almost all cases. There are several different types of systemic therapies routinely used for the treatment of primary breast cancer. The type used depends on a number of factors including the molecular subtype of the tumour (Higgins and Baselga, 2011), and the overall health of the patient (Barroso-Sousa et al., 2016). I have divided the relevant systemic therapies into endocrine therapy, HER2-targeted therapy, cytotoxic chemotherapy and other targeted therapies in the following sections, in which I discuss each therapy separately.

Systemic therapies are also typically used in conjunction with radiotherapy with or without surgery (Miller et al., 2016). The various combinations of treatment options that can be used in the treatment of breast cancer partly depend on stage, with lower stage tumours more frequently being treated by local treatments alone, while increasing proportions of patients with the higher stage tumours receive systemic treatments in addition. These are the proportions of patients treated with different combinations of therapies, broken down by stage - according to the National Cancer Data Base statistics from 2013 (Miller et al., 2016); of all patients with either stage I or II breast cancer, the largest proportion (34%) underwent BCS and ionising radiation with no systemic therapy. The next two treatment options of equal proportions (17%) were BCS with both ionising radiation and systemic therapy and mastectomy alone. In contrast, of patients with stage III breast cancer, nearly 50% underwent mastectomies with both ionising radiation and systemic therapy. The next two largest proportions of patients were treated with BCS and both ionising radiation and systemic therapy (15%) and a mastectomy and systemic therapy (13%). Patients with stage IV breast cancer usually do not undergo surgery as distant metastasis has already occurred, therefore surgery is often not thought to have any overall benefits (Blanchard et al., 2008). Therefore, the largest proportion of patients (48%) receive ionising radiation and/or systemic therapy. The next largest proportion of patients (28%) did not receive any treatment, likely due to the conditions indicating no benefits would be gained from any treatments. As these statistics indicate, systemic therapies are a major component of therapy regimens in the treatment of breast cancer and have the advantage of being able to reach all areas of the body, unlike ionising radiation.

1.2.2 Systemic therapies in the treatment of breast cancer

1.2.2.1 Endocrine therapy

Endocrine therapy is a form of targeted systemic therapy that can be used to treat breast cancer by targeting the oestrogen responsive component of tumours. This means that endocrine therapy can only be used to treat a subset of breast cancers – the luminal A and B breast cancers that express the oestrogen receptor (Table 1.1.3). It is important to note, however, that

endocrine therapies are not always used alone to treat these subtypes of breast cancer, since these can also be treated with non-targeting, cytotoxic chemotherapies (see section 1.2.2.3) (Colleoni and Montagna, 2012). However, some of the criteria that are taken into consideration when making the choice concerning whether to include chemotherapy with (or – rarely – instead of) endocrine therapy is the patients' health and choice. Endocrine therapies are more well tolerated than chemotherapies and so endocrine therapies are sometimes the only appropriate treatment option available for patients who are frail and/or elderly (Barroso-Sousa et al., 2016).

There are multiple classes of endocrine therapies available for the treatment of breast cancer. Anti-oestrogens, also known as selective oestrogen receptor mediators (SERMs), such as tamoxifen block the ER (Figure 1.2.1), thus preventing oestrogen from binding and activating the receptor (Nass and Kalinski, 2015). Tamoxifen has long been used to treat ER positive breast cancers in both premenopausal and postmenopausal women (MacCallum et al., 2000, Davies et al., 2011, Dowsett et al., 2015), and trials have even shown that breast cancer incidence is decreased in healthy women with a high risk of developing breast cancer that take tamoxifen (Rutqvist et al., 1995, Kisanga et al., 2004, Cuzick et al., 2015). However, there are several worries associated with the use of this drug. Several studies have shown that whilst tamoxifen can be used successfully to treat ER positive breast cancers, patients who have been administered this treatment also have an increased risk of developing second primary cancers, most notably endometrial cancer (Curtis et al., 1996, Bernstein et al., 1999). There has also been a suggestion that tamoxifen may increase the risk of developing gastrointestinal cancers (Rutqvist et al., 1995). It is thought that this increased risk is due to tamoxifen having partial oestrogenic effects rather than anti-oestrogenic effects in specific tissues including in the endometrium (Shang, 2006).

A second drug that can be used to treat ER positive breast cancers is fulvestrant, a selective oestrogen receptor degrader (SERD) (Figure 1.2.1) (McDonnell et al., 2015). As with tamoxifen and other SERMs, fulvestrant competitively binds the ER, however it has a much higher binding affinity to the ER than tamoxifen (89% that of oestradiol (the prototypical endogenous ligand) vs 2.9% that of oestradiol respectively) (Osborne et al., 2004). Tamoxifen binds

to the ER and induces a conformational change, reducing the ability of the receptor to interact with coactivators. However, alterations in the cell such as increased expression of these coactivators can lead to the inhibition by tamoxifen being overcome and activation of the ER to occur (McDonnell and Wardell, 2010). Fulvestrant however, accelerates the degradation of ER protein without affecting ER transcript levels, ultimately preventing ER function influencing oncogenesis (Nicholson et al., 1995, Osborne et al., 2004). It has also been noted that fulvestrant does not have oestrogenic properties in other tissues such as endometrial tissues as tamoxifen does (Carlson, 2005).

Finally, aromatase inhibitors (AIs) can also be used to treat ER positive breast cancers (Figure 1.2.1), although their use is limited to postmenopausal patients (Maughan et al., 2010). Instead of targeting the ER as SERMs and SERDs do, AIs target oestrogen production by blocking its conversion from androgens. By blocking this process, oestrogen levels fall to virtually undetectable levels in plasma, leading to less activation of the ER by oestrogens (Howell and Dowsett, 2004, Dowsett et al., 2015). Oestrogen is largely produced by the ovaries in premenopausal women (Goss and Strasser, 2001, Miller, 2003). In this subset of patients, inhibition of oestrogen synthesis by AIs activates a pathway involving gonadotrophin that ultimately leads to signals to the ovaries that stimulate aromatase levels, thus limiting the efficacy of the AIs. In postmenopausal women, however, after ovarian failure, oestrogen is largely synthesised in peripheral tissues and circulates at lower levels. In these circumstances, ovarian stimulation no longer plays a role, resulting in AIs remaining effective in this subset of patients. Studies comparing AIs with anti-oestrogens, usually tamoxifen, have revealed that AIs are the superior endocrine therapy in terms of response rates, durations of response, and recurrence rates (Howell and Dowsett, 2004, Dowsett et al., 2015). However, tamoxifen and AIs are often both used to treat different groups of patients (Brufsky, 2017), and tamoxifen remains the appropriate choice in the premenopausal context.

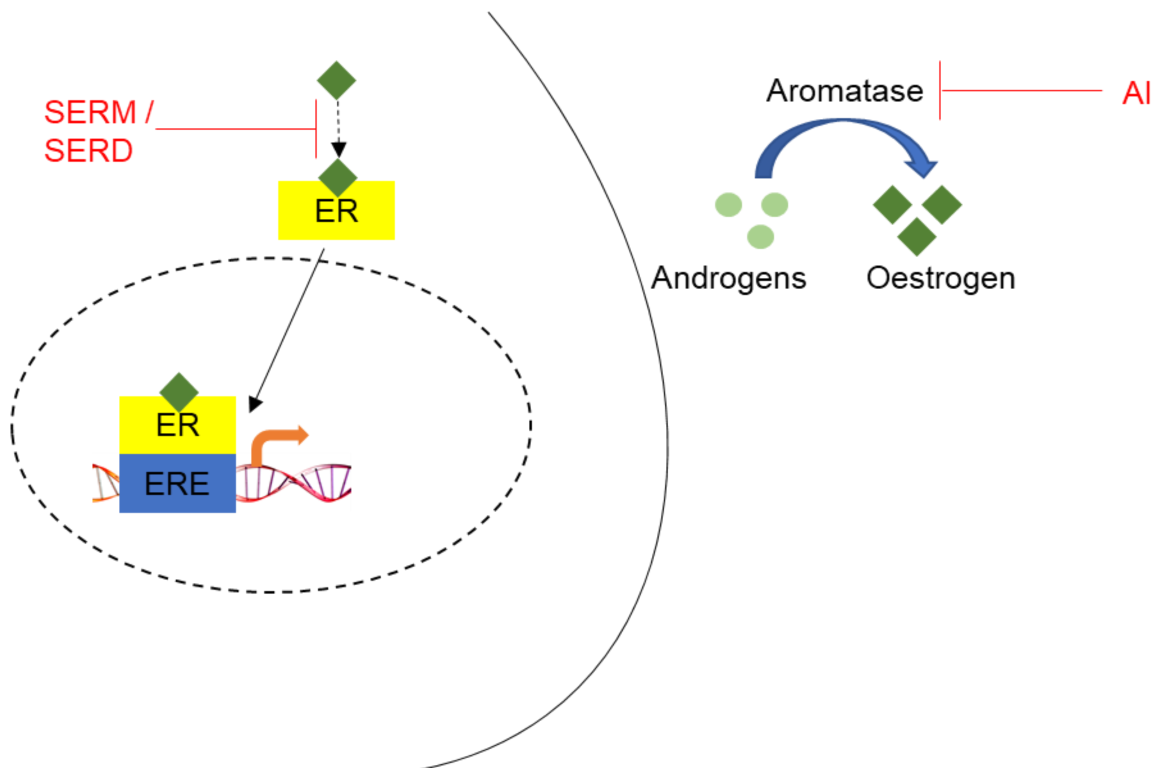

Figure 1.2.1: Endocrine therapy targets of the ER pathway
Aromatase inhibitors (AI) target aromatase enzymes involved in the conversion of androgens to oestrogen. Selective oestrogen receptor modulators (SERM) and degraders (SERD) competitively bind the oestrogen receptor (ER), thus preventing binding of oestrogen to the receptor, and consequently inhibiting the transcription of oestrogen-regulated genes.

1.2.2.2 HER2-targeted therapy

Since the development of endocrine therapies that target the hormonal component of luminal type breast cancers, other targeted therapies have also been developed that work on a similar principle of attempting to inhibit the action of a specific receptor. In breast cancer therapy, the most widely used of these novel targeted therapies are the HER2-targeted therapies. These were developed after it was discovered that overexpression of HER2 protein was observed in almost a quarter of all breast cancers and was associated with a particularly aggressive subtype (Gschwind et al., 2004, Ross et al., 2009). HER2 is a tyrosine kinase receptor (Nielsen et al., 2009). It has been shown to form heterodimers with HER1 (also known as EGFR) and HER3, leading to activation of the PI3K pathway. Numerous clinical trials investigating the effects of HER2 targeting therapies on HER2 positive breast cancers in both the preoperative and postoperative settings have revealed significant benefits

associated with HER2 targeted therapies in terms of overall survival and disease free survival (Dent et al., 2013, Mendes et al., 2015).

The first HER2-targeted therapy was trastuzumab, a monoclonal antibody still in use today (Nielsen et al., 2009, Higgins and Baselga, 2011). A newer monoclonal antibody, pertuzumab, has since been developed. These monoclonal antibodies function by inhibiting the dimerization of the receptors, thus inhibiting the activation of downstream pathways. There is also evidence that monoclonal antibodies including trastuzumab also function by stimulating elements of the immune system such as natural killer cells to target cancer cells (Clynes et al., 2000, Arnould et al., 2006). Trastuzumab has, however, been associated with cardiotoxicity although newer HER2-targeted therapies are associated with a significantly lower risk of cardiac dysfunction (Sendur et al., 2013). Evidence has suggested that treatment regimens consisting of a combination of trastuzumab and one of the newer agents do not have significantly worse cardiotoxicity than trastuzumab alone. However, considering that HER2-targeted therapies are often administered together with cytotoxic chemotherapy and cardiotoxicity is associated with both trastuzumab and the widely used class of therapeutics, the anthracyclines (see section 1.2.2.3.1), it has been suggested that anthracyclines should not be administered in combination with trastuzumab (Popat and Smith, 2008). Small molecule tyrosine kinase inhibitors also exist to treat HER2 positive breast cancers including lapatinib, which has been shown to inhibit both HER1 and HER2, leading to inhibition of the downstream AKT and MAPK pathways (Nielsen et al., 2009). It has been shown that this agent is even able to restore sensitivity to tamoxifen in tamoxifen resistant tumours. There is even modest evidence that lapatinib may be able to cross the blood brain barrier (Lin et al., 2008), providing hope that it could be effective at targeting sub-clinical brain metastases.

1.2.2.3 Cytotoxic chemotherapy

Cytotoxic chemotherapy was the first chemical means of treating cancers (DeVita and Chu, 2008) and is still used today to treat a large proportion of primary breast cancers. According to a study that calculated various statistics associated with a number of cancers, including breast cancer, in the USA, 40%

of patients with stage I or II breast cancer were treated with a therapeutic regimen including chemotherapy (DeSantis et al., 2014). For the higher stages of breast cancer, approximately 70% of patients were treated with a regimen including chemotherapy. Cytotoxic chemotherapy drugs have the ability to kill cancer cells using multiple mechanisms. In general, these drugs target various mechanisms used by cells to proliferate. The inhibition of proliferation can lead to activation of other pathways that ultimately lead to cell death (Waldman et al., 1997, Pucci et al., 2000). As such, chemotherapeutic drugs largely target highly proliferative cells, as is often the case for tumour cells, but also target other proliferating cells in the body that are completely healthy. This leads to off-target toxicities that can have adverse effects on patients (Cheok, 2012, Liu et al., 2015a). This is a disadvantage of chemotherapy as it is administered systemically and not targeted specifically to tumour cells. There is however an advantage to this systemic administration. As mentioned previously, unlike surgical interventions and radiotherapy, chemotherapy as well as other systemic therapies have the benefit of being able to target cells that have disseminated from the primary tumour. This is therefore the primary choice of treatment in the context of metastatic disease (Liu et al., 2015a), although palliative radiotherapy may be indicated in specific circumstances such as cases of bone metastases that may cause fractures and/or neurological complications, extensive brain metastases, and fungating tissue masses (Cardoso et al., 2012).

Chemotherapy also has the advantage of being able to treat all subtypes of breast cancer as it is not targeted to any specific molecules present in only certain subtypes of breast cancer (see Table 1.1.3, for example, since these receptors provide targets for endocrine therapy and HER2 targeted therapy). Instead, chemotherapeutic drugs target various generic cell processes such as DNA replication and mitotic segregation during cell division (Siddik, 2002). It is for this reason that chemotherapy is the only form of systemic treatment that can be used to treat triple negative breast cancers (Foulkes et al., 2010). However, cytotoxic chemotherapy can also be used alone or in conjunction with targeted therapies to treat receptor positive breast cancers (Gnant et al., 2015).

For patients with ER positive tumours – luminal A or luminal B-type breast cancers – endocrine therapies are often sufficient (see section 1.2.2.1). In

these patients, chemotherapy can, however, be recommended for patients with large or node-positive tumours, and is commonly recommended for luminal B-type tumours (Gnant et al., 2015), which are more highly proliferative and may also receive chemotherapy in combination with trastuzumab if HER2 positive. Interestingly, it has previously been noted that despite the fact that both luminal A-type tumours and luminal B-type tumours express the oestrogen receptor, luminal A-type tumours are generally less responsive to chemotherapy drugs than luminal B-type tumours (Ignatiadis and Sotiriou, 2013, Gnant et al., 2015), which probably relates to the difference in proliferation rate. Chemotherapy is also generally administered to patients with HER2 subtype tumours in conjunction with HER2-targeted therapies (see section 1.2.2.2) (Arteaga et al., 2012). It has previously been noted that when administered preoperatively, triple negative and HER2 subtype breast tumours are more sensitive to anthracycline and taxane-containing chemotherapies (see section 1.2.2.3.1) than the luminal breast tumours, although gene expression profiles obtained from the triple negative or HER2 subtype tumours following chemotherapy suggest that the molecular mechanisms employed that contribute to this sensitivity vary between them (Rouzier et al., 2005).

1.2.2.3.1 Multiple classes of chemotherapy drugs with different mechanisms of action are used in the treatment of breast cancer

Different classes of chemotherapeutic drugs can be used, each of which primarily target different cellular processes. One of the first classes of drug developed, which is still commonly used today, is alkylating agents (Ralhan and Kaur, 2007). These directly target DNA using three different mechanisms. Alkylating agents have the ability to cross-link DNA, resulting in the inability of the double strands to unwind during replication and transcription. Secondly, alkylating agents can cause the mispairing of nucleotides leading to mutations. The final mechanism involves the drug adding alkyl groups to DNA bases. When DNA repair enzymes attempt to replace these alkylated bases, the DNA is fragmented. The most commonly used alkylating agent in the treatment of breast cancer is cyclophosphamide (Stoll, 1970, Wisinski et al., 2013).

Anthracyclines are another class of chemotherapy drug frequently used in the treatment of breast cancers. These antibiotics have been used to treat breast cancer for half a century, although more recent retrospective studies have

suggested that this class of chemotherapeutic drug should be used less frequently in favour of newer classes of drug that have fewer associated toxicities (Robson and Verma, 2009). The most commonly used anthracyclines in the treatment of breast cancer are doxorubicin and epirubicin. There appear to be multiple possible mechanisms of action used by anthracyclines to kill tumour cells. However, the two most prominent mechanisms are through the inhibition of topoisomerase II and the production of free radicals (Bonadonna et al., 1993, Gewirtz, 1999). Topoisomerase II is an enzyme required for the unwinding of DNA during replication and transcription by creating double strand DNA breaks (Nitiss, 2009a). Anthracyclines function by intercalating with the topoisomerase II:DNA covalent complex and inhibit the DNA from being re-ligated (Nitiss, 2009b). Anthracyclines can also be oxidised and then converted back in a reaction that releases reactive oxygen species, which can activate a number of processes such as apoptosis and cause membrane damage as well as DNA damage (Thorn et al., 2011). A particular disadvantage of anthracyclines is associated toxicities, in particular cardiotoxicities as anthracycline-induced cardiomyopathy is largely irreversible (Hortobagyi, 1997, Thorn et al., 2011), although evidence suggests that epirubicin is less toxic than doxorubicin (Khasraw et al., 2012).

Taxanes are a newer class of chemotherapeutic drug that have been suggested to be less toxic in terms of side effects than anthracyclines and it has therefore been suggested that these be used preferentially (Robson and Verma, 2009). These chemotherapeutic drugs target microtubule dynamics, preventing chromosome segregation, and thereby causing mitosis to halt (Risinger et al., 2009). They have also been shown to promote apoptosis; paclitaxel is a common taxane used in the treatment of breast cancer and has been shown to activate the apoptosis signal-regulating kinase (*ASK1*), leading to the inactivation of the anti-apoptotic protein Bcl2 (McGrogan et al., 2008). Docetaxel is also commonly used to treat breast cancers (Baselga and Tabernero, 2001). Finally, taxanes have also been shown to inhibit angiogenesis by reducing cell motility. This inhibition was associated with decreased activity of Rac1 and Cdc42 activity, thought to be caused by cytoskeletal changes induced by the drugs observed in endothelial cells (Bijman et al., 2006).

The final class of chemotherapeutic drugs used to treat breast cancers are platinum-based drugs such as cisplatin (Decatris et al., 2004). Clinically, this class of drugs has been limited to the triple negative subtype of breast cancers as studies have shown significant benefits associated with these drugs when administered in this subtype (Guan et al., 2015, Zhang et al., 2015). Their primary mechanism of action is very similar to that of alkylating agents in that they too form intra-strand adducts and inter-strand crosslinks in DNA. Cisplatin has also been shown to bind RNA and cellular proteins. Interestingly, platinum-based chemotherapy has been shown to be particularly effective in patients with *BRCA1* mutations or silencing of *BRCA1* (Stefansson et al., 2012).

1.2.2.4 Other targeted therapies

Other targeted therapies also exist that can be used to treat breast cancers. Cyclin dependent kinases (CDKs) are essential for cell cycle progression and it has previously been shown that CDK4 and CDK6 in particular are overexpressed in breast cancer and may play a particular role in hormone receptor positive and HER2 positive breast cancers (Malumbres and Barbacid, 2009, Finn et al., 2009, DeMichele et al., 2015). These two CDKs are required for transition from G_1 to S phase of the cell cycle, and several drugs have been developed to target these kinases, including palbociclib (O'Leary et al., 2016). This agent has been shown to be well tolerated by patients with significant increases in progression free survival (PFS) in hormone receptor positive breast cancer resistant to endocrine therapies compared with hormone receptor negative breast cancers (DeMichele A et al., 2015). In pre-clinical models, CDK4/6 inhibitors have also been shown to work well in combination with HER2-targeted therapies in a bid to overcome resistance to the latter therapy (Witkiewicz et al., 2014). These two kinases function downstream of the HER2 receptor and simultaneous inhibition of these kinases and the receptor should lead to increased cell cycle arrest. Similarly, inhibition of PI3K has also been shown to be successful in the treatment of breast cancers with mutant *PIK3CA* as well as those with resistance to HER2-targeted therapies, and treatments consisting of combinations of CDK4/6 inhibitors with PI3K inhibitors have been shown to work synergistically (Vora et al., 2014, Wilks, 2015). However, these targeted inhibitors have not yet entered standard

practice, with PI3K inhibitors still undergoing clinical trials and further development (Massacesi et al., 2016). Palbociclib has been approved by the Food and Drug Administration, but only for the treatment of hormone receptor positive, metastatic breast cancer when given in combination with the endocrine therapy letrozole (Vidula and Rugo, 2016). Further clinical trials in breast cancer are ongoing.

Another promising targeted therapy is poly(ADP-ribose) polymerase (PARP) inhibitors (Lord and Ashworth, 2017). PARP enzymes are DNA damage sensors and signal transducers that bind damaged DNA and recruit DNA repair enzymes. As such, PARP inhibitors have been of particular interest in the context of *BRCA* mutations, as these mutations adversely affect DNA repair capabilities (Livraghi and Garber, 2015). Multiple clinical trials investigating the efficacy of various PARP inhibitors such as olaparib have been performed in breast cancers, the majority of which only included breast cancers with *BRCA* mutations (Livraghi and Garber, 2015, Kaufman et al., 2015, Michalarea et al., 2016). These clinical trials have showed promising results in terms of a favourable response of the tumours to the inhibitors and limited off-target toxicities. Olaparib has been approved for the treatment of ovarian cancer, but clinical trials are still ongoing for the treatment of breast cancer (Lord and Ashworth, 2017, Yamaguchi et al., 2018).

1.2.2.5 Therapeutic drugs can be administered as single agents or in combination

Systemic therapies can be administered as single agents or as combination therapies. Not only can multiple chemotherapeutic agents be administered together for example, but chemotherapeutic agents can also be administered in combination with endocrine therapies or other targeted therapies (Bergh et al., 2001, Slamon et al., 2001). There are multiple advantages associated with administering polytherapies. These include increased rates of survival, higher rates of overall response and longer durations of response. Another potential benefit of administering multiple therapies is a greater chance of overcoming resistance or preventing its development (Masui et al., 2013). A well-known example of this is in HER2-targeted therapies. Addition of a HER2 targeting agent such as trastuzumab to chemotherapy led to increased patient response

rates compared with chemotherapy or HER2 targeting therapies alone (see section 1.2.2.2).

There are, however, disadvantages to administering multiple therapeutic agents as opposed to only single agents. Administration of polytherapies is also associated with increased toxicities such as leukopenia and anaemia. For example, when the HER2-targeted therapy trastuzumab was given in combination with a chemotherapy regimen consisting of an anthracycline and cyclophosphamide, when compared with chemotherapy alone, 27% versus 8% of patients experienced some cardiac dysfunction. For patients who were administered paclitaxel alone, 1% experienced cardiac dysfunction whereas 13% experienced this side effect when administered a combination of paclitaxel and trastuzumab (Slamon et al., 2001, Beslija et al., 2003). As such, recommendations have been made regarding the use of single agent therapies versus polytherapies. In metastatic breast cancer, these involve taking into consideration patient prognosis, symptom control and toxicity profiles with the ultimate goal being optimising the quality and quantity of life (Beslija et al., 2003). It has been suggested that in metastatic breast cancer, single agent therapies are a reasonable option when the primary goals are to limit drug toxicity whilst maximising the quality of life. This was considered particularly relevant for those patients with tumours that are ER negative and whose disease is only slowly progressive.

There are two main approaches that can be taken when administering polytherapies, and the use of these is partly dependent on the patient (Miles et al., 2002, Beslija et al., 2003, Bergh et al., 2001). Therapeutic drugs can be administered simultaneously or sequentially. Studies comparing the efficacies of these two methods of administration have not discovered any significant differences between the two in terms of overall survival (Bergh et al., 2001). However, trends have been noticed specifically in patients with liver metastases that they had better survival rates when administered simultaneous combination therapies as opposed to sequential therapies (Joensuu et al., 1998). It was also noticed that in the former approach, the response and time to progression tended to be better, but these benefits were accompanied by increased toxicity. Therefore, as with the decision between administering single agents versus polytherapies, the choice between administering combination

therapies simultaneously or sequentially is partially dependent on the patient (Miles et al., 2002). It has been suggested that in the context of metastatic breast cancer, administration of therapeutic agents simultaneously may be preferable to administering them sequentially if an urgent reduction in tumour burden is required. In contrast, sequential therapies may be especially appropriate in patients who are frail and/or elderly and may not be able to tolerate the toxicity associated with simultaneous administration of the therapies. This second approach may also be beneficial for those patients with slow growing tumours as this method also allows optimal delivery of each single drug and therefore potentially reduces the risk of toxicity, which may improve the quality of life (Miles et al., 2002).

The issue of sequential or simultaneous treatments is of particular relevance to my work here, since in Chapter 6 I have attempted to study the consequences of initial treatment with one therapy (endocrine) on response to a subsequent therapy (cytotoxic chemotherapy). In addition, the remainder of my work involves identification of changes in expression within tumour cells that are induced by chemotherapy – and it would be possible to consider the implications of these changes on any subsequent therapies (in the case of my work, this would most likely be adjuvant endocrine therapy).

1.2.2.6 Adjuvant therapies versus neoadjuvant therapies

Traditionally, systemic therapies are administered post-surgery in the hope of eradicating any tumour cells remaining locally after removal of the main tumour mass, as well as any distant sub-clinical metastatic cells (Sonnenblick and Piccart, 2015). This therapy is known as adjuvant therapy (Zhang et al., 2013a). However, in more recent years, the administration of systemic therapy prior to surgery, known as neoadjuvant therapy, has increased (Kaufmann et al., 2012, Kesmodel, 2016). Chemotherapy and endocrine therapy as well as other targeted therapies can be administered in the neoadjuvant setting although chemotherapy is the most commonly administered neoadjuvant option (Barroso-Sousa et al., 2016).

1.2.2.6.1 The advantages of neoadjuvant systemic therapies

There are several potential advantages to neoadjuvant therapies. The first is that the response of the tumour to the administered drug can be monitored using non-invasive imaging (Kaufmann et al., 2012, Park et al., 2015). This allows clinicians to observe whether the tumour is sensitive or resistant to the therapy and allows the opportunity to change the treatment regimen if the tumour is non-responsive (Porkka et al., 1994, Polyzos et al., 2009). This of course is not possible when administering adjuvant therapies, since the primary tumour is not present, and assessments of therapy response can only be made subsequently based on whether recurrences occur or not (Gonzalez-Angulo et al., 2007, Sotgia et al., 2017), at which point it is already too late to avoid these recurrences.

A benefit of being able to monitor tumour response in the neoadjuvant setting is that there is a high correlation between response and change in tumour size (Gajdos et al., 2002, Samuel et al., 2018). It is inferred that a good response in the primary tumour likely indicates a good response in any disseminated tumour cells as well. Therefore, if there is a large degree of shrinkage of the primary tumour post-neoadjuvant therapy, it is likely that any disseminated tumour cells will also have responded very well to the therapy. The association between incidences of pathological complete response (pCR) and the associated improved survival of these patients supports this (Mieog et al., 2007, Gianni et al., 2014). The term pCR broadly refers to the absence of the primary tumour, although various groups use differing specific definitions. Two of the definitions used to define pCR are where no residual cancer at all is left and where no invasive cancer is detected with various degrees of residual cancer in axillary nodes and non-invasive cancer cells (von Minckwitz et al., 2012).

Neoadjuvant therapies are also beneficial in cases where the tumour is responsive to the treatment and results in tumour shrinkage (Goble and Bear, 2003). At diagnosis, some tumours are deemed inoperable due to advanced lymph node involvement (El-Charnoubi et al., 2012). Neoadjuvant therapy may successfully treat affected lymph nodes, thus rendering the tumour operable. Similarly, a tumour that once required a complete mastectomy could only require breast-conserving surgery following neoadjuvant treatment (Mauri et al.,

2005). In the best cases, neoadjuvant therapies can sometimes even result in pCR. This occurs in 3-18% of cases. The likelihood of pCR depends on a large number of factors such as tumour subtypes, stage of disease, the patient and definition of pCR used (Cortazar et al., 2012, von Minckwitz et al., 2012).

A third advantage for administering systemic therapies in the neoadjuvant setting does not have immediate clinical benefits, but rather benefits the research community. It is clear that the administration of systemic therapies leads to alterations in gene expression (Magbanua et al., 2015, Klintman et al., 2016). Some of these alterations cause the tumour to be more sensitive to the treatment whereas others can cause the tumour to be more resistant (Kim et al., 2013a, Magbanua et al., 2015). The combinations of all these changes put together results in tumours responding to the treatment to various degrees or not responding at all. These changes in gene expression can be investigated only in the neoadjuvant setting as samples from both pre-treatment (biopsy taken for initial diagnosis) and/or post-treatment (resection from surgical removal of the tumour) are available, in cases where pCR is not achieved (Gonzalez-Angulo et al., 2012, Bhola et al., 2013).

The ability to determine gene expression changes associated with systemic therapies has many advantages. Together with clinical follow-up data, different gene expression changes can be used as signatures to determine what changes are associated with numerous factors such as disease free survival, overall survival and response to subsequent therapies if any (Ayers et al., 2004, Magbanua et al., 2015). As such, the molecular response of tumours can be correlated with the observed clinical response and give a greater understanding of phenomenons such as how resistance is acquired and what combinations of therapies result in the best outcomes (Rouzier et al., 2005, Colleoni and Montagna, 2012). Understanding how resistance occurs could also lead us to strategies that can be used to overcome this resistance such that more cancers can be treated more successfully.

1.2.2.6.2 Comparing neoadjuvant and adjuvant systemic therapies in the clinical setting

Several clinical trials have been performed to analyse whether there were any significant differences between adjuvant and neoadjuvant administration of

systemic therapies for primary breast cancer. In one of these studies, chemotherapy administered to premenopausal patients with tumours too large for BCS was compared in these two settings (Scholl et al., 1994). In this study, patients also received radiation therapy and also underwent surgery if tumour masses remained post-irradiation, irrespective of whether systemic therapies were administered in the adjuvant or neoadjuvant settings. Where tumour masses remained post-treatment, surgery was aimed to be as conservative as possible. Results showed a statistically significant difference in overall survival of patients between the two treatment arms, with patients who received NAC having better outcomes than those who received adjuvant chemotherapy. However, there was no difference in disease free survival (DFS) or rates of local recurrence.

A meta-analysis published in 2005 (Mauri et al.) considered results from nine separate randomised trials, including the study described above (Scholl et al., 1994), in which breast cancer patients were treated either in the neoadjuvant or adjuvant settings with the same regimen. This study concluded that there were no significant statistical differences between these two settings in terms of rates of death, disease progression or recurrences in the form of distant disease. However, it was noted that neoadjuvant therapies were statistically significantly associated with an increased risk of loco-regional disease recurrences compared to adjuvant therapies. This was noted to be of particular importance if patients received radiation therapy but no surgery. The results from this meta-analysis could therefore lead to the conclusion that administering systemic therapies in the adjuvant setting may be superior in terms of disease control, than in the neoadjuvant setting. It is important to note however, that a treatment regimen for breast cancer that does not involve surgery is very rare (between 1% and 4% in stages I, II and III) and usually only occurs in stage IV breast cancer when distant metastasis is present (Miller et al., 2016).

However, another meta-analysis published two years later (Mieog et al., 2007) that included data from fourteen randomised trials comparing neoadjuvant and adjuvant systemic therapies had slightly different conclusions. This meta-analysis included the studies that were in the former meta-analysis (Mauri et al., 2005). As with the previous meta-analysis, overall survival was equivalent in both treatment groups (Mieog et al., 2007). It was noted though, that rates of

mastectomy were lower in the neoadjuvant group than in the adjuvant group and that this had no effect on the ability to control local recurrences. NAC was also associated with fewer side effects such as infectious complications and cardiotoxicity. When comparing loco-regional recurrences between the two groups, this meta-analysis excluded trials where it was deemed that patients received inadequate loco-regional treatment. In the first meta-analysis (Mauri et al., 2005), data from these trials were included (see above). However, in the latter meta-analysis (Mieog et al., 2007), data from these trials were removed from the analysis. Data from the remaining eight studies that reported data on loco-regional recurrences demonstrated no difference in the rate of loco-regional recurrences between the neoadjuvant and adjuvant groups. The results from this meta-analysis could therefore lead to the conclusion that administering systemic therapies in the neoadjuvant setting may be superior to the adjuvant setting when considering patient psycho-social outcomes in terms of increased rates of BCS in the former setting – an outcome usually preferred by patients. Combining these data with the advantage of being able to monitor tumour response to the administered therapy in the neoadjuvant setting, allowing the regimen to be changed if necessary (Porkka et al., 1994, Kaufmann et al., 2006), it seems there are numerous advantages to neoadjuvant therapies compared to adjuvant therapies.

1.3 An introduction to microRNAs

MicroRNAs (miRNAs) are short, non-coding RNA sequences typically between 20 and 24 nucleotides in length (Chekulaeva and Filipowicz, 2009). These single stranded RNA sequences were first identified in 1993 in *Caenorhabditis elegans* (Wightman et al., 1993, Lee et al., 1993) and have since been discovered to regulate gene expression post-transcriptionally (Ambros, 2001). MiRNAs are now known to repress gene function by inhibiting the translation of messenger RNAs (mRNAs) and/or targeting mRNAs for degradation (Fabian et al., 2010, Meijer et al., 2013). However, in more recent years, evidence has suggested that miRNAs may also be able to up-regulate gene expression (Ørom et al., 2008).

1.3.1 MiRNAs are expressed as primary transcripts and are processed to form mature miRNAs

Genes encoding miRNAs have been located in transcription units containing clusters of distinct miRNAs as well as transcription units that also encode proteins (Carthew and Sontheimer, 2009). MiRNA genes can be found in the introns of protein-coding genes and both introns and exons of non-coding genes (Rodriguez et al., 2004). MiRNA genes can also be found in intergenic regions (Kim and Nam, 2006). Genes are typically transcribed by RNA polymerase II, and transcripts are capped and polyadenylated and form double-stranded structures known as primary miRNAs (pri-miRNAs) (Carthew and Sontheimer, 2009). These products are processed by the Drosha-DGCR8 complex (Han et al., 2004). Drosha is a nuclear RNase III enzyme that cleaves the pri-miRNA to form precursor mRNAs (pre-miRNAs) that form hairpin structures. DGCR8 is an essential component of the complex: it contains two double-stranded RNA binding sites. The pre-miRNAs are exported from the nucleus in a Ran-GTP dependent manner, mediated by Exportin-5 (Murchison and Hannon, 2004).

Once in the cytoplasm, the pre-miRNAs are further cleaved by a second RNase III enzyme known as Dicer, to form the mature miRNA duplex. As with the Drosha-DGCR8 complex, the involvement of TRBP, known to contain three double-stranded RNA binding domains, has been shown to be essential in this process (Chendrimada et al., 2005). One strand of the duplex (the guide strand) is designated to be loaded onto the RNA-induced silencing complex (RISC) to form the miRISC, whilst the second strand (the passenger strand) is degraded (Winter et al., 2009). There is a bias present as to which strand is selected as the guide strand. This bias has been shown to be influenced by the thermodynamic properties of the two strands of the pre-miRNA duplex (Khvorova et al., 2003).

The process of miRNA biogenesis is shown in Figure 1.3.1.

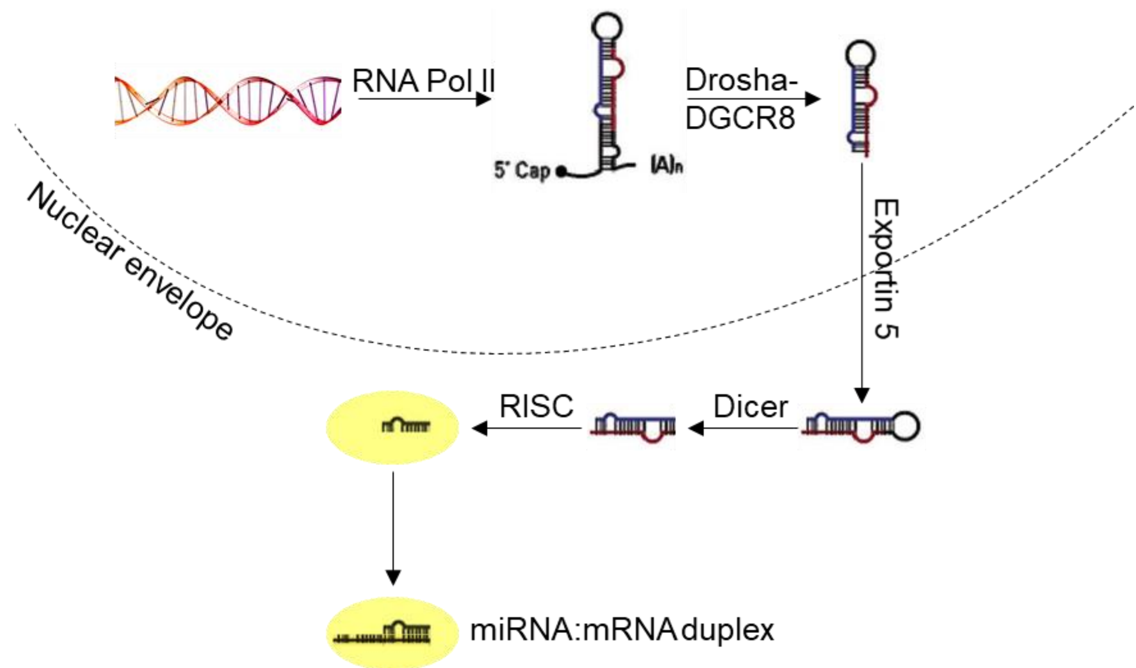

Figure 1.3.1: Biogenesis of miRNAs

MiRNAs are transcribed by RNA polymerase (Pol) II and capped and polyadenylated. They are then processed by the Drosha-DGCR8 complex before being exported from the nucleus in a process mediated by Exportin 5. The mature miRNA duplex is formed by the Dicer enzyme before the guide strand is loaded into the RISC. This complex containing the mature miRNA strand can then target mRNA transcripts.

1.3.1.1 MiRNA nomenclature

When the guide and passenger strands of miRNAs were first identified, the standard nomenclature used to differentiate between the two strands was a '*' symbol following the miRNA name to denote the passenger strand (Griffiths-Jones, 2004). However, after it was discovered that different strands were used as a passenger or guide in different tissues (Bhayani et al., 2012), the '*' system was found to be unhelpful. Therefore, the '-5p' and '-3p' suffix system was put in place to denote the 5' strand and the 3' strand of the duplex respectively (Kozomara and Griffiths-Jones, 2014).

MiRNA nomenclature is further complicated by the fact that some mature miRNAs derived from different genes can differ at only one or two positions in their sequence (Griffiths-Jones, 2004). These miRNAs are differentiated by a letter suffix, for example miR-26a and miR-26b. Alternatively, some identical mature miRNAs have different genomic origins. These are differentiated by a

number suffix, for example miR-19b-1 and miR-19b-2 (Moreno-Moya et al., 2014).

Finally, miRNA families also exist. A family refers to a group of miRNAs that derive from a common evolutionary ancestor and normally have similar physiological functions (Zou et al., 2014). However, their primary sequence is not always conserved, nor is their secondary structure.

1.3.1.2 MiRNAs identify their targets via a seed sequence

The miRISC complex, composed in part by argonaute proteins (Ago) (Liu et al., 2005), recognises the 3' UTR of target mRNAs by virtue of the seed sequence of the miRNA (the sequence of nucleotides in positions 2-8 numbering from the 5' end) (Brennecke et al., 2005, Wilczynska and Bushell, 2015). More recently, miRNAs have also been shown to recognise sequences in the 5' UTR (Ørom et al., 2008) as well as the coding region (Hausser et al., 2013). Evidence also exists of miRNAs binding their targets via their central regions rather than the seed region (Martin et al., 2014). However, most binding is still thought to occur between the seed sequence of the miRNA and the 3'UTR of the target mRNA. Because sequence complementarity between a miRNA and its target mRNA is only required in a limited number of positions (the prototype being the seven positions in the seed region), each miRNA has many potential targets in terms of complementarity alone (Wilczynska and Bushell, 2015). It has previously been noted however, that a surprising number of miRNA genes are located under the transcriptional control of their target genes (Carthew and Sontheimer, 2009). The location and transcription of miRNA genes could therefore provide clues as to some of their mRNA targets beyond simple sequence complementarity.

1.3.2 MiRNAs post-transcriptionally regulate gene expression

Translational repression of target mRNAs by miRNAs is still a relatively poorly understood process. However, evidence has shown that this step is essential for mRNA degradation (Meijer et al., 2013), but that not all mRNAs are ultimately degraded – some miRNA-repressed mRNAs can be translationally reactivated (Wilczynska and Bushell, 2015). Inhibition of translation involves miRNAs preventing the formation of the eIF4F initiation complex. This is

achieved by the binding of the RNA helicase eIF4A2 to the CCR4:NOT deadenylase complex (Meijer et al., 2013). This process requires not only the presence of the miRNA target site in the 3’ UTR of the mRNA, but also secondary structures in the 5’ UTR. These secondary structures are hairpin loops, the complexity of which seems to contribute to the efficiency of miRNA-mediated repression of mRNAs (Ricci et al., 2013). In some cases, translational repression is followed by the deadenylation and eventual degradation of the mRNA (Djuranovic et al., 2012). This process occurs in specific processing bodies (P bodies) and the complex is directed to these sites by the Ago proteins (Behm-Ansmant et al., 2006). The degradation of mRNAs by miRNAs requires the GW182 protein, which has been shown to interact with the Ago proteins, as well as the decapping DCP1:DCP2 complex and the CCR4:NOT deadenylase complex. It has previously been shown that a switch can be made between translational repression and degradation of mRNAs when bound by miRNAs (Horman et al., 2013). Phosphorylation of Ago2 at S387 was shown to facilitate the interaction of this protein with GW182, resulting in increased localisation of miRNA:mRNA complexes to P bodies. This phosphorylation, by Akt3, increased translational repression of target mRNAs and decreased degradation.

1.3.3 MiRNAs in cancer

1.3.3.1 Differential expression of miRNAs in breast cancer

Dysregulation of miRNA expression has been observed in multiple cancer types including chronic lymphocytic leukaemia, lung cancer and breast cancer (Calin and Croce, 2006). Despite the thousands of miRNAs that we now know exist, we still know relatively little about what role individual miRNAs play in the pathogenesis of cancers (Palanichamy and Rao, 2014). However, individual miRNAs have been labelled as oncogenic, as their expression is generally increased compared to normal levels and are known as oncomiRs (Esquela-Kerscher and Slack, 2006). Other miRNAs have been labelled as tumour suppressors, with their expression generally decreased compared to normal levels. The functions of these miRNAs are presumed to be the results of the

miRNAs acting through their mRNA targets: either tumour suppressors or oncogenes respectively.

In breast cancer, many different miRNAs have been implicated in influencing tumour progression, either by promoting or by inhibiting metastasis and by increasing sensitivity or resistance to systemic therapies (Corcoran et al., 2011). Numerous miRNAs have been associated with breast cancer including increased expression of miR-21 and miR-155 (Volinia et al., 2006), and decreased expression of let-7 and miR-10b (Iorio et al., 2005) relative to non-cancerous expression. Interestingly, specific miRNAs have been associated with the differential receptor expression used for subtyping breast cancer (Iorio et al., 2005). These include the miR-26 and miR-30 families with increased expression in ER positive tumours compared to ER negative tumours, and let-7c and specific members of the miR-30 family with increased expression in PR positive tumours compared to PR negative tumours. It is important to note that in both of these profiling studies, total RNA was isolated from whole tumour samples containing all cell types, therefore these differential expressions cannot confidently be assigned to the tumour epithelial cells alone.

MiRNA expression profiling studies have also been performed on breast tumours that have taken into consideration which cellular components of tumours express the dysregulated miRNAs. In one of the first of such studies, whole tumour samples initially underwent miRNA expression profiling compared with normal tissue as described above (Sempere et al., 2007). This led to the identification of numerous dysregulated miRNAs in the tumour tissue including increased expression of miR-21 and decreased expression of miR-145 and miR-451 relative to normal tissue expression. In situ hybridisation (ISH) experiments were then performed on tissue microarrays (TMAs) containing breast tissue cores. The miRNAs of interest were targeted in order to view their localisation. MiR-21 was expressed primarily in luminal epithelial cells and was occasionally detected in fibroblasts. In contrast, miR-145 was expressed primarily in myoepithelial cells of lobules and ducts and in the smooth muscle cells within blood vessels. Of significance, miR-451 was exclusively expressed in erythrocytes and not in any cancer cells. It was hypothesised that the significant down-regulation of this miRNA observed in the whole tumour sample may have simply reflected the fact that tumour

vasculature is inherently different to normal vasculature. This observation underscored the importance of assessing not only miRNA expression but also the localisation of this expression within the tumour mass.

More recent profiling studies have taken the observation of the importance of miRNA localisation into consideration. In a study investigating whether circulating miRNA profiles were reflective of miRNA expression profiles in the breast tumour, miRNA microarrays were performed using RNA extracted from plasma samples and tumour tissue samples that had been macro-dissected for areas enriched for tumour epithelial cells (Cookson et al., 2012). This led to the identification of let-7b, let-7g and miR-18b as potential biomarkers, with higher expression levels associated with breast tumours to normal breast tissue. In a separate study investigating the different miRNA expression profiles associated with breast cancers in different ethnic backgrounds, RNA was also extracted from tumours that had been macro-dissected for areas enriched for tumour epithelial cells (Pollard et al., 2018). Nine miRNAs were differentially expressed across the four ethnic groups. Tumour epithelial cells taken from patients of Nigerian origins had increased expression of miR-140-5p, miR-194 and miR-423-5p relative to other ethnic groups, whereas increased expression of miR-101 was observed in the Indian group. It may, however, be worth emphasising that although macro-dissection was performed to select areas of tumour enriched for epithelial cells, other cells present in these areas were also included in the analyses and may therefore skew some results.

It has been noted that many miRNA genes are located in fragile chromosome locations and cancer-associated genomic regions (Calin et al., 2004), and that this may contribute to aberrant expression of miRNAs. For example, miR-335, located at 7q32.2, is commonly genetically deleted in breast cancers (Png et al., 2011). However, it was also observed that the promoter also underwent hypermethylation in every metastatic derivative obtained from the patients' malignant cell populations (Png et al., 2011). Since then, polymorphisms in the miRNAs themselves have been identified, such as in miR-196a2, where a specific single nucleotide polymorphism (SNP) was observed to increase the risk of breast cancer in individuals who were homozygous for this allele (Gao et al., 2011). However, evidence linking specific polymorphisms in miRNAs with

breast cancer risk is controversial as meta-analyses and confirmatory studies often do not obtain the same results (Mulrane et al., 2013).

Polymorphisms in miRNA target binding sites have also been identified. A SNP in *ITGB4* at a potential binding site of miR-34a correlated with decreased survival in ER negative breast cancers (Brendle et al., 2008). Interestingly, polymorphisms in genes coding for proteins required in the miRNA biogenesis pathway, as well as other causes leading to aberrant expression and/or function of proteins, such as *DICER1* and *AGO2* have also been associated with breast cancer risk (Blenkiron et al., 2007, Sung et al., 2012). It is evident that aberrant miRNA expression contributes to numerous processes in cancer and evidence has shown that regulation of miRNA expression is complex. This involves genetic and epigenetic factors of not only the miRNAs themselves but also the genes required for miRNA processing and function. Further study is clearly required to understand the exact mechanisms behind miRNA function in cancer, with a view to identifying novel therapeutic targets (Corcoran et al., 2011, Liu, 2012, Mulrane et al., 2013).

1.3.3.2 Functions of dysregulated miRNAs in breast cancer

Some miRNAs function as suppressors of metastasis, including miR-335 (Tavazoie et al., 2008). Expression of this miRNA was absent in the majority of breast cancer patients who suffered relapses and this loss of expression was associated with poor metastasis-free survival. Investigations into the mechanism by which miR-335 supresses metastasis led to the identification of two mRNA targets. These were the products of the metastasis-associated genes *SOX4* and *TNC*.

Other miRNAs are oncomiRs, including miR-155 (Jiang et al., 2010). Expression of this miRNA has been shown to be increased in a number of cancer types including breast. As such, a search for mRNA targets of this miRNA resulted in the identification of *SOCS1*. Investigating miR-155 and *SOCS1* expression in both breast cancer cell lines and primary breast tumours showed an inverse correlation in expression of these two RNAs. *In vitro* studies showed that increased expression of miR-155 led to increased cell proliferation. Similar results were observed when *SOCS1* was knocked down. Interestingly, in tumours from two patients, increased expression of both miR-155 and

SOCS1 was observed. Further investigations revealed a SNP in the 3'-UTR of *SOCS1* at the miR-155 binding site, thus demonstrating that although miRNA expression may be dysregulated, expression of target mRNAs may not be affected if binding by the miRNA is inhibited.

However, dysregulation of miRNA can be complex. Although miR-10b had previously been shown to have decreased expression in breast cancer relative to normal breast tissue (Iorio et al., 2005), subsequent studies revealed that this miRNA acts as a promoter of metastasis, with over-expression resulting in non-metastatic breast cancer cells converting to a metastatic phenotype (Ma et al., 2007). This phenotype was a result of miR-10b targeting *HOXD10*, leading to increased expression of the pro-metastatic gene *RHOC*. In the initial profiling study where decreased expression of miR-10b was observed relative to normal tissue, RNA was extracted from whole tumour tissues (Iorio et al., 2005). However, the subsequent functional study was performed only in tumour epithelial cells (Ma et al., 2007). This again highlights the importance of performing miRNA expression profiling studies on RNA extracted from specific tumour components, such as tumour epithelial cells.

1.4 Mechanisms of resistance to systemic therapies

Resistance to systemic therapies accounts for 90% of cases of treatment failure in metastatic breast cancer (Hodges, 2011). The origins of resistance are not fully understood, although there are two over-arching categories to this phenomenon. These are the principles of *de novo* resistance, where tumour cells are intrinsically resistant to the administered drugs (Li et al., 2008), and acquired resistance, which refers to tumours where cells initially respond to the administered therapy but gradually respond less over time. This is due to the tumours modifying their reliance on the function of the drug targets in order to escape death, ultimately leading to increased chances of recurrence and can also cause tumours to become more aggressive (Pogribny et al., 2010, Higgins and Baselga, 2011, Hazlehurst et al., 2003).

One aspect of *de novo* resistance is the phenomenon of clonal selection. This relates to intra-tumour heterogeneity: the fact that tumours are composed of different populations of cells and that even within cell populations, there are

differences between cells (Navin et al., 2011, Gerlinger, 2012, Tudoran et al., 2016). The hypothesis is that within a tumour, certain clones are intrinsically resistant to the administered therapy, thus surviving and preventing a complete response to treatment (Burrell and Swanton, 2014). A contributing factor to both *de novo* resistance as well as acquired resistance is the presence of cancer stem cells, which are often resistant to therapies, and cells that undergo the epithelial-to-mesenchymal transition (EMT) process (Liu and Wicha, 2010, Giatromanolaki et al., 2011, Jia et al., 2017). The EMT process has been proposed to be a highly dynamic process, with cells able to pause the process such that they exhibit properties of both states and can switch back and forth. This cellular plasticity mediated by EMT allows cells to switch between drug resistant and sensitive states dynamically (Jia et al., 2017).

Some other contributors to the development of acquired resistance are therapy-induced changes in gene expression, alternative splicing events, and mutagenesis of genes with numerous possible consequences including alterations of target sites and changes in expression (Poulikakos et al., 2011, Wood, 2015, Nakazawa et al., 2018). These events can lead to altered gene function, altered drug binding sites, and/or activation and repression of molecular pathways that contribute to cell survival and cell death respectively. These therapy-induced changes ultimately lead to increased cell survival, as a result of acquired resistance to the administered therapy.

Another important mechanism of acquired resistance is therapy-induced up-regulation of xenobiotic drug transporter expression (Di Nicolantonio et al., 2005, Kim et al., 2015). Expression of these pumps can be increased in response to presence of the therapeutic drug. This is a result of the therapeutic drug inducing expression of drug pumps that then function to pump out any drugs in the cell, thus preventing the drug from reaching its target (Vasiliou et al., 2009). Considering the heterogeneous nature of tumours, it seems likely that acquired resistance is the result of both intrinsic resistant features and acquired resistance mechanisms.

1.4.1 Molecular effectors of therapy resistance

Alterations in gene expression are a possible mechanism cells use to avoid destruction by systemic therapies. One example of this is decreased

expression of the breast cancer susceptibility gene 1 (*BRCA1*). This gene contributes to the double strand DNA break repair mechanism (Scully et al., 1999). Decreased expression of this gene leads to different responses depending on the treatment administered. For several drugs, deficiencies in *BRCA1* expression results in hypersensitivity, whereas for microtubule-targeting drugs, such as paclitaxel, resistance is encountered (Chabalier et al., 2006). Deficiencies in *BRCA1* are found in many cases of familial breast cancer and are also found in a small percentage of sporadic breast cancer cases (Kennedy et al., 2004). In some cases this is due to epigenetic changes where hyper-methylation of the promoter has occurred, and in other cases is due to genomic changes where copy number deletions and loss of heterozygosity occur (Rice et al., 2000, Staff et al., 2003, Chabalier et al., 2006). Determining *BRCA1* status of patients is therefore important when deciding which systemic therapies should be administered in suspected cases of familial breast cancer. However, since *BRCA1* aberrations are only found in a small percentage of sporadic breast cancer cases (Ford et al., 1995), patients diagnosed with this breast cancer type are not routinely tested (Levy et al., 2011, Grindedal et al., 2017).

Another example of altered gene expression conferring resistance is the increased expression of the transcription factor FOXM1 in endocrine therapy resistance in ER positive breast cancer (Bergamaschi et al., 2014). In this case, the increased expression of FOXM1 is a result of altered transcription rates. A previous study had noted that increased expression of 14-3-3ζ (gene name *YWHAZ*) in primary tumours was associated with earlier time to recurrence and metastasis in breast cancer (Bergamaschi et al., 2013) and increased expression following tamoxifen treatment was found to be a marker of poor prognosis (Frasor et al., 2006). 14-3-3ζ was subsequently observed to regulate FOXM1, acting upstream of this transcription factor (Bergamaschi et al., 2011). 14-3-3ζ has also been previously shown to influence expression of other genes that contribute to chemoresistance such as the xenobiotic drug transporter *ABCB1* (protein name P-glycoprotein) (Liang et al., 2014). Increased FOXM1 protein expression was correlated with significantly reduced survival in ER positive breast cancer patients and was also associated with an expansion of the cancer stem-like cell population as well as resistance to endocrine

therapies (Bergamaschi et al., 2014). FOXM1 regulates the expression of genes involved in cell proliferation, metastasis and stem cell-like properties, implying several mechanisms by which tumour cells would be able to overcome tamoxifen-induced death.

A more indirect mechanism of conferring resistance to therapeutic drugs is altered expression and function of enzymes that metabolise the drugs. These include cytochrome P450 enzymes, which are predominantly expressed in the liver but have also been shown to be expressed in tumour tissues including breast (Oyama et al., 2004, van Schaik, 2008). These enzymes are responsible for converting pro-drugs to active drugs and active drugs to their inactive metabolites. Increased expression of several of these metabolic enzymes has been observed in breast cancers, including CYP1B1 (Husbeck and Powis, 2002) and CYP2E1 (Kapucuoglu et al., 2003). Evidence shows a role for CY1B1 in the metabolism of the anti-oestrogen tamoxifen (Crewe et al., 2002), whereas CYP2E1 has been shown to contribute to the metabolism of the chemotherapy agents cyclophosphamide and etoposide (Kawashiro et al., 1998, Huang et al., 2000). Numerous polymorphisms in the genes encoding these enzymes have also been identified. The consequences of these polymorphisms on enzyme function vary. For example, CYP2D6, another enzyme involved in tamoxifen metabolism (Coller et al., 2002) has numerous alleles with different functions (van Schaik, 2008). Patients administered tamoxifen who were carriers of the alleles with reduced or no activity (examples include CYP2D6*9, *10, *41 and CYP2D6*3, *4, *5 respectively) had shorter times to recurrence compared with patients administered tamoxifen who were carriers of alleles with normal functionality (examples include CYP2D6*1, *2, *35) (Bonanni et al., 2006, Goetz et al., 2007, Schroth et al., 2007). This was proposed to be due to the decreased ability of the poor metabolisers to metabolise tamoxifen into its more active forms, such as endoxifen.

A further mechanism by which cells can become resistant to chemotherapeutic agents is by expressing xenobiotic efflux pumps, which pump out substrates such as cytotoxic drugs. A well-known family of proteins that perform this function is the ABC transporter family (Sharom, 2008). These proteins actively transport substrates across the plasma membrane against the concentration gradient. The ABC protein family has a wide variety of substrates, although

there are many overlaps in substrate specificity between individual members (Massey et al., 2014). These genes are regulated by a number of mechanisms including epigenetic changes (Reed et al., 2008, Spitzwieser et al., 2016), genomic changes (Reed et al., 2008, O'Brien et al., 2008) and transcriptional changes (Ee et al., 2004, Wang et al., 2008).

1.4.1.1 The role of ABC transporters in multi-drug resistance

The ATP-Binding Cassette (ABC) proteins are membrane bound proteins involved in the efflux of many compounds across the cell membrane. Substrates include amino acids, metal ions, and sugars, demonstrating the important role these transporters play in normal cell function. There are forty-nine known human ABC genes (Vasiliou et al., 2009, Hodges et al., 2011). The ABC family is subdivided into seven subfamilies, designated A-G, each of which has specific characteristics. For example, ABCA subfamily members are predominantly expressed in the central nervous system and several members of the ABCB subfamily facilitate intracellular peptide transport (Robey et al., 2009, Fletcher et al., 2010).

However, in cancer, the presence of the ABC transporters can pose a major problem during systemic treatment as some of the many substrates that can be transported across the cell membrane by these pumps include chemotherapeutic drugs (Eckford and Sharom, 2009). This can lead to multi-drug resistance as these drug pumps lead to an efflux of drug from the cell, resulting in lower intracellular drug concentrations and increasing the ability of the cell to overcome damage-induced cell death (Hazlehurst and Dalton, 2006, Zahreddine and Borden, 2013). Three ABC genes in particular have been shown to contribute to multi-drug resistance in breast cancer. These are *ABCB1*, known also as MDR1 or P-glycoprotein (Pgp), *ABCC1*, also known as MRP1, and *ABCG2*, known also as MXR or Breast Cancer Resistance Protein (BCRP) (Eckford and Sharom, 2009, Fletcher et al., 2010).

ABCB1 has numerous chemotherapeutic substrates that include anthracyclines, taxanes, mitoxantrone and irinotecan. *ABCC1* also has several chemotherapeutic substrates, some of which are in common with *ABCB1* such as anthracyclines and others that are unique such as antifolate antineoplastic agents. As with the previous two ABC family members, *ABCG2* substrates

include anthracyclines, mitoxantrone and etoposide (Staud and Pavek, 2005, Gillet et al., 2007, Fletcher et al., 2010). Of these, *ABCG2* / BCRP is of particular relevance to my work since I study its expression and potential importance in Chapter 6.

1.4.2 Molecular pathways with indirect influence on resistance to systemic therapies

Resistance to systemic therapies can also be conferred indirectly by altered activity of molecular pathways. One such pathway is the mitogen-activated protein kinases (MAPK) pathway, increased activity of which has been implicated in resistance to numerous systemic therapies. This pathway is involved in multiple cellular processes including cell proliferation (Zhang and Liu, 2002). Some of the effectors activated by this pathway that contribute to this function are c-fos, c-myc and c-jun (Sanchez et al., 1994, Price et al., 1996, Zhu et al., 2008). Increased expression of these proteins has previously been observed in breast cancer cells (Walker and Cowl, 1991, Berns et al., 1992, Zajchowski et al., 2001). Molecular profiling of residual breast tumours post-NAC identified decreased expression of *DUSP4*, which was associated with high rates of cell proliferation post-NAC and the basal-like subtype of breast cancers (Balko et al., 2012). The *DUSP4* protein (MKP2) is a dual-specificity MAPK phosphatase, which dephosphorylates ERK1/2, p38 and JNK (Lake et al., 2016). Reduced expression of MKP2 led to increased activation of the MAPK pathway and reduced response to chemotherapy (Balko et al., 2012). Further investigations revealed that in the basal-like subtype of breast cancer, the *DUSP4* promoter is more highly methylated than in other breast cancer subtypes, suggesting one mechanism, an epigenetic change, for the decreased expression of this protein post-NAC. Increased activity of the MAPK pathway has also been associated with endocrine therapy resistance (Haagenson and Wu, 2010).

Another dysregulated pathway implicated in systemic therapy resistance is the activation of the PI3K pathway, attributed in part to the over-expression and activation of growth factor receptors and their coactivators, including integrin receptors and JAK proteins (Garcia-Becerra et al., 2013, Martz et al., 2014). Similarly to the MAPK pathway, the PI3K pathway also contributes to the

regulation of cell proliferation, and also aids in the process of evasion of apoptosis (Ghebeh et al., 2014). Genomic changes involving mutations in *PIK3CA* have been observed in breast cancer, mostly consisting of SNPs (Isakoff et al., 2005). Several of the most studied mutations result in increased PI3K activity (Altomare and Testa, 2005), thus providing another possible mechanism by which PI3K pathway activity can be increased, leading to resistance to systemic therapies.

1.4.2.1 Molecular pathways as novel therapeutic targets

Identification of these dysregulated pathways and understanding the mechanisms behind the activities of these pathways that lead to chemoresistance, potentially allows the identification of novel therapeutic targets. Specific inhibitors of these molecular pathways have already been developed, although it has been observed that targeting only one pathway can result in another pathway being activated instead, resulting in continued resistance to therapies (Mirzoeva et al., 2009). This was reflected when inhibitors of the MAPK pathway tested in clinical trials yielded limited positive results in multiple cancers including breast cancer (Rinehart et al., 2004), and this has since been attributed to tumours being able to circumvent the effects of these inhibitors on their target expression (Duncan et al., 2012). Simultaneous administration of inhibitors of the MAPK and PI3K pathways had a synergistic effect, increasing apoptosis and cell cycle arrest (Mirzoeva et al., 2009). A more recent phase Ib clinical trial investigating the efficacy of alpelisib, an inhibitor of the PI3K catalytic subunit p110α (*PI3KCA*), in combination with the endocrine therapy letrozole, showed promising results, particularly in patients with a mutation in *PIK3CA* (Mayer et al., 2017). Phase II and III trials are ongoing and will hopefully show limited ability of tumours to circumvent the inhibition of the PI3K pathway.

1.4.3 Altered miRNA expression in systemic therapy resistance

MiRNAs are regulators of gene expression that act at post-transcriptional levels (see section 1.3.2). A wide range of miRNAs have also been implicated in therapeutic resistance, typically through their roles in controlling expression of protein-coding targets that themselves have roles in resistance, although the

specific targets have not always been identified for each miRNA. MiR-487a was down-regulated in a variant of the ER positive, breast cancer cell line MCF7 that was resistant to mitoxantrone (Ma et al., 2013). These resistant cells had increased expression of the drug efflux pump breast cancer resistance protein (BCRP), which confers multidrug resistance (see section 1.4.1.1). Several validation experiments demonstrated that miR-487a was able to down-regulate BCRP expression by binding to the 3'UTR of the transcript, therefore that the down-regulation of miR-487a in the resistant cells was contributing to the resistance by leading to up-regulation of BCRP.

MiRNAs also influence chemosensitivity via other less direct mechanisms such as evasion of apoptosis and epithelial-mesenchymal transition (EMT) (Kutanzi et al., 2011, Wang et al., 2015). One of the mechanisms the chemotherapeutic doxorubicin uses to kill tumour cells is by increased production of reactive oxygen species (ROS) (see section 1.2.2.3.1). Increased expression of let-7a increased the chemosensitivity of doxorubicin resistant triple negative breast cancer cells, an effect that was attenuated when cells were pre-treated with an antioxidant (Serguienko et al., 2015). This implicated let-7a in ROS production, although the precise molecular mechanisms were not identified. In a slightly different mechanism of inhibition of apoptosis, decreased expression of miR-149 in doxorubicin resistant ER positive breast cancer cells led to increased expression of its target *NDST1* (He et al., 2014a). This increased expression led to activation of a heparan sulphate pathway that triggered inhibition of apoptosis.

An example of a miRNA that influences EMT is miR-125b, which was observed to be down-regulated in paclitaxel resistant ER positive MCF7 and HER2 positive SKBR3 breast cancer cells (Yang et al., 2015). This down-regulation resulted in mesenchymal features, apparently including drug resistance, due to increased expression of the miR-125b target *SEMA4C*.

Interestingly, multiple miRNAs can target the same transcript. *PTEN* is a tumour suppressor gene and over-expression of this gene sensitises ER positive breast cancer cells to doxorubicin by enhancing caspase-3-dependent apoptosis (Wang et al., 2011). Both miR-19 (Liang et al., 2011) and miR-21 (Wang et al., 2011) have been shown to mediate chemoresistance via *PTEN*. Altogether, over sixty miRNA families have been identified as mediators of

resistance to systemic therapies in breast cancer (Kutanzi et al., 2011). This emphasises the vast network of small RNAs that could have a major impact on breast cancer response to systemic treatments that could be exploited when searching for novel therapeutic targets.

1.5 Hypothesis and Aims

The hypothesis of this project is that the specific molecular changes that occur during systemic therapy can lead to resistance to chemotherapy. There are two main aims to this project:

1. To identify molecular changes that occur during neoadjuvant chemotherapy and determine which of these changes contributes to chemoresistance and what their mechanisms of function are.
2. To determine whether the up-regulation of the Breast Cancer Resistance Protein (BCRP) post-neoadjuvant endocrine therapy could lead to resistance to subsequent chemotherapy.

Chapter 2: Materials & Methods

2.1 Ethical considerations and patient samples

Ethical approval was obtained from the Leeds (East) Research Ethics Committee, reference 06/Q1206/180. Tumour samples were available for research in the form of formalin-fixed paraffin-embedded (FFPE) cancer tissue blocks archived as part of routine clinical management at St James' University Hospital, Leeds (Leeds Teaching Hospitals NHS Trust). Tissue was available for two patient cohorts: 1) 5 patients diagnosed with ER positive and HER2 negative primary breast cancer at St James's University Hospital, and who were treated with a combination of epirubicin and cyclophosphamide (EC) in a neoadjuvant setting and showed partial resistance to this treatment, as defined by MRI assessments during treatment and histopathology assessment of the resection samples. Matched tissues representing diagnostic core biopsies (pre-neoadjuvant chemotherapy (NAC)) and resections (post-NAC) were available. 2) 305 patients diagnosed with primary breast cancers of all molecular subtypes at St James's University Hospital. Patients were diagnosed with primary breast cancer between 2005 and 2010. Tumour samples were taken from patients during surgical resection of the tumour without prior systemic therapies. Patients with incomplete follow-up data including time of recurrence (if any) and/or death were excluded. Areas of tumour enriched for tumour epithelial cells were marked by a breast histopathologist (Dr Eldo Verghese, project supervisor and consultant pathologist at Leeds Teaching Hospitals NHS Trust) and three cores with a diameter of 0.6mm were taken from each sample to construct tissue microarrays (TMAs).

2.2 Tissue sectioning and staining of tissues with Haematoxylin and Eosin (H&E)

Sectioning procedures were different for samples destined for Laser Capture Microdissection (LCM), or for immunohistochemistry. For LCM, samples were sectioned at 10µm and placed on MembraneSlide NF 1.0 PEN (415190-9211-000; Carl Zeiss; Oberkochen, Germany) slides. Samples (whole tissue blocks

for optimisation or tissue microarrays) for immunohistochemistry were sectioned at 5μM and placed on SuperFrost Plus™ Adhesion Slides (12625336; Fisher Scientific; Hampton, USA). Following sectioning, slides were placed on a hot plate at 70°C for 15 minutes to ensure tissue attachment.

Slides to be Haematoxylin and Eosin (H&E) stained were placed in xylene three times for 3 minutes each. Tissues were then rehydrated using dilutions of ethanol (100%, 75% and 50%) for 1 minute each followed by distilled water. Slides were then placed in filtered Mayer's haematoxylin (008011; Thermo Fisher Scientific; Waltham, USA) for 2 minutes followed by washing with distilled water until excess haematoxylin had been removed. This was followed by staining with 1% eosin for 2 minutes and washing again to remove excess eosin. Tissues were dehydrated again using dilutions of ethanol (75% and 2x 100%) for 2 minutes each and then xylene for a total of 5 minutes.

2.3 Isolation of tumour epithelial cells using Laser Capture Micro-dissection (LCM)

All samples were viewed by breast histopathologists (Dr Eldo Verghese, consultant breast histopathologist at Leeds Teaching Hospitals NHS Trust and co-supervisor for this work, or Professor Andrew Hanby, consultant breast histopathologist at Leeds Teaching Hospitals NHS Trust) and areas containing cancer cells were identified. LCM was performed using the PALM® Robot Microbeam laser microdissection system (P.A.L.M. Microlaser Technologies GmbH; Bernried, Germany) using the PALM RoboSoftware 3.2, or the ArcturusXT™ Laser Capture Microdissection System (Thermo Fisher Scientific; Waltham, USA). Two approaches were used to isolate epithelial cancer cells. When samples consisted predominantly of epithelial cancer cells, any unwanted elements such as stroma or Ductal Carcinoma In Situ (DCIS) were removed from the slides by LCM (Figure 2.3.1A and B) and tumour cells were later macro-dissected from the slides into micro-centrifuge tubes using a scalpel. When samples contained smaller patches of epithelial cancer cells, dissection occurred using LCM directly (Figure 2.3.1C and D) into an Adhesive Cap 500 clear (415190-9211-000; Carl Zeiss; Oberkochen, Germany) (for use with the PALM system, or an Arcturus® CapSure® Macro LCM Cap (10284-04;

Applied Biosystems; Foster City, USA) (for use with the Arcturus system). For most samples, 10 to 15 sections were used except where very few cells were present, in which case up to 25 sections were used. When samples contained smaller patches of epithelial cancer cells, the total areas of tissue that were LCM dissected were 7-54mm^2.

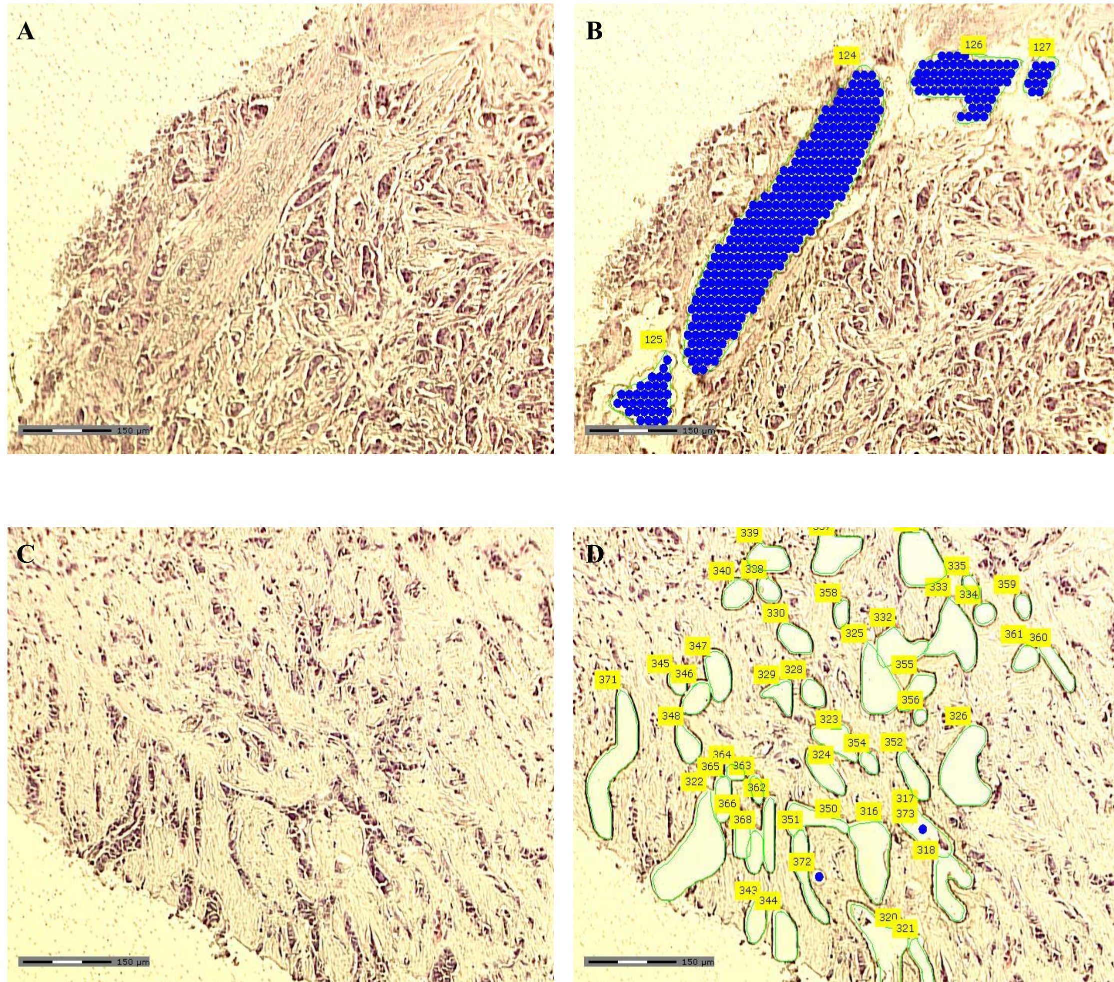

Figure 2.3.1: Isolation of tumour epithelial cells using two different LCM approaches

Two approaches were taken when performing LCM to isolate tumour epithelial cells – representative pairs of images for each approach are shown. (A) An example section of tissue highly enriched with tumour epithelial cells with only small areas of unwanted elements, in this case stroma. (B) The same section of tissue shown in (A) after removal of stroma by LCM – the epithelial cancer cells left behind were collected manually for RNA extraction. (C) An example section of tissue with small groups of tumour epithelial cells interspersed with many areas of unwanted elements. (D) The same section of tissue shown in (C) after direct isolation of the tumour epithelial cells by LCM; RNA was extracted from these directly isolated cells.

2.4 Extraction of total RNA from isolated tumour cells from patient samples

RNA was extracted from cells using the AllPrep DNA/RNA FFPE Kit (80234; Qiagen; Venlo, the Netherlands). In brief, cells were re-suspended in Buffer PKD with proteinase K and incubated at 56°C for 15 minutes followed by a 3 minute incubation on ice. Samples were centrifuged for 15 minutes at 20000g and the supernatant incubated for a further 15 minutes at 80°C. Buffer RLT was added together with ethanol before applying the sample to an RNeasy MiniElute spin column. The spin column was washed with Buffer FRN before a mixture of DNaseI and Buffer RDD was added directly to the membrane and allowed to incubate at room temperature for 15 minutes. The spin column was washed again with Buffer FRN and the flow-through reapplied to the membrane. Washing occurred twice with Buffer RPE before being centrifuged for 5 minutes to dry the membrane. RNA was eluted in RNase-free water and stored at -80°C.

2.5 Preparation of cDNA for analysis of miRNA expression in patient samples

To perform reverse transcription of miRNAs, the TaqMan™ MicroRNA Reverse Transcription Kit (4366597; Applied Biosystems; Foster City, USA) was used following the manufacturer's protocol. In brief, up to 200ng total RNA in a volume of 3µl was mixed with the reverse transcription reaction mixture containing Megaplex™ RT Primers, Human Pool A v.2.1. (4399966; Applied Biosystems; Foster City, USA) in 0.2ml PCR® Tubes (321-02-501; Axygen; Corning, USA). The mixture was incubated on ice for 5 minutes before being placed in a controlled temperature heat block to undergo reverse transcription (Table 2.5.1). The cDNA was stored at -20^{0}C.

Table 2.5.1: Conditions for reverse transcription of cDNAs (from miRNAs)

Number of cycles	Temperature (°C)	Time (mins)
40	16	2
	42	1
	50	1 second
1	85	5
1	4	∞

Since low levels of RNA were extracted from the FFPE samples, pre-amplification of the cDNA was required. This was performed using Megaplex™ PreAmp Pool A Primers (4399830; Applied Biosystems; Foster City, USA) and the TaqMan® PreAmp Master Mix (4384266; Applied Biosystems; Foster City, USA) following the manufacturer's protocol. In brief, the cDNA was placed in a mixture with the Pool A primers and master mix into 0.2ml PCR Tubes (321-02-501; Axygen; Corning, USA) before being placed in a controlled temperature heat block to undergo pre-amplification (Table 2.5.2). The PCR products were then diluted with 0.1X TE buffer pH8.0 and stored at -20^{0}C.

Table 2.5.2: Conditions for pre-amplification of miRNAs

Number of cycles	Temperature (°C)	Time (min)
1	95	10
1	55	2
1	72	2
12	95	15 seconds
	60	4
1	99.9	10
1	4	∞

2.6 Expression profiling of miRNAs in patient samples

To analyse miRNA content, samples were mixed with TaqMan® Universal PCR Master Mix (4324018; Applied Biosystems; Foster City, USA) and loaded onto TaqMan® MicroRNA Array A cards (4398977; Applied Biosystems; Foster City, USA). These were amplified on the 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, USA). Each individual sample was normalised to the mean of all miRNAs expressed within that sample. Those

miRNAs detected in only one of the paired samples for each patient were also included in the number of miRNAs either up- or down-regulated on the condition that the Ct value of the detected sample was a maximum of 38.5.

2.7 Analysis of mRNAs in patient samples using NanoString

Total RNA was either analysed directly or was first reverse transcribed followed by pre-amplification. Reverse transcription was performed using 1µl SuperScript® VILO™ Master Mix (11755050; Thermo Fisher; Waltham, USA) and 50-110ng total RNA, in a total volume of 5µl. Equal amounts of total RNA were used for matched pre- and post-NAC samples for each patient. The mixture was incubated at 25°C for 10 minutes, then 42°C for 60 minutes followed by 85°C for 5 minutes.

Pre-amplification was performed using TaqMan® PreAmp Master Mix (4384266; Applied Biosystems; Foster City, USA) and pooled MTE primers (containing primers for all genes in the NanoString codeset chosen). Together with all of the reverse transcription product (5µl), this mixture, in a total volume of 11µl, was placed in a controlled temperature heatblock (see Table 2.7.1 for conditions).

Table 2.7.1: Conditions under which Multiplexed Target Enrichment of cDNA was performed in preparation for analysis by NanoString Technologies

Step	**Temperature (^{o}C)**	**Time**
Denaturation	94	10 mins
MTE cycles (10x)	94	15 s
	60	4 mins
Hold	4	∞

Total RNA or cDNA was sent for analysis using the nCounter® PanCancer Pathway panel (NanoString Technologies) to the NanoString service, Human Dendritic Cell Lab, Newcastle University, UK (contact individual was Ms Anastasia Resteu; Newcastle University, UK). Data were analysed using the nSolver Analysis Software 3.0 (NanoString Technologies).

2.8 Drugs used in tissue culture and their storage conditions

Epirubicin hydrochloride (E9406; Sigma; St Louis, USA) was dissolved in autoclaved water at a stock concentration of 100mM. It was stored at -20°C in aliquots of 10µl. Docetaxel (01885; Sigma; St Louis, USA) was dissolved in dimethyl sulphoxide (DMSO) at a stock concentration of 10mM. It was stored at -20°C in aliquots of 200µl. Tamoxifen (T5648; Sigma; St Louis, USA) was dissolved in 100% ethanol at a stock concentration of 53.8mM. It was stored at -20°C in aliquots of 1ml.

2.9 Cell culture

MCF7, T47D and MDA-MB-175 cells were available within the Hughes group, having been originally purchased from the European Collection of Animal Cell Cultures. Cell line identities were confirmed (STR profiles, Leeds Genomics Service) and lines were consistently negative for mycoplasma (MycoAlert Mycoplasma detection assay, Lonza, Basel, Switzerland). MCF7 and T47D cells were routinely cultured in Dulbecco Modified Eagle's Medium (DMEM) (31966; Gibco® by Life Technologies™; Waltham, USA), and MDA-MB-175 cells were routinely cultured in Leibovitz's L-15 Medium (11415049; Gibco® by Life Technologies™; Waltham, USA), all supplemented with 10% Foetal Calf Serum (FCS) (Sigma-Aldrich; St Louis, USA) and 1% Penicillin/Streptomycin (15070-063; Gibco® by Life Technologies™; Waltham, USA). MCF7, T47D and MDA-MB-175 cells were routinely grown at 37°C and MCF7 and T47D cells were also kept in 5% CO_2. Cells were passaged when they had reached over 70% confluency. The media was removed and the cells washed with Dulbecco's Phosphate Buffered Saline (DPBS) (14190; Gibco® by Life Technologies™; Waltham, USA). Trypsin solution (0.05% v/v) (15400054; Gibco® by Life Technologies™; Waltham, USA) was added and cells were incubated for up to 5 minutes at 37°C. To inactivate the trypsin, fresh media was added to the cells and – typically – one-twentieth of this volume for MCF7 and T47D cells (split ratio of 1:20) and one-third of this volume for MDA-MB-175 cells (split ratio of 1:3) was added to a new flask containing fresh media.

2.9.1 Development of epirubicin resistant MCF7 and T47D cell lines

MCF7 and T47D cells were treated initially with low doses of epirubicin hydrochloride (E9406; Sigma) or vehicle control (water) and cultured continuously as described above. Fresh drug was added once a week, with doses gradually increased over many months such as to avoid excessive cell killing. MCF7 cells were initially treated with 1nM epirubicin and T47D cells were initially treated with 10nM epirubicin. Doses were no longer increased once MCF7 cells were treated regularly with 350nM epirubicin and T47D cells with 1µM epirubicin.

2.10 Transfection of cells with microRNA mimics and hairpin inhibitors, and siRNAs

Initial studies were performed using microRNA mimics and hairpin inhibitors (Dharmacon; Lafayette, USA) with mature sequences listed in Table 2.10.1. Different numbers of cells in different sized wells were used depending on the end-point assays to be used, as follows. For subsequent RNA extractions and colony forming assays, cells were plated into each well of 24-well plates, or for MTT assays, cells were plated into each well of 96-well plates. Cells were plated for each assay such that they were 70-80% confluent at the time of transfection. The following day, Lipofectamine 2000 Transfection Reagent (11668019; Invitrogen; Carlsbad, USA) was used to transfect cells with the mimics or inhibitors (final concentrations in each complex for each well are shown in Table 2.10.1) together with equimolar amounts of the appropriate controls in Opti-MEM Reduced Serum Medium (31985; Gibco® by Life Technologies™; Waltham, USA). For MCF7 cells, 0.4 times of the final transfection mixture volume was added of Lipofectamine 2000 (2:5 ratio). For MDA-MB-175 cells, 0.25 times of the final transfection mixture volume was added of Lipofectamine 2000 (5:20 ratio). The mixture containing Lipofectamine 2000 and miRNA mimic or inhibitor was in a final volume of 70µl for 24-well plates and 15µl for 96-well plates. Twice this volume of Opti-MEM Reduced Serum Medium and one times of this volume of normal media (DMEM for MCF7 cells and Leibovitz’s L15 for MDA-MB-175 cells) was also added to each

well. Cells were incubated in transfection media for 24 hours, at which point the transfection media was replaced with normal media.

Biotinylated miRCURY LNA™ microRNA Mimics or a negative control (47997-671; Exiqon; Venlo, the Netherlands) were used to transfect MCF7 cells in T150 tissue culture flasks (CLS430825; Sigma; St Louis, USA) with Lipofectamine 2000 as described above. This was performed using mimics of miR-26b-5p and miR-195-5p and were transfected into cells at final concentrations of 25nM and 15nM respectively in each complex for each flask.

The siRNAs were purchased from IDT (San Jose, USA) and transfected into cells using the same protocol as was used for the miRNA mimics and inhibitors. Sequences are found in Table 2.10.2.

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Table 2.10.1: MicroRNA mimics and hairpin inhibitors used in transfections

MiRNA (product number)	Mimic / Inhibitor	Sequence of mature target miRNA	MCF7 (nM)	MDA-MB-175 (nM)
hsa-miR-26b-5p (C-300501-07-0005)	Mimic	UUCAAGUAAUUCAGGAUAGGU	5	25
hsa-miR-195-5p (C-300643-03-0005)	Mimic	UAGCAGCACAGAAAUAUUGGC	0.5	5
hsa-miR-10a-5p (C-300549-03-0005)	Mimic	UACCCUGUAGAUCCGAAUUUGUG	0.05	5
hsa-miR-26b-5p (IH-300501-08-0005)	Inhibitor	UUCAAGUAAUUCAGGAUAGGU	12.5	25
hsa-miR-195-5p (IH-300643-05-0005)	Inhibitor	UAGCAGCACAGAAAUAUUGGC	25	25
hsa-miR-10a-5p (IH-300549-05-0005)	Inhibitor	UACCCUGUAGAUCCGAAUUUGUG	50	50
Negative Control (CN-001000-01-05)	Mimic	Propriety sequence		
Negative Control (IN-001005-01-05)	Inhibitor	Propriety sequence		

Table 2.10.2: Sequences of the siRNAs used in transfections

mRNA (reference number)	Sequence of antisense strand
CCDC6 (161436520)	5’ - UGAAAUUCAGACUAAGCUCAUGCAUUA
REEP4 (76776114)	5’ - GUAUCUGACCAACACUCAUCCUCGGUG
SEMA6D (76776117)	5’ – UUCUAAGGUACUCAACCUGUAGUAUCU
PRKCD (76776108)	5’ - GAUGUUGAAGCGUUCUUUCUGGAAUAU
ARL2 (76776111)	5’ - UUUAUUUCACAACUGAGUGAAGGAUGA

2.11 Harvesting cells transfected with non-biotinylated miRNA mimics or inhibitors or siRNAs for RNA extraction

At the appropriate time points, media was removed and the cells washed with PBS. The cells were then covered with 100µl of BL buffer (Promega; Madison, USA) supplemented with 1-Thioglycerol (TG) according to the manufacturer’s instructions (Promega; Madison, USA). Cells were incubated in the buffer for 20 minutes at room temperature with occasional shaking to allow cell lysis to occur, followed by collection of the buffer mixture that was stored at -80°C until RNA extraction occurred.

2.12 Extracting total RNA from cells

RNA was extracted using the ReliaPrep™ RNA Cell Miniprep System (Z6012; Promega; Madison, USA) according to the manufacturer’s instructions. In brief, isopropanol was added to the BL+TG buffer cell lysate at a final concentration of 34%. Samples were placed into a ReliaPrep™ Minicolumn. All centrifugation steps occurred at 12000g unless stated otherwise. Minicolumns were then washed with RNA Wash Solution. A DNaseI mix was freshly prepared containing Yellow Core Buffer, $MnCl_2$ and DNaseI enzyme that was added to the membrane and left to incubate for 15 minutes followed by addition of Column Wash Solution and centrifugation for 15 seconds. This was followed by two washes with RNA Wash Solution. The final centrifugation step lasted 2 minutes. Nuclease-free (NF) water was then added to the membrane and left for 1 minute before centrifugation for 1 minute at 5500g. Extracted RNA was stored at -80°C.

2.13 Analysing miRNA expression using real-time quantitative PCR (individual miRNAs)

RNA samples first underwent reverse transcription, resulting in stable cDNA samples. This was achieved using the TaqMan Reverse Transcription Kit (4366597; Applied Biosystems; Foster City, USA) according to the manufacturer's instructions. In brief, 1-10ng of total RNA was mixed with a master mix containing dNTPs, reverse transcriptase, RNase inhibitor and reverse transcription buffer in a total volume of 15µl. Reactions were placed in a controlled temperature heat block to allow reverse transcription to occur (see Table 2.13.1 for conditions).

Table 2.13.1: Conditions for miRNA reverse transcription

Temperature (°C)	Time (mins)
16	30
42	30
85	5

MiRNA qPCR was performed by combining TaqMan 2x Universal PCR Master Mix, No AmpErase UNG (4324018; Applied Biosystems; Foster City, USA), NF water, the primers for the miRNA of interest, and the reverse transcription product in 10µl total volumes, in technical triplicate reactions. The reference miRNA used was RNU48.

Reactions occurred in MicroAmp® Optical 96-Well Reaction Plates (N8010560; Applied Biosystems; Foster City, USA) using a QuantStudio 5 Real-Time PCR System (Applied Biosystems; Foster City, USA). The conditions for the reactions are shown in Table 2.13.2.

Table 2.13.2: Conditions for miRNA real-time quantitative PCR

<table>
<tr><th>Step</th><th>Temperature (°C)</th><th>Time (mins)</th></tr>
<tr><td>HOLD</td><td>95</td><td>10</td></tr>
<tr><td rowspan="2">40 cycles</td><td>95</td><td>15 seconds</td></tr>
<tr><td>60</td><td>1</td></tr>
</table>

2.14 Analysing mRNA expression using real-time quantitative PCR

RNA samples first underwent reverse transcription, resulting in stable cDNA samples. This was achieved using the GoScript™ Reverse Transcription System (A5001; Promega; Madison, USA) according to the manufacturer's instructions. In brief, 0.5-1µg of total RNA was diluted in NF water before addition of Random Primers in a total volume of 5µl. Samples were then incubated at 70ºC for 5 minutes and placed in ice water immediately after. Reactions were combined in a total volume of 20µl containing NF water, GoScript™ 5X Reaction Buffer, $MgCl_2$, PCR Nucleotide Mix and GoScript™ Reverse Transcriptase. Two control reactions were performed: one lacking Reverse Transcriptase, and one lacking RNA template. Reactions were placed in a controlled temperature heat block to allow reverse transcription to occur (see Table 2.14.1 for conditions).

Table 2.14.1: Conditions for mRNA reverse transcription

Temperature (ºC)	Time (mins)	Function
25	5	Annealing
42	60	Extension
70	15	Thermal inactivation of reverse transcriptase

Quantification of mRNA levels of genes of interest was performed using the GoTaq® qPCR Master Mix kit (6001; Promega; Madison, USA) according to the manufacturer's instructions. In brief, separate mixtures were prepared containing GoTaq® qPCR Master Mix (2X), CXR Reference Dye and forward and reverse primers for each gene of interest (Table 2.14.2**Error! Reference source not found.**). The reference gene used was beta-actin. Reactions occurred in MicroAmp® Optical 96-Well Reaction Plates (N8010560; Applied Biosystems; Foster City, USA) in a total volume of 10µl in technical triplicates. 96-well plates were centrifuged for 1 minute at 1000g. Analyses were performed in the 7500 Real Time PCR System machine (Applied Biosystems; Foster City, USA) or QuantStudio 5 Real-Time PCR System (Applied Biosystems; Foster City, USA), using the 7500 System SDS Software (v1.2) or the QuantStudio 5 Real-Time PCR Software. The conditions under which the reactions took place are shown in Table 2.14.3.

Table 2.14.2: qPCR primer sequences for mRNAs

Primer name (Supplier)	**Primer sequence**
Beta-actin Forward (Sigma)	5' – TTCTACAATGAGCTGCGTGTG
Beta-actin Reverse (Sigma)	5' – GGGGTGTTGAAGGTCTCAAA
ABCG2 Forward (Sigma)	5' – CAGGTGGAGGCAAATCTTCGT
ABCG2 Reverse (Sigma)	5' – ACACACCACGGATAAACTGA
CCDC6 Forward (IDT)	5' - CTCCAGAAAATATGATGCGTCAC
CCDC6 Reverse (IDT)	5' – CCTCCAGATACTGTGCCATT
PRKAR2B Forward (IDT)	5' – TCTCTTTTTGGCATTGTTTTTCAC
PRKAR2B Reverse (IDT)	5' - CTACAATCACTGCTACCTCTCC
GNAI3 Forward (IDT)	5' - CATCCTCTGAATAGCCATCCTC
GNAI3 Reverse (IDT)	5' - AAAAAGCGGCCAAAGAAGTG
E2F7 Forward (IDT)	5' - CAATGTCATAGATGCGTCTCCT
E2F7 Reverse (IDT)	5' - GCTCGCTATCCAAGTTATCCC
PRKCD Forward (IDT)	5' - GGATACATGGTCGGCTTCTTC
PRKCD Reverse (IDT)	5' - CATCGCCTTCAACTCCTATGAG
SEMA6D Forward (IDT)	5' - ACTGGACTTCCCATCGTACA
SEMA6D Reverse (IDT)	5' - TGCTGTTAACCGAAGACTTCT
ARL2 Forward (IDT)	5' - TTAGCAAAGATGAGGAGGGTTG
ARL2 Reverse (IDT)	5' - GAACTACTTTGAGAGCACCGAT
REEP4 Forward (IDT)	5' - GTACATCATCCACCGCACAT
REEP4 Reverse (IDT)	5' - ATGATCTGTCGCCTGGTG

Table 2.14.3: Conditions for real-time quantitative PCR for mRNAs

Number of cycles	**Temperature (°C)**	**Time (mins)**
1	50	2
1	95	10
40	95	15 seconds
	60	1

2.15 Chemo-survival assays using MTT assays

Cells were seeded at the appropriate density into 96-well plates. For transfections, target confluency for the next day was 70-80%. For drug treatments, target confluency for the next day was 90%. The following day, cells were either transfected with miRNA mimics/inhibitors/controls or target siRNAs or controls, treated with epirubicin or docetaxel, or transfected and then treated with epirubicin forty-eight hours later. Survival was assessed using MTT assays up to 48 hours after chemotherapy treatment. Medium was removed

and 25µl of 5mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazdium Bromide) reagent (M6494; Invitrogen; Carlsbad, USA) was added. Cells were then incubated at 37°C in 5% CO_2 in the dark for four hours followed by removal of the MTT reagent and addition of 50µl 100% isopropanol. Plates were then placed on a shaker for 15-30 minutes and absorbance was read at 570nm using a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies; Bad Wildbad, Germany).

2.16 Colony forming survival assays

Cells were transfected with miRNA mimics/inhibitors/controls or targeting siRNAs or controls as described in section 2.10. Forty-eight hours post-transfection, MCF7 cells were treated with 30nM epirubicin for twenty-four hours, or MDA-MB-175 cells were treated with 300nM or 600nM epirubicin for twenty-four hours. After treatment, cells were removed from the wells using trypsin as described in section 2.9, and re-seeded at low density in 6-well plates (technical duplicates for each condition). For MCF7 cells, 100 cells were plated per well, while for MDA-MB-175 cells 200 cells per well were plated. Importantly, MDA-MB-175 cells were re-plated in medium that had been pre-conditioned by MDA-MB-175 cells; this medium was prepared by being placed on confluent MDA-MB-175 cells for twenty-four hours and then being removed and centrifuged to ensure no carry-over of cells. This conditioned medium was prepared fresh where possible. When not possible, conditioned medium was prepared and stored at -20°C. Following plating, cells were incubated under standard culture conditions for fourteen days to allow colonies to grow. These plates were incubated in an incubator dedicated to colony forming assays, which was opened minimally during experiments; this was important to ensure that growing colonies were not disturbed and thereby avoid single colonies being split into multiple smaller colonies.

After fourteen days, growth media was removed and cells washed with DPBS. Cells were then fixed and stained simultaneously using the following solution containing 5mg/ml crystal violet (V5265-500ML; Sigma; St Louis, USA): 30% water, 50% methanol, and 20% ethanol. After four minutes, the solution was removed and colonies were washed twice with de-ionised water. The plates

were then air-dried before the number of colonies for each condition was manually counted. Tight groups of at least 30 cells were considered to be colonies. To validate the reproducibility of colony counts, a second count of ten plates was performed by Lisa Allinson (LA; a PhD student colleague at the University of Leeds) who had also performed colony forming survival assays in MCF7 cells. Results are shown in Figure 2.16.1, with a R^2 value of 0.999, demonstrating that counting of colonies was highly reproducible.

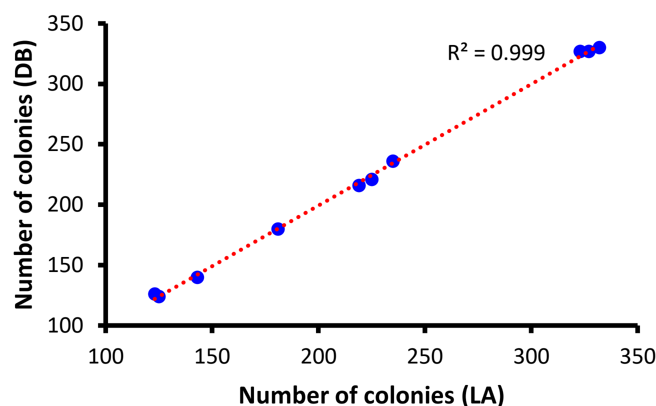


Figure 2.16.1: Raw colony counts are highly reproducible

Ten plates of colony forming assays were counted by both Lisa Allinson (LA) and the author (DB). Raw counts were plotted and a line of best fit drawn, with the correlation coefficient calculated.

2.17 Pulldowns of biotinylated-miRNA mimic transfected cells and RNA preparation

Initially, it was necessary to block the streptavidin-coated agarose beads to be used in the pulldowns with non-specific RNA and protein, in order to reduce background recovery. Pierce High Capacity Streptavidin Agarose beads (20357; Thermo Fisher Scientific; Waltham, USA) were blocked in aliquots of 50μl of slurry. 500μl of ice-cold lysis buffer was added to each aliquot in a 1.5ml microcentrifuge tube. These were centrifuged at 12000g for 30 seconds before being washed in lysis buffer and centrifuged again. The beads were then resuspended in 50μl lysis buffer and final concentrations of 1mg/ml tRNA (AM7119; Ambion; Foster City, USA) and 1mg/ml albumin (acetylated from bovine serum) (B2518-10MG; Merck; St Louis, USA) were added. This mixture was incubated with rotation at 4°C for 3 hours. The beads were then washed

twice with 300μl ice-cold lysis buffer before being resuspended in 100μl ice-cold lysis buffer.

MCF7 cells were transfected with biotinylated miRNA mimics as described in section 2.10. Twenty-four hours post-transfection, cells were harvested with trypsin and washed twice with ice-cold PBS and centrifuged at 400g for 5 minutes to form a pellet that was resuspended in ice-cold 1.5ml lysis buffer (see Appendix A.1). This resuspension was vortexed, placed on ice for 20 minutes and vortexed again. The mixture was then centrifuged at 12000g for 15 minutes at 4°C. The majority of the supernatant (except 50μl that was kept aside as an input control) was added to 100μl blocked Streptavidin Agarose beads and incubated whilst rotating for 4 hours at 4°C. The mixture was then centrifuged at 12000g for 1 minute at 4°C and the supernatant removed. The beads were washed five times with 1ml ice-cold lysis buffer. After the final wash, beads (and the input control samples) were resuspended in 100μl ice-cold lysis buffer.

To extract total RNA, 500μl TRI-Reagent (T9424; Sigma; St Louis, USA) was added to each sample and incubated at room temperature for 5 minutes whilst mixing occasionally. Then, 100μl chloroform (10332702; ACROS Organics; Hampton, USA) was added and the mixture was shaken vigorously for 30 seconds before centrifuging at 4°C for 15 minutes at 16000g. The aqueous phase was transferred to a fresh tube and 5μl GlycoBlue Coprecipitant (AM9515; Ambion; Foster City, USA) was added. RNA was precipitated in 2.5 volumes of 100% ethanol and 0.1 volume of sodium acetate (S7899; Sigma; St Louis, USA) at -80°C overnight.

The next day, the mixtures were centrifuged at 4°C for 30 minutes at 16000g. The supernatant was removed and the pellet washed in 1ml 75% ethanol followed by vortexing and centrifugation at 4°C for 15 minutes at 16000g. This wash step was repeated once. After the second, the ethanol was removed and the pellet was air-dried before being resuspended in 13μl NF water.

2.18 Library preparation for RNA-Seq

Samples of total RNA were first depleted of rRNA using the Ribo-Zero Gold rRNA Removal Kit (MRZG12324; Illumina; San Diego, USA) according to manufacturer's instructions. In brief, rRNA binding buffer and removal mix were added to total RNA before denaturation at 68°C for 5 minutes, in a total volume of 15µl. For the input RNA samples, 438-511ng total RNA was used, and for the pulldown RNA samples, 102-122ng total RNA was used. rRNA removal beads were then added and binding allowed to occur for 1 minute at room temperature before the supernatant was cleaned using RNAClean XP beads and washing with 70% ethanol followed by elution in 11µl Elution Buffer. This elution was further processed by the addition of an equal volume of Elute, Prime, Fragment High Mix and the mixture incubated at 94°C for 8 minutes followed by a brief centrifugation to mix any condensation with the rest of the mixture.

The whole volume of rRNA-depleted RNA samples then underwent first strand cDNA synthesis using SuperScript II Reverse Transcriptase and First Strand Synthesis Act D Mix in a total volume of 25µl followed by incubation at 25°C for 10 minutes, 42°C for 15 minutes and 70°C for 15 minutes.

Second strand cDNA synthesis was then performed by adding 20µl Second Strand Marking Master Mix to the first strand cDNA synthesis product and incubation at 16°C for 1 hour. The sample was then cleaned using 90µl AMPure XP beads and 80% ethanol followed by resuspension in 17.5µl Resuspension Buffer.

Following cDNA synthesis, adenylation of the 3' ends was performed in order to facilitate ligation to the unique sequence adapters using 12.5µl A-Tailing Mix in a total volume of 30µl followed by incubation at 37°C for 30 minutes and 70°C for 5 minutes. This was followed by ligation of the indexing adapters unique to each sample using 2.5µl RNA Adapter Indexes and 2.5µl Ligation Mix, followed by incubation at 30°C for 10 minutes. 5µl Stop Ligation Buffer was added to inactivate the ligation, followed by two washes using 80% ethanol and AMPure XP Beads. Samples were eluted in 20µl Resuspension Buffer.

Samples then underwent PCR amplification specific for those DNA fragments with adapter molecules on both ends using the PCR Primer Cocktail and PCR

Master Mix in a total volume of 50µl and incubation at 98°C for 30s and 15 cycles of 98°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds followed by incubation at 72°C for 5 minutes. Samples were cleaned using 50µl AMPure XP Beads and 80% ethanol and eluted in 30µl Resuspension Buffer.

Samples were quantified using the Quanti-iT™ High Sensitivity dsDNA Assay Kit (Q33120; Life Technologies; Carlsbad, USA) and Qubit® 2.0 Fluorometer (Q32866; Life Technologies; Carlsbad, USA). To assess the presence of only library prepared fragments and no primer dimers in the final samples, the quality of the samples was assessed using the Agilent 2200 TapeStation using the High Sensitivity D1000 ScreenTape (Agilent; Santa Clara, USA). Samples were sequenced combined in a single lane of paired end sequencing using the Illumina HiSeq 3000 sequencing platform (San Diego, USA).

2.19 Analysis of RNA-Seq data

All analyses described here were performed by Dr James Poulter (a colleague at the University of Leeds). FASTQ files were aligned to the human genome using a FASTA reference file using the Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al., 2013). This was performed using the two pass alignment method that has been shown to permit higher sensitivity of novel splice junction detection than the single pass alignment method (Veeneman et al., 2016). Header and Read Groups were then manually added to the SAM files using Picard Tools (Broad Institute; Cambridge, USA) to label individual samples. Quality control of data was performed using FastQC (Babraham Institute; Cambridge, UK) before and after removal of adapter sequences, primers and poly-A tails using Cutadapt software (Martin, 2011). All samples passed quality control.

Differential expression of genes in samples containing targeting miRNA bait compared with samples with a non-targeting miRNA bait was performed using two different methods. The first method involved using the Model-based Analysis of ChIP-Seq (MACS2) algorithm (Zhang et al., 2008), which identifies enriched regions of sequence, detecting these regions as peaks. A p value for each peak is calculated using a dynamic Poisson distribution that takes into consideration read background levels. This software was designed to analyse

ChIP-Seq data. My RNA-Seq data was analysed using this software on the basis that the experiment was performed using a pulldown method and included samples from both input and pulldown samples. As this software identifies peaks of read counts above the background level, another factor taken into consideration was the fact that there would be many more reads of genes bound to the miRNA bait than any other reads and should therefore be easily identified by the MACS2 algorithm as peaks.

The second method used DESeq (EMBL; Heidelberg, Germany), an R package that can be used to test for differential expression of genes by use of the negative binomial distribution (see Appendix B for commands used). This also involved the use of featureCounts to annotate the reads with the corresponding gene identification using hg38 as a reference, to ensure only read pairs were counted as paired end sequencing was performed, and to ensure chimeric fragments (fragments with two reads mapped to different chromosomes) were not counted. Differential expression of genes was calculated after read counts were normalised to the total number of reads in each sample i.e. pseudocounts for each test (miR-26b targets compared with non-targeting miRNA targets, and miR-195 targets compared with non-targeting miRNA targets). Differential expression of genes was calculated between input samples and between pulldown samples.

2.20 Staining for REEP4 and SEMA6D in breast cancer tissue samples by immunohistochemistry (IHC)

Immunohistochemistry was performed on tissue microarrays (TMAs) containing breast cancer tissue samples. TMAs were constructed by Ms Stacey Jones (colleague at University of Leeds).

Immunohistochemistry was performed by the author and by Dr Filomena Esteves (colleague at University of Leeds). Sections were dewaxed and hydrated by being placed in xylene three times for 3 minutes each followed by 100% ethanol for 1 minute each and finally running tap water for 5 minutes. Antigen retrieval was performed using a 10mM citrate buffer pH6.0 and heating in a 900W microwave for 10 minutes followed by rinsing in tap water. Endogenous peroxidase was then blocked using 0.3% hydrogen peroxide

solution for 10 minutes before being rinsed in running tap water for 5 minutes. Sections were then blocked using 100µl antibody diluent (003218; Invitrogen; Carlsbad, USA) before addition of the primary antibody and incubation in a humidified chamber. See Table 2.20.1 for concentrations of primary antibody used.

Table 2.20.1: Primary antibodies used for IHC and the dilutions at which they were used

Antigen	Antibody	Dilution (Time)
REEP4	Anti-REEP4 (rabbit) (ab237689; Abcam; Cambridge, UK)	1:1000 (1 hour)
SEMA6D	Anti-SEMA6D (N-terminal) (rabbit) (ab198745; Abcam; Cambridge, UK)	1:25 (overnight at 4°C)

The following day, sections were washed twice for 5 minutes each with 1x Tris-Buffered Saline with 0.1% Tween 20 (TBST) (see Appendix A.5) followed by washing for 5 minutes with 1x Tris-Buffered Saline (TBS) before addition of the secondary antibody: SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) (8114P; Cell Signaling Technology; Danvers, USA) was added so as to cover the section and allowed to incubate in a humidified chamber for 30 minutes at room temperature. Sections were then washed as before with TBST and TBS followed by addition of 3,3'-diaminobenzidine (DAB) working solution using the SignalStain® DAB Substrate Kit (8049P; Cell Signaling Technology) and incubation at room temperature for 1-5 minutes until brown staining developed. Sections were then rinsed in running tap water for 5 minutes before counterstaining with Mayer's Haematoxylin (HST011; Solmedia; Shrewsbury, UK), washing in running tap water for 1 minute and blueing in Scott's tap water (see Appendix A.4) for 1 minute and another 1 minute wash in running tap water. Finally, sections were dehydrated once again using 3x 100% ethanol for a total of 4 minutes and 3x xylene for 1 minute each before coverslips were mounted using DPX. Slides were scanned using an Aperio Digital Pathology Scanner (Leica; Wetzlar, Germany).

2.21 Analysis of REEP4 and SEMA6D expression in patient samples

Of the 305 cases, 42 were scored for each antigen separately by myself and Dr Eldo Verghese (consultant breast histopathologist at Leeds Teaching Hospitals NHS Trust and co-supervisor for this work) from the same digital images. Staining for both antigens was largely cytoplasmic, with scoring an assessment of staining intensity. Scoring of REEP4 was divided into three groups: weak, moderate and strong, with scores of 1, 2 and 3 given respectively. Scoring of SEMA6D was divided into two groups: weak and strong, with scores of 1 and 2 given respectively.

Linear weighted kappa statistics were calculated to determine the concordance between the two scorers using VassarStats (Lowry, 1998). Correlations between replicate antigen expression scores and between antigen expression scores and clinical factors were calculated using Spearman rho correlation coefficients using the statistical software SPSS version 25 (IBM SPSS Statistics; Armonk, USA). Associations between antigen expression and disease free survival and overall survival were analysed by Kaplan-Meier survival curves and log rank tests also using SPSS. All tests were 2-tailed. The limit of significance was 0.05 for all tests.

2.22 Extraction of matched protein and RNA from cells

Cells were removed from culture plastic using trypsin as described previously (see section 2.9). Following this procedure, 4ml fresh media was added to the cell suspension. This was then divided into two 15ml falcon tubes: 1ml (for RNA extraction) and 3ml (for protein extraction) and centrifuged at 400g for 5 minutes. The pellet to be used for protein extraction was washed twice with PBS before adding 200µl RIPA buffer (see Appendix A.3) supplemented with the chelating agents 1mM EDTA (E6758; Sigma; St Louis, USA) and 0.5mM EGTA (E3889; Sigma; St Louis, USA) and 1mM of the protease inhibitor PMSF (36978; Thermo Fisher Scientific; Waltham, USA). This mixture was left on ice for 15 minutes to allow cell lysis to occur, followed by centrifugation at 14 000g for 10 minutes at 4°C. The supernatant containing the extracted protein was stored at -20°C.

The smaller pellet was washed once with PBS before being re-suspended in BL buffer (Promega; Madison, USA) supplemented with 1-Thioglycerol (TG) according to the manufacturer's instructions (Promega; Madison, USA) and stored at -80°C until RNA extraction occurred (see section 2.12).

2.23 SDS-PAGE and Western blotting

A final concentration of 1x Laemmli sample buffer (see Appendix A.2) supplemented with 0.2M dithiothetriol (DTT) (Y00122; Invitrogen; Carlsbad, USA) was added to the protein sample for a final volume of 50µl. This mixture was denatured at 105°C for 5 minutes before being transferred to ice for a further 5 minutes. The samples were then centrifuged at 8000g for 30 seconds followed by re-suspension and storing on ice until ready to load on the gel.

Samples were loaded onto 4-12% Bis-Tris Gels (NP0321BOX; Novex by Life Technologies; Carlsbad, USA). A PageRuler Plus Prestained Protein Ladder (26619; Thermo Fisher Scientific; Waltham, USA) was used to confirm the correct molecular weights of identified proteins at a later stage. Samples were separated using the XCell SureLock Mini-cell electrophoresis system (Invitrogen; Carlsbad, USA) in 1x NuPage MOPS SDS Running Buffer (NP0001; Invitrogen; Carlsbad, USA). Gels were run at 180V for 60 minutes.

Proteins were transferred onto 0.45µm Polyvinylidene fluoride (PVDF) membranes (88518; Thermo Fisher Scientific; Waltham, USA) using a wet method. PVDF membranes were activated by soaking in methanol for 30 seconds followed by rinsing with running water for 5 minutes. A sandwich was formed using sponges and filter paper (88600; Thermo Fisher Scientific; Waltham, USA) to flank the gel and the membrane; all soaked in 1x NuPage Transfer Buffer (NP0006-1; Invitrogen; Carlsbad, USA). The sandwich was placed in an XCell II Blot Module (359577-063; Invitrogen; Carlsbad, USA), which was locked into the XCell SureLock Mini-cell electrophoresis system. The blot module was filled with 1x Transfer Buffer and the outer chamber was filled with de-ionised water. Transfer occurred at 30V for 90 minutes.

Once transfer was complete, the membrane was blocked with the appropriate blocking buffer (Table 2.23.1) for 1 hour at room temperature to inhibit non-specific antibody binding. The blot was washed for 5 minutes with TBST to

remove excess milk proteins. All antibodies were diluted in 1% milk powder dissolved in TBST. Primary antibodies were added and incubated for the appropriate times (Table 2.23.1). The blots were then washed 3 times for 10 minutes each with TBST, followed by incubation with appropriate secondary antibody (Table 2.23.1) diluted in 1% milk powder for 1 hour at room temperature. The secondary antibody used was a polyclonal rabbit anti-mouse immunoglobulin conjugated with a horseradish peroxidase (HRP) molecule (P0260; Dako; Santa Clara, USA) and used at a 1:2000 dilution. A further 3 washes with TBST for 10 minutes each ensured removal of unbound secondary antibody. Blots were developed using the SuperSignal West Pico Trial kit (34079; Thermo Fisher Scientific; Waltham, USA) and imaged using the Bio-Rad Gel Doc Imaging system together with the Image Lab software (version 5.2.1).

If blots were to be re-probed with a different antibody, blots were re-blocked in either 5% or 1% milk powder (Table 2.23.1).

Table 2.23.1: Primary antibodies and the conditions in which they were used for Western blots

Antigen	**Antibody**	**Dilution (incubation conditions)**	**Blocking buffer**
β-actin	Anti-β-actin (mouse monoclonal) (A5441; Sigma-Aldrich)	1:10 000 (1 hour, room temperature)	5% milk powder in TBST
BCRP	BXP-21 (mouse monoclonal) (ab3380; Abcam)	1:250 (overnight, 4°C)	1% milk powder in TBST

2.24 Cell cycle profiles analysis

Cells were harvested using trypsin (see section 2.9) so as to detach cells from the flask followed by addition of media to inactivate trypsin. Cells were then counted using a haemocytometer and 500000 cells were washed in PBS before being resuspended in 1ml ice-cold 70% ethanol in PBS. Cells were fixed on ice for 1-2 hours before being transferred to polystyrene round-bottom tubes (352052; Corning life sciences; Corning, USA). Pellets were formed by

centrifugation at 500g for 3 minutes at 4ºC followed by washing with 500μl ice-cold PBS and finally resuspension in 470μl ice-cold PBS. Propidium iodide (PI) was added at a final concentration of 0.02mg/ml (P4864; Sigma; St Louis, USA) and RNase A (EN0531; Thermo Fisher Scientific; Waltham, USA) at a final concentration of 0.4mg/ml. Samples were incubated at room temperature in the dark for 20 minutes before being placed on ice. Samples were analysed, with 10000 events counted for each sample using the Attune® Acoustic Focusing Cytometer (Applied Biosystems; Foster City, USA) and accompanying Attune Cytometric Software Version 2.1. The gating of cells using forward, side and PI stain scatter parameters is shown in Figure 2.24.1A. Data was analysed using the ModFit LT software (Verity Software House; Topsham, USA) with a representative histogram shown in Figure 2.24.1B.

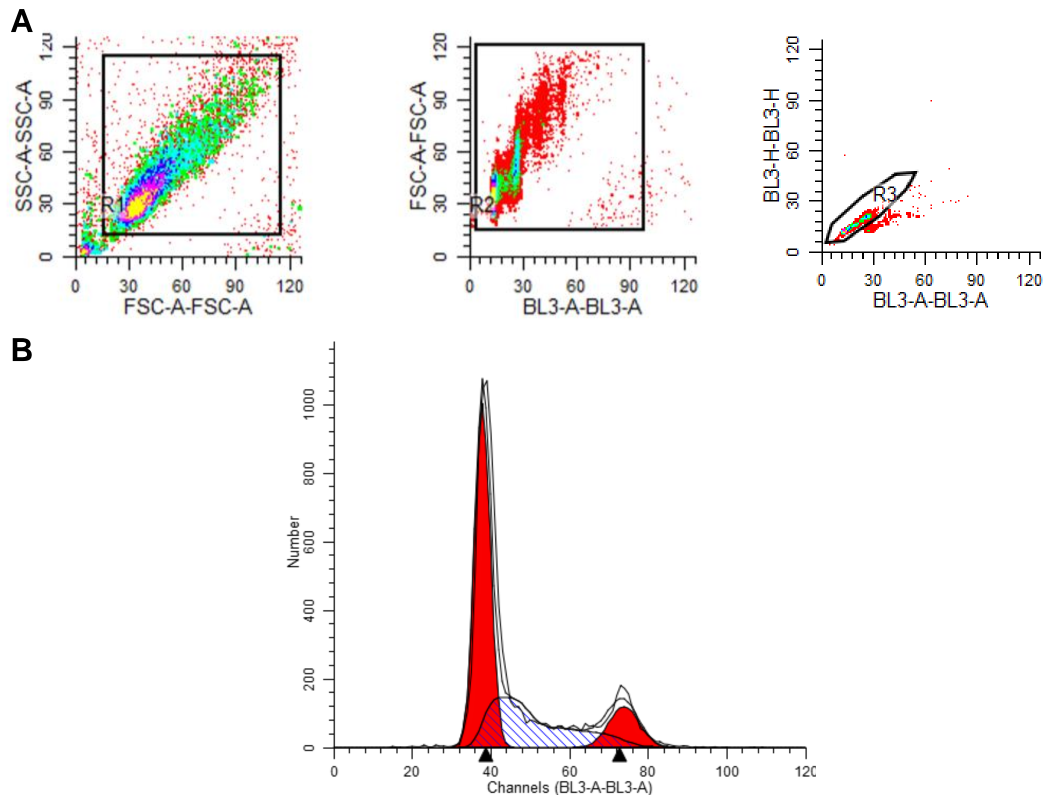


Figure 2.24.1: Representative density plots with gatings used to identify cell populations of interest

(A) The left panel shows the forward and side scatter plots with the black square showing the gate used to identify live cells. The middle panel shows the propidium iodide (PI) (BL3-A) and forward scatter plots with the black square showing the gate used to identify nuclei successfully stained with PI. The right panel shows the PI area (BL3-A) and height (BL3-H) scatter plots with the black outline showing the gate used to identify single cells. (B) Histogram of PI stained cells selected by gates above.

Chapter 3: Consistent changes in microRNA and mRNA expression are apparent post-neoadjuvant chemotherapy in breast cancer

3.1 Abstract

Resistance to chemotherapy in tumours is a common problem encountered in cancer patients. The aim in this chapter was to identify potential mediators of chemoresponse using tumour samples from patients with primary breast cancer taken before and after neoadjuvant chemotherapy (NAC) treatment. My hypothesis was that in patients showing only a partial response to the treatment, expression changes between these matched samples may be associated with chemoresistance.

Formalin-fixed paraffin embedded (FFPE) tissues comprising matched core biopsy (pre-NAC) and resection (post-NAC) tumour samples were selected from five primary breast cancer patients. These patients all had tumours that expressed the oestrogen receptor and displayed only a partial response to the administered chemotherapy regimen consisting of epirubicin and cyclophosphamide (EC). Tumour epithelial cells were isolated from these samples using laser capture microdissection (LCM) and RNA was extracted. Expression of miRNAs and mRNAs was profiled using low-density qPCR arrays and NanoString mRNA quantification respectively; fold changes post-NAC were determined. All miRNAs and mRNAs that were consistently deregulated post-NAC across all five patients were identified. For miRNAs, thresholds were set for fold changes of interest, allowing identification of miR-26b (mean fold change post-NAC of 4.9), miR-195 (2.6) and miR-10a (-4.15) for further analyses. For mRNAs, pathway analysis using the whole dataset demonstrated up-regulation of the MAPK and PI3K-AKT pathways, partial up-regulation of the JAK-STAT pathway and partial down-regulation of the TGF-Beta pathway post-NAC. I concluded that these miRNA and mRNA expression analyses identified several potential mediators of chemoresistance for further testing in other assays.

3.2 Introduction

Whether patients with primary breast cancer are administered chemotherapy, endocrine therapy or both depends in part on the molecular characteristics of their tumours (see section 1.2.2). Patients with hormone receptor positive breast cancers are only sometimes administered chemotherapy, since endocrine therapies are often sufficient in combatting the tumour and are generally less toxic (Semiglazov et al., 2007). However, luminal B-type tumours are generally treated with chemotherapy and chemotherapy is also usually recommended for those patients with larger or node-positive tumours (Gnant et al., 2015, Ignatiadis and Sotiriou, 2013). In some cases, tumour cells are intrinsically resistant (*de novo* resistance), or alternatively cells can develop resistance during the course of the treatment (Li et al., 2008). It is estimated that of all patients diagnosed with primary breast cancer, 20-50% of these will eventually suffer a recurrence and develop metastatic disease (Brufsky, 2017). In the context of metastatic disease, chemoresistance is thought to contribute to over 90% of cases of treatment failure (Coley, 2009).

Chemoresistance in breast cancer has been associated with various changes in gene expression (see section 1.4), such as increased expression of xenobiotic export pumps (Wilson et al., 2006). More recently, changes in microRNA (miRNA) expression have also been associated with chemoresistance (Kutanzi et al., 2011, Wang et al., 2015). It has been observed that overall, miRNAs are down-regulated in cancers as compared to normal cells (Lu et al., 2005). However, individual miRNA levels have now been shown to increase in breast cancer, leading to resistance. The gene *PTEN* is known to have a tumour suppressive function. Evidence has shown that inhibition of this gene’s function leads to resistance to chemotherapy in breast cancer (Steelman et al., 2008). Several miRNAs have now been shown to contribute to this inhibition; with increased miR-21 expression in doxorubicin-resistant MCF7 cells compared with parental cells concurrent with reduced expression of *PTEN* (Wang et al., 2011). Similar cases of increased miR-19 (Liang et al., 2011) and miR-130b (Miao et al., 2017) expression in chemoresistant cells and/or patient samples, together with a decrease in *PTEN* expression have also been published.

Treatment with chemotherapy leads to numerous responses in breast cancer cells such as changes in gene expression (Klintman et al., 2016) that can lead either directly to cell death or to the activation or inhibition of specific molecular pathways that function to increase the probability of cell survival.

The administration of neoadjuvant chemotherapy provides matched tumour samples before and after chemotherapy. Analysing gene expression of such samples taken from patients who responded poorly to the treatment (indicative of relative chemoresistance) could lead to the identification of changes in gene expression that are relevant to the resistance phenotype, and therefore the identification of novel drug targets to use to attempt to overcome the resistance. This approach has been used previously. One example of such an approach is a study in which biopsy samples were taken from gastric cancer patients prior to treatment with cisplatin and fluorouracil. Of these, 22 patients developed resistance to the treatment and a second biopsy was taken. Both pre- and post-treatment samples were analysed using a microarray and compared. The acquired resistance signature identified consisted of 633 genes that function in multiple pathways known to contribute to chemoresistance when deregulated, including DNA repair pathways, drug metabolism and embryonic stem cell signatures (Kim et al., 2011). A similar approach was taken in this chapter where the transcriptomes of cancer cells were analysed from five breast cancer patients both before and after chemotherapy. As these patients had all displayed only a partial response to the chemotherapy, the aim was to identify molecular pathways that were altered post-chemotherapy that could contribute to chemoresistance.

3.2.1 Specific objectives

There were three key objectives in the work described in this chapter:

1. To determine whether FFPE samples of cancer tissue pre-chemotherapy (core biopsy samples) and post-chemotherapy (resection samples) could be used successfully to extract RNA of sufficient quality to perform miRNA and mRNA analyses.
2. To determine miRNA and mRNA expression profiles of a group of breast tumours that were partially resistant to the administered neoadjuvant chemotherapy before and after therapy.

3. To determine a prioritised list of miRNA and mRNA expression changes of interest associated with chemotherapy treatment to direct future studies on these molecules as potential mediators of chemoresponse.

3.3 Results

3.3.1 RNA of sufficient quantity and quality for downstream analyses can be extracted from small clinical (FFPE) cancer samples

Fixing tissue in formalin results in multiple modifications of several key components of cells that are also of interest in research studies. This includes RNA, which is susceptible to degradation even in the absence of formalin (Auer et al., 2003, Schroeder et al., 2006) and suffers further damage when fixed (Li et al., 2007, Masuda et al., 1999). As the purpose of this study was to identify changes in miRNA and mRNA expression that occur during chemotherapy, a preliminary optimisation study was performed to investigate whether the quantity and quality of RNA extracted from FFPE tissue samples would be potentially sufficient for the planned down-stream analysis methods.

Small, representative, clinical FFPE samples were used for this preliminary study – namely two biopsy samples and two resection samples from colon cancers (at this optimisation stage, the specific cancer type was not felt to be important. However, the aim was to use samples of similar overall size, cell type (carcinoma) and clinical fixation procedures). These were sectioned, stained with haematoxylin and eosin (H&E) and total RNA was extracted from the whole tissue sections. Biopsy samples were of a comparable size to those of the breast cancer core biopsies to be used for later analyses whereas, these resection samples were on average slightly smaller than the breast cancer resection samples, thereby representing a more stringent test. In order to assess the impact of using different numbers of sections for extraction on total RNA recovery, different numbers of sections were used: three (Biopsy 1 and Resection 1) or four (Biopsy 2 and Resection 2) whole sections for each sample were used for the final RNA extraction. The RNA quantity and quality were analysed using the Agilent TapeStation automated analysis machine

(Figure 3.3.1 and Appendix C). The total RNA yields obtained are shown in Table 3.3.1. Total RNA yield was consistently greater when four sections of tissues were used as opposed to three tissue sections. By contrast, the larger tissue areas of the resections did not consistently yield more RNA than the smaller biopsies (compare Resection 2 and Biopsy 2). The two platforms chosen to be used for subsequent miRNA and mRNA expression analyses on breast tissues require an absolute minimum of 60ng total RNA combined, although a minimum of 150ng is preferred. Of the four optimisation samples tested, one biopsy sample and one resection sample had RNA yields that were too low (38.96ng and 59.92ng respectively) but the other two samples had RNA yields large enough for further analyses.

Another metric of interest was the RIN^e value, quantified on a scale of 1 for complete degradation to 10 for no degradation (Table 3.3.1) (Padmanaban et al., 2012). The RIN^e values obtained from these samples are low compared with values obtained from RNA extracted from fresh samples, but were similar to those in the literature for other FFPE tissues (Ribeiro-Silva et al., 2007).

It has been debated that no minimum RIN value is required for miRNA expression analysis using RT-qPCR as the assays are inherently robust due to the short lengths of miRNAs themselves (Dijkstra et al., 2012). Expression analysis of mRNAs extracted from FFPE tissues with low RIN values comparable with those obtained here has previously been successful using NanoString technologies (Chen et al., 2016a). It was therefore concluded that RNA of sufficient quality as well as quantity could be extracted from FFPE tissues with the chosen extraction method.

Table 3.3.1: Concentration and quality of RNA extracted from four FFPE tumour samples

Tissue sample	RNA yield (ng)	RIN^e
Biopsy 1	39.0	1.7
Biopsy 2	196.0	2.1
Resection 1	59.9	2.1
Resection 2	113.6	2.3

Total RNA was extracted from either three (Biopsy 1 and Resection 1) or four (Biopsy 2 and Resection 2) whole FFPE tissue sections stained with H&E. The RNA was then analysed using the Agilent TapeStation automated analysis machine to determine yield and RNA quality (RIN^e).

The sizes of the RNA fragments was also a metric of interest as this measure of quality might determine which downstream analysis technologies would be likely to succeed. RNA fragments obtained from these optimisation samples were largely over 200 nucleotides long in all samples. A representative graph showing the range of fragment lengths is shown in Figure 3.3.1. These fragment sizes are much larger than is required for analysis of miRNA expression (miRNAs are themselves only 18-22 nucleotides long) and were potentially suitable for analysis of mRNA expression using NanoString technology, for which RNA fragments of only 100 nucleotides long are required and have previously been shown to give data from FFPE tissue representative of those from matched fresh frozen samples (Chen et al., 2016a, Reis et al., 2011).

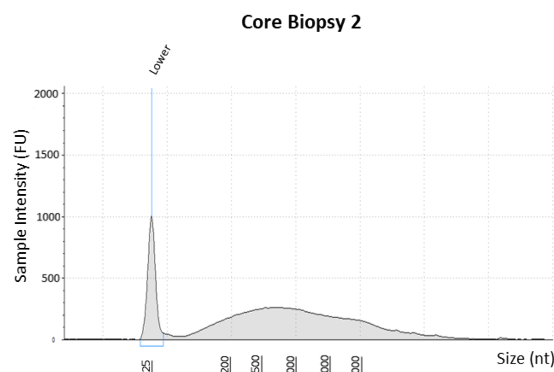


Figure 3.3.1: Representative graph showing range of nucleotide fragment lengths of RNA extracted from test FFPE samples

RNA was extracted from four FFPE tumour samples; two from biopsy samples and two from resection samples. The RNA was run on the Agilent 2200 TapeStation using the High Sensitivity RNA ScreenTape to determine average lengths of RNA fragments extracted from tissues stored as FFPE samples. This graph shows results from the Biopsy 2 sample, with the size of the fragments shown on the x-axis and the quantity of fragments shown on the y-axis. The large peak at 25nt is a marker used for reference.

3.3.2 Total RNA was successfully extracted from isolated cancer epithelial cells of five pairs of matched pre-NAC and post-NAC primary breast tumour samples

Breast cancer patients who could potentially be included in the study were identified. Patients were included if they were diagnosed with primary breast cancer, invasive ductal carcinoma of no special type, of the clinically-defined luminal A subtype (tumours expressing ER but not HER2). These criteria were

chosen in order to reduce inherent heterogeneity within the cohort associated with different tumour subtypes. A second reason for these criteria was that tumours of this subtype typically respond relatively poorly to NAC compared with other subtypes such as luminal B (Ignatiadis and Sotiriou, 2013), and very rarely achieve pCR (Colleoni and Montagna, 2012). This maximised the chances of having sufficient cancer cells post-treatment to allow analysis.

Within the above subgroup of patients, the search was further limited to those patients who displayed only a partial response to the administered NAC. An incomplete response to the chemotherapy would suggest that the tumour is partially resistant to the therapy. As one of the main objectives of this study was to determine the molecular changes that are associated with chemoresistance, this limitation was necessary.

Finally, the search was also limited to patients treated only with the epirubicin and cyclophosphamide (EC) regimen. This additional filter was performed in order to reduce further the heterogeneity within the results obtained as different drugs would potentially activate different pathways of resistance and may therefore make identifying potential therapeutic targets more difficult if certain pathways were affected in some patients treated with one therapy but were not affected in other patients treated with a different therapy. The EC regimen was chosen as that was the most common initial regimen used in this patient group at St James's University Hospital, Leeds at the time of sample retrieval.

Limiting the search to only patients who displayed a partial response and were treated with EC significantly reduced the number of eligible patients. This was because patients who display no response or poor response to the therapy are usually switched to a different chemotherapy regimen (typically taxanes). The final limitation was the availability of sufficient tissue for both pre-NAC and post-NAC samples.

Once patients had been identified, tumour histology was examined, using either diagnostic archival H&E slides or new H&E slides produced for the purpose, in order to assess whether the tissue itself was suitable for the planned analysis. This was important in terms of the presence of sufficient tumour epithelial cells (the cells of interest) for downstream analyses as opposed to large amounts of unwanted elements such as lymphocytes and other stromal cells. Combining all

of the above criteria, five patients and their tumours were determined to be appropriate. Table 3.3.2 shows the clinico-pathological features of the patients whose tumours were used in this study. One of the patients showed a very good response to NAC during treatment, however the tumour resumed growing once treatment had ended.

Table 3.3.2: Clinico-pathology features of patients/tumours that were used in this study

<table>
<tr><th>Characteristic</th><th>Categories</th><th>Number</th></tr>
<tr><td>Mean Age (Range)</td><td></td><td>51 (41-64)</td></tr>
<tr><td rowspan="2">Positive receptor status (pre-NAC)</td><td>ER</td><td>5</td></tr>
<tr><td>HER2</td><td>0</td></tr>
<tr><td rowspan="3">Grade (pre-NAC)</td><td>1</td><td>1</td></tr>
<tr><td>2</td><td>2</td></tr>
<tr><td>3</td><td>2</td></tr>
<tr><td rowspan="2">Response to NAC (as assessed at surgery)</td><td>Very good</td><td>1</td></tr>
<tr><td>Very limited</td><td>4</td></tr>
</table>

As the purpose of this study was to determine molecular changes that occur in the cancer cells specifically, isolation of these tumour epithelial cells was required. This was achieved by sectioning the tissue, staining with haematoxylin and eosin (H&E) in order to distinguish between tumour cells and the surrounding stroma, and then isolating the epithelial cells using laser capture microdissection (LCM). It was felt that this step was particularly necessary since NAC treatment is known to reduce cancer cellularity within the tumour mass (Sahoo and Lester, 2009, Masood, 2016), therefore analyses comparing pre-NAC and post-NAC samples without purifying the tumour cells risk identifying expression changes simply associated with differences in the tumour-stroma ratio, rather than true expression differences in the tumour cells present. LCM protocols, with representative images, are described in detail in section 2.3. These protocols were optimised in order to yield the maximum amount of RNA possible, largely by isolating tumour epithelial cells available from up to twenty tissue sections. The settings on the LCM equipment were also optimised to ensure optimal collection of tissue whilst avoiding tissue charring.

Total RNA was extracted from the isolated tumour epithelial cells from pre-NAC and post-NAC samples from the five cases, and was quantified (using Nanodrop analysis; assessment of RNA amount/quality using the TapeStation automated analysis machine was not performed in order to preserve the maximum amount of RNA for downstream analyses). Total RNA amounts obtained for each pre-NAC and post-NAC sample per patient are shown in Table 3.3.3. These met the minimum required amounts for the downstream analyses: a minimum of 100ng total RNA is preferred for the low density miRNA RT-qPCR arrays and at least 50ng total RNA is required for NanoString analysis of mRNA expression; although in one sample (patient 4, pre-NAC) this was only just the case.

Table 3.3.3: Total RNA extracted from LCM-isolated tumour epithelial cells from each patient sample

Patient	Pre-NAC (ng)	Post-NAC (ng)
1	1999.4	16814.2
2	469.3	575.63
3	785.2	942.95
4	153.4	401.8
5	305.4	1944.6

A summary of the details of the patient cohort used in this study and the processes used to analyse RNA expression is shown in Figure 3.3.2.

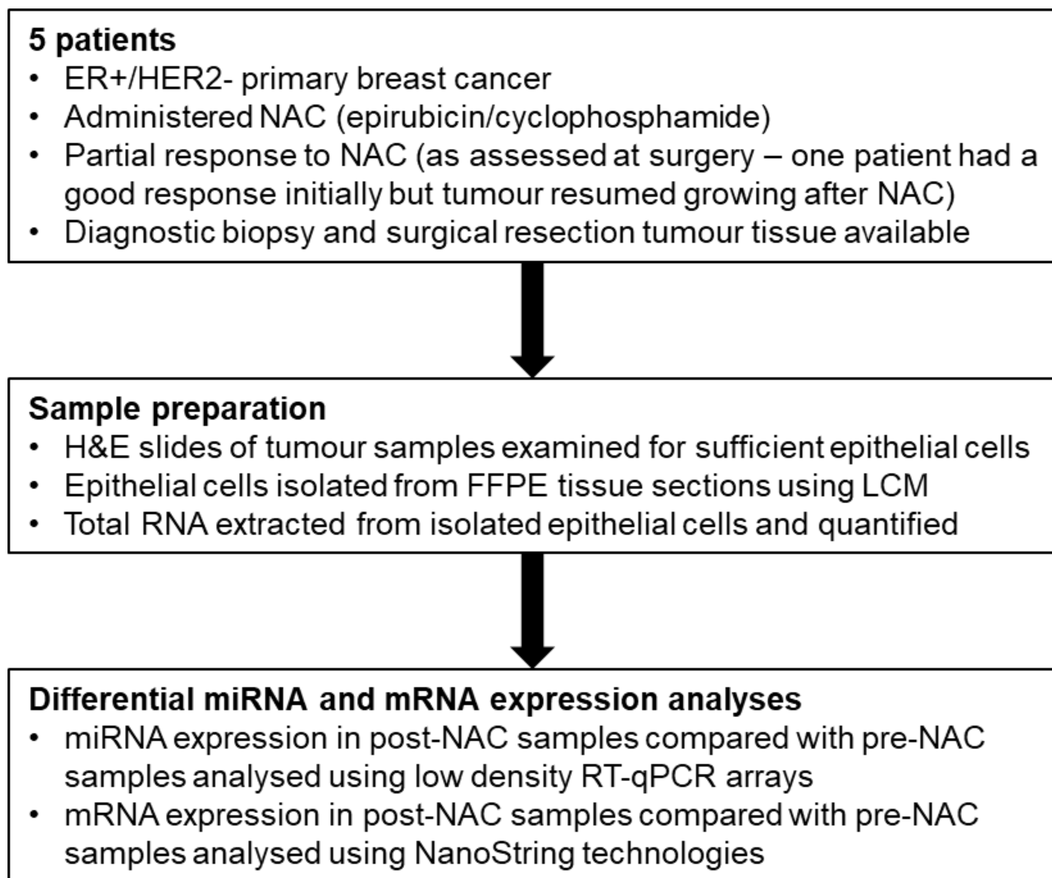


Figure 3.3.2: CONSORT diagram of samples used in this study
Five patients were identified who met all the required criteria (top box), whose samples were prepared for total RNA extraction (middle box) for miRNA and mRNA differential expression analysis between pre- and post-NAC samples (bottom box).

3.3.3 MiRNAs miR-26b, miR-195 and miR-10a were consistently deregulated post-NAC in five breast cancer samples

Expression levels of 384 RNAs, including a very large panel of commonly expressed miRNAs and several RNAs traditionally used as housekeeping RNAs when investigating miRNA expression such as U6 and RNU48, were analysed using low density RT-qPCR arrays. Data were normalised to the mean expression of all RNA species detected.

In my first analysis, I used unsupervised hierarchical clustering to investigate which miRNA expression profiles were most similar across the samples. It was possible that the predominant similarities between samples would be between matched pre-NAC and post-NAC samples, suggesting that tumour specific differences were relatively more important compared with changes induced by chemotherapy, or would be within the post-NAC group, suggesting that

chemotherapy-induced expression changes were predominant. This analysis is presented as a heat map in Figure 3.3.3. Results from this analysis showed that there is not a predominant post-NAC miRNA expression profile associated with NAC. This is demonstrated in the fact that post-NAC samples were not clustered together. This analysis also shows that there is no clear relationship within individual tumour samples as pre-NAC and post-NAC samples from the same patient do not cluster together. Indeed, the results of this analysis show a combination of these two possible clusters occurs. For example, the two samples from Patient 4 cluster together but the pre-NAC samples from Patients 1 and 5 also cluster together. This shows that differences between both tumours and treatment effects can vary largely.

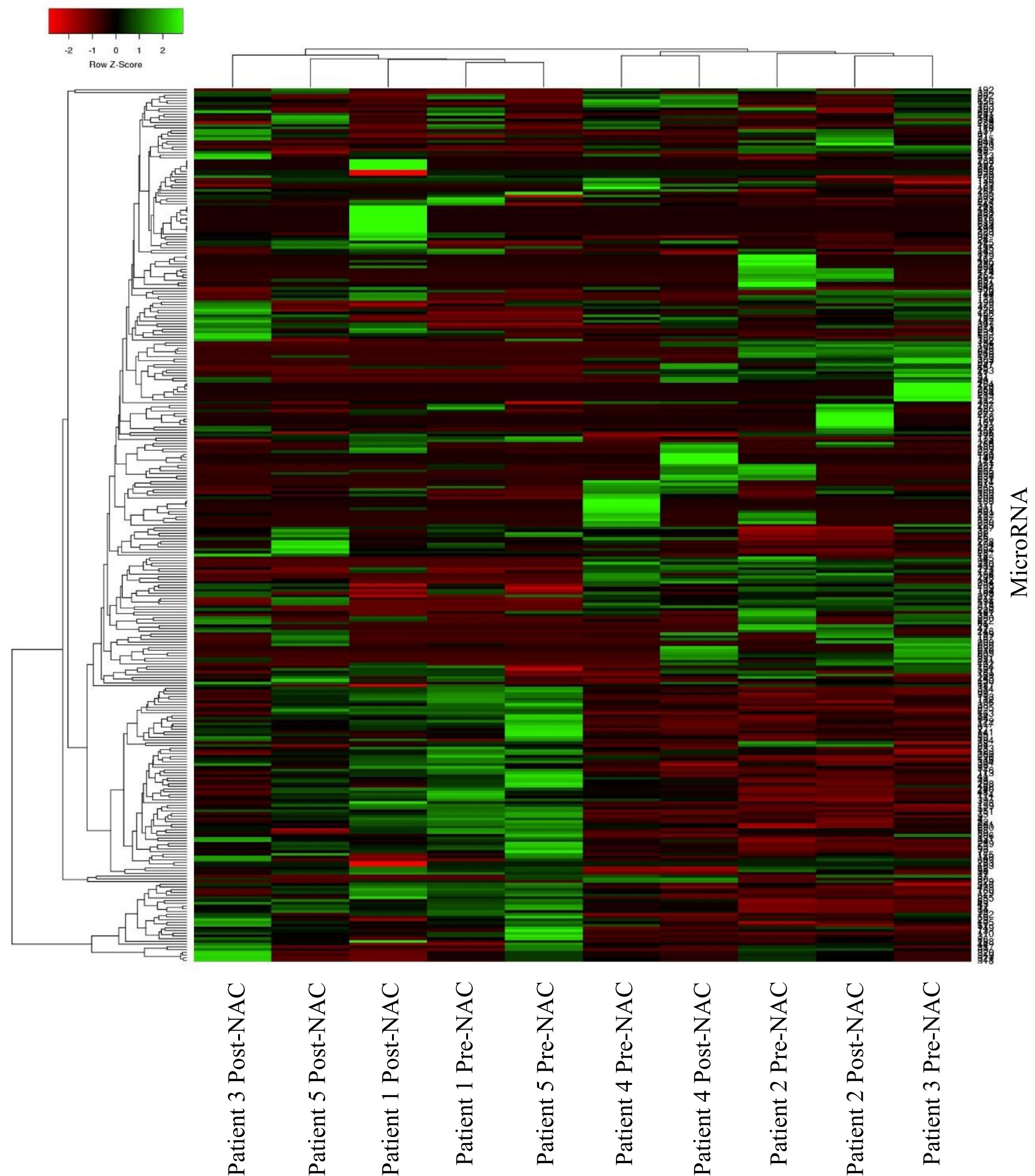


Figure 3.3.3: Unsupervised hierarchical clustering of patient samples pre-NAC and post-NAC using miRNA expression data does not show definite relationships between sample types

RNA was extracted from tumour epithelial cells from pre-NAC and post-NAC samples from five patients and expression of an array of miRNAs was determined using low-density miRNA microarrays. Results were normalised to the average Ct value of each sample and unsupervised hierarchical clustering was performed. The resultant heat map is shown, with the dendrogram showing the relationships between the ten samples at the top.

Following this initial analysis, miRNAs with altered expression between the matched pre-NAC and post-NAC samples from each individual patient were identified and miRNAs that were consistently either up-regulated or down-

regulated across all five patient samples post-chemotherapy were then selected as possible candidates for further study. Eleven miRNAs were consistently up-regulated post-NAC whereas two miRNAs were consistently down-regulated post-NAC. In order to prioritise those miRNAs for further study, a minimum fold change in each patient was used as a threshold to define the miRNAs of interest. However, previous studies have suggested that even small changes in miRNA expression can have large impacts downstream (St. Laurent et al., 2013, Calin and Croce, 2006). Therefore, conservative thresholds were set, requiring fold changes of more than 1.3 for up-regulation or less than -1.25 for down-regulation. Using these criteria, three miRNAs were selected for further analysis: miR-26b, miR-195 and miR-10a with mean fold changes of 4.9, 2.6 and -4.15 respectively. Fold changes for each individual patient are shown in Table 3.3.4. A further nine miRNAs were identified that were up-regulated in all five patients but the fold-change in one patient sample did not reach the minimum threshold or was not detected in one patient (Appendix D). One other miRNA was consistently down-regulated but did not meet the chosen criteria (Appendix D). Tests for statistical significance of deregulated miRNA expression were considered. However, the cohort was too small for these tests to be meaningful, adjustments for multiple testing were severe because of the large panel of RNAs assessed and the suitability of parametric/non-parametric tests was uncertain. Therefore, I decided to proceed with candidates based on consistency of deregulation and my (arbitrary) thresholds for size of fold changes.

Table 3.3.4: Fold changes of selected miRNAs post-chemotherapy for each patient

MicroRNA	**Patient**					
	1	**2**	**3**	**4**	**5**	**Mean**
miR-26b	3.389	1.537	1.922	1.793	15.707	4.870
miR-195	3.464	1.837	1.7	1.333	4.497	2.566
miR-10a	-2.33	-1.42	-14.39	-1.27	-1.36	-4.154

MicroRNAs that were consistently deregulated post-NAC across all five patients were identified. For up-regulated miRNAs, a minimum threshold for the fold change for each individual patient was 1.3 and for down-regulated miRNAs, a maximum threshold for the fold change for each individual patient was -1.25. The mean fold change across all patients for each miRNA is also shown.

3.3.4 Identifying a mRNA expression signature associated with chemotherapy treatment

3.3.4.1 Multiple assays failed and conclusions may not be reliable

For mRNA analyses, RNA samples were sent to the Institute of Cellular Medicine, Newcastle University where they were analysed using the nCounter PanCancer Pathways Panel (NanoString Technologies) to asses expression of 730 cancer-related genes and 40 internal reference controls. This technology is based on fluorescent counting of individual target molecules based on their attachment to barcoded probes. Raw data were normalised relative to a panel of housekeeping genes; followed by calculation of fold changes for genes post-NAC compared with pre-NAC. It was, however, noted that two samples failed quality control. These displayed either low counts for the internal positive controls within the analysis (patient 3; post-NAC), potentially indicating the presence of chaotropic contaminants, or low counts for the genes themselves (patient 3; post-NAC and patient 5; pre-NAC), potentially suggesting excessive RNA fragmentation or low sample input.

However, for both samples, expression of a few genes was successfully quantified and levels of these were taken into account in downstream analyses, although the fold changes in these two patients (3 and 5) should be treated with some caution.

In an attempt to overcome these issues, I decided to pre-amplify the pairs of samples from these two patients (Patients 3 and 5) and to repeat the NanoString analysis with this amplified input. As a positive control, the pair of samples from Patient 1 were also pre-amplified, which had previously worked well, in order to allow analysis of how non-amplified (initial - Run 1) and pre-amplified (repeat - Run 2) analyses compare. Samples underwent pre-amplification using a pool of primers specific for the genes on the nCounter PanCancer Panel, before being re-analysed by the Institute of Cellular Medicine, Newcastle University, on the same nCounter PanCancer Pathways Panel.

Initial observations of the new data from the two samples that had failed in the first run showed that even after pre-amplification, these samples failed again.

Analysis of the new data for Patient 1, which was repeated with pre-amplification as a control, also presented concerns. Only eighteen more genes were detected in the pre-amplified second analysis compared with the first analysis. This represents only 0.02% of all genes on the panel and one-hundred and eighty four genes were still undetected in both the pre-NAC and post-NAC samples after pre-amplification, representing 24% of all genes on the panel. This suggested that pre-amplification had not substantially increased sensitivity. To determine whether pre-amplification introduced any bias and whether results between the two runs were comparable, the correlation between the fold changes determined in the two runs of Patient 1's samples were first determined (Figure 3.3.4).

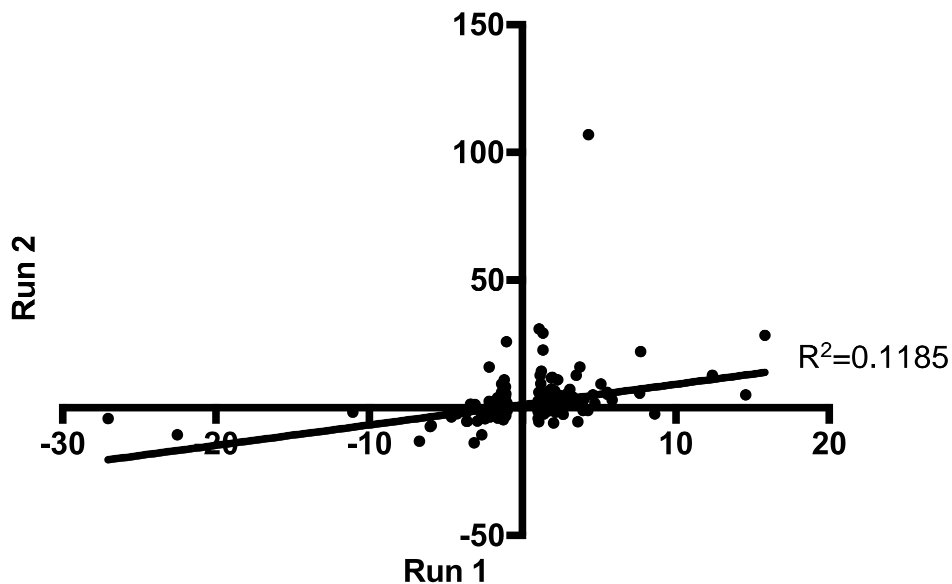


Figure 3.3.4: Fold changes obtained from pre-amplified samples (Run 2) correlate poorly with those obtained from non-amplified samples (Run 1)

Tumour epithelial cells were isolated from pre-NAC and post-NAC samples from a single patient (Patient 1) and RNA was extracted. Gene expression profiles were determined using this extracted RNA either without pre-amplification (Run 1) or with pre-amplification (Run 2) using NanoString PanCancer Pathway analyses. Fold changes in gene expression comparing post-NAC to pre-NAC in samples that underwent pre-amp (Run 2) were plotted against fold changes in the same genes as determined with RNA that did not undergo pre-amplification (Run 1). This includes the expression of all genes that were detected in all samples (398 genes). A correlation line is shown along with the R-squared value.

The correlation between the gene expression fold changes obtained in the two separate runs for samples from Patient 1 was low: the correlation coefficient

was only 0.1185. This suggests that results obtained from samples that underwent pre-amplification were not similar to those obtained from samples that did not undergo pre-amplification. However, it should be noted that any genes that were not detected in one or more samples were not included as values for fold changes could not be calculated; therefore this correlation may under-represent the actual concordance.

3.3.4.2 Changes in gene expression post-NAC lead to alterations in signalling cascades

Since results obtained after pre-amplification were not similar to those obtained before pre-amplification and the pre-amplified samples were considered to be most prone to be unrepresentative due to amplification biases, further analyses were limited to the results obtained from samples that did not undergo pre-amplification. Of the samples that successfully passed quality control (eight samples in total; three matched pairs and individual samples from Patients 3 and 5), a mean of 547 genes (of the 730 targets) were detected. Of the samples that did not pass quality control, 62 genes were successfully detected in the Patient 3 post-NAC sample, and 261 genes were successfully detected in the Patient 5 pre-NAC sample. Once data were normalised and fold changes calculated, genes consistently changed across all five patients (Table 3.3.5) or across only the patients for whom the assays worked more reliably (Patients 1, 2 and 4; 209 genes in total) were noted.

Table 3.3.5: Genes consistently changed post-NAC across all five patient samples

Gene	Expression change post-NAC	Mean fold change
KITLG	Up	1.654
NR4A1	Up	11.380
WEE1	Up	1.802
COL5A1	Down	-1.471
EFNA3	Down	-3.816
MYB	Down	-2.066
NOTCH3	Down	-2.028
PPP3R1	Down	-1.771
PRKDC	Down	-1.836
RHOA	Down	-2.095

Tumour epithelial cells were isolated from pre-NAC and post-NAC samples from five patients and RNA extracted. Gene expression analyses were performed using the NanoString PanCancer Pathway Panel. Expression of genes that were successfully quantified in all five patients both pre-NAC and post-NAC were identified and those consistently changed were noted. Mean fold changes for each of these genes were calculated.

Analysis was then performed to determine whether consistently deregulated genes were clustered in specific molecular pathways. This analysis was limited to the 209 genes found to be consistently deregulated in the three patients for whom the NanoString analyses were most reliable. Genes were grouped into their functional pathways, as defined by the classifications associated with the NanoString PanCancer Pathways Panel, and the directions of fold changes were assessed for each individual gene to determine whether it represented up-regulation or down-regulation of the pathway overall. Pathways where most consistently deregulated genes identified were representative of the same overall change in pathway activity were noted.

3.3.4.2.1 MAPK pathways are activated post-NAC

Of the genes on the NanoString PanCancer Pathways Panel that are part of the MAPK signalling pathway (157 genes in total), forty-six genes were consistently deregulated post-NAC. Taking into consideration the function of these genes (see Figure 3.3.5) and whether they were up-regulated or down-regulated post-NAC, the global changes in gene expression suggested that this pathway was activated post-NAC. Of the forty-six consistently deregulated genes, thirty-four were changed in such a way as to contribute to this

activation. In order to make an assessment of whether this expression pattern showed characteristics of statistically significant activation, a binomial probability test was performed. The result was a p value of 0.00055, suggesting that this distribution is not likely to have occurred by chance. Genes of note included several of the MAPK genes themselves as well as several downstream targets such as *FOS* and *JUN*.

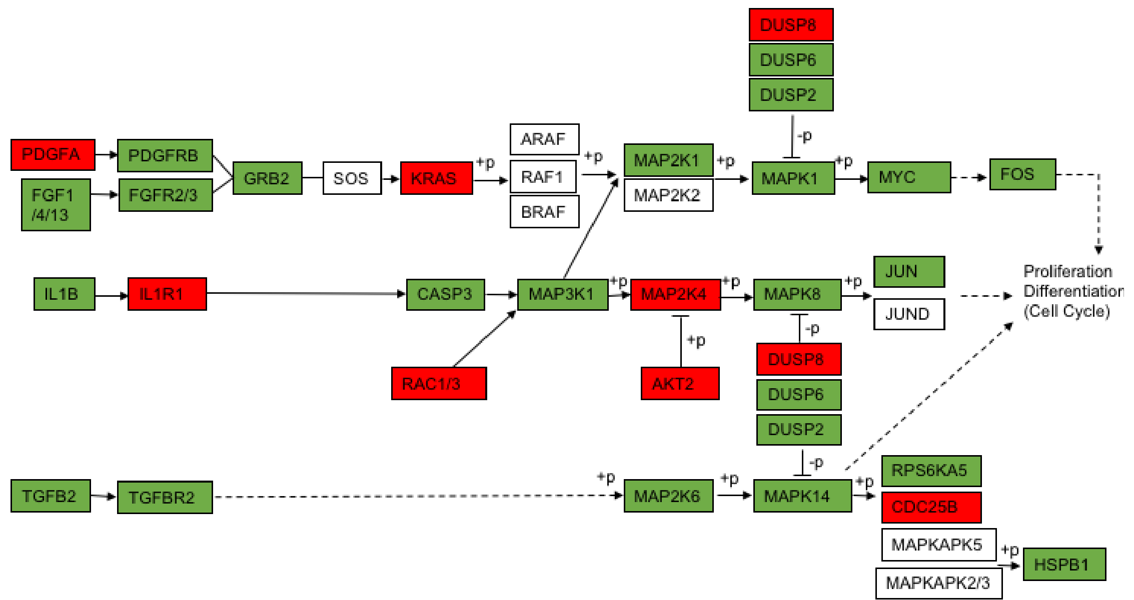


Figure 3.3.5: The MAPK pathway is activated post-NAC

Diagram showing the major elements that function in the MAPK pathway. Genes consistently up-regulated post-NAC are shown in green whereas genes consistently down-regulated post-NAC are shown in red. Genes shown in white are either not consistently changed or are not present on the NanoString PanCancer Pathways Panel. Overall, changes in gene expression suggest increased activation of this pathway post-NAC (p=0.00055).

3.3.4.2.2 The PI3K-AKT pathway is activated post-NAC

Of the genes on the NanoString PanCancer Pathways Panel that are part of the PI3K-AKT pathway (201 genes in total), fifty-three genes were consistently deregulated post-NAC. As this pathway affects many processes downstream of AKT including several other pathways, for the purposes of determining whether this pathway was significantly altered post-NAC, only genes upstream of and including AKT were included in this analysis. Of these genes (201 in total), forty-two were consistently de-regulated post-NAC. Taking into consideration the function of these genes (see Figure 3.3.6) and whether they were up-

regulated or down-regulated post-NAC, the global changes in gene expression suggested that this pathway was activated post-NAC. Twenty-nine consistently deregulated genes were changed in such a way as to contribute to this activation. As with the analysis of the MAPK pathway, a binomial probability test was performed to determine whether this expression pattern showed characteristics of statistically significant activation. The result was a p value of 0.0058, suggesting that this distribution is not likely to have occurred by chance. Genes of note included several extracellular matrix components such as *COL1A2*, integrin receptor components such as *ITGA2* and *ITGB4* as well as *PIK3CG*.

Of the downstream effectors, several proteins involved in regulating processes such as the cell cycle and apoptosis were either up-regulated or down-regulated, resulting in certain arms of the PI3K-AKT pathway being activated and others being suppressed.

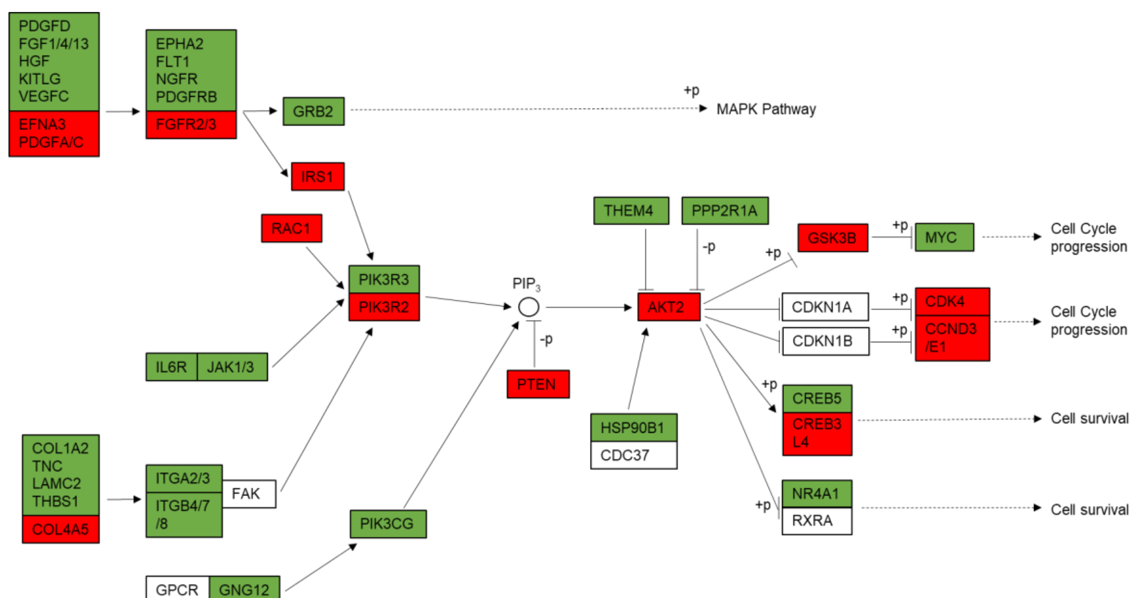


Figure 3.3.6: The PI3K-AKT pathway is activated post-NAC

Diagram showing the major elements that function in the PI3K-AKT pathway. Genes consistently up-regulated post-NAC are shown in green whereas genes consistently down-regulated post-NAC are shown in red. Genes shown in white are either not consistently changed or are not present on the NanoString PanCancer Pathways Panel. Overall, changes in gene expression of genes upstream of and including AKT suggest an increase in activation of this pathway post-NAC (p=0.0.0058).

3.3.4.2.3 Genes involved in regulating proliferation and metastasis are up-regulated post-NAC

Fifty-one genes that influence cell proliferation and/or metastasis processes were consistently deregulated post-NAC, from a total of 137 genes defined as contributing to this pathway overall on the analysis panel. Of these, thirty-two (62.7%) were either up-regulated or down-regulated in such a way as to contribute to increased cell proliferation and/or metastasis post-NAC. A binomial probability test was performed to determine whether this expression pattern showed characteristics of statistically significant activation of these processes. The result was a p value of 0.022, suggesting that this distribution is potentially not likely to have occurred by chance, although this significance is marginal in the context of testing multiple potentially significant pathways. Genes of note included *JAK3, MEN1, BCL2A1* and *CDKN2A*.

3.3.4.2.4 Although individual genes show consistent deregulation, a number of other pathways do not demonstrate evidence of overall significant activation or repression

Many other genes included in the NanoString PanCancer Pathway Panel can be defined as components of a number of further molecular pathways and were found to be consistently deregulated post-NAC. These include components of the RAS, JAK-STAT, WNT and TGF-Beta pathways. Assessments were made as to whether these pathways were overall potentially activated or repressed post-NAC, using the methodology as above for the MAPK, PI3K-AKT and proliferation/metastasis pathways. However, when binomial probability tests were performed as before, there was no evidence of significant overall pathway activation or repression. These observations are summarised in Table 3.3.6.

Table 3.3.6: Pathways that were not significantly altered post-NAC

Pathway	Activation / Repression post-NAC	No. of genes consistently deregulated post-NAC	No. of genes that contribute to overall activation or repression	p value
RAS	Activation	36	22	0.055
JAK-STAT	Activation	16	11	0.067
WNT	Activation	24	14	0.117
TGF-Beta	Repression	16	9	0.175

Individual genes on the NanoString PanCancer Pathway Panel that were consistently deregulated post-NAC were noted. According to their function within pathways and whether they contributed to the overall activation or repression of these pathways, binomial probability tests were performed to determine whether these overall activations or repressions were statistically significant.

3.4 Discussion

Resistance to chemotherapy can present in patients as either *de novo* or acquired resistance (Hazlehurst and Dalton, 2006). In the neoadjuvant setting, this results in primary tumours failing to reduce in size substantially or even growing bigger. For sub-clinical metastatic cells that have already seeded into pre-metastatic niches, this chemoresistance can lead to metastatic recurrences and poor prognoses (Abdullah and Chow, 2013, Kajiyama et al., 2007). Therefore, it is important to identify the mechanisms behind this resistance in order to discover alternative molecular targets that can be used in cancer treatment.

3.4.1 Methodologies for investigating chemoresistance

3.4.1.1 The use of clinical samples versus breast cancer cell lines

A variety of approaches have been used by researchers in attempts to identify molecular mechanisms that contribute to chemoresistance. Part of this research includes the attempt to identify gene expression signature patterns associated not only with chemoresistance but also with chemoresponse. These approaches have been used to identify altered expression patterns of both mRNAs and miRNAs.

The majority of studies that have been performed to identify mRNAs and miRNAs with altered expression associated with chemoresistance in breast cancer utilised various breast cancer cell lines, usually with matched parental and drug-resistant cells. This approach is popular likely due to the ease of obtaining the required samples. It was used to identify miR-298 as a modulator of chemoresistance; with reduced expression in doxorubicin resistant breast cancer cells compared with the sensitive parental cell line. This reduced expression was associated with increased expression of the xenobiotic drug transporter P-glycoprotein, thereby conferring resistance (Bao et al., 2012). A similar approach of using drug-resistant breast cancer cells was used in the identification of miR-19 (Liang et al., 2011) and miR-21 (Wang et al., 2011), both of which have increased expression in resistant cell lines compared with parental controls and were associated with decreased *PTEN* expression. As a slight variation of this approach, creating drug-resistant cell lines by manipulating gene expression has also been performed. For example, Martz et al. produced cells expressing pre-identified mutant cDNAs (activating or inhibiting) that function in pathways known to enhance survival of cancer cells, and investigated the response to several targeted therapies (Martz et al., 2014).

There are limitations to this approach, however, as there are many other cell types and extracellular components present in tumour that may influence response to chemotherapy that are not present under *in vitro* conditions. A second approach that uses tumour samples taken from breast cancer patients would provide a more accurate conclusion in this respect. There have been a small number of studies that have taken pre-treatment breast cancer samples, analysed gene expression and correlated these expression data with responses to subsequent chemotherapy. The primary objective of one such study was to distinguish separate gene expression patterns for response and resistance to the chosen chemotherapy regimen. In this study, pre-NAC core biopsy samples were used for analysis (Cleator et al., 2006). It is important to note that core biopsy samples are not the only pre-NAC samples available. Fine needle aspirations have also been used in similar studies (Ayers et al., 2004, Gonzalez-Angulo et al., 2012), although the identification of genes associated with chemoresistance was not the purpose of these studies.

In this study, I have used samples taken from tumours of primary breast cancer. These were matched samples taken pre-NAC (core biopsy) and post-NAC (resection) from tumours that showed only a partial response to the administered chemotherapy that consisted of a regimen of epirubicin and cyclophosphamide, suggesting a degree of chemoresistance. This allowed the identification of changes in mRNA and miRNA expression post-NAC compared with pre-NAC of a large panel of genes. It is possible that these changes are associated with the observed chemoresistance. Such an approach of using matched pre-NAC and post-NAC samples has, although rarely, previously been used in an attempt to identify genes associated with chemoresistance.

In one such study, pre-treatment core biopsy samples and post-treatment resection samples (multiple therapy regimens) were used to investigate expression of multiple genes known to be involved in conferring resistance in order to observe expression patterns of these genes with respect to response or non-response to the administered chemotherapies. Interestingly, different combinations of genes had altered expression post-NAC depending on type of chemotherapy regimen given (Litviakov et al., 2013). This demonstrates the fact that different therapies result in different molecular responses. This means that if a particular change in expression is associated with resistance to a particular chemotherapeutic drug, it does not translate into the same change in expression being associated with resistance to all chemotherapeutic drugs. It is for this reason that I have used tumour samples taken from patients who were all treated with the same chemotherapy regimen so as to minimise variations due to different treatments. This study by Litviakov et al. however, used this approach to determine expression of pre-selected genes rather than for screening to identify new genes of interest (Litviakov et al., 2013).

3.4.1.2 Whole tumour, microdissection and laser capture microdissection tissue samples for analysis

Multiple approaches have been used previously when using breast cancer tissue samples to analyse gene expression in order to identify genes associated with chemoresistance. The simplest of these approaches is to use the entire tissue sample available for analysis. In some cases, no mention is made of samples being dissected in any way (Bhola et al., 2013), however, in

some instances, specific criteria were set as to what samples were eligible for analysis. One such criteria was the cellularity of tumour samples, with minimum thresholds set (Cleator et al., 2006).

A more popular method used by researchers when using clinical samples is the microdissection of highly cellular regions of available breast tumour samples. In these cases, one section was used to demarcate areas of tumour cells ranging between minimum thresholds of 20% to 50% cellularity and these demarcations were used to guide the manual microdissection of further sections using needles or razor blades (Balko et al., 2012, Magbanua et al., 2015, Klintman et al., 2016). The advantage of using samples enriched for tumour cells only is important when attempting to discover genes specifically associated with chemoresistance of tumour cells as the influence of gene expression in other cell types may confound results. This differential expression between sample types has previously been reported on, where differentially expressed genes were compared in ER positive tumour samples that had been manually microdissected with tumour epithelial samples and tumour stroma samples (Xu et al., 2015). Comparing those genes deregulated in epithelial and stroma samples, 86% of them were consistently deregulated, with the remaining 14% of genes inconsistently deregulated. Of these genes consistently deregulated in epithelial and stroma samples, 91% of genes were found to overlap when comparing with deregulated genes in the microdissected samples. However, when comparing deregulated genes in the microdissected samples with those inconsistently deregulated in the epithelial and stroma samples, the consistency score was only 52%. These results reinforce the importance of using only the cell populations of interest for analysis.

The most accurate but much more time consuming method of laser capture microdissection to isolate tumour cells has also been used previously. This is the method that I used in this study, although few other studies have used this technique in a similar context. Other than the time aspect, one possible reason for this method not being widely used is the amount of tumour sample required for sufficient material for analysis. In this study, I have used up to twenty sections of one case for laser capture microdissection in order to collect enough material for downstream analyses. In the studies where manual microdissection was used, authors have used three to five sections for each

case (Balko et al., 2012), four sections for each case (Magbanua et al., 2015) or one to six sections for each case (Klintman et al., 2016). In contrast, to gain enough RNA to perform microarray analysis, another group required three sections and two rounds of RNA amplification (Ma et al., 2003). If avoidance of RNA amplification is desired, many more sections are required as only a limited amount of RNA can be extracted from microdissected cells (Fuller et al., 2003).

3.4.2 Specific microRNAs are consistently dysregulated post-NAC in partially chemoresistant tumours

MicroRNAs have previously been shown to be dysregulated in breast cancer (Mulrane et al., 2013). Although dozens of dysregulated miRNAs have been identified, multiple studies investigating the same miRNA sometimes observe differences in expression and/or function, therefore only those where multiple studies have shown similar results are concluded to definitely be involved in breast cancer. These are largely subdivided into two functional groups: tumour suppressors and oncogenic miRNAs, with eighteen and sixteen major miRNAs respectively including the miR-200 family in the former group and miR-221/222 in the latter considered as being involved in breast cancer in 2015 (van Schooneveld et al., 2015, Starlard-Davenport et al., 2015), although these numbers may have expanded since then. More recently, miRNAs have also been linked with resistance to chemotherapy (Wang et al., 2015, Kutanzi et al., 2011). One such miRNA is miR-21. This miRNA was noted to be involved in the resistance of breast cancer cells to chemotherapeutic taxols, as inhibition of this miRNA led to increased sensitivity of cells to this treatment (Mei et al., 2010). Further studies with breast cancer cells have also shown that up-regulation of miR-21 also leads to resistance to doxorubicin (Wang et al., 2011). However, it has been noted that the same miRNAs may be dysregulated in different ways depending on the context such as type of cancer and treatment. An example of this is with miR-195 expression, which when up-regulated in glioblastoma multiforme cells contributes to resistance to temozolomide but increases sensitivity of breast cancer cells to adriamycin treatment (Ujifuku et al., 2010, Yang et al., 2013). It is therefore important to identify expression signatures for each cancer type and treatment regimen.

An array of 384 miRNAs was chosen and miRNAs that were consistently either up- or down-regulated post-NAC when compared with pre-NAC were identified. MicroRNAs that met the chosen requirements were selected.

3.4.2.1 MiR-26b, -195 and -10a are potential mediators of chemoresistance

Three miRNAs were selected that had large enough fold changes across all five patient samples. These were miR-26b, miR-195 and miR-10a. The first two were up-regulated post-NAC, with mean fold change expressions of 4.87 and 2.57 respectively, whereas miR-10a was down-regulated post-NAC, with a mean fold change of -4.15. Considering the fact that these patients all displayed partial resistance to the administered chemotherapy, it is possible that these consistently changed miRNAs may contribute to the chemoresistance.

Previous studies have shown that all three of these miRNAs do play roles in chemoresistance in other cancer types and in some cases in breast cancer treated with other chemotherapeutics such as platinum-based therapies (Zhao et al., 2014, Yang et al., 2013, Pogribny et al., 2010), although evidence is conflicting. Evidence in hepatocellular carcinoma suggests that miR-26b expression enhances the chemosensitivity of these cells to docetaxel, TNFα and doxorubicin, in part by targeting *TAK1* and *TAB3* and suppressing NF-κB signalling (Rui et al., 2010, Zhao et al., 2014). In contrast, evidence obtained from work with non-small cell lung carcinoma, both *in vitro* and *in vivo*, suggests that over-expression of miR-26b leads to increased resistance to the chemotherapeutic drug cisplatin (Liang et al., 2015). These apparent conflicting data support the theory that, as with other miRNAs, the function of miR-26b is dependent on context. Previous studies in breast cancer have shown that miR-26b expression is decreased compared with normal tissue and up-regulation of this miRNA contributes to suppressed cell proliferation and the promotion of apoptosis (Liu et al., 2011, Li et al., 2013, Li et al., 2014a). However, there is as yet no direct evidence linking aberrant expression of miR-26b in tumour epithelial cells and resistance to anthracyclines such as epirubicin in breast cancer.

As with miR-26b, miR-195 has previously been shown to have decreased expression in tumour tissues including breast cancer and non-small cell lung carcinoma. As such, miR-195 is generally considered to be a tumour suppressor (Li et al., 2011, Luo et al., 2014, Liu et al., 2015b). Also similarly, the role of miR-195 in chemoresistance is not fully understood. Up-regulation of this miRNA has been shown to result in increased chemosensitivity to adriamycin in breast cancer (Yang et al., 2013), doxorubicin in colon cancer (Qu et al., 2015) and 5-FU in hepatocellular carcinoma (Yang et al., 2012). However, increased expression of miR-195 has also been implicated in acquired temozolomide resistance in glioblastoma multiforme cells (Ujifuku et al., 2010) and resistance to doxorubicin in small cell lung cancer cells (Guo et al., 2010b). This again supports the theory that miRNAs have differing functions depending on context.

Finally, miR-10a has been shown to have numerous functions. An interesting function includes the ability of this miRNA to enhance the translation of ribosomal protein mRNAs (Ørom et al., 2008). This function is the opposite of the classically defined function of miRNAs where the binding of target mRNAs results in the reduced expression of these targets (Farh et al., 2005, Guo et al., 2010a, Huntzinger and Izaurralde, 2011). In cancers, miR-10a has been shown to result in increased cell growth in a subset of chronic myeloid leukaemia cells (Agirre et al., 2008). It is also down-regulated in colorectal cancer tumour tissues and cells (Eyking et al., 2016). However, decreased expression of miR-10a blocks the metastatic behaviour of pancreatic cancer (Weiss et al., 2009) and increased expression has been observed in lymph node metastases of gastric cancer (Chen et al., 2012) and in medullary thyroid carcinoma (Hudson et al., 2013). As with miR-26b and miR-195, miR-10a also seems to play various roles with regards to sensitivity to chemotherapeutic agents. Silencing of this miRNA reverses cisplatin resistance in a resistant lung cancer cell line (Sun et al., 2015) and increased expression of miR-10a was found in a cisplatin resistant ER positive breast cancer cell line (Pogribny et al., 2010). In contrast, miR-10a was down-regulated in gemcitabine resistant non-small cell lung cancer cell lines (Zhang et al., 2013b). In breast cancer, increased expression of miR-10a was associated with a longer relapse-free time in ER positive

tumours following treatment with the anti-oestrogen tamoxifen (Hoppe et al., 2013).

In view of all this conflicting evidence, miR-26b, miR-195 and miR-10a are all possible candidates for contributing to resistance to anthracycline-based therapies in primary ER positive breast cancers.

3.4.3 Multiple pathways are dysregulated post-NAC in partially resistant tumours

Several genes have previously been shown to contribute to chemoresistance in a variety of cancers. These include the MAPK and PI3K pathways as well as drug efflux pumps (Haagenson and Wu, 2010, Martz et al., 2014, Sun et al., 2012). In order to identify the pathways that contribute to chemoresistance of primary ER positive breast cancers to anthracycline-based therapies, total RNA extracted from five patients who displayed only a partial response to NAC that was used to perform a miRNA microarray was also used to determine changes in mRNA expression using NanoString technology.

3.4.3.1 Identifying candidate mRNA genes that contribute to chemoresistance using pre-NAC and post-NAC samples

As mentioned briefly in 3.4.1.1, the use of pre-NAC and post-NAC samples to identify genes associated with chemoresistance in breast cancer is not as widely used as the use of matched parental and chemoresistant cell lines, or the use of pre-NAC samples and associated correlations with subsequent clinical data. However, there are some studies that have used pre-NAC and post-NAC samples, although the primary purpose of these studies was not always to investigate genes associated with chemoresistance.

Creighton et al. examined breast cancer biopsy samples and matched post-treatment samples to determine the molecular features of surviving tumour cells (Creighton et al., 2009). Patients were treated with either endocrine therapy (letrozole) or chemotherapy (docetaxel). It had previously been noted that post-chemotherapy, breast cancer samples had an increased proportion of cells with a $CD44^{+}/CD24^{-/low}$ phenotype compared with pre-chemotherapy samples and that these cells also have an increased ability to form

mammospheres. These cells were classed as tumour-initiating cells and deemed more chemoresistant (Li et al., 2008). Subsequently, this was tested with samples post-NAET and post-NAC compared with their matched pre-treatment samples and the increased presence of such cells was confirmed post-treatment. Several mesenchymal associated genes that had previously been found to be expressed in this cell population using microarray analysis were also found to be more highly expressed; a feature usually associated with the claudin-low subset of breast cancers (Creighton et al., 2009). Claudin-low breast cancers are largely hormone-receptor negative, have a poor prognosis and of all the breast cancer subtypes most closely resemble epithelial stem cells (Prat et al., 2010).

Other studies have also used microarray profiling using pre-NAC and post-NAC samples. One such study used this method and these samples as well as samples taken during chemotherapy to analyse gene expression changes that occur during the course of chemotherapy and then related these changes with either a response to the chemotherapy (anthracycline followed by a taxane) or subsequent recurrences. Both a decreased expression of cell cycle inhibitors together with increased expression of cell proliferation genes and an increase in interferon signalling were associated with poor response and reduced recurrence free survival (Magbanua et al., 2015).

3.4.3.2 NanoString Technologies: limitations to sensitivity and reproducibility

It has been well established that changes in gene expression are associated with chemoresistance. However, investigations into these changes in patients using FFPE tissue samples are limited due to this storage method resulting in cross-linking and fragmentation of RNA as well as modifications (Masuda et al., 1999, Gnanapragasam, 2010, Kokkat et al., 2013). Unlike with microarrays where a total signal is measured for each gene, NanoString Technologies uses probes attached to a barcode unique for each gene and these are then counted individually. Sample fragment lengths must be at least 100 nucleotides for the probes (Geiss et al., 2008). As shown in Figure 3.3.1, total RNA extracted from my FFPE tissues largely met this requirement.

The technology offered by NanoString is relatively new compared with more standard technologies used in gene expression analysis, such as microarrays. The use of NanoString technology is attractive when using RNA sourced from FFPE samples as very little input of total RNA is required and it can be the case that no reverse transcription and subsequent amplification of the RNA is required, thus removing any potential biases associated with these processes (Reis et al., 2011). NanoString technologies have also been shown to be more sensitive than other comparable technologies, such as microarrays (Geiss et al., 2008).

NanoString assays have been used for a number of purposes in the last decade, although literature searches have revealed that input RNA is usually derived from tumour samples rather than cells used *in vitro*. This perhaps reflects the idea that this technology may provide more accurate results compared with microarray technology when using limited samples. A customised NanoString Panel was used to characterise a previously defined side population of cells in pancreatic tumour samples. Both tumour samples and adjacent normal tissue fresh frozen samples were used, with some samples excluded as the RNA extracted had to have a RIN value of greater than 7. This side population of cells was found to express cancer stem cell associated and prognostic genes (Van den Broeck et al., 2013). This minimum RIN value is markedly higher than the RIN values of RNA that I have used in this study. However, in the above study, RNA was extracted from fresh frozen tissue whereas I have extracted RNA from FFPE tissue.

In a study of locally advanced head and neck squamous cell carcinoma (HNSCC), FFPE samples of pre-treatment tissues were collected. Patients were treated with a combination of chemotherapy (cisplatin or mitomycin C) and radiotherapy. Of the 158 FFPE samples that matched the specific criteria, twenty samples had to be omitted due either to insufficient material or low yield of RNA. Gene expression profiles were identified using a customised NanoString Panel and together with HPV status, tumour volume and stem cell marker expression, subgroups of patients with a good prognosis were identified (Linge et al., 2016).

NanoString Technologies have also been used to analyse gene expression using breast cancer samples. Klintman et al. used pre-NAC core biopsy and

post-NAC resection samples to analyse gene expression using a customised NanoString Panel in order to identify genes with altered expression post-treatment and associate these changes with prognostic implications (Klintman et al., 2016). At first, 220 patients were identified, although this number was reduced due to a number of factors including low cellularity of the tumour. Three samples were excluded due to insufficient RNA yield where 50-100ng total RNA was used for NanoString analysis. These are similar amounts of RNA to the amounts that I have used in this study.

Gene expression analysis using NanoString Technologies of post-NAC breast cancer samples to identify genes associated with chemoresistance was performed on 98 samples. One of these samples failed quality control after being analysed using the NanoString Panel. Using data from the remaining 97 samples, this study concluded that decreased expression of *DUSP4* post-NAC was associated with chemoresistance (Balko et al., 2012).

On running the RNA extracted from the patient samples in this study, two samples failed quality control following the run, although for different reasons to that described previously (Balko et al., 2012). This was determined to be likely due to chaotropic contamination, low concentrations of RNA and highly fragmented RNA. This demonstrates that although NanoString Technologies may be more sensitive than other similar technologies such as microarrays (Geiss et al., 2008), low RNA quantity and quality are still important limiting factors when analysing gene expression using RNA extracted from FFPE tissues.

Samples from one patient that were successfully analysed using the NanoString assays without pre-amplification were pre-amplified together with the samples that failed the initial analyses in order to allow comparison of reproducibility with and without pre-amplification, and thereby determine if any bias was introduced between samples that underwent pre-amplification and those that didn’t. Fold changes of genes differentially expressed between pre-NAC samples and post-NAC samples were plotted comparing between values obtained without pre-amplification (Run 1) and those obtained after pre-amplification (Run 2). The correlation coefficient had a very low value indicating that results obtained from samples that underwent pre-amplification were largely not comparable with those obtained from samples that were not pre-

amplified. This suggests that the process of pre-amplification does introduce bias, and therefore should be avoided. This has recently been observed in a study investigating whether circulating tumour cells could be reliably detected using NanoString assays (Porras et al., 2018).

Combining all the studies mentioned as well as my own, it is clear that whilst NanoString assays may indeed be more sensitive and require less input RNA, as well as being able to analyse gene expression using RNA extracted from FFPE tissue samples (Geiss et al., 2008, Reis et al., 2011), there are still limitations of this technology.

3.4.3.3 Consistent changes in gene expression are observed post-NAC and suggest altered activity of several pathways leading to chemoresistance

Using the available complete and incomplete datasets, many genes were identified that were consistently either up- or down-regulated post-NAC across at least the three patient samples that were successfully analysed. Genes that were successfully detected in the two patient samples that largely failed analysis were also taken into account. Using the KEGG database (Kanehisa and Goto, 2000, Kanehisa et al., 2015, Kanehisa et al., 2016) and the annotations provided by NanoString Technologies that indicated which pathways each gene functions in, each pathway was manually annotated depicting whether individual genes were up-regulated or down-regulated. Each pathway was then examined to determine whether overall, the changes in gene expression would lead to activation or suppression of the pathway as a whole and statistical tests using the binomial test were performed to determine if these changes were significant. Investigating the functions of these pathways together with literature searches revealed whether the altered activities of these pathways may contribute to the partial chemoresistant phenotype observed in these patients.

MAPK and PI3K-AKT pathways were activated post-NAC and many genes contributing to alterations in proliferation and metastasis were also involved. Increased activity of the MAPK pathway, in particular the JNK and p38 arms, has previously been linked with chemoresistance (Igea and Nebreda, 2015, Leung et al., 2008, Suzuki et al., 2015). Previous studies have also indicated

that activation of the PI3K-AKT pathway has previously been linked with chemoresistance (West et al., 2002). The activation and suppression of distinct arms of this pathway downstream of AKT in these patient samples post-NAC may reflect the fact that these patients do partially respond to chemotherapy but also display some resistance. The activation of these two pathways and the changes in gene expression of 25% of genes that are altered in such a way as to contribute to increased cell proliferation and/or metastasis indicate that these pathways contribute to the chemoresistance observed in these patients.

As with the PI3K-AKT pathway, elements of the RAS pathway such as genes involved in cell motility and cell survival are down-regulated, reflecting the fact that these tumours showed a partial response to chemotherapy. Other elements of this pathway such as genes involved in cytoskeletal remodelling and activating components of the MAPK pathway are up-regulated, reflecting the partial resistance exhibited by these tumours. Increased activation of the RAS pathway has previously been shown to contribute to chemoresistance and has also been shown to act via the MAPK and PI3K-AKT pathways (Jin et al., 2003).

3.4.4 Conclusions

In this chapter, I have used breast tumour samples taken from patients before and after NAC, who displayed only a partial response to the chemotherapy. Consistent changes in mRNA and miRNA expression were observed, with results suggesting activation of the MAPK and PI3K-AKT pathways, and up-regulation of miR-26b and miR-195, and down-regulation of miR-10a. The next aim was to determine whether any of these changes in expression were associated with changes in chemoresponse *in vitro*. Further studies were first designed to investigate the dysregulated miRNAs as these provided individual molecules to study, rather than the whole molecular pathways provided by the mRNA expression data.

Chapter 4: Deregulation of microRNAs in ER positive breast cancer causes altered chemosensitivity

4.1 Abstract

I have identified miR-26b, miR-195 and miR-10a as consistently deregulated in luminal A breast cancer cells that survived chemotherapy treatment; this suggested that these miRNAs may contribute to defining chemoresponse. In this chapter, I investigated firstly whether these miRNAs alter chemosensitivity of ER positive breast cancer cells *in vitro* using miRNA mimics and inhibitors with two separate chemoresponse assays, and secondly, whether it was possible to identify the targets of these miRNAs that may be responsible for their influences.

Increased expression of miR-26b or miR-195 using miRNA mimics resulted in significantly increased resistance to the anthracycline epirubicin in two cell lines representative of luminal A breast cancers (MCF7 and MDA-MB-175); this was in accordance with the observed increase in expression of these miRNAs in cancer cells surviving NAC in patients. Surprisingly, increased expression of miR-10a also resulted in increased chemoresistance *in vitro*, an observation apparently in conflict with the down-regulation of miR-10a that was observed post-NAC in patients. Decreasing expression of miR-26b, miR-195 and miR-10a using inhibitors generally had less effect, although when significant findings were made, the results were the opposite of over-expression, as would be expected. Also, investigation of expression of miR-26b and miR-195 in two ER positive breast cancer cell lines that had been selected to become resistant to epirubicin revealed that the expression of these two miRNAs was increased in the resistant cell lines compared with parental controls.

Next, attempts were made to identify the mRNA targets through which miR-26b and miR-195 contribute to chemoresistance using predictive algorithms, literature searches and mining of public datasets. Six putative targets of miR-26b and five putative targets of miR-195 were identified, including *CCDC6* as a potential target of both. Expression analysis of *CCDC6* after transfection with miR-26b or miR-195 mimics and inhibitors confirmed that *CCDC6* is indeed downstream of the miRNAs but chemosensitivity assays after transfection with

CCDC6 siRNA demonstrated that this gene did not alone influence chemoresistance.

I concluded that miR-26b and miR-195 can contribute to chemoresistance in breast cancer, but that other assays will be required to identify the pathways downstream.

4.2 Introduction

4.2.1 The role of miRNAs in chemoresistance

MiRNAs are short, non-coding sequences of RNA typically between twenty and twenty-four nucleotides long. They were first discovered twenty-five years ago (Wightman et al., 1993, Lee et al., 1993) and have since been found to regulate gene expression at post-transcriptional levels (Obernosterer et al., 2006). There are two principal mechanisms used by miRNAs in this function. These are the ability to sequester mRNAs such that the transcripts are no longer accessible to translational machinery (Pillai, 2005) and the ability to mark the transcripts for destruction (Orang et al., 2014). Ultimately, both of these mechanisms lead to decreased protein production (see section 1.3.2).

Since their discovery, evidence has shown that individual miRNAs have the potential to bind to multiple mRNA targets. An example of this is miR-21, which has been shown to regulate the expression of *PTEN* (Wang et al., 2011), *PDCD4* (Asangani et al., 2008) and *BCL2* (Dong et al., 2011). Increasing the complexity of miRNA function, evidence has also shown that a single mRNA transcript can be targeted by multiple miRNAs. This is evidenced by the fact that *PTEN* has been shown to be regulated by both miR-19 (Liang et al., 2011) and miR-21 (Wang et al., 2011).

The observation that miRNAs generally have decreased expression in cancer tissue compared to normal tissue (Lu et al., 2005) has led to increased interest in their regulation and function in multiple contexts, including their ability to contribute to the classification of tumour subtypes (Blenkiron et al., 2007) or to determine whether tumours are likely to respond to specific systemic therapies (van Schooneveld et al., 2015, Muluhngwi and Klinge, 2015).

Several miRNAs have been identified as regulators of chemoresponse, for example miR-298 expression has been implicated in response to doxorubicin in breast cancer (Bao et al., 2012). It is important to note, however, that the same miRNA may have differing functions depending on context. This has been shown with miR-195, which when up-regulated results in resistance to temozolomide in glioblastoma multiforme cells but is also associated with increased sensitivity to adriamycin in breast cancer (Ujifuku et al., 2010, Yang et al., 2013). This highlights the importance of identifying molecular profiles associated with sensitivity and resistance to specific treatment regimens rather than expecting findings to be generic. It is also important that these molecular profiles are identified for each tumour subtype.

The validation of miRNAs that contribute to chemoresistance has previously involved the use of miRNA mimics and inhibitors. Such a method was used after it had been established that miR-130b was up-regulated in primary breast tumours compared with adjacent normal tissue and the discovery that this miRNA was also up-regulated in MCF7 cells that had been selected to become resistant to adriamycin as compared to parental cells. With the use of a miR-130b mimic, it was discovered that the up-regulation of this miRNA in normal MCF7 cells was sufficient to increase chemoresistance and proliferation, and to decrease apoptosis. The down-regulation of this miRNA using an inhibitor in MCF7 cells that were resistant to adriamycin increased chemosensitivity and apoptosis, and reduced proliferation (Miao et al., 2017). A similar approach was taken in this chapter. MiR-26b, miR-195 and miR-10a were identified as consistently deregulated in terms of expression in the cancer cells surviving NAC in ER positive tumours that displayed a partial response to the chemotherapy (see section 0). In this chapter, I have used mimics and inhibitors of these miRNAs to assess whether changes in their expression levels are sufficient to cause altered chemosensitivity of ER positive breast cancer cells *in vitro*. I have also attempted to identify targets downstream of the miRNAs.

4.2.2 Specific objectives

There were two specific objectives in this chapter:

1. To identify whether miR-26b, miR-195 or miR-10a influence chemoresponse of ER positive breast cancer cells *in vitro*.
2. To use predictive algorithms together with literature searches and public datasets in an attempt to identify putative mRNA targets of the miRNAs that contribute to chemoresponse and determine if these genes contribute to the altered chemosensitivity.

4.3 Results

4.3.1 Deregulated miRNAs affect chemo-sensitivity *in vitro*

Three miRNAs, miR-26b, miR-195 and miR-10a, were identified in Chapter 3 (section 0) as potentially involved in defining chemoresponses of breast cancers, based on their consistent deregulation of expression in breast cancer cells that had survived chemotherapy treatment. My next aim was to investigate *in vitro* whether these miRNAs individually play roles in contributing to chemoresponse of breast cancer cells. To do this, I used two different cell lines representative of the luminal A subtype of breast cancer, since this was the subtype of breast cancer examined in Chapter 3: these were MCF7 and MDA-MB-175 cells. The MCF7 cell line is a well-established luminal A representative (Holliday and Speirs, 2011), whereas MDA-MB-175 cells were classified as luminal B cells according to the PAM50 classification although there appear to be results that suggest that this cell line is also luminal A (Jiang et al., 2016). In addition, two separate methods of determining chemosensitivity were employed: MTT assays to observe short-term survival of cells after treatment with an appropriate chemotherapy agent, and colony forming assays to determine long-term effects on cell viability as assessed by ability to proliferate sufficiently after treatment to generate a colony of progeny cells. The anthracycline epirubicin was used, as this was the key component of the clinical regimen in the treatment of the patients studied in Chapter 3.

4.3.1.1 The two luminal A representative cell lines MCF7 and MDA-MB-175 have very different sensitivities to epirubicin

First, it was necessary to determine the sensitivities of the two cell lines to be used to epirubicin, so that cells could be treated with doses in the appropriate range after manipulation of miRNA expression. In this context, an appropriate range meant doses resulting in approximately 20-80% survival, and thereby suitable for assessment of any induced changes in survival, as opposed to doses causing very dramatic death or having little effect, which may be relatively impervious to protection or sensitisation from single gene influences. MCF7 or MDA-MB-175 cells were treated with a wide range of doses of epirubicin for 24 hours and MTT assays were performed (Figure 4.3.1). As expected, epirubicin induced dose-dependent reductions in survival. MCF7 cells were considerably more sensitive than MDA-MB-175 cells, with respective IC50 doses of 1.1μM and 178μM.

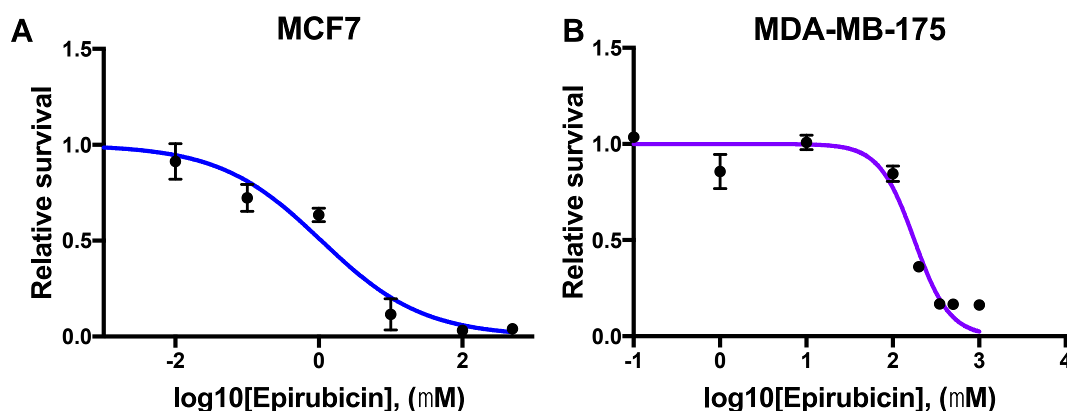


Figure 4.3.1: Different epirubicin doses are appropriate in the two different representative breast cancer cell lines

MCF7 or MDA-MB-175 cells were seeded and treated with a range of epirubicin concentrations for 24 hours. Cell viability was then measured using MTT assays. Cell survival at each dose was calculated relative to viability of vehicle control (water) treated cells for each cell line. (A) The dose-response curve for MCF7 cells. Data represent means of two biological repeats with error bars showing SEM. (B) The dose-response curve for MDA-MB-175 cells. Data represent means of three biological repeats with error bars showing SEM.

4.3.1.2 MiRNA mimics and inhibitors successfully modify miRNA expression levels

Next, I aimed to modify the cellular expression levels of the miRNAs of interest by transfecting with miRNA mimics or inhibitors, therefore transfections were performed and RT-qPCR used to assess expression levels. MCF7 or MDA-MB-175 cells were transfected with targeted miRNA mimics or inhibitors for miR-26b, miR-195, or miR-10a or appropriate non-targeting mimic or inhibitor controls. RNA was extracted seventy-two hours later and expression of these miRNAs was then quantified by RT-qPCR (Figure 4.3.2). Transfection with miRNA mimics or inhibitors resulted in successful over-expression (by 145 to 4500 fold) or down-regulation (to 0.4 or lower relative to control levels) of the miRNAs respectively.

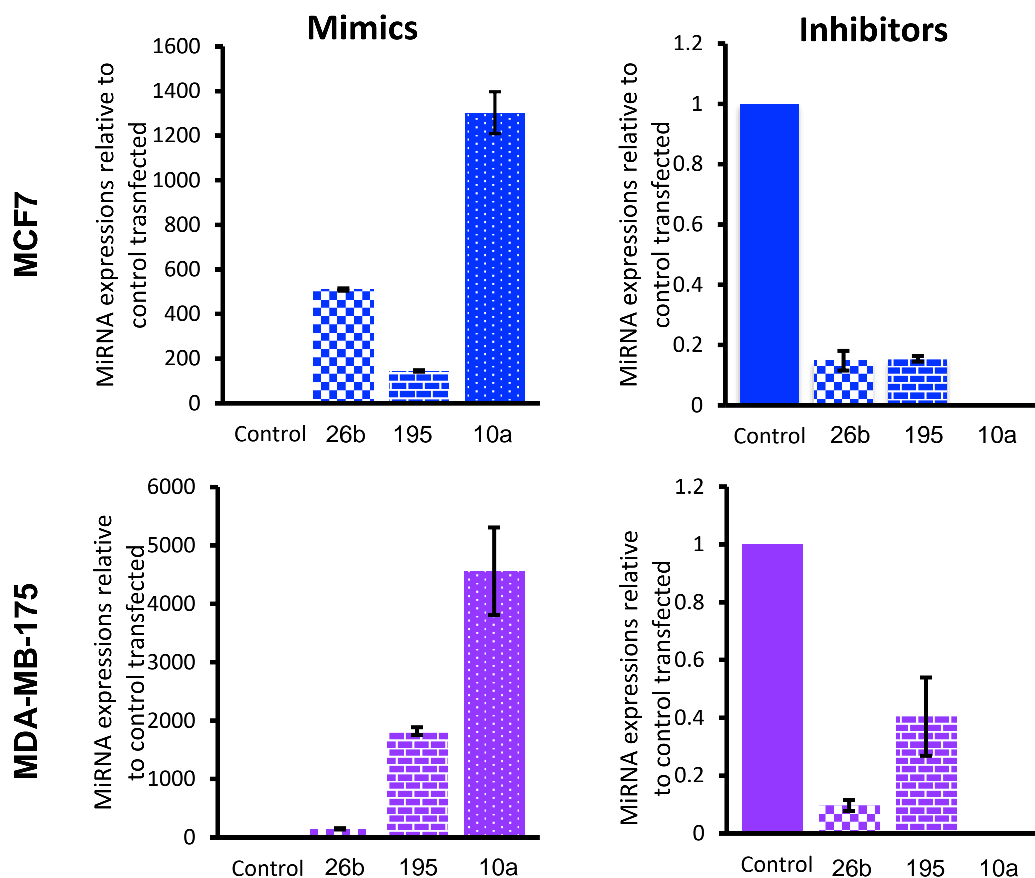


Figure 4.3.2: MiR-26b, miR-195 and miR-10a were successfully up- or down-regulated in MCF7 and MDA-MB-175 cells

MCF7 (top panels) or MDA-MB-175 (bottom panels) cells were transfected as shown with individual miRNA mimics (left panels) or inhibitors (right panels) or controls, and total RNA extracted seventy-two hours post-transfection. Expression of the targeted miRNAs was quantified by RT-qPCR relative to RNU48 and is presented relative to levels in cells transfected with control mimics or inhibitors. These graphs represent means of three technical replicates from one biological repeat, with error bars showing SD.

4.3.1.3 Over-expression of miR-26b or miR-195 increases resistance of MCF7 and MDA-MB-175 cells to chemotherapy

Having successfully confirmed the altered expressions of the miRNAs of interest with the miRNA mimics and inhibitors, I then wanted to assess the influence of over-expression and knockdown of these miRNAs individually on chemoresponse. Initially, I focussed on miR-26b and miR-195, both of which were up-regulated in the cancer cells that survived NAC in patients. MCF7 or MDA-MB-175 cells were transfected with either miRNA mimics or inhibitors, or

appropriate controls. Forty-eight hours post-transfection, cells were treated with two different doses of epirubicin for twenty-four hours and then MTT assays were performed to determine effects of the miRNA mimics or inhibitors on cell viability. Note this forty-eight plus twenty-four hours protocol means that MTT assays were performed at the same time point as I used previously to test the degrees of over-expression or knockdown (Figure 4.3.2). Epirubicin doses were chosen so as to result in approximately 75% or 25% cell viability in the control transfected samples.

Data obtained from MTT assays in MCF7 cells (Figure 4.3.3) and in MDA-MB175 cells (Figure 4.3.4) showed that when cells over-expressed miR-26b or miR-195, cells were significantly more viable after treatment with epirubicin ($p<0.05$), suggesting that they were more resistant to the epirubicin treatment compared with their control counterparts. By contrast, the inhibitors of miR-26b or miR-195 did not cause compelling changes in chemoresponse, with no significant differences seen in MCF7 cells, while in MDA-MB-175 cells a slight (but significant, $p<0.05$) chemo-protection was seen from the miR-195 inhibitor at one epirubicin dose only.

The data from these MTT assays in both cell lines suggested that an increase in either miR-26b or miR-195 expression in ER positive breast cancer cells caused increased resistance to chemotherapy. Despite these increases in cell survival being relatively small, the suggestion that the up-regulation of these miRNAs did contribute to chemoresistance appeared convincing based on the facts that: 1) the increases in cell survival were significant at various doses and in both cell lines; 2) doses that did not show significant differences appeared to show similar trends; and 3) these data were consistent with expectations from patient data where increased expression of these miRNAs was observed in cells that had survived chemotherapy treatment (see section 0).

Conversely, data obtained using inhibitors of these miRNAs were unexpected considering the effects of the mimics as it might be expected that the inhibitors would have the opposite effects on cell survival after epirubicin treatment. However, data obtained from these MTT assays suggested that decreased expression of these miRNAs had little effect. In MDA-MB-175 cells, the inhibitor for miR-195 (Figure 4.3.4D) led to a significant increase in cell survival at the higher dose of epirubicin ($p<0.00005$). However, this was the only significant

result obtained with the miRNA inhibitors and was only observed in one cell line at one dose of epirubicin, and could therefore be considered an anomaly. In particular, it may be worth noting that transfection of MDA-MB-175 cells with the miR-195 inhibitor resulted in the least effective change in expression seen across the whole panel of miRNA mimics and inhibitors (a decrease in expression of approximately 60%; Figure 4.3.2), therefore, the result of increased survival following inhibition of miR-195 and treatment with epirubicin in MDA-MB-175 cells may be especially questionable.

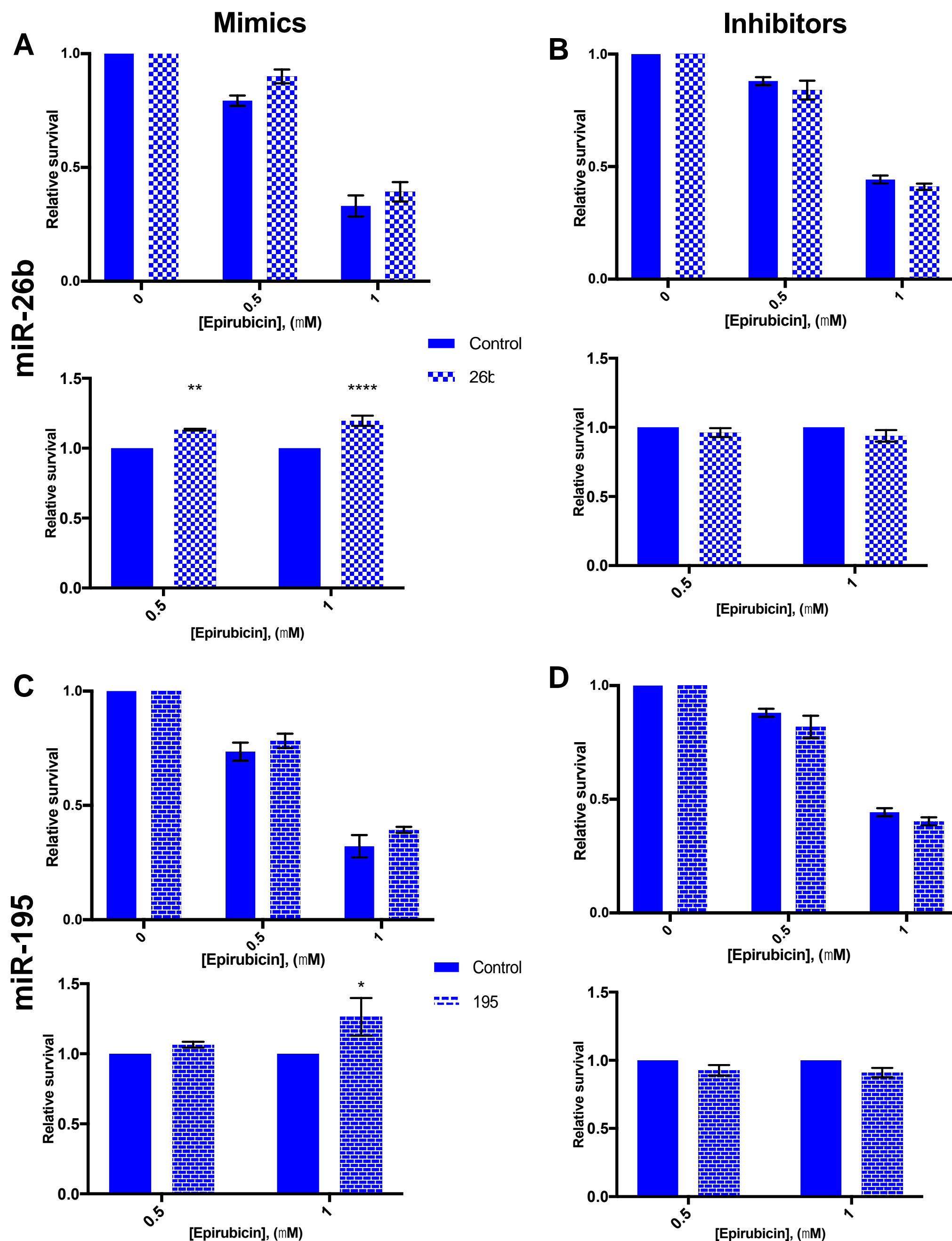


Figure 4.3.3: Increased miR-26b and miR-195 expression in MCF7 cells confers chemoresistance

MCF7 cells were transfected with either mimics (left panels) or inhibitors (right panels) of miR-26b (A and B) or miR-195 (C and D). Forty-eight hours post-transfection, cells were treated with two concentrations of epirubicin (0.5μM or 1μM) or vehicle control (water). Twenty-four hours post-treatment, MTT assays were performed. Raw absorbance values were normalised to the control transfected and control untreated samples. The top panels show the effects of epirubicin on overall survival whereas the bottom panels are normalised to allow focus on the effects of the targeted miRNA mimic or inhibitor on survival in epirubicin-treated samples. Three biological repeats were performed for each experiment, with error bars showing SEM (*p<0.05, **p<0.005, ****p<0.00005).

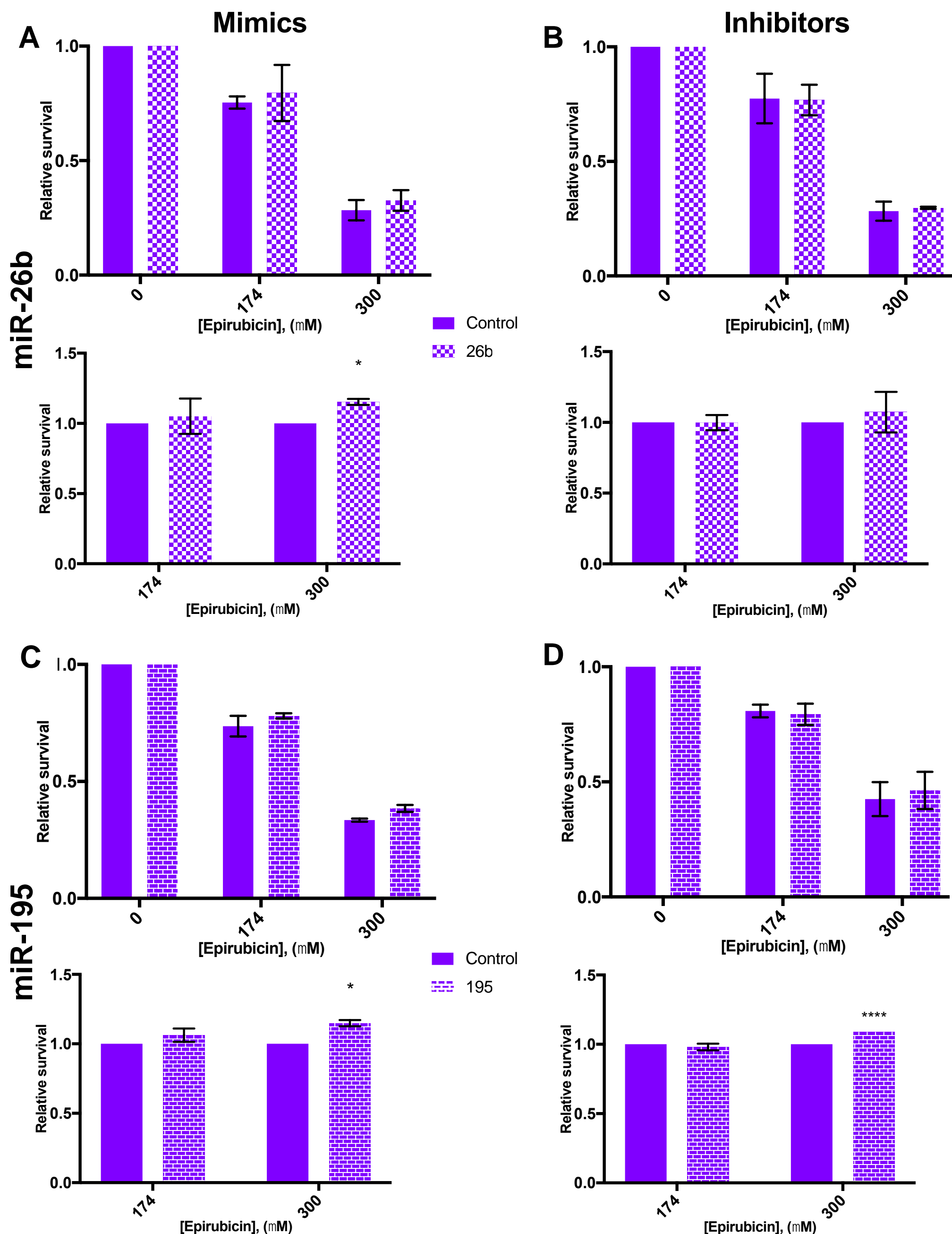


Figure 4.3.4: Increased miR-26b and miR-195 expression in MDA-MB-175 cells confers chemoresistance

MDA-MB-175 cells were transfected with either mimics (left panels) or inhibitors (right panels) of miR-26b (A and B) or miR-195 (C and D). Forty-eight hours post-transfection, cells were treated with two concentrations of epirubicin (174μM or 300μM) or vehicle control (water). Twenty-four hours post-treatment, MTT assays were performed. Raw absorbance values were normalised to the control transfected and control untreated samples. The top panels show the effects of epirubicin on overall survival whereas the bottom panels are normalised to allow focus on the effects of the targeted miRNA mimic or inhibitor on survival in epirubicin-treated samples. Two biological repeats were performed for each experiment, with error bars showing SEM (*p<0.05, ****p<0.00005).

4.3.1.4 Over-expression of miR-10a increases resistance of MCF7 and MDA-MB-175 cells to chemotherapy

Next, I took the same approach to investigate the role of miR-10a in chemoresponse; miR-10a had been identified as down-regulated in cancer cells that survived NAC in patients. MCF7 and MDA-MB-175 cells were transfected with mimics and inhibitors, treated with epirubicin, and survival was assessed as before (Figure 4.3.5). MiR-10a mimics induced increased survival after epirubicin treatment, which was significant in one cell line at one dose ($p<0.05$), while – as previously – the inhibitors had little effect.

The data from these MTT assays in both cell lines suggested that, as with miR-26b and miR-195, increased expression of miR-10a contributed to increased chemoresistance compared with control transfected cells. Despite the fact that there was only one significant result in one cell line at one dose, all of the other results consistently showed an apparent increase in cell viability in both cell lines, supporting the conclusion that increased miR-10a expression led to increased cell survival. However, these results were surprising since they were not concordant with expectations based on patient data, as miR-10a was down-regulated in cells that survived chemotherapy treatment (see section 0), leading to an expectation that increased miR-10a expression would lead to decreased cell survival.

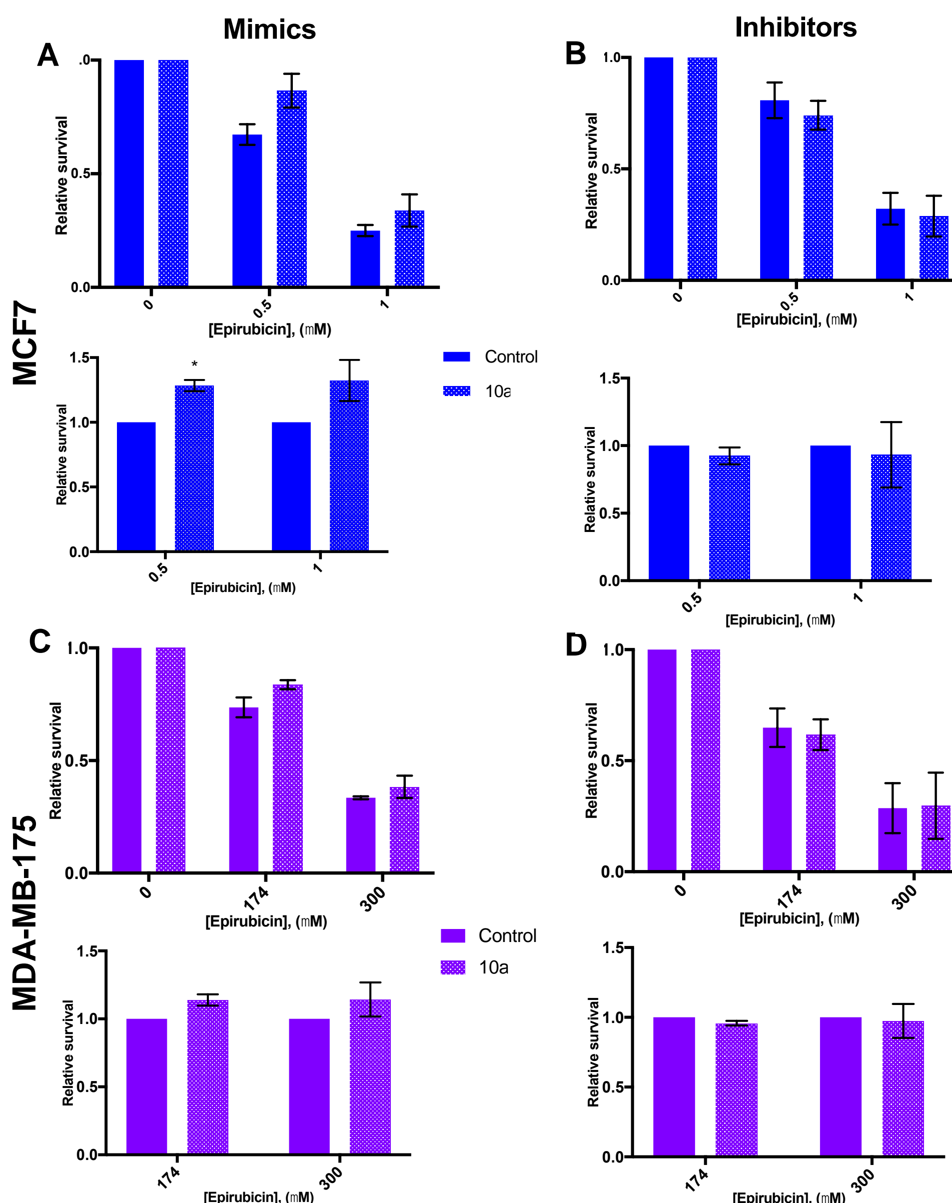


Figure 4.3.5: Increased miR-10a expression in MCF7 and MDA-MB-175 cells confers chemoresistance not chemosensitivity

MCF7 (A and B) or MDA-MB-175 (C and D) cells were transfected with either miR-10a mimic (left panels) or miR-10a inhibitor (right panels) or appropriate control mimic/inhibitor. Forty-eight hours post-transfection, cells were treated with two concentrations of epirubicin or vehicle control (water). Twenty-four hours post-treatment, MTT assays were performed. Raw absorbance values were normalised to the control transfected and control untreated samples. The top panels show the effects of epirubicin on overall survival whereas the bottom panels are normalised to allow focus on the effects of the targeted miRNA mimic or inhibitor on survival in epirubicin-treated samples. Three biological repeats were performed using MCF7 cells and two biological repeats were performed using MDA-MB-175 cells, with error bars showing SEM (*p<0.05).

4.3.1.5 Expression levels of miR-26b, miR-195 and miR-10a influence clonogenic survival following chemotherapy in MCF7 and MDA-MB-175 cells

The data obtained from these short-term MTT assays suggested that over-expression of miR-26b, miR-195 or miR-10a individually contribute to chemoresistance. However, it is important to consider that this assay simply observes cell viability directly after twenty-four hours treatment with the chemotherapy drug and does not take into account longer-term influences on proliferative ability. For this reason, colony forming assays were performed as these assess whether cells retain proliferative capacity, as opposed to merely staying alive, and therefore these assays may be more sensitive to influences of some regulators of response.

These experiments were performed using the same initial procedure as that used in the previous MTT protocol (sections 4.3.1.3 and 4.3.1.4), where cells (MCF7 or MDA-MB-175) were transfected with individual miRNA mimics or inhibitors or controls. Forty-eight hours post-transfection, cells were treated with doses of epirubicin for twenty-four hours. Instead of performing an MTT assay, however, cells were seeded at low densities in fresh media without epirubicin and left in culture for two weeks to observe what proportion of cells retained the ability to form colonies following the epirubicin treatment (Figure 4.3.6 and Figure 4.3.7).

Epirubicin doses were again selected to allow approximately 50% of cells to survive epirubicin treatment (ie grow into a colony); note these doses are considerably lower than the previous short-term assay, since long-term proliferative ability is sensitive to much lower doses. Initially, experiments were performed targeting miR-26b or miR-195 (Figure 4.3.6), since for these miRNAs MTT data were concordant with patient data therefore these represented the most promising candidate mediators of clinically relevant chemoresistance.

Over-expression of miR-195 caused a significant increase in cell survival in MCF7 cells after epirubicin treatment ($p<0.005$) and increased miR-26b also caused an increase in cell survival, although this was not significant (Figure 4.3.6A). In marked contrast to the MTT assays, the miRNA inhibitors also had

functional effects in this assay. When MCF7 cells were transfected with miR-26b or miR-195 inhibitors, clonogenic survival was decreased, which was significant with the miR-195 inhibitor (Figure 4.3.6B, $p<0.0005$).

Similar results were observed when miRNA mimics or inhibitors were transfected into MDA-MB-175 cells, with significant increases in relative survival when cells were transfected with miRNA mimics (Figure 4.3.6C, $p<0.0005$) and significant decreases in survival when cells were transfected with miRNA inhibitors (Figure 4.3.6D, $p<0.005$).

The data obtained from colony forming assays suggested that increased expression of miR-26b and miR-195 led to an increased ability of both MCF7 and MDA-MB-175 cells to survive and form colonies after chemotherapy treatment. Conversely, decreased expression of these miRNAs led to both cell lines becoming more sensitive to chemotherapy treatment resulting in fewer colonies being formed. These results are concordant with those obtained from MTT assays and also from patient data, providing strong evidence that miR-26b and miR-195 modulate chemoresistance.

Clonogenic survival assays were also performed after transfection with mimics or inhibitors for miR-10a in exactly the same way (Figure 4.3.7 [page 110]). MiR-10a over-expression caused a significant increase in cell survival after epirubicin treatment in both MCF7 and MDA-MB-175 cells ($p<0.05$), while miR-10a down-regulation caused a significant decrease in cell survival after epirubicin treatment in both cell lines ($p<0.05$). These data were concordant with those obtained from MTT assays, but again did not meet expectations based on patient data where miR-10a expression was decreased in cells that had survived chemotherapy treatment.

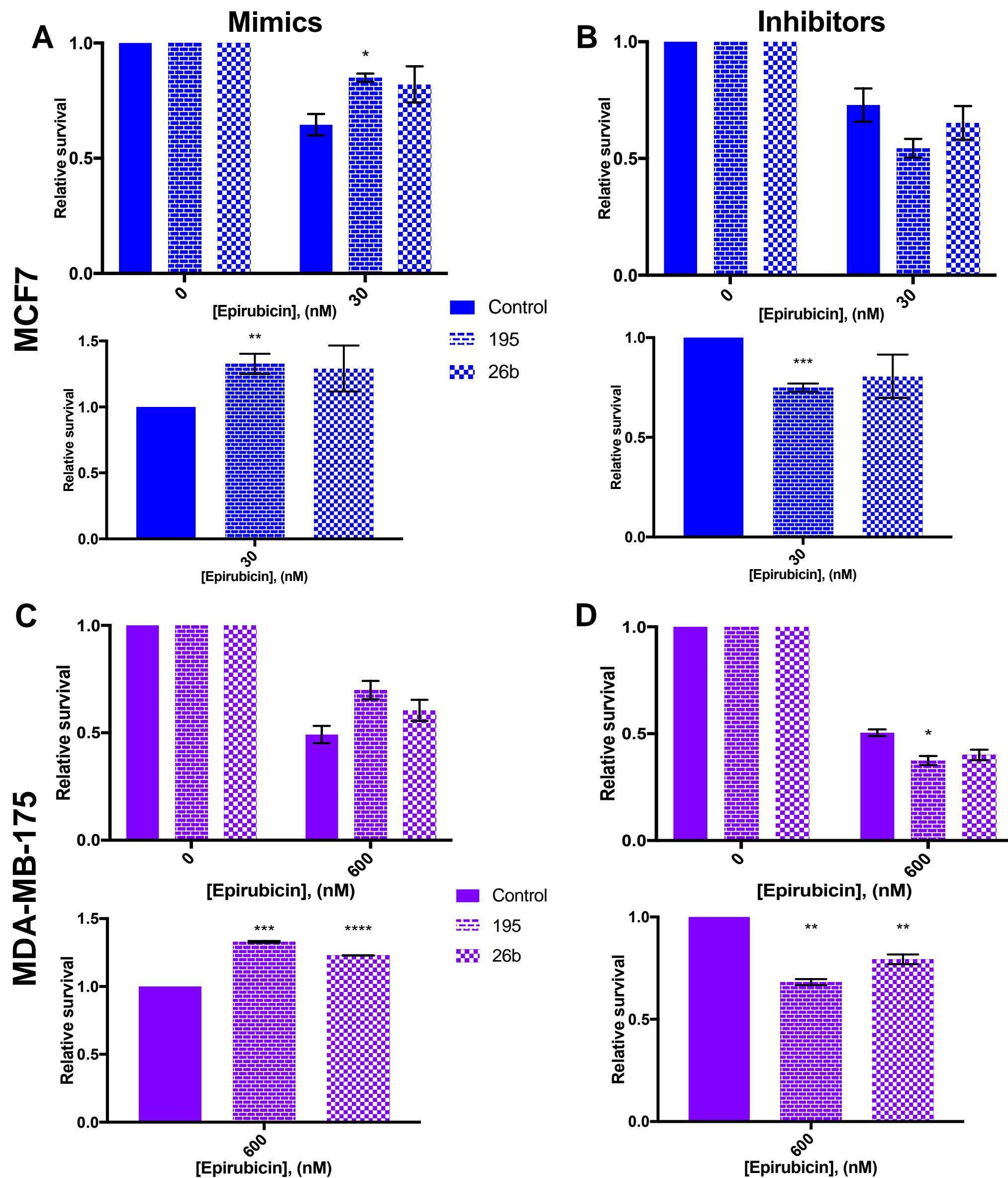


Figure 4.3.6: Increased miR-26b and miR-195 expression in MCF7 and MDA-MB-175 cells confers clonogenic survival

MCF7 (A and B) or MDA-MB-175 (C and D) cells were transfected with either miRNA mimics (A and C) or inhibitors (B and D) or a miRNA control mimic or inhibitor. Forty-eight hours post-transfection, MCF7 cells were treated with 30nM epirubicin and MDA-MB-175 cells were treated with 600nM epirubicin for twenty-four hours before being seeded at low densities and left to grow for fourteen days. Colonies were fixed and stained with a mixture of methanol, ethanol and crystal violet solution before being counted. The top panels show for each condition, epirubicin-treated samples were normalised to their untreated counterparts. The bottom panels show the miRNA transfected and treated samples normalised to the control transfected and epirubicin-treated sample. Three biological repeats were performed with MCF7 cells and two biological repeats were performed with MDA-MB-175 cells, with error bars showing SEM (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.00005$).

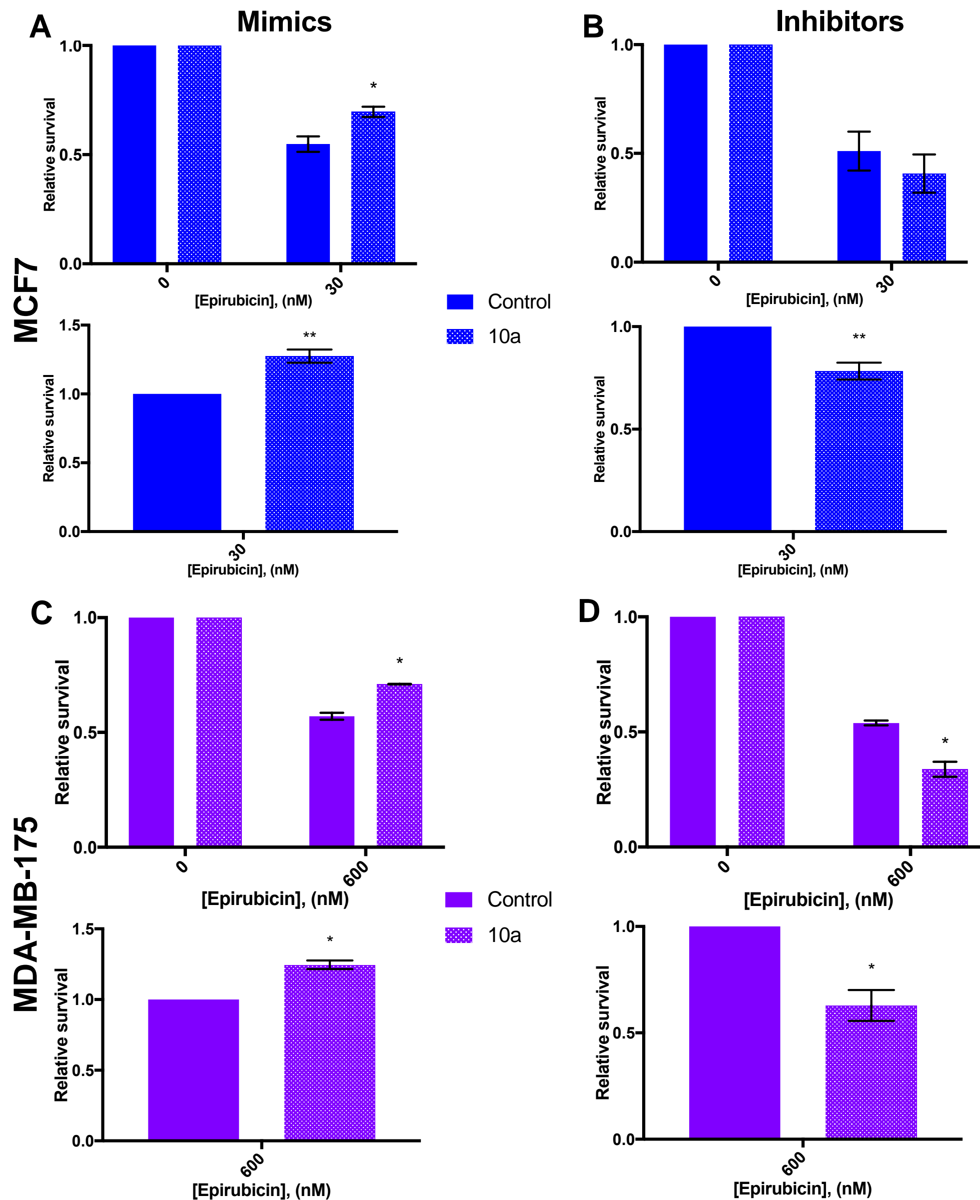


Figure 4.3.7: Increased miR-10a expression in MCF7 and MDA-MB-175 cells confers clonogenic survival

MCF7 (A and B) or MDA-MB-175 (C and D) cells were transfected with either a miR-10a mimic (A and C) or inhibitor (B and D) or a miRNA control mimic or inhibitor. Forty-eight hours post-transfection, MCF7 cells were treated with 30nM epirubicin and MDA-MB-175 cells were treated with 600nM epirubicin for twenty-four hours before being seeded at low densities and left to grow for fourteen days. Colonies were fixed and stained with a mixture of methanol, ethanol and crystal violet solution before being counted. The top panels show for each condition, epirubicin-treated samples were normalised to their untreated counterparts. The bottom panels show the miRNA transfected and treated samples normalised to the control transfected and epirubicin-treated sample. Three biological repeats were performed with MCF7 cells and two biological repeats were performed with MDA-MB-175 cells, with error bars showing SEM (*p<0.05, **p<0.005).

Combining data from both short-term and long-term survival analyses, I concluded that over-expression of all three miRNAs can individually contribute to increased chemoresistance and that down-regulation of these miRNAs individually can contribute to increased chemosensitivity. As miR-26b and miR-195 were observed to be up-regulated in breast cancer cells post-chemotherapy in patients, the *in vitro* data I have obtained are concordant with these observations. However, miR-10a was observed to be down-regulated post-chemotherapy in patients, thus the data I have obtained do not conform with expectations. As such, further work was performed on miR-26b and miR-195 only, and not miR-10a.

4.3.1.6 Combined manipulation of expression of miR-26b and miR-195 does not strikingly enhance their effects on chemoresponse

Since miR-26b and miR-195 both showed promising results in multiple assays individually, I decided to observe whether manipulating the expression of both miRNAs simultaneously would have any additive or synergistic effects on chemoresponse in the assays previously used. Therefore, I initially transfected MCF7 cells with either both miRNA mimics or both inhibitors or relevant controls using the same method described previously (section 4.3.1.2) and quantified expression levels of both miRNAs post-transfection relative to control transfected samples (Figure 4.3.8). Simultaneous transfection with both miRNA mimics or inhibitors resulted in successful over-expression (by 8 and 1200 fold) or down-regulation (to 0.05 and 0.03 relative to control levels) of the miRNAs.

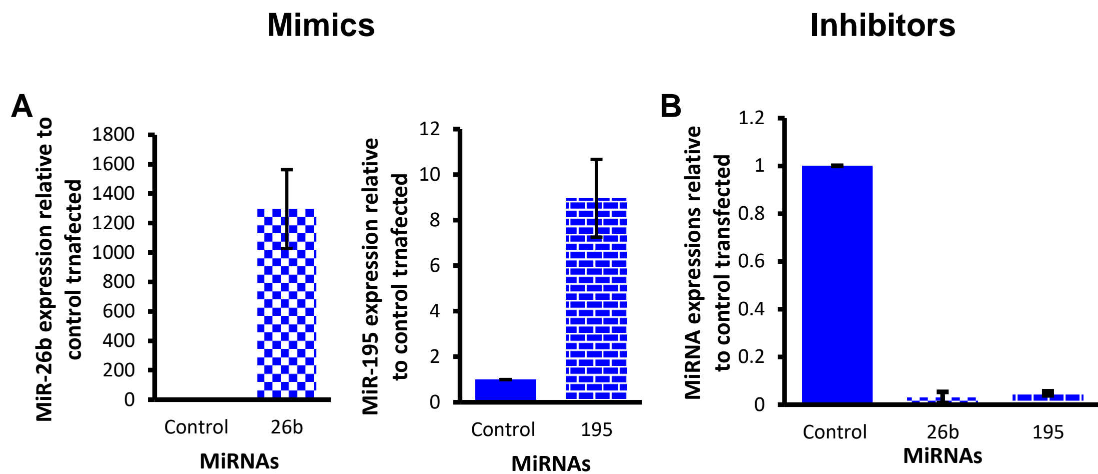


Figure 4.3.8: MiR-26b and miR-195 were successfully up- and down-regulated in MCF7 cells

MCF7 cells were simultaneously transfected with miR-26b and miR-195 mimics (A) or inhibitors (B) or relevant controls and total RNA extracted seventy-two hours post-transfection. Expression of the targeted miRNAs was quantified by RT-qPCR relative to RNU48, and are presented relative to levels in cells transfected with control mimics or inhibitors. These graphs represent mean of three technical replicates from one biological repeat, with error bars showing SD.

Having successfully confirmed the altered expressions of the miRNAs of interest, I then wanted to assess the influence of over-expression and knockdown of these miRNAs together on chemoresponse. MCF7 cells were transfected with either both miRNA mimics or inhibitors, or appropriate controls. Forty-eight hours post-transfection, cells were treated with epirubicin for twenty-four hours and then either MTT or colony forming assays performed as previously described (sections 4.3.1.3 and 4.3.1.5) (Figure 4.3.9).

Data from these MTT assays and colony forming assays suggested that when cells over-expressed miR-26b and miR-195 together, significantly more cells survived after epirubicin treatment ($p<0.05$) (Figure 4.3.9A and C). Decreased expression of both of these miRNAs in MCF7 cells did not have any effect on cell survival in either of the assays (Figure 4.3.9B and D).

The data obtained from these two assays suggested that increased expression of both miR-26b and miR-195 led to an increased ability of MCF7 cells to survive chemotherapy treatment over both a short term and long term. These results are concordant with those obtained from MTT assays and colony forming assays after over-expression of individual miRNAs and are also

concordant with patient data. However, over-expression of both miRNAs simultaneously did not appear to have any additive or synergistic effects on chemoresistance compared with over-expression of individual miRNAs. Conversely, decreased expression of both miRNAs together did not affect the chemoresponse of MCF7 cells, even in colony forming assays. This was in contrast with data obtained from colony forming assays following decreased expression of individual miRNAs where significant increases in chemosensitivity were observed (Figure 4.3.6). I therefore concluded that manipulation of both miRNAs simultaneously did not provide any additional information regarding their roles in chemoresponse. As such, further work was performed on miR-26b and miR-195 individually.

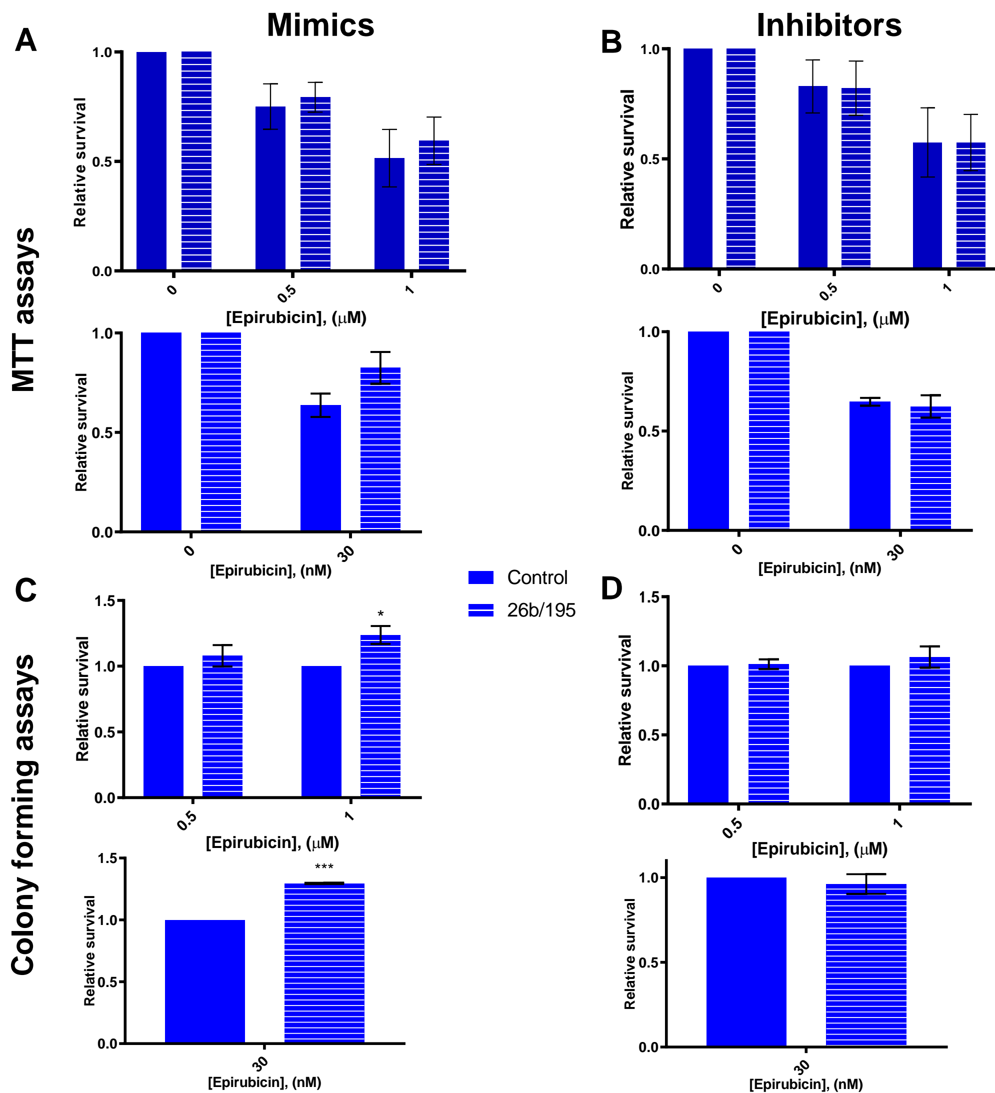


Figure 4.3.9: Increased expression of miR-26b and miR-195 together in MCF7 cells confers chemoresistance

MCF7 cells were transfected with either both mimics (left panels) or both inhibitors (right panels) of miR-26b and miR-195 or controls. Forty-eight hours post-transfection, cells were treated with epirubicin or vehicle control (water). Twenty-four hours post-treatment, either MTT assays (A and B) or colony forming assays (C and D) were performed. For MTT assays, raw absorbance values were normalised to the control transfected and control untreated samples. The top panels show the effects of epirubicin on overall survival whereas the bottom panels are normalised to allow focus on the effects of the targeted miRNA mimic or inhibitor on survival in epirubicin-treated samples. For colony forming assays, cells were seeded at appropriate densities and left to grow for fourteen days. Colonies were fixed and stained with a mixture of methanol, ethanol and crystal violet solution before being counted. The top panels show for each condition, epirubicin-treated samples were normalised to their untreated counterparts. The bottom panels show the miRNA transfected and treated samples normalised to the control transfected and epirubicin-treated sample. Three biological repeats were performed for MTT assays and two biological repeats were performed for colony forming assays, with error bars showing SEM (*p<0.05, ***p<0.0005).

4.3.1.7 MiR-26b and miR-195 are up-regulated in two ER positive epirubicin resistant breast cancer cell lines

As a further tool for the study of resistance to epirubicin in ER positive breast cancer, I developed two cell lines with stable constitutive epirubicin resistance. To achieve this, I treated the breast cancer cell lines MCF7 and T47D with initial low doses of epirubicin and gradually increased the drug concentration over many months of continuous culture, while avoiding increasing excessively and thereby killing all the cells. I also continuously grew parental cell lines without epirubicin in parallel, to provide appropriate control cells. I assessed the degree of resistance of each selected cell line by performing epirubicin dose responses for the resistant cells as compared to their untreated parental controls using MTT assays (Figure 4.3.10), exactly as previously described in section 4.3.1.1. The resistant MCF7 cells had an estimated IC50 12.9 times greater than that of their parental control cells (Figure 4.3.10A) and the resistant T47D cells had an estimated IC50 16.7 times greater than that of their parental control cells (Figure 4.3.10B).

Having concluded that transient over-expression of miR-26b or miR-195 can lead to increased resistance to epirubicin (sections 4.3.1.3 and 4.3.1.5), and that these miRNAs were up-regulated in relatively chemotherapy resistant breast cancer cells in patients, I was next interested in testing whether up-regulation of these miRNAs was potentially playing a role in these resistant cell lines.

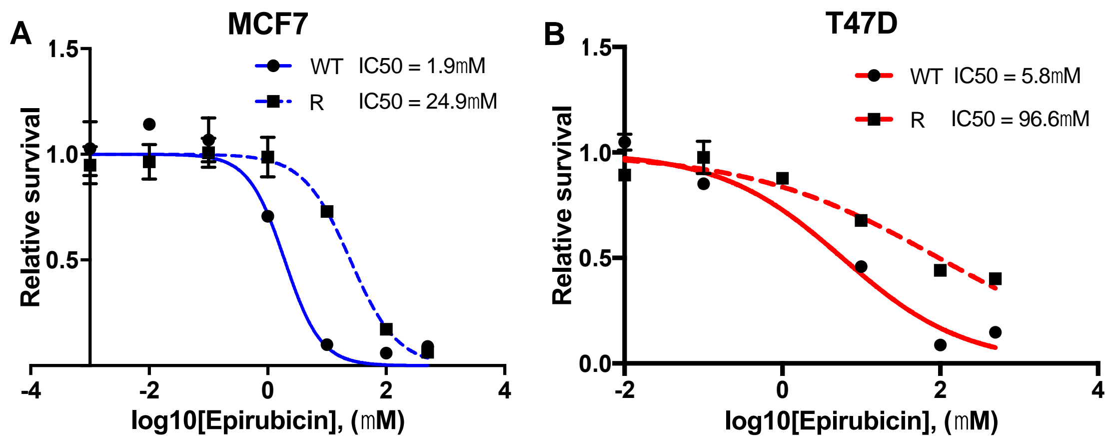


Figure 4.3.10: MCF7 and T47D cells are more chemoresistant than their parental lines after long-term exposure to epirubicin

Epirubicin resistant cell lines were developed by continuous exposure to increasing concentrations of epirubicin in both MCF7 and T47D cells. Resistant (R) and parental (wild type, WT) MCF7 (A) or T47D (B) cells were then exposed to a wide range of doses of epirubicin for 24 hours before cell viability was measured using MTT assays. Each drug dose was measured in triplicate. Cell survival at each dose was calculated relative to cell viability of vehicle control (water) treated cells for each cell line. The dose-response curves show one biological repeat with error bars showing +/- SD of technical replicates.

I performed RT-qPCR to determine whether there was differential expression of miR-26b or miR-195 in the resistant cell lines compared to their parental controls (Figure 4.3.11). Expression of both miR-26b and miR-195 was increased in the epirubicin resistant cells compared with their parental controls. These data further support the hypothesis that both of these miRNAs contribute to chemoresistance in breast cancer cells.

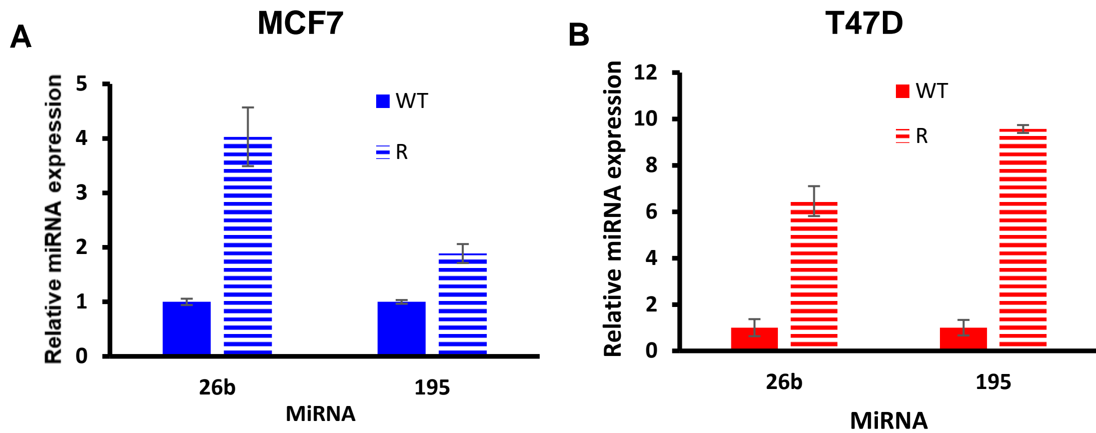


Figure 4.3.11: MiR-26b and miR-195 were up-regulated in epirubicin resistant MCF7 and T47D cells

Total RNA was extracted from MCF7 (A) and T47D (B) epirubicin resistant (R) or parental control cell lines (wild type, WT). Relative expressions of miR-26b and miR-195 were quantified by RT-qPCR, using RNU48 as the control. Data are expressed relative to wild type, and represent one biological repeat with error bars showing +/- SD of technical replicates.

4.3.1.8 Epirubicin resistance does not confer resistance to a taxane

Breast cancer patients who are found to have tumours that are substantially resistant to an initial chemotherapy regimen are often switched to a different regimen to assess whether responses can be improved by different agents that may act in different ways (Porkka et al., 1994, Thomas et al., 2004, Polyzos et al., 2009). In the context of breast cancer neoadjuvant chemotherapy, initial therapies are often anthracycline-based (such as epirubicin), whereas the alternative may be taxane-based (such as docetaxel) (Esteva and Hortobagyi, 2008, Rivera and Gomez, 2010). I was therefore interested to investigate whether the epirubicin resistant cell lines that I had developed were also more resistant to an alternative chemotherapeutic drug with a different mode of action. Therefore, epirubicin (an anthracycline) resistant and parental MCF7 and T47D cells were treated with a range of doses of the taxane docetaxel and relative survival was determined using MTT assays (Figure 4.3.12).

Interestingly, neither epirubicin resistant line was also resistant to docetaxel; in T47D cells there was no difference in sensitivity to docetaxel between the epirubicin resistant and parental control cells, while – surprisingly – in MCF7 cells, the epirubicin resistant cells were actually more sensitive to docetaxel than the parental line. This result emphasises that resistance pathways for

different agents can be unrelated and do not necessarily give multi-drug resistance.

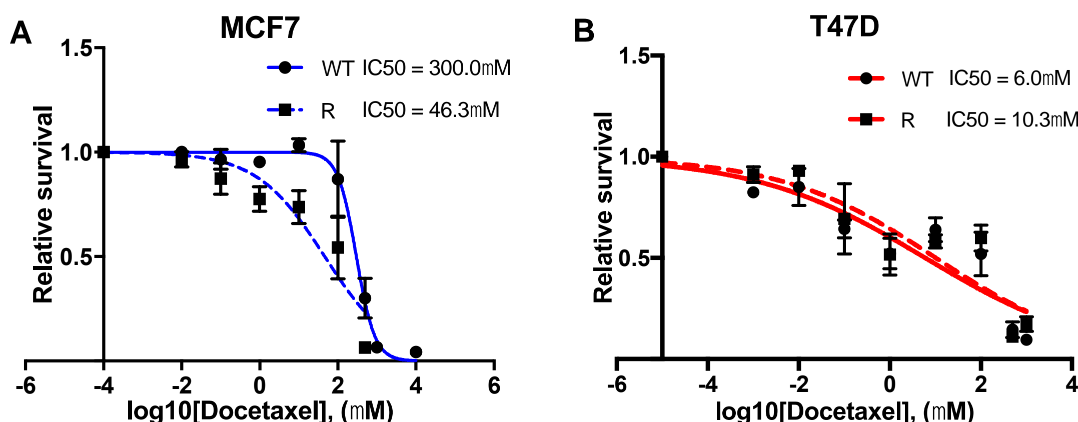


Figure 4.3.12: Epirubicin resistant MCF7 and T47D cells are not resistant to docetaxel

MCF7 (A) and T47D (B) epirubicin resistant (R) and wild type (WT) cells were exposed to increasing doses of docetaxel. MTT assays were performed twenty-four hours post-treatment to measure cell viability. Each drug dose was measured in triplicate. Cell survival at each dose was calculated relative to cell viability of vehicle control (DMSO) treated cells for each cell line. Graphs show means of two biological repeats (+/- standard error).

4.3.2 Identification of miRNA targets that potentially contribute to the chemoresistance phenotype

Having established that over-expression of miR-26b and miR-195 contributes to chemoresistance, and that increased expression of these miRNAs is observed in epirubicin resistant cell lines, I then attempted to identify the possible mechanisms of action of these miRNAs by investigating their potential mRNA targets.

4.3.2.1 Identifying putative mRNA targets of miR-26b-5p and miR-195-5p using predictive algorithms and literature searching

Initially, the on-line resource starBase v2.0 (Yang et al., 2011, Li et al., 2014b) was used to identify mRNAs that are potentially targeted by either miR-26b or miR-195. This program gathers data from five prediction algorithms (miRanda, PicTar, TargetScan, RNA22 and PITA) that use multiple factors to predict targets including sequences in the mRNAs that match the seed region of

miRNAs and the accessibility of these sequences to the miRNAs using free energy assessments of secondary structures, and also incorporates experimental evidence, if any. This led to the identification of several thousand possible targets for both miRNAs.

To narrow down the number of possible targets, those mRNAs predicted by all five prediction algorithms were given priority. Twenty-four mRNAs were predicted by all five algorithms for miR-26b and fifty-five mRNAs were predicted for miR-195. A search of the literature for all seventy-eight potential targets was performed, to identify genes within the list that had previously been linked or suggested to be linked to chemoresistance when down-regulated. This criterion was used since an up-regulation of miRNA expression would most likely lead to a down-regulation of target mRNA and/or protein. In addition, transcripts consistently down-regulated in all the original patients used for initial miRNA discovery according to NanoString mRNA expression data (see section 3.3.4) were also investigated if predicted to be targets of miR-26b or miR-195 by any of the predictive algorithms. Transcripts were also considered if consistently down-regulated in the three patients with complete datasets but not in one of the other patients with incomplete datasets if they were predicted by all five predictive algorithms. The final list of candidate targets of interest is shown in Table 4.3.1 (miR-26b) and Table 4.3.2 (miR-195), along with a summary of the evidence that led to the inclusion of each entry. It was particularly interesting to note that *CCDC6* was a predicted target of both miR-26b and miR-195.

Table 4.3.1: Top predicted mRNA targets of miR-26b

Gene	No. of predictive algorithms	Expression post-NAC in all patients (NanoString)	Function in chemoresponse
PPP3R1	4	Down (5/5 patients)	Activates mitochondrial apoptosis pathway in human hepatoma HepG-2 cells (Yang et al., 2017)
PRKAR2B	1	Down (3/3 patients)	Down-regulated in etoposide and cisplatin resistant small cell lung cancer H446 cell line compared with parental cell line (Chen et al., 2017)
GSK3B	5	Down (3/4 patients)	Inactivation leads to increased resistance to doxorubicin and tamoxifen in MCF7 breast cancer cells (Sokolosky et al., 2014)
CCDC6	5	Not on panel	Reduced expression confers resistance to cisplatin in non-small cell lung cancer but confers sensitivity to olaparib (Morra et al., 2015)
ULK1	5	Not on panel	Down-regulated in most breast cancers, addition of synthetic agonist induced cell death associated with autophagy (Zhang et al., 2017)
WNT5A	5	Down (3/3 patients)	Decreased expression in poor responders to chemotherapy in osteosarcoma patients and in chemoresistant cell line compared with responders (Walters et al., 2008)

Potential targets for miR-26b were selected for further study using a combination of bioinformatics predictions (five different algorithms used), mRNA expression data concerning down-regulation in breast cancers in patients post-NAC (Chapter 3), and literature searching for published roles contributing to chemoresistance.

Table 4.3.2: Top predicted mRNA targets of miR-195

Gene	No. of predictive algorithms	Expression post-NAC patients (NanoString)	Function in chemoresponse
APH1B	1	Down (3/3 patients)	Down-regulated in MCF7 breast cancer cells resistant to adriamycin and paclitaxel (Zhang et al., 2016)
MYB	5	Down (5/5 patients)	Knockdown in MCF7 breast cancer cells increased tumourigenesis and resistance to tamoxifen (Thorner et al., 2010)
E2F7	5	Not on panel	Reduced expression in ovarian cancer patients resistant to platinum-based therapies compared with platinum-sensitive tumours (Reimer et al., 2007)
CCDC6	5	Not on panel	Reduced expression confers resistance to cisplatin in non-small cell lung cancer but confers sensitivity to olaparib (Morra et al., 2015)
GNAI3	5	Not on panel	Identified in gene expression signature - decreased expression in taxane-based resistant breast cancer patients (He et al., 2014b)

Potential targets for miR-195 were selected for further study using a combination of bioinformatics predictions (five different algorithms used), mRNA expression data concerning down-regulation in breast cancers in patients post-NAC (Chapter 3), and literature searching for published roles contributing to chemoresistance.

At this point, public datasets were mined in order to determine whether there were significant negative correlations between miR-26b or miR-195 expression and their predicted mRNA targets in breast cancers, as might be expected if the miRNA really does target the mRNA in actual breast cancer tissues. These data are shown in Figure 4.3.13, in the form of scatter plots with correlation best-fit lines.

Of the six predicted targets for miR-26b, only one (*PRKAR2B*) showed a significant negative correlation between its expression and miR-26b expression. There were also negative correlations between miR-26b expression and both *CCDC6* and *ULK1* expression although these were not significant (p values 0.1824 and 0.1089 respectively). These analyses support the proposal that in breast cancer, miR-26b targets *PRKAR2B* and may target *CCDC6* and *ULK1*.

Of the five predicted targets for miR-195, there were significant negative correlations between miR-195 expression and expression of *CCDC6, E2F7* and *GNAI3* expression supporting the proposal that miR-195 targets these three genes.

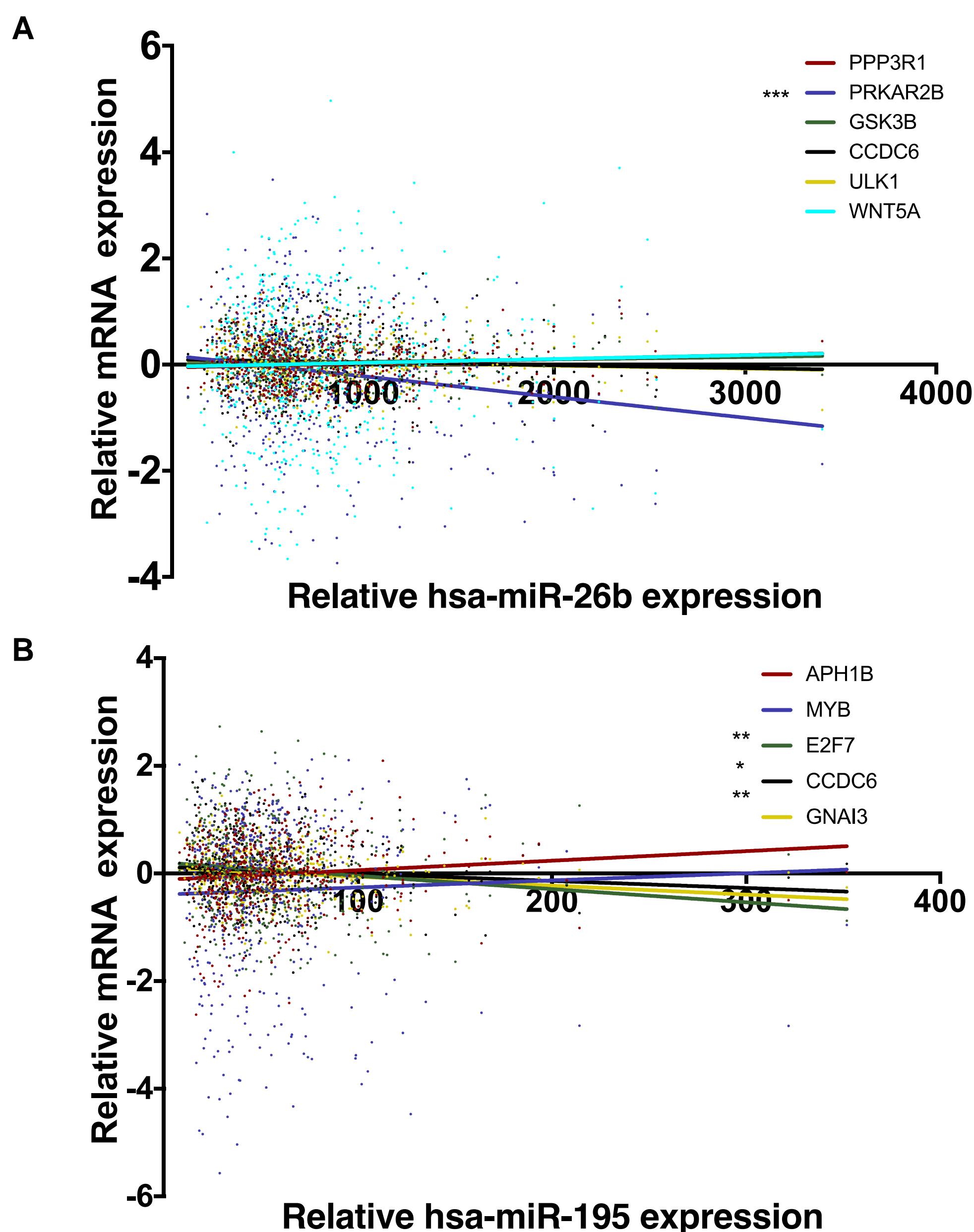


Figure 4.3.13: Correlations between miR-26b and miR-195 expression and expression of their predicted mRNA targets

Expression data for miRNAs and mRNAs of interest in breast cancers were accessed from a public dataset (TCGA, 2012) by Dr J Thorne (University of Leeds). Data were obtained from matched miRNA sequencing and Agilent mRNA expression arrays respectively from 489 breast cancer tumours. The x axes show expression levels of the miRNA of interest (relative read depth) whereas the y axes show expression levels (gene-median centered and log_2 transformed) of the mRNAs of interest. (A) Pearson correlations between miR-26b expression and the six selected predicted mRNA targets (***p<0.0005). (B) Pearson correlations between miR-195 expression and the five selected predicted mRNA targets (*p<0.05, **p<0.005).

4.3.2.2 *CCDC6* is the only potential target gene that can be validated in MCF7 and MDA-MB-175 cells

In vitro assays were next used to assess whether the miRNA-mRNA pairs supported by significant negative correlations above were reproduced in cell lines. In addition, since *CCDC6* was to be tested as a target for miR-195, I also tested whether it was a target for miR-26b even though this latter relationship was not significant in Figure 4.3.13. MCF7 and MDA-MB-175 cells were transfected with either miRNA mimics or inhibitors or their controls as previously, and RNA was extracted seventy-two hours later. Expression levels of mRNAs were determined using RT-qPCR (Figure 4.3.14 and Figure 4.3.15). A confirmed canonical miRNA target would be down-regulated by the presence of the miRNA mimic, and up-regulated by the miRNA inhibitor.

In MCF7 cells, *PRKAR2B* expression was not significantly altered by over-expression or inhibition of miR-26b. Similarly, expression of neither *E2F7* or *GNAI3* was significantly altered by over-expression or inhibition of miR-195. However, data concerning *CCDC6* looked more promising: *CCDC6* expression was reduced by mimics of both miRNAs (significantly for miR-26b) and was increased by inhibitors of both miRNAs (significantly for miR-195).

The same experiments were also performed in MDA-MB-175 cells. Results (Figure 4.3.15) were more variable but did suggest that *CCDC6* is a potential target of miR-26b in these cells. However, results also appeared to demonstrate that *CCDC6* is not targeted by miR-195 in MDA-MB-175 cells, unlike in the MCF7 cells.

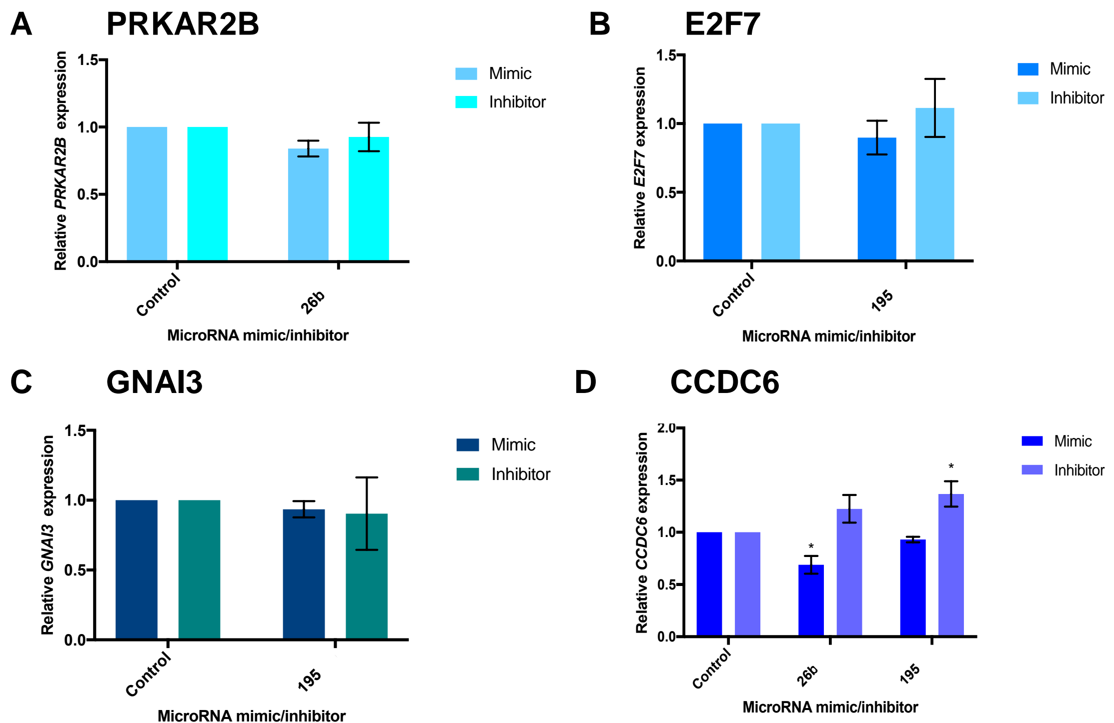


Figure 4.3.14: *CCDC6* is a target of miR-26b and miR-195 in MCF7 cells but *PRKAR2B, E2F7* and *GNAI3* are not

MCF7 cells were transfected with either the miRNA mimic or inhibitor of interest or appropriate controls. Cells were harvested seventy-two hours post-transfection and RNA extracted followed by RT-qPCR to quantify expression of the potential miRNA targets of interest. These graphs show three biological repeats and error bars show SEM (*p<0.05). (A) *PRKAR2B* expression following transfection with either miR-26b mimic or inhibitor. (B) *E2F7* expression following transfection with either miR-195 mimic or inhibitor. (C) *GNAI3* expression following transfection with either miR-195 mimic or inhibitor. (D) *CCDC6* expression following transfection with either miR-26b or miR-195 mimic or inhibitor.

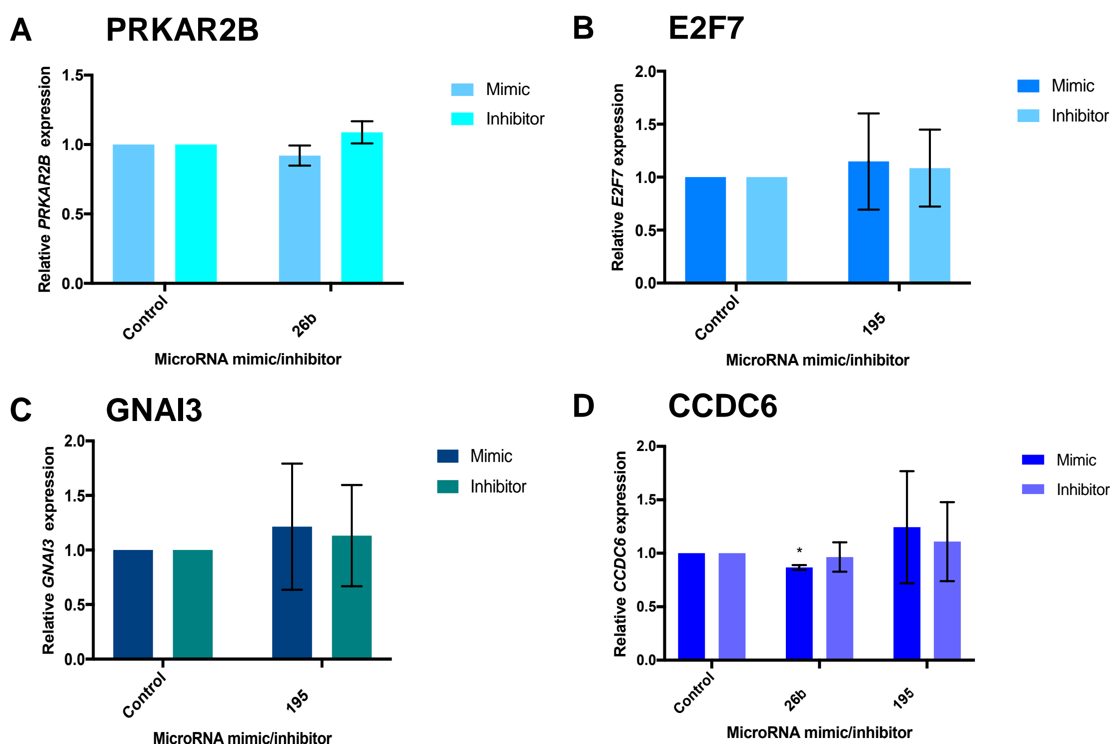


Figure 4.3.15: *CCDC6* appears to be targeted by miR-26b but not miR-195 in MDA-MB-175 cells, while *PRKAR2B, E2F7* and *GNAI3* are not targeted

MDA-MB-175 cells were transfected with either the miRNA mimic or inhibitor of interest or appropriate controls. Cells were harvested seventy-two hours post-transfection and RNA extracted followed by RT-qPCR to quantify expression of the potential miRNA targets of interest. These graphs show two biological repeats and error bars show SEM (*p<0.05). (A) *PRKAR2B* expression following transfection with either miR-26b mimic or inhibitor. (B) *E2F7* expression following transfection with either miR-195 mimic or inhibitor. (C) *GNAI3* expression following transfection with either miR-195 mimic or inhibitor. (D) *CCDC6* expression following transfection with either miR-26b or miR-195 mimic or inhibitor.

4.3.2.3 CCDC6 expression does not negatively correlate with miR-26b and/or miR-195 expression in MCF7 and T47D epirubicin resistant cell lines

As *CCDC6* appeared to be a potential target of miR-26b and miR-195, *CCDC6* expression was quantified in the MCF7 and T47D epirubicin resistant cells in which I had already shown that both of these miRNAs were overexpressed (Figure 4.3.11). RNA was extracted from the epirubicin resistant and parental control cell lines and *CCDC6* expression in resistant cells relative to parental control cells was determined by RT-qPCR (Figure 4.3.16). Expression of this gene was not significantly different in the MCF7 resistant cell line compared to

its matched control, but was significantly *increased* in the T47D resistant cell line. These results suggest that although *CCDC6* may be targeted by either or both of miR-26b and miR-195, this influence is not the main determinant of relative *CCDC6* expression and decreased expression of this gene does not contribute to chemoresistance in these two epirubicin resistant lines.

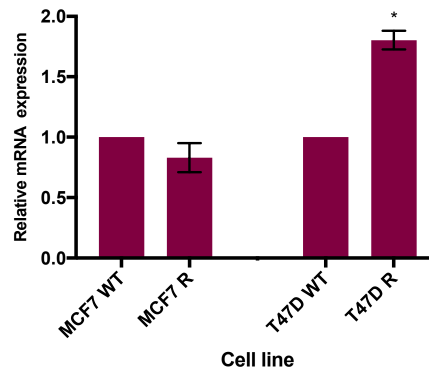


Figure 4.3.16: *CCDC6* is not differentially expressed in epirubicin resistant cell lines in accordance with a role downstream of miR-26b or miR-195 in chemoresistance

CCDC6 expression was quantified by RT-qPCR in two ER positive epirubicin resistant (R) breast cancer cells relative to expression in sensitive parental cells (WT). This graph shows two biological repeats and error bars show SEM (*p<0.05).

4.3.2.4 *CCDC6* does not modify chemoresponse in MCF7 cells

Although *CCDC6* was not significantly down-regulated in the stable epirubicin resistant cell lines and therefore its down-regulation was seemingly not contributing to this stable resistance, it remained possible that down-regulation of *CCDC6* was contributing to the miR-26b or miR-195 induced transient resistance seen in Figure 4.3.3, Figure 4.3.4 and Figure 4.3.6. In addition, *CCDC6* has previously been linked with chemoresponse (Morra et al., 2015). Therefore, siRNA against *CCDC6* was used to knock-down expression and chemosensitivity assays were performed to determine whether this decreased expression would result in increased resistance of breast cancer cells to epirubicin, thereby supporting miR-26b and miR-195 acting via *CCDC6*.

To confirm down-regulation of *CCDC6* by a targeted siRNA, MCF7 cells were first transfected with the siRNA or a non-targeting control. RNA was extracted seventy-two hours post-transfection and *CCDC6* expression was measured by

RT-qPCR (Figure 4.3.17). Results show that expression of *CCDC6* was successfully reduced by the siRNA by approximately 90%.

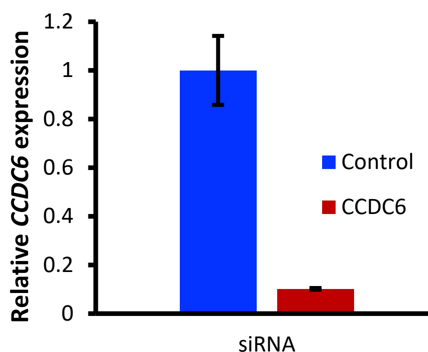


Figure 4.3.17: *CCDC6* was successfully inhibited by a targeted siRNA

MCF7 cells were transfected with a siRNA targeting *CCDC6* or a control non-targeting siRNA. RNA was extracted seventy-two hours post-transfection and *CCDC6* expression was quantified by RT-qPCR relative to expression in non-targeted siRNA transfected MCF7 cells. This graph represents one biological repeat with error bars showing +/- SD of three technical replicates.

To assess chemosensitivity, MCF7 cells were transfected with the siRNA targeting *CCDC6* or a non-targeting control, and forty-eight hours post-transfection were treated with epirubicin for twenty-four hours. Response to epirubicin was then assessed by performing either MTT assays or colony forming assays as previously (Figure 4.3.18).

Results from both MTT assays and colony forming assays indicated that MCF7 breast cancer cells with decreased expression of *CCDC6* had almost exactly the same sensitivity to epirubicin treatment as their control transfected counterparts – with no suggestion of resistance.

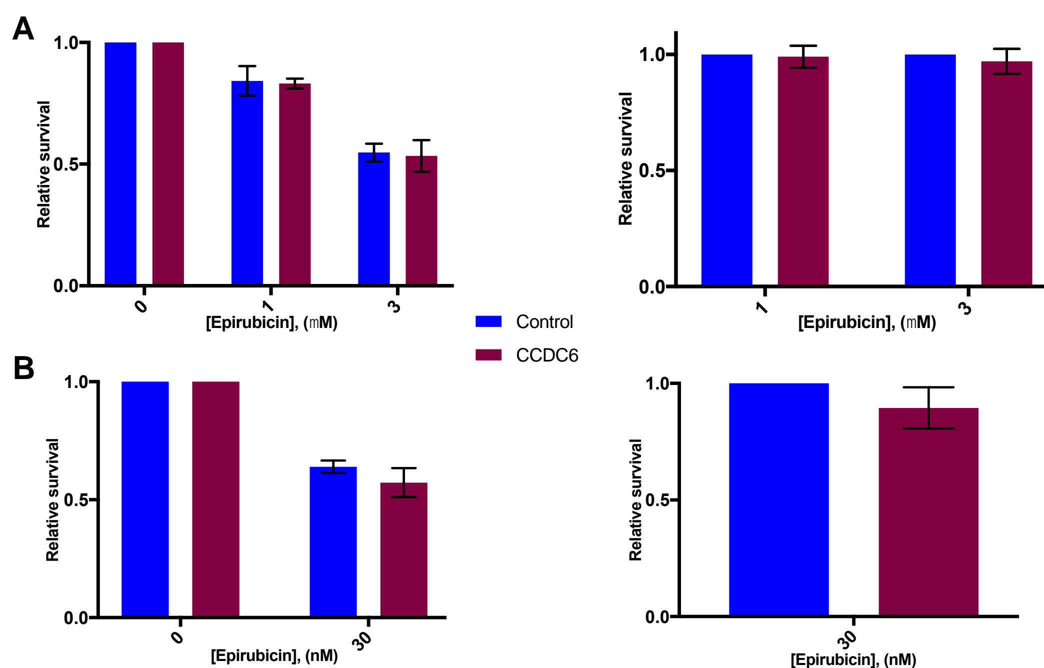


Figure 4.3.18: Reduced *CCDC6* expression is not sufficient to induce chemoresistance in MCF7 cells

MCF7 cells were transfected with siRNAs targeting *CCDC6* or control non-targeting siRNAs. Forty-eight hours post-transfection, cells were treated with either 1µM or 3µM epirubicin for MTT assays or 30nM epirubicin for clonogenic survival assays for twenty-four hours. MTT assays (A) or clonogenic survival assays (B) were then performed. These graphs show the results of two biological repeats and error bars show the standard error of the mean. (A) Each sample was first normalised to its untreated counterpart (left panel), then the *CCDC6* siRNA transfected samples were normalised to the control transfected samples for each concentration of epirubicin in order to determine the effects of *CCDC6* knockdown on chemosensitivity (right panel). (B) Colonies were counted and numbers were normalised to each untreated sample (left panel), or relative survival after epirubicin treatment in *CCDC6* siRNA transfected samples was normalised to relative survival in control siRNA transfected samples after treatment with epirubicin (right panel).

4.4 Discussion

4.4.1 Mimics and/or inhibitors of specific miRNAs have successfully been used to investigate chemosensitivity

In this chapter I have used mimics and inhibitors of my three miRNAs of interest, as identified in the screens performed in section 0, to investigate their roles in chemoresponse. This was performed by transfecting ER positive breast cancer cells with these mimics and inhibitors followed by treatment with

epirubicin. This was the same chemotherapeutic drug that was used to treat the patients in the cohort used for the initial screens (see Chapter 3). Then, by assessing the cell viability and clonogenic ability of the cells, I successfully identified miR-26b and miR-195 as contributors to chemoresistance when up-regulated. This was in accordance with data obtained from the initial screens performed in section 0 where expression of these two miRNAs was increased post-NAC compared with pre-NAC in partially chemoresistant breast cancers.

My use of miRNA mimics and/or inhibitors to investigate roles for miRNAs in chemosensitivity of cancer cells has a few highly-related published precedents. A mimic for the miRNA let-7a was used to investigate *in vitro* what effect increased expression of this miRNA would have on the sensitivity of epirubicin resistant HER2 positive breast cancer cells to epirubicin (Wu et al., 2015). It was noted that following transfection of this mimic into these drug resistant cells, the IC50, determined by MTT cell viability assays, was significantly less than their control transfected counterparts (1.53µg/ml compared with 2.25µg/ml). Although this shift in IC50 is comparatively small, compared to the difference between parental and stably resistant cell lines in my work, it should be noted that I have not formally assessed IC50s after transient transfections with mimics and inhibitors; the differences I have seen in this context by MTT or colony forming survival assays have generally been small and could be of similar magnitude. Let-7a was previously identified as a miRNA of interest in breast cancer as it was noted to be rarely expressed in breast tumour initiating cells but was more highly expressed in differentiated breast tumour cells (Yu et al., 2007). As this population of tumour initiating cells is generally known to be more chemoresistant than their differentiated counterparts, the authors hypothesised that let-7a expression may be reduced in patients with chemoresistant tumours when compared to those with chemosensitive tumours and that modulation of let-7a levels may influence chemoresponse (Wu et al., 2015). In my dataset, let-7a was not consistently changed across all patients and was therefore not investigated further.

A second example is the use of both mimics and inhibitors of miR-195, the exact same miRNA I have tested, to investigate the role of miR-195 in the chemosensitivity of ER positive breast cancer cells to adriamycin (Yang et al., 2013). This study showed that miR-195 mimic reduced cell viability following

adriamycin treatment in both MCF7 wild-type and adriamycin resistant cells, as measured by MTT assays. Increased miR-195 expression had a greater effect on cell viability in MCF7 wild-type cells compared with resistant cells. However, when expression of this miRNA was reduced by the inhibitor, no corresponding increase in cell viability was observed. This is in marked contrast to my study, where I found miR-195 mimic to protect MCF7 cells from epirubicin, although my data concur that inhibitors appear to be less successful in inducing changes in response at least in short-term survival assays. This difference between the efficacy of mimics and inhibitors may relate to their differing abilities to induce fold changes in expression of the miRNA – with mimics commonly leading directly to much greater over-expression than the fold-inhibition of inhibitors (Figure 4.3.2). Having defined the role of miR-195 in chemoresponse, the authors then identified Raf-1 as a potential target of this miRNA, and observed increased protein expression in chemoresistant cell lines compared to parental cell lines. However, unlike my work, no attempts were made to test functionally whether the mRNA is a target of miR-195.

These two studies combined emphasise the fact that miRNAs can have different functions depending on context – in this case the chemotherapy agent being the difference. Adriamycin, also known as doxorubicin, is a member of the same chemical family as epirubicin, the anthracyclines. The only difference between these two drugs is a slight structural modification in epirubicin resulting in the reorientation of a hydroxyl group. However, multiple clinical trials have revealed that therapeutically, epirubicin is as effective as doxorubicin but that off-target toxicities are less harsh (Khasraw et al., 2012). It is evident that despite the fact that these two drugs are both anthracyclines and are therefore thought to have similar mechanisms of action, the seemingly minor structural alteration in epirubicin does appear to have far reaching effects. It is possible that miR-195 is one of the factors that responds differently to these slight differences in structure that ultimately leads to the differences in toxicity observed in patients.

Interestingly, in a previous study in which MCF7 cells were selected for resistance to individual chemotherapy drugs including doxorubicin and epirubicin, gene expression microarray analyses revealed more genes were differentially expressed in doxorubicin resistant cells relative to parental cells

than in epirubicin resistant cells (Hembruff et al., 2008). Analyses of expressions of various ABC drug transporters revealed that epirubicin resistant MCF7 cells, but not doxorubicin resistant cells, had increased expression of *ABCB1* whereas the latter had increased expression of *ABCC1*. This provides further evidence that doxorubicin and epirubicin affect different molecular pathways.

A final, highly-related example is an investigation into the role of miR-452 in adriamycin resistant MCF7 cells, in which a mimic and inhibitor were used to demonstrate that decreased expression of this miRNA was associated with resistance to adriamycin (Hu et al., 2014). The authors also identified IGF-1R as a target of miR-452 by using multiple prediction algorithms and observing transcript and protein expression in response to manipulation of miRNA levels. This is a similar method to my work to identify and confirm targets of miR-26b and miR-195, although I only considered transcript expression and not protein expression. As with let-7a, in my dataset, miR-452 was not consistently changed post-NAC across all patients and therefore I did not study it further.

4.4.1.1 Using colony forming assays to determine chemosensitivity

I also used miRNA mimics and inhibitors in the context of colony forming survival assays. Other studies have also previously used this method to determine the effect of particular miRNAs on chemoresistance, usually in parallel with MTT assays. In a study investigating the role of miR-27b in the chemoresponse of ER negative and HER2 positive breast cancer cells, both MTT and colony forming survival assays were used (Chen et al., 2018). In this study, the inhibition of miR-27b resulted in cells being more resistant to paclitaxel. Inhibition of this miRNA also resulted in increased resistance to cisplatin, doxorubicin, gemcitabine and fluorouracil, although this was only performed with MTT assays. The authors also identified *CBLB* and *GRB2* as targets of miR-27b using multiple prediction algorithms, as I have used, although they were confirmed as direct targets using luciferase assays instead of quantifying transcript expression in response to altered miRNA expression. In my dataset, miR-27b was not consistently changed across all patients post-NAC, therefore this miRNA was not studied in my work.

Similarly, a study investigating the role of miR-4443 in chemoresponse also used both MTT and colony forming survival assays (Chen et al., 2016b). In this study, transfection of the triple negative breast cancer cell line MDA-MB-231 with a miR-4443 mimic and treatment with epirubicin resulted in increased chemoresistance compared with their control transfected counterparts. This was shown with both MTT and colony forming survival assays, although the reciprocal experiment with a miR-4443 inhibitor was only performed with MTT assays. *TIMP2* was successfully identified as a target of miR-4443 using only a single predictive algorithm and quantification of transcript levels following manipulation of miRNA levels. MiR-4443 was not included on the panel used to quantify miRNA expression in the patient samples in my study.

In these two studies, where both MTT and colony forming survival assays were performed, results from the two assays showed the same changes in chemoresponse. In my dataset however, the measurement of cell viability using MTT assays following transfection with miRNA inhibitors did not show changes in chemoresponse whereas colony forming survival assays frequently did. However, when viewed together, the results from my MTT assays and colony forming assays indicated that miR-26b and miR-195 contribute to chemoresistance. This was further supported by the observation that expression of these miRNAs was also up-regulated in two epirubicin resistant cell lines. The fact that the MTT assays and the colony forming assays were also performed in two different cell lines using both mimics and inhibitors and that all results were consistent, suggests that these results are true indicators of the role these miRNAs contribute to chemoresponse in ER positive breast cancer. The other studies discussed here did not perform both assays using both mimics and inhibitors in multiple cell lines – and in this regard my study could be viewed as unusually thorough.

4.4.2 Predictive algorithms can successfully identify target mRNAs of specific miRNAs

Multiple algorithms exist that use various factors such as nucleotide sequences, the number of predicted binding sites and free energies of complexes to predict mRNA targets of miRNAs (Witkos et al., 2011). Combined with experimental CLIP-Seq (where mRNA fragments bound to argonaute

proteins that form part of the RISC and the associated miRNA are sequenced) data obtained from multiple studies to validate these predictions, these algorithms have demonstrated that of the hundreds of conserved miRNAs and protein coding genes, there are several thousand potential interactions between miRNAs and their target mRNAs (Li et al., 2014b).

Some studies use only a single predictive algorithm coupled with data such as associations between miRNA expression and observed pathway activity obtained previously to identify putative target genes of specific miRNAs. In one such study, miR-26b was found to suppress TNFα-induced NF-κB signalling in hepatocellular carcinoma cells. In order to identify target mRNAs that may mediate this phenomenon, the predictive algorithm TargetScan was used. Two genes, *TAK1* and *TAB3*, which are upstream positive regulators of the NF-κB pathway, were predicted to have putative miR-26b binding sites in their 3'UTRs. Validation of these genes as targets of this miRNA was performed using luciferase reporter assays. Sequences containing the wild-type 3'-UTRs or mutated 3'-UTRs (where potential binding sites were removed) were co-expressed with the miRNA, and levels of endogenous proteins and transcripts after up- or down-regulation of the miRNA were assessed. Very interestingly, in this case target protein levels were significantly reduced when miR-26b expression was increased, whereas transcript levels were not affected (Zhao et al., 2014). This is in contrast with results I have obtained as I have shown that increased expression of miR-26b leads to a significant reduction in the level of *CCDC6* transcript. This observation suggests that not only do miRNAs have different functions depending on context but that their mechanism of action may also differ. As such, previous studies have also observed a down-regulation of target mRNA expression when miR-26b expression was increased (Gennarino et al., 2009).

The contrast between miR-26b affecting transcript levels versus protein levels does highlight the risk presented by the strategy that I have used – observing the effects of miRNA expression on potential target mRNA levels only and not protein levels as well. I thus risked identifying false negative targets that are not affected at the transcript level but are functionally targeted at the protein level. This has previously been observed not just with miR-26b but also miR-519 (Abdelmohsen et al., 2008) and miR-19b (Thorne et al., 2018), which were

shown to affect HuR and P-glycoprotein protein levels respectively but not transcript levels.

However, it is more common to investigate putative target mRNAs that are predicted by multiple predictive algorithms. Four databases were used in a study to identify mRNA targets of miR-27b (Chen et al., 2018). These were miRanda, PicTar, TargetScan and miRDB. Even focusing only on those genes predicted by all four algorithms, 277 mRNAs were putative targets. Therefore, the authors performed functional analyses using KEGG and Gene Oncolcogy (GO) databases to narrow down the list of genes of interest. Most target genes functioned in cancer-related pathways and drug-response pathways. Of the thirteen top candidates, the transcript levels of five genes were found to be down-regulated by miR-27b in further validations. Further analyses were performed in which mRNA levels in tumour tissues and matched normal tissues were compared, which led to the identification of *CBLB* and *GRB2* as high priority candidate targets. Expression of these two genes was significantly higher in tumour tissues than in normal tissues and there was a significant negative correlation between miR-27b expression and *CBLB* and *GRB2* expression in tumour tissues of breast cancer patients. Similarly, I focused on genes predicted by five different algorithms: TargetScan, miRanda, PicTar2, PITA and RNA22 together with miRNA and mRNA expression data, all presented in the starBase v2.0 database (Yang et al., 2011, Li et al., 2014b). I then performed literature searches to identify those genes that may play a role in contributing to chemoresponse, and then investigating further only genes of interest with significant negative correlations between transcript expression and miRNA expression in clinical breast cancers using public datasets. The final stage was *in vitro* validation by assessing whether potential target transcript levels were modified by over-expression or inhibition of miRNA expression, as was performed in the previous study (Chen et al., 2018). As such, I identified *CCDC6* as a target of both miR-26b and miR-195.

4.4.3 *CCDC6*: a function in chemoresponse?

I have shown that *CCDC6* is targeted by both miR-26b and miR-195 and therefore was potentially a mediator of their roles in chemoresponse, however reduction of *CCDC6* expression alone did not appear to reproduce the altered

chemosensitivity seen with over-expression of miR-26b or miR-195. *CCDC6* was first identified as part of a fusion gene with the protooncogene *RET* in papillary thyroid carcinoma (Fusco et al., 1987, Grieco et al., 1990). *CCDC6* has since been shown to be involved in the DNA damage response, specifically in the ATM pathway. In the event of ATM activation, *CCDC6* is phosphorylated, which ultimately promotes apoptosis (Cerrato et al., 2018). It has previously been noted that attenuation of *CCDC6* confers sensitivity to PARP inhibitors in prostate cancer cells (Morra et al., 2017) as well as in non-small cell lung cancer and confers resistance to cisplatin (Morra et al., 2015). I therefore considered *CCDC6* to be a strong candidate regulator of epirubicin resistance in my experiments. However, in the context of my study, this does not appear to be the case, although I cannot rule out that changes in its expression may contribute to the resistance seen with over-expression of these miRNAs only in the context of the other expression changes they also cause.

4.4.4 Resistance to one class of chemotherapeutic drug does not always result in resistance to a different class of drug

My results suggest that epirubicin resistance does not alter the chemosensitivity of T47D cells to docetaxel. However, in MCF7 cells, results suggest that instead of epirubicin resistant cells being more resistant to docetaxel, they are actually more sensitive to the second chemotherapeutic drug (Figure 4.3.12). These data indicate that resistance to one class of chemotherapeutic drug does not necessarily indicate resistance to other classes of chemotherapeutic drugs with other mechanisms of action.

Such a phenomenon has previously been observed in MCF7 cells (Wang et al., 2014). In this study, the response of MCF7 cells resistant to docetaxel ($MCF7_{TXT}$) or doxorubicin ($MCF7_{DOX}$) were compared to parental MCF7 cells in the presence of docetaxel, paclitaxel or doxorubicin. As expected, $MCF7_{TXT}$ cells were more resistant to docetaxel and paclitaxel and $MCF7_{DOX}$ cells were more resistant to doxorubicin. However, $MCF7_{DOX}$ cells were not resistant to either taxane, indeed these cells were actually slightly more sensitive to these drugs than parental MCF7 cells. Similarly, $MCF7_{TXT}$ cells were not resistant to doxorubicin. The fact that such resistance mechanisms do not necessarily overlap, strongly support the typical clinical practice of combining

chemotherapy agents, either concurrently or in sequence (Masui et al., 2013). This has the benefit of targeting multiple cell processes, thus decreasing the possibility of cells being able to develop resistance to multiple agents instead of one. An important clinical consideration however is that the wrong combinations and/or doses of agents may prove too toxic for patients.

An example where multiple therapies has proved to be the most successful is in the treatment of HER2 positive breast cancer. Before the development of HER2-targeted therapies, HER2 expression was considered a marker of poor prognosis and was treated with chemotherapy (Muss et al., 1994). With the development of the monoclonal antibody trastuzumab that targets HER2, it became common clinical practice to combine HER2-targeted therapies with chemotherapy when treating HER2 positive breast cancer after it had been shown that addition of this targeting drug was associated with numerous benefits including increased disease-free survival and longer survival when compared with chemotherapy alone (Slamon et al., 2001). This highlights the benefits of targeting multiple cell processes at the same time instead of only a single process.

4.4.5 Conclusions

I have determined that up-regulation of miR-26b and miR-195 contribute to resistance to the chemotherapeutic drug epirubicin in ER positive breast cancer but that down-regulation of miR-10a does not. I have attempted to identify mRNA targets of miR-26b and miR-195 that function to contribute to this chemoresistance. I identified *CCDC6* as a target of both miR-26b and miR-195, however down-regulation of this gene was not sufficient to reproduce the altered chemosensitivity, and its expression profile in constitutively resistant cell lines did not implicate it as a key mediator of resistance in this context. In the next chapter, I undertake alternative experimental approaches to identify the targets downstream of miR-26b and miR-195, with a view to thereby gaining understanding of the mechanisms by which these miRNAs define chemoresponse.

Chapter 5: *REEP4* and *SEMA6D* are targets of miR-26b and miR-195 and may contribute to chemoresponses in breast cancer

5.1 Abstract

MiR-26b and miR-195 were identified as potential mediators of chemoresponse in breast cancer cells in Chapters 3 and 4. Attempts were also made to identify mRNA targets of miR-26b and miR-195 that contribute to this function, by using predictive algorithms and public miRNA and mRNA expression datasets. This approach, however, did not allow successful identification of any candidate genes with relevant functions in chemoresponse. Therefore, in this chapter I have taken an unbiased approach to identifying the targets of these miRNAs, by performing a pulldown assay using biotinylated miRNA mimics of miR-26b and miR-195 as bait, and RNA-Seq to identify RNAs bound to the target miRNAs. Putative mRNA targets of these miRNAs were identified by assessing pulldown of transcripts relative to control pulldowns, and by taking into account relative expression in the input samples based on the hypothesis that true targets may show reduced overall expression in miRNA mimic-transfected samples. Potential target genes were also prioritised if they were predicted by a minimum of two different prediction algorithms to be targets of these miRNAs. Finally, a literature search was performed on candidate genes to identify those that potentially contribute to chemoresistance when down-regulated.

Four genes were identified as candidates suitable for further experimental follow up: *REEP4* and *PRKCD* as targets of miR-26b and *SEMA6D* and *ARL2* as targets of miR-195. Cell viability assays were performed following knockdown of these genes and treatment with chemotherapy. *REEP4* and *SEMA6D* knockdown contributed to increased chemoresistance in accordance with roles in chemoresponse down-stream of the miRNAs, but *PRKCD* and *ARL2* knockdown did not. However, analysis of REEP4 and SEMA6D protein expression in a cohort of breast cancer patients that had received adjuvant chemotherapy revealed a significant, but unclear relationship between REEP4 expression and patient survival, and no significant relationships between SEMA6D expression and patient survival.

I concluded that further experimentation is required to define the targets of miR-26b and miR-195 that are relevant to their roles in defining chemoresponse in breast cancer.

5.2 Introduction

Aberrant expression of miRNAs has been observed in cancer (Lu et al., 2005), with more recent evidence showing that aberrant expression of specific miRNAs such as miR-298 in breast cancer influence chemoresponse (Bao et al., 2012). However, when searching for novel drug targets to overcome chemoresistance, it could be potentially dangerous to target miRNAs directly due to the fact that individual miRNAs have the potential to bind multiple mRNA targets (Asangani et al., 2008, Dong et al., 2011, Wang et al., 2011). It is possible that in targeting the expression of a specific miRNA, not only could the expression of mRNAs that contribute to chemoresistance be affected, but the expression of other mRNAs with different functions could also be affected, potentially leading to unforeseen negative consequences.

Evidence has also shown that the same mRNA can be targeted by multiple miRNAs, such as miR-19 and miR-21, which target *PTEN* (Liang et al., 2011, Wang et al., 2011). This poses a second challenge to targeting miRNAs directly as altering expression of one miRNA may not significantly alter target mRNA expression if a second miRNA can also regulate its expression. These two potential challenges demonstrate the importance of identifying the target mRNAs of aberrantly expressed miRNAs that contribute appropriately to chemoresponse when identifying novel drug targets to overcome chemoresistance.

Algorithms that predict mRNA targets of miRNAs are constantly being developed to reflect new discoveries involving the relationships between these two RNA species, in order to be more accurate. However, predicted mRNA targets still require experimental validation. Multiple methods have been proposed to either prove or discover new miRNA:mRNA binding partners (Thomson et al., 2011). These include immunoprecipitation of RISC components, with target mRNAs being co-immunoprecipitated, with or without prior cross-linking of RNA to RNA-binding proteins by ultra-violet radiation

(Hendrickson et al., 2008, Hafner et al., 2010). Another method proposed to show direct miRNA:mRNA binding involves the use of synthetic biotinylated miRNAs transfected into cells and performing a pulldown, followed by identification of any mRNAs that were pulled down with the miRNA (Ørom et al., 2008). There are of course advantages and disadvantages associated with all of these methods (Thomson et al., 2011).

Identification of mRNA targets of a specific miRNA by using a synthetic biotinylated miRNA as bait, performing a pulldown, and identifying RNA transcripts by RNA-Seq, has previously been used for miR-522 (Tan et al., 2014). Over-expression of this miRNA had been observed in triple-negative breast cancer, with increased expression associated with poor prognosis. To identify how miR-522 contributed to this phenomenon, a synthetic biotinylated mimic of miR-522 was transfected into the triple negative breast cancer cell line MDA-MB-468, a pulldown performed, and RNA-Seq performed to identify the transcripts bound to the miRNA bait. To confirm results obtained from the pulldown experiment, microarrays were also performed using samples where miR-522 was either over-expressed or not. There was a large overlap between mRNAs identified by the pulldown method and those down-regulated when miR-522 expression was increased. To discover the functions of miR-522, pathway analysis tools were used to identify biological interactions and functions of the genes. This led to the conclusions that miR-522 regulates cellular processes such as proliferation, migration and epithelial-mesenchymal transition, which explain why over-expression of this miRNA was associated with a poor prognosis. In this chapter, a similar approach was taken to identify mRNA targets of miR-26b and miR-195 that contribute to chemoresponse. I have used synthetic biotinylated mimics of these miRNAs, performed a pulldown, and identified bound transcripts using RNA-Seq. I then attempted to validate functional roles for potential targets using chemosensitivity assays *in vitro*, and assessing correlations between expression and cancer outcomes in patients.

5.2.1 Specific objectives

There were three key objectives in the work described in this chapter:

1. To identify mRNA targets of miR-26b and miR-195 by performing pulldown assays using biotinylated-miRNA mimics as bait and performing RNA-Seq on the bound mRNAs;
2. To determine the function of these target mRNAs with respect to chemoresistance;
3. To assess the clinical importance of the proteins encoded by these target mRNAs in breast cancer patients treated with chemotherapy.

5.3 Results

5.3.1 RNA was successfully recovered using miR-26b and miR-195 mimics as baits, and samples were prepared for RNA-Seq

In Chapter 4, I demonstrated that miR-26b and miR-195 were regulators of chemotherapy response in breast cancer cells, but I failed to identify the mRNA targets of the miRNAs that were responsible for these functional effects. My aim here was to identify the targets by purifying and sequencing the mRNAs bound by the miRNAs. This involves transfecting relevant cells with miRNA mimics tagged with biotin at the 3' end, pulling down the biotinylated miRNAs and subsequently performing RNA-Seq to identify RNA molecules bound to the miRNAs. Previous studies have successfully identified target mRNAs of miRNAs using this method (Krishnan et al., 2013, Tan et al., 2014). Therefore, biotinylated miRNA mimics of miR-26b and miR-195 were obtained.

5.3.1.1 Biotinylated microRNA mimics affect chemosensitivity in a similar manner to their non-biotinylated counterparts

Prior to performing pulldown experiments using biotinylated miRNA mimics, chemosensitivity assays were performed using MCF7 cells transfected with these new biotinylated mimics and treated with epirubicin to confirm that the addition of the biotin tag did not affect the function of these mimics in this context (previously shown with unbiotinylated mimics in Figure 4.3.3). MCF7 breast cancer cells were transfected with either the biotin-tagged miRNA mimic or a biotin-tagged mimic control. Cells were treated with epirubicin for twenty-

four hours and MTT assays performed (Figure 5.3.1). Data supported the conclusion that the cells transfected with biotinylated miR-26b or miR-195 mimics were more resistant to chemotherapy than controls, as was the case for the unbiotinylated mimics, and therefore that the biotin tag had no or little effect on miRNA function, although it should be noted that the resistance seen in this experiment was not statistically significant after the two repeats shown.

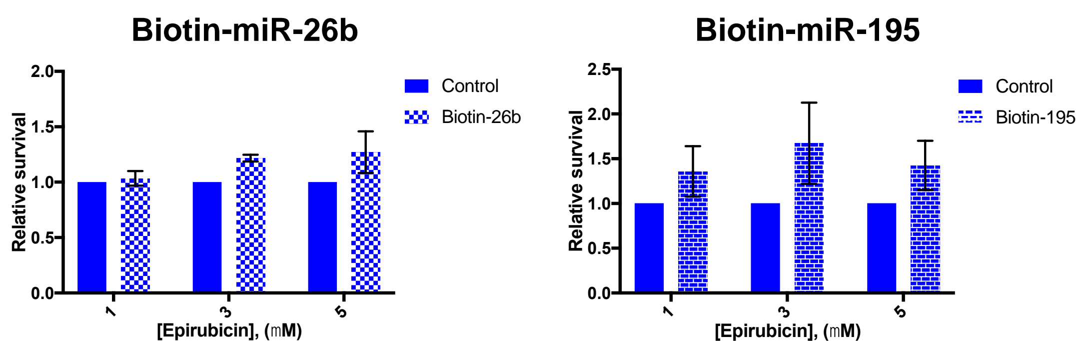


Figure 5.3.1: Biotinylated miRNA mimics cause increased resistance to chemotherapy

Cells were transfected with either biotinylated miR-26b, miR-195 or negative control mimics. Forty-eight hours post-transfection, cells were treated with epirubicin or vehicle control (water). Twenty-four hours later, MTT assays were performed. Data for epirubicin-treated samples were first normalised to untreated counterparts, then the biotinylated miRNA mimic transfected samples were normalised to the control transfected samples for each concentration of epirubicin, in order to determine the effects of the biotinylated miRNA mimic on chemosensitivity. This graph shows the results of two biological repeats +/- SEM.

5.3.1.2 RNA was successfully pulled down using biotinylated miRNA mimics as bait

Having shown that the addition of the biotin tag to the miRNA mimics did not obviously inhibit the function of these mimics with respect to chemoresistance, MCF7 cells were transfected with the biotinylated mimics of either miR-26b or miR-195 or biotinylated controls. Twenty-four hours later, the cells were harvested, a pulldown assay was performed using streptavidin-coated beads to capture the biotinylated miRNA mimics, samples were washed, and total RNA was extracted. Also, a small amount of transfected and lysed cells were not used in the pulldown, in order to allow extraction of total RNA in the cells, which represents the input RNA for the pulldown. RNA was then quantified using the Qubit High Sensitivity Assay and fluorometer (Table 5.3.1).

Table 5.3.1: Total RNA extracted from MCF7 cells transfected with biotinylated miRNA mimics before and after a pulldown assay was performed

Sample	MiRNA	RNA (μg)
Input	**Control**	8.76
	26b	10.22
	195	9.64
Pulldown	**Control**	0.122
	26b	0.102
	195	0.113

MCF7 cells were transfected with biotinylated mimics of either miR-26b or miR-195 or a control. Twenty-four hours later, cells were harvested. A small volume of lysate (50μl from a total of approximately 2ml) from each sample was kept separate and total RNA extracted directly as an input sample. The remaining lysate was added to streptavidin-coated beads and the mixture left to bind. Beads were collected, washed, and the pulled-down RNAs were then extracted. RNA was quantified using the Qubit High Sensitivity Assay and fluorometer.

A minimum of 0.1μg total RNA was required for RNA-Seq protocols leading to analysis by the HiSeq 3000 Sequencing System. The RNA extracted from all samples was therefore sufficient to perform the desired analyses.

5.3.1.3 Libraries of sufficient quantity and quality were successfully prepared for RNA-Seq

Having extracted a sufficient quantity of RNA for the desired analyses, libraries of the samples were prepared for RNA-Seq. Since miRNAs have previously been shown to interact with not only mRNAs but other non-coding RNA species as well (Jalali et al., 2013, Li et al., 2014b), polyA selection, which selects only mRNAs with polyA tails, was not performed. Therefore, only rRNA depletion was performed prior to library preparation, so as to minimise the influence of an overabundance of these rRNAs during subsequent analyses. This was performed on the pulldown samples and the input samples. Once the libraries had been prepared, samples were analysed to ensure sufficient quantities and qualities. A minimum of 3.5ng cDNA was required and single populations with fragment lengths of 200-400 base pairs. These parameters were assessed using a Qubit fluorometer (Table 5.3.2) and an Agilent TapeStation automated analysis machine respectively. Representative graphs showing library-prepared

samples from input and pulldown samples are shown in Figure 5.3.2, with remaining graphs shown in Appendix E.

Table 5.3.2: Total cDNA after rRNA removal and library preparation

<table>
<tr><th>Sample</th><th>MiRNA</th><th>cDNA (ng)</th></tr>
<tr><td rowspan="3">Input</td><td>Control</td><td>261.9</td></tr>
<tr><td>26b</td><td>122.4</td></tr>
<tr><td>195</td><td>197.1</td></tr>
<tr><td rowspan="3">Pulldown</td><td>Control</td><td>6.08</td></tr>
<tr><td>26b</td><td>3.62</td></tr>
<tr><td>195</td><td>10.68</td></tr>
</table>

Total RNA extracted from MCF7 cells transfected with biotinylated miRNA mimics was used to make libraries for RNA-Seq analysis. First, RNA samples underwent rRNA removal to remove the overabundance of these RNA species, and then cDNA synthesis was performed. 3’ ends were then adenylated and adapters ligated onto the ends in order to distinguish between samples by adding unique labels, before the cDNA samples were amplified. The final products were quantified using the Qubit High Sensitivity Assay and fluorometer.

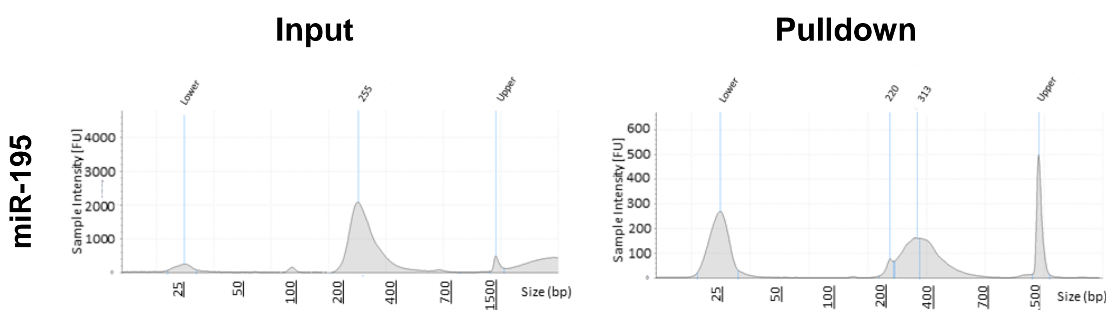


Figure 5.3.2: Representative graphs showing the fragment populations of library prepared samples

Libraries that were quantified in Table 5.3.2 were also analysed using the Agilent 2200 TapeStation using the High Sensitivity DNA ScreenTape to ensure that only library cDNA fragments were present in the samples and no primer dimers were present. The x-axis shows fragment length (base pairs) and the y-axis shows abundance. The ‘lower’ and ‘upper’ peaks at 25nt and 1500nt are markers used as internal references. The left panel shows the library prepared from total RNA extracted from cells transfected with the miR-195 mimic that did not undergo the pulldown process (input) and the right panel shows the library prepared from RNA extracted following the pulldown process (pulldown).

Having confirmed that libraries of sufficient quantity (minimum 3.5ng, see Table 5.3.2) and quality (populations with fragment lengths of 200-400 base pairs,

see Figure 5.3.2) had been prepared, samples underwent paired end RNA sequencing using the Illumina HiSeq 3000 sequencing platform.

5.3.2 Potential targets of miRNAs were successfully identified following RNA-Seq analysis

5.3.2.1 Analysis of RNA-Seq data using the MACS2 algorithm successfully identified cancer-related genes

Analysis of RNA-Seq data was performed using multiple methods. In the first method, the Model-based Analysis of ChIP-Seq algorithm version 2 (MACS2) (Zhang et al., 2008) was used to identify enriched regions of sequences present in the miRNA bait pulldown samples compared with the control pulldown sample. Pulldown samples were also compared with input samples, to account for biases such as pulldown efficiency and reads sequenced that also originate from other areas of the genome. Thus, input samples were used as normalisation samples, such that for each transcript, the number of reads in the pulldown sample was normalised to the number of reads in the input sample. For each miRNA, the top 100 most abundant sequences in the pulldown samples compared with the control pulldown sample and compared with the input samples were selected. These sequences represented the most abundant transcripts, rather than corresponding genes. As a brief validation method, the genes encoding these transcripts were analysed by the pathway enrichment analysis software Reactome (Fabregat et al., 2017) to observe whether they may contribute to chemoresponse. Table 5.3.1 shows that several targets of both miR-26b and miR-195 are involved in the cell cycle, DNA repair and DNA replication pathways as well as gene expression (transcription) and metabolism of proteins, thus suggesting that the methodology has led to identification of cancer-relevant genes, which may contain contributors to chemoresistance – with DNA repair being of particular potential relevance to response to DNA-damaging chemotherapy agents. All 100 sequences, representing 80 genes as miR-26b mRNA targets, and 86 genes as miR-195 mRNA targets, were included in further analyses.

Table 5.3.3: Selected pathways and the number of genes identified as potential targets of miR-26b and miR-195 that function in them

Pathway	Number of miR-26b mRNA targets	Number of miR-195 mRNA targets
Cell Cycle	12	13
DNA Repair	7	2
DNA Replication	1	3
Gene Expression (Transcription)	22	30
Metabolism of Proteins	27	42

Genes identified as the top 100 sequences targeted by miR-26b and miR-195 were analysed by the pathway enrichment analysis software Reactome. Pathways where multiple miRNA targets were involved were identified, with a focus on those that may contribute to the response of cells to chemotherapy.

5.3.2.2 Analysis of RNA-Seq data using the DESeq package combined with comparisons with MACS2 results and predictive algorithms showed several genes in common with multiple methods of analysis

In the second method, the input and pulldown samples were initially analysed separately. Differential expressions between samples derived from biotinylated miRNA transfected cells and the samples derived from biotinylated control transfected cells were calculated for both input and pulldown samples, using a R package called DESeq (Anders, 2010). To narrow down the number of targets for further investigation, thresholds were applied. Both fold differences and p value parameters were considered. These p values were calculated taking into account several factors including fold differences and number of reads in each sample, although it should be noted this statistical assessment is still based on only one biological sample. Calculations were performed using the parameters suggested by the creators of the DESeq package (Anders, 2010) when only one repeat was performed. Initially, for the pulldown comparisons, the threshold for minimum fold difference was 100, and the threshold for significance was $p<0.05$. For the input samples the threshold for fold difference applied was 0.7, thereby selecting as candidate genes those that were down-regulated by more than this cut off after transfection with the mimics, and the threshold for significance was $p<0.1$. Interestingly, there were

no transcripts that met both thresholds for fold change and significance within the pulldown samples or within the input samples, therefore subsequent analyses were performed separately for those transcripts that met one of these thresholds. The numbers of genes that met each of the above criteria are shown in Table 5.3.4.

Table 5.3.4: Number of genes that complied with chosen criteria

MiR-26b (pulldown)	**No. of genes**	**MiR-26b (input)**	**No. of genes**
Fold difference >100	787	Fold difference <0.7	8523
p<0.05	86	p<0.1	0
MiR-195 (pulldown)	**No. of genes**	**MiR-195 (input)**	**No. of genes**
Fold difference >100	787	Fold difference <0.7	8530
p<0.05	134	p<0.1	63

Following differential expression analysis of pulldown and input samples, lists of genes were compiled that met the chosen criteria of minimum and maximum fold differences and p values.

Comparisons were then made between transcripts identified by the MACS2 algorithm, and those downregulated in input samples (whether those with a maximum fold difference of 0.7 or p<0.1), and upregulated in pulldown samples (whether those with a minimum fold difference of 100 or p<0.05) to identify any that were in common. In Chapter 4 (section 4.3.2.1), I used the multiple predictive algorithms included in Starbase in attempts to identify putative mRNA targets of miR-26b and miR-195 – different numbers of gene targets were predicted depending on whether I set requirements that individual genes should be predicted by 2, 3, 4 or all 5 specific algorithms available. The comparisons between transcripts identified by MACS2 and those identified by DESeq were also compared with those identified by these predictive algorithms, first to corroborate whether transcripts identified in my experiment may be true targets of miR-26b and miR-195, and secondly to narrow down the list of potential genes of interest. For this reason, transcripts identified by MACS2 and those identified by DESeq were initially compared with those genes predicted by 2, 3, 4 and 5 algorithms. Following this, the number of predictive algorithms used in these comparisons depended on the number of genes common with the other three methods. The aim of these analyses was to

initially identify a small number of genes (up to 10 genes of interest) that could be searched in the literature for any links with chemoresponse.

Multiple comparisons were made between genes identified as up-regulated in the pulldown samples with those down-regulated in the input samples, those predicted by between 2 and 5 predictive algorithms, and the top 100 transcripts identified by MACS2. As noted before, there were no genes in common in the pulldown samples between those with a minimum fold difference of 100 and those with $p<0.05$, and no genes in common in the input samples between those with a maximum fold difference of 0.7 and those with $p<0.1$. Therefore, separate comparisons were made for each of these thresholds. There were no genes in common between those representing the top 100 transcripts identified by MACS2 and those with a minimum fold difference of 100 for potential targets of miR-26b, and only one gene in common for miR-195, which was *C12orf60*.

Following these comparisons between two groups, further comparisons were made between three separate groups, with focuses largely on those genes identified in the pulldown samples and those predicted by a variable number of algorithms, with a preference for those predicted by a larger number of algorithms. The focus on genes identified in the pulldown samples was due to the fact these should only have been present in the sample if they were attached to the miRNA bait. The focus on genes predicted by as large a number of predictive algorithms as possible was due to the fact that, in principle, genes predicted by numerous algorithms would have a larger probability of being real targets of the miRNA of interest. Combined with those genes identified in the pulldown samples, this method was thought to be an effective method of narrowing down the number of potential targets, such that the majority of these would be true targets. Since not all mRNAs are down-regulated by their targeting miRNAs, and since MACS2 used a different method for analysing data compared with DESeq, both the genes identified as down-regulated in the input samples and those representing the transcripts identified by MACS2 were used as the third comparison group separately. This was an attempt to avoid removing true targets identified by one of these methods from the list of genes for final consideration.

To narrow down the list of potential targets of interest even further, four comparisons were made using all four methods. As mentioned previously,

there were no genes identified by MACS2 that were also identified in the pulldown samples with a minimum fold difference of 100 for miR-26b and only one for miR-195, therefore all comparisons with four groups were performed using genes identified in the pulldown samples with $p<0.05$.

5.3.2.3 Final comparisons between different methods of analysis showed seven genes of interest as potential targets of miR-26b and miR-195

The final multiple comparisons made were designed to identify genes identified commonly by all four methods (Figure 5.3.3, top panels), which represent genes highly likely to be true targets of their targeting miRNAs, and those identified by three methods (Figure 5.3.3, bottom panels), which represent targets with a slightly lower but still high probability of being true targets. The comparisons made between three groups were done such as to avoid identifying the same targets identified by the comparisons between all four groups, therefore either the criteria for the pulldown identification was varied between fold change or p value thresholds, and/or the number of Starbase algorithms included was altered. For this reason, the comparisons made for targets of miR-26b and miR-195 were not the same. The final comparisons made between three groups were chosen as these identified a small number of genes of interest for further investigation. The combination of comparisons with four groups and with three groups finally led to the identification of three genes as targets of miR-26b and four genes as targets of miR-195 (within the central overlaps in Figure 5.3.3, and listed in Table 5.3.5).

Figure 5.3.3: Final comparisons made between multiple methods of identifying miRNA target genes of interest

These Venn diagrams show the numbers of genes identified by up to four different methods. Comparisons were made using genes predicted to be targets of miR-26b and miR-195 by the predictive algorithms included in Starbase (numbers in brackets indicate how many algorithms predicted those targets), differentially expressed genes identified in the input samples (with a fold reduction in expression of at least 0.7), differentially expressed genes identified in the pulldown samples (either where $p<0.05$ or a minimum fold change of 100), and genes identified by the MACS algorithm (version 2). Those genes identified by all shown methods were shortlisted for further investigation.

Table 5.3.5: List of potential miRNA targets of interest, and brief details of published data relating to roles in cancer biology and/or chemoresponse

Gene	Evidence for role in cancer biology and/or chemoresponse	No. of analyses in which gene was identified
Potential miR-26b targets		
REEP4	Microtubule binding protein required for cell division (Schlaitz et al., 2013). Under-expressed in cancer (Hornstein et al., 2008, Doyen et al., 2014).	4
PRKCD	Tumour suppressor. Functions as pro-apoptotic protein during DNA damage-induced apoptosis (Liu et al., 2007).	3
BLOC1S2	Expression promotes apoptosis, decreased expression observed in multiple cancers (not tested in breast cancer) (Gdynia et al., 2008).	3
Potential miR-195 targets		
CHAC1	Elevated expression associated with a poor outcome in breast cancer (Goebel et al., 2012).	4
CCNE1	Down-regulated in breast cancer cells resistant to doxorubicin (AbuHammad and Zihlif, 2013). Amplification observed in residual chemoresistant breast tumours post-NAC (Balko et al., 2014).	4
ARL2	Reduced expression in breast cancer cells results in proliferative advantage (Beghin et al., 2009). Expression has been associated with chemosensitivity in breast cancer (Beghin et al., 2008).	4
SEMA6D	Tumour suppressor function suggested. High expression has been associated with a favourable outcome in breast cancer (Chen et al., 2015).	3

Following identification of likely targets of miR-26b and miR-195, a literature search was performed to determine whether these genes may contribute to chemoresponse. Published data concerning the functions of these genes are summarised in Table 5.3.5. Of these genes, *REEP4* and *PRKCD* seemed to be promising targets of miR-26b that may contribute to chemoresponse. *REEP4* was chosen because it is required in cell division, a process that is targeted by chemotherapy. *PRKCD* was chosen because of the evidence of its function as a pro-apoptotic protein during DNA damage-induced apoptosis, since one of the targets of the chemotherapy agent used in this project is DNA replication. *BLOC1S2* was not chosen because of the fact that although expression of this

gene was decreased in multiple cancers, it had not been observed in breast cancer. *ARL2* and *SEMA6D* seemed to be promising targets of miR-195 that may contribute to chemoresponse. *CHAC1* was not chosen due to the fact that elevated expression was associated with a poor outcome in breast cancer, whereas as a target of a miR-195, decreased expression would be expected to be associated with a poorer outcome. *CCNE1* was not chosen due to the conflicting evidence between increased and decreased expression in chemoresistant breast cancers. *ARL2* and *SEMA6D* were both chosen due to the evidence suggesting lower expression would result in poorer outcomes in breast cancer.

5.3.3 Confirming mRNA targets of miR-26b and miR-195 and their functions in chemoresponse

5.3.3.1 Increased miR-26b expression significantly decreased *REEP4* transcript levels, and decreased miR-195 expression significantly increased *SEMA6D* transcript levels

Having identified two potential mRNA targets of miR-26b and miR-195 each, an initial validation step was performed. Although *PRKCD* was not identified as down-regulated post-miR-26b mimic transfection in the input sample, the other mRNA targets were identified in the pulldown samples as well as identified as down-regulated post-miRNA mimic transfection in the input samples. Therefore, it was likely that the up-regulation of these miRNAs in ER positive MCF7 cells using miRNA mimics would lead to down-regulation of the target transcripts. I tested this by transfecting MCF7 cells with miRNA mimics or inhibitors or relevant controls, extracting RNA and performing RT-qPCR to investigate target mRNA expression (Figure 5.3.4). The effect of the miR-26b mimic or inhibitor on *PRKCD* expression was also tested as a change in expression may have been present in the sample analysed by DESeq that did not meet the required criteria.

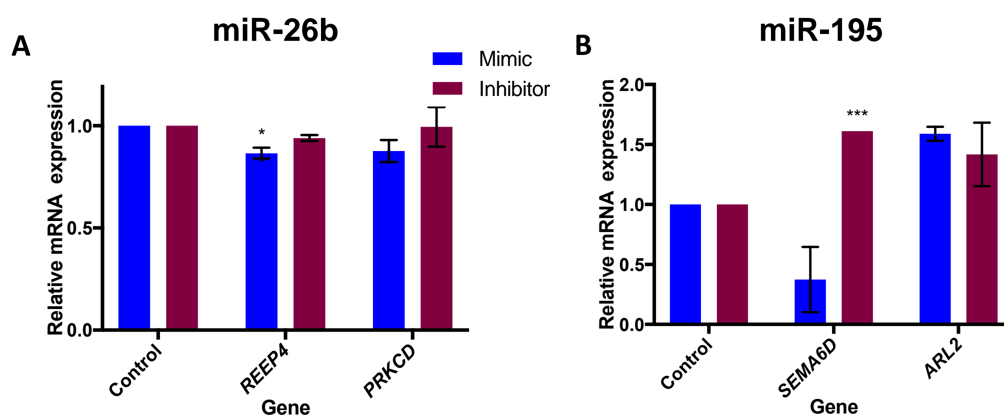


Figure 5.3.4: *REEP4* and *SEMA6D* are targeted by miR-26b and miR-195 respectively but not *PRKCD* and *ARL2*

MCF7 cells were transfected with either the miRNA mimic or inhibitor of interest or appropriate controls. Cells were harvested seventy-two hours post-transfection and RNA extracted followed by RT-qPCR to quantify expression of the potential miRNA targets of interest. (A) Gene expressions of *REEP4* and *PRKCD* following transfection with either miR-26b mimic or inhibitor. This graph shows three biological repeats and error bars show SEM (*p<0.05). (B) Gene expressions of *SEMA6D* and *ARL2* following transfection with either miR-195 mimic or inhibitor. This graph shows two biological repeats and error bars show SEM (***p<0.0005).

REEP4 showed a significant decrease in expression when miR-26b was up-regulated, and no change in expression when this miRNA was down-regulated. *SEMA6D* showed a decrease in expression after transfection with the miR-195 mimic, and a significant increase in expression when miR-195 was inhibited. Whereas, neither *PRKCD* or *ARL2* expression changed significantly in the presence of either miR-26b mimic or inhibitor, or miR-195 mimic or inhibitor respectively. These data provided support for the hypothesis that *REEP4* and *SEMA6D* were regulated by miR-26b and miR-195 respectively, but did not support that *PRKCD* and *ARL2* were so regulated, although it should be noted that miRNAs do not always impact on the mRNA expression levels of their targets.

5.3.3.2 Expression of miRNA target genes in epirubicin resistant MCF7 and T47D cells

In Chapter 4 (section 4.3.1.7), I described the development of two epirubicin resistant ER positive breast cancer cell lines. In both resistant cell lines, miR-26b and miR-195 were both up-regulated (see Figure 4.3.11). Since I was attempting to identify mRNA targets of miR-26b and miR-195 that contribute to

this chemoresistance, I investigated expression of my four putative targets in these epirubicin resistant cells in comparison to the parental lines with standard epirubicin sensitivity, with the expectation that true targets of the miRNAs that were contributing to the chemoresistance they cause would be down-regulated in the resistant lines. Total RNA was extracted from MCF7 and T47D parental and epirubicin resistant cells and expression of the genes of interest were determined by RT-qPCR (Figure 5.3.5). Data showed that in MCF7 cells, expressions of *REEP4* and *PRKCD* were not changed in resistant cells compared with parental cells, whereas *SEMA6D* and *ARL2* both had decreased in expression in resistant cells, although these decreases were not significant. In T47D cells, however, all transcripts were over-expressed in epirubicin resistant cells compared with parental cells, with significant increases for *REEP4* and *PRKCD*.

For the potential miR-26b targets *REEP4 a*nd *PRKCD*, these data provide no evidence of potential roles down-stream of miR-26b in chemoresistance, since the genes were not down-regulated in the resistant lines. For the potential miR-195 targets *SEMA6D* and *ARL2*, the result is less clear, with down-regulation in resistant MCF7 cells supporting a role in resistance down-stream of miR-195, but the up-regulation in T47D being compatible with these genes being regulated by different unrelated mechanisms in this line. Data from these two cell lines highlight the presence of large differences between different cell lines, even those representative of the same cancer subtype.

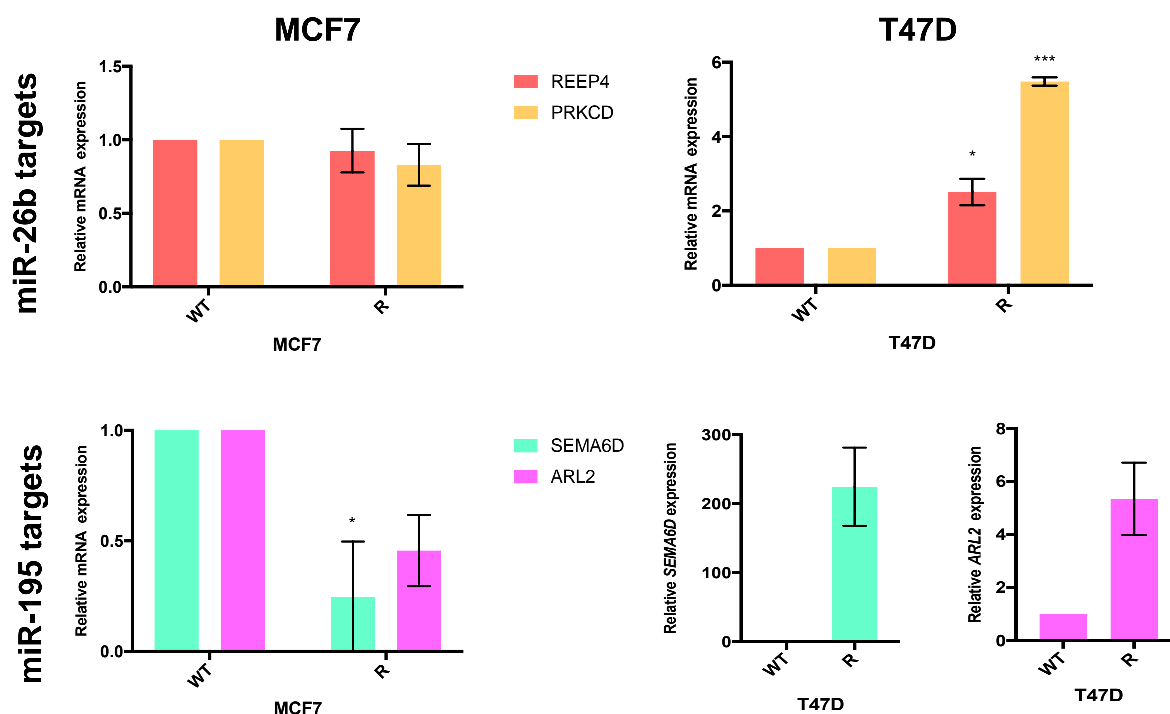


Figure 5.3.5: *REEP4, PRKCD, SEMA6D* and *ARL2* are differentially expressed in epirubicin resistant cell lines

Total RNA was extracted from MCF7 (left panel) and T47D (right panel) epirubicin sensitive (WT) and resistant (R) cells and RT-qPCRs were performed to determine expression of *REEP4* and *PRKCD,* putative targets of miR-26b (top panels) and *SEMA6D* and *ARL2,* putative targets of miR-195 (bottom panels). These graphs show two biological repeats and error bars show SEM (*p<0.05, ***p<0.0005).

5.3.3.3 Knockdown of *SEMA6D* caused increased chemoresistance

As increased expression of miR-26b and miR-195 was observed in cells surviving chemotherapy (Chapter 3, section 0) and was also observed to contribute to chemoresistance *in vitro* (Chapter 4, section 4.3.1), decreased expression of their target mRNAs that are relevant for this function may also lead to increased chemoresistance. To observe whether down-regulation of the genes identified by the pulldown assay was sufficient to induce chemoresistance, MCF7 cells were transfected with siRNAs targeting these genes. Forty-eight hours post-transfection, cells were treated with the chemotherapeutic drug epirubicin and chemosensitivity assays were performed (Figure 5.3.6).

Cells transfected with siRNAs targeting *SEMA6D* were significantly more resistant to chemotherapy at one dose of epirubicin than their control siRNA

transfected counterparts. Cells transfected with siRNAs targeting *REEP4* were also potentially more resistant to chemotherapy although this difference was not significant. The knockdown of *PRKCD* and *ARL2* however did not result in increased chemoresistance. There was no change in chemosensitivity of cells following transfection with a siRNA targeting *ARL2* suggesting that this gene did not contribute to chemoresponse in this context. However, there was a significant *increase* in chemosensitivity when cells were transfected with siRNAs targeting *PRKCD* followed by treatment with 1μM epirubicin, although this was not maintained following treatment with 3μM epirubicin.

These results supported the hypothesis that decreased expression of *SEMA6D* contributed to chemoresistance, and were in accordance with data from epirubicin resistant MCF7 cells, where expression of this transcript was also decreased (Figure 5.3.5). There was also a suggestion that decreased expression of *REEP4* might contribute to chemoresistance, although this result was not significant.

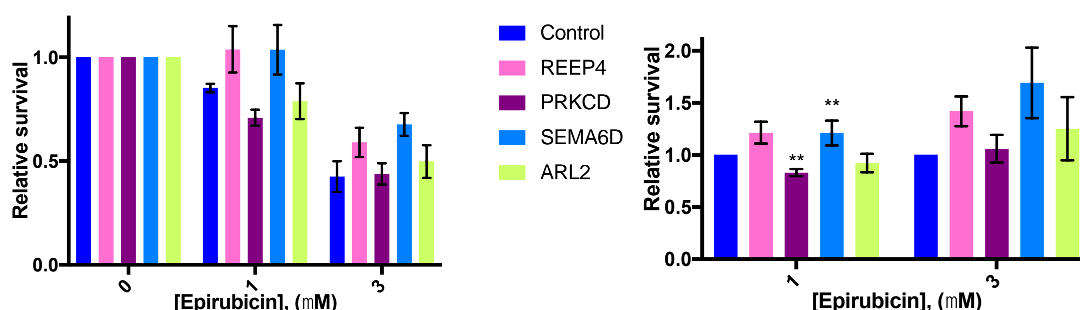


Figure 5.3.6: Knockdown of target mRNA genes caused altered chemosensitivity of MCF7 cells

MCF7 cells were transfected with siRNAs targeting the genes of interest (or negative control). Forty-eight hours post-transfection, cells were treated with two concentrations of epirubicin (1 or 3μM) or vehicle control (water). Twenty-four hours post-treatment, MTT assays were performed. Raw absorbance values were normalised to the control transfected and control untreated samples. The left panel shows the effects of epirubicin on overall survival whereas the right panel is normalised to allow focus on the effects of the targeted miRNA mimic or inhibitor on survival in epirubicin-treated samples. Two biological repeats were performed for each experiment, with error bars showing SEM (**p<0.005).

5.3.3.4 Knockdown of *REEP4* and *SEMA6D* but not *PRKCD* or *ARL2* caused increased colony forming ability

Colony forming assays were also performed to assess the impact of decreased expression of my four target mRNAs on chemoresistance, in order to confirm the results obtained in 5.3.3.3 in a longer term assay. As before, MCF7 cells were transfected with siRNAs targeting each of the four genes of interest or with a control siRNA. Forty-eight hours post-transfection, cells were treated with 30nM epirubicin or vehicle control for twenty-four hours before being seeded at a low density and cultured to allow colonies to grow. Colonies were then counted (Figure 5.3.7). As with the short term cell viability assays, knockdown of *SEMA6D* in MCF7 cells resulted in chemoresistance, shown by the ability to form significantly more colonies than the control transfected counterpart. Again, similarly to the short term assay, knockdown of *REEP4* also resulted in increased ability to form colonies although again this difference was not significant. Knockdown of *PRKCD* and *ARL2* again did not affect colony forming ability after epirubicin treatment.

These data further support the model that *REEP4*, as a target of miR-26b, and *SEMA6D*, as a target of miR-195, may mediate the increased chemoresistance observed on over-expression of these miRNAs.

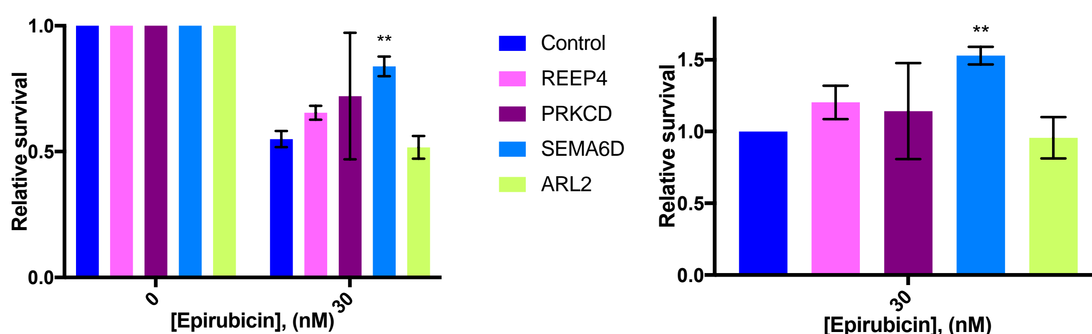


Figure 5.3.7: Knockdown of target mRNA genes altered colony forming ability of MCF7 cells

MCF7 cells were transfected with siRNAs targeting the genes of interest (or negative control). Forty-eight hours post-transfection, cells were treated with 30nM epirubicin or vehicle control (water) for twenty-four hours before being seeded at low density and left to grow for fourteen days. Colonies were counted. The left panel shows epirubicin-treated samples normalised to their untreated counterparts. The right panel shows the miRNA transfected and treated samples normalised to the control transfected and epirubicin-treated sample. Three biological repeats were performed, with error bars showing SEM (**$p<0.005$).

5.3.4 Expression of *REEP4* and *SEMA6D* in clinical breast cancer samples

Having identified *REEP4* and *SEMA6D* as target genes of miR-26b and miR-195 respectively that potentially contribute to the response of breast cancer cells *in vitro* to chemotherapy, further investigations were performed to determine whether expression levels of these two proteins provide prognostic or therapy predictive insights in patients. Therefore, tissue microarrays (TMAs) were constructed using breast cancer samples and immunohistochemistry (IHC) performed, followed by correlation tests to determine whether expression of the REEP4 and SEMA6D proteins correlated with disease-free survival (DFS) and/or overall survival (OS).

Breast cancer patients who could potentially be included in the study were identified by Ms Stacey Jones (Clinical Research Fellow, Leeds Teaching Hospitals NHS Trust / University of Leeds). Patients were included if they were diagnosed with primary breast cancer between 2005 and 2010, received no neoadjuvant systemic therapy, received adjuvant cytotoxic chemotherapy, and had complete follow-up data on DFS and OS at the time of sample selection. These criteria were chosen in order to allow for a wide variety of breast cancer subtypes, such that differences in protein expression between subtypes may be noted if present, and also so that the majority of breast cancer subtypes were represented. Only patients who received adjuvant chemotherapy were selected in order to test the impact of expression of these proteins on outcomes specifically after this therapy, although it should be noted that the patients received a wide variety of additional therapies including endocrine and HER2-targeted agents (see Table 5.3.6).

Once patients had been identified, tumour histology was examined, using haematoxylin and eosin (H&E) stained slides in order to assess whether the tissue itself was suitable for the planned analysis. This was important in terms of the presence of sufficient tumour epithelial cells since chemoresistance was attributed to altered expression of the genes of interest in these cells, although the presence of stromal cells within cores was not avoided. This procedure was also required to ensure three representative cores of tissue were available from regions of each tumour. Combining all of the above criteria, 305 patients and

their tumours were determined to be appropriate. Table 5.3.6 shows the clinico-pathological features of these patients and tumours.

Table 5.3.6: Clinico-pathology features of patients/tumours that were used in this study

Characteristic	Categories	Number (%)
Age Mean 52, range 25-74		
Positive receptor status	ER	212 (69.5%)
	PR	160 (52.5%)
	HER2	68 (22.3%)
Grade	1	16 (5.2%)
	2	113 (37%)
	3	161 (52.8%)
Number of lymph nodes involved	0	115 (37.7%)
	≥1	190 (62.3%)
Cytotoxic chemotherapy	EC-containing	300 (98.4%)
HER2-targeting therapy	Trastuzumab	68 (22.3%)
Endocrine therapy	Tamoxifen	126 (41.3%)
	Anastrozole	104 (34.1%)
	Others	19 (6.2%)
	None	92 (30.2%)

All patients were treated with adjuvant cytotoxic chemotherapy.

Once patients had been identified, three cores were taken from FFPE tumour blocks by Ms Stacey Jones (Leeds Teaching Hospitals NHS Trust / University of Leeds) to construct the TMAs. These TMAs were then stained by IHC using antibodies directed against the REEP4 and SEMA6D proteins.

5.3.4.1 Scoring of immunostained TMAs for REEP4 and SEMA6D

Once tissues had been immunostained, slides were viewed to determine the appropriate scoring methods for each antigen, taking advice from Dr Eldo Verghese (project supervisor and consultant breast histopathologist within Leeds Teaching Hospitals NHS Trust). Staining of both antigens was largely cytoplasmic in the epithelial cells, with approximately equal proportions of epithelial cells being stained in all cases. Scoring therefore consisted only of staining intensity for both antigens. Representative images of different staining intensities are shown in Figure 5.3.8.

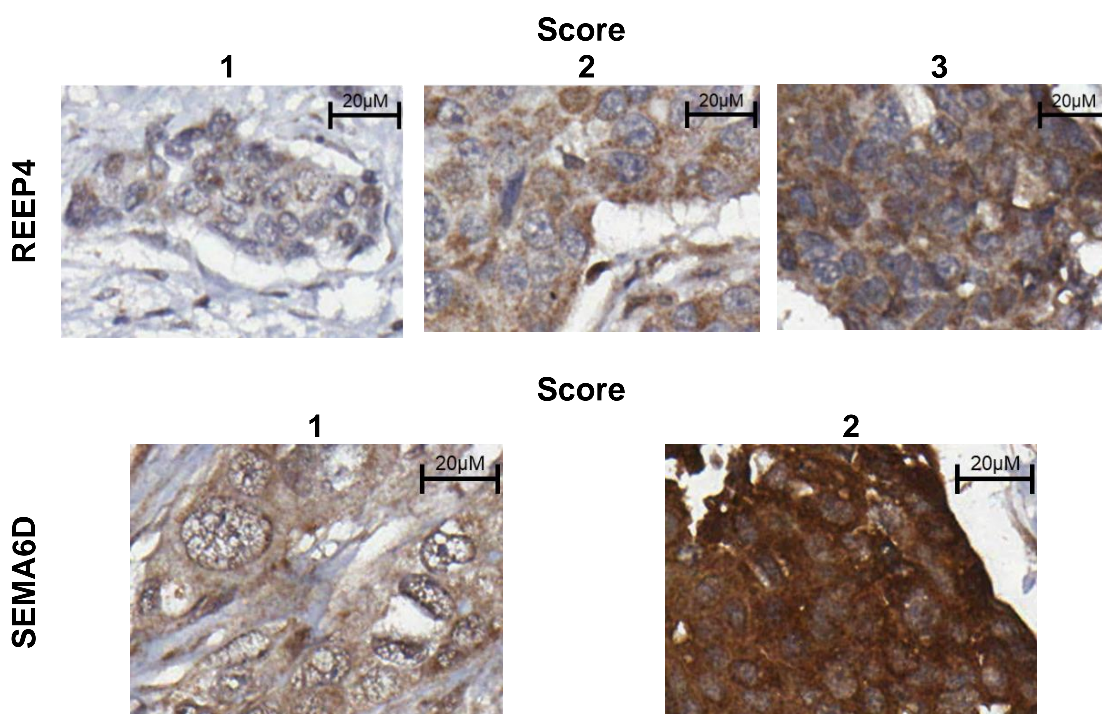


Figure 5.3.8: Representative images of staining intensities for REEP4 and SEMA6D

Representative images of tissues stained for REEP4 or SEMA6D (brown) and counterstained with haematoxylin (blue) at 40x magnification, with corresponding scores assigned on the basis of intensity staining.

Of the 915 cores representing 305 cancer cases, 765 cores were assessable for REEP4 and 773 cores were assessable for SEMA6D. Of the 305 cases, these represent 293 cases for REEP4 and 291 cases for SEMA6D. Reasons for cores being non-assessable included staining artefacts, absence of tumour epithelial cells in the tissue, or core loss.

5.3.4.2 Scoring concordance between scorers and within cases was moderate to high

To determine whether reproducible scores were assigned for each antigen, 42 cases were double scored independently by myself and Dr Eldo Verghese. Weighted kappa statistics were calculated to determine the degree of concordance between the two sets of scores. Weighted kappa values of 0.41-0.60 indicate moderate agreement, and values of 0.61-0.80 indicate substantial agreement (Landis and Koch, 1977), with actual values shown in Table 5.3.7. These values demonstrated moderate agreement between scorers for REEP4 and substantial agreement between scorers for SEMA6D.

Table 5.3.7: Weighted kappa values for each antigen with corresponding 95% confidence intervals (CI) values

Antigen	κ_w	95% CI
REEP4	0.55	0.41-0.68
SEMA6D	0.69	0.55-0.83

Variation between cores from the same cases was also determined by calculating Spearman's rho correlation coefficients. This was to determine the probability of selected cores being representative of the whole tumour with respect to antigen expression – poor correlations would suggest substantial heterogeneity within the tissue and would suggest use of TMAs could be problematic. For REEP4, 269 cases were assessed, and for SEMA6D, 264 cases were assessed, as these had a minimum of two assessable cores. Correlations for both antigens were strong and significant, indicating little variation within cases (Table 5.3.8). This indicated that scoring was highly likely to be representative of the whole tumour.

Table 5.3.8: Correlation coefficients indicate strong correlations between cores of the same cases for REEP4 and SEMA6D

Antigen	No. of cases	Correlation coefficient
REEP4	269	0.92*
SEMA6D	264	0.91*

Spearman's rho correlation coefficients were calculated for replicate cores for each antigen, where *$p<0.05$.

5.3.4.3 REEP4 and SEMA6D show variable expression in different breast cancer cases

To determine the frequency of expression scores for each antigen, the mean expression (rounded to the nearest whole number) was first calculated for each case from the cores available. Following this, the frequency of each expression score was then calculated (Figure 5.3.9). For both proteins, each available score was well represented, demonstrating that expression varied substantially across the cohort.

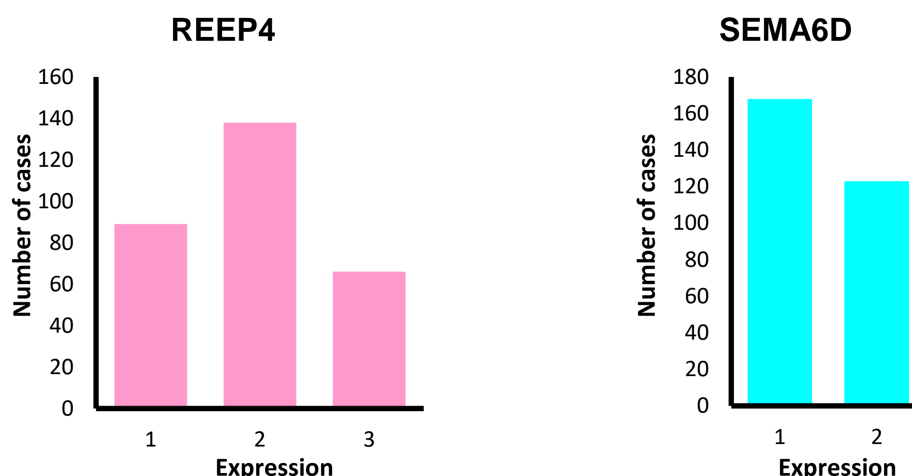


Figure 5.3.9: Frequencies of each expression score for REEP4 and SEMA6D show variable expression

Once cases had been scored for the expression of each antigen, the total number of cases were calculated for each expression group. The left panel shows the number of cases in each expression group for REEP4 and the right panel shows the number of cases in each expression group for SEMA6D.

5.3.4.4 Expression of REEP4 and SEMA6D does not correlate with prognostic factors

Correlations between expression of REEP4 and SEMA6D and prognostic factors were assessed. These prognostic factors included histological grade, ER status and presence of lymph node metastasis. Spearman's rho correlation coefficients were calculated for each factor with each antigen. Results are shown in Table 5.3.9 and showed no significant correlations between REEP4 or SEMA6D expression and any of the prognostic factors.

Table 5.3.9: Correlation coefficients (r) indicate no significant correlations between antigen expression and prognostic factors

Antigen		Lymph node metastasis	Histological grade	ER status
REEP4	r	-0.009	0.074	-0.104
	p	*0.874*	*0.204*	*0.077*
SEMA6D	r	0.042	-0.046	-0.002
	p	*0.472*	*0.433*	*0.978*

Spearman's rho correlation coefficients (r) were calculated for individual antigen expression and the prognostic factors. Prognostic factors included the presence of metastasis in at least one lymph node, histological grade of the tumour and ER status.

5.3.4.5 Expressions of REEP4 and SEMA6D correlate with each other

Although REEP4 and SEMA6D do not have the same functions, they do have one factor in common; in this project, *REEP4* and *SEMA6D* have been shown to be targeted by miR-26b and miR-195 respectively and both of these miRNAs were up-regulated post-NAC in patients who displayed partial resistance to chemotherapy, therefore it can be inferred that both *REEP4* and *SEMA6D* may have been down-regulated in these resistant cells. If so, a positive correlation between the expressions of these two proteins may exist. Therefore, the Spearman's rho correlation coefficient was calculated for the correlation between the two proteins. A moderate but statistically significant correlation coefficient of 0.494 was calculated ($p=4.97 \times 10^{-19}$). This suggests that in this context, there may be a relationship, perhaps in terms of co-regulation of gene expression, between the two proteins.

5.3.4.6 No significant differences are seen in disease free or overall survival between different expression values of REEP4 and SEMA6D

Although no correlations were observed between REEP4 and SEMA6D expression and various prognostic factors, Kaplan-Meier survival analyses were performed to determine whether expression of either of these proteins had a significant impact on disease free survival (DFS) or overall survival (OS). Log rank tests were performed to test the null hypotheses that there were no significant differences between the different expression groups of REEP4 and SEMA6D with respect to the probability of a recurrence (in the case of DFS) or death (in the case of OS). Mean survival times with 95% confidence intervals were also calculated. The graphs showing Kaplan-Meier survival curves are shown in Figure 5.3.10 and mean survival times are shown in Table 5.3.10.

Results show there were no significant differences between different expression groups of either protein for DFS or OS. There is a trend for patients with higher expressions of REEP4 to have a poorer outcome in terms of DFS (mean times of 4143 and 3595 days for the low and high groups respectively), but this was not significant ($p=0.051$). These results suggest that neither REEP4 nor SEMA6D have prognostic value in terms of DFS or OS in breast cancer.

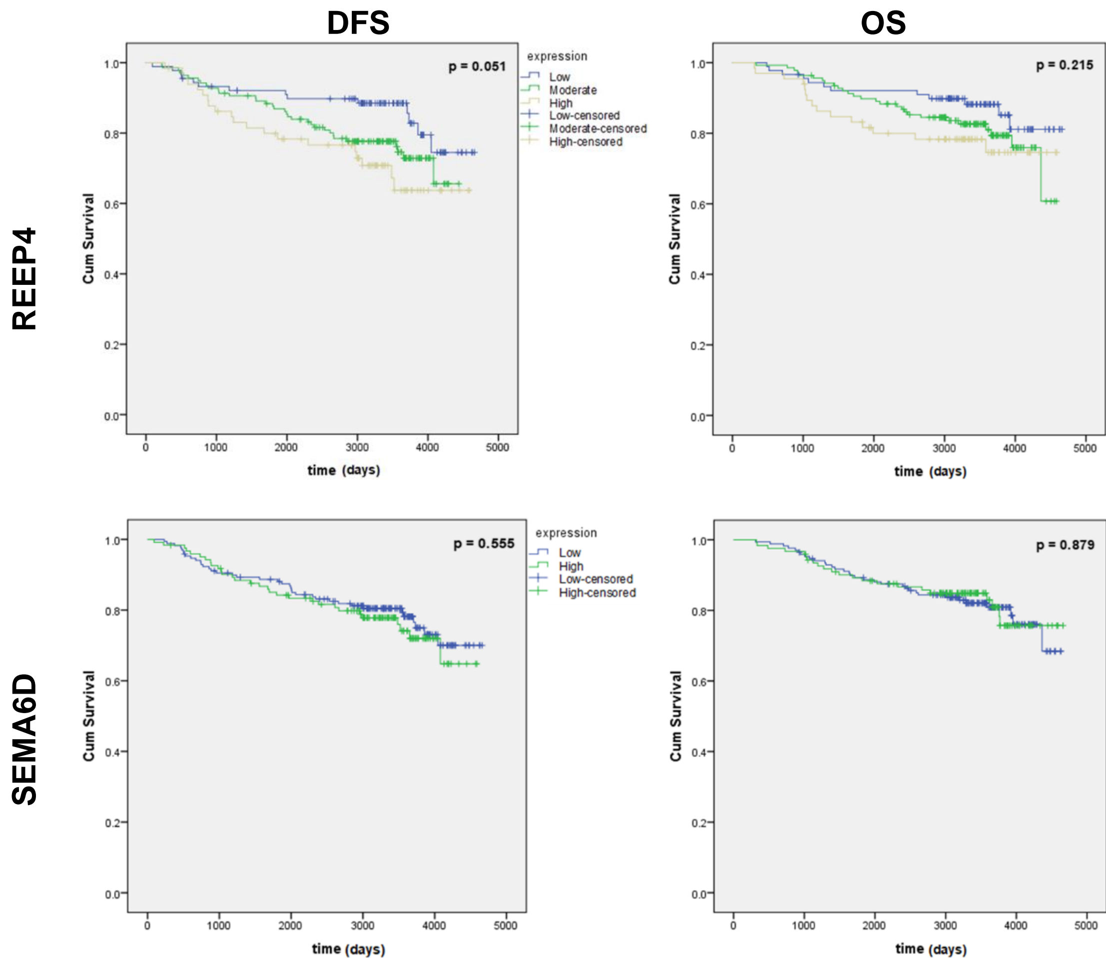


Figure 5.3.10: Kaplan-Meier survival curves showing DFS and OS for different expression groups of REEP4 and SEMA6D

Kaplan-Meier survival analyses were performed showing DFS (left panel) and OS (right panel) for low, moderate and high expression groups of REEP4 (top panel), and low and high expression groups of SEMA6D (bottom panel). The x-axis shows survival time in days and the y-axis shows cumulative survival.

Table 5.3.10: Mean DFS and OS times for different expression groups of REEP4 and SEMA6D show no significant differences

		DFS		OS	
		Mean (95% CI)	**p value**	**Mean (95% CI)**	**p value**
REEP4	Low	4143 (3887-4399)	0.051	4240 (4012-4469)	0.215
	Moderate	3726 (3510-3943)		4008 (3811-4206)	
	High	3595 (3223-3968)		3823 (3479-4168)	
SEMA6D	Low	3928 (3717-4139)	0.555	4040 (3855-4224)	0.879
	High	3793 (3540-4046)		4077 (3853-4302)	

Log rank tests were performed to compare DFS and OS times between different expression groups of REEP4 and SEMA6D. Mean survival times with 95% confidence intervals are shown for each group with significance values shown for each test.

5.3.4.7 Further analyses reveal significant differences in disease free survival between expression values of REEP4

Since there was almost a significant difference in DFS between the expression values for REEP4, and since REEP4 expression was divided into three groups, I tried to combine the three groups into two groups in an attempt to improve the statistical power of the analysis. To do this, I combined the moderate expression group with either the low expression group or with the high expression group. Kaplan-Meier survival analyses and log rank tests were then performed as before (see section 5.3.4.6). Mean survival times with 95% confidence intervals were also calculated. The graphs showing Kaplan-Meier survival curves are shown in Figure 5.3.11 and mean survival times are shown in Table 5.3.11.

Results showed significant differences between high and low expression groups of REEP4 for both combinations of groups, that is when the low and moderate groups were combined, and when the moderate and high groups were combined. The analysis with the combination of the low and moderate groups showed patients with lower expression of REEP4 to have a significantly better outcome in terms of DFS than those with high expression of this protein (mean times of 3990 and 3598 days for the low and high groups respectively;

p=0.042). The analysis with the combination of the moderate and high groups also showed patients with low expression of REEP4 to have a significantly better outcome in terms of DFS than those with higher expression of this protein (mean times of 4143 and 3765 days for the low and high groups respectively; p=0.036). These results suggest that REEP4 does have prognostic value in terms of DFS in breast cancer. However, these results were the opposite to expectations, when considering the fact that REEP4 is a target of miR-26b, which is up-regulated in chemoresistant tumours, therefore low expression of REEP4 would be associated with chemoresistance and therefore a poorer outcome after chemotherapy in terms of DFS.

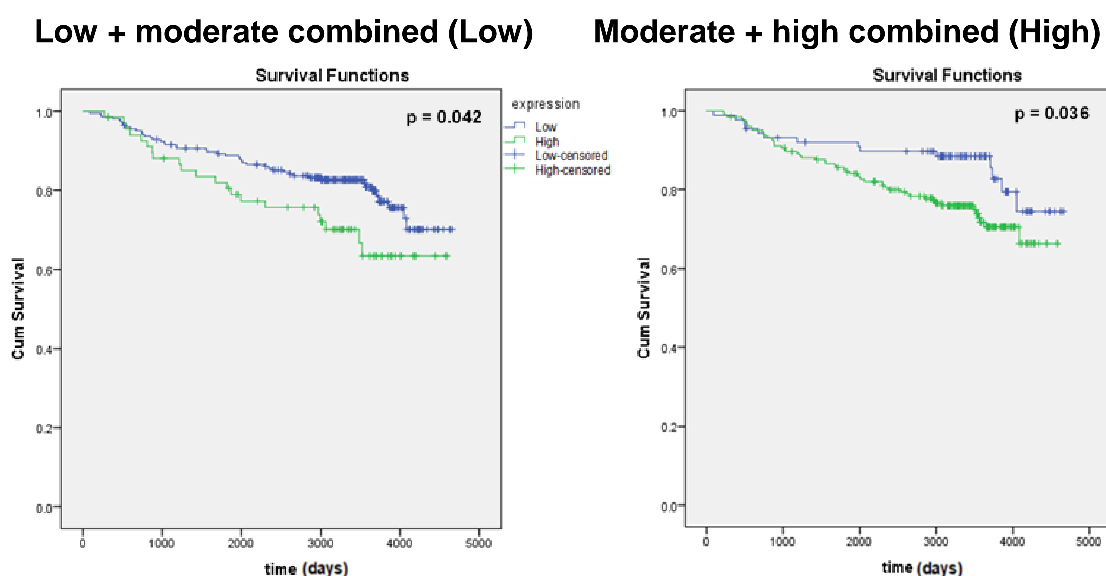


Figure 5.3.11: Kaplan-Meier survival curves showing DFS for the two combined expression groups of REEP4

Kaplan-Meier survival analyses were performed showing DFS for the combined expression groups of low + moderate (Low) versus high expression (left panel), and the combined expression groups of moderate + high (High) versus low expression (right panel) for REEP4. The x-axis shows survival time in days and the y-axis shows cumulative survival.

Table 5.3.11: Mean DFS times for different combined expression groups of REEP4 show significant differences for both combinations

		DFS	
		Mean (95% CI)	**p value**
REEP4	Low (low + moderate)	3990 (3813-4167)	0.042
	High	3598 (3237-3960)	
REEP4	Low	4143 (3887-4399)	
	High (moderate + high)	3765 (3568-3961)	0.036

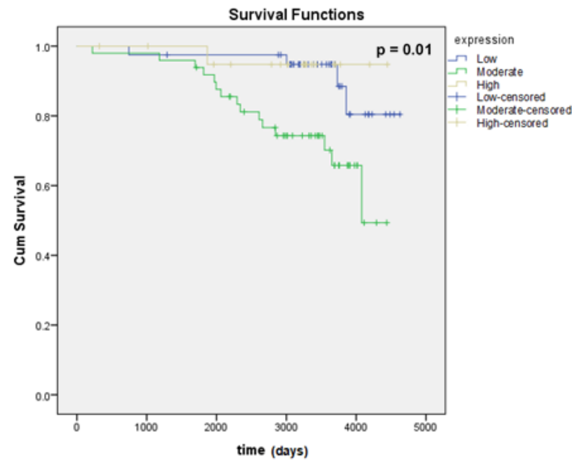
Log rank tests were performed to compare DFS between the combined expression groups of low + moderate (Low) versus high expression, and the combined expression groups of moderate + high (High) and low expression for REEP4. Mean survival times with 95% confidence intervals are shown for each analysis with significance values shown for each test.

The result that REEP4 expression correlated with DFS in the opposite direction compared to expectations was sufficiently surprising that further analyses were warranted. In particular, I noted that the cohort assembled for this analysis included many patients treated with ER negative cancers and patients treated with taxane-based therapy, while my initial cohort (Chapter 3) and subsequent *in vitro* work (Chapter 4 and this Chapter) focused on ER positive cancers treated with epirubicin. I therefore performed Kaplan-Meier survival curves and log rank tests using data only from those patients with tumours that were ER positive and were treated with the epirubicin and cyclophosphamide (EC) chemotherapy regimen, since these patients were more representative of my previous work. Mean survival times with 95% confidence intervals were also calculated. This analysis was performed using three different methods. In the first, all three expression groups were analysed separately as in section 5.3.4.6. In the second and third methods, the moderate expression group was again combined with either the low or high expression groups. The graphs showing Kaplan-Meier survival curves are shown in Figure 5.3.12 and mean survival times are shown in Table 5.3.12.

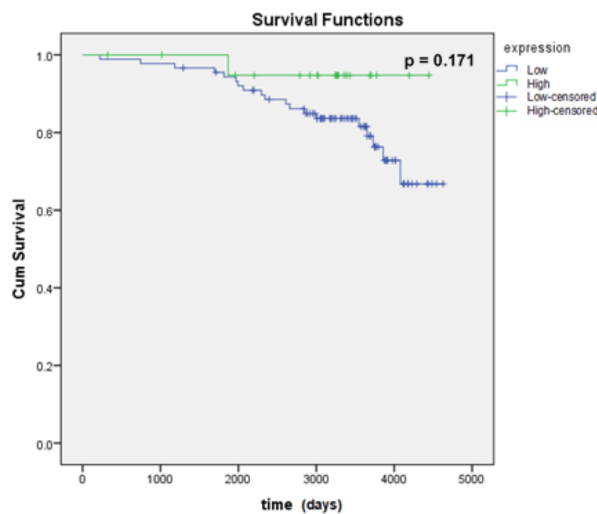
Results showed a significant difference in DFS between the three expression groups of REEP4 (mean times of 4367, 3681 and 4309 days for the low, moderate and high groups respectively, p=0.01). Results also showed a significant difference in DFS when the moderate group was combined with the

high expression group (4367 and 3848 days for the low and high groups respectively, p=0.044). There was no significant difference in DFS when the moderate group was combined with the low expression group. However, when considering the Kaplan-Meier survival curves for the three separate expression groups (Figure 5.3.12, top panel), the moderate expression group appeared to have the poorest outcome in terms of DFS with both high and low expression groups apparently having better outcomes, leaving some uncertainty as to the true answer regarding how levels of REEP4 expression relates to DFS. In pairwise analyses of these groups, the low expression and the moderate expression groups, and the moderate expression and the high expression groups were both independently significantly different (p=0.01 and p=0.045 respectively), hinting at a subtle dose-dependent relationship where the extremes of expression share good prognosis, and intermediate expression defined significantly poor outcomes. Although these three groups can be combined into two groups (Figure 5.3.12, bottom panels), these analyses probably do not reflect the complexity of the situation and this was demonstrated by the result differing depending on whether the moderate expression group was combined with the high or low group.

Three separate expression groups



Low + moderate combined (Low) **Moderate + high combined (High)**



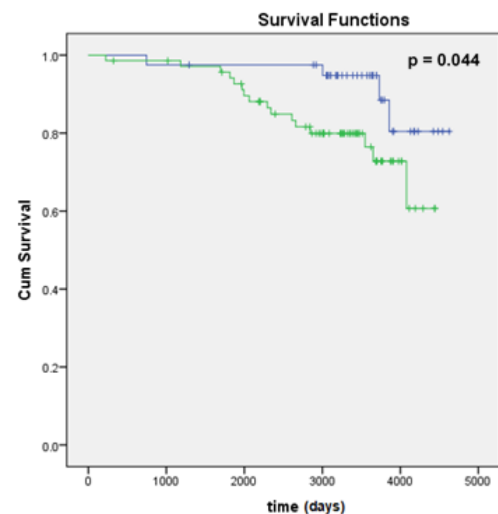


Figure 5.3.12: Kaplan-Meier survival curves showing DFS for different expression groups of REEP4 where tumours were ER positive and treated with the EC chemotherapy regimen

Kaplan-Meier survival analyses were performed showing DFS for low, moderate and high expression groups of REEP4 (top panel), combined expression groups of low + moderate (Low) versus high expression (bottom left panel), and combined expression groups of moderate + high (High) versus low expression (bottom right panel). Data analysed was from patients whose tumours were ER positive and treated with a chemotherapy regimen containing only EC. The x-axis shows survival time in days and the y-axis shows cumulative survival.

Table 5.3.12: Mean DFS times for different expression groups of REEP4 where tumours were ER positive and treated with the EC chemotherapy regimen show significant differences between expression groups

		DFS	
		Mean (95% CI)	**p value**
REEP4	Low	4367 (4118-4615)	0.01
	Moderate	3681 (3356-4006)	
	High	4309 (4051-4568)	
REEP4	Low (low + moderate)	4062 (3832-4292)	0.171
	High	4309 (4051-4568)	
REEP4	Low	4367 (4118-4615)	0.044
	High (moderate + high)	3848 (3590-4107)	

Data from ER positive tumours, treated with the EC chemotherapy regimen, were used to perform log rank tests to compare DFS times between either all three expression groups of REEP4, the combined expression group of low + moderate (Low) versus high expression, or the combined expression group of moderate + high (High) versus low expression. Mean survival times with 95% confidence intervals are shown for each group with significance values shown for each test.

5.4 Discussion

5.4.1 Using synthetic biotinylated miRNA mimics as bait to identify target mRNAs

In Chapter 4, I attempted to identify mRNA targets of miR-26b and miR-195 that contribute to chemoresistance by using predictive algorithms and literature searches. Despite identifying *CCDC6* as a target of both miRNAs of interest, this gene did not contribute to chemoresistance as expected. Another method was therefore required to identify mRNA targets of miR-26b and miR-195. The use of synthetic biotinylated miRNA mimics as bait to capture mRNA transcripts has previously been described (Ørom and Lund, 2007). This method has the advantage of identifying the mRNA targets that are actually bound within the cell type of interest – and could therefore include both those potentially

calculated by predictive algorithms as well as those that may bind in non-canonical ways and are not predicted by current methods (Thorne et al., 2018).

Synthetic biotinylated miRNA mimics have previously been successfully used to identify mRNA targets of miR-139-5p (Krishnan et al., 2013). Expression of this miRNA was deregulated in tissue samples of triple negative breast cancer, with down-regulation frequently observed in a cohort of invasive breast carcinomas. To identify the function of miR-139-5p in this context, the authors used a biotinylated mimic of this miRNA as bait, to perform a pulldown in order to identify target mRNAs in MCF7 breast cancer cells. RNA that had been pulled down was analysed using microarrays, instead of RNA-Seq as I have used. Following identification of target mRNAs, of which there were 1356 that were significantly enriched compared to the control sample, these were compared with those predicted by the predictive algorithm TargetScan, of which there were 346, in order to determine whether the pulldown was successful. Of these genes, 46 were common to the two methods, which was significantly more than expected by chance. These results reinforce the fact that predictive algorithms are limited in their capacity to predict all mRNA targets, and indeed may predict false targets. This may explain the limited success I had in identifying targets of miR-26b and miR-195 using predictive algorithms in Chapter 4 (section 4.3.2). Following confirmation that the pulldown was successful, gene set enrichment analysis was performed using the genes encoding the transcripts that were pulled down. These results led to the conclusion that miR-139-5p expression regulated metastasis but not proliferation, with further *in vitro* studies showing that expression of this miRNA suppressed invasion and migration of MDA-MB-231 breast cancer cells.

In another study, the authors used this method specifically together with RNA-Seq to identify the functions of miR-522 based on its mRNA targets as it allowed unbiased identification of targets without having to rely on any assumptions based on seed pairing (Tan et al., 2014). Targets identified by this pulldown method were also compared with those genes identified by microarray analysis that were down-regulated when miR-522 was over-expressed. This was similar to my methodology, although down-regulated mRNAs were identified by RNA-Seq in my experiment, instead of microarray analysis. The use of RNA-Seq has the advantage of not being limited to the

detection of genes for which probes are present on the microarray. Following this comparison, mRNAs that had been pulled down were also compared with those predicted by five individual predictive algorithms, with the most overlaps found between mRNAs enriched in the pulldown and the predictive algorithm TargetScan. As with the previous study (Krishnan et al., 2013), the authors in this study (Tan et al., 2014) compared their results with those from predictive algorithms as a method to ensure the pulldown was successful. Using pathway analysis with those genes identified by the pulldown, miR-522 was found to regulate processes including cell cycle progression, cell survival and cell motility.

In the above mentioned studies, lists of genes corresponding to the transcripts pulled down with the bait miRNA were compared with those provided by predictive algorithms as a form of quality check to ensure that the pulldown was successful. Further analyses were performed using the list of genes identified by the pulldown alone. As I had already concluded that miR-26b and miR-195 contribute to chemoresistance in Chapter 4 (section 4.3.1), the aim of my experiment in this chapter was to identify individual genes that contribute to this phenomenon that are regulated by these miRNAs. Therefore, I compared genes whose transcripts had been pulled down with the bait miRNA with those provided by predictive algorithms not only as a quality check to ensure the pulldown was successful, but also as a means to shorten the list of genes of interest. For this reason, I also took into account those genes that were down-regulated in the presence of the miRNAs of interest, unlike in the second study, where authors used this method as a second quality check (Tan et al., 2014). Using biotinylated miRNA mimics as bait and comparing results with these two other methods, followed also by literature searching, I identified two genes of interest as targets for each miRNA: *REEP4* and *PRKCD* for miR-26b and *ARL2* and *SEMA6D* for miR-195.

5.4.2 The roles of *REEP4* and *SEMA6D* in chemoresponse

Having identified potentially relevant miRNA targets, I performed a number of assays to determine their actual relevance in chemoresistance; these experiments followed a similar flow to those examining the potential targets studied in Chapter 4 (section 4.3.2). First, expression of potential targets was determined in parental and epirubicin resistant MCF7 and T47D cells, which

suggested *SEMA6D* and *ARL2* as genes with potential roles in chemoresistance in MCF7 cells but not in T47D cells. These results reinforce the concept discussed in more detail in Chapter 6 (section 6.4.2.3) that although both of these cell lines are ER positive and represent the luminal A breast cancer subtype (Holliday and Speirs, 2011), they do have differential protein expression profiles (Aka and Lin, 2012). Short term cell viability assays and long term colony forming assays were then performed, using ER positive MCF7 cells transfected with *REEP4* or *SEMA6D*-targeted siRNAs and treatment with the chemotherapeutic drug epirubicin. These two assays suggested a role for *SEMA6D* in chemoresistance, supporting the results obtained from the MCF7 epirubicin resistant cells. Results for *REEP4* were not significant, but were consistent, and also suggested a role in chemoresistance.

Results obtained for both of these genes were consistent with expectations, as expression of their miRNA regulators miR-26b and miR-195 was increased post-NAC in chemoresistant patients. As targets of these two miRNAs, increased miRNA expression should cause decreased expression of *REEP4* and *SEMA6D*, and therefore should also lead to chemoresistance.

5.4.2.1 The function of REEP4 in cancer

Very little is known about *REEP4* specifically in the context of cancer. It was identified as a microtubule-binding protein six years ago as part of a known protein family, with the protein localised to the endoplasmic reticulum (EnR) (Schlaitz et al., 2013) as with the localisation of other REEP family members (Park et al., 2010). It has several isoforms, the largest of which is 29.4kDa (Saito et al., 2004, Schlaitz et al., 2013). REEP proteins contain two conserved hydrophobic regions in their N-terminal domain (Voeltz et al., 2006), and the amino acids between these two domains in REEP proteins 1-4 are positively charged, which represent the microtubule-binding region (Schlaitz et al., 2013). Investigations into the function of this protein revealed that when depleted together with REEP3, defects in the structure of the nuclear envelope were observed (Schlaitz et al., 2013). In normal mitosis, the EnR membrane aids in the formation of the nuclear envelope, and is cleared from mitotic chromatin after the nuclear envelope is formed. However, when REEP4 and REEP3 were depleted, aberrant association of the EnR with mitotic chromatin was observed,

as well as other mitotic defects. The authors concluded that REEP4, together with REEP3, promotes the separation of daughter nuclei through its binding with microtubules, thereby facilitating the process of mitosis.

The *REEP4* gene is situated on chromosome 8p, a region frequently deleted in several cancers including prostate and rectal carcinomas (Hornstein et al., 2008, Doyen et al., 2014). Microarray analyses performed on samples of prostate cancer tissue and benign tissue revealed significant down-regulation of *REEP4* in tumour tissue compared with healthy tissue (Hornstein et al., 2008). Since *REEP4* contributes to the fidelity of the mitotic process, one possible consequence for lower expression of this gene is less efficient and accurate mitosis, thus contributing to tumorigenesis. It may also lead to aberrant expression of other genes in response to inefficient and inaccurate mitosis, which may contribute to the chemoresistance observed *in vitro* in this chapter when *REEP4* was silenced in breast cancer cells treated with chemotherapy.

5.4.2.2 The function of SEMA6D in cancer

Semaphorins are a family of signalling proteins that regulate the morphology and motility of many cell types, and function largely through the plexin family of receptors (Alto and Terman, 2017). The domain essential for signalling, the sema domain, is located at the N-terminus, and is also the domain that mediates dimerization with other semaphorin proteins (Klostermann et al., 1998, Gherardi et al., 2004). Structurally, proximal to the sema domain is the plexin-sema-integrin (PSI) domain (Siebold and Jones, 2013). *SEMA6D* was first identified as a new member of the class 6 transmembrane semaphorin protein family almost two decades ago, with a predicted molecular weight of 113kDa (Qu et al., 2002, Taniguchi and Shimizu, 2004, Alto and Terman, 2017). It was detected in a variety of human tissues (Qu et al., 2002). However, when several cancer cell lines were tested, including those representing leukaemia, colorectal adenocarcinoma and lung carcinoma, no *SEMA6D* was detected, suggesting a loss of expression in cancer. Further studies in gastric cancer, however, revealed significantly higher protein and mRNA expression in gastric carcinoma tissue compared with normal gastric mucosa (Zhao et al., 2006). Further examination revealed that the expression of both SEMA6D and

its receptor plexin-A1 was high in the endothelial cells (Lu et al., 2016). Together with the observation that when plexin-A1 and VEGFR2 were complexed together and SEMA6D was bound, leading to VEGFR2 being phosphorylated, it was concluded that SEMA6D may play a role in tumour angiogenesis.

With respect to chemoresistance, there is no direct published evidence that *SEMA6D* is a contributor, but it has been noted as a target of several miRNAs that do confer resistance. MicroRNA expression profiling was performed on ovarian cancer cells that were resistant to either paclitaxel or cisplatin, and was compared to the parental cells (Sorrentino et al., 2008). MiR-26a and miR-30c were both down-regulated in both resistant cell lines compared with their sensitive counterparts. *SEMA6D* was predicted to be a target of both miRNAs by the predictive algorithms TargetScan and PicTar. As such, it would be inferred that up-regulated *SEMA6D* would contribute to chemoresistance. It is worth noting that in my results, *SEMA6D* mRNA was pulled down by miR-26b, with a fold difference of at least 100, and was predicted as a target of this miRNA by four different predictive algorithms. MiR-26a and miR-26b have highly similar sequences, and they have many mRNA targets in common (Trompeter et al., 2013, Hu et al., 2018). The inference that up-regulated *SEMA6D* would contribute to chemoresistance is in contrast to the results that I have obtained in this chapter, although it is important to note that my experiments were performed in breast cancer cells rather than ovarian cancer cells. It is also important to note, however, that no further investigations were performed to verify that *SEMA6D* was a target of miR-26a and miR-30c in the ovarian cancer cells. As I have shown in Chapter 4 (section 4.3.2.2), where *CCDC6* appeared to be targeted by both miR-26b and miR-195 in MCF7 cells but only miR-26b in MDA-MB-175 cells, cellular context is important when considering mRNA targets of miRNAs.

5.4.3 REEP4 and SEMA6D expression as prognostic factors in breast cancer

Since reduced expression of both *REEP4* and *SEMA6D* appeared to contribute to chemoresistance, expression of the corresponding proteins in patients was tested to determine if they had any prognostic and potentially therapy predictive

value. This potential therapy predictive value originates from the fact that in this cohort, all patients received adjuvant chemotherapy. However, this value remains only potentially predictive when considering all treatments, as no comparisons were made with patients who received no adjuvant chemotherapy. One caveat to this, is that differences could potentially be observed when analyses are separated between different chemotherapy regimens, in which case, the protein would have a therapy predictive value specific to that regimen.

There were no significant correlations between expression of either protein with ER status, histological grade of the tumour or involvement of lymph nodes, which is important as it suggests that any correlation with outcome for these patients could be independent of known prognostic factors – and would therefore provide new prognostic information not otherwise available. There were also no significant relationships between protein expression and DFS or OS, although there was a trend for poorer DFS when REEP4 expression was higher (p=0.051). It is important to note however, that having started with 305 patients represented on the TMAs, there were only 68 incidences of recurrence with corresponding REEP4 expression data available. It is possible that with a larger number of recurrences and therefore larger statistical power, that there may indeed be a significant difference in DFS between patients with low, moderate and high expression of REEP4. Since this analysis was performed using three expression groups, further analyses were performed where the moderate expression group was combined with either the low or high expression groups in an attempt to improve the statistical power. This did indeed lead to significant differences in DFS between the two expression groups, both when the moderate group was combined with the low group (p=0.042) and when it was combined with the high group (p=0.036). This led to the conclusion that REEP4 expression did have prognostic value and potentially therapy predictive in terms of DFS.

However, although differences in DFS between the two expression groups of REEP4 were significant, the relationships between REEP4 expression and DFS were the opposite to expectations based on all the preceding work in the thesis. Since all previous work was conducted in ER positive breast cancer cells, and the chemotherapy agent administered was epirubicin, further

analyses were performed to determine whether expression of REEP4 had a prognostic value in accordance with expectations in this subgroup of patients. Once again, significant differences in DFS were observed between the three expression groups of REEP4 (p=0.01), and when the moderate expression group was combined with the high expression group (p=0.044). However, these analyses did not provide simple evidence of how REEP4 expression relates to DFS, since the moderate expression group appeared to have much poorer DFS than either the low or the high expression groups (verified in separate analyses where p=0.01 and p=0.045 respectively). It is important to note however, that despite performing these analyses using the subgroup of patients with ER positive tumours and treated with a chemotherapy regimen consisting only of EC, the majority of these patients were also treated with endocrine therapies, and some were also treated with radiotherapy. Both of these other forms of cancer treatment could have influenced the effect of REEP4 expression on DFS. Therefore, this subgroup of patients, although representing as closely as possible the context in which all previous analyses were performed, did not represent the precise context of all previous analyses.

According to the online database The Human Protein Atlas, under the pathology section (Uhlen et al., 2017), there is no difference in OS between patients with high and low expression of REEP4 (p=0.24). This database uses RNA-Seq data taken from TCGA datasets, with data available for 1075 patients for REEP4 expression. My data also shows no difference in OS between different expression groups of REEP4 (p=0.215). The Human Protein Atlas database does not provide data regarding DFS. The same result is observed regarding the prognostic value of SEMA6D in breast cancer, where no significant difference is observed in OS between the different expression groups (p=0.2). It is important to note, however, that The Human Protein Atlas database performed these analyses using RNA expression data, whereas I used protein expression data. This is particularly important, as mRNA and protein expression levels do not always perfectly correlate (Riches et al., 2015). A second important factor to consider is that I identified these proteins as targets of miRNAs, and miRNAs do not always alter mRNA expression (Horman et al., 2013, Wilczynska and Bushell, 2015).

However, in a study investigating SEMA6D expression in breast cancer, a difference was observed in OS (Chen et al., 2015). Data was taken from a TCGA dataset and expression of SEMA6D was split into three groups: low, medium and high. The primary aim of this study was to identify genes differentially expressed between the low and high expression groups and determine the functions of these genes. These functions included the cell cycle process, response to drugs and the G-protein coupled receptor (GPCR) pathway, of which the latter function plays an essential role in cancer metastasis. Contributing to the results of this analysis, was also the observation that FOS and FOXO1, which both contribute to oncogenesis and metastasis, and are also potentially regulated by SEMA6D, both having increased expression in the high SEMA6D expression group. Kaplan-Meier analyses revealed that SEMA6D expression was significantly associated with OS ($p=0.0156$), with further analyses revealing a particularly large difference in the triple negative subtype of breast cancer ($p=0.0083$). Although these data do not correspond with my Kaplan-Meier survival analyses results, they do support my results that suggest a role for SEMA6D in chemoresistance. If lower expression of SEMA6D results in increased chemoresistance, this could translate to patients with lower expression of SEMA6D not surviving as long due to more severe therapy failure than those with higher expression.

5.4.4 Conclusions

I have successfully used synthetic biotinylated mimics of miR-26b and miR-195 as bait to pull down mRNA targets of these miRNAs. By using multiple methods, I selected two candidate targets of interest for each miRNA. Observing their expression following manipulation of miR-26b and miR-195 expression and performing short and long term cell viability assays, I concluded that *REEP4* is a target of miR-26b and *SEMA6D* is a target of miR-195, contributing to chemoresistance when silenced. Further analyses revealed that SEMA6D expression, however, had no prognostic value in terms of DFS or OS. REEP4 expression did have prognostic value in terms of DFS in a subgroup of patients with ER positive tumours, treated with the chemotherapy regimen EC. However, the exact relationship between REEP4 expression and DFS remains unclear.

Chapter 6: Endocrine therapy can induce resistance to subsequent chemotherapy

6.1 Abstract

Neoadjuvant systemic therapy for breast cancer is not limited to chemotherapy, since some patients with ER positive tumours can receive neoadjuvant endocrine therapy (NAET) as an alternative. This is most commonly the case in patients who are relatively frail and/or suffer from co-morbidities, therefore may not be suitable for neoadjuvant chemotherapy or for surgery at that time. However, a proportion of these patients go on to have surgery and some have adjuvant chemotherapy. Studies on the impact of NAET on response to subsequent adjuvant chemotherapy have not been performed. Previous work in the Hughes lab has shown that NAET is associated with significant increases in expression of Breast Cancer Resistance Protein (BCRP) in the tumour cells. BCRP is a protein that acts as a xenobiotic pump and expression has previously been linked with chemoresistance. The aim of this chapter was to examine whether this increase in BCRP occurs in appropriate cell line models, and to assess its impact on sensitivity to subsequent chemotherapy.

Expression of BCRP at both protein and mRNA levels (gene name *ABCG2*) was significantly increased in the ER positive cell line T47D following daily treatment with the endocrine therapeutic 4-hydroxy-tamoxifen (tamoxifen) for fifteen days ($p<0.05$). This pre-treatment with tamoxifen was associated with significantly increased resistance to subsequent treatment with the chemotherapeutic epirubicin ($p<0.05$). Cell cycle analysis using flow cytometry revealed no significant alterations in the proportion of cells in each cycle phase after this tamoxifen pre-treatment, thus eliminating reduced proliferation as a potential mechanism of resistance and thereby implicating the increased BCRP expression as the likely resistance mediator.

These results suggest that the impact of NAET on patients' response to adjuvant chemotherapy is worthy of further study.

6.2 Introduction

Oestrogen receptor positive breast cancers can be treated with endocrine therapies, of which there are two main classes: anti-oestrogens and aromatase inhibitors (Lumachi et al., 2011). However, patients with ER positive tumours can also be treated with cytotoxic chemotherapy, or a combination of both (Fisher et al., 1997, Paik et al., 2006). Previous studies have noted that of the ER positive breast cancers, luminal A-type tumours are generally less responsive to chemotherapies whereas luminal B-type tumours are generally less responsive to endocrine therapies (Ignatiadis and Sotiriou, 2013, Gnant et al., 2015).

In a subset of patients, neoadjuvant endocrine therapy (NAET) is being increasingly recommended instead of neoadjuvant chemotherapy (NAC) as previous studies have shown similar overall response rates with less associated toxicity (Semiglazov et al., 2007, LeVasseur et al., 2017). This subset of patients includes those who are elderly and/or too frail to be treated with chemotherapy or to undergo surgery (Barroso-Sousa et al., 2016, Spring et al., 2016), and also includes patients with comorbidities (Macaskill et al., 2006). However, the consequences of administering adjuvant chemotherapy following NAET have not been investigated. As the use of NAET for the treatment of breast cancer is increasing, the incidence of such situations will also increase. Therefore, investigations into these possible consequences should be performed.

The expression of drug efflux pumps on tumour cells is a known mechanism for resistance to systemic therapies (Sharom, 2008). A well known family of proteins with the ability to export therapeutic drugs is the ABC family, which has a wide variety of substrates (Massey et al., 2014). One member of this family, is the Breast Cancer Resistance Protein (BCRP) and the gene encoding it is *ABCG2* (Staud and Pavek, 2005) (see section 1.4.1.1). BCRP is known to be associated with chemoresistance (Kovalev et al., 2013, Mao and Unadkat, 2015); one of the first discoveries of this drug pump was in a chemotherapeutic drug resistant subline of MCF7 breast cancer cells that did not overexpress the previously known drug transporters P-glycoprotein or the multidrug resistance protein (Doyle et al., 1998, Doyle, 1998). *ABCG2*/BCRP is of particular interest

in the context of NAET as it has been shown to have an oestrogen response element in the promoter of the gene (Ee et al., 2004, Zhang et al., 2006, Pradhan et al., 2010), therefore expression of the protein may be influenced by endocrine therapies. Previous studies have shown that NAC has the ability to induce changes in expression of this protein in breast cancers and that these induced levels were predictive of survival (Kim et al., 2013a). Altered expression of this protein post-NAET may therefore influence the sensitivity of tumour cells to any subsequent chemotherapy treatments.

Previous work in the Hughes lab had demonstrated that increased BCRP expression was associated with NAET for breast cancer (Figure 6.2.1A and B). This was further supported by analysis of patient data available from two separate public datasets. These datasets included data from patients treated with various NAET regimens where microarray analyses were performed on samples before and after treatment. Figure 6.2.1C shows the increase in *ABCG2* transcript expression post-NAET relative to pre-NAET expression levels. As a useful control, it was also shown that in patients who received no neoadjuvant therapy, tumours did not show any change in *ABCG2* expression over time.

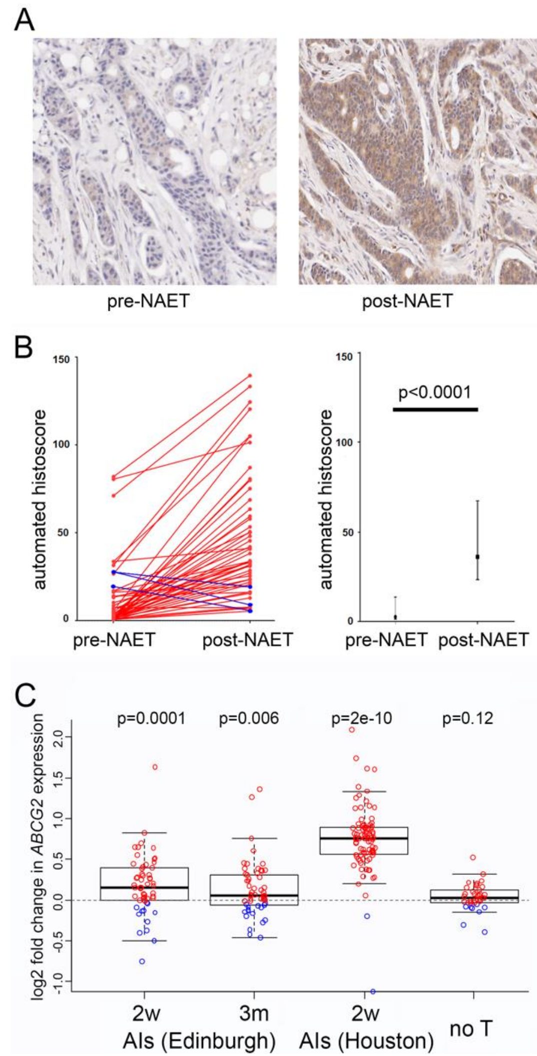


Figure 6.2.1: *ABCG2*/BCRP expression is increased in patients post-NAET (Baxter et al., 2018)

In A and B, fifty-one matched pre- and post-NAET breast tumour samples were stained for BCRP (clone BXP-21, Abcam) using immunohistochemistry. These tumours were from patients with primary breast cancer that had received NAET for a period of time between one month and one year. These patients received no other treatment. Expression of BCRP was quantified in tumour cells by weighted histoscores using a semi-automated protocol, validated previously (Kim et al., 2013a). (A) Representative images of matched pre- and post-NAET breast tissues showing stained BCRP (brown). (B) The left panel shows individual histoscores for pre- and post-NAET tissue samples with lines connecting matched samples. The red lines indicate increases in BCRP expression post-NAET and blue lines indicate decreases in BCRP expression. The right panel shows median histoscore values with interquartile range. Significance was assessed using the Wilcoxon signed rank test. (C) *ABCG2* (transcript) expression was assessed using microarray expression arrays in matched pre- and post-NAET breast cancer samples in two separate cohorts. In the Edinburgh cohort, fifty-five patients were treated with the endocrine therapy letrozole. The change in *ABCG2* expression was assessed after two weeks and three months of treatment relative to pre-treatment expression levels. In the Houston cohort, ninety-four patients were treated with aromatase inhibitors (no information as to which) and change in *ABCG2* expression was assessed after two weeks relative to pre-treatment expression levels. *ABCG2* expression was also assessed in thirty-seven matched diagnostic biopsy and surgical excision samples from patients with breast cancer who did not receive any neoadjuvant therapies (no T). *ABCG2* expression in post-NAET samples is shown relative to pre-NAET samples as log2-fold changes, with red and blue circles indicating up- or down-regulation for individual samples respectively. The median change in expression is shown by the black line, the boxes show the upper and lower quartiles and the whiskers show 1.5x the interquartile range. Significance of changes in expression was assessed using paired Wilcoxon tests. This figure was used in accordance with rights retained by the authors of this article from the publishers, Elsevier.

6.2.1 Specific objectives

There were three key objectives in the work described in this chapter:

1. To determine whether increased expression of BCRP in response to endocrine therapy could be replicated *in vitro* using the anti-oestrogen 4-hydroxy-tamoxifen and an appropriate cell line.
2. To determine whether pre-treatment of an ER positive cell line with 4-hydroxy-tamoxifen led to increased resistance to subsequent chemotherapy.

3. To determine whether treatment with 4-hydroxy-tamoxifen leads to an altered cell cycle profile that could explain any altered chemosensitivity rather than it relating to changes in BCRP expression.

6.3 Results

6.3.1 BCRP expression is up-regulated post-tamoxifen treatment in an ER positive breast cancer cell line

BCRP expression has previously been shown to be significantly increased post-neoadjuvant endocrine therapy (NAET) in tumours taken from a cohort of primary breast cancer patients with ER positive tumours (Figure 6.2.1) (Baxter et al., 2018). As the main aim of this chapter was to determine whether NAET leading to increased BCRP expression could potentially lead to increased chemoresistance, the first objective was to identify a cell line that reproduced *in vitro* the up-regulation of BCRP seen in patients post-NAET. Therefore, the ER positive cell line T47D was treated with 4-hydroxy-tamoxifen (TAM), an agent that competitively binds the oestrogen receptor, inhibiting its activation and BCRP expression was investigated post-treatment at different timepoints. This form of tamoxifen is the more biologically active form of the drug.

6.3.1.1 BCRP expression is up-regulated post-tamoxifen treatment within 24 hours and is maintained for several days

The ER positive cell line T47D was initially treated with 1µM TAM for a period of up to 24 hours. At various timepoints, protein was extracted for analysis of BCRP expression by western blot (Figure 6.3.1). BCRP expression was increased even after only 4 hours of TAM treatment and continued to increase over time to approximately 2.5 times more than in the control sample after seventeen hours of exposure to the drug. These results confirmed that BCRP expression was increased in the presence of TAM in T47D cells and therefore that T47D may represent a suitable cell line model.

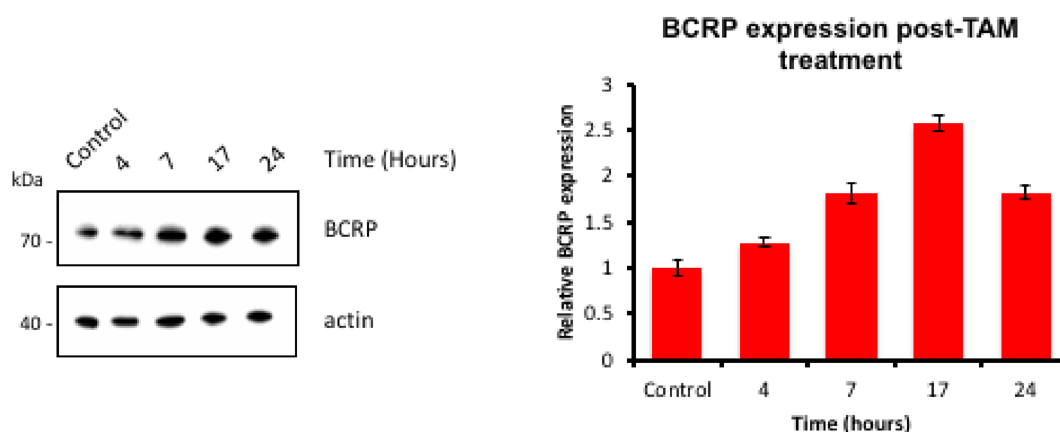


Figure 6.3.1: BCRP expression is up-regulated post-TAM treatment over a 24 hour period

T47D cells were treated once with 1μM TAM (or vehicle control) and protein was extracted at the indicated timepoints. Western blots were performed, probing for BCRP or beta-actin used as a loading control. BCRP was quantified relative to actin using Image Lab software three separate times for each blot. One representative western blot is shown in the left panel, with an accompanying graph in the right panel showing means of three separate quantifications (+/- standard deviations).

A second ER positive cell line was also tested. MCF7 cells were treated with TAM using the same method as with the T47D cells. However, BCRP expression decreased after 4 hours of TAM treatment and slowly returned back to the original levels after 24 hours of TAM treatment (Figure 6.3.2). As no increase in BCRP expression was observed in these cells, further experiments were performed in T47D cells only.

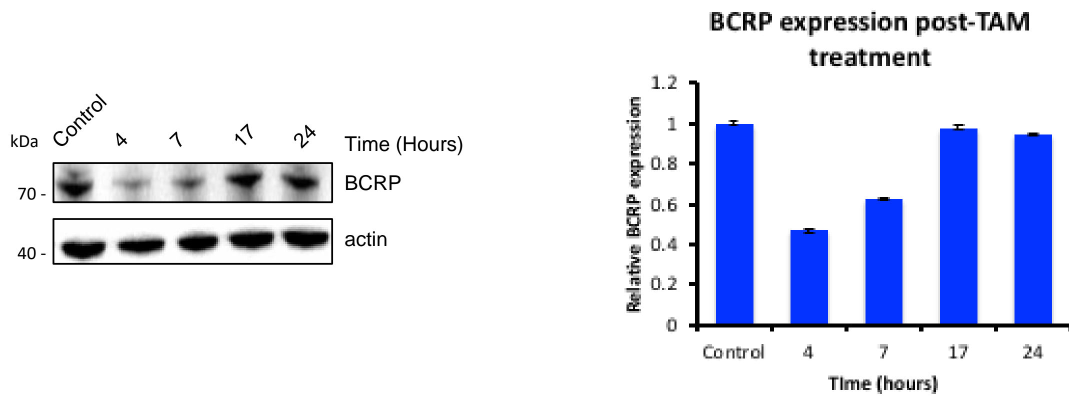


Figure 6.3.2: BCRP expression is not up-regulated post-TAM treatment over a 24 hour period

MCF7 cells were treated once with 1μM TAM (or vehicle control) and protein was extracted at the indicated timepoints. Western blots were performed, probing for BCRP or beta-actin used as a loading control. BCRP was quantified relative to actin using Image Lab software three separate times for each blot. One representative western blot is shown in the left panel, with an accompanying graph in the right panel showing means of three separate quantifications (+/- standard deviations).

Next, BCRP expression in T47D cells was also analysed up to four days after a single dose of TAM to determine whether the rapid increase in expression seen previously was sustained. A similar increase in BCRP expression was observed to that observed over a twenty-four hour period and it was sustained throughout the extended time period (Figure 6.3.3).

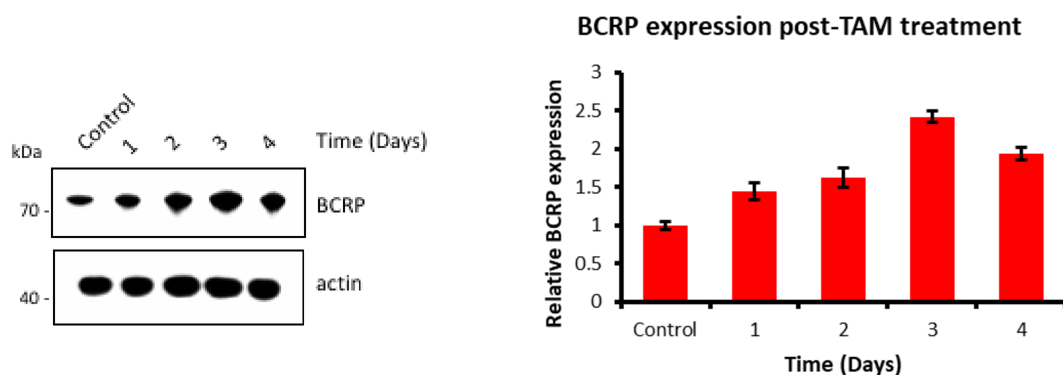


Figure 6.3.3: Up-regulated BCRP expression was maintained up to 4 days post-TAM treatment

T47D cells were treated on Day 0 with 1µM TAM (or vehicle control) and protein was extracted once a day. Western blots were performed, probing for BCRP or beta-actin used as a loading control. BCRP was quantified relative to actin using Image Lab software three separate times for each blot. One representative western blot is shown in the left panel, with an accompanying graph in the right panel showing means of three separate quantifications (+/- standard deviations).

6.3.1.2 *ABCG2* mRNA and protein expression are up-regulated post-daily TAM treatment for up to fifteen days

Patients who are administered tamoxifen receive a daily oral dose of the drug (Clarke et al., 1998, Davies et al., 2011). Therefore, to replicate these conditions *in vitro*, T47D cells were treated daily with either of two different doses (1 or 5µM) of TAM for a period of up to fifteen days. *ABCG2* (transcript) and BCRP (protein) expression were analysed by RT-qPCR and western blots respectively (Figure 6.3.4).

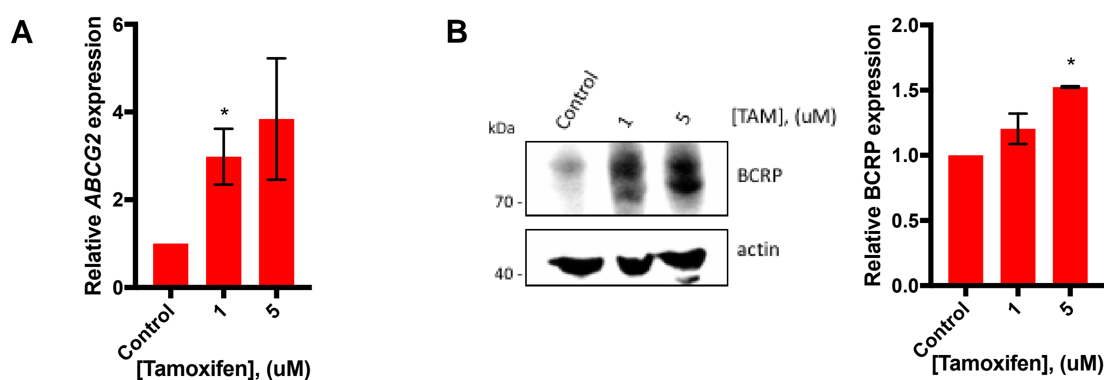


Figure 6.3.4: Daily TAM treatment for fifteen days increases *ABCG2* mRNA and protein levels

T47D cells were treated with either 1 or 5µM of TAM (or vehicle control) every twenty-four hours for fifteen days. Total RNA and protein were then extracted and analysed by RT-qPCR (A) or western blot (B) respectively, with beta-actin used for normalisation. (A) Data represent means of three biological repeats (+/- standard error); *p<0.05. (B) One representative western blot is shown in the left panel. The accompanying graph in the right panel shows means of two biological repeats (+/- standard error); *p<0.05.

Both *ABCG2* mRNA and BCRP protein expression were significantly up-regulated. This coincides with observations in patients administered NAET (Figure 6.2.1) (Baxter et al., 2018). The T47D cell line was therefore an appropriate model cell line that could be used for subsequent tests investigating the effect of this BCRP up-regulation on chemosensitivity.

6.3.2 Pre-treatment with TAM leads to increased resistance to subsequent chemotherapy

Having observed in section 6.3.1 that daily treatment of T47D cells with TAM for a period of fifteen days led to increased expression of BCRP, chemosensitivity assays were performed on these treated cells to determine whether they had become more resistant to chemotherapy – as might be predicted with increased expression of a chemoresistance-associated xenobiotic pump. Cells were treated with 1 or 5µM of tamoxifen daily for fifteen days as before. TAM was then removed and cells were cultured for twenty-four hours in fresh (drug-free) media before being assessed in chemosensitivity assays. Two concentrations of the chemotherapy drug epirubicin were used to treat these cells for twenty-four hours, and an MTT assay was performed to determine cell viability following drug treatments (Figure 6.3.5).

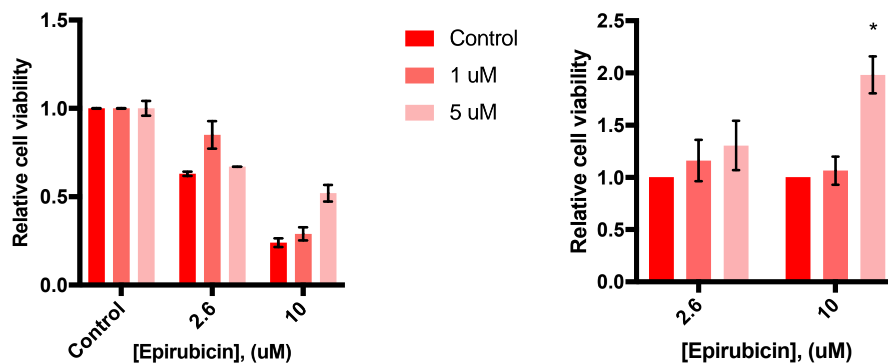


Figure 6.3.5: T47D cells pre-treated with TAM were more resistant to chemotherapy

T47D cells were treated daily for fifteen days with either 1 or 5μM of TAM (or vehicle control) before being treated with the chemotherapy drug epirubicin at either 2.6μM or 10μM (or vehicle control) for twenty-four hours. Cell viability assays were then performed. The left panel shows one biological repeat (data represent means of three technical replicates +/- standard deviation). The right panel shows means of two biological repeats (+/- standard error), normalising to survival in the control without tamoxifen to allow focus on the effect of tamoxifen pre-treatment (*$p < 0.05$).

T47D cells were more resistant to epirubicin when pre-treated with TAM. In addition, the higher dose of TAM appeared to confer a higher degree of chemoresistance, which correlates with the higher induced levels of BCRP seen previously (Figure 6.3.4).

6.3.3 Pre-treatment with TAM does not significantly alter the cell cycle profile

Pre-treatment with TAM leads to T47D cells having increased expression of BCRP (Figure 6.3.4) and becoming more resistant to chemotherapy (Figure 6.3.5). While this is an association, it does not demonstrate a causal link. One alternative possible explanation for the resistance observed would be that it could be attributed to an altered cell cycle profile induced by TAM – in particular, tamoxifen can induce reduced proliferation (Sutherland et al., 1983, Osborne et al., 1983). This may be especially important in this context if there is a significantly altered proportion of cells in the S phase as the chemotherapeutic drug epirubicin induces cell death by causing DNA damage, thus primarily affecting cells during DNA replication (Bell and Dutta, 2002). Therefore, after daily treatment with TAM for fifteen days as before, the cell

cycle profiles of the cells were analysed by propidium iodide (PI) staining and flow cytometry (Figure 6.3.6).

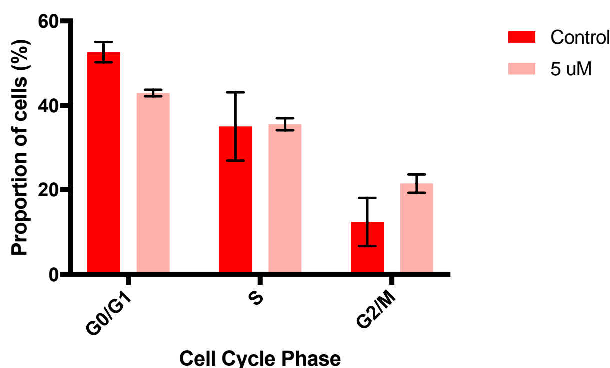


Figure 6.3.6: TAM did not induce significant alterations in proportions of cells in different cell cycle stages

T47D cells were treated daily for fifteen days with 5μM of TAM (or vehicle control). Cells were then stained with PI and analysed by flow cytometry. Data were analysed using the ModFit LT software (Verity Software House) to determine the proportions of cells in each stage of the cell cycle. Data represent means of two biological repeats (+/- standard error).

There were no significant alterations in the cell cycle profile of T47D cells pre-treated with TAM when compared with control cells, with no suggestion of a difference in proportions in S phase – the cycle stage targeted by epirubicin. Taken in this context, these results suggested that an alteration in the cell cycle profile was unlikely to be the direct cause of the chemoresistance observed in pre-treated cells and it is therefore possible that the up-regulation of BCRP post-tamoxifen treatment may indeed be the cause behind this chemoresistance.

6.4 Discussion

6.4.1 Oestrogen and anti-oestrogens influence *ABCG2*/BCRP expression

Neoadjuvant endocrine therapy is becoming more commonly used in the treatment of a subset of patients with ER positive breast cancers. However, there is as yet no evidence of there being any consequences on the sensitivity of remaining cancer cells to adjuvant chemotherapies. BCRP, a xenobiotic drug transporter, has previously been shown to confer resistance to a number of

chemotherapeutic drugs (Staud and Pavek, 2005). Previous work had shown that in a statistically significant number of patients treated with NAET, there is increased expression of this drug pump (Figure 6.2.1) (Baxter et al., 2018). This means there is certainly a possibility that these patients would not respond optimally to adjuvant chemotherapy if administered.

I treated an ER positive cell line, T47D, with the anti-oestrogen tamoxifen to observe initially whether the up-regulation of BCRP could be replicated *in vitro* in order to perform subsequent chemosensitivity assays. This was successfully achieved even after only twenty-four hours after treatment and was maintained after daily exposure to tamoxifen for a period of fifteen days. This suggests that lack of oestrogen signalling somehow leads to increased expression of BCRP, which is certainly possible as putative EREs have previously been identified in the promoter region of the *ABCG2* gene (Ee et al., 2004, Zhang et al., 2006).

Similar increases in BCRP expression have previously been observed in the absence of oestrogen signalling. In a study that included the ER positive breast cancer cell lines T47D, which I have used in my study, and MCF7, BCRP expression was found to be down-regulated in response to oestrogen. This decrease in BCRP expression was reversed by tamoxifen. This study concluded that the down-regulation of BCRP by oestrogen occurred post-transcriptionally (Imai et al., 2005). However, there is also conflicting evidence from other studies that have shown the opposite effects of oestrogens and anti-oestrogens on *ABCG2* and/or BCRP expression. In one such study where T47D were also used as in my study, expression of *ABCG2* was found to be increased in the presence of 17β-oestradiol, which was reversed in the presence of 4-hydroxy-tamoxifen (Ee et al., 2004), the same anti-oestrogen that I have used. Similarly, both *ABCG2* transcript and BCRP protein levels increased in response to 17β-oestradiol in MCF7 cells. This effect was abolished in the presence of tamoxifen. The ERE in the promoter region of the gene and the presence of the alpha isoform of the ER were found to be essential for the transcriptional activation of *ABCG2* by 17β-oestradiol (Zhang et al., 2006). Following on from this study, the anti-oestrogen toremifene was found to lead to decreased expression of both *ABCG2* transcript and BCRP protein in MCF7 cells (Zhang et al., 2010). Taken together, all of these studies suggest that the regulation of *ABCG2* transcript and protein expression is

complex and can differ depending on culture conditions. This complexity has previously been commented on (Bailey-Dell et al., 2001, Nakanishi and Ross, 2012) with work still ongoing in attempting to understand the regulation of this gene. These conflicting data may explain why in my initial experiments treating cells with TAM for 4-24 hours, the MCF7 cells did not show an increase in BCRP expression.

It is important to note that all of the work presented in the studies above was all performed on cell lines *in vitro,* with no mentions made of observing the effects of anti-oestrogens in the treatment of breast cancer on *ABCG2* and/or BCRP expression in patients, on which my study was based (Baxter et al., 2018). In this context, it may be worth emphasising that the regulation I see *in vitro* mirrors that in patients and may therefore be more representative.

6.4.1.1 Other mechanisms of BCRP expression regulation

The *ABCG2* gene also has three peroxisome proliferator-activated receptor response elements (PPARE) (Szatmari et al., 2006) and a progesterone response element (PRE) (Wang et al., 2008). In a study investigating the effects of progesterone on BCRP expression in human placental BeWo cells, progesterone increased expression of this protein (Wang et al., 2006). In this same study, oestrogen decreased expression of BCRP. In a very similar study investigating the effects of oestrogen and progesterone on BCRP expression in BeWo cells, the opposite results were obtained: progesterone decreased expression of BCRP and oestrogen increased expression (Yasuda et al., 2006). It is evident from these two studies that regulation of BCRP expression is a complex process that likely is dependent on numerous factors.

MiRNAs have also previously been shown to regulate BCRP expression. As mentioned previously, miR-487a has been shown to regulate expression by targeting the 3'UTR, thus influencing the chemosensitivity of ER positive breast cancer cells (see section 1.4.3) (Ma et al., 2013). In a colon cancer cell line, miR-519c was also observed to target the 3'UTR of the *ABCG2* transcript, leading to translational repression and eventual degradation of the mRNA (To et al., 2008). MiR-328 has been reported to target BCRP in ER positive breast cancer cells (Pan et al., 2009) and miR-520h regulates BCRP expression in pancreatic cancer cells (Wang et al., 2010).

6.4.1.2 Investigating correlations between *ABCG2* expression and other oestrogen-regulated genes in breast tumours treated with endocrine therapies could clarify the regulation of *ABCG2* expression *in vivo*

Previous work shown in Figure 6.2.1 (Baxter et al., 2018), together with the *in vitro* work I have performed, demonstrated increased *ABCG2* transcript and/or protein expression post-endocrine therapy. These observations however, do not demonstrate a direct mechanism of endocrine therapies regulating BCRP expression. The Edinburgh dataset used in the analysis shown in Figure 6.2.1C (Baxter et al., 2018), consists of gene expression profiles of tumour samples taken from ER positive primary breast cancer patients, before and after treatment with the endocrine therapy letrozole (Miller et al., 2009). A similar study, the Functional Aromatase Inhibitor Molecular Study (FAIMoS), was also conducted, in which patients were treated with the endocrine therapy anastrozole, with and without the addition of the EGFR inhibitor gefitinib (Smith et al., 2007). In a subsequent study performed by Gao et al. using samples from the FAIMoS, gene expression profiles were investigated pre- and post-anastrozole only therapy (Gao et al., 2014). It would therefore be possible using these datasets, to assess correlatations between *ABCG2* expression and oestrogen-regulated genes in order to gain understanding into the mechanisms by which endocrine therapy affects the expression of *ABCG2* in patients.

The gene *TFF1* is an oestrogen-responsive gene that is frequently expressed in breast tumours (Prest et al., 2002). As such, *TFF1* expression would be expected to be down-regulated in response to endocrine therapies. With the observations made of increased BCRP expression post-endocrine therapy in Figure 6.2.1, together with my *in vitro* work, a negative correlation between *TFF1* and *ABCG2* would be expected. It is important to note, however, that despite *TFF1* being considered an oestrogen-responsive gene, it has been observed *in vitro* that in the presence of tamoxifen, a small increase in *TFF1* expression occurred in MCF7 breast cancer cells (Kuske et al., 2006), rather than the expected decrease. In addition, an increase in expression of both *TFF1* and *ABCG2* was observed in tamoxifen resistant MCF7 cells compared with parental cells (Zheng et al., 2018). These results suggest the possibility that the regulation of *TFF1* expression is not simply dependent on the presence

of oestrogen. Therefore, any correlation between *TFF1* and *ABCG2* expression would need to be treated with caution.

Another oestrogen-responsive gene is that encoding PR (gene name *PGR*). A negative correlation between *PGR* and *ABCG2* expression would again support the hypothesis of endocrine therapies inducing *ABCG2* expression. However, as with *TFF1*, any correlation must be treated with caution, as a positive correlation between *PGR* and *ABCG2* expression has previously been observed in untreated primary breast tumour samples (Burger et al., 2003).

PDZK1 and *GREB1* are also oestrogen-responsive genes (Xue et al., 2019) that have been observed to overexpressed in ER positive breast cancers compared with ER negative breast cancers (Ghosh et al., 2000). As oestrogen-responsive genes, a negative correlation between the expression of *ABCG2* and *PDZK1* and *GREB1* individually would be expected. This would support the hypothesis that endocrine therapies induce *ABCG2* expression. This expectation is supported by previous observations made of the expression of both *PDZK1* and *GREB1* being reduced in the presence of tamoxifen *in vitro* (Ghosh et al., 2000, Kim et al., 2013b).

6.4.2 Increased BCRP expression post-tamoxifen treatment causes increased resistance to chemotherapy in breast cancer

6.4.2.1 A regimen of NAET followed by adjuvant chemotherapy is very rare in the treatment of breast cancer

In this study, I have shown that post-tamoxifen treatment, expression of both *ABCG2* transcript and BCRP protein are increased compared with cells that were not pre-treated. I have also shown that cells pre-treated with tamoxifen are more resistant to chemotherapy treatment with epirubicin than their non-treated counter parts. Unfortunately, assessing whether this observation is relevant clinically is difficult because the administration of tamoxifen in the neoadjuvant setting followed by adjuvant chemotherapy treatment is not standard practice in the treatment of ER positive breast cancer, although it is used fairly rarely. Indeed, from the initial patient cohort in which increased BCRP expression was observed post-NAET that my study was based on, of the fifty-one patients, only six received adjuvant chemotherapy. Therefore, no

studies have been performed to compare the outcomes of patients treated with NAET followed by adjuvant chemotherapy versus similar patients treated with adjuvant chemotherapy alone. However, with NAET becoming a more commonly used treatment regimen for post-menopausal patients (Chia et al., 2010, Chiba et al., 2017), an increase in the proportion of patients treated with NAET followed by adjuvant chemotherapy is probable. It is therefore important - in future - to determine whether this therapy regimen is safe to use. Unfortunately, even when suitable patient cohorts are available, the follow-up time required for outcomes is likely to be sufficiently long that it will be many years before a clear answer is available.

6.4.2.2 Increased BCRP expression causes decreased sensitivity to chemotherapy

BCRP has previously been shown to be induced by neoadjuvant chemotherapy (NAC) and that these post-NAC expression levels predict disease free survival (Kim et al., 2013a). In this study, patients were treated with a regimen consisting of anthracyclines with or without taxanes. A more expansive study revealed that there was a correlation between higher BCRP expression and poor clinical outcomes. In this study, it was observed that when classifying tumour subsets into either high or low expressions of BCRP, the high expressing subset had a much shorter time of progression free survival than the low expressing group, but only if treated with anthracycline-based therapies (5-fluorouracil, epirubicin and cyclophosphamide) and not when treated with cyclophosphamide, methotrexate and 5-fluorouracil (Burger et al., 2003). This observation of different treatment regimens leading to different clinical responses highlights the fact that each treatment regimen should be investigated individually with respect to tumour responses as each treatment regimen has the potential to cause unique responses.

The basis of my study was that previous work had shown an increase in BCRP expression following NAET (Baxter et al., 2018). No other such studies have previously been performed. There are however, a large number of studies that have confirmed that increased BCRP expression leads to increased chemoresistance. BCRP was first identified in MCF7 cells resistant to doxorubicin and verapamil when it was noticed that the two transporters known

previously to confer multi-drug resistance, P-glycoprotein and MRP1, were not involved (Doyle, 1998). Subsequent studies confirmed the role of BCRP in conferring resistance to the chemotherapeutic agent mitoxantrone in the placenta (Allikmets et al., 1998) and in a colon cancer cell line (Miyake et al., 1999). Since these initial studies, BCRP has also been found to confer resistance to several other chemotherapeutic drugs including topotecan and SN-38 (Yang et al., 2000). Importantly, epirubicin, the chemotherapy drug that I used in this study to determine if cells pre-treated with tamoxifen were more resistant to chemotherapy treatment than their untreated counterparts, is also a substrate of BCRP (Brangi et al., 1999). It is therefore possible that the resistance to epirubicin displayed by the cells pre-treated with tamoxifen in my study is mediated by the increase in BCRP expression that occurs as a consequence of the tamoxifen treatment.

6.4.2.3 Changes in cell cycle profile post-TAM treatment do not contribute to chemoresistant phenotype

Anthracyclines, such as epirubicin, the chemotherapeutic drug used in this study, function by inhibiting topoisomerase II (Zunino and Capranico, 1990). Topoisomerase II enzymes function by creating transient breaks in both strands of DNA in order to allow the strands to unwind so that processes such as DNA replication and transcription can occur (Nitiss, 2009a). If topoisomerase II enzymes are inhibited for example by anthracyclines, the DNA can no longer unwind and replication cannot occur, thus inhibiting cell cycle progression. DNA replication occurs during the S phase of the cell cycle (Bell and Dutta, 2002). Therefore, a possible mechanism of resistance to epirubicin of cells after pre-treatment with tamoxifen other than an increase in drug efflux by BCRP is that tamoxifen could cause fewer cells to be in S phase. This would mean that fewer cells would require topoisomerase II activity, thereby resulting in fewer active targets of epirubicin, resulting in fewer cells being affected by the chemotherapeutic drug and an apparent resistant phenotype.

Previous studies have shown that treatment with tamoxifen causes reduced rates of proliferation of ER positive breast cancer cells (Sutherland et al., 1983, Osborne et al., 1983). However, the majority of these experiments have been performed with MCF7 cells. Evidence has shown that there can be marked

differences between these two ER positive cell lines such as different functional protein expression profiles (Aka and Lin, 2012) and differences in responses to oestrogens and anti-oestrogens (Karey and Sirbasku, 1988, Radde et al., 2015). This is despite the fact that both cell lines are representative of luminal A-type breast cancers (Holliday and Speirs, 2011). Not only do different cell lines display differences, but different culture conditions have also been shown to give rise to different responses to drug treatments in the same cell lines (Reddel et al., 1985). Interestingly, it has been observed that in the absence of oestrogen, MCF7 cells increase expression of the ER whereas T47D cells decrease expression of the ER in the absence of oestrogen (Sweeney et al., 2012).

All the apparent differences between cell lines and within cell lines perhaps explain why despite previous studies showing reduced cell proliferation with fewer cells in S phase and a greater proportion of cells in the G_0/G_1 phases, I did not observe any significant differences in cell cycle profiles between those cells treated with TAM and those that were not treated. Therefore, since my results suggested TAM did not induce any changes in the cell cycle profile, it is unlikely that the chemoresistance observed in cells pre-treated with TAM was due to fewer cells being in the cell cycle phase most affected by the chemotherapy drug. Coupled with the increase in *ABCG2* transcript and BCRP protein expression post-TAM treatment, these data suggest that the increased chemoresistance observed post-TAM treatment is potentially due, perhaps in part, to the increased expression of the xenobiotic drug transporter BCRP.

6.4.2.4 Investigating a correlation between *ABCG2* expression and response to endocrine therapies would indicate whether increased *ABCG2* expression is a marker of poor prognosis

As mentioned in section 6.4.1.2, gene expression profiles of primary breast tumour samples before and after endocrine therapy are available from the Edinburgh and FAIMoS datasets (Miller et al., 2009, Gao et al., 2014). In the Edinburgh study, tumour size was also measured during therapy, and patients were determined to be either good or poor responders based on whether there was a minimum of a 50% reduction in tumour size or not post-therapy (Miller et al., 2009). In the FAIMoS, Ki67 was measured before and after therapy, with

patients who had an increase in Ki67 of more than 10% post-therapy classed as poor responders (Smith et al., 2007). As such, correlations between *ABCG2* expression and outcome could be performed to determine whether increased *ABCG2* expression is associated with a poor prognosis, as would be expected based on work presented in this chapter. However, it is important to note that neither the Edinburgh or FAIMoS studies reported on whether patients were administered adjuvant chemotherapy, which is the scenario posited in this chapter. Therefore, while *ABCG2* expression could be correlated with response to the endocrine therapies, it could not be correlated with response to subsequent chemotherapies.

6.4.3 Conclusions

Previous work has shown that BCRP expression was increased in patients post-NAET (Baxter et al., 2018). I have successfully replicated this phenomenon using the ER positive cell line T47D and the anti-oestrogen tamoxifen. Subsequent chemotherapy treatment demonstrated increased resistance to this second therapy compared with non-pre-treated cells. Therefore, it is possible that the increase in BCRP expression post-tamoxifen treatment causes this increased chemoresistance. These results suggest that a regimen of NAET followed by adjuvant chemotherapy should be administered to patients with caution, and outcomes following this regimen should be assessed in detail when data are available.

Chapter 7: General discussion and conclusions

7.1 The molecular mechanisms of chemoresistance in breast cancer

Resistance to systemic therapies, in particular cytotoxic chemotherapy, poses a major obstacle to the successful treatment of breast cancer due to a number of factors. The first is that systemic therapies have the benefit of targeting tumour cells in most areas of the body simultaneously, rather than being focused to specific body sites at one time. This is particularly advantageous in the treatment of sub-clinical micro-metastases, which cannot be specifically targeted using local therapies when they have not yet been detected. This inability to detect sub-clinical micro-metastases remains an ongoing problem (Pantel et al., 2009). The second factor specific to cytotoxic chemotherapy is that this treatment option is the only fully-established choice of systemic therapy for certain primary breast tumours, such as those of the triple negative subtype, which lacks expression of any proteins targeted by the common targeted therapies (Foulkes et al., 2010). It is also the only choice for those tumours in the metastatic setting, that were previously treated with targeting drugs and that developed resistance (Reinert and Barrios, 2015). In such cases, if resistance to the cytotoxic chemotherapy arises, no alternative types of systemic therapy exist, other than different classes of chemotherapy drugs, which may not work if the tumour has developed multi-drug resistance (Zahreddine and Borden, 2013).

However, it is important to note that newer targeted therapies have been developed and may soon be approved for primary breast cancer, potentially providing alternatives to cytotoxics. For example, CDK4/6 inhibitors have recently been approved for the treatment of metastatic, ER positive breast cancers in combination with endocrine therapy (Pernas et al., 2018), and the PARP inhibitor olaparib was recently approved by the FDA for the treatment of *BRCA*-mutated metastatic breast cancers (McCann and Hurvitz, 2018). It is, however, vital that we understand the molecular mechanisms of chemotherapy resistance in order that we may be able to identify not just novel drug targets so that resistance may be overcome or prevented, but also that we know which

successions of treatment regimens can be used successfully if resistance arises to the initial therapies.

Numerous studies have demonstrated that resistance to cytotoxic chemotherapy is a result of numerous molecular changes, such as changes in the expression of miRNAs (Kutanzi et al., 2011), molecular pathway activity (Balko et al., 2012), and expression of xenobiotic drug pumps (Sharom, 2008). In this project, I have attempted to identify some of these molecular changes that contribute to chemoresistance during treatment of ER positive breast cancers with a cytotoxic chemotherapy regimen consisting of epirubicin and cyclophosphamide (EC). MiRNA and mRNA expression profiling was performed on samples taken from breast cancer patients both before and after neoadjuvant chemotherapy (NAC), who displayed only a partial response to the administered therapy, thus implying a degree of chemoresistance. Changes in mRNA expression that occurred during chemotherapy suggested that the activities of both the MAPK and the PI3K-AKT pathways were increased (Chapter 3, section 3.3.4.2). This result was in accordance with previous studies that demonstrated increased activity of these pathways in tumour cells resistant to chemotherapy (West et al., 2002, Leung et al., 2008, Igea and Nebreda, 2015). Three miRNAs were consistently dysregulated post-NAC (Chapter 3, section 0), with further investigations revealing expression of miR-26b and miR-195 was associated with chemoresistance (Chapter 4, section 4.3.1). Expression of these miRNAs had previously been associated with chemoresistance, although evidence was conflicting, with results suggesting that these miRNAs play specific roles in chemoresponse depending on context (Rui et al., 2010, Ujifuku et al., 2010, Yang et al., 2013, Liang et al., 2015).

MiRNAs execute their function by manipulating gene expression post-transcriptionally (Ambros, 2001). It would therefore follow that changes in miRNA expression lead to alterations in expression of their targets. *REEP4* was identified as a target of miR-26b, and *SEMA6D* was identified as a target of miR-195 (Chapter 5, section 5.3.3.1). REEP4 is a protein required for cell division (Schlaitz et al., 2013), whereas SEMA6D is a protein whose function has been linked with angiogenesis (Lu et al., 2016). While the first function relates directly to division and therefore DNA replication, a process that is the main target of the chemotherapeutic drug epirubicin (Nitiss, 2009b), the second

function also relates, albeit indirectly, to tumour growth as tumours require a blood supply to survive and grow (Tozer et al., 2005).

Resistance to chemotherapy is not just a result of changes in gene expression that directly alter the effectiveness of the function of the drug. In order to perform their function, these cytotoxic drugs must first reach their targets. Increased expression of xenobiotic drug pumps whose substrates include cytotoxic drugs can pump these cytotoxic drugs out of the cells (Eckford and Sharom, 2009), thus preventing the drug from reaching its target. Increased expression of the xenobiotic drug pump BCRP was observed in patients following neoadjuvant endocrine therapy (NAET) (Baxter et al., 2018). This phenomenon was replicated *in vitro*, and resulted in an increase in chemoresistance following subsequent challenge with epirubicin (Chapter 6, sections 6.3.1 and 6.3.2). These results demonstrated that not only can cytotoxic chemotherapy drugs induce resistance to themselves by altering gene expression, but that targeted therapies such as endocrine therapies, can also induce resistance to cytotoxic chemotherapies. This leads back to the concept of multi-drug resistance (MDR), and the importance of knowing which successions of treatment regimens can be used successfully. The results in this thesis indicate that the efficacy of cytotoxic chemotherapy may be reduced if administered after endocrine therapy, and that this sequence of therapies should potentially be avoided.

It is important to consider, however, that although I investigated increased BCRP expression in terms of endocrine therapy, previous studies have shown that not only is expression of the BCRP gene *ABCG2* regulated by the presence of oestrogens (Ee et al., 2004, Zhang et al., 2006), it has also been shown to be targeted by miRNAs (Pan et al., 2009, Wang et al., 2010). With work presented here demonstrating altered miRNA expression contributing to chemoresistance being induced by cytotoxic chemotherapy, it is possible that *ABCG2* expression, as well as that of other xenobiotic drug pumps, can also be induced by this form of systemic therapy. Indeed, the PI3K-AKT pathway, the activity of which was increased post-NAC in my work, and has previously been shown to confer resistance (West et al., 2002), has also been shown to be essential for the localisation of BCRP to the plasma membrane (Mogi et al., 2003, Takada et al., 2005). It has also been shown that cytotoxic chemotherapy

drugs induced expression of the *ABCG2* gene by demethylating the promoter (Bram et al., 2009). This resulted in a dramatic increase in transcription of this gene within twelve hours of treatment.

Combining all results together, it is clear that resistance to cytotoxic chemotherapy is caused by multiple contributing factors, with therapies inducing changes in miRNA, mRNA, and xenobiotic drug pump expression, which in turn confer chemoresistance. This highlights the importance of understanding the molecular changes that occur during chemotherapy, so that we may be better able to treat breast cancer successfully by preventing and overcoming chemoresistance. This involves optimising treatment regimens, and knowing which therapies can be used successfully in second line treatments if resistance to the first line treatment develops.

7.2 Novel targeted therapies for the treatment of chemoresistant breast cancers

7.2.1 MiRNA-targeted therapies

In this thesis, I have demonstrated that increased expression of miR-26b and miR-195 contribute to increased chemoresistance in ER positive breast cancer. It may therefore be beneficial to inhibit these miRNAs during chemotherapy treatment of these breast cancers, in order to potentially reduce this resistance.

With the development of miRNA mimics and inhibitors used *in vitro* to manipulate miRNA expression in order to discover their functions, therapies targeting miRNAs also become potentially available. However, it was noted soon after the discovery of miRNAs as potential therapeutic targets that delivering these miRNA mimics and inhibitors *in vivo* to the correct locations was problematic (Bader et al., 2011, Braicu et al., 2019). This was due to a number of factors including the inherent nature of these mimics and inhibitors to be susceptible to nucleases, to be cleared by the kidneys, and to be taken up by target cells inefficiently. For this reason, numerous studies have investigated a number of potential delivery agents that could be used to transport these nucleotide sequences to their targets, as well as modifications to the sequences themselves.

As I have demonstrated in this thesis, some miRNAs have increased expression in cancer, and therefore require suppression. This can be achieved by antisense inhibition using synthetic oligonucleotides that have been modified so as to resist degradation, and to promote their uptake into cancer tissues (Esau, 2008, Lennox and Behlke, 2011). These anti-miRNA oligonucleotides (AMOs) primarily function by sterically blocking miRNA function. Second generation modifications involve the use of locked nucleic acids (LNAs) (Walayat et al., 2018). These nucleic acids have been modified such that a methylene bridge was formed between the 2'O and the 4'C atoms of the ribose, thus reducing the flexibility of the structure, effectively 'locking' it into a rigid conformation (Obika et al., 1997). These LNAs have been shown to increase the binding affinity of the AMOs to their target miRNAs, and are more resistant to nuclease degradation (Elmen et al., 2008). MiR-155 has been shown to have elevated expression in cutaneous T cell lymphoma (CTCL) (Ralfkiaer et al., 2011). Preliminary results for a phase I clinical trial have been published in which patients with CTCL were included (Querfeld et al., 2016, Querfeld et al., 2017). In this study, a LNA-modified AMO targeting miR-155 named MRG-106 was used. Results so far have suggested that MRG-106 is well tolerated and is effective in reducing the effects of miR-155 over-expression. The most advanced LNA-modified AMO targets miR-122, also known as miravirsen (Janssen et al., 2013, van der Ree et al., 2016), which was successfully used in a phase II clinical trial. However, this trial was performed using patients with a chronic infection of the hepatitis C virus, rather than cancer.

Increasing expression of specific miRNAs that are down-regulated in tumours could also be therapeutically beneficial. The delivery of miRNA mimics in the clinical setting is more developed than the delivery of inhibitors. However, such deliveries investigated have so far included the use of delivery agents. One such delivery agent involves the use of nanoparticles, of which there are many types (Farina et al., 2018). One of these involves the use of liposomes, which encapsulate the nucleotide sequences. These liposomes are largely composed of phospholipids, and greatly resemble cell membranes and are thus highly biocompatible (Bozzuto and Molinari, 2015). This also allows them to incorporate themselves into the cell membrane in order to deliver their contents. In order to minimise effects such as low specificity to cancer cells, as

well as off-target toxicity, surface modifications can be made such that the liposomes are targeted to cancer cells (Ganju et al., 2017). Such liposomes loaded with a miRNA mimic of miR-34 have successfully been used in a phase I clinical trial, which included patients who were diagnosed with one of many cancers, including hepatocellular cancer, pancreatic cancer and breast cancer (Beg et al., 2017). These liposomes were optimised to ensure the maximum possible time in circulation before degradation, and uptake of the contents into the target cells (Daige et al., 2014). This clinical trial was the first of its kind to successfully deliver a miRNA mimic using this formulation, named MRX34, to the desired target site in human patients (Beg et al., 2017).

A second delivery agent involved the use of what were named TargomiRs, which are comprised of non-viable minicells loaded with a nucleotide sequence (MacDiarmid and Brahmbhatt, 2011, Lin et al., 2014). These minicells use a bacterially-derived system to deliver their contents into target cells. As with the liposomes mentioned previously, additions to the surface of these minicells are used for targeting to cancer cells. These minicells were successfully used in a phase I clinical trial which included patients with mesothelioma (van Zandwijk et al., 2017). A high proportion of mesothelioma patients have high expression of the epidermal growth factor receptor (EGFR) (Destro et al., 2006). For this reason, the TargomiR used in the clinical trial was coated with an antibody to EGFR, thereby targeting the minicells to the mesothelioma cells (van Zandwijk et al., 2017). The purpose of this clinical trial was to determine whether levels of miR-16 could be successfully restored, and the safe range of doses of these minicells that could be administered to patients. MiR-16 has a tumour suppressor function in mesothelioma, and has been observed to be frequently down-regulated (Reid et al., 2013). Results from this clinical trial were promising, and the possibility of additional studies using TargomiRs in combination with chemotherapy or immune checkpoint inhibitors was mentioned (van Zandwijk et al., 2017).

Other methods have been proposed to deliver miRNA mimics and inhibitors efficiently to cancer cells, as well as other methods to inhibit miRNA function. These include miRNA sponges (Ebert et al., 2007), which are RNAs that contain multiple, tandem binding sites for the target miRNA, miR-masks (Wang, 2011), anti-AMOs that bind the 3’UTR of target mRNAs, thus inhibiting binding

by the miRNA of interest, and adeno-associated viral (AAV) vectors (Kota et al., 2009, Miyazaki et al., 2012), which contains the miRNA sequence conjugated with an expression plasmid, and is highly efficient in transducing these miRNA sequences into the target cell. However, these other methods have not yet reached the development stage where they can be tested in the clinical setting.

7.2.2 BCRP-targeted therapies

Since BCRP expression can be induced by both endocrine therapy (Chapter 6) and cytotoxic chemotherapy (Bram et al., 2009), and confers MDR (Allen and Schinkel, 2002), BCRP is a logical target for novel drugs that could be used to reverse chemoresistance (Doyle and Ross, 2003). Many drugs have been identified as potential inhibitors of this xenobiotic drug pump.

Dofequidar fumarate was initially identified as a quinolone derivative that inhibited another of the ABC drug pumps, Pgp (Suzuki et al., 1997). It was used in a phase III clinical trial where patients with breast cancer were also treated with a chemotherapy regimen of cyclophosphamide, doxorubicin and fluorouracil (CAF) (Saeki, 2004). It was discovered that patients had significant improvements in disease free survival (DFS) and overall survival (OS) if they had not received prior therapies. In further *in vitro* and *in vivo* studies, dofequidar fumarate was used to treat chemoresistant side populations of cells, consisting of cancer stem-like cells, from several cancer types including breast, that over-expressed BCRP but not any other ABC drug pumps (Katayama et al., 2009). It was discovered that this drug suppressed the function of BCRP. When xenografts composed of irinotecan-resistant side population cells were treated with irinotecan together with dofequidar fumarate, tumour growth was significantly reduced as opposed to irinotecan treatment alone. These results suggest that dofequidar fumarate could also be used in combination with cytotoxic chemotherapy in the presence of BCRP over-expression.

Lapatinib, a kinase inhibitor, is a HER2-targeting drug that has also been shown to inhibit BCRP function (Perry et al., 2010). In an *in vitro* study using several cancer cell lines including breast, significant synergy was observed when lapatinib was administered in combination with the cytotoxic chemotherapy drug SN-38, resulting in increased apoptosis. This increase in cell death was attributed to increased accumulation of SN-38 inside the cells.

Lapatinib was also shown to inhibit the efflux of a BCRP-specific substrate mitoxantrone. Two phase II trials investigating the combination of lapatinib with cytotoxic chemotherapy agents in ovarian, peritoneal and breast cancers did not show promising results however (Lin et al., 2011, Weroha et al., 2011). Excess toxicity was a major problem, and lapatinib showed only a limited ability to inhibit BCRP function.

Other kinase inhibitors have also been identified as potential inhibitors of BCRP function. Sunitinib malate has been shown to target multiple tyrosine kinase receptors (Mendel et al., 2003). A phase III clinical trial was conducted to determine whether the addition of this drug to the chemotherapy regimen fluorouracil, leucovorin and irinotecan (FOLFIRI) would be beneficial in the treatment of metastatic colon cancer (Carrato et al., 2013). This trial had to be discontinued, however, due to severe adverse effects, including toxicity-related death. Available results demonstrated no beneficial effect of the addition of sunitinib malate to OS. A phase I/II trial of sorafenib in combination with irinotecan for the treatment of metastatic colon cancer and *KRAS*-mutated tumours did, however, show some promising results (Samalin et al., 2014).

It seems although there are numerous potential BCRP inhibitors, the few that do reach the point of being tested in a clinical setting fail due to excess toxicity. As such, the search for inhibitors of the BCRP drug pump that can reverse chemoresistance is ongoing (Kathawala et al., 2017, Guo et al., 2018). It is possible, that as with miRNA-targeted therapies, BCRP inhibitors may be required to be delivered to cancer cells specifically, rather than be allowed to inhibit all BCRP pumps, as BCRP is expressed in a variety of healthy tissues that require this drug pump for normal function (Doyle and Ross, 2003). Previous BCRP inhibitors that failed in clinical trials, failed due to excess toxicity (Lin et al., 2011, Carrato et al., 2013), and it is possible that these toxicities may be prevented if the BCRP inhibitors were delivered specifically to cancer cells.

7.2.3 Combining existing cytotoxic chemotherapies with novel targeting therapies

When it was observed that HER2 expression played a significant role in the prognosis of breast cancer (Gschwind et al., 2004, Ross et al., 2009), HER2-

targeted therapies were developed that significantly improved DFS and OS (Dent et al., 2013, Mendes et al., 2015). These HER2-targeted therapies were administered in combination with existing cytotoxic chemotherapy agents, as before the development of these agents, chemotherapy alone was still beneficial. This highlights the possibility of adding novel therapies to existing therapies to improve outcomes, creating additive or even synergistic effects. Since individual miRNAs do not cause tumour growth or chemoresistance alone, any miRNA-targeted therapies should be administered in combination with existing therapies.

Preliminary studies have already been performed where miRNA-targeted therapy was combined with the cytotoxic chemotherapy agent paclitaxel to treat melanoma cells in a mouse model (Shi et al., 2014). In this study, miR-34a mimic was encapsulated in a lipid nanoparticle that also contained paclitaxel. These nanoparticles provided good protection for the miR-34a mimic and paclitaxel against degradation in serum. *In vitro* studies revealed this miRNA-chemotherapy combination had a synergistic anti-cancer effect. *In vivo* studies showed a significant increase in inhibition of tumour growth and elimination of cancer cells present in the lung using the combination as compared to nanoparticles loaded only with paclitaxel.

As demonstrated in section 7.2.2, all clinical trials investigating the effects of potential BCRP inhibitors were performed comparing cytotoxic chemotherapy alone with a combination of cytotoxic chemotherapy and the potential inhibitor. The benefit of improved efficacy of combination therapies is, however, accompanied by the disadvantage of more adverse side effects (Bergh et al., 2001, Slamon et al., 2001, Beslija et al., 2003). It is therefore important that the correct combinations of therapies are used so as to limit adverse effects and maximise positive outcomes. This leads back to the importance of knowing the molecular changes that are induced by different therapies so that we have a better understanding of which combinations would be most effective.

7.3 Conclusions

In this thesis I have discovered several molecular mechanisms that contribute to chemoresistance. In particular, I identified miRNAs, miR-26b and miR-195,

that enhance chemoresistance, but I failed to identify the specific molecules downstream of these that cause these functional effects. Nevertheless, the miRNAs themselves present potential therapeutic targets. I also determined that increased expression of the xenobiotic drug pump BCRP induced by endocrine therapy, also confers chemoresistance, highlighting the concerns about the sequence of therapies of endocrine therapy followed by chemotherapy. In summary, it is clear from my results that resistance to chemotherapy is a result of multiple molecular changes that all contribute to overall resistance, and that novel miRNA and BCRP targeting therapies could be used in combination with existing systemic therapies to prevent and/or overcome chemoresistance.

Appendix A: Recipes

A.1 Cell lysis buffer for pulldown experiment

- 20mM Tris (pH7.5) (93362; Sigma; St Louis, USA)
- 200mM NaCl (S3014; Sigma; St Louis, USA)
- 2.5mM $MgCl_2$ (M8266; Sigma; St Louis, USA)
- 0.05% Igepal (I8896; Sigma; St Louis, USA)
- Add fresh: 60U Superase-In RNase Inhibitor (AM2694; Ambion; Foster City, USA), 1mM DTT (D1532; Invitrogen; Carlsbad, USA), 1mM PMSF (36978; Thermo Fisher; Waltham, USA)

A.2 Laemmli sample buffer (4x)

- 9.6ml 1M Tris-HCl (93363; Sigma; St Louis, USA)
- 3.2g SDS (436143; Sigma; St Louis, USA)
- 116ml glycerol (BP229-1; Fisher Scientific; Hampton, USA)
- 11.2ml water
- Pinch of Bromophenol blue (ICNA04805732; VWR; Radnor, USA)
- Add fresh on the day: 0.2M DTT (D1532; Invitrogen; Carlsbad, USA)

A.3 RIPA buffer

- 10mM Tris-HCl pH8.0 (93363; Sigma; St Louis, USA)
- 140mM NaCl (S3014; Sigma; St Louis, USA)
- 0.1% SDS (436143; Sigma; St Louis, USA)
- 1% Triton X-100 (T8787; Sigma; St Louis, USA)
- 0.1% sodium deoxycholate (89904; Thermo Fisher; Waltham, USA)
- 1mM EDTA (E6758; Sigma; St Louis, USA)
- 0.5mM EGTA (E3889; Sigma; St Louis, USA)
- Add fresh on the day: 1mM PMSF (36978; Thermo Fisher; Waltham, USA), 1mM DTT (D1532; Invitrogen; Carlsbad, USA)

A.4 Scott's tap water

- 20g sodium bicarbonate (S5761; Sigma; St Louis, USA)
- 3.5g magnesium sulphate (MX0075; Sigma; St Louis, USA)
- Make up to 1l

A.5 Tris-Buffered Saline (10x) + 0.1% Tween-20 (TBST)

- 60ml 2.5M NaCl (S3014; Sigma; St Louis, USA)
- 20ml 1M Tris-HCl (93363; Sigma; St Louis, USA)
- Make up to 1l
- Check pH 7.5
- 0.1% Tween-20 (BP337-100; Fisher Scientific; Hampton, USA) in 1x TBS

Appendix B: Commands used for analysis of RNA-Seq data using DESeq

```
> library (“Rsubread”)
> library (“DESeq”)


> input_files <-
c(“BAM files”)


> Design <- data.frame(condition = c(“1”, “2”, “C”),
test1= c(“y”,”n”,”y”), test2= c(“n”,”y”,”y”))
```

For comparing 1 target miRNA sample with control sample:

```
> test2 = Design$test2 == ”y”
> countTable2 = fc$counts[ , test2]
> condition = factor( c(“biotin”,”scrambled”))
> cds2 = newCountDataSet(countTable2, condition)
> cds2 = estimateSizeFactors(cds2)
> sizeFactors(cds2)
> head( counts(cds2, normalized=TRUE))
> cds2 = estimateDispersions(cds2, method=”blind”,
sharingMode=”fit-only”)
> res3 = nbinomTest(cds, “biotin”, “scrambled”)
> write.csv(res3, file=”B2vsCon.csv”)
> res3 = nbinomTest(cds2, “biotin”, “scrambled”)
> write.csv(res3, file=”B2vsCon.csv”)
```

Appendix C: Quality assessments of RNA extracted from FFPE colon cancer samples using TapeStation automated analysis

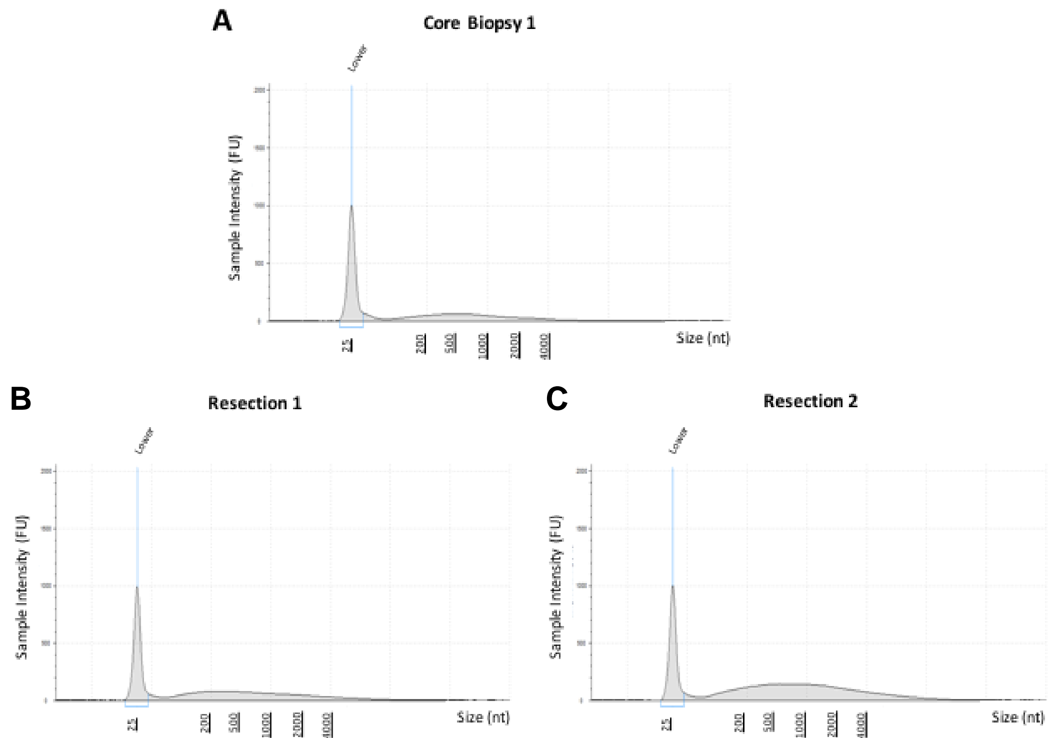


Figure C.1: Graphs showing range of nucleotide fragment lengths of RNA extracted from test FFPE samples

RNA was extracted from three other FFPE tumour samples; one from a biopsy samples (A) and two from resection samples (B and C). The RNA was run on the Agilent 2200 TapeStation using the High Sensitivity RNA ScreenTape to determine average lengths of RNA fragments extracted from tissues stored as FFPE samples. The size of the fragments are shown on the x-axis and the quantity of fragments are shown on the y-axis. The large peak at 25nt is a marker used for reference.

Appendix D: MiRNAs consistently changed in all five patients that did not meet the thresholds for fold change

Table D.1: MiRNAs consistently deregulated post-NAC that did not meet the minimum or maximum thresholds for fold change in all patients

MiRNA	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
let-7c	1.708	2.759	1.989	1.147	1.941
miR-26a	1.718	1.18	2.83	1.544	2.686
miR-330	1.695	1.328	1.112	1.822	>30*
miR-335	3.147	4.941	1.156	1.547	>120*
miR-362	1.683	1.031	1.266	2.85	>100*
miR-483-5p	7023.331	3.336	1.127	2.27	19.93
miR-493	>880*	>110*	Not detected	>40*	>30*
miR-885-5p	>870*	1.144	42.026	2.686	>20*
miR-625	27.755	1.237	1.102	4.038	>10*
miR-365	-1.18	-1.47	-4.03	-1.19	-1.66

MicroRNAs that were consistently deregulated post-NAC across all five patients were identified. A minimum threshold for the fold change for each individual patient of 1.3 was chosen for up-regulated miRNAs and a maximum threshold for fold change for each individual patient of -1.25 was chosen for down-regulated miRNAs. Fold changes marked as '>number*' are where the miRNA was not detected in the pre-NAC sample and a Ct value of 40 was used to determine an approximate fold change.

Appendix E: Quality assessments of library-prepared samples for RNA-Seq

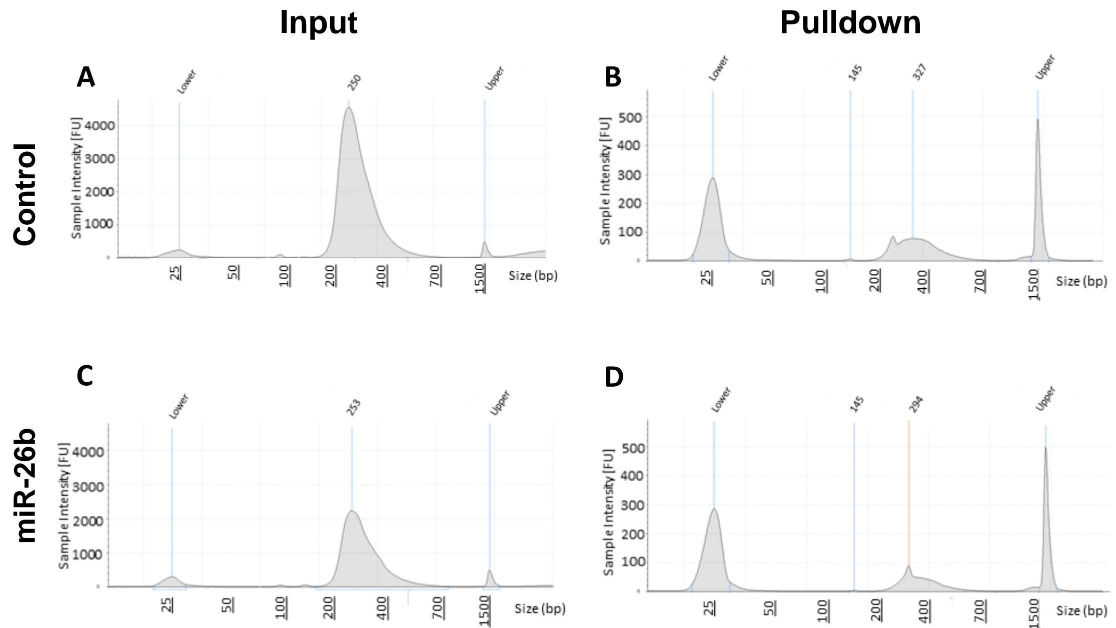


Figure E.1: Graphs showing populations of library prepared samples

Library-prepared samples were analysed using the Agilent 2200 TapeStation using the High Sensitivity DNA ScreenTape to ensure that only library cDNA fragments were present in the samples and no primer dimers were present. The x-axis shows fragment length (nucleotides) and the y-axis shows abundance. The 'lower' and 'upper' peaks at 25nt and 1500nt are markers used as internal references. The left panels show libraries prepared from total RNA extracted from cells that did not undergo the pulldown process (input) and the right panels show libraries prepared from RNA extracted following the pulldown process (pulldown). A and B show control transfected samples, and C and D show miR-26b mimic transfected samples.

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