The role of fibroblasts in chemoresistance in triple negative breast cancer

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Intellectual property and publication statement

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Thesis abstract

Triple negative breast cancer (TNBC) accounts for 10-15% of breast cancers and is associated with relatively poor prognosis. TNBC is characterised by lack of ER, PR and Her2 expression, and consequently does not respond to the targeted treatments routinely used to treat other breast cancers. Therefore, cytotoxic chemotherapy is the only systemic treatment for primary TNBC in routine use. Resistance to chemotherapy in TNBC is a major clinical issue and is reflected in the relatively high rate of early recurrences. Cancer associated fibroblasts (CAFs) are the most abundant cell type in the tumour stroma and have been shown to influence behaviour of the tumour cells. My aims were to determine whether CAFs modify responses of TNBC cells to chemotherapy, to identify molecular pathways responsible for this cross-talk, and to develop these pathways as potential therapeutic targets to enhance chemotherapy responses.

I have shown that breast CAFs, but not normal fibroblasts, dose-dependently protect the claudin-low TNBC cell lines MDA-MB-231 and MDA-MB-157 from the chemotherapeutic epirubicin. By contrast, the basal claudin-high TNBC line MDA-MB-468 was not protected, demonstrating that the CAFs' influence is specific to certain TNBC characteristics. Transcriptome profiling demonstrated that CAF-induced protection of MDA-MB-231 cells was associated with deregulation of specific genes and pathways, including activation of the interferon-signaling pathway. Recombinant interferons were sufficient to protect MDA-MB-231 and MDA-MB-157 cells, implicating IFN as the key mediator of CAF-induced protection. Inhibition of the activation of interferon signaling using blocking antibodies, JAK/STAT inhibitors or under-/over-expression of specific signaling intermediates (miR-155) reduced CAF-dependent protection of MDA-MB-231 and MDA-MB-157 cells, suggesting that CAF-induced up-regulation of interferon signaling is necessary for CAF-induced protection.

I concluded that CAF-induced activation of interferon signaling in certain TNBCs is a potential mechanism of chemoresistance, and therefore that addition of inhibitors of interferon signaling to chemotherapy regimens in patients with stromal-rich TNBCs may improve chemotherapy responses.

Contents

List of Tables

List of Figures

Abbreviations

Ago2- Argonaute 2 protein
ALDH- Aldehyde dehydrogenase
Bad- Bcl-2-associated death promoter protein
BASC- BRCA1-associated genome surveillance complex
Bax- Bcl-2-like protein 4
Bcl-2- B cell lymphoma 2
Bcl-xl- B cell lymphoma-extra large
BCRP- Breast cancer resistance protein
BCS- Breast conserving surgery
Bid- BH3 interacting-domain death agonist
Bim- Bcl-2-like protein 11
BL1- Basal-like-1
BL2- Basal like-2
BLIA- Basal-like immune-activated
BLIS- Basal-like immune-suppressed
CAF- Cancer associated fibroblast
CDK- cyclin-dependent kinase
CRK/L- Crk adaptor protein/ligand
CT- Computerised tomography
CXCR/L- CXC-motif chemokine receptor/ ligand
CYP- Cytochrome P450
DC- Dendritic cells
DCIS- Ductal Carcinoma *in situ*
DGSCR8- DiGeorge syndrome critical region gene 8
DNA- Deoxyribonucleic acid
ECM- Extracellular matrix
EGF- Epidermal growth Factor
EGFR- Epidermal growth factor receptor 1
EMT- Epithelial mesenchymal transition
ER- Oestrogen receptor
FACS- Fluorescence activated cell sorting
FAK-Focal adhesion kinase
FAP- Fibroblast activated protein
FGF- Fibroblast growth factor
FOXO3a- Forkhead box O3
GAS- IFN-γ- activated site
GDP/GTP- Guanine di/triphosphate
GFP- Green fluorescent protein
HDAC- Histone deacetylase
HER2- Human epidermal growth factor receptor 2
HGF- Hepatocyte growth factor
ICAM1- Intra-cellular adhesion molecule I
IFITM1- Interferon induced transmembrane protein 1
IFN- Interferon
IFNAR- Interferon-α receptor
IFNGR- IFN-γ receptor
IFNLR- IFN- λ receptor 1
IGF/R- Insulin growth factor/receptor

IGFBP- Insulin-like growth factor binding proteins
IKKε- IκB kinase ε
IL-6- Interleukin-6
IM- Immunomodulatory
INPP4B- Inositol polyphosphate 4-phosphotase Type II
IRDS- IFN-related DNA damage resistance signature
IRF- IFN response factors
IRF- Interferon regulatory factor
ISG- Interferon stimulated gene
ISGF3- ISG Factor 3
ISRE- IFN-stimulated response element
JAK- Janus activated kinase
LAR- Luminal androgen receptor
LOX- Lysyl oxidase
M- Mesenchymal
MAL- MYD88-adaptor like
MAPK- Mitogen activated protein kinase
Mcl-1- Myeloid leukemia cell differentiation protein 1
MDR1- Multidrug resistance protein 1
MDSC- Myeloid-derived suppressor cells
MEK- Mitogen activated protein kinase
miRNA- microRNA
MLH1- MutL homolog 1
MMP- Matrix metalloproteinase
MRI- Magnetic resonance imaging
MRP1- Multi-drug resistance protein-1
MSC- Mesenchymal stem cells
MSH2- MutS protein homolog 2
MSL- Mesenchymal-like
mTOR- Mammalian targets of rapamycin
MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MX- Myxovirus resistance
MYD88- Myeloid differentiation primary response 88
NAC- Neoadjuvant chemotherapy
NF(1/2)- Immortalised normal fibroblasts(1/2)
NF-κB- nuclear factor-κB
NHS- National Health Service
NK cells- Natural killer cells
NSCLC- Non small cell lung carcinoma
NST- No special type
OAS- Oligoadenylate synthase
PACT- protein kinase R-activating protein
PAMP- Pattern-associated molecular patterns
PARP- Poly-ADP ribose polymerase
pCAF- Primary cancer-associated fibroblast
pCR- Pathological complete response
pNF- Primary normal fibroblasts
PD-1/PDL-1- Programmed cell death protein/ ligand 1
PDCD- Programmed cell death
PDGF- Platelet derived growth factor
PI3K- Phosphatidylinositide-3-kinase

$PIP_{2/3}$- Phosphatidylinositol-4,5- bis/triphosphate
PR- Progesterone receptor
PRR- Pattern recognition receptors
PTEN- Phosphatase and tensin homolog
q/RT/PCR- Quantitative/ Real time polymerase chain reaction
RAP1A- Ras related protein Rap-1A precursor
RISC- RNA-induced silencing complex
RNA- Ribonucleic acid
SDF-1- Stromal derived factor-1
SOCS- Suppressor of cytokine signaling
SPARC- Secreted protein, acidic and rich in cysteine
STAT- Signal transducers and activator of transcription proteins
TBK- TANK-binding kinase 1
TDLU- Terminal duct lobular unit
TGF-β- Transforming growth factor- β
TLR-Toll-like receptor
TNBC- Triple negative breast cancer
TNM- Tumour node metastasis
TPM1- Tropomyosin1
TRAM- TRIF-related adaptor molecule
TRBP- Trans-activating response RNA binding protein
TRIF- TIR-domain-containing adapter-inducing interferon-β
TYK- Tyrosine kinase
UNC-Unclassified
USP18- Ubiquitin specific peptidase 18
UTR- Un-translated region
VCAM1- Vascular adhesion molecule I
VEGF- Vascular endothelial growth factor
WNT- Wingless/Integrated
α-SMA- α-smooth muscle actin

Chapter 1– Introduction

1.1 Breast cancer epidemiology and development

Breast cancer is the most common cancer in the UK, with around 54,900 cases a year accounting for 15% of all new cancer cases in 2015; this means there are approximately 150 new breast cancer diagnoses every day (Cancer Research UK). Breast cancer is very prevalent in females, but also presents, rarely, in males amounting to approximately 380 new cases of male breast cancer each year (Cancer Research UK). Breast cancer is responsible for the 4th highest mortality of all cancer types and accounts for 7% of all cancer related deaths. In females, it is the 2nd highest cause of cancer related deaths, accounting for 15% of female cancer mortalities with 11,563 deaths associated with breast cancer in 2016 (Cancer Resarch UK). Breast cancer survival rates have almost doubled in the last 40 years. In the 1970s, 40% of women diagnosed with breast cancer survived longer than 10 years, however now this figure is now 78%. Currently 95% of women diagnosed with breast cancer survive a year, with 65% surviving 20 years or more (Cancer Research UK).

Cancers, including breast cancer, are formed from the poorly controlled division of cells, which is driven by an accumulation of genetic and epigenetic changes. Mammary epithelial cells that have acquired mutations over time divide rapidly and have the ability to sustain proliferation, evade growth suppression signals and resist cell death (Hanahan et al, 2000).

Breast cancers are initially confined to epithelial-lined compartments of the breast termed the lobules and ducts and in most cases are believed to arise in the Terminal Duct Lobular Unit (TDLU) (Wellings et al,1973) (Figure 1.1). Whilst confined to epithelial-lined compartments, the cancer is known as *in-situ* breast cancer and is non-invasive (Cowell et al, 2013). Further proliferation of the breast cancer cells, confined in the lobules and ducts, leads to the integrity of the basement membrane of these breast structures being lost or fragmented, and it is at this stage that the cancer becomes invasive (Duffy et al, 2000). Cancer cells are then able to infiltrate into the surrounding breast tissue and

can continue to grow as well as migrate throughout the breast tissue (Weigelt et al, 2005). It is in the surrounding breast tissue where the tumour will create its own niche and microenvironment, which in turn will aid the tumour's growth and spread into lymph and blood vessels (Wiseman et al, 2002). Infiltration into blood and lymph vessels and circulatory systems can enable tumour cells to travel to secondary sites such as the brain, lung and bone (Lacroix et al, 2006). Here, the tumour cells can begin to grow and form metastases, which impair the normal functions of distant organs. It is commonly the formation of secondary breast cancers that is responsible for the death of breast cancer patients (Hart et al, 1980).

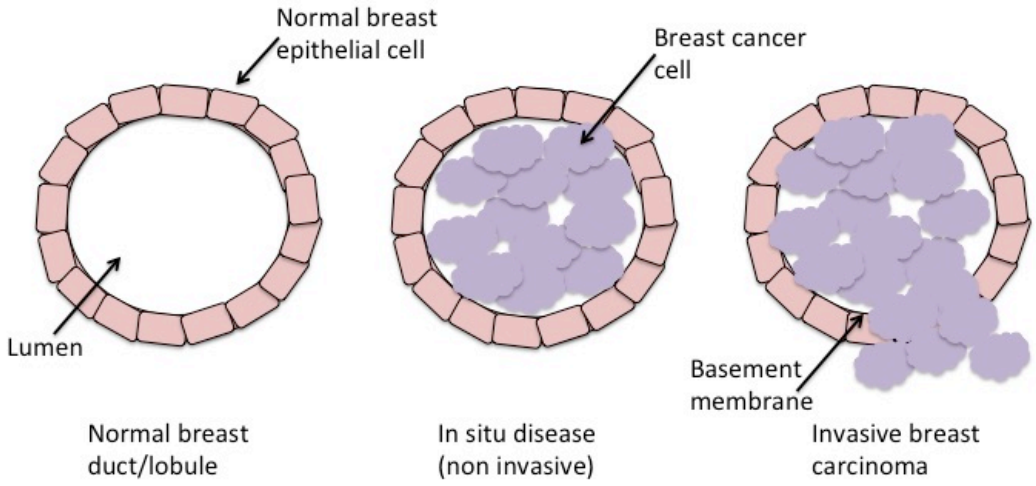


Figure 1.1: Breast cancer development from normal breast structures to invasive carcinoma
Normal breast tissues include normal epithelial cells (pink) surrounding open lumens. Breast cancer cells (purple) are initially confined to within the lumen of the breast ducts and lobules, and are referred to as carcinoma *in situ*. Breast carcinomas become invasive when they break down the basement membrane of the ducts/lobules, and invade into the surrounding tissue.

1.2 Breast cancer classification

Breast cancer is not one homogenous cancer type, and it is now commonly recognized that there are multiple different classes of breast cancer (Perou et al, 2000), which can be defined both histologically and molecularly. Both classifications are discussed in this section.

1.2.1 Histological classification of breast cancers

One major classification system for breast cancer is based on their microscopic appearance, as determined by histopathologists (Rosai, 2011). Importantly, histological classification is not just taxonomical, but the different subtypes it defines correlate with prognosis, and patients with these different disease subtypes are recommended for different treatment strategies (Makki et al, 2015).

Initially, malignant lesions are split into 2 groups: *in situ* carcinomas and invasive carcinomas (see Figure 1.1) (Burstein et al, 2004). This distinction is not one of type but how far the disease has progressed. *In situ* disease occurs prior to invasive carcinomas (Hu et al, 2008).

1.2.1.1 Classification of *in situ* disease

Whilst it was historically believed that *in situ* carcinomas originated from ductal (confined to breast ducts) or lobular (confined to breast lobules) compartments, this is not the case (Wellings et al, 1973). The difference between what is called lobular and ductal is merely derived from different molecular repertoires governing the morphology of lobular neoplasia in particular, where inactivation of the adhesion complex, normally via malfunction of E-cadherin, occurs (Mastracci et al, 2005). Nearly all breast cancer derives from the TDLU (Wellings et al, 1973).

At a practical/clinical level the most important determinant in ductal carcinoma *in situ* (DCIS) is nuclear grade (Lagios et al, 1989). DCIS also can grow in a variety of morphologies; according to Makki et al (Makki et al, 2015), DCIS can be further classified as:

- Comedo or solid (cancer cells have completely filled the duct)
- Cribform (gaps between cancer cells within the duct)
- Micropapillary (dilated ducts lined by stratified population of monotonous cells)
- Papillary (asymmetrical fern like pattern within the duct)

However, mixed patterns often occur making the usefulness of the architectural classifications minimal (Vinay et al, 2010). Modern practice would be to record the nuclear grade of the DCIS, which is generally mandatory in minimum data sets. The pattern is generally not mandatory data (Pinder et al, 2010).

1.2.1.2 Classification of invasive disease

Invasive carcinomas are also highly heterogenous and can be further classified into tumours with very distinct morphological features known as 'specical type'. (Shi et al, 2017). These 'special type' tumours include tubular, ductal lobular, invasive lobular, mucinous (colloid) and medullary. They all contain some reproducible characteristics that can be recognized down the microscope with varying degrees of agreement (Page, 2003).

The remainder of invasive carcinomas (70-80%) are put in the category of invasive ductal (infiltrating ductal) of no special type (NST) (Lakhani et al, 2012). It is important to recognize that invasive ductal carcinoma NST is not a specific type of invasive carcinoma but any carcinoma for which no specific morphological profile has yet been determined (Malhotra et al, 2010).

To attempt to predict better the behavior of breast cancers, histological grading was divised by Bloom and Richardson (Bloom et al, 1957) and later modified by Elston and Ellis (Elston et al, 1991). This grading system gives some guide to the biological aggression of the tumour. Grade is determined by a combined score derived from the degree of tubule formation, the number of mitotic figures in the tumour site, and the nuclear pleomorphism. Each are scored 1-3. Tumour grading is determined from the additive score from each of the components; 3-5 is assigned grade I, 6-7 grade II and 8-9 Grade III (Bloom et al, 1957, Elston et al, 1991).

As described by Bloom and Richarson, and Elston and Ellis, grade 1 breast cancer cells are normally well differentiated with a high proportion of the tumour (above 75%) showing normal breast tissue structures. Grade 1 tumours have a low number of mitotic cells and the cells are small and uniform with minimal

variation in size and shape. Grade 2 tumours are moderately differentiated, where the tumour has between 10%-75% of normal breast structures and a moderate mitotic rate. The tumour cells have moderate nuclear variation so are slightly bigger than normal cells and display some variation in shape. Grade 3 tumours are poorly differentiated with less than 10% of the tumour displaying normal breast structures. Grade 3 tumours have a high mitotic rate and the tumours cells look markedly different to normal cells with varied size and shape. Histological grading can be a strong predictor of patient outcome; for example, a study of 2219 breast cancer cases demonstrated that histological grade was strongly associated with both breast cancer specific survival and disease-free survival (Rakha et al, 2008).

The stage of the cancer is an assessment of the size of the tumour, lymph node involvement and spread of the cancer beyond the breast. The staging has been classified by the tumour, node, metastasis (TNM) system, which has been characterized by the American joint committee of cancer (American Cancer Society). Stage 1 breast cancer tumours are classically no larger than 2cm and in some cases there are also small clusters of cancer cells in the lymph node. Stage 2 breast tumours are typically between 2-5cm in size normally with axillary lymph node involvement. Stage 3 tumours are progressively larger than Stage 2 tumours and are more likely to have spread to multiple lymph nodes. Finally stage 4 breast cancers are commonly referred to as metastatic or advanced breast cancer. The breast cancer in stage 4 has spread beyond the breast tissue into secondary sites such as the brain, bone and lungs (American Cancer Society).

1.2.2 Clinical molecular classification of breast cancers

In seminal work by Perou, it was shown that molecular profiles could also be used to separate subtypes of breast cancer (Perou et al, 1999). Four subtypes of breast cancer are the mainstay of the current molecular classification used in clinical practice; these are Luminal A and B, HER2 subtype and triple negative breast cancers (Perou et al, 2000). Breast cancer subtypes were originally defined based on microarray-based mRNA expression profiles, but are

classically defined clinically using the surrogate markers oestrogen receptor (ER), progesterone receptor (PR), HER2 and Ki67 (Perou et al, 2000). The characteristics of all these subtypes, and their standard treatment regimens, are summarized in Table 1.1.

1.2.2.1 Luminal A and B breast cancer

ER positive breast cancers account for around 70% of all invasive breast cancers and can be further divided in to Luminal A and Luminal B breast cancers. Luminal A and B cancers can also have progesterone receptor (PR) expression (Vallejos et al, 2010). Luminal A and B cancers are distinguished between using Ki67 expression, which is a proliferation marker. Luminal B breast cancers have higher levels of Ki67 and can also express high levels of HER2 (Inic et al, 2014). Both Luminal A and B breast cancer patients tend to have a relatively good prognosis, however Luminal B tends to have slightly worse prognosis than Luminal A, which is presumably related to the higher proliferation rate (Parise et al, 2014).

1.2.2.2 HER2 positive breast cancer.

HER2 invasive breast cancer accounts for 15% of all breast cancers and, likened to its name, is characterised by high gene and protein expression of HER2 and most, but not all, are ER negative. (Cooke et al, 2001).

1.2.2.3 Triple negative breast cancer

Another classified group of invasive breast cancer is triple negative breast cancer (TNBC); these will be discussed in more detail further on in section 1.4. TNBCs are ER and PR negative, and are also HER2 negative. Many TNBC have basal characteristics and as such express high molecular weight cytokeratins CK 5 and 14 and also EGFR1 (Sørlie et al, 2001). However, 25% of TNBC do not fall into the basal-subtype despite a substantial overlap of characteristics (Rakha et al, 2008). As I discuss later, the taxonomy of these tumours is quite complex with a great deal of hidden diversity.

1.2.2.4 Basal-like cancer

As indicated above, basal-like cancers express genes that are commonly associated with normal breast epithelial cells. In order for identification of basal like breast cancer, expression of growth factors and growth factor receptors (EGFR1, VEGF and IGFR) are determined as well as having a specific cytokeratin profile (Nielsen et al, 2004).

	Luminal A and B breast cancer	**HER2 breast cancer**	**Triple negative breast cancer**
Occurrence	70% of all invasive breast cancers	15% of all invasive breast cancers	10-15% of all invasive breast cancers
Classification	Presence of Oestrogen (ERα) and Progesterone (PR) receptors	No ER/PR HER2 genomic amplification, and high expression of Her2 protein	Lack of ER, PR and Her2
Systemic Treatment	Endocrine therapy (tamoxifen or aromatase inhibitors) Chemotherapy (mainly high grade or stage tumours)	Targeted Her2 treatment, such as trastuzumab Chemotherapy	No targeted treatment Chemotherapy
Typical Prognosis	Good	Poor	Poor

Table 1.1: Characteristics of the main clinical subtypes of invasive breast cancer
The main clinical molecular subtypes of invasive breast cancer are shown highlighting their occurrence, classification, treatment options and prognosis.

1.3 Breast cancer treatment

A diverse range of treatments are recommended for primary breast cancer patients, with the treatment plan dependent substantially on the breast cancer subtype as well as the patient's choice, age and overall health. The various treatment options available to patients will be covered in 3 different subsections below.

1.3.1 Local therapy

Most breast cancer patients will be treated surgically by either a mastectomy or breast conserving surgery (BCS). Mastectomies remove all breast tissue including the nipple and most of the skin that covers the breast (Sarrazin et al, 1989). In breast conserving resections, the surgery is not as drastic as a mastectomy. The cancerous cells are removed as well as a margin of normal tissue surrounding the cancer, but the whole breast is not removed (Chen et al, 2016). The recommendation to either undergo breast conserving surgery or breast mastectomy is determined by clinicopathological findings and radiology. Patient choice is equally as important with the patients' quality of life also taken into account regarding which surgery suits their needs best (Morrow et al, 2009). For larger tumours, patients could be advised to have a mastectomy, although in some cases shinking the tumour with neoadjuvant chemotherapy (NAC) can allow for more conservative surgery if the lesion shrinks sufficiently (Trimble et al, 1993).

Smaller tumours are more likely to be treated with BCS. In BCS, adjuvant (after surgery) radiotherapy is recommended for BCS patients to provide a localized therapy to target any remaining tumour cells within the breast tissue (Dean et al, 2009). Trials have shown that this combination of BCS and radiotherapy has a local recurrence rate that is not different from mastectomy (Chen et al, 2017), demonstrating that the approach is safe. Mastectomy patients may also receive radiotherapy if they show factors that are indicative of poor prognosis such as positive lymph nodes (Tsoutsou et al, 2013).

1.3.2 Cytotoxic chemotherapy

Despite targeted treatments being available for specific breast cancer subtypes, some breast cancer patients also receive traditional cytotoxic chemotherapy in addition to targeted treatments (Slamon et al, 2001). Around 34% of breast cancer patients receive chemotherapy for treatment of primary breast cancer (National Cancer Registration & Analysis Service and Cancer Research UK, 2017). The decision to undergo chemotherapy treatment is strongly influenced by stage, grade and size at diagnosis. For example, only 17% of stage I breast cancer patients will receive chemotherapy in comparison to 70.5% of stage III breast cancer patients (National Cancer Registration & Analysis Service and Cancer Research UK, 2017).

Breast cancer patients can either receive systemic treatment prior to surgery (neoadjuvant) or after surgery (adjuvant); in both cases, the primary aim is to reduce the chances of metastatic recurrences by targeting sub-clinical micrometastases (EBCTCG, 2018). In addition, neoadjuvant chemotherapy (NAC) aims to reduce the primary tumour size to enable the option of breast conserving surgery instead of mastectomy. NAC can also be used to gauge drug response of the tumour (Thompson et al, 2012). An advantage of NAC is the monitoring of the primary tumour response to chemotherapy as a different chemotherapy drug could be used if the response in the primary tumour is minimal; the assumption being that the response of micrometastases will mirror the primary tumour (Buchholz et al, 2003).

Response rates can be determined through non-invasive imaging (MRI or ultrasound), to determine if the primary tumour has changed in size (Chen et al, 2007). In the metastatic setting, response can also be determined by imaging of overall tumour burden using scintigraphy (bone scan), MRI and CT scans as well as assessing circulating tumour markers (Woolf et al, 2015). In the case of adjuvant chemotherapy it is not possible to monitor response as the primary tumour has been removed and any sub-clinical micrometastases are, by definition, not detectable (Chew et al, 2001). Effectiveness of adjuvant

treatment can only be assessed from subsequent frequency and time post treatment of any recurrences (Isakoff et al, 2010).

Luminal A and B patients receive chemotherapy as well as endocrine therapy if there are indications of poor prognosis such as involved lymph nodes, which are indicative of likely cancer metastasis (NCCN guidelines, 2016). HER2 positive patients are given chemotherapy in combination with targeted Herceptin treatment (Piccart-Gebhart et al, 2005). However, because of the lack of molecular targets for precision therapy in TNBC patients, chemotherapy is the only systemic therapy routinely available to them. TNBC patients will therefore receive chemotherapy, while chemotherapy is only given to a minority of patients with other subtypes of primary breast cancer (Yershal et al, 2014).

1.3.3 Targeted systemic treatments

Molecularly-targeted treatments are also available to breast cancer patients but they are dependent on the subtype of breast cancer. Two classes of targeted agent are commonly used: endocrine agents, or HER2-targeted agents (Masoud et al, 2017).

Luminal A and B breast cancer patients can receive targeted endocrine therapy because Luminal A and B breast cancers express the ER, which is the target of endocrine agents. An example of endocrine therapy is tamoxifen, which is a selective ER modulator (Nolvadex Adjuvant Trial Organisation, 1998). Tamoxifen recognizes and competitively binds to the ER thereby reducing ER driven proliferation of the cancer cells (McDonnell et al, 2010).

HER2 expression, in Luminal B and HER2 subtypes of breast cancer, also provides a route for targeted therapy. Herceptin (trastuzumab) targets HER2 positive cells and binds to the extracellular domain of HER2 on the membrane of the cell (Baselga et al, 1998). Upon binding to HER2, Herceptin prevents receptor dimerization and therefore prevents the activation of the HER2 signaling pathway (Clarke et al, 2015). Consequently, inhibition of HER2 dimerization prevents activation of the MAPK and PI3K/Akt pathway leading to

an increase in cell cycle arrest and suppression of tumour growth and survival (Vu et al, 2012). Herceptin also has the ability to attract immune cells to HER2 positive cancer cells, such as natural killer cells, which leads to antibody dependent cellular cytotoxicity and lysis of HER2 positive breast cancer cells (Park et al, 2010). Herceptin can be given alongside chemotherapy (Baselga et al, 2001) and can also be used in combination with another agent, pertuzumab (Harbeck et al, 2013). Pertuzumab is a targeted therapy that binds specifically to HER2 to prevent HER2 dimerisation. Pertuzumab, therefore, prevents activation of signaling pathways that normally stimulate cell proliferation and tumour growth (Baselga et al, 2012). The use of Herceptin and pertuzumab when combined with chemotherapy in both the neoadjuvant and adjuvant setting have been shown to increase cell death in early HER2 positive breast cancer patients (Gianni et al, 2016, von Minckwitz et al, 2017).

1.4 Triple negative breast cancer

TNBC epidemiology, classification, treatment and prognosis will be discussed in the next sections to highlight the importance of further investigation into treatment options for TNBC patients.

1.4.1 Triple negative breast cancer epidemiology and clinical characteristics

TNBC accounts for 10-15% of all breast cancers in caucasians (Badve et al, 2011), and commonly affects a younger population of women who are often pre-menopausal. The mean diagnosis age of TNBC is 46 years (±12.2 years) in comparison to other breast cancer patients for whom the mean diagnosis age is 53 years (±9.78 years) (Sajid et al, 2014). In the TNBC group, 65.9% were younger than 50 years old with 34.1% older than 50 years old (Sajid et al, 2014).

Studies have also shown a higher proportion of TNBC cases in African-American who are pre-menopausal. 39% of pre-menopausal breast cancer cases in African-American women are TNBC. This is compared to 14% in post-

menopausal African-American women and 16% any aged non-African-American women (Carey et al, 2006).

BRCA1/2 mutation carriers have also shown to have a link to TNBC with a lifetime risk of developing TNBC at 50%-80%. From a recent study analyzing 3,791 BRCA1/2 mutation carriers diagnosed with invasive breast cancer, 68% were TNBC and 32% non-TNBC (Mavaddat et al, 2012). However, it is important to note that not all TNBCs are derived from BRCA1/2 mutations (Young et al, 2009, Peshkin et al, 2010). In addition, somatic mutations of TP53 (82%) frequently occur in TNBC cancers in comparison to Luminal A, Luminal B and HER2 cancers (12%, 29% and 72%) (Cancer Genome Altas Network, 2012).

TNBCs are highly aggressive with high proliferation and migratory properties. Because of their high proliferation rate, TNBCs grow quickly in comparison to other breast cancer subtypes (Matos et al, 2005). TNBC patients commonly present with metastatic recurrences, with secondary tumours presenting themselves in common breast metastases sites such as the brain, bone and lungs (Fulford et al, 2007). 5-year disease free survival of TNBC is the worst for all breast cancer subtypes, at 69.1% in comparison to the highest 5-year disease free survival of Luminal A cancers at 92.1%. At 5 years, overall survival of TNBC is 78.5% in comparison to Luminal A 95.1% (Hennigs et al, 2016, Zhang et al, 2013).

1.4.2 Triple negative breast cancer classification

As previously described, TNBCs lack expression of ER, PR and HER2 but even with these characteristics in common, TNBCs define a very diverse group of tumours, which are heterogenous in morphological and molecular terms (Perou et al, 2000). In previous years, there has been large debate over the subclasses within TNBC even now there is still some dispute between scientists on the classifications.

For example, using gene expression profiles and clustering analysis, Lehmann et al (Lehman et al, 2011) determined there are 6 TNBC subtypes: luminal androgen receptor (LAR), basal-like 1 (BL1), basal-like 2(BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and unclassified (UNC). However, using RNA and DNA profiling analysis, Burstein et al (Burstein et al, 2015) distinguished 4 groups; LAR, M, basal-like immune-suppressed (BLIS) and basal-like immune-activated (BLIA) (see Table 1.2).

The LAR subtype (in both papers) is the most distinct and abundant subtype In TNBC, with estrogen/androgen pathways up-regulated compared to other TNBC subtypes. It is characterized by luminal gene expression and is driven by the androgen receptor.

M (in both papers) and MSL (Lehman et al, 2011) tumours have high levels of certain epithelial genes, particularly those involved in epithelial mesenchymal transition (EMT) and signaling pathways involved in cell motility, growth and differentiation. For example, increased levels of genes involved in the TGF-β pathway, growth factor pathways (FGF, IGF and PDGF) and Wnt pathway. A further subtype, “claudin-low”, has also been identified within the M and MSL subtypes (Dias et al, 2017). The claudin-low subtype is characterized by a lack of cell-cell junction proteins and has high expression of EMT, immune response (interferon pathway) and cancer stem-like genes (Dias et al, 2017). The M and MSL groups were distinguished by the MSL group showing decreased expression of genes required for cell proliferation and increased expression of genes associated with stem cells (eg ABC genes (A8 and B1) and PROCR).

The BL1 subtype (Lehmann et al, 2011) has increased cell cycle and DNA damage response gene expression, which differs from BL2 (Lehmann et al, 2011), which has high expression of growth factor signaling components and myoepithelial genes. IM (Lehmann et al, 2011) mainly has genes involved in immune and cytokine signaling pathways as well as immune antigens. The basal like groups, defined by Lehmann et al, are comparable to the BLIS (BL1 and M) and BLIA (BL2 and IM) groups identified by Burstein et al (see Table 2).

Lehmann et al recently redefined their 6 groups to take into account transcripts from normal stromal cells and immune cells in the tumour microenvironment, thereby creating a reduced number of only 4 distinct groups (BL1, BL2, LAR and M), with cases from the previous IM and MSL groups being re-assigned between these 4 groups (Lehmann et al, 2016).

TNBC subtype	Classified by	Description
Luminal androgen receptor (LAR)	Lehman et al (2011) Burstein et al (2015)	Hormone receptor pathway expression
Mesenchymal (M)	Lehman et al (2011) Burstein et al (2015)	High level of epithelial genes and growth factor pathways
Mesenchymal stem-like (MSL)	Lehman et al (2011)	Similar to M subtype with high level of epithelial genes Decreased expression of genes required for cell proliferation compared to other TNBC subtypes
Basal-like 1 (BL-1)	Lehman et al (2011); Comparable to Basal-like immune suppressed (BLIS) in Burstein et al (2015)	Increased cell cycle and DNA damage response gene expression
Basal-like 2 (BL-2)	Lehman et al (2011); Comparable to some features of Basal-like immune-activated (BLIA) in Burstein et al (2015)	High growth factor signaling and myoepithelial genes.
Immunomodulatory (IM)	Lehman et al (2011); Comparable to some features of BLIA in Burstein et al (2015)	Immune and cytokine signaling as well as immune antigen presence
Unclassified (UNC)	Lehman et al (2011)	The miscellaneous TNBCs that do not fit into the other subtypes

Table 1.2: Triple negative breast cancer subtypes
A comparison of the different TNBC subtypes, according to Lehmann et al (2011) and Burstein et al (2015) classifications.

1.5 Triple negative breast cancer treatment

Unlike other breast cancer subtypes that have targeted treatments, there are currently no effective targeted treatments routinely available for primary TNBCs, and the targeted therapies used for other breast cancers are ineffective in this context because of the lack of ER and HER2 expression (Chittaranjan et al, 2014). Along with radiotherapy and the surgical options available to them, TNBC patients will therefore receive cytotoxic chemotherapy (Wahba et al, 2015).

1.5.1 Triple negative breast cancer responses to treatment and prognosis

Chemotherapy treatment can be very effective in reducing the size of the primary tumours when given in the neoadjuvant context, presumably because of the relatively high proliferation rate of TNBC (Carey et al, 2007). In the context of primary disease treated with NAC, either a decrease in tumour size (partial response) or in some cases a pathological complete response (pCR) can be seen (Rouzier et al, 2005). A pCR is determined by a lack of all morphological evidence of residual cancer. pCRs are important as patients who have achieved a pCR are more likely to have a better long-term prognosis (Cortazar et al, 2014). NAC is more effective in TNBC than any other subtype of breast cancer as TNBC cancers showed a pCR of 45% when treated with NAC in comparison to a pCR of 6% for luminal tumours (Rouzier et al, 2005). In another study carried out, TNBC patients again showed higher pCR rates (22%) compared to non-TNBC patients (11%). If pCR was achieved, TNBC patients, in this study, had similar survival to non-TNBC patients (Liedtke et al, 2008).

However, although patients may have a decrease in tumour size and partial response, more than 50% of patients will not achieve a pCR (Cortazar et al, 2014). Patients who have residual disease have worse overall survival if they had TNBC compared to non-TNBC (68% vs 88%) (Liedtke et al, 2008), which is likely due to chemoresistance in TNBC. Unfortunately, if only a partial response is seen, it is these patients who are more likely to relapse within 3 years of completion of their chemotherapy (Cortazar et al, 2014), as TNBC patients have

significantly decreased 3-year progression free survival rates when compared to non-TNBC patients (63% vs 76%) (Liedtke et al, 2008). The prognosis is also normally poor with 3-year overall survival lower than non-TNBC patients (74% vs 89%) (Liedtke et al, 2008). Treatment of the metastatic secondary tumour is always incomplete and TNBC patients can expect less than 14 months median survival (Kassam et al, 2009).

Therefore, an aim in development of neoadjuvant therapy currently is to improve pCRs of TNBC, and thereby kill any distant metastatic cells to prevent metastatic recurrences. Once a clinical metastatic reoccurrence is present, regardless of the primary response of the cancer, the prognosis is poor.

1.5.2 Current therapies for triple negative breast cancer

Chemotherapy commonly comprises of a combination of cytotoxic drugs that target proliferating cells. There are different families of chemotherapeutic agents, for example the anthracyclines, the taxanes and the alkylating agents (Isakoff et al, 2010). As determined by MRI, clinical response to neoadjuvant anthracycline treatment in TNBC cancer was 85%, compared to 70% in HER+ ER-, and 47% in luminal subtypes (Carey et al, 2007).

1.5.2.1 Mechanism of action of breast cancer cytotoxics

The anthracycline drugs that are commonly used for breast cancer treatment are doxorubicin and epirubicin (Kaklamani et al, 2003). Both doxorubicin and epirubicin target the cancer cells, along with any other actively proliferating cells, by inhibiting DNA synthesis and thereby replication, by intercalating between the base pairs in cellular DNA (Reinert, 1983). Anthracyclines can also inhibit topoisomerase activity. Topoisomerase enzymes are required for DNA strand cleavage and unwinding for efficient DNA replication to take place (Osheroff, 1989). Anthracyclines intercalate into the DNA and inhibit the DNA-topoisomerase complex after the nicking phase, and prevent the resealing stage in DNA synthesis (Han et al, 2015).

Taxanes are also commonly used in breast cancer chemotherapy, with the two specific agents being paclitaxel or docetaxel (Nabholtz et al, 2005). Taxanes have an effect on the microtubules required for cell division by stabilizing GDP-tubulin, which prevents microtubule depolymerisation and subsequent chromosome separation in mitosis (Sachdev et al, 2016).

Alkylating agents, such as cyclophosphamide, are also used in breast cancer therapy and are commonly given with anthracyclines and taxanes (Fisher et al, 1990). Alkylating agents function by addition of an alkyl group to nitrogen in the purine ring of the guanine base of the DNA, forming intra-molecular DNA strand crosslinks between guanine bases to prevent the unwinding of DNA during DNA replication (Hall et al, 1992).

Multiple combinations of these drugs are commonly used for TNBC treatment. Treatment options are largely based on the NICE guidelines, the practice within the NHS trust where treatment is occurring, and the preference of individual oncologists in consultation with the patient (NICE, 2009, NICE, 2018). Drug classes anthracyclines (doxorubicin and epirubucin) and taxanes (docetaxel and paclitaxel) can be used separately or in combination with one another. In addition, cyclophosphamide can also be given in combination with either anthracyclines or taxanes. All 3 drug classes can also be combined for TNBC patients (Cleator et al, 2009).

1.5.3 Therapies under investigation for triple negative breast cancer

Because of the relatively high recurrence rate and poor prognosis, there is still an unmet clinical need for new and targeted therapies for TNBC. Poly-ADP ribose polymerase (PARP) inhibitors are one of the emerging therapies and are currently undergoing clinical trials in TNBC (Pettitt et al, 2018). PARP proteins play an important role in the repair of DNA strand breaks by being responsible for the recruitment of DNA repair enzymes (Chambon et al, 1963). The inhibitors of PARP work as cytotoxics by inhibiting the recruitment of DNA repair enzymes, leading to an accumulation of further double-stranded breaks resulting in apoptosis and cell death (Carey, 2011). PARP inhibitors look to be

promising drugs in TNBC with BRCA1 or BRCA2 mutations (Bryant et al, 2005). BRCA1/2 mutated cells are unable to form the BRCA1-associated genome surveillance complex (BASC) which therefore prevents the interaction of the BASC complex with RNA polymerase II (Wang et al, 2000, Scully et al, 1997). Subsequently, DNA strand breaks are therefore not fixed through homology directed repair. PARP inhibitors are effective in BRCA1/2 mututated tumours as the BRCA1/2 mutations render the cells uniquely dependent on DNA repair pathways that require PARP (Silver et al, 2012). PARP inhibitor, olaparib has been used in the treatment of metastatic breast cancer patients with BRCA1/2 mutations. Patients, who received olaparib, response rate was 59.9% when compared to 28.8% response rate in patients who received standard therapy. Additionally, patient progression free survival increased from 4.2 months to 7 months (Robson et al, 2017). Therefore, olaparib has recently been FDA approved for treatment of BRCA1/2 mutated metastatic breast cancers (FDA, 2018). However there does not appear to be any benefit of PARP inhibitors to other TNBC patients who do not have BRCA1/2 mutated TNBC (Tutt et al, 2010).

The addition of platinum salts to adjuvant chemotherapy has also been explored. Platinum based drugs such as cisplatin and carboplatin have shown promising responses. In primary TNBCs, cisplatin addition to docetaxel, following prior epirubicin/cyclophosphamide treatment, produced overall response rate of 77% compared to overall response rate without the addition of cisplatin to docetaxel of 70% (Alba et al, 2012). For metastatic TNBC, cisplatin addition to chemotherapy improved overall survival by 4 months (Bhattacharyya et al, 2009) and carboplatin has increased patients response rate by 11% in advanced TNBC patients and improved overall survival by 3 months (Carey et al, 2012).

PIK3CA mutations are present in 10% of TNBC and are attributed to the LAR and M subtype as described by Lehmann et al (Lehmann et al, 2011) (section 1.4.2). PIK3CA mutations lead to activation of the PI3K/Akt pathways, which can mediate many of the hallmarks of cancer (Fruman et al, 2014). In addition, expression of the tumour suppressors inositol polyphosphate 4-phosphotase

type II (INPP4B) and phosphatase and tensin homolog (PTEN) are frequently lost in TNBC, also contributing to activation of the PI3K/Akt pathway (Li et al, 2017). PIK3CA mutations and loss of INPP4B and PTEN provided the impetus for the development of ipataserib, which is an ATP competitive small molecule inhibitor of Akt (Saura et al, 2017). Ipataserib is currently being used in clinical trials to determine efficacy in combination with other chemotherapeutic agents. Ipataserib clinical trials are in the early stages at phase Ib and II in patients with TNBC (Lehmann et al, 2015).

Multiple other therapeutic strategies are being explored, such as blockade of the androgen receptor in LAR TNBC (Lehmann et al, 2015), histone deactylase (HDAC) inhibitors (Uehara et al, 2012), mammalian targets of rapamycin (mTOR) inhibitors (Basho et al, 2017) and the development of immunotherapies (Stagg et al, 2013). It is hoped that blockade of AR, HDAC and mTOR inhibitors will increase the options and effectiveness of treatments available to TNBC patients.

With a lack of well-established targeted treatment available to TNBC patients, chemotherapy remains the predominant treatment for TNBC patients. Although response to chemotherapy can be promising, chemoresistance is a real clinical problem in TNBC so there is a need to understand chemoresistance pathways. Targeting cancer specific chemoresistance pathways in combination with traditional chemotherapy, could be a viable clinical route to improve survival of TNBC patients. One particular opportunity for such a combination therapy is explored in Chapter 5.

1.6 Mechanisms of chemoresistance in breast cancer

Chemotherapy remains the predominant treatment for TNBC patients and chemoresistance in TNBC, reflected in cancer recurrences and deaths, is a major clinical problem. Identification of chemoresistance pathways, and potentially inhibition of these pathways in combination with chemotherapy could represent a treatment option for TNBC patients and forms the basis of research in this thesis.

1.6.1 Physical mechanisms of chemoresistance

Extracellular matrix (ECM) proteins, particularly collagens, have been implicated in chemoresistance. Increased levels of collagen have been implicated in limited delivery and diffusion of chemotherapeutic into cancer tissues (Senthebane et al, 2017). In breast cancer cells, high levels of expression of collagen genes is associated with increased drug resistance (Iseri et al, 2010). Collagen type I has also recently shown to promote survival of breast cancer cells *in vitro* as well as enhancing metastasis (Badaoui et al, 2018). Increased levels of collagen and expression of lysyl oxidase (LOX), a protein involved in collagen cross-linking (Herchenhan et al, 2015), can increase ECM stiffness in breast cancer. It is thought that ECM stiffness can induce chemoresistance and increase cell survival through activation of PI3K and adhesion signaling (Levental et al, 2009).

Cytoskeletal proteins have also been implicated in chemoresistance, in particular β tubulin. β tubulin III subunit overexpression has correlated with increased resistance to taxane-based chemotherapy (paclitaxel and docetaxel) in advanced breast cancer (Paradiso et al, 2005).

1.6.2 Drug inactivation mechanisms of chemoresistance

Chemoresistance can occur in epithelial cells through drug inactivation/detoxification or through increased expression of drug pumps. ABC transporters such as the P-glycoprotein pump MDR1 and breast cancer resistant protein (BCRP/ABCG2) have been shown to play a role in chemoresistance in TNBC by increasing the efflux of chemotherapeutics (Zheng, 2017). MDR1 has the ability to pump out multiple different types of chemotherapeutics in breast cancer, including paclitaxel and docetaxel (Mechetner et al, 1998). In addition, chemotherapy induced activation of Notch leads to the up-regulation of multi-drug resistance protein-1 (MRP1), with inhibition of Notch-dependent MRP1 sensitizing cells to chemotherapy (Kim et al, 2015).

BCRP/ABCG2 can also pump out chemotherapeutics including anthracyclines such as doxorubicin (Doyle et al, 1998). More recently, NAC has been shown to increase expression of BCRP and has been implicated in determining disease-free survival (Kim et al, 2013). Patients displaying low expression of BCRP, following chemotherapy, had significantly longer disease free survival in comparison to patients with high expression of BCRP (Kim et al, 2013).

Drug inactivation or detoxification is also a mechanism employed by breast cancer cells to enable chemoresistance. ALDH1A1 and ALDH3A1 have been shown to lead to inactivation of cyclophosphamide, which is commonly used in combination with anthracyclines (Sladek et al, 2002). Drug inactivation has also been attributed to altered metabolism of drugs by cytochrome P450s (CYPs). CYP2D6 is responsible for the metabolism of tamoxifen in breast cancer and therefore reduced the efficacy of tamoxifen (Ali et al, 2016). CYP3A/5 has been shown to metabolize docetaxel into less effective compounds for the treatment of breast cancer (Bedard et al 2010).

1.6.3 Cellular mechanisms of chemoresistance

Alterations in genes involved in apoptosis have been implicated in chemoresistance. In TNBC, mutations in p53 are often present and over-expression of anti-apoptotic proteins Bcl-2 is also observed (Vegran et al, 2006, Aas et al, 1996). Loss of p53 and up-regulation of Bcl-2 have been linked to chemoresistance in breast cancer cells following anthracycline chemotherapy (p53) or cyclophosphamide, epirubicin and 5-fluorouracil (Bcl-2) respectively (Vegran et al, 2006, Aas et al, 1996). Loss of the mismatch repair proteins MSH2 and MLH1 has also been linked to resistance to epirubicin and doxorubicin (Fedier et al, 2001).

1.7 Tumour stroma

The role of the tumour stroma, in particular of cancer-associated fibroblasts, in chemoresistance in TNBC is a key focus throughout this thesis. I will, therefore, provide background on what is already known about the tumour stroma and cancer-assocoiated fibroblasts as well as their role in cancer progression.

For a cancer to survive, grow and thrive it requires the right environment. The tumour stroma is responsible for this as the tumour stroma infiltrates in and around the tumour mass and provides an environmental niche for the tumour cells (Egebald et al, 2008). Tumour stroma consists of a large number of different types of stromal cells, including fibroblasts, pericytes, adipocytes and immune cells. In addition, to deliver the nutrients that the tumour requires, the tumour stroma contains blood vessels and also contains extracellular matrix proteins such as collagen, fibronectin and laminin (Kharaishvili et al, 2014). In the presence of tumour cells, components of the stroma undergo complex alterations, which alter the characteristics of the stroma. The altered characteristics of the tumour stroma are exploited by tumours to aid in cancer progression (Lu et al, 2012).

The ratio of tumour stroma to tumour cells in TNBC has been investigated to see if it correlates with prognosis of TNBC patients. TNBC patients with cancers containing greater than 50% stroma, as determined by point-counting, had greater chances of recurrences within 5 years in comparison to those with tumours that had less than 50% tumour stroma (85% vs 45%) (Moorman et al, 2012). High tumour stroma content in TNBC was also associated with poorer overall survival (89% vs 65%), however in ER positive disease, the influence on overall survival was reversed, with prolonged survival associated with stromal rich tumours (Downey et al, 2015).

The most abundant cell type within the tumour stroma is the stromal fibroblasts (Bussard et al, 2016), and fibroblasts are a key focus of my work. Fibroblasts are present normally within the body, however fibroblasts in tumour stroma show an altered phenotype and are referred to as cancer-associated fibroblasts

(CAFs) (Sadlonova et al, 2009). Investigation into the role of CAFs has increased understanding of the role that CAFs play in the progression of breast and other carcinomas. CAFs can enhance cell proliferation, angiogenesis, invasion and cancer metastasis to secondary sites (Orimo et al, 2005, Dumont et al, 2013).

1.8 Fibroblasts

Fibroblasts are non-vascular, non-epithelial and non-inflammatory cells that are commonly found with the fibrillar matrix of the connective tissue (Tarin et al, 1970). Fibroblasts remain quiescent until they are required for their functions, which include deposition of the ECM, control of epithelial cell differentiation, regulation of inflammation, and most commonly wound healing (Parsonage et al, 2005). Fibroblasts also have the ability to produce multiple types of collagen (I, III, IV and V) as well as fibronectin and laminin (Qureshi et al, 2017). Matrix metalloproteinases (MMPs) are another product that fibroblasts secrete to enable ECM degradation and cell movement through the extracellular matrix (Tomasek et al, 2002).

A key role of fibroblasts in normal physiology is in wound healing. When a wound occurs, fibroblasts are responsible for invading into the wound, producing ECM components such as collagen, fibronectin and laminin to provide a scaffold for epithelial cells to move onto and grow (Darby et al, 2014). Fibroblasts are also responsible for producing cytoskeletal components to enable contraction to pull the wound together (Gabbiani et al, 1971). As previously mentioned, fibroblasts remain quiescent until they are activated and required for their function (Gabbiani et al, 1971). When fibroblasts are in this quiescent state, they are spindle-shaped and metabolically relatively inactive, with little active secretome (Tarin et al, 1970). Fibroblasts also do not actively divide to any great extent, being commonly in G_0/G_1 arrest, although there is a slow rate of self-renewal (Marthandan et al, 2014). In addition, fibroblasts are non-migratory, and are unable to produce components of the ECM (Kalluri, 2016). Fibroblasts are activated in order to carry out their cellular function from multiple stimuli such as growth factors, chemokines, cytokines as well as

physical stress and hypoxic conditions (Bainbridge et al, 2013). Transforming growth factor- β (TGF-β) is well established as an activator of fibroblasts, particularly for fibroblast involvement in wound healing (Montesano et al, 1998). Platelet-derived growth factors are also important for both fibroblast activation and the wound healing process. Release of platelet-derived growth factor (PDGF), recruits and activates fibroblasts at the wound site for effective wound healing (Rajkumar et al, 2006). When activated, the morphology of fibroblasts changes as well as the secretory phenotype. Fibroblasts become cruciform or steallate in shape as well as becoming metabolically active, proliferative, and motile (Ravikanth et al, 2011). Once activated, fibroblasts have a dynamic secretome that includes growth factors (eg EGF, FGF, HGF) as well as the ECM components previously described. Fibroblasts may go through several cycles of active remodelling, healing or inflammatory functions, followed by successive periods of quiescence and activation depending on external stimuli (Kalluri and Zeisberg, 2006).

By contrast, CAFs are constantly active and never revert back to the quiescent inactive fibroblast state. CAFs are thought to have undergone irreversible epigenetic modifications from the normal activated fibroblast phenotype where they have acquired a higher proliferation rate and enhanced secretory phenotype (Littlepage et al, 2005). CAFs also have the ability to self-renew in comparison to the activated fibroblast phenotype as well as producing a greater amount of growth factors, cytokines and chemokines. CAFs also produce more ECM proteins, such as tenascin C (Hanamura et al, 1997), periostin (Kikuchi et al, 2008) and SPARC (Gao et al, 2017) as well as collagen (Pankova et al, 2016). Both activated fibroblasts and CAFs express enhanced levels of vimentin and α-SMA (Shiga et al, 2015). However, CAFs produce fibroblast-activated protein (FAP) (Park et al, 1999), which is not present in activated fibroblasts.

1.9 Origins of cancer-associated fibroblasts

The origins of CAFs have been debated with multiple different suggestions as to where they arise from and how they become activated.

Firstly, it is thought that the majority of CAFs are derived from the conversion of normal activated fibroblasts into CAFs, potentially driven by cancer cell secreted cytokines (Kojima et al, 2010). The mechanisms by which this conversion is induced are thought to be similar to activation of normal fibroblasts in wound healing as well as some epigenetic changes occurring (Qiu et al, 2008). TGF-β is responsible for activation of fibroblasts in wound healing but is also commonly secreted by cancer cells (Knabbe et al, 1987). Cancer cell secretion of TGF-β activates TGF-β signaling in CAFs, which in turn leads to an activated CAF phenotype (Kojima et al, 2010). Epigenetic changes are also required for CAF activation, with loss of p53 and PTEN required. Interestingly, loss of p53 and PTEN occurred in CAFs in close proximity to tumour cells indicating the potential cancer cell driven activation of CAFs (Mao et al, 2013). Direct cell contact between cancer cells and fibroblast can also lead to the activation of CAFs through adhesion molecules such as intra-cellular adhesion molecule I (ICAM1) and vascular adhesion molecule I (VCAM1) (Clayton et al, 1998). CAFs have a different miRNA expression profile in comparison to normal fibroblasts, which can also play a regulatory role in transformation of normal activated fibroblasts to CAFs. Recent studies have implicated miR-31, miR-214 and miR-155 in the transition from normal fibroblasts to CAFs (Mitra et al, 2012).

There is also evidence that bone marrow derived mesenchymal stem cells (MSCs) can differentiate into CAFs. It is thought that this differentiation could also be TGF-β driven. Characteristic CAF markers like α-SMA, FAP and tenascin-C are expressed in these MSC derived differentiated CAFs (Spaeth et al, 2009).

Finally, another mechanism by which fibroblast-like cells are produced is when malignant epithelial cells undergo epithelial mesenchymal transition (EMT).

EMT is commonly triggered by the expression of TGF-β, EGF, PDGF, FGF2 and CXC-motif chemokine ligand (CXCL) (Spaeth et al, 2009). Not only can epithelial cells transition into CAF-like cells but endothelial cells have also undergone endothelial to mesenchymal transition to produce a CAF phenotype, which has been confirmed by the expression of CAF markers (Zeisberg et al, 2007).

Despite all these potential origins of CAFs, the precise origin of CAFs in breast cancer is not fully understood and it is plausible that it is a combination of CAFs from multiple origins that comprise the tumour mass.

1.10 Cancer associated fibroblasts in breast cancer progression

CAFs have been implicated in multiple aspects of cancer progression including tumour growth, angiogenesis and metastasis.

In terms of tumour growth, CAFs can secrete the growth factors EGF, HGF and FGF, which can activate growth factor signaling in the cancer cells and thereby enhance tumour growth (Matsumoto et al, 2006). Other novel secretory factors from CAFs have been implicated in tumour growth and survival. Secreted frizzled related protein 1 and IGF like family member (IGF) have also been shown to directly or indirectly enhance tumour growth (Ostman et al, 2009). Stromal derived factor-1 (SDF-1) can also contribute to tumour growth and can also have an impact on migration and invasion to secondary sites (Orimo et al, 2005). SDF-1 binds to its receptor CXCR4 on the surface of breast cancer cells to activate signaling pathways to enhance proliferation of the breast cancer cells (Orimo et al, 2005).

CAFs can also aid in migration, invasion and metastasis (Verghese et al, 2013). Interleukin-6 (IL-6) has been shown to promote migration in ER positive breast cancer and IL-6 also has the ability to induce EMT in ER positive breast cancer cells (Hugo et al, 2012). One of the main classes of secretory factors that CAFs release that aid in migration, invasion and metastasis are MMPs (Singer et al, 2002). MMPs degrade ECM proteins such as collagen and laminin and remodel

the ECM to allow cancer cells to migrate through the stroma and surrounding tissue to aid cells in reaching the lymph vessel and blood vessels to travel to secondary sites (Gianneli et al, 1997). MMP2, MMP9 and MMP14 have been shown to have increase migration and invasion in breast cancer (Kessenbrock et al, 2010, Rothschild et al, 2015).

Another area CAFs can aid in is angiogenesis. CAFs can secrete VEGF, which upon binding to its receptor, activates VEGF signaling in endothelial cells, which in turn promotes the formation of new blood vessels to deliver oxygen and nutrients to the tumour to aid in its growth and survival (Bachelder et al, 2002).

The roles of CAFs, in breast cancer, are best understood in growth, migration, invasion and angiogenesis, however there is still a lack of understanding of how CAFs contribute to other hallmarks of cancer such as evading growth suppression, immune destruction and resisting cell death. In addition, there are large gaps in knowledge of whether and how CAFs contribute to chemoresistance.

1.11 The interferon family

Interferons, their signaling pathways, and their roles in cancer progression will be highlighted as it became evident in my results that interferon signaling could play a role in chemoresistance. The interferon (IFN) family is a group of secreted cytokines that are widely expressed, predominantly by immune cells, and are typically produced in response to viral infection to induce potent antiviral and anti-proliferative effects (Le Page et al, 2000).

There are multiple different interferon paralogues, each expressed from its own gene. These can be categorised into 3 different classes: Type I, Type II and Type III (Pestka et al, 1987) (summarized in Table 1.3). Within Type I, there are nine different paralogues including IFN-α (13 known isoforms), IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ, IFN-v and IFN-ζ (Pestka et al, 2004). Interestingly, the genes for Type I interferons are found within a single cluster on one chromosome (9p21) (Diaz et al, 1994). IFN-γ is the only representative of Type

II interferon (Pestka et al, 1987). IFN-λ1, IFN-λ2 and IFN-λ3 form the Type III paralogues (Ank et al, 2006). Each class of interferon binds to its own subset of receptors. Type I interferons bind to the heterodimeric receptor complex of IFN-α receptor 1 (IFNAR1) and IFNAR2 subunits (Mogensen et al, 1999). Type II interferons bind to the IFN-γ receptor IFNGR1 and IFNGR2 complex, which is a tetramer of 2 molecules of each of the two subunits (Pestka et al, 1997). Finally, the Type III class binds to the IFN- λ receptor 1 (IFNLR1) (Kotenko et al, 2003)

1.12 Interferon biogenesis

Type I IFNs are produced by a number of cell types in response to various stimuli (Gibbert et al, 2013), whereas expression of Type II and III IFNs are far more limited and only commonly expressed in immune cells, normally T cells and NK cells (Samuel et al, 2001).

Pattern-associated molecular patterns (PAMP), from viruses or other foreign bodies, are recognized by cells, which leads to interferon production (Baig et al, 2008). PAMPs then activate pattern recognition receptors (PRRs) (Medzhitov et al, 1997). The most predominant PRR are the Toll-like receptors (TLR). TLR1, TLR2, TLR4, TLR5 and TLR6 can react to bacterial lipopolysaccharides, whereas TLR3, TLR7, TLR8 and TLR9 have the ability to react to nucleic acids (Uematsu et al, 2007). MYD88, MAL, TRIF and TRAM are adaptors of response and are recruited to the TLRs when TLRs become activated (O'Neill et al, 2007). Recruitment of adaptors to the TLRs leads to the activation of TANK-binding kinase 1 (TBK) and IKKε and IκB (Ma et al, 2012), which then bind and activate transcription factors; IFN response factors (IRFs) and nuclear factor-κB (NF-κB) (Ma et al, 2012, Pomerantz et al,1999). IRFs and NF-κB transcription factors upregulate the expression of IFNs (Randall and Goodbourn, 2008).

1.13 Interferon signaling

Following interferon production, interferons carry out the late phase viral response through activation of the interferon signaling pathway using the classical JAK-STAT pathway (Silvennoinen et al, 1993). The different interferon

/interferon receptor pathways also lead to varying activation of the classical JAK-STAT pathway (summarized in Table 1.3). Activation of the JAK-STAT pathway occurs relatively quickly as there is no need to synthesize new protein for the signaling cascade to proceed (Pestka, 2007).

	Type I Interferon	**Type II Interferon**	**Type III Interferon**
Interferons	IFN-α (13 subtypes), IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-τ, IFN-ν and IFN-ζ	IFN-γ	IFN-λ1, IFN-λ2, IFN-λ3
Receptor	IFNAR1 and IFNAR2	IFNGR1 and IFNGR2	IFNLR1 and IL10R2
STATs	STAT1, STAT2, STAT3 and STAT5 STAT4 and STAT6 - IFN-α only Must form ISGF3 complex with IRF9 for signaling	STAT1	STAT1, STAT2, STAT3, STAT4 and STAT5
Promoter element	ISRE and GAS	GAS	ISRE and GAS

Table 1.3: Classes of interferons
A comparison of the 3 different types of interferon, their receptors, the STATS used for signal transduction and the promoter elements (IFN-stimulated response element (ISRE) and IFN-γ- activated site (GAS)) the STATs are able to bind to for transcription of interferon-stimulated genes (ISGs)

1.13.1 Type I interferon signaling

Upon binding of the Type I IFNs to the IFNAR1 and IFNAR2 receptor complex, ligand-dependent rearrangement and dimerization of the receptor occurs (Pattyn et al, 1999). Receptor dimerization leads to trans-phosphorylation events of the receptor associated JAK kinases, TYK2 and JAK. Activation of TYK2 and JAK enables further phosphorylation events and phosphorylation of STATs (Darnell et al, 1994) (Figure 1.2). There are multiple forms of STATs that can be phosphorylated in the Type I signaling pathway which include STAT1, STAT2, STAT3 and STAT5. In addition, IFN-α can also activate STAT4 and STAT6. All of these STAT subunits are able to form homodimers and heterodimers with one another upon phosphorylation by TYK2 and JAK (Darnell et al, 1997). STAT1 and STAT3 are known to be phosphorylated on serine 727 (Wen et al, 1995), however the location of phosphorylation of the other STATs has not been explored. STAT homo or heterodimers can translocate to the nucleus and act as transcription factors, binding at specific binding sites in target promoters (Meinke et al, 1996). There are 2 distinct canonical promoter elements that STAT dimers bind to: either the IFN-stimulated response element (ISRE) or IFN-γ- activated site (GAS) element (Yang et al, 2005). Most of the STAT homo and heterodimers have the ability to bind to the GAS element and enable transcription. However, only STAT1-STAT2 heterodimers have the ability to bind to ISREs in combination with IRF9, as the ISG Factor 3 (ISGF3) complex (Fu et al, 1992). IRF9 does not require phosphorylation to bind to the STAT1-STAT2 heterodimer. The ISGF3 complex then translocates to the nucleus to bind to the ISRE and enable transcription of interferon stimulated genes (ISGs) (Tsuno et al, 2009).

1.13.2 Type II interferon signaling

IFN-γ is the only Type II interferon and upon binding to its receptors, IFNGR1 and IFNGR2, IFN-γ also activates the JAK-STAT pathway shown in Figure 1.2. However, in this case the 2 receptor subunits, IFNGR1 and IFNGR2, are each constitutively associated with JAK1 and JAK2 respectively (Bach et al, 1997). Activation of JAK1 and JAK2 leads to the phosphorylation of STAT1 on tyrosine

701, which enables the formation of a STAT1 homodimer. The STAT1 homodimer then translocates to the nucleus and binds to the GAS elements within target promoters to permit transcription of ISGs (de Weerd and Nguyen, 2012).

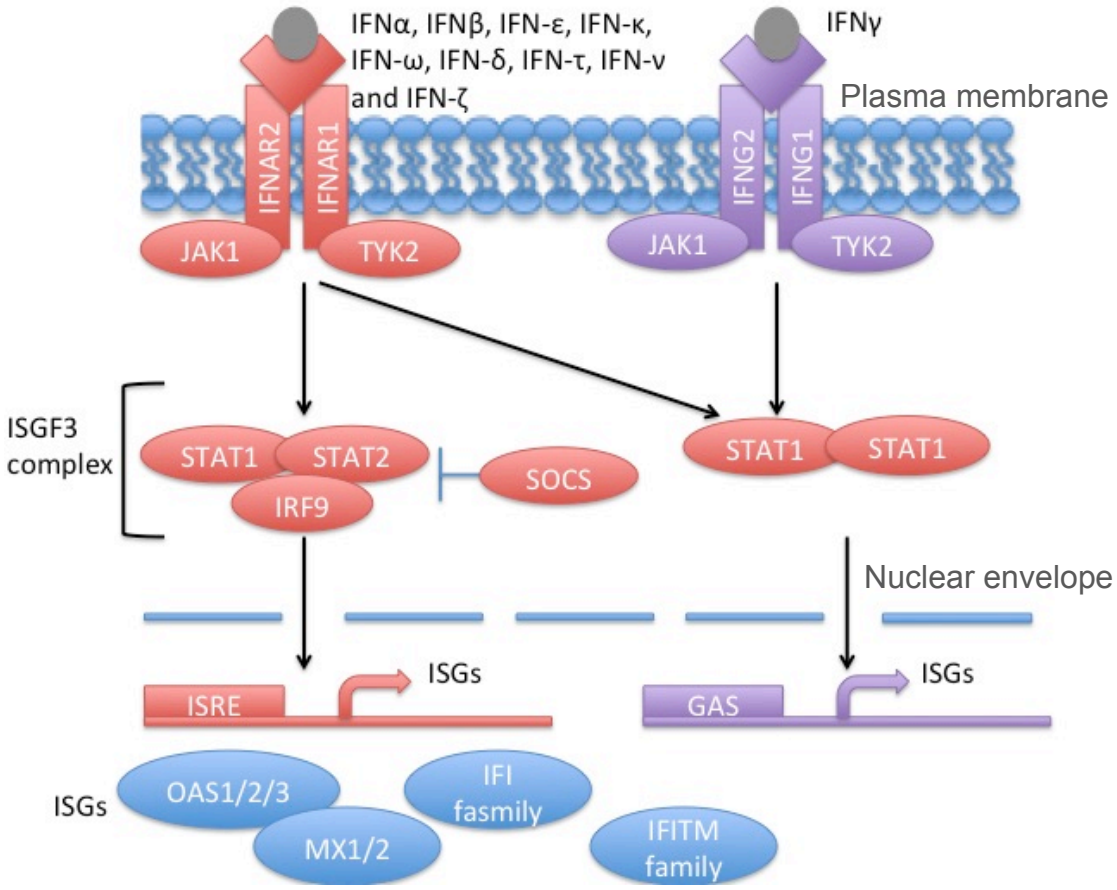


Figure 1.2: Type I and II Interferon signaling
The interferon signaling pathway is represented schematically from extracellular at the top, down to intra-nuclear at the bottom, with the plasma membrane (blue double-barrier) and nuclear membrane (non-continuous blue line). Purple elements are specific to Type II with red components specific with Type I, although cross-talk with Type II should be noted.

1.13.3 Regulation of interferon signaling

Interferon signaling is tightly regulated, which may be important for overall organism homeostasis since, for example, prolonged signaling has been associated with autoimmune diseases (Feng et al, 2012). Part of interferon regulation involves negative feedback, as some ISGs that are up-regulated by interferon signaling act to inhibit the JAK-STAT pathway. The 2 main examples

of this are USP18 and SOCS (Ivashkiv et al, 2014). USP18 is an isopeptidase that is normally responsible for the degradation of proteins (Honke et al, 2016). However, this role of USP18 is not responsible for the inhibition of the interferon signaling pathway. USP18 specifically binds to the IFNAR2 subunit, thereby preventing the binding of JAK1 to IFNAR2 and subsequently inhibiting JAK-STAT activation and downstream interferon signaling (Sarasin-Filipowicz et al, 2009). The family of SOCS proteins are also inhibitors of the JAK-STAT pathway, particularly SOCS1 and 3. SOCS proteins bind to phosphorylated residues on the IFN receptors or on JAK components, and prevent subsequent binding of STATs and thereby inhibit activation of the signaling pathway (Yoshimura et al, 2007).

1.13.4 Interferon stimulated genes

Transcription of many different genes is stimulated upon binding of STATs to either the ISRE or GAS elements of their promoters, which are known as interferon stimaulted genes (ISGs).

One family of genes that is up-regulated by IFN signaling is the oligoadenylate synthase (OAS) family. This comprises OAS1, OAS2 and OAS3 (Rebouillat et al, 1999). The OAS family stimulates cleavage of double stranded RNA structures. OAS enzymes convert adenosine triphosphate (ATP) into 2'-5'-linked oligoadenylate (2-5A) (Kerr et al, 1978). 2-5A in turn promotes dimerization and activation of the latent endoribonuclease, RNaseL (Zhou et al, 1993). Activated RNaseL is then responsible for degradation of double-stranded viral and cellular RNA, as well as promoting apoptosis, attenuating proliferation and inhibiting protein synthesis (Sadler et al, 2008).

Another family of proteins that are prominent ISGs is the myxovirus resistance (MX) family (Horisberger et al, 1991). MX family is made up of MX1 and MX2, and the proteins are responsible for the inhibition of virus entry into cells. MX1 and MX2 are structurally similar to dynamin-like large guanosine triphosphatase (GTPases) (Haller et al, 2007). MX1 and MX2 have N-terminal GTPase domains, bundle signaling elements and C-terminal stalk domains. Both N-

terminal and C-terminal domains are critical for the function of MX1 and MX2 as they enable self-oligomerisation for the formation of multiple ring like structures (Gao et al, 2011). It is thought that these ring like structures interact with and surround viral nucleocaspids, which activates the GTPase activity of the MX proteins. Activation of GTPase activity leads to the degradation of the viral nucleocaspids, however this mechanism has yet to be fully determined (Haller et al, 2011).

Both the OAS and MX family of proteins are classical ISGs that are activated upon interferon signaling and are involved in viral response. OAS1 and MX1 have been used in my work to confirm interferon signaling activation.

1.14 Interferons in cancer

In addition to interferons role in anti-viral response, interferons have also been implicated in cancer. It is thought that they can play a role in extrinsic and intrinsic mechanisms of cancer cells. Extrinsic mechanisms have an impact on other cell types that are not directly the tumour cell (section 1.14.1 and Figure 1.3), whereas intrinsic mechanisms impact the tumour cells directly (section 1.14.2 and Figure 1.4) (Platanais et al, 2013).

1.14.1 Extrinsic roles of interferons in cancer

Interferons can modulate immunoregulatory effects. Interferons have been shown to regulate the activity of a number of different classes of immune cells and can potentially activate anti-tumour immunity. Activation of natural killer cells (NK) cells and CD8-positive cytotoxic T cells by interferons can reduce tumour burden and enable anti-metastatic effects to prevent cancer spread (Swann et al, 2007).

Interferons can also suppress proliferation of effector T cells preventing immune response against cancer cells. The presence of interferons inhibits the ability of T-regulatory cells (Tregs) to divide, thereby reducing their suppression of cytotoxic T cell activity (Pace et al, 2010). Interferons can also have an impact

on myeloid-derived suppressor cells (MDSC) by reducing their local levels, and therefore reducing MDSC subsequent suppressive function on immune responses (Zoglmeier et al, 2011). Production of interferons can also influence the production of further chemokines required for immune responses against cancer cells, such as IL-15. IL-15 is responsible for activation and proliferation of T and NK cells as well as providing survival signals for memory T cells to maintain the lymphocyte population (Munger et al, 1995).

Interferon driven activation of dendritic cells (DC) can also increase presentation of tumour antigens to T cells as well as enhancing the expression of tumour antigens on the surface of cells (Schiavoni et al, 2013). Interferons can also increase the expression of programmed cell death protein 1 (PD1) and its ligand PD-L1 on immune cells and tumour cells respectively. Upon binding of PD1 to its ligand PD-L1, T-cell receptor signaling is inhibited and subsequently T cell proliferation is stalled. It is currently thought that up-regulation of PD-L1 and PD1 enable evasion of immune responses (Parker et al, 2016).

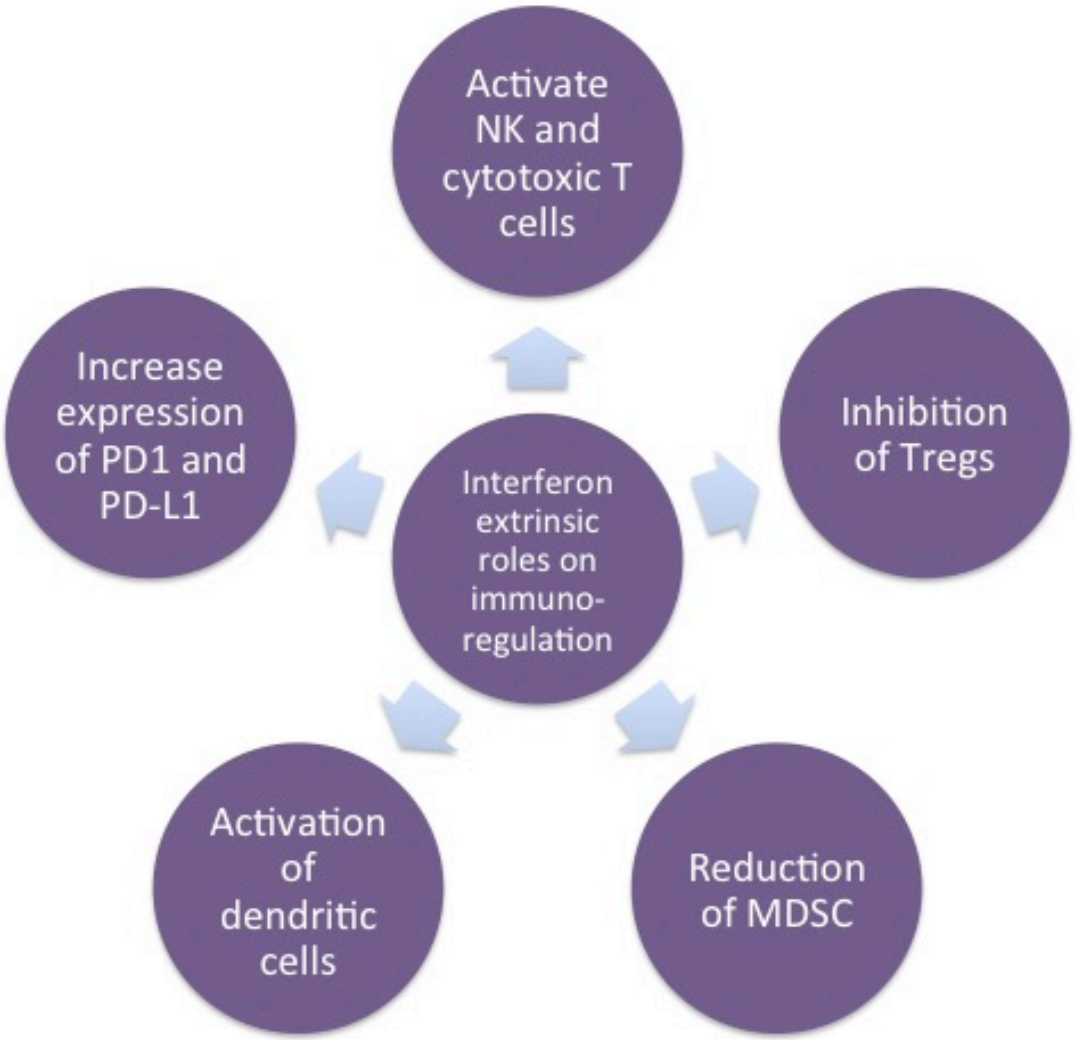


Figure 1.3: Extrinsic roles of interferons on immune-regulation in cancer

1.14.2 Intrinsic roles of interferons in cancer

Interferons can also have an impact on the tumour cells themselves (Figure 1.4) and have been implicated in many core biological functions. Interferons have the ability to prolong and stall the cell cycle. Interferons have been shown to delay entry into S phase and G2/mitosis by lengthening the time cells spend in G1, S and G_2/M, therefore inhibiting proliferative ability of tumour cells. In addition, quiescent cells in G_0/G_1 were delayed entry into the cell cycle in the presence of interferons (Balkwill et al, 1978). Interferons have also been shown to activate another pathway by activating CRK proteins. The CRK protein CRKL interacts with RAP1A, which has previously been shown to be a tumour suppressor of RAS GTPases, which are predominantly activated in cancer and activate multiple signaling pathways (Fathers et al, 2012). Presence of both Type I and Type II interferons has been shown to block cell cycle progression through up-regulation of the cyclin-dependent kinase inhibitor (CDK1) p21. p21 is able to block cell cycle progression by binding directly to cyclin-dependent kinase 2-cyclin complexes (cyclin A and E), which are required for passage through G1 and the S phase to G2/M transition (Hobeika et al, 1997).

Interferons can also have an impact on apoptosis of tumour cells. IFN-α has been shown to induce apoptosis of cells. In order to do this, IFN-α activated multiple different caspases (-1,-2,-3, -8 and -9). Caspases are required for apoptosis, with caspase-8 and -9 being the major initiator caspases (McIlwain et al, 2013). Normally caspase-8 is activated by death ligands binding to their receptors and caspase-9 is activated by mitochondrial cytochrome c. However, IFN-α has been shown to activate casapase-8 and -9 in the absence of death ligands and thereby induce apoptosis (Thyrell et al, 2002).

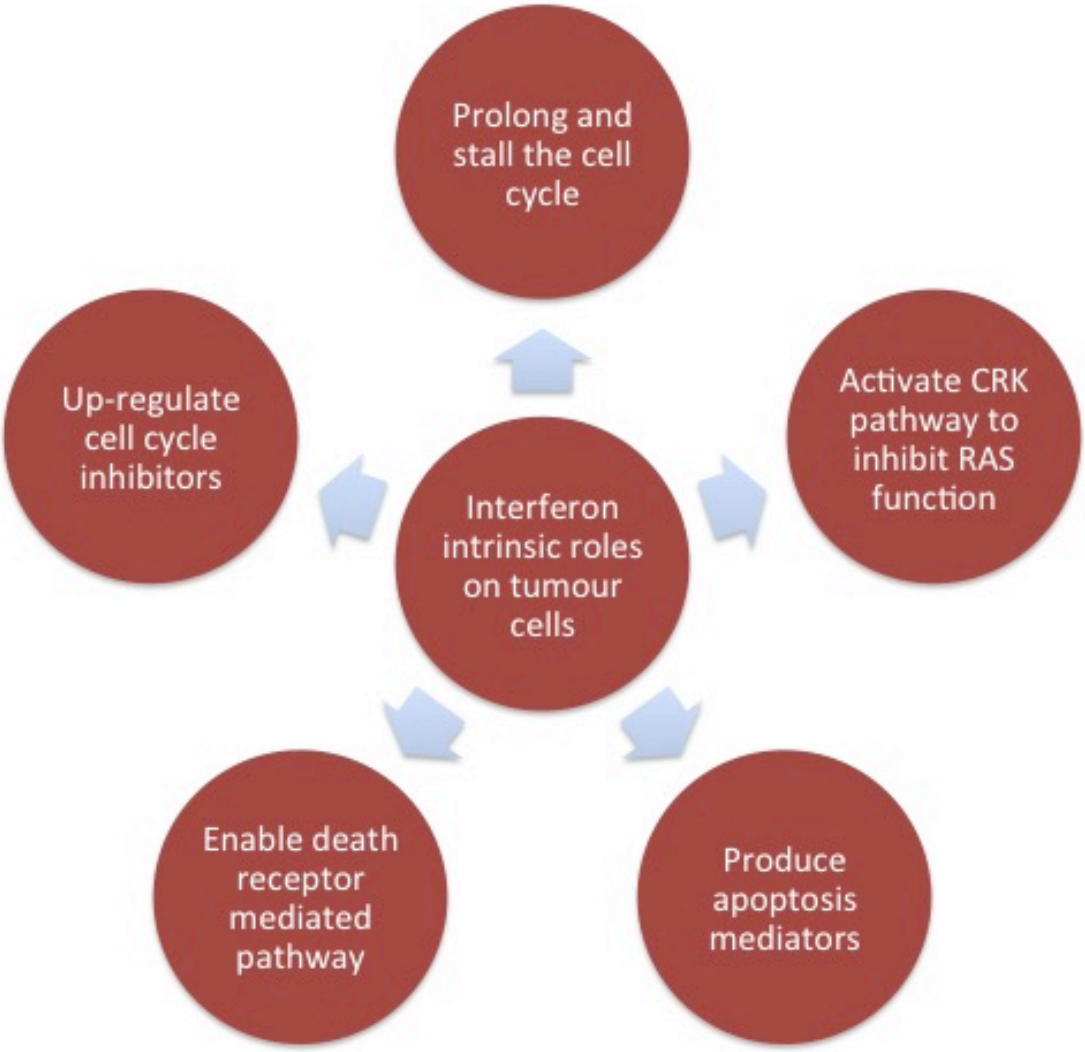


Figure 1.4: Intrinsic roles of interferons on tumour cells

1.15 MiRNAs

In my results, I have attempted to identify miRNAs with potential roles in chemoresistance, therefore, here I describe miRNA biogenesis, the function of miRNA in gene silencing and the relevance of their expression in cancer.

MiRNAs (miRNAs) are a subset of the transcriptome, being short non-coding RNAs of approximately 20-22 nucleotides in length, with 5'- phosphate and 3' hydroxyl ends (Krol et al, 2004). MiRNA genes are found in multiple different regions of the genome, including in the introns of coding genes as well as in inter-genic regions. MiRNA genes are also commonly found in clusters within both these regions (Lagos-Quintana et al, 2001). It is thought that transcription of miRNAs within a gene is driven by the same promoter that is responsible for the transcription of the host gene. However, transcription of miRNAs present in inter-genic regions is driven by their own promoters (Bartel et al, 2004).

1.16 MiRNA biogenesis

A number of modification and processing stages are required to convert the initial transcript of a miRNA into a mature miRNA (summarized in Figure 1.5). Transcription of miRNAs normally occurs using RNA polymerase II (Lee at al, 2004).

Initial miRNA transcripts that are produced following gene transcription are referred to as primary miRNAs, or pri-miRNAs. Pri-miRNAs have a specific structure as they have 2 segments of single stranded RNA, that are complimentary to one another, which link together by a hairpin loop and 3 spiral turns (Cai et al, 2004). In addition, the pri-miRNA must contain a 5' end cap and a poly A tail (Cai et al, 2004). If the pri-miRNA does not contain the hairpin loop and 5' and 3' modifications, the RNA will not be processed correctly for the formation of mature functional miRNA (MacFarlane and Murphy, 2010).

Nuclear processing of the pri-miRNA is then undertaken. Nuclear protein DiGeorge syndrome critical region gene 8 (DGSCR8) recognizes the double

stranded hairpin loops in the pri-miRNA and binds to the double-stranded RNA (Han et al, 2004). DGSCR8 associates with the RNA III endonuclease Drosha to form the microprocessor complex (Denli et al, 2004). Drosha then cuts around 11 nucleotides from the pri-miRNA to produce an isolated hairpin species, referred to as the pre-miRNA (Lee et al, 2003).

MiRNA transcripts can also be produced from an intron in a host-coding gene. The production of these miRNAs is initially different from the transcripts described above. Intronic miRNAs are again transcribed by RNA polymerase II when the host-coding gene is transcribed (Kim et al, 2007). This therefore produces precursor mRNA (pre-mRNA). Pre-mRNA contains the 5' UTR, the host gene protein-coding region, non-coding introns (in which the intronic miRNA is present) and the 3'UTR (Lin et al, 2006). The pre-mRNA then must undergo splicing by the spliceosome to remove the intron region from the host gene-coding region to produce the intron containing miRNA transcript and the mRNA for protein translation (Ruby et al, 2007). Further splicing and processing enables the spliced intron to function as the pri-miRNA. Following production of the pri-miRNA, the processing of the intronic and inter-genic miRNA is exactly the same (Shomron et al, 2009).

For further processing, the pre-miRNA hairpins must be exported to the cytoplasm (Lund et al, 2004). For this to occur the pre-miRNA assembles into a complex with the nucleo-cytoplasmic transporter factor exportin-5. Exportin-5 recognises a 2 nucleotide overhang on the 3' end of the hairpin (Bohnsack et al, 2004). For exportin-5 mediated transport across the nuclear barrier, GTP is required, which is bound to a Ran protein. The Ran-GTP is converted to Ran-GDP to actively transport the pre-miRNA to the cytoplasm (Yi et al, 2003).

For the production of mature miRNA, the pre-miRNA is cleaved by the RNase III endonuclease, Dicer (Bernstein et al, 2001). Dicer forms a complex with argonaute 2 protein (Ago2) and trans-activating response RNA binding protein (TRBP) (Chendrimada et al, 2005). Dicer interacts with the 5' and 3' ends of the pre-miRNA hairpin and cuts the long flexible loop that joins the 5' and 3' arms of the hairpin to create an imperfect miRNA: miRNA* duplex (Zhang et al, 2002).

Unwinding of the miRNA: miRNA* duplex is then required, which is performed by the Dicer, TRBP and Ago2 complex, to produce mature miRNA (Kawamata et al, 2009). The single-stranded mature miRNA that is bound to the Dicer, TRBP and Ago2 complex is then incorporated into the RNA-induced silencing complex (RISC) for miRNA silencing (Provost et al, 2002).

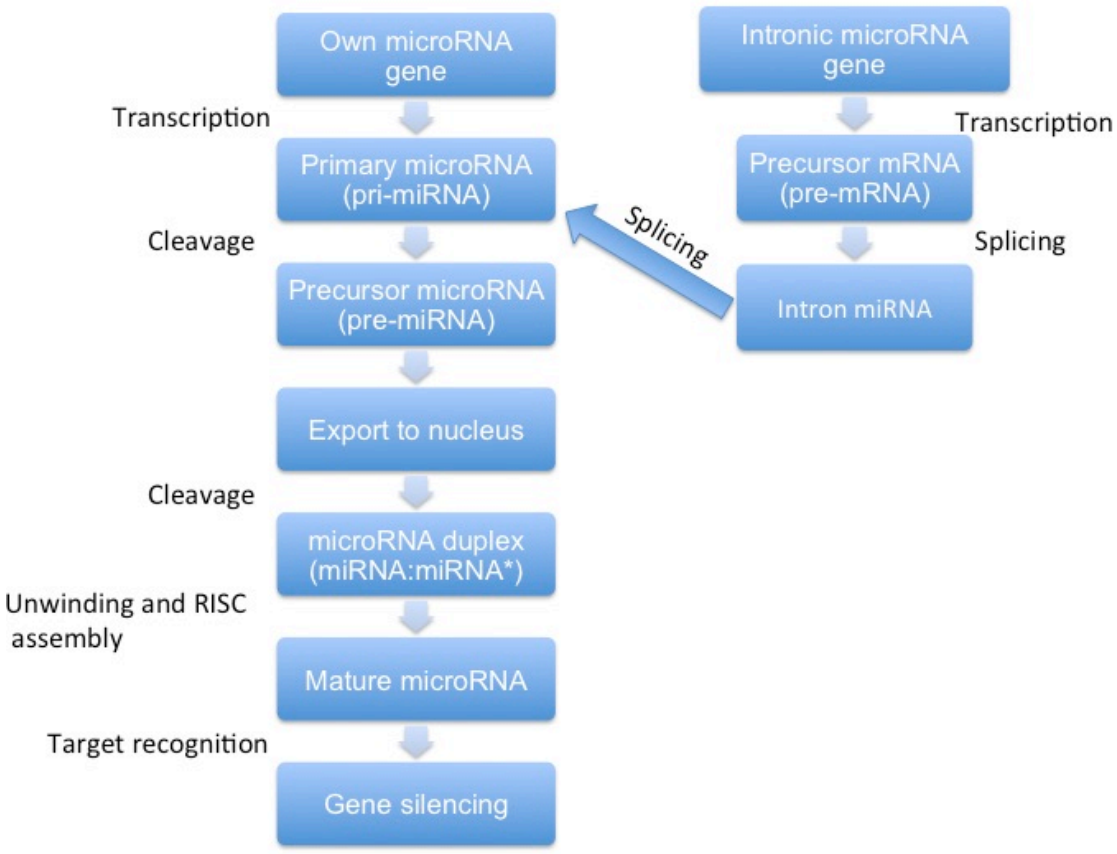


Figure 1.5: MiRNA biogenesis
A schematic flow chart of miRNA biogenesis starting from miRNA transcribed from within its own miRNA gene or intronic miRNA gene, and the processing and export steps until the miRNA binds to its target for gene silencing.

1.17 MiRNA silencing of RNA

The major role of miRNAs is in RNA silencing and post-transcriptional regulation of gene expression. MiRNAs have the ability to impair the production of proteins from target mRNAs and thereby reduce expression of particular mRNA targets (Figure 1.6) (Shivdasani et al, 2006). It is thought that 60% of all mRNAs are targets for regulation by miRNAs (Friedman et al, 2009). For RNA silencing to occur, the formation of the RISC complex is vital as the RISC complex guides the miRNA towards its correct target mRNA (Pratt et al, 2009).

The core components of the RISC complex are Dicer, Ago2, TRBP and protein kinase R-activating protein (PACT) (Lee et al, 2006). Once the mature miRNA has been formed, the mature miRNA is loaded into the RISC complex that then guides the miRNA to target sequences in mRNAs, normally in the 3' UTRs (Chendrimada et al, 2005).

MiRNA driven mRNA silencing can occur in different ways. Firstly, miRNA can be responsible for the cleavage of the mRNA strand and subsequent degradation (MacFarlane et al, 2010). Here the miRNA, that has been guided to the target mRNA by the RISC complex, is usually – but not always - fully complimentary and base pairs with the mRNA (Meister et al, 2004). Cleavage of the mRNA strand is then catalyzed by Ago2 in the RISC complex leading to degradation of the RNA (Liu et al, 2004)

Secondly, miRNAs can be responsible for inhibition of mRNA translation (Valencia-Sanchez et al, 2006). Inhibition of mRNA translation can occur when nucleotides 2-7 of the miRNA, which is known as the seed region, are complementary to the mRNA while the remainder of the miRNA is only partially complementary and the base pairing is mismatched (Meister et al, 2004). It is thought that complimentary and mismatched base pairing creates bulges in the miRNA and mRNA pairing to prevent translation occurring. However, further understanding is required (Bartel et al, 2004). Complimentary and mismatched base pairing between the miRNA and mRNA can also lead to an increase in the speed of deadenylation and removal of the mRNA poly A tail leading to more rapid mRNA degradation (Pillai, 2005).

1.18 MiRNAs in cancer

Cancer is driven by mutations and dysregulation of genes, so, given the roles miRNAs play in gene regulation in normal cells, the roles miRNAs have in gene dysregulation in cancer have been studied intensively (Peng et al, 2016).

Firstly, expression profiles of miRNAs have been assessed to identify any increased or decreased expression of miRNAs in cancers (Espinosa et al,

2006). MiRNA expression profiles are commonly carried out using sequencing platforms (miRNA-SEQ) to screen a large number of miRNAs, with a large number of changes seen in miRNA expression in many different tumour types (Zhu et al, 2014). For example, miR-155 has been shown to overexpressed in many types of cancer such as haematopoietic cancers, breast, lung and colon (Faraoni et al, 2009). In addition, miR-21 has been found to be overexpressed in almost all cancers studied such as breast, colon, liver, brain, pancreas and prostate (Volinia et al., 2006). The miR-17-92 cluster, which encodes 6 different miRNAs, is overexpressed in multiple cancers such as lung, colon and gastric cancers (Concepcion et al, 2012).

It is evident that the expression of miRNAs change during malignant transformation and further work has been carried out to identify what role miRNAs have in cancer progression (Adams et al, 2014). Because of miRNAs role in dysregulation of gene expression, miRNAs can act as either oncomiRs or tumour suppressor miRs. MiRNAs act as oncomiRs when they aid in cancer progression and promote tumor development, normally by targeting inhibitory regulators of cancer as well as genes that stimulate cell differentiation and apoptosis (Jansson et al, 2012). On the other hand, tumour suppressor miRs prevent cancer progression by reducing expression of oncogenes (Price et al, 2014).

There are a large number of examples of oncomiRs in a variety of different cancers that can play a role in different hallmarks of cancer. miR-21 is responsible for the down-regulation of four tumour suppressor genes: mapsin, programmed cell death 4 (PDCD4), tropomyosin1 (TPM1) and phosphatase and tensin homolog (PTEN) (Volinia et al., 2006). Additionally, it is thought that PTEN is also a target of the miR-17-92 cluster (Zhu et al, 2014).

The miR-17-92 cluster contains the genes for the following six miRNAs: miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a and miR-92-1. The miR-17-92 cluster has also been shown to be an oncomiR (Mendell, 2008). The miR-17-92 cluster has been linked to the expression of c-myc, which is required for proliferation. Both c-myc and the miR-17-92 cluster regulate the expression of the

transcription factor E2F1 leading to increased cell survival (O'Donnell et al, 2005). There are also numerous examples of miRNAs acting as tumour suppressors. For example, let-7, which is one of the first-identified members of the miRNA family, has been shown to negatively regulate the oncogene RAS (Johnson et al, 2005).

1.19 Hypothesis and aims

Having reviewed the current literature, my hypothesis was that CAFs enable chemoresistance in TNBC through the release of specific mediators that act on epithelial cancer cells.

I aimed to:

- Identify whether CAFs enable chemoresistance in TNBC cells;
- Characterize potential mediators (miRNA) and signaling pathways responsible for CAF-induced chemoresistance;
- Determine if the CAF-induced chemoresistance pathways could be inhibited to improve TNBC response to chemotherapy;
- Highlight any potential cellular mechanisms responsible for CAF-induced chemoresistance.

Chapter 2: Methods and materials

2.1 Cell lines and tissue culture

Triple negative (ER, PR and HER2 negative) breast epithelial cancer cell lines MDA-MB-231 (wild-type [without either luciferase or GFP expression]), MDA-MB-468 and MDA-MB-157 cells were acquired from American Type Culture Collection (ATCC HTB-26, HTB-132 and HTB-24, Manassas, USA). MDA-MB-231 cells that stably express firefly luciferase (MDA-MB-231-luc) were purchased (Cat# AKR-231, Cell Biolabs, San Diego, USA), and MDA-MB-231 cells that stably express GFP and firefly luciferase (MDA-MB-231-GFP/luc) were developed in-house by transduction with lentiviruses coding for over-expression of the appropriate reading frames (Lorger et al, 2010). MDA-MB-468 that stably express firefly luciferase (MDA-MB-468-luc) or GFP (MDA-MB-468-GFP) were also developed in house following transduction with lentiviruses (Lorger et al, 2010). Normal breast fibroblasts (NFs) or breast cancer-associated fibroblasts (CAFs) were extracted from breast cancer patient samples by the Hughes group and immortalised by viral transduction to allow stable over-expression of hTERT (Verghese et al, 2013) to create the lines NF1 and NF2 (normal fibroblasts) or CAF1 (cancer-associated fibroblasts). CAF1-GFP cells were also developed by transduction with lentiviruses coding for over-expression of the appropriate reading frame (Verghese et al, 2013). Primary NF (pNF) and CAF (pCAF) were also extracted from breast cancer patient samples and were used at relatively early passage (between 5 and 10 passages). Both the pNFs and pCAFs were taken from a TNBC patient with the pCAFs being taken from within the tumour mass, and the pNFs being extracted from at least 1cm outside the tumour margin. Fibroblasts were extracted from samples provided by breast cancer patients undergoing treatment within the Leeds Teaching Hospitals NHS Trust; patients were recruited and informed consent was taken under ethical permission from the Leeds (East) Research Ethics Committee reference 09/H1326/108.

MDA-MB-231 (all derivatives), MDA-MB-468 (all derivatives), NF1, NF2, CAF1 and CAF1-GFP cells were all grown in the same media: DMEM (Cat# 31966-

021, Thermofisher Scientific, Waltham, USA), 10% FCS (Cat# F7524, Sigma, St Louis, USA) and 1% penicillin/streptomycin at final concentrations of 100 U/ml penicillin and 100µg/ml Streptomycin (Cat# 15070-063, Thermofisher Scientific, Waltham, USA). Cells were cultured at 37^0C in 5% CO_2/air and were kept sub-confluent by sub-culturing every 2-4 days as appropriate. MDA-MB-157 cells were grown in Leibovitz L-15 media (Cat# 11415-049, Thermofisher Scientific, Waltham, USA) with 10% FCS and 1% penicillin/streptomycin as above, and were cultured at 37^0C in sealed flasks or plates in 100% air. MDA-MB-157 cells were kept sub-confluent by sub-culturing every 7 days, with 2 media changes during this period. pNFs and pCAFs were cultured in DMEM F12 media (Cat# 31331-028, Thermofisher Scientific, Waltham, USA) supplemented with 10% FBS, 1% penicillin/streptomycin as above, and 1% Fungizone at a final concentration of 5µg/ml (Cat# A2942, Sigma, St Louis, USA). Cells were cultured at 37^0C in 5% CO_2/air and were kept sub-confluent by sub-culturing every 2-4 days as appropriate. All cells were maintained routinely in 150cm^2 tissue culture flasks (Cat# CLS430825, Corning, Sigma, St Louis, USA).

For the passaging of cells, medium was removed and cells were washed with 10ml Dulbecco's phosphate-buffered saline (DPBS, Cat# 14190-094, Thermofisher Scientific, Waltham, USA). 2ml of 0.05% trypsin (Cat#1540054, Thermofisher Scientific, Waltham, USA) was added and incubated for 5min at 37^0C, or until all the cells had dissociated from the plastic. To inactivate the trypsin, 10ml of the correct culture media was added to the cells. 2-10ml of the cell suspension was then added to 20ml of fresh culture media in a new 150cm^2 tissue culture flask. The volume of cell suspension varied dependent on the cell line used, with – for example - MDA-MB-157 cells and primary fibroblasts requiring higher seeding densities, therefore 10ml of cell suspension was added to the 20ml fresh culture media.

To remove cells from plastic mid-experiment for replating or for further down-stream analysis, cells were trypsinised using 2ml of 0.05% trypsin and resuspended in 10ml cell culture media. Cells were then centrifuged at 400g for 5 min to pellet the cells. Cells were then resuspended in a further 10ml of

culture media ready for replating. For down-stream analysis, following pelleting, cells were resuspend in 10ml PBS instead.

2.2 Transfection of cell lines

ISRE/GAS reporter plasmids and renilla plasmid (pRL-TK using HSV thymidine kinase promoter (Mankouri et al, 2010)) were kind gifts from Dr Andrew Macdonald (University of Leeds). MiRNA mimics and inhibitors and appropriate controls were purchased from Dharmacon (Lafayette, USA), and are listed in Table 2.1.

MDA-MB-231 cells (all derivatives) were seeded for transfection in 24 well plates (Cat# CLS3527, Corning Costar, Sigma, St Louis, USA) at 50,000 cells per well, or in 96-well plates (Cat# CLS9102, Corning Costar, Sigma, St Louis, USA) at 20,000 cells per well and were incubated in standard culture for 24h before transfection. MDA-MB-157 cells were seeded for transfection in 96-well plates at 40,000 cells per well 24h prior to transfection. Cells were then transfected in OPTI-MEM (Cat# 31985-070, Thermofisher Scientific, Waltham, USA) without serum, with varying concentrations of miRNA inhibitors/mimics or plasmids as appropriate, using Lipofectamine 2000 (Cat#11668019, Thermofisher Scientific, Waltham, USA). Prior to addition to wells, miRNA mimics or inhibitors, or plasmids, were combined with OPTI-MEM and Lipofectamine 2000 for 20min at room temperature; 2µl of Lipofectamine 2000 was used per 24-well in 100µl of OPTI-MEM or 0.5µl of Lipofectamine 2000 was used per 96-well in 50µl of OPTI-MEM. Transfection mixtures were added to cells, with medium already on the cells, for 6h and cells were incubated under standard culture conditions. After this 6h, medium was then replaced with full fresh medium and cells were incubated in standard culture conditions at least overnight before further manipulation (for example, trypsinisation for re-plating in co-culture experiments). MiRNA mimics or inhibitors and their associated controls were used at concentrations of between 500pM to 500nM in the transfection mix (mimics/inhibitors and controls at the same concentration in each individual experiment), while ISRE/GAS reporter plasmids were used at 1µg/ml.

Target	Dharmacon catalogue number
Control mimic	CN-001000-01-05
Control inhibitor	IN-001005-01-05
miR 27a-3p mimic	C-300502-03-0002
miR 27a-3p inhibitor	IH-300502-05-0002
miR 155-5p mimic	C-300647-05-0002
miR 155-5p inhibitor	IH-300647-06-0002

Table 2.1: MiRNA mimics and inhibitors used for transfection

2.3 Chemotherapy agents, chemical inhibitors, recombinant interferons, blocking antibodies

Epirubicin hydrochloride (Cat# E9406, Sigma, St Louis, USA) was dissolved in DMSO at a stock concentration of 100mM, and was used at a wide range of doses, depending on cell line and end-point assay, (short-term survival or colony forming assays) of 10nM to 79μM. Docetaxel (Cat#01885-5MG-F, Sigma, St Louia, USA) was dissolved in DMSO at a stock concentration of 10mM, and was used at 350pM for colony forming assays. Ruxolitinib (Cat#S1378, Selleckchem, Munich, Germany) was dissolved in DMSO at a stock of 10mM and was used at 10nM for colony forming assays. Recombinant IFN-α and IFN-γ were kind gifts from Matthew Holmes and Fiona Errington-Mais (University of Leeds) and were manufactured by Peprotech (Cat # 300-02A and 300-02 respectively, Rocky Hill, USA). Anti-Human Interferon Alpha/Beta receptor chain 2 (clone MMHAR-2, Cat# 21385-1, PBL Assay Science, Piscataway, USA) or anti-human Interferon gamma R1 neutralising antibodies (CD119, Cat# AF673, R&D Systems, Minneapolis, USA) were added to cultures with a final concentration of 1μg/ml or 5μg/ml respectively. Appropriate isotype controls for both the IFN-α and IFN-γ blocking antibodies were used respectively: mouse IgG_{2A} isotype control (Cat#MAB003, R&D Systems, Minneapolis, USA) and goat IgG control (Cat# AB-108-C, R&D Systems, Minneapolis, USA) at the same concentrations.

2.4 Dual luciferase reporter assays

MDA-MB-231 cells that had been transfected with the ISRE or GAS reporter plasmids were then trypsinised and re-plated in culture/co-culture with varying proportions of NF1 or CAF1 cells at 0, 8, 20 or 55% fibroblasts within a total of 20,000 cells per well in 96-well plates. Cultures were incubated with or without epirubicin as described in the appropriate figure legend. Cells were then washed twice with PBS and lysed using 50µl of 1x passive lysis buffer per well (Cat# E194A, Promega, Madison, USA) and left on a rocker for 15min at room temp. Analysis of luciferase activity followed the protocol in section 2.5.1, however *renilla* activity was also measured with 20µl Stop and Glo reagent (Cat#E640A and E641A, Promega, Madison, USA) added to each well after the LARII (Cat# E151A and E195A, Promega, Madison, USA) reading was taken. The plate was shaken for 2s, delay 2s and read for 5s.

2.5 Luciferase-based chemo-survival assays

2.5.1 Dose determination

For simple mono-culture experiments, MDA-MB-231-luc or MDA-MB-468-luc were plated in 96-well plates at 20,000 cells per well and were cultured for 24h as normal. Cells were then treated in triplicate with epirubicin hydrochloride at doses ranging from 1pM to 500µM, or without epirubicin treatment (control), by replacing the media with 50µl of dosed media. Following culture for 24h, cells were washed twice with PBS and lysed using 100µl of 1x passive lysis buffer (Cat# E194A, Promega, Madison USA). Plates were left on a rocker for 15min at room temperature for the cells to lyse. 10µl of lysate was added in triplicate to wells of a new 96-well opaque wall plate, and were analysed for luciferase activity using the Berthold technologies Mithras LB 940 (Bad Wildbad, Germany) 96-well plate reader. The following luminescence protocol was used: 20µl LARII (Cat# E151A and E195A, Promega, Madison, USA) was added to each well, shaken for 2s, delay 2s and read for 5s. For calculating cell survival,

mean treated readings were expressed relative to the no treatment (control) average. IC25, 50 and IC75 doses were estimated from dose response curves.

2.5.2 Co-culture assays

For co-culture experiments, MDA-MB-231-luc or MDA-MB-468-luc cells were plated in 96-well plates in triplicate with varying proportions of NF1, NF2, or CAF1 cells (from 0% fibroblasts to 55% fibroblasts) within a total of 20,000 cells per well. After 24h of culture, cells were treated with approximate IC10, 50 or 75 concentrations of epirubicin hydrochloride (as determined by mono-culture dose-response assays (see section 2.5.1), or with vehicle only, for a further 24h. For 24h treatments only, medium was then removed and cells washed twice with PBS and lysed in 1x passive lysis buffer (Cat# E194A, Promega, Madison, USA) as above. For 72h incubations, after 24h of drug treatment, medium was replaced with drug-free medium for a further 48h before washing and lysis as above. Luciferase activity within lysates was assessed using the Berthold plate reader as above. Readings from epirubicin-treated cultures were normalised to readings from matched cultures with the same fibroblast proportion and treated with vehicle control. Normalisation this way allowed focus on differences in response to epirubicin, and to avoid the confounding factors of differences in the total numbers of epithelial cells seeded and the potential influences of fibroblasts on epithelial growth.

2.6 MTT assays

Cells were seeded/transfected in 96-well plates as appropriate for individual experiments. 25µl of 5mg/ml MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Cat# M6494, Thermofisher Scientific, Waltham, USA) was added to each well for 4h in the dark at 37^0C in 5% CO_2/air, and then removed. 50µl of propan-1-ol (Cat# 6775-25, Macron fine chemicals, Center Valley, USA) was added to each well and plates were rocked until all the precipitate had dissolved. Absorbances were then measured at 570nm using the Berthold 96-well plate reader.

2.7 Mono-culture colony forming assays

For initial chemotherapy dose-finding experiments and for recombinant interferon colony formation experiments, mono-culture colony forming assays were performed. Cells (MDA-MB-231-GFP/luc, MDA-MB-468-GFP, or MDA-MB-157) were seeded in 6-well plates at 200,000 cells per well and allowed to adhere overnight. Cells were treated with chemotherapy agents (see section 2.3) or recombinant interferons as appropriate for up to 48h. The final concentrations of IFN-α ranged from 500-5000pg/ml and IFN-γ ranged from 500-3000pg/ml. Cells were then trypsinised, counted using a haemocytometer and plated in duplicate 10cm tissue culture dishes (Cat# CLS430167, Corning, Sigma, St Louis, USA) at 500 cells per plate in fresh medium lacking chemotherapeutics or recombinant interferons. Plates were incubated for 14 days undisturbed (in an isolated incubator dedicated to these assays with minimal opening/closing – critical to minimise cells from individual colonies becoming dispersed into multiple colonies). For MDA-MB-231-GFP/luc and MDA-MB-468-GFP cells, cells were then fixed/stained using 1ml of 5mg/ml Crystal Violet (Cat# V5265, Sigma, St Louis, USA) in 50% methanol, 20% ethanol and 30% water for 30s, followed by 3 washes in distilled water. For MDA-MB-157 cells, cells were fixed/stained twice in succession by two full repeats of 2ml of 12.5mg/ml Crystal Violet in 50% methanol, 20% ethanol and 30% water, for 5min and followed by 3 washes in distilled water. Isolated colonies estimated to be of more than 40 cells were counted manually.

The reproducibility and objectivity of these manual colony counts was confirmed as follows. The author and a fellow researcher (Melina Teske, University of Leeds) independently counted colonies on 16 plates, representing a range of different number of colonies. The two independent values for the number of colonies on each plate were compared, and the correlation between the sets of data was calculated (Figure 2.1). The correlation coefficient of 0.949 indicates near perfect agreement between the independent scores, demonstrating excellent reproducibility of these data.

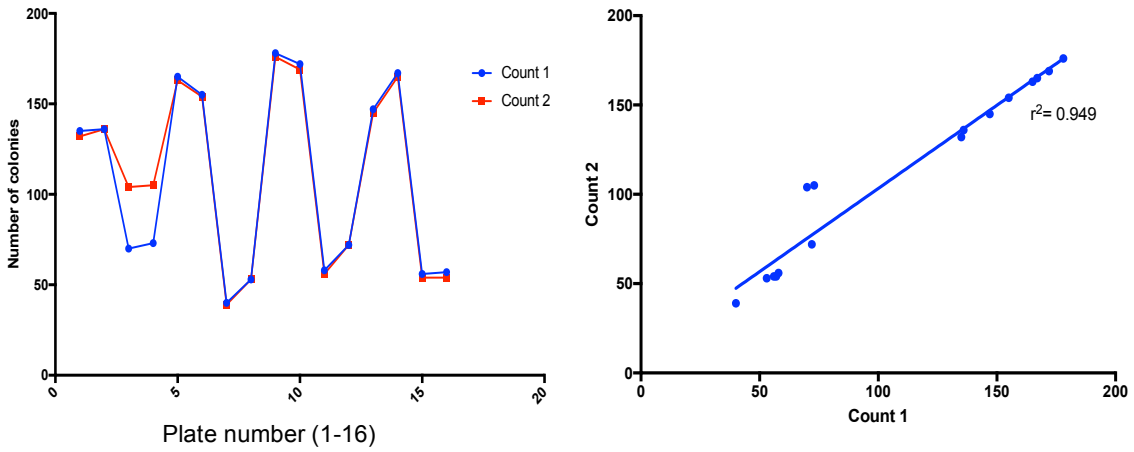


Figure 2.1: Comparison of 2 independent counts of colony forming assay plates of MDA-MB-468 cells
Colonies on 16 separate plates of MDA-MB-468 cells with differing numbers of colonies following recombinant interferon and/or epirubicin treatments were counted independently by the author, RB (count 1) or a colleague, Melina Teske (count 2). Counts for each plate were plotted independently (left panel) or relative to each other (right panel).

2.8 Co-culture colony forming assays

For co-culture experiments, MDA-MB-231-GFP/luc, MDA-MB-468-GFP, or MDA-MB-157 cells were seeded with varying proportions of NF1, CAF1, CAF1-GFP, pNF or pCAF cells at 0, 8, 20 or 55% fibroblasts within the same total number of cells ($1x10^6$ cells in a T25 flask [Cat# CLS430639, Corning, Sigma, St Louis, USA] or 400,000 in each well for 6-well plates). MDA-MB-231-GFP/luc and MDA-MB-468-GFP were co-cultured with NF1 or CAF1 cells (which are GFP negative), but the GFP negative MDA-MB-157 cells were cultured with CAF1-GFP (GFP positive). Epithelial cells may have previously been transiently transfected immediately prior to seeding for these co-cultures, as previously described in section 2.2. Cultures were treated with a variety of chemotherapy agents, chemical inhibitors, or antibodies as appropriate (see section 2.3). After up to 48h, cultures (including the 0% fibroblast culture, which is an epithelial mono-culture) were treated for fluorescence-activated cell sorting (FACS), and epithelial cells were re-plated to form colonies as described for section 2.7.

FACS was used to separate components of cultures for subsequent down-stream analyses of the separate cell types (by colony forming assay, or qPCR

for example). Importantly, mono-cultures were also sorted in experiments where mono-cultures were compared with co-cultures, to ensure that the cells were treated similarly in both cases. Cultures were trypsinised and washed twice with PBS on ice, and resuspended in 1ml RPMI phenol red free media (Cat# 11835-030, Gibco, Thermofisher Scientific, Waltham USA). Cells were then strained using a 70µM nylon cell strainer (Cat#352350, Corning, Sigma, St Louis, USA) before sorting. The BD Influx 6-way cell sorter was used, identifying GFP positive cells using the 488nm blue laser, and gating on live cells using FSC/SSC. Only single cells were collected. Representative flow plots are shown for the cell combinations separated: MDA-MB-231-GFP/luc cells combined with fibroblasts (GFP negative), MDA-MB-468-GFP combined with fibroblasts (GFP negative), and CAF1-GFP cells combined with MDA-MB-157 cells (Figures 2.2, 2.3 and 2.4 respectively). Up to 100,000 cells of the desired population were collected on ice for re-plating (section 2.7) or other down-stream analysis (section 2.12).

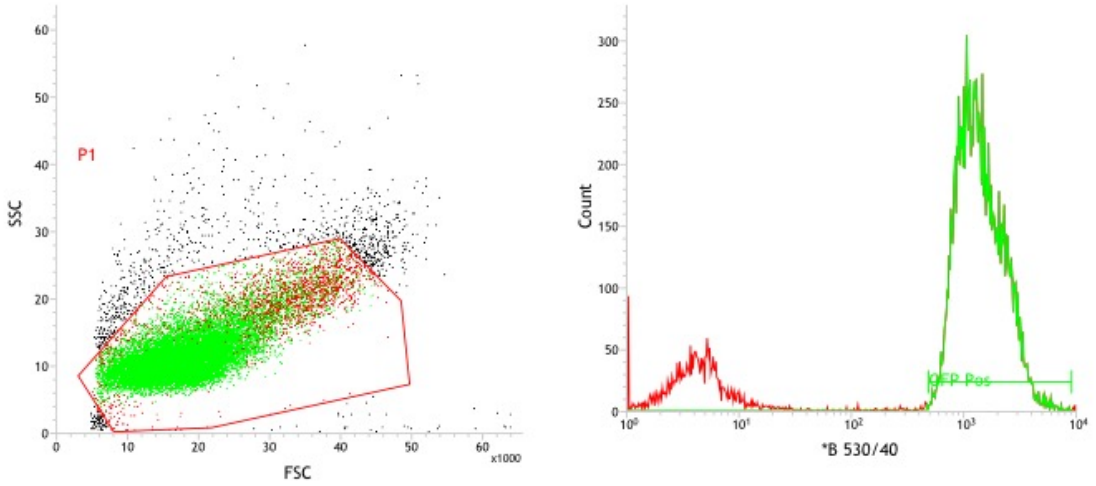


Figure 2.2: FACS cell sorting plots for MDA-MB-231 GFP/luc cells co-cultured with 20% CAF1 cells
Viable cells were first gated (FSC vs SSC left panel) followed by gating on GFP expression: GFP negative CAF1 cells (red peak in right panel) and GFP positive MDA-MB-231 cells (green peak in right panel). Cells were sorted from the appropriate peaks on the right plot.

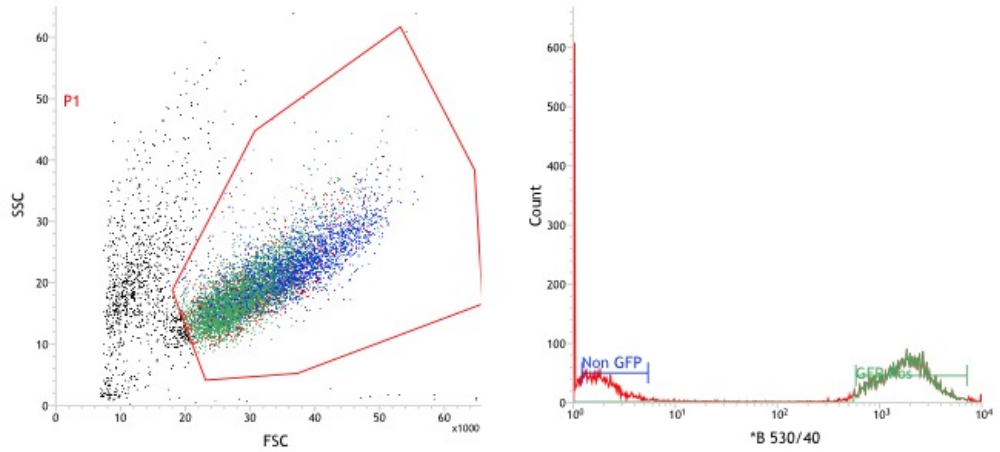


Figure 2.3: FACS cell sorting plots for MDA-MB-468 GFP cells co-cultured with 20% CAF1 cells

Viable cells were first gated (FSC vs SSC left panel) followed by gating on GFP expression: GFP negative CAF1 cells (red peak in right panel) and GFP positive MDA-MB-231 cells (green peak in right panel). Cells were sorted from the appropriate gated peak on the right plot.

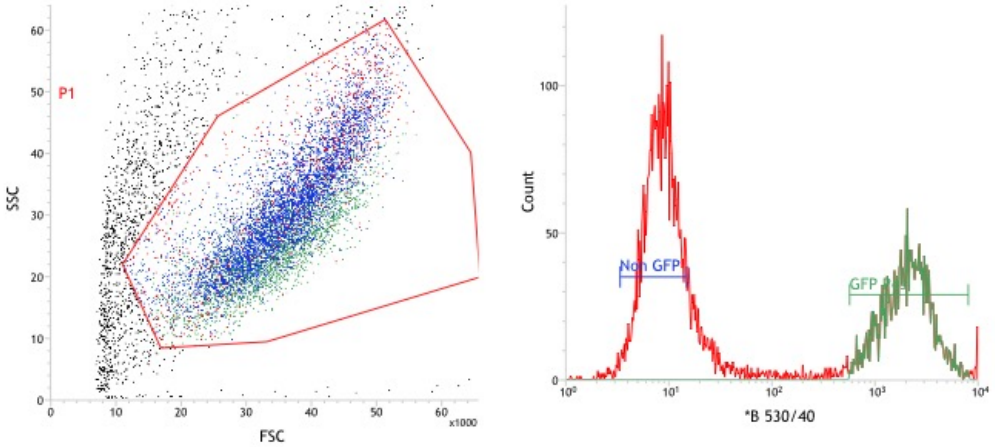


Figure 2.4: FACS cell sorting plots for MDA-MB-157 cells co-cultured with 20% CAF1-GFP cells

Viable cells were first gated (FSC vs SSC left panel) followed by gating on GFP expression: GFP negative MDA-MB-157 cells (red peak in right panel) and GFP positive CAF1-GFP (green peak in right panel). Cells were sorted from the appropriate gated peak on the right plot.

2.9 Preparation of fibroblast conditioned media, and use in colony forming chemo-survival assays

In order to prepare fibroblast-conditioned media (with and without epirubicin), T150 tissue culture flasks of NF1 and CAF1 cells were seeded so as to be just sub-confluent. Flask were treated with 10nM epirubucin hydrochloride for 24h (induced conditioned media) or left without drug (conditioned media). Medium was collected from these flasks, centrifuged at 400g for 5min, and the supernatent was used on epithelial cells immediately without storage, meaning that multiple flasks of fibroblasts had to be established and treated at different times in order to supply fresh conditioned media when required. Epithelial cells (MDA-MB-231-GFP/luc, MDA-MB-157 or MDA-MB-468-GFP) were seeded for 8h in standard culture conditions at $1x10^6$ cells in T25 tissue culture flasks in either normal fresh medium or a mix of 50% conditioned medium/50% normal fresh medium using conditioned medium from either NF1 or CAF1 cells. Medium was then replaced with fresh medium or 50% conditioned medium as appropriate and cells were cultured for a further 16h. Cells were then treated with 10nM epirubicin hydrochloride in either 0% or 50% conditioned media from NF1 or CAF1 cells or 50% induced conditioned media from NF1 or CAF1 cells. Cells were left for 8h at 37^0C 5% CO_2 with media again replaced for a further 16h. Cells were then trypsinised, counted and re-plated to allow colonies to form exactly as described in section 2.7.

2.10 Anoikis assays

MDA-MB-231-GFP/luc were seeded with NF1 or CAF1 cells in T75 flasks (Cat# CLS430641, Corning, Sigma, St Louis, USA) with varying proportions of fibroblasts, including 0, 8, 20 or 55% fibroblasts at a total cell number per flask of $2x10^6$. Cultures were incubated for 24h, followed by a media change and a further incubation for 24h. Cultures were then trypsinised, washed twice with ice cold PBS and GFP-positive MDA-MB-231-GFP/luc cells were collected by FACS exactly as described in section 2.8; $1.5x10^5$ MDA-MB-231-GFP/luc cells were collected. The remainder of the protocol followed the CytoSelect 96-well anoikis assay manufacturer's protocol (Cat#CBA-081, Cell Biolabs, San Diego, USA). All MDA-MB-231-GFP/luc cells collected from FACS were spun at 400g

for 5min in a centrifuge and resuspended in 100µl of media and added to 96-well anchorage resistant plates (part 108101 from kit named above). Cells were incubated for 36h under normal culture conditions. In order to quantify surviving cells, 10µl of the MTT reagent (part 113502), was added to each well for 3h, and the plate was incubated as for normal culture. Following this incubation, 100µl of detergent solution (part 108004) was added to each well and plates were incubated for 3h at room temperature in the dark. The 150µl of solution in the wells was transferred to a 96-well tissue culture plate and the absorbance of each well was measured at 570nm on the Mithras LB 940 96-well plate reader (Berthold Technologies).

2.11 Analysis of transcriptomes for MDA-MB-231 cells cultured with and without CAF1 cells

MDA-MB-231-GFP/luc cells were seeded alone (0% fibroblasts) or with 20% CAF1 cells in T75 tissue culture flasks, giving a total cell number of $2x10^6$ in both cases. Cultures were incubated (24h), treated with 10nM epirubicin (24h), and then treated for FACS to purify GFP-positive MDA-MB-231-GFP/luc cells as described above (section 2.8). 900,000 MDA-MB-231-GFP/luc cells were collected. Sorted MDA-MB-231-GFP/luc cells were then collected by centrifugation at 400g for 5min, washed with PBS and collected again by centrifugation at 400g for 5min. RNA was extracted using the ReliaPrep RNA miniprep system (Cat#Z6012, Promega, Madison, USA) following the manufacturer's protocol, and suspended in 30µl of nuclease free water (part#P119E). RNA concentrations were measured using the Nanodrop (ND-1000 spectrophotometer). Three separate biological repeats of this experiment were completed in a three week period to provide robust biological replicates. The same RNA samples were used for transcriptome analyses of both miRNAs and mRNAs (section 2.11.1 and 2.11.2).

2.11.1 mRNA transcriptome analyses

Samples were sent to the Sheffield Microarray/Genomics core facility at the University of Sheffield to be analysed on Affymetrix Clariom D microarrays

(Santa Clara, USA) by Dr Paul Heath (University of Sheffield). Initial data quality examination was then performed using Affymetrix Expression Console software and probe level summarisation files were then used for further analysis using the Affymetrix transcriptome analysis console v3.0. Using the transcriptome analysis console, the three biological repeats were grouped into 0% CAF and 20% CAF samples and a paired ANOVA was performed for each individual mRNA probe. Thresholds were put in place where fold changes between the 0% and 20% groups were greater than +/- 2 with p value from the ANOVA less than 0.05. The list of genes that fit these criteria were then analysed in ToppGene (https://toppgene.cchmc.org, Chen et al, 2009) to identify pathways of interest. For ToppGene software, the ToppFun programme was used and the full gene list was added with the entry type Ensembl ID.

2.11.2 miRNA analyses

The three separate samples representing independent biological replicates for 0% CAF or 20% CAF were pooled for each group. These pools were diluted to equal RNA concentrations (as determined by Nanodrop) of 136ng/µl. Expression of 377 miRNAs was determined using TaqMan low-density arrays: human miRNA array A (Thermofisher Scientific, Waltham, USA). RNA was reverse transcribed using MegaPlex RT pool A primers using the TaqMan miRNA reverse transcription kit (Cat# 4366597, Thermofisher Scientific, Waltham, USA): the RT reaction mix was made up of 0.8µl of MegaPlex RT pool A primer, 0.2µl of 100mM dNTPs, 1.5µl of 50U/µl Multiscribe Reverse transcriptase, 0.8µl 10X RT buffer, 0.9µl of 25nM $MgCl_2$, 0.1µl of 20U/µl RNase Inhibitor, 0.2µl of nuclease free water per sample. 4.5µl of the RT reaction mix was mixed with 3µl of RNA per sample and incubated on ice for 5min. The tubes were then placed on the Dyad Peltier Thermocycler (Bio-rad, Hercules, USA) following the thermal-cycling conditions shown in Table 2.2.

Stage	Temp	Time
Cycle (40 Cycles)	16°C	2min
	42°C	1min
	50°C	1sec
Hold	85°C	5min
Hold	4°C	∞

Table 2.2: Thermal cycling protocol required for Taqman MegaPlex miRNA cards reverse transcription

Following the RT, 6µl of the RT product was combined with 450µl of Taqman Universal PCR master mix (Cat# 4304437, Thermofisher Sceintific, Waltham, USA) and 444µl of nuclease free water per sample. 100µl of PCR reaction Mix was added into each port of a Taqman human miRNA array A plate and reactions were performed on the 7900 HT real time PCR machine using a standard curve (AQ) and 384 well TaqMan Low density array. These data were normalised in two alternative ways: to RNU48, a snoRNA commonly used as an invariant normaliser (Gee et al, 2011), or to the array mean including every CT value less than 40 (Park et al, 2003). Undetermined CTs were set to 40 to allow an estimation of minimum fold change when compared to the detected values. Fold changes were determined using $\delta\delta$ct method (Livak et al, 2001).

2.12 RNA extractions and qPCR

RNA was extracted using the ReliaPrep RNA miniprep kit (Promega, Madison, USA) following the manufacturer's protocol.

2.12.1 miRNA qPCR

TaqMan miRNA assays were used. A minimum of 50ng of RNA was used per sample, with equal amounts of RNA used for all samples in the same analysis. RNA volumes were made up to 10µl using nuclease free water (giving 5µl each for detection of target and normaliser). Reverse transcription reactions were carried out in a total volume of 15µl per sample comprising of 5µl RNA, 3µl target or control primer and 7µl RT master mix. The RT master mix, using the

TaqMan miRNA reverse transcription kit (Cat#4366596, Thermofisher Scientific, Waltham, USA), was comprised of 0.15µl of 100mM dNTPs with dTTP, 1µl of 50U/µl Multiscribe Reverse transcriptase, 1.5µl 10X RT buffer, 0.19µl of 20U/µl RNase Inhibitor, 4.16µl of nuclease free water per sample. Samples were incubated on ice for 5min and the tubes were placed on the Dyad peltier thermocycler using the thermal-cycling conditions shown in Table 2.3.

Stage	Temp	Time
Hold	16°C	30min
Hold	42°C	30min
Hold	85°C	5min
Hold	4°C	∞

Table 2.3: Thermal cycling protocol required for TaqMan miRNA assays reverse transcription

For real-time PCRs, total reaction volumes were 10µl. This was made up of 0.5µl target or control primer, 0.67µl RT product (which had been diluted 1:3 with nuclease free water), 5µl TaqMan 2x Universal PCR master mix (Cat# 4304437, Thermofisher Scientific, Waltham, USA) and 3.83µl of nuclease free water. This was added to a microAmp optical 96 well reaction plates (Cat # 4316813, Thermofisher Scientific, Waltham, USA) and qPCR analysis was performed using the QuantStudio 5 system on the TaqMan chemistry setting. Target primers included miR-27a-5p (Cat# 4427975 ID 002445, Thermofisher Scientific, Waltham, USA), miR-27a-3p (Cat# 4427975 ID 000408, Thermofisher Scientific, Waltham, USA), miR-32-5p (Cat# 4427975 ID 002109, Thermofisher Scientific, Waltham, USA), miR-155-5p (Cat# 4427975 ID 002623, Thermofisher Scientific, Waltham, USA), miR-422a (Cat# 4427975 ID 002297, Thermofisher Scientific, Waltham, USA) and miR-454-3p (Cat# 4427975 ID 002323, Thermofisher Scientific, Waltham, USA). For each individual analysis, RNU48 (Cat# 4427975 ID 001006, Thermofisher Scientific, Waltham, USA) was performed in parallel and was used as the normaliser; relative expression levels of target miRNAs were calculated by the δδct method (Livak et al, 2001).

2.12.2 mRNA qPCR

The Promega GoTaq 2-Step RT-qPCR system (Cat# A5000, Promega, Madison, USA) was used. A minimum of 50ng in 4µl per sample was used. 1µl of random primers was added. The RNA/primer mix was preheated to 70°C for 5min then immediately chilled to 4°C for at least 5min. The reverse transcription reaction mix (Promega, Madison, USA) was then added to the RNA primer mix, with the following volumes per sample; 5µl nuclease free water, 4µl GoScript 5x reaction buffer, 4µl of 25M $MgCl_2$, 1µl PCR nucleotide mix for final concentration of 0.5mM and 1µl GoScript reverse transcriptase. The tubes were then placed on the Dyad peltier thermocycler and incubated at the thermal cycling temperatures shown in Table 2.4.

Stage	Temp	Time
Hold	25°C	5min
Hold	42°C	1hr
Hold	70°C	15min
Hold	4°C	∞

Table 2.4: Thermal cycling protocol required for SYBR green mRNA assays reverse transcription

The resulting cDNA was diluted with 40µl nuclease free water prior to real-time PCR. For the real-time PCR, 5µl of the GoTaq qPCR master mix was used per sample with 0.2µl of 40X target primer and 0.05µl CXR reference dye used per sample. Target primers used were OAS1 (Cat# 74007036, IDT, Coralville, USA), MX1 (Cat# 74007039, IDT, Coralville, USA), IFNA2 (Cat# 74849839, IDT, Coralville, USA), IFNB1 (Cat# 74849836, IDT, Coralville, USA), and IFNG (Cat#74849833, IDT, Coralville, USA), with ACTB (Cat# 74007033, IDT, Coralville, USA) used as a control normaliser (see Table 2.5 for primer sequences). Master mix and primer solution were combined with 5µl of cDNA per well in a microAmp optical 96 well reaction plate. qPCR was performed using the QuantStudio 5 system using the pre-programmed SYBR chemistry setting. Expression was determined relative to ACTB using the δδct method (Livak et al, 2001).

Target	Primer
OAS1	5’-GATGAGCTTGACATAGATTTGGG-3’
	5’-GGTGGAGTTCGATGTGCTG-3’
MX1	5’-CGAAACATCTGTGAAAGCAAGC-3’
	5’-CAGGCTTTGTGAATTACAGGAC-3’
IFNA2	5’-TTGACTTGCAGCTGAGCA-3’
	5’-CCCATTTCAACCAGTCTAGCA-3’
IFNB1	5’-GCCATCAGTCACTTAAACAGC-3’
	5’-GAAACTGAAGATCTCCTAGCCT-3’
IFNG	5’-CGACAGTTCAGCCATCACTT-3’
	5’-GCAACAAAAAGAAACGAGATGAC-3’
ACTB	5’-CCTTGCACATGCCGGAG-3’
	5’-ACAGAGCCTCGCCTTTG-3’

Table 2.5: Primer sequences of target and control mRNA qPCR primers

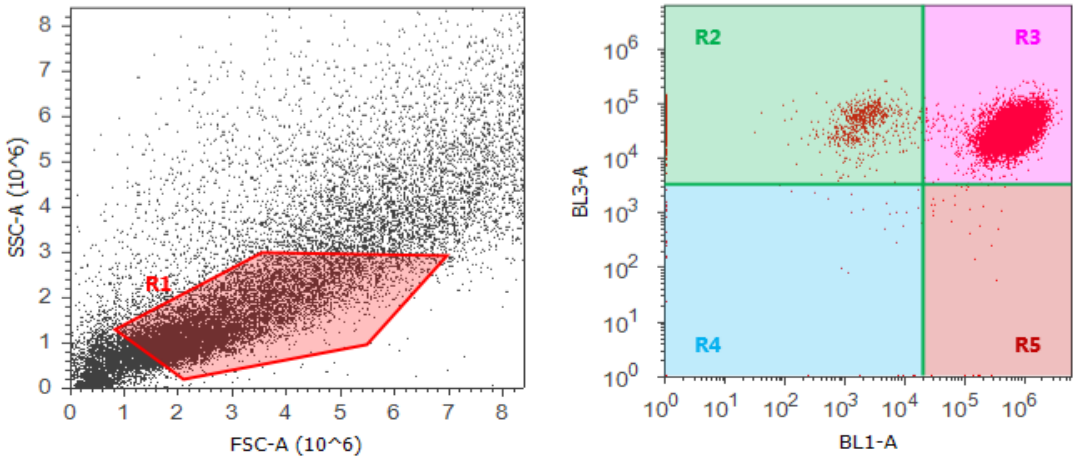
2.13 Flow cytometric assessment of epirubicin loading or cell cycle

To assess epirubicin loading, cells (mono-cultures or co-cultures) were prepared for flow-cytometry by being removed from the plastic by trypsin, and being resuspended in PBS (see section 2.1). The Attune flow cytometer (Thermofisher, Waltham, USA) was used. The BL-1 channel was used to detect GFP expression, while the BL-3 channel was used to detect epirubicin. Analysis was gated on live cells on the basis of FSC/SSC, and then on either the GFP-positive MDA-MB-231-GFP/luc cells or the GFP-negative fibroblasts (representative flow-cytomtery dot plots are shown in Figure 2.5). Epirubicin loading was quantified as BL-3 median level from a minimum of 10,000 events. The specific cell populations are in the following sections; R2- GFP negative fibroblasts loaded with epirubicin, R3- GFP positive MDA-MB-231 cells loaded with epirubcin, R4- GFP negative fibroblasts not loaded with epirubicin, R5-GFP positive MDA-MB-231 cells not loaded with epirubicin. The following voltages for each channel and compensation matrix were used (Table 2.6).

	FSC	SSC	BL1	BL3
Voltage	2700	3400	1200	1400

Compensation matrix	BL1	BL3
BL1	100	-1.5
BL3	-3	100

Table 2.6: Voltages for channels and compensation matrix used for drug loading



Parameters: **BL1-A vs BL3-A**
Gate: **R1**
Experiment Name: **250118 Drug loading**
Specimen Name: **Specimen**
Sample Name: **20 T**
Showing **25,802** of **25,802** events.

Name	Event Count	% Parent	% Total	BL1-A Media	BL3-A Media
All Events	25,802	100.000%	100.000%	548,991	46,632
R1	10,000	38.757%	38.757%	588,333	32,750
R2	986	9.860%	3.821%	1,600	50,236
R3	8,898	88.980%	34.486%	654,863	32,006
R4	86	0.860%	0.333%	-5,224	-115
R5	30	0.300%	0.116%	118,224	1,008

Figure 2.5: Flow cytometry gating strategy for epirubicin loading assessments
MDA-MB-231-GFP/luc were co-cultured with CAF1 cells and treated with epirubicin. First, a gate was determined to select viable cells using FSC vs SSC (top left panel). Then cells were analysed based on BL-1 (GFP positive, x-axis) and BL-3 (epirubicin, y-axis) with the dot plot (top right panel) split into quarters. R2 represents GFP negative epirubicin loaded cells and R3 represents GFP positive epirubicin loaded cells. The median absorbance for BL-3 (epirubicin) in the separate quadrants was determined as shown in the table.

For cell cycle analysis, cells (mono-cultures or co-cultures) were prepared for flow-cytometry by being removed from the plastic by trypsin, and being resuspended in 1ml complete medium with 1µl of Dye Cycle violet (Cat# V35003, Thermofisher Scientific, Waltham, USA). Samples were incubated for at least 30min at 37^0C in the dark. Samples were then analysed on the Attune flow cytometer. Analysis was gated on Dye cycle violet viable cells, on the basis of FSC/VL1 and single cells, on the basis of VL1-H vs VL1-A. GFP positive (MDA-MB-231-GFP/luc) or negative (CAF1 cells) were gated separately using VL1 channel vs BL1 channel. Finally, cell cycle profiles from the Dye cycle violet were extracted using VL1-H. For the analysis, the following compensation and settings were used.

	FSC	SSC	BL1	VL1
Voltage	2700	3600	1200	1300

Compensation matrix	BL1	VL1
BL1	100	-0.1
VL1	-0.6	100

Table 2.7: Voltages for channels and compensation matrix used for cell cycle analysis

Following the formation of the cell cycle profile, these data were analysed using ModFit LT (Verity Software) to determine the percentage of cells that were in each stage of the cell cycle (G1, S, or G2/M).

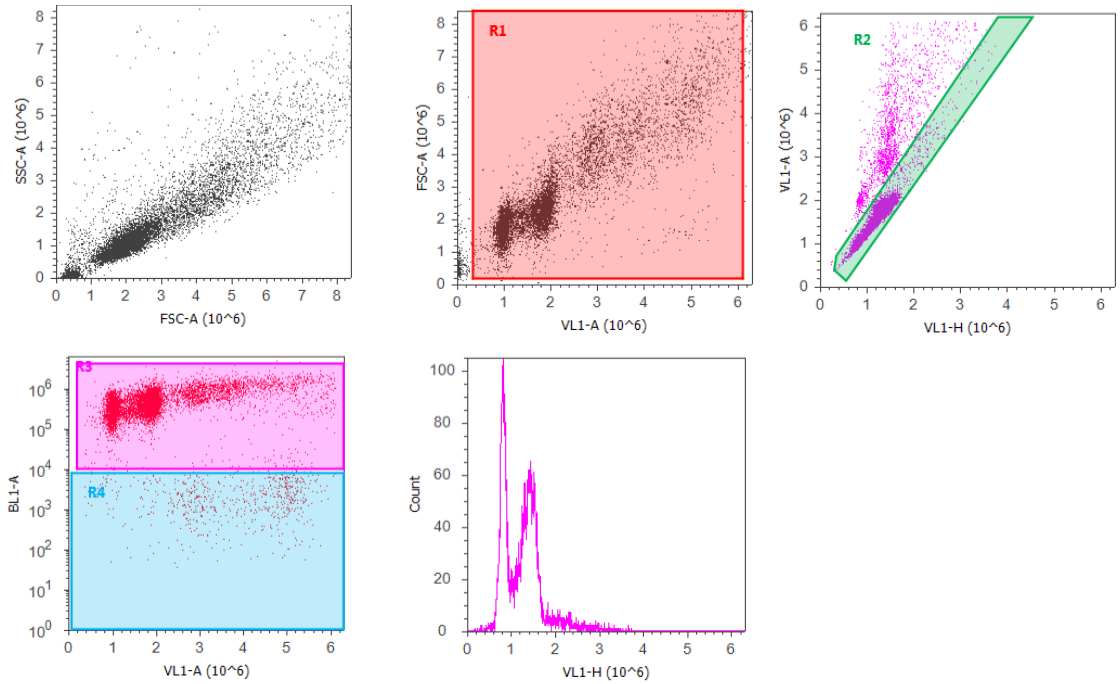


Figure 2.6: Flow cytometry gating strategy for cell cycle assessments
MDA-MB-231-GFP/luc were co-cultured with CAF1 cells and treated with epirubicin. DyeCycle Violet dye was added to all cultures. Cells were analysed based DyeCycle violet loading VL1 (y-axis) in all viable cells vs FSC (x-axis top middle plot). Violet loaded cells (R1) were then analysed based on singlet cells (top right panel). BL-1 (GFP positive, y-axis) was then plotted against VL1 (DyeCycle violet x-axis) to determine GFP positive cells loaded with DyeCycle Violet (bottom left panel, labeled R3). DyeCycle violet count of R3 was produced to create cell cycle profile required for analysis (bottom right panel).

Chapter 3- Cancer-associated fibroblasts protect some triple negative breast cancer cell types from chemotherapeutics

3.1 Abstract

Cytotoxic chemotherapy is the only routine systemic treatment for primary triple negative breast cancer (TNBC). Resistance to chemotherapy in TNBC is a clinical problem reflected in the relatively high rate of early recurrences. Cancer-associated fibroblasts (CAFs) are the most abundant cell type in breast tumour stroma, and have been shown to influence behaviour of tumour cells. My aims were to determine whether the presence or amount of CAFs or normal fibroblasts (NFs) modifies responses of TNBC cells to chemotherapy.

I have shown that the short-term survival of MDA-MB-231 cells after treatment with the anthracycline chemotherapeutic epirubicin was significantly enhanced by the presence of immortalized human breast CAFs, and that this protection was dose-dependent in terms of the proportion of CAFs in the culture. This survival advantage was not observed in MDA-MB-468 cells, which represent a different sub-class of TNBC – the claudin-high cancers. Using clonogenic survival as the end point, claudin-low TNBC cell lines MDA-MB-231 or MDA-MB-157 cells were both significantly protected from epirubicin-induced death by immortalized CAFs, while claudin-high MDA-MB-468 cells were again not protected. Immortalised breast NFs did not protect any cell lines from the effects of epirubicin in this assay. Clonogenic assays were also performed using MDA-MB-231 cells in combination with a matched pair of primary breast CAFs or NFs. As previously, CAFs protected the cancer cells from epirubicin, while NFs did not. Immortalised CAFs also protected MDA-MB-231 cells from the taxane chemotherapeutic docetaxel. Interestingly, conditioned media from CAFs did not offer significant protection from epirubicin in MDA-MB-231, MDA-MB-157 or MDA-MB-468 cells, suggesting that physical contact or bi-directional signaling between the CAFs and epithelial cells is required to induce protection.

I concluded that CAFs, but not NFs, were capable of protecting some types of TNBC cells from chemotherapy, and that the mechanisms involved were worthy of further study.

3.2 Introduction

For TNBC patients, there are no routinely used targeted treatments currently available, so chemotherapy is the only systemic therapeutic option (Chittaranjan et al, 2014). Chemotherapy can be given prior to surgery (neoadjuvant) or post-surgery (adjuvant), with TNBC patients showing relatively good responses in comparison to other breast cancer groups in the neoadjuvant setting (Rouzier et al, 2005). Up to 50% of TNBC patients can achieve pCR, however, responses in the remainder of patients are highly variable. Some patients' tumours will decrease in size but will not achieve a pathological complete response, while others may even continue to grow. It is these patients, those that do not respond to chemotherapy, that are more likely to have a poorer prognosis, with relapses common within 1-3 years post-treatment (Cortazar et al, 2014). Therefore, chemoresistance in these patients is a clinical problem and an understanding of chemoresistance would be useful in order to design improved therapies, as there are currently limited alternative treatment options available.

Tumour stroma is usually defined as comprising the non-cancer cell components of tumours, such as pericytes, adipocytes, immune cells, blood and lymph vessel cells, and fibroblasts, along with the extracellular matrix. Typically, the most abundant cell type present in the tumour stroma is the cancer-associated fibroblasts (CAFs) (Madar et al, 2013). However, the number of fibroblasts present can vary greatly from patient to patient. Although studies focusing solely on fibroblasts are lacking, it has previously been shown that TNBC patients with a higher proportion of stroma (>50%) have a poorer prognosis than patients with a lower proportion of stroma (<50%) (Moorman et al, 2012), with – surprisingly - opposite results reported in ER positive breast cancers (Downey et al, 2014). This suggests that stromal elements may have roles to play in defining treatment responses.

CAFs have been shown to have influences on tumour progression, including at the specific levels of tumour growth, angiogenesis and metastasis (Gascard et al, 2016). In addition, it has been reported that CAFs may have a protective role for the epithelial cancer cells that enables drug resistance, through remodeling of collagen I within the extra-cellular matrix thereby providing a physical barrier for chemotherapeutic perfusion of the cancer cells (Netti et al, 2000). In addition to structural changes, secretory factors released by CAFs to induce drug resistance in breast cancer have also been explored. For example, CAFs have been shown to be responsible for de-sensitization of ER positive breast cancer to the anti-estrogen fulvestrant (Leyh et al, 2015). In TNBC, CAFs have been shown to release to HGF, which activates Met signaling and can induce resistance to EGFR inhibitors (Mueller et al, 2012). However, there is no firm consensus concerning the main pathways by which CAFs influence therapy-response of breast cancer cells, particularly within the TNBC subtype and with respect to cytotoxic chemotherapeutics.

In this chapter, I have explored whether the presence and differing amounts of breast fibroblasts (NFs or CAFs) have an impact on chemoresistance in TNBC cells. The chemotherapeutic agent that I have used mainly is the anthracycline epirubicin. Epirubicin is routinely used in treatment of TNBC, usually in combination with other chemotherapeutic agents, such as cyclophosphamide, and sometimes in sequence with taxanes. My aim was to provide insights into whether different fibroblast proportions confer changes in chemo-response and therefore, potentially, outcomes after chemotherapy.

3.3 Results

3.3.1 Breast fibroblasts can protect TNBC cell lines from the chemotherapeutic epirubicin

My first aim was to determine whether the presence of normal breast fibroblasts, or breast CAFs was able to influence the immediate sensitivity of TNBC epithelial cells to cytotoxic chemotherapy. Therefore, I developed a short-term, co-culture survival assay where luciferase expressing TNBC cells (MDA-MB-231-GFP/luc or MDA-MB-468-luc cells) were co-cultured with either immortalised breast NFs or CAFs in varying proportions, ranging from a majority of fibroblasts in the culture (seeded as 55% fibroblasts: 45% epithelial cells) through to a small minority of fibroblasts (seeded as 8% fibroblasts) or only epithelial cells (0% fibroblasts), with the same total cell number seeded in each case in order to achieve comparable overall confluencies. Two different breast NF lines were used (NF1 and NF2), while only one CAF line was used (CAF1). Cultures were treated for 24h with the chemotherapeutic epirubicin at 3 different doses approximating to the IC10, IC50 and IC75 doses, which had previously been estimated for each epithelial cell line when treated alone, or were treated with vehicle control (Appendix Figure 1). Survival of epithelial cells only was assessed using luciferase assays and is expressed relative to the untreated cultures of the same fibroblast: epithelial ratio (Figure 3.1). This normalisation method enabled a comparison of epithelial survival following treatment in the cultures of differing ratios of cells, and excluded the differences in actual numbers of epithelial cells plated to create these differing ratios.

Epirubicin dose-dependently reduced survival of the epithelial cells, which was evident at the higher doses of epirubicin, although less consistently for the lower doses. In the case of MDA-MB-231 cells and MDA-MB-468 (Figure 3.1A and 3.1B), the introduction of increasing proportions of breast NFs (NF1 or NF2) or CAFs did not have any signifcant impact on the survival of the epithelial cells, with no significant differences observed between the 0% fibroblast cultures and any other proportion. Although not significant (p values 0.571, 0.881 and 0.956), CAF1 cells appeared to provide some protection to MDA-MB-231 against all

epirubicin doses at the lower proportions of CAF1 cells (4%,8% and 20%). It is also interesting to note that at some doses, the ability of the epithelial cells to survive in the presence of CAF1 cells increased above the level of the matched untreated culture (ie above 1 on the y-axis). Therefore, CAF1 cells appeared to enable an epirubicin-dependent growth stimulatory effect.

Next, I modified this assay to be more sensitive to the toxic effects of epirubicin that might not be evident immediately after such a short treatment with the drug. In this modified assay, following the 24h treatment with epirubicin, drug was removed and cells were cultured for an additional 48h before luciferase measurement (Figure 3.2). My hypothesis was that this extended culture time might allow cells that were severely damaged but still assessed as alive after 24h to break-down fully, and also might allow more time for fibroblast-dependent protection.

As expected, the same doses of epirubicin proved to be more toxic to both epithelial cell lines after this extended incubation (compare the positions on the y-axis in Figure 3.2 to Figure 3.1). More interestingly, significant fibroblast-dependent protection of epithelial cells was seen. This was most striking for MDA-MB-231 cells with CAF1 cells (Figure 3.2A, $p<0.01$), and from CAF1s with MDA-MD-468 cells (Figure 3.2B, $p<0.01$). In the case of CAF1 cells and MDA-MB-231 cells, 55% fibroblasts provided *complete* protection from the effects of the lowest dose of epirubicin.

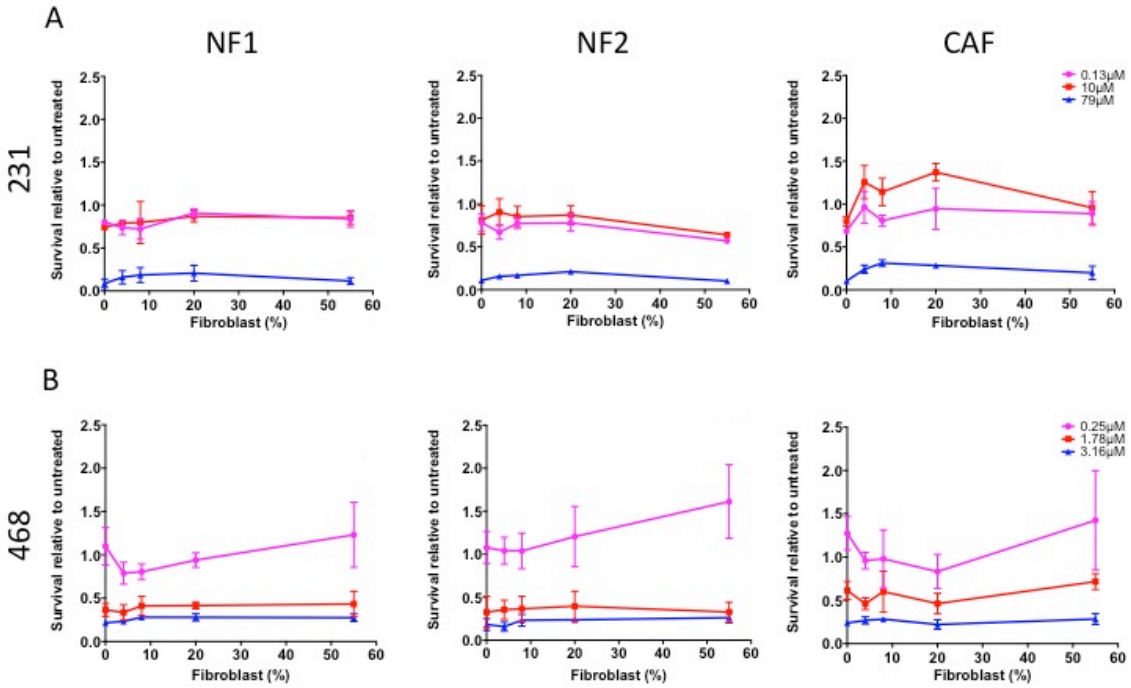


Figure 3.1: CAF1s enhance chemo-survival in MDA-MB-231 cells with various proportions of fibroblasts after 24h epirubicin treatment

MDA-MB-231-GFP/luc (A) or MDA-MB-468-luc (B) luciferase-positive cells were plated in mono-culture, or in co-culture with two alternative immortalised normal breast fibroblast lines (NF1 or NF2) or the immortalised breast CAF line (CAF1) in the proportions shown. Cultures were treated with their respective estimated IC10, 50 and 75 epirubicin doses for 24h. Cells were lysed, and luciferase assays were performed. Values were normalised to untreated cultures of the same fibroblast proportion. Data represent the means (+/- SE) of 3 independent experimental repeats except for MDA-MB-468 with NF (2 replicates only), while each independent repeat itself comprised 3 replicate wells. Linear regression analysis was carried out with significant differences in the overall trend across the fibroblast proportions for the appropriate doses calculated. No significant differences were observed.

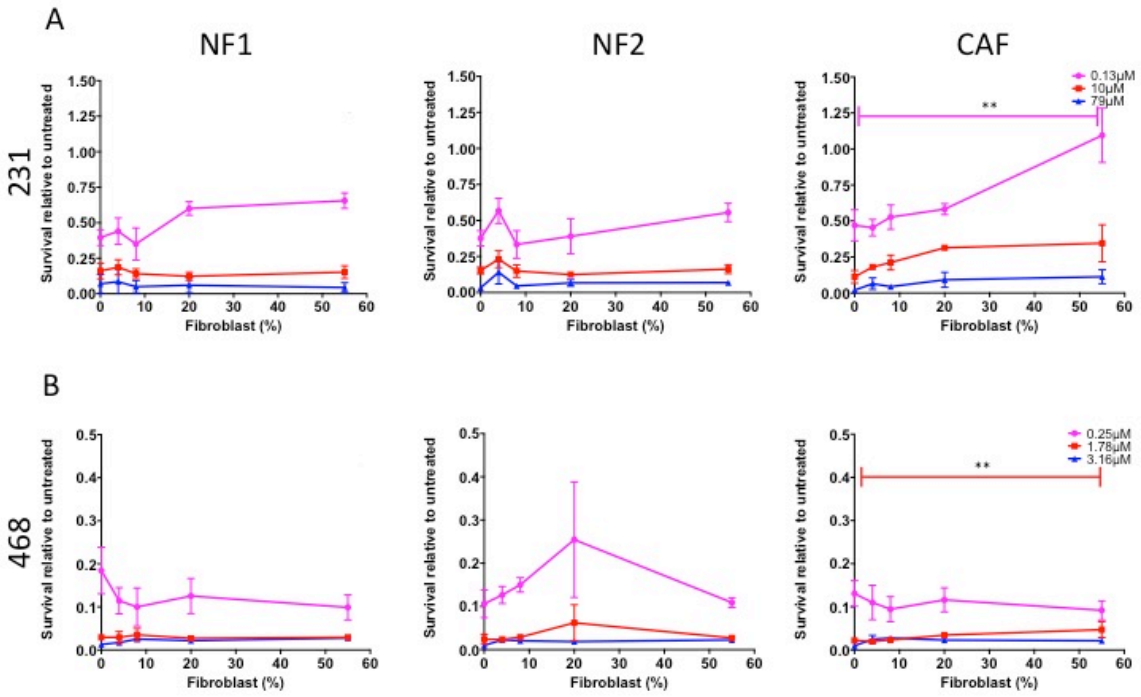


Figure 3.2: CAF1s enhance chemo-survival in MDA-MB-231 cells with various proportions of fibroblasts after 72h epirubicin treatment

MDA-MB-231-GFP/luc (A) or MDA-MB-468-luc (B) luciferase-positive cells were plated in mono-culture, or in co-culture with two alternative immortalised normal breast fibroblast lines (NF1 or NF2) or the immortalised breast CAF line (CAF1) in the proportions shown. Cultures were treated with their respective estimated IC10, 50 and 75 epirubicin doses for 24h. Drug media was then removed and replaced with drug free media and left for a further 48h. Cells were lysed, and luciferase assays were performed. Values were normalised to untreated cultures of the same fibroblast proportion. Data represent the means (+/- SE) of 3 independent experimental repeats except for MDA-MB-468 with NF (2 replicates only), while each independent repeat itself comprised 3 replicate wells. Linear regression analysis was carried out with significant differences in the overall trend across the fibroblast proportions for the appropriate doses calculated. P values ** <0.01.

3.3.2 Immortalised CAF1s protect claudin-low TNBC cells (MDA-MB-157 and MDA-MB-231), but not claudin-high MDA-MB-468 cells from epirubicin

Having identified that breast fibroblasts can enhance the survival of TNBC cells using a short-term assay, my next aim was to extend this observation to examine the influence of fibroblasts on epithelial survival using an alternative assay. This was done using clonogenic survival assays, which are more sensitive to lesser degrees of chemotherapy-induced damage, as for cells to count as “having survived” they must be capable of repeated cell divisions.

A similar principle was used as previously for the short-term chemo-survival assays, whereby triple negative cancer cell lines (MDA-MB-231-GFP/luc or MDA-MB-468-GFP) were cultured alone or with increasing proportions of NF1 or CAF1 cells. Cultures were established, and epirubicin or vehicle control was added. GFP-positive epithelial cells were separated from fibroblasts using fluorescence activated cell sorting (FACS) and re-plated to assess clonogenic potential. Cultures without fibroblasts (i.e. 0% fibroblast / 100% epithelial) were also sorted to control for any FACS-induced technical artifacts and to allow accurate comparisons. 500 cells were plated, cultured for 2 weeks, fixed and stained before individual colonies were counted (Figure 3.3). Data are expressed as numbers of colonies (Figure 3.3A and B “Colony counts”), and as colony numbers after epirubicin treatment *relative* to matched cultures that were not treated with epirubicin (Figure 3.3 A and B “Relative to no chemo”), to allow focus on the influence of fibroblasts on epithelial survival after chemotherapy treatment.

Firstly, an unexpected observation was noted in the colony number data in the absence of epirubicin treatment, which was not the initial aim of this set of experiments. Although not significant (p value 0.087), clonogenic plating efficiency of MDA-MB-231 cells was improved as the proportion of CAF1 cells increased, while - interestingly - NF1 cells significantly decreased clonogenicity of MDA-MB-231 cells (Fig 3.3A; ‘colony counts’ compare pink and orange bars $p<0.05$). CAF1 cells did not confer this increased clonogenic ability on MDA-MB-468 cells (Figure 3.3B ‘colony counts’).

Next, as expected, epirubicin reduced clonogenic survival by between 48 and 52% in both MDA-MB-231 and MDA-MB-468 cells in the absence of fibroblasts (Fig 3.3A and 3.3B 'relative to no chemo', 0% bars: note position on y axis). CAF1 cells, but not NF1 cells, protected MDA-MB-231s from this epirubicin-mediated impairment to colony formation in a dose-dependent manner (Fig 3.3A purple bars; $p<0.05$). However, CAFs did not exert protection on MDA-MB-468 cells (Figure 3.3B, dark blue bars). At the highest proportion of CAF1s (55%), MDA-MB-231 cells were very substantially protected from epirubicin; survival was 83% relative to the MDA-MB-231 cells cultured with 55% CAF1s that were not treated with epirubicin.

It is important to note that although the MDA-MB-231 and MDA-MB-468 cell lines are both classed as triple negative cell lines, they can be sub-classified into claudin-low (MDA-MB-231) and claudin high (MDA-MB-468) (Ricardo et al, 2012, Dias et al, 2017), and these sub-classes may respond and act differently (see section 1.4.2 for an introduction to sub-classes of TNBCs). In order to examine whether the effect of CAF1s on the MDA-MB-231 cells was potentially related to the claudin-low phenotype, the experiment was repeated with a second claudin-low TNBC cell line, MDA-MB-157. Some methodological changes were required as GFP-positive MDA-MB-157 cells were not available. Therefore, GFP-negative MDA-MB-157 cells were used with GFP-positive fibroblasts, with FACS again used to separate the cell types. GFP-positive CAF1s, but not NFs, were available therefore this experiment was performed only with GFP-CAF1. Otherwise, the experiment was performed and data were expressed as previously (Figure 3.4A).

As previously, increasing proportions of CAF1s enhanced the clonogenic plating efficiency of the cancer cells in the absence of epirubicin, however this was not found to be statistically significant (compare light green bars in Figure 3.4 A "Colony counts", p value 0.337). The findings in MDA-MB-157 cells with respect to chemotherapy response mirrored those found with MDA-MB-231 cells. Epirubicin treatment reduced clonogenic survival in the purely epithelial culture, 0% fibroblasts, to 32% compared to untreated. As CAF1s were

introduced, MDA-MB-157 survival increased but the overall trend in survival was deemed not significant by linear regression (p value 0.242). However comparison of survival of 0% vs 55% cultures, using Mann-Whitney, was significant in isolation ($p<0.05$) (Figure 3.4 A "Relative to no chemo", compare across the dark green bars).

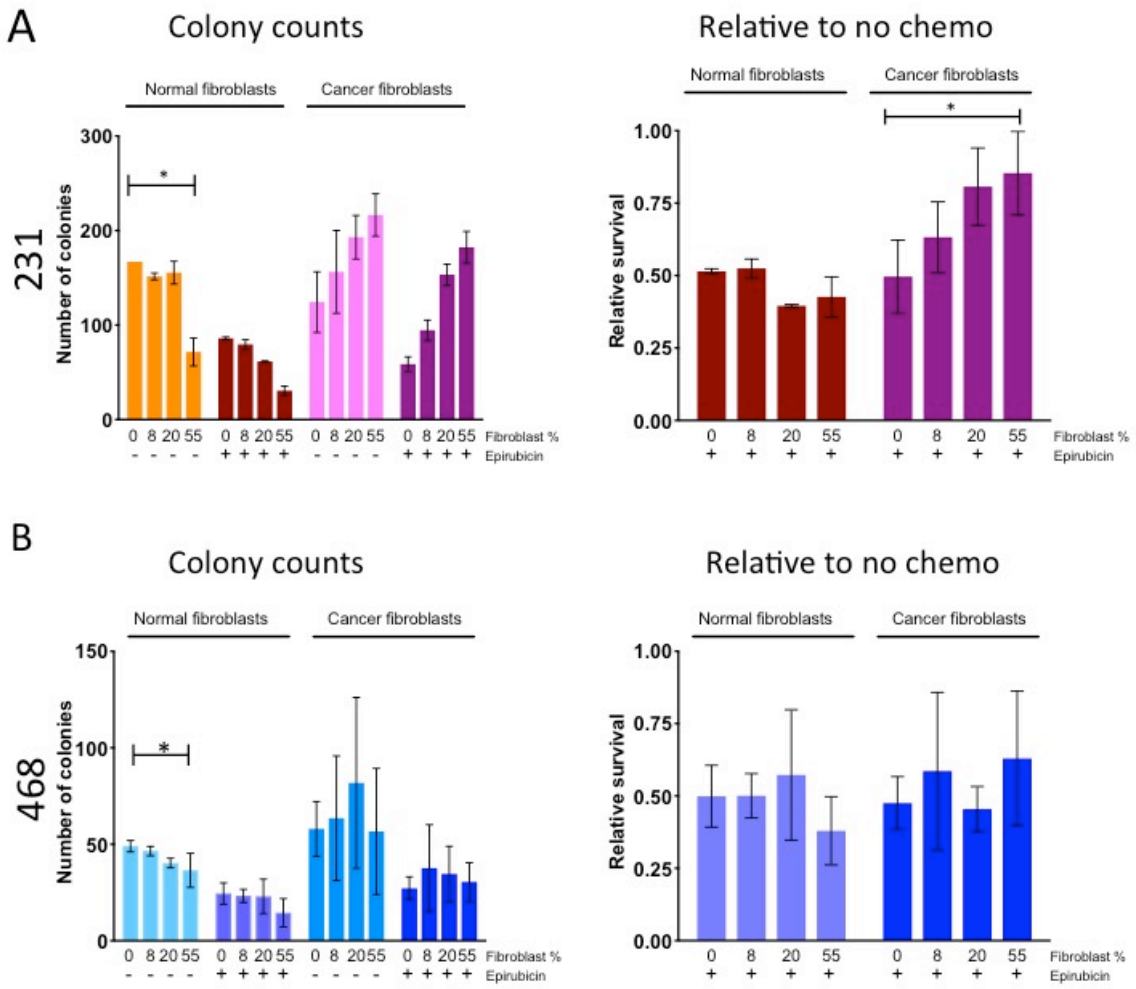


Figure 3.3: CAF1s, but not NF1s, protect MDA-MB-231 cells, but not MDA-MB-468 cells, from epirubicin

Cultures of MDA-MB-231-GFP/luc or -468-GFP cells were established with various proportions of either immortalised normal breast fibroblasts (NF1) or immortalised breast CAFs (CAF1). Epithelial cells had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Cultures were treated with 10nM epirubicin or untreated for 24h. Cultures were then treated by FACS to prepare pure epithelial populations. 500 epithelial cells were seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are normalised to the no treatment value for each individual fibroblast proportion and represent the means (+/- SD) of 3 independent experimental repeats. Linear regression analysis was carried out on no treatment for colony counts and normalised treated data with significant differences in the overall trend across the fibroblast proportions calculated. P values * <0.05

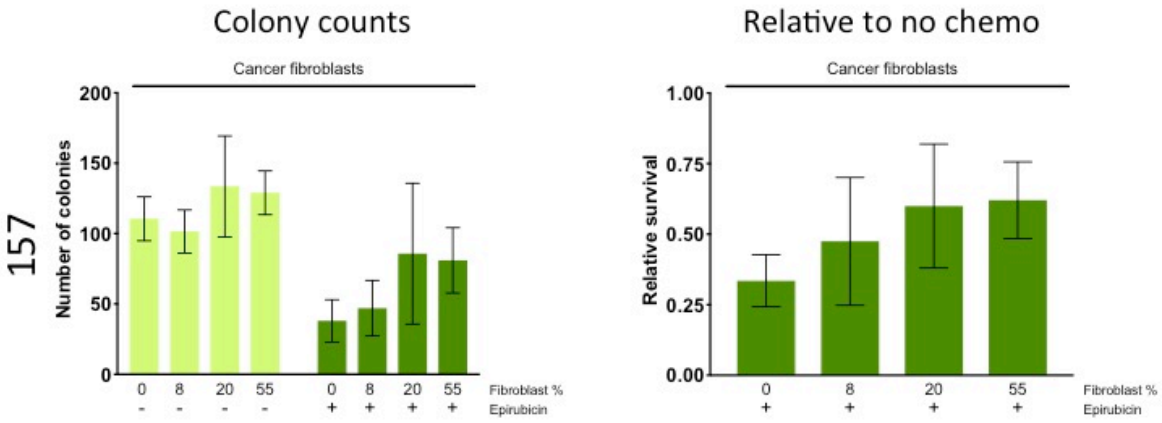


Figure 3.4: CAF1s protect MDA-MB-157 cells from epirubicin
Cultures of MDA-MB-157 cells were established with various proportions of GFP positive immortalised breast CAFs (CAF1-GFP). Fibroblasts had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Cultures were treated with 25nM epirubicin or untreated for 24h. Cultures were then treated by FACS to prepare pure epithelial populations. 500 epithelial cells were seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are normalised to the no treatment value for each individual fibroblast proportion and represent the means (+/- SD) of 3 independent experimental repeats. Linear regression analysis was carried out on no treatment for colony counts and normalised treated data with significant differences in the overall trend across the fibroblast proportions calculated. No significant differences were observed.

3.3.3 Primary breast CAFs, but not primary NFs, protect the claudin-low TNBC cell line MDA-MB-231 from epirubicin

To assess further whether CAF-induced protection from epirubicin was generalizable, I next tested whether primary breast fibroblasts could enable protection from epirubicin. A matched pair of primary NFs (pNF) and primary breast CAF (pCAF) cultures was obtained: NFs were extracted from a breast cancer resection specimen at least 1cm outside the tumour margin, and CAFs were taken from within the tumour mass itself. The influence of this matched pair of primary fibroblasts on clonogenic survival after epirubicin treatment was assessed in MDA-MB-231 cells exactly as previously. MDA-MB-231-GFP/luc cells were used, to allow fluorescent cell sorting, while the experiment was not performed with MDA-MB-157 cells as neither GFP-positive primary fibroblasts, nor GFP-positive MDA-MB-157 cells were available.

In the absence of epirubicin treatment, fibroblasts again induced a dose-dependent increase in clonogenic plating efficiency (Figure 3.5A 'colony counts', bright orange and pink bars). This was evident both with pNFs and with pCAFs, although the pCAFs achieved near maximal increases in survival at a lower percentage (compare 8% bars), suggesting their influence was stronger. After normalization to allow focus on responses to epirubicin, all proportions of pCAFs increased protection of MDA-MB-231 cells, but pNFs did not (Figure 3.5 'relative to no chemo').

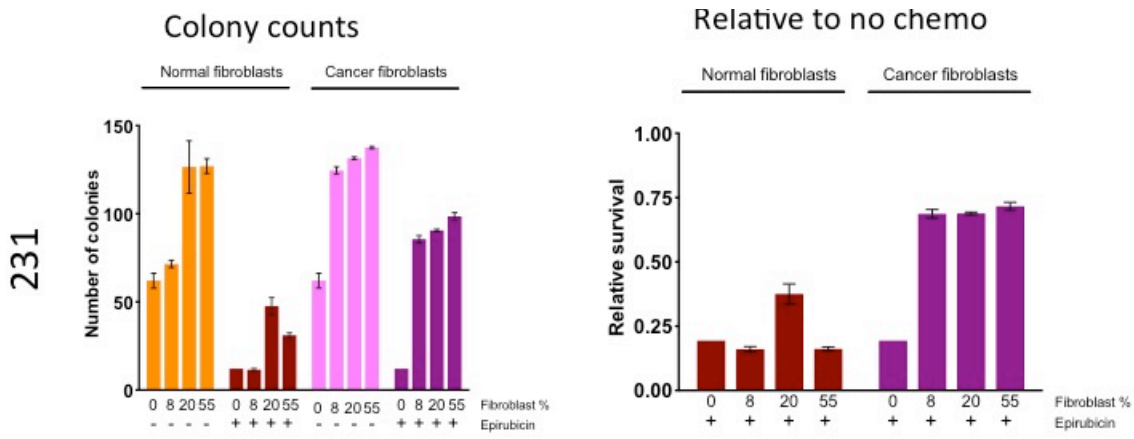


Figure 3.5: pCAFs, but not matched pNFs, protect MDA-MB-231 cells from epirubicin

Cultures of MDA-MB-231-GFP/luc cells were established with various proportions of either primary normal breast fibroblasts (pNF) or primary breast CAFs (pCAF). Epithelial cells had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Cultures were treated with 10nM epirubicin or untreated for 24h. Cultures were then treated by FACS to prepare pure epithelial populations. 500 epithelial cells were seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are normalised to the no treatment value for each individual fibroblast proportion and represent the means (+/- SD) of 1 independent experimental repeat.

3.3.4 Immortalised CAF1 cells protect MDA-MB-231s from an alternative chemotherapeutic, docetaxel

Next, I wanted to investigate if CAFs could also confer protection from another, functionally-unrelated, chemotherapeutic. I chose to examine the taxane, docetaxel, which is – like epirubicin – routinely used in treatment of TNBC. The same clonogenic survival methodology was used as above, with only MDA-MB-231 and immortalized CAF1s as the cells under test (Figure 3.6), since these had previously demonstrated strong CAF-dependent protection from epirubicin.

Docetaxel treatment reduced clonogenic survival to 22% in the 0% fibroblasts MDA-MB-231 cultures ("Relative to no chemo" plot, Figure 3.6), while the presence of CAF1 cells dose-dependently increased survival ($p<0.05$) after docetaxel treatment to a maximum of 61% survival with the highest proportion of CAF1 cells relative to the untreated.

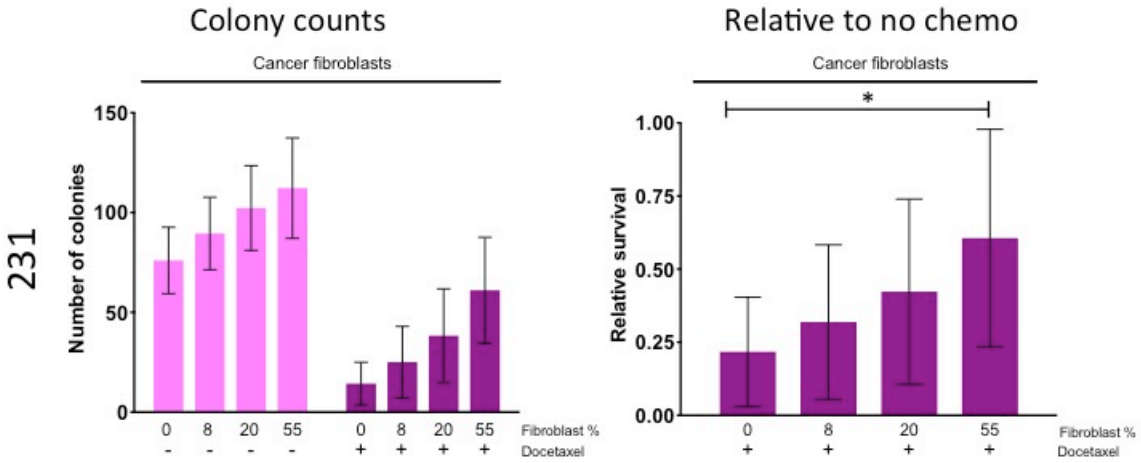


Figure 3.6 CAF1s protect MDA-MB-231 cells from docetaxel
Cultures of MDA-MB-231-GFP/luc cells were established with various proportions of immortalised breast CAFs (CAF1). Epithelial cells had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Cultures were treated with 350pM docetaxel or untreated for 24h. Cultures were then treated by FACS to prepare pure epithelial populations. 500 epithelial cells were seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are normalised to the no treatment value for each individual fibroblast proportion and represent the means (+/- SD) of 3 independent experimental repeats. Linear regression analysis was carried out on no treatment for colony counts and normalised treated data with significant differences in the overall trend across the fibroblast proportions calculated. P values * <0.05

3.3.5 Fibroblast-dependent protection of claudin-low TNBC cell lines MDA-MB-157 and MDA-MB-231 is not reproduced by fibroblast conditioned medium

Next, I wanted to investigate mechanisms by which CAFs were able to protect selected epithelial breast cancer lines from chemotherapy. My first hypothesis was that the fibroblasts release a specific secreted factor(s) from CAFs, but not from NFs, that acts on the target epithelial cells. In order to test this, I collected conditioned medium from fibroblast mono-cultures (either NFs, NF1, or CAFs, CAF1) and assessed its ability to stimulate chemoresistance in epithelial cultures.

Clonogenic survival assays were again performed using either MDA-MB-231, MDA-MB-157 or MDA-MB-468 cells. In these experiments, mono-cultures of these epithelial cells were treated with conditioned media taken from fibroblast cultures to simulate the presence of fibroblasts (labelled “M” on the Figures), or were left untreated to simulate the 0% fibroblast cultures. A further layer of complexity was also added to the experiment to take account of the fact that it was possible that fibroblasts were only induced to release the relevant secreted factor(s) by epirubicin treatment itself. Therefore, conditioned medium was also collected from fibroblast mono-cultures that had been pre-treated with epirubicin for 24h, and had then been swapped into epirubicin-free medium for conditioning of this fresh medium to take place; I have referred to this as “induced” conditioned medium (“iM").

In all cell lines used, MDA-MB-231, MDA-MB-157 and MDA-MB-468 no significant differences were observed on treatment with either NF1 or CAF1 conditioned media in either clonogenic plating efficiency (Figure 3.7 A, B, C left panel) or survival after epirubicin treatment (Figure 3.7 A, B, C right panel).

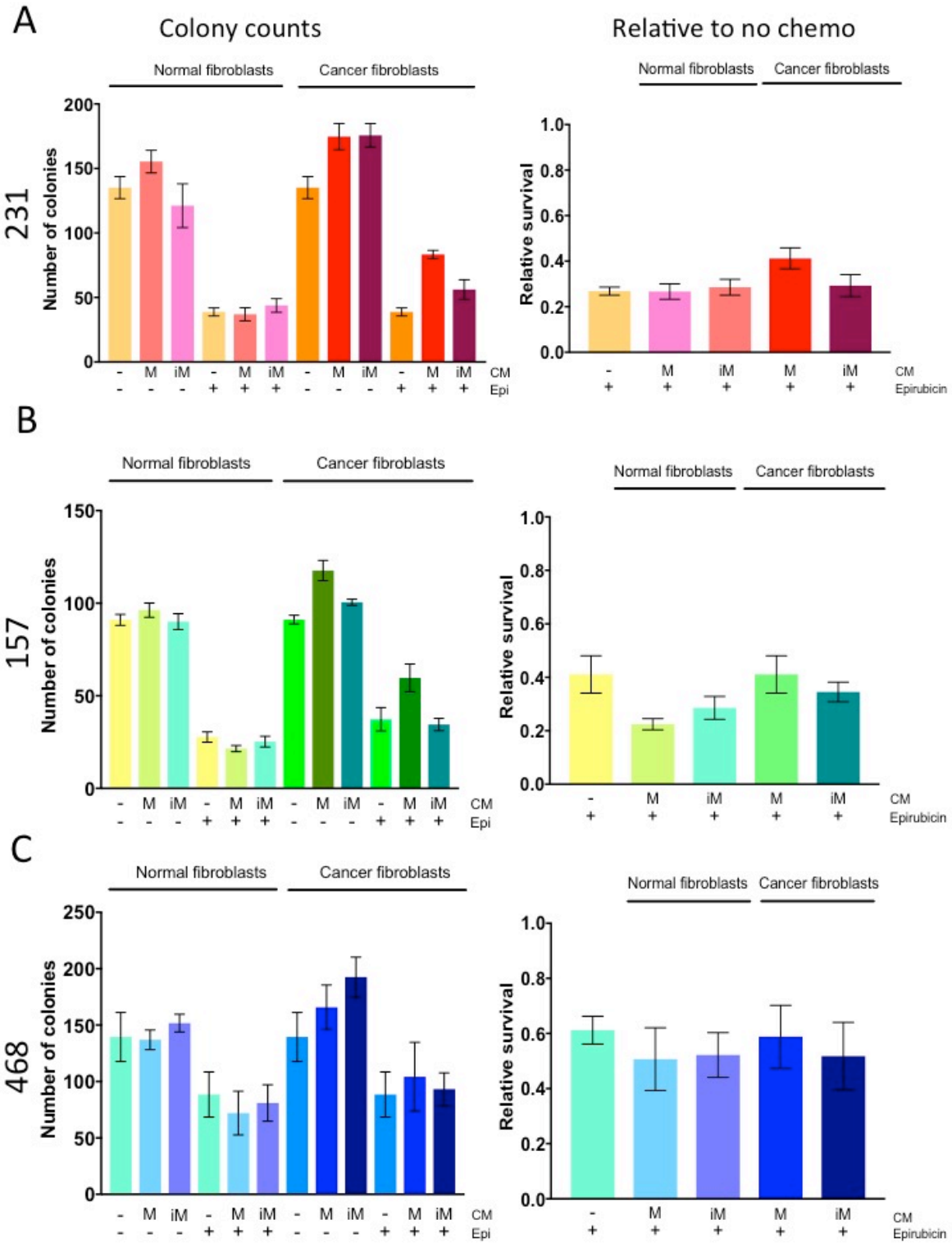


Figure 3.7 Fibroblast conditioned media does not protect MDA-MB-231, -468 and -157 cells when treated with epirubicin

Cultures of MDA-MB-231-GFP/luc, 468-GFP and -157 cells were established in media containing 50% fibroblast conditioned medium that had been taken from NF1 or CAF1 after 24h of culture (either without, “M”, or after pre-treatment of fibroblasts with epirubicin, “iM”). Epithelial cultures were then treated with 10nM epirubicin, with conditioned media replenished for 24h. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted (left panel). Data were also normalised to the no epirubicin treatment values for each

conditioned medium treatment. Data represent the means (+/- SE) of 3 (-231) or 2 (-468 and -157) independent experimental repeat. Two-tailed Mann-Whitney U tests were carried out to no conditioned with no significant differences observed.

3.4 Discussion

The aim of the work in this chapter was to identify whether the presence of fibroblasts (NFs or CAFs) had an impact on chemo-response of TNBC cells, and to determine if any impact was mediated by a secreted factor potentially contained within conditioned medium.

3.4.1 Methodology for assessing chemo-response

In order to study chemo-response *in vitro*, the ability to measure cancer cell survival following chemotherapeutic treatment was required. Over the years, various techniques to determine cell survival have been developed to assess not only chemotherapy response but also targeted drug and radiation responses (Menyhart et al, 2016). However, the particular circumstances of the research question I aimed to answer – ie the influence of one cell type on another in co-culture, defined that I needed to use slightly adapted assays.

The most common and well-established assay for assessing chemo-response *in vitro* are MTT assays. Importantly, MTTs can measure drug sensitivity in multiple different cancer cell lines (Alley et al, 1988). MTT assays are commonly used as they are cheap, convenient, quick and reliable at assessing survival (Kratze et al, 1996). Although use of MTT assays would enable assessment of chemo-response, MTT assays could not be used in the work described here for this purpose as co-culture experiments were carried out and the aim was to assess response of only the breast cancer cell lines through the background of differing amounts of fibroblasts.

Therefore, in order to assess survival of purely the epithelial cells in co-culture instead of the MTT readout, stable luciferase expression in the epithelial cells was exploited as a readout method. While uncommon *in vitro*, this methodology is commonly used for assessment of cell numbers, or cell survival after

treatments using *in vivo* models. Luciferase positive human breast cells have frequently been used in monitoring tumour development in localised and disseminated disease through bioluminescent imaging using IVIS imaging systems (Kalra et al, 2011). Cellular luciferase expression has also been used to quantify total cell numbers in novel *in vitro* 3D tissue engineered breast cancer models (Goliwas et al, 2017). Therefore, the use of luciferase as a read out was a viable option for assessment of purely epithelial cells in a mixed co-culture of cells.

However, short-term assays commonly only last a few days due to cultures becoming confluent, therefore the use of a longer-term assay was also appropriate to determine the cellular fate of the cells in terms of potentially continuing to proliferate; therefore, I also used colony formation/clonogenic assays. Clonogenic assays are based on the ability of single cells to divide and continue to grow into individual colonies. Clonogenic assay show the reproductive ability following cytotoxic treatment and, therefore, the effectiveness of the treatment (Crowley et al, 2016). Clonogenic assays are deemed more representative of what occurs clinically; firstly, the ability of a tumour to respond to treatment and secondly the potential of relapse and formation distant metastases from the primary tumour (Franken et al, 2006).

Interestingly, and in support of my preferred use of clonogenic assays here, responses of breast tumours to chemotherapy have been successfully determined from patient samples using clonogenic assays (Jones et al, 1985). Among breast cancer patients who were deemed *in vitro* sensitive to treatment, 59% of patients responded clinically to chemotherapeutics, with resistance correctly predicted in 100% patients who were *in vitro* resistant to treatment. Thus clonogenic assays were found to reproduce clinical findings with respect to chemo-response impressively effectively (Jones et al, 1985). The clinical relevance of the long-term clonogenic assays in addition to the ability to assess response, cellular fate and cell regrowth following treatment were key features in choosing clonogenic assays for use in this chapter to assess chemoresistance.

For my clonogenic experiments, I needed to sort the epithelial cancer cells, the cells of interest in terms of chemo-response, away from the co-cultured fibroblasts, which I achieved using FACS. As far as I am aware, this combination of co-cultures, FACS, and clonogenic assays have not previously been published. The only related assay I have found is where breast cancer cells were seeded for clonogenic assays onto monolayers of fibroblasts (Samoszuk et al, 2005), which support their subsequent proliferation, but their initial response to chemotherapeutics was not assessed.

One of the difficulties for both of the assays I performed was deciding how best to show the treatment effects. In particular, since different numbers of epithelial cells were used in each condition under test, it was necessary to avoid this confounding experimental artifact to allow focus on the influence of the CAFs on epithelial survival. In many cases, in both short-term assays and long-term clonogenic assays, data are routinely normalised to the no treatment sample rather than using absolute values (Maycotte et al, 2012). In the case of my experiments, separate no treatment data were available for each individual fibroblast proportion under test, which led to more complex normalisation of each individual experimental value to its matched no treatment sample. The value of this method of normalisation was particularly evident in the clonogenic assays, where the increasing proportions of CAFs had an impact on the clonogenic plating efficiency of the cells in the absence of chemotherapy agents (visible in the colony counts), but also additionally on chemo-response (visible in the colony counts, but more clearly in data that were normalised relative to untreated samples).

3.4.2 Conditioned media clonogenic assays – a soluble mediator of cross-talk between CAFs and cancer cells?

Conditioned medium has frequently been used experimentally to show how secretory factors can impact on growth (Cullen et al, 1989), migration and invasion (Walter et al, 2009) of breast cancer cells. Therefore, I attempted to use the same approach to identify if CAFs were secreting a soluble factor that was driving the chemoresistance I had seen in the TNBC cells (section 3.2.5).

Previously, conditioned media from CAFs has been shown to increase migration and invasion of MDA-MB-231 cells significantly, through the chemokine CXCL12 (Dvorak et al, 2018). CAFs have also been shown to induce phenotypic transformation of breast cancer cells through use of CAF conditioned media on MCF-7 cells (Wang et al, 2018). Conditioned media from NFs and CAFs have also been shown to increase growth in SKBR-3, T47D and MDA-MB-468 cells using MTT assays (Merlino et al, 2017). It is therefore evident that CAFs could impact on breast cancer progression through conditioned media. However, here in this chapter CAF conditioned media had no significant impact on chemoresistance in MDA-MB-231, -157 or -468 TNBC cells (Figure 3.7).

The simplest interpretation of these results is that a secreted factor is not responsible for the CAF-induced chemoresistance in section 3.3.2, however there is a risk that the result is a false negative. It is possible that the secretory factor is a labile factor that is not well maintained within the conditioned medium, or that the concentration of the secretory factor in the conditioned media is at a low concentration. In order to minimise the risk of these false negatives, I ensured that my conditioned medium was prepared fresh for every experiment (rather than being stored), and I reduced the medium volume on the conditioning cells in an effort to concentrate any secreted factors. A further possibility is that the CAFs may need to be induced to produce the proposed secretory factor, perhaps by the epirubicin treatment itself, or by the epithelial cells, representing bi-directional cross-talk. With respect to the hypothesis that the epirubicin treatment could induce the CAFs to start secreting the relevant factor(s), there are published precedents for this. Treatment induced changes in CAF secretory molecules and subsequent effects on cancer cells have been shown in colorectal cancer, where chemotherapy activated CAFs to increase secretion of IL-17A that in turn led to remodelling of the tumour microenvironment to self-renew colorectal cancer-initiating cells (Lotti et al., 2013). In breast cancer, chemotherapy has been shown to induce CAFs to secrete IL-6 inducing a pro-inflammatory microenvironment, which subsequently activated interferon-mediated signaling as well as stemness

pathways (Sonic Hedgehog signaling) in breast cancer cells (Peiris-Pagès et al, 2015). In addition, chemotherapy induced damage of the microenvironment and CAFs in breast cancer led to increased levels of WNT16B, which drove breast cancer resistance to docetaxel / mitoxantrone combination therapy (Sun et al, 2012). To take into account the potential influence of epirubicin directly on the CAFs themselves, in my experiments (section 3.2.5) I also pre-incubated CAFs with epirubicin and collected "induced" conditioned medium following this treatment. Interestingly induced CAF induced conditioned media didn't behave significantly differently from the non-induced conditioned media, suggesting that epirubicin treatment alone did not induce CAFs to secrete a relevant factor. However, making induced conditioned media was slightly problematic as it was essential to pre-treat CAFs with epirubicin, then wash out epirubicin and collect induced conditioned media following this treatment, not during the treatment. If media was collected during epirubicin treatment, induced conditioned media could have added an additional substantial dose of epirubicin to cells and led to further cell death. However, it is possible sustained chemotherapy treatment is required to induce CAFs correctly but by collecting media following treatment, this could have been missed.

Therefore, because of these unavoidable experimental weaknesses with the design of the conditioned medium experiments undertaken, it is not possible to rule out a role for a simple secreted factor. Nevetheless, conditioned media data also supports the model of a more complex interaction between the CAFs and epithelial cells, where bi-directional signaling or physical contact between the cell types is required.

3.4.3 CAFs induce enhanced clonogenicity

One unexpected finding, which was not an initial aim of my experiments, was the discovery that CAFs, but not NFs, induced increased clonogenic survival in cancer cells in the absence of chemotherapy treatment. This effect was often normalised away to allow focus on chemo-response (for example, in Figure 3.3 compare the "Relative to no chemo" and the "Colony counts" plots). However, it is worthy of consideration in its own right. During these clonogenic assays, the

cells are detached from their substrate and are “rounded up”; cells remain suspended in solution until they are re-plated and are able to reattach. This can be a traumatic insult to many cell types – particularly epithelial cells – and can induce apoptotic cell death, termed anoikis in this context (Ishikawa et al, 2015). One interpretation of my observation regarding CAF-induced increased clonogenicity is that CAFs had induced anoikis-resistance in the co-cultured epithelial cancer cells. In terms of cancer biology, resistance to this type of induced apoptosis is of great interest, as some resistance is required to survive metastatic dissemination via blood or lymph (Cao et al, 2017). Therefore, an interesting hypothesis that could follow on would be that epithelial cancer cells from fibroblast-rich tumours might survive longer in the blood and could therefore be more metastatic. The potential role of CAFs in modifying anoikis in epithelial cells is explored experimentally in Chapter 6.

3.5 Conclusions and aspects of future work

I have demonstrated that CAFs, but not NFs, are capable of inducing enhanced clonogenic survival, and relative chemoresistance in selected TNBC cell lines. The protection from chemotherapy is not reproduced by conditioned medium taken from fibroblasts, indicating that either the relevant secreted factors are too labile or low in concentration to be transferred in this context, or that physical contact or bi-directional signaling is required between the two cell types. In the next chapter, I have aimed to explore the potential mechanisms of increased chemoresistance in CAF-protected epithelial cells, using transcriptomic approaches, with a view to identifying the signaling mechanism between the cell types and/or the effectors of the resistance itself.

Chapter 4- Cancer-associated fibroblasts induce up-regulation of interferon signaling in breast epithelial cancer cells, leading to chemoresistance

4.1 Abstract

CAF-dependent mechanisms of chemoresistance are poorly understood, with no firm consensus on the signaling pathways involved. Identifying potential signaling pathways driving chemoresistance in TNBC could pave the way for exploitation of these pathways to improve chemotherapy responses. In the previous chapter, I demonstrated that breast CAFs, but not NFs, can induce chemoresistrance in the TNBC cell lines MDA-MB-231 and MDA-MB-157.

I next used array technologies to investigate the changes in the transcriptome of MDA-MB-231 cells induced by the presence of CAFs during treatment with the chemotherapeutic epirubicin. A signature of up-regulation of interferon signaling was observed, as reflected in the up-regulation of multiple components and targets of the pathway, including MX1, OAS1 and miR-155. CAFs, but not NFs, were capable of inducing interferon signaling in MDA-MB-231 cells. Also, CAF-induced up-regulation of interferon signaling in breast cancer epithelial cells only occurred in the cell lines that were protected from chemotherapy by CAFs (MDA-MB-231 and MDA-MB-157 cells), but not in a line in which protection was not induced (MDA-MB-468 cells). In the absence of fibroblasts, recombinant interferons were sufficient to induce chemoresistance in MDA-MB-231 and MDA-MB-157 cells, but not in MDA-MB-468 cells. The expression profile of CAFs when co-cultured with breast cancer epithelial cells and treated with epirubicin implicated interferon-β as the activator of the pathway.

I concluded that identification of interferon signaling, as a key driver of CAF-induced chemoresistance in TNBC, could provide future scope for development of treatments to improve patient responses to chemotherapy.

4.2 Introduction

In Chapter 3, I showed that CAFs can stimulate chemoresistance in MDA-MB-231 and MDA-MB-157 TNBC cells, but not in the MDA-MB-468 TNBC cell line. My next interest was to try to understand how CAFs enable this chemoresistance.

Various potential mechanisms of chemoresistance have been identified. Of particular interests is the fact that stromal components have previously been implicated in chemoresistance, since this is similar to my observation of stromal fibroblasts inducing chemoresistance. The published data, however, appear to support chemoresistance through a physical barrier to chemotherapy penetration of the tumour that is enabled by production of collagen and increased interstitial pressure as well as increased ECM stiffness (Netti et al, 2000). Type I collagen production, that is commonly produced by fibroblasts, can also directly induce chemoresistance in cancer cells through integrin signaling (Armstrong et al, 2004). It is not obvious that these could be the mechanisms occurring in the context of my experiments, as it seems unlikely that CAFs could provide a physical barrier to drug delivery, or secrete substantial ECM proteins in standard two-dimentional co-culture on plastic.

It is also possible to speculate about other potential mechanisms by which CAFs could directly impact on the chemotherapy dose reaching the epithelial cells. For example, it is plausible that fibroblasts themselves could be responsible for inactivation of the chemotherapy agents. The majority of drug inactivation is driven by phase I and/or phase II enzymes, which are present in the liver, intestines and tumour cells (Pan et al, 2015). An important phase I group of enzymes is the human cytochrome P450s (CYPs), and these have been seen to detoxify anticancer drugs (Fujita, 2006). CYP3A4 is responsible for metabolizing targeted breast cancer treatments such as tamoxifen as well as

chemotherapeutic agents like taxanes (Miyoshi et al, 2002). Mutations in CYPs have also been shown to be associated with poor responses to treatment in breast cancer. Changes in CYP2B6 and CYP2D6 have lead to poor responses to cyclophosphamide chemotherapy and tamoxifen respectively (Patel, 2015). A phase II set of enzymes, glutathione s-transferases (GSTs) are responsible for metabolizing a large number of chemotherapeutic drugs including cisplatin and cyclophosphamide, which can be used in breast cancer treatment (Sau et al, 2010, Allocati et al, 2018). However, data implicating CAFs in direct inactivation of chemotherapy agents are lacking.

I have taken the view that it may be more likely that CAFs induce changes in gene expression within the epithelial cells, perhaps by paracrine signaling, that allow the epithelial cells themselves to become more resistant. A number of activated signaling pathways within epithelial cells have been associated with chemoresistance, such as the EGFR pathway (Kuroda et al, 2010), which in turn can activate the MAPK (Zhang et al, 2014) and PI3K/AKT pathways (Page et al, 2000), or the NF-κB signaling pathway (Weldon et al, 2001). However, activation of these in breast cancer cells has not been attributed to the presence of CAFs, therefore these do not appear to present likely candidate pathways in the context of my findings. Various effectors of chemoresistance, such as xenobiotic drug pumps or enzymes capable of inactivating chemotherapy agents (Zheng et al, 2017), are relatively well established within the literature, but there is little evidence that the presence of CAFs can lead to up-regulation of their expression or activity leading to chemoresistance.

In the absence of clear and definitive candidate pathways that have been shown to enable CAFs to induce chemoresistance, I chose to examine changes in the gene expression profile that CAFs induce in co-cultured breast epithelial cells in the hope that these would provide clues about the key mechanisms of CAF-induced chemoresistance in this system. I focused on both mRNA and miRNA expression changes in order to gain a comprehensive insight, and have tested the functional role in chemoresistance of candidates identified from these screens.

4.3 Results

4.3.1 CAF1s induce changes to the mRNA transcriptome of MDA-MB-231 cells during epirubicin treatment

My first aim was to identify the gene expression changes in TNBC cells induced by the presence of CAFs that could be responsible for CAF-induced chemoresistance.

To achieve this, MDA-MB-231-GFP/luc breast cancer cells were cultured on their own (0% fibroblasts) or were co-cultured with CAF1 cells (seeding the fibroblasts as 20% of the initial culture) exactly as previously described (section 3.3.2). The 20% CAF proportion was chosen, as opposed to 8% or 55%, as this induced strong and reproducible chemoresistance in the epithelial cells and was most representative of the proportion of CAFs commonly seen in breast tumours (unpublished data from the Hughes group). Cultures were treated with epirubicin for 24h, and the epithelial cancer cells were isolated by FACS (on the basis that MDA-MB-231-GFP/luc cells expressed GFP) from both the epithelial alone (0% fibroblasts) and 20% fibroblast cultures as previously. RNA was then extracted from the MDA-MB-231-GFP/luc cells. This entire experiment was performed three times over three separate weeks (importantly, not simply using three separate concurrent parallel cultures) to provide robust biologically separate repeats. Expression profiling was performed on the three pairs of samples using Affymetrix Clariom D microarrays, and data were normalized to array medians.

Following successful completion of the Affymetrix clariom D microarray analyses, supervised hierarchical clustering was performed to determine what differences there were between epithelial expression in the 0% and the 20% cultures (Figure 4.1). It was clear that between the 0% and 20% groups there were differing expression levels for many genes with triplicates within each group also similar, thereby allowing robust comparison between the groups.

Expression data from the 3 samples for 0% or 20% cultures were grouped and paired ANOVA tests were performed to identify significant changes in gene expression of either up- or down-regulation by at least 2-fold. The numbers of genes showing such changes are summarized in Table 4.1, and their names and fold-changes are listed in appendix Table 1.

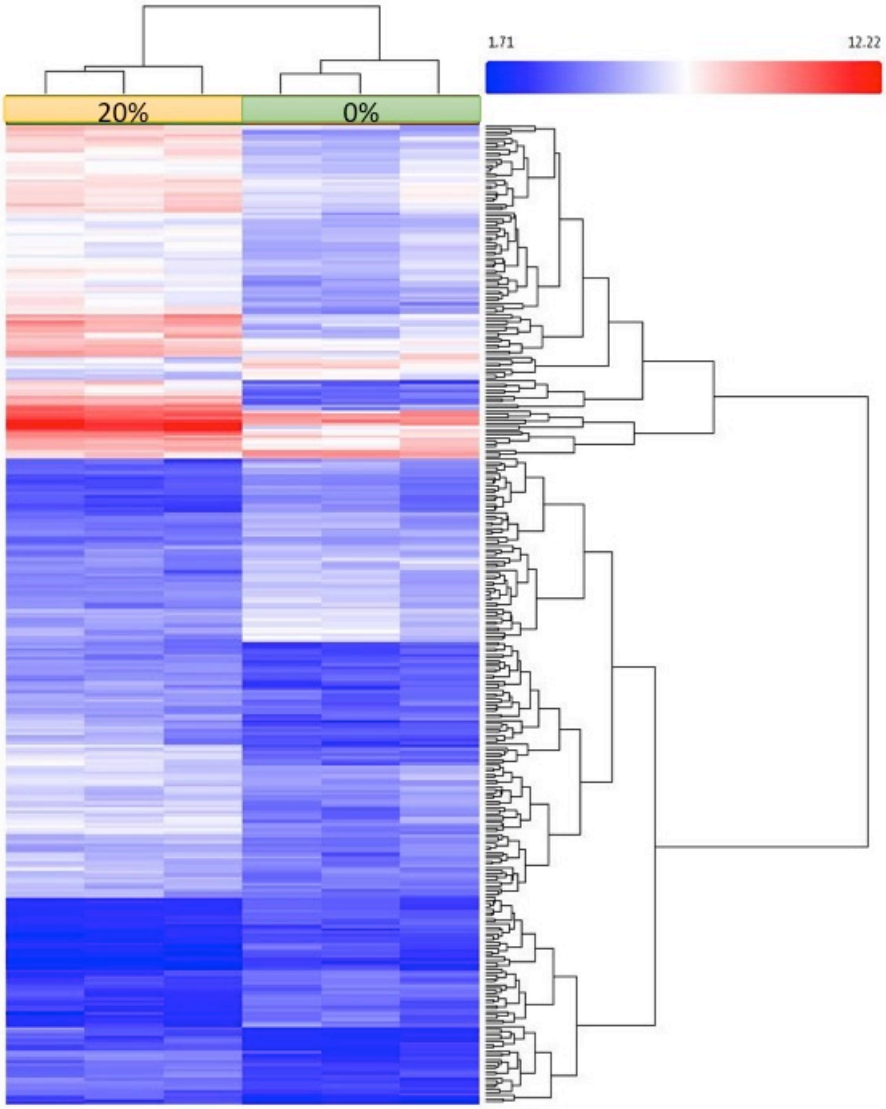


Figure 4.1: Supervised hierarchical clustering of expression profiles from biological triplicate samples of MDA-MB-231 cells when in culture alone (0%) or with CAF1 cells (20%) following epirubicin treatment

MDA-MB-231-GFP/luc cells were cultured alone (0%) or with CAF1 cells (20%) and were treated with 10nM epirubicin. MDA-MB-231-GFP/luc cells were purified by FACS on the basis of positive GFP expression and RNA was prepared. Three separate biological repeats were performed giving three pairs of samples. Gene expression was assessed using Affymetrix Clariom D microarrays, and comparisons were made between the 0% and 20% using the Affymetrix transcriptome analysis console (paired ANOVA tests with fold changes greater than +/- 2; supervised hierarchical clustering analyses).

Total number of gene probes	135750
Differentially expressed genes	184
Genes expressed more highly in 20% compared to 0%	127
Genes expressed at lower levels in 20% compared to 0%	57

Table 4.1: Summary of gene expression changes in MDA-MB-231 cells between the 0% fibroblast and 20% fibroblast cultures
MDA-MB-231-GFP/luc cells were cultured alone (0%) or with CAF1 cells (20%) and were treated with 10nM epirubicin. MDA-MB-231-GFP/luc cells were purified by FACS on the basis of positive GFP expression and RNA was prepared. Three separate biological repeats were performed giving three pairs of samples. Gene expression was assessed using Affymetrix Clariom D microarrays, and comparisons were made between the 0% and 20% samples (paired ANOVA tests with fold changes greater than +/- 2).

4.3.2 CAF1s induce changes to the miRNA transcriptome of MDA-MB-231 cells during epirubicin treatment

The same RNA samples as described above (section 4.3.1) were also used for analysis of miRNA expression profiles in order to identify miRNAs that were differentially expressed in the presence of CAFs. For this analysis, the three separate biological replicates for the two groups were pooled into single samples representing MDA-MB-231 cells with 0% fibroblasts or with 20% CAF1 cells. Expression was assessed using Taqman miRNA array cards, testing levels of 377 individual RNA species. Expression levels were normalized to expression of the small nucleolar RNA, RNU48, which is commonly used as an appropriate normalizer (Gee et al, 2011), and were compared between the 0% and 20% cultures. Differences in miRNA expression were categorised into four groups: present in the 0% culture but not in the 20% culture, present in the 20% culture but not the 0% culture, and increased or decreased relative expression from 0% to 20%. The numbers of miRNAs in each of these categories are shown in Table 4.2 (“Number of differentially expressed miRNAs” column), the total being 145.

In order to reduce the number of candidate miRNAs of interest to a number that was experimentally tractable, thresholds were incorporated to define minimum fold-changes and maximum cT values. For all four groups categorised, I imposed a maximum threshold for qPCR cT values of 35, thereby reducing the chances of including miRNAs detected in error or expressed at very low levels. For miRNAs in the increased (from 0% to 20%) or decreased (from 0% to 20%) expression groups, I set a further arbitrary threshold of requiring a minimum of 2-fold change to remain of interest. The numbers of miRNAs remaining of interest after incorporating these thresholds are shown in Table 4.2 ("…after thresholds" column), with the total being 45; their identities are listed in appendix Tables 2 and 3.

Category	Number of differentially expressed miRNAs	Number of differentially expressed miRNAs after thresholds
Present in 0% but not 20%	14	3
Present in 20% but not 0%	45	18
Increase from 0% to 20%	54	13
Decrease from 0% to 20%	32	11

Table 4.2: Numbers of miRNAs differentially expressed between MDA-MB-231 cells cultured alone (0%) or with CAF1 cells (20%) during epirubicin treatment
RNA was prepared, as described for Figure 4.1, from triplicate cultures of MDA-MB-231-GFP/luc cells alone or co-cultures with CAF1 cells. Triplicate samples were combined, and miRNA expression was profiled using TaqMan qPCR arrays. The numbers of miRNAs showing differential expression in the categories described are listed, either including all data and any degree of differential expression, or setting thresholds for detection level (maximum cT of 35 for all four groups) and fold difference (2-fold for increase and decrease groups).

Following the thresholds that had been put in place to reduce the number of differentially expressed miRNAs, literature searching was also carried out. For literature searching, each miRNAs expression in breast cancer was searched in addition to whether the miRNA had been previously been implicated in cancer progression or chemoresistance. This resulted in 5 miRNAs for further study (see Table 4.3, which names the miRNAs, categorises them, and gives a fold-change where appropriate).

MiRNA and group	Fold change
miR-32 (present in 0% not 20%)	-
miR-27a (present in 20% not 0%)	-
miR-155 (increase from 0% to 20%)	Fold change 108.93
miR-422a (increase from 0% to 20%)	Fold change 51.52
miR-454 (increase from 0% to 20%)	Fold change 2.63

Table 4.3: Five miRNAs that were differentially expressed between MDA-MB-231 cells cultured alone (0%) or with CAF1s (20%) were selected for further study
5 miRNAs were selected for further study as described in the main text. For miRNAs expression in only one sample, the sample in which the miRNA was present is stated. For miRNAs expressed in both samples, the extent and direction of differential expression is stated.

These final 5 miRNAs were then validated using qPCR to support the up- or down-regulation observed in the microarray experiments. The same pooled RNA from the 3 individual biological repeats (as previously) was used (Figure 4.2). Data for miR-27a, miR-155 and miR-422a broadly reproduced the findings from the microarray – with the microRNAs up-regulated in the presence of CAF1s. MiR-32 expression was not reliably detected by qPCR, and these data are excluded from the figure. For miR-454, down-regulation in the presence of CAF1s was seen in the qPCR data, which did not match the microarray observations. After these analyses, I concluded that miR-27a and miR-155 were the strongest candidates as regulators of chemoresistance, as they were dysregulated in the presence of CAFs during epirubicin treatment to the greatest extent and were consistent between the microarray and validation qPCR.

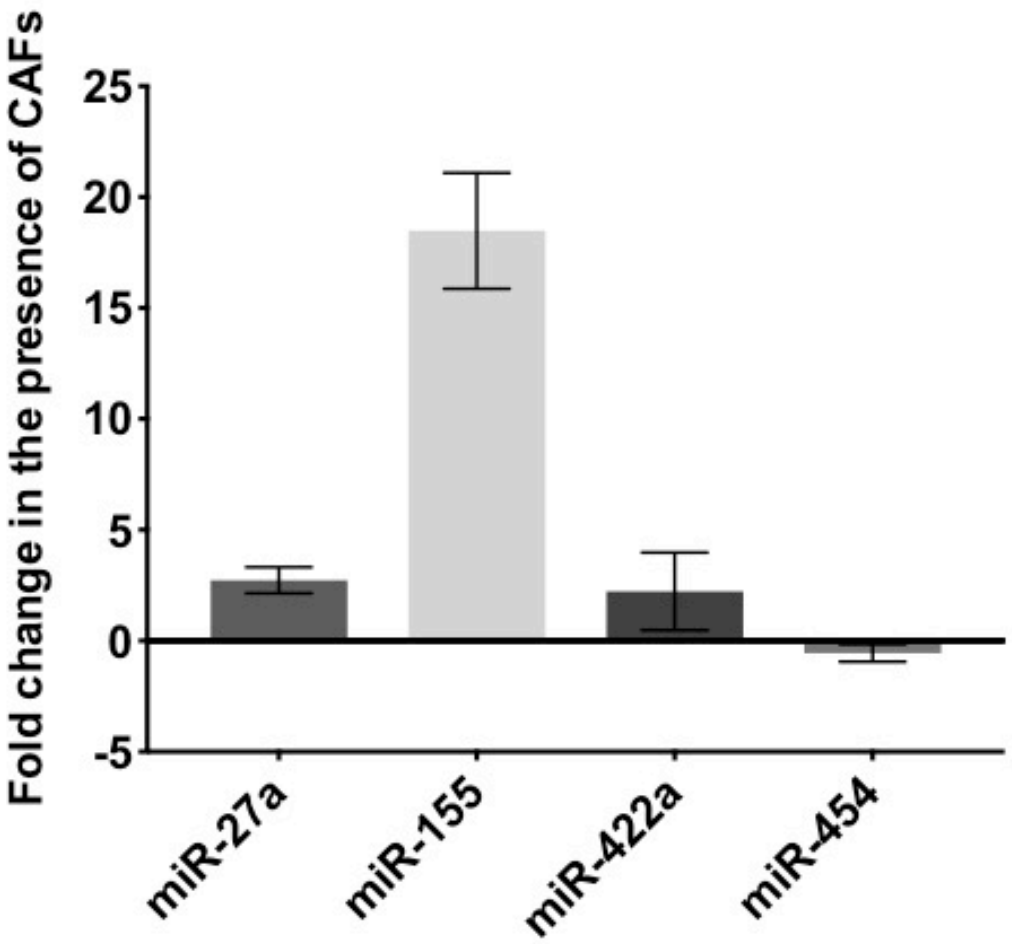


Figure 4.2: MiR-155 and miR-27a are up-regulated in MDA-MB-231 cells in the presence of CAF1s during epirubicin treatment

RNA was prepared, as described for Figure 4.1, from triplicate cultures of MDA-MB-231-GFP/luc cells alone or from co-cultures with CAF1 cells. Triplicate RNA samples were combined, and miRNA expression was assessed using TaqMan qPCR assays (normalised to RNU48). Data represent mean fold changes (+/- SD for technical triplicate analysis of one biological replicate).

4.3.3 MiR-155 and miR-27a do not have consistent, significant influences on survival of MDA-MB-231 cells in the absence or presence of epirubicin treatment, although miR-155 demonstrated a potentially relevant trend

Having identified miRNAs that were differentially expressed in cancer cells in the presence of CAFs (section 4.3.2), I was next interested to test whether the dysregulation of these miRNAs potentially contributed to the CAF-dependent chemo-protection of epithelial cells observed.

MiRNA mimics or inhibitors for miR-27a and miR-155 were transfected into MDA-MB-231-GFP/luc cells in mono-culture to allow manipulation of their expression levels and therefore testing of whether changes in the levels of these miRNAs were sufficient to alter chemo-response. After optimisation of transfections, appropriate dose of miRNA mimics and inhibitors for each of miR-27a and miR-155 were identified, and their abilities to cause successful over-expression or knockdown of miRNA expression respectively were validated by qPCR (Figure 4.3).

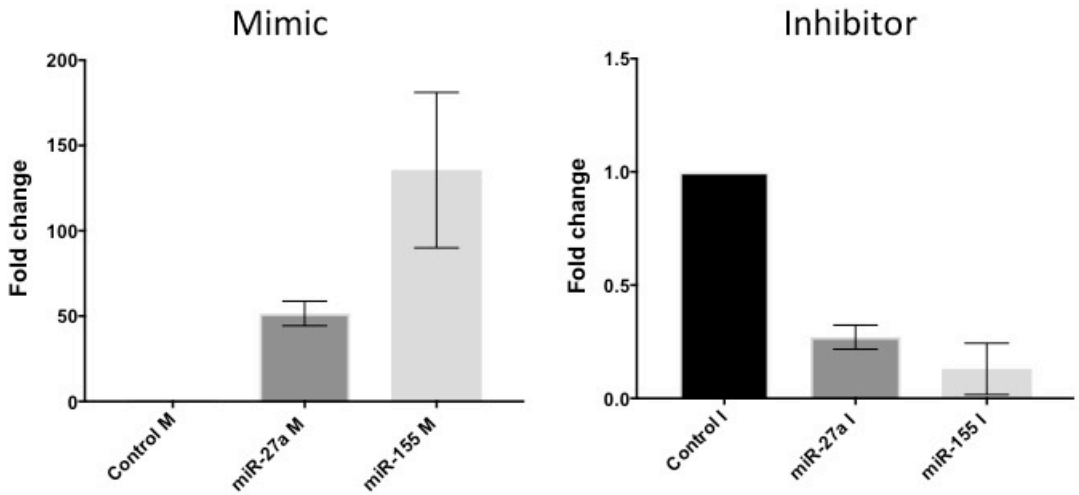


Figure 4.3: MiR-155 and miR-27a mimics and inhibitors successfully over-express or knockdown appropriate miRNA
MDA-MB-231-GFP/luc cells were transfected in triplicate with different doses of miR-27a and miR-155 mimics or inhibitors as well as control mimic or inhibitor and left for 24h. miR2-27a mimics and inhibitors concentrations were 500nM and 50nM respectiviely with miR-155 mimics and inhibitors 500pM and 10nM RNA was then extracted from triplicate wells and combined. MiRNA levels were assessed using TaqMan qPCR assays (normalized to RNU48). Data represent mean fold change (+/- SD for technical triplicates, with biological n=1).

To investigate chemo-responses, cells were transfected with targeted or control mimics or inhibitors, and then incubated without a chemotherapy agent, or with 10μM or 50μM epirubicin for 24h. Cells were then cultured for a further 24h in fresh medium without drug, before MTT assays were performed to quantify viable cells remaining in the culture (Figure 4.4).

To determine the influence of these miRNAs on survival and proliferation in the absence of chemotherapy, raw MTT assay values from the untreated transfections were plotted (Figure 4.4, left panel). The mimics and inhibitors, for both miR-27a and miR-155 had only very small, and non-significant, influences on survival/proliferation. In the context of epirubicin treatment, miR-27a mimics or inhibitors (Figure 4.4A and B right panel) showed no significant effects on chemotherapy-response. Similarly, miR-155 mimics and inhibitors showed no significant effects (Figure 4.4C and D right panel).

However, these data were worthy of closer inspection. Cells transfected with miR-155 mimics showed a marginal increase in relative cell survival at both doses of epirubicin (Figure 4.4C right panel, p values 0.310 and 0.093), while those transfected with the miR-155 inhibitor showed a marginal decrease in relative cell survival at least at one dose (Figure 4.4D right panel, p values 0.937 and 0.132). These marginal changes were consistent with the hypothesis that increased miR-155 expression, as seen in the CAF-induced chemo-protected cancer cells, could contribute to chemo-resistance. Therefore, miR-155 remained of interest as a potential mediator of CAF-induced chemoresistance, even though the results from these assays were not significant.

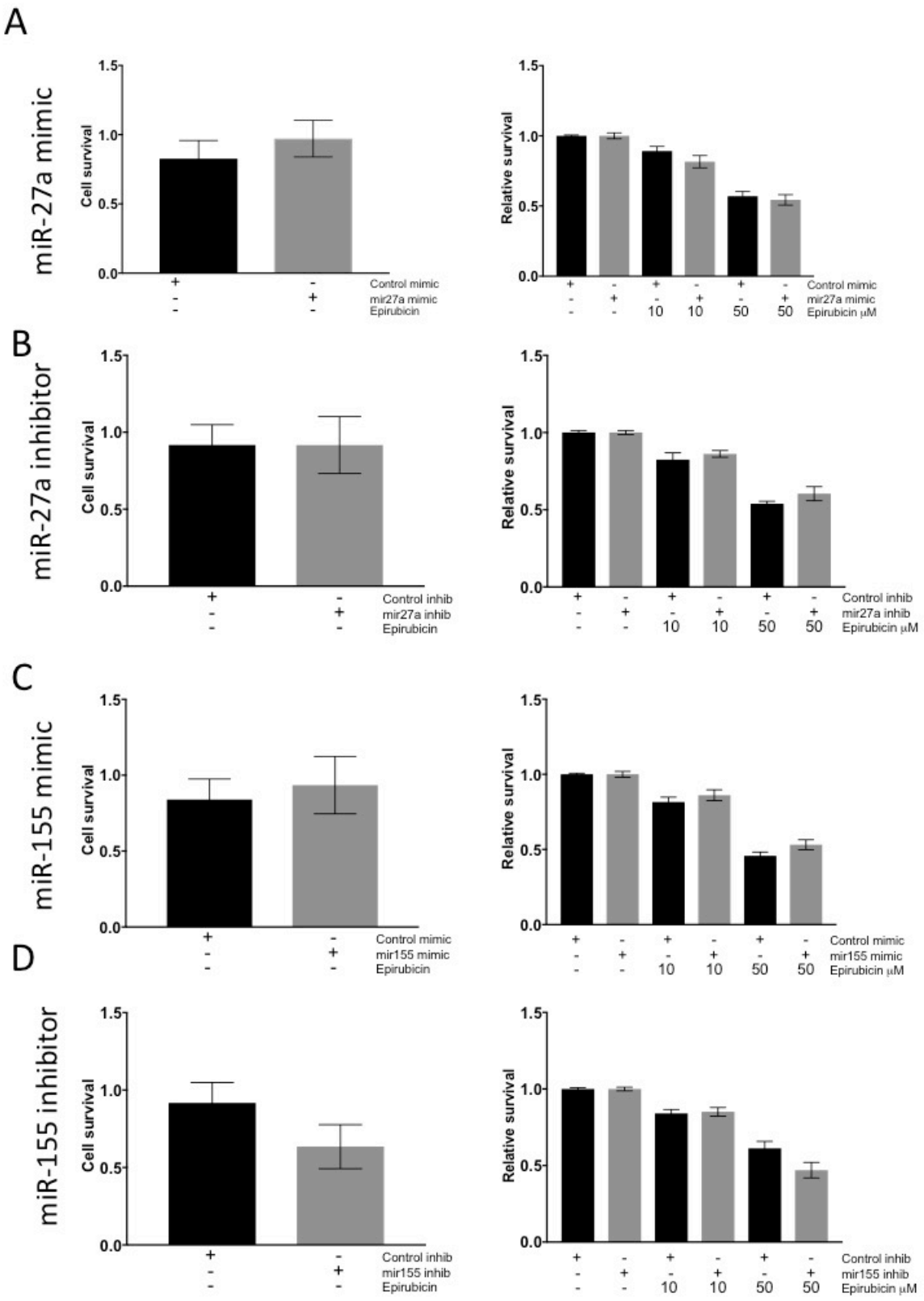


Figure 4.4: MiR-155 and miR-27a do not have consistent, significant influences on survival of MDA-MB-231 cells in the absence or presence of epirubicin treatment using short-term assays

MDA-MB-231-GFP/luc cells were transfected with miR-27a mimics (500nM, A), miR-27a inhibitors (50nM, B), miR-155 mimics (500pM, C), miR-155 inhibitors (10nM, D) or matched mimic/inhibitor controls (concentrations matched to targeted mimics/inhibitors, A-D). 24h later, transfected cells were treated with 10 or 50μM epirubicin or were untreated for 24h. MTT assays were performed. Plots on the left show raw absorbance values for cells transfected with targeted mimics/inhibitors or matched controls in the absence of epirubicin. Plots on the right show data relative to samples without epirubicin treatment. Data represent means (+/- SE) for three independent biological repeats. Two-tailed Mann-Whitney U tests were carried out with no significant differences observed between control or mimic/inhibitor transfected.

4.3.4. MiR-155 may have a role in chemo-response in MDA-MB-157 cells

Having shown that miR-155 was a potential interest in MDA-MB-231 cells, the assay was repeated in MDA-MB-157 cells. In the absence of epirubicin treatment, miR-155 mimics or inhibitors had no impact on cell survival (Figure 4.5A and B left panels). However, as for MDA-MB-231 cells, the miR-155 mimic and inhibitors had marginal, but non-significant, influences on chemo-response (Figure 4.5 A and B right panel). Consistently with data in MDA-MB-231 cells, miR-155 mimic increased chemoresistance (p values 0.818) and miR-155 inhibitors significantly sensitized the cells to chemotherapy (Figure 4.5B right panel $p<0.01$) therefore miR-155 remained potentially of interest.

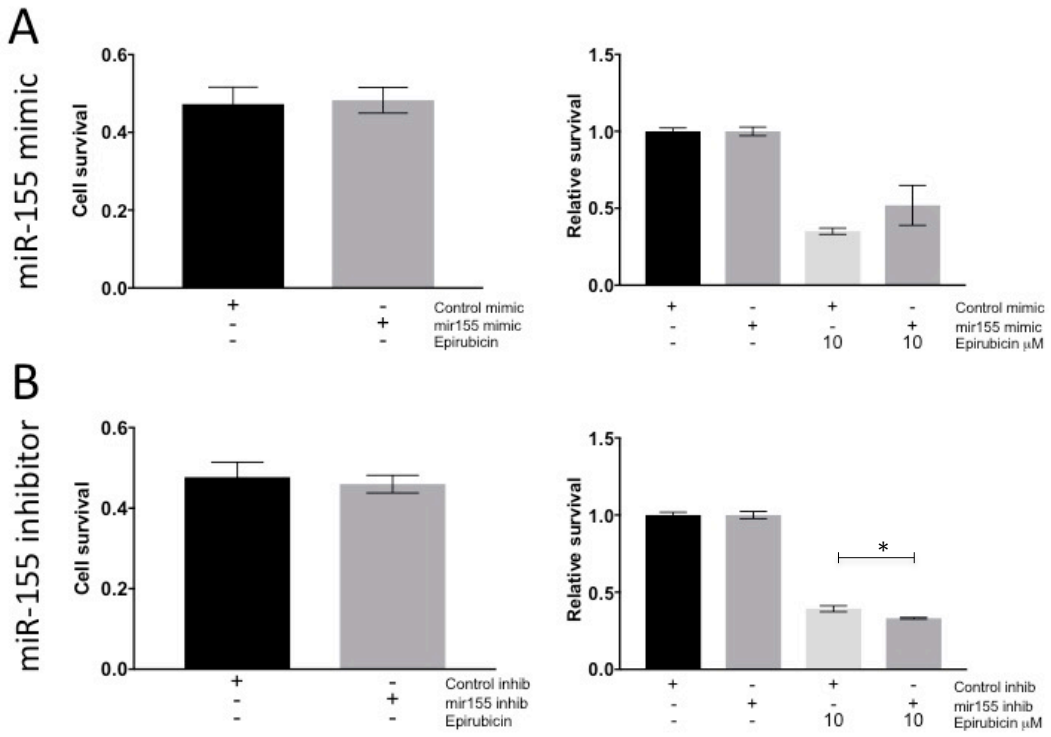


Figure 4.5: MiR-155 did not have significant influences on survival of MDA-MB-157 cells in the absence or presence of epirubicin treatment using short-term assays

MDA-MB-157 cells were transfected with miR-155 mimics (A), miR-155 inhibitors (B) or matched mimic/inhibitor controls (A-B). 24h later, transfected cells were treated with 10μM epirubicin or were untreated for 24h, followed by a further 24h culture in drug free media. MTT assays were performed. Plots on the left show raw absorbance values for cells transfected with targeted mimics/inhibitors or matched controls in the absence of epirubicin. Plots on the right show data relative to samples without epirubicin treatment. Data represent means (+/- SE) for n=3. Two-tailed Mann-Whitney U tests were carried out with no significant differences observed between control or mimic/inhibitor transfected.

4.3.5 Co-culture with CAF1s during epirubicin treatment induces dysregulation of two major biological pathways in MDA-MB-231 cells

Returning to the mRNA analyses using Affymetrix arrays, one way of gaining biological insights from large lists of differentially expressed genes is to analyse these genes for any changes (up-regulation or down-regulation) within specific pathways, thereby potentially defining dysregulated pathways. In my case, I was interested to identify pathways that could potentially play roles in the CAF-induced chemoresistance of MDA-MB-231 cells. Therefore, all the genes that were defined as differentially expressed between the 0% fibroblast MDA-MB-231 cultures and the 20% CAF1 MDA-MB-231 cultures (184 genes, Table 4.1) by the Affymetrix expression arrays, were analysed using a pathway enrichment platform, ToppGene (Chen et al, 2009). Analysis identified two notable and significantly dysregulated pathways (Table 4.4): interferon signaling and genes responsible for encoding components of the extracellular matrix. Differentially expressed genes from within these pathways are listed in Table 4.5 (interferon signaling) and Appendix Table 4 (extracellular matrix) with a full list of dysregulated pathways in Appendix table 5. For both pathways, all the identified pathway components were up-regulated in the co-culture with CAF1s (20%), as compared to the mono-culture (0%), suggesting that CAFs induce comprehensive up-regulation of these pathways.

Pathway	P Value	Number of genes from 184 input	Number of genes in pathway
Interferon alpha/beta signaling	5.127×10^{-13}	12	69
Ensemble of genes encoding extracellular matrix and extracellular matrix proteins	3.676×10^{-12}	28	1028

Table 4.4: Molecular pathways enriched in the differentially expressed genes in MDA-MB-231 cells after co-culture with CAF1s, as identified using the ToppGene portal

Genes showing significant differential expression of greater than 2-fold between a mono-culture of MDA-MB-231-GFP/luc cells (0% fibroblasts) and the MDA-MB-231-GFP/luc cells within a co-culture with 20% CAF1 cells were analysed using ToppGene gene enrichment analysis. The top two significant pathways identified are listed, with the number of genes from the inputted list within that pathway, and the total number of genes identified as part of the specified pathway. All other dysregulated pathways from ToppGene analysis are listed in appendix table 5. The individual genes from the interferon signaling pathway are listed in Table 4.5.

Target	Fold change 1	Fold change 2	Fold change 3
IRF9	8.41	5.17	3.42
STAT2	4.3	4.6	2.06
OAS1	36.36	21.93	37.03
OAS2	15.52	4.71	5.95
MX1	46.38	32.23	15.66
MX2	18.43	13.66	10.79
IFI6	11.91	13.35	3.25
IFI44L	12.04	8.37	11.83
IFI27	12.92	7.87	6.2
IFITM1	5.56	4.71	3.09
USP18	7.13	9.15	5.15
XAF1	7.83	3.93	3.74

Table 4.5: Genes in the interferon signaling pathway that were significantly up-regulated in MDA-MB-231 cells by co-culture with CAF1s during epirubicin treatment
See Table 4.4 legend for experimental details. Genes are listed with the fold changes associated with each of the three biological replicate analyses.

As discussed in section 4.2, it has already been reported that CAF-induced changes in the ECM may have roles in chemoresistance, therefore I chose to look further into the potential role of the interferon signaling pathway in chemoresistance as this appeared to have more novelty. In addition, *in vitro* work investigating the interferon signaling pathway would potentially be experimentally easier to manipulate and analyse since a range of chemical inhibitors are available and the pathway is comparatively simple compared to the very diverse signaling induced by the ECM and complexity involved in using 3D ECM scaffolds. In addition, the interferon pathway was chosen given that miR-155 (from the miRNA screen in section 4.3.2 and 4.3.3/4) has been reported as a positive regulator of the interferon pathway (Forster et al, 2015).

4.3.6 Interferon signaling is stimulated by CAF1s in epithelial cell types that are protected from epirubicin treatment by CAF1s, but not in cells that are not

To confirm my proposed CAF-dependent up-regulation of interferon signaling, I selected key genes from those identified as up-regulated to act as endogenous "reporter genes" for the pathway. OAS1 and MX1 were selected as these are very well-established interferon-responsive genes (Schoggins et al, 2011), and they had very high fold-changes (means >25) in the microarray data (Table 4.5). I also selected miR-155 that I demonstrated to be deregulated previously (section 4.3.2 and Figure 4.2), since it is both a positive regulator of the interferon pathway and is itself up-regulated by the pathway (Forster et al, 2015).

Next, I repeated the experiment where MDA-MB-231-GFP/luc cells were grown in mono-culture (0%) or in co-culture with 20% CAF1 for 24h. However, in this repeat, I treated cultures either with or without epirubicin to allow assessment of whether epirubicin impacts on the CAF-induced interferon signaling stimulation. As before, epithelial cells were purified by FACS, and RNA was extracted for qPCR expression analysis of OAS1, MX1 and miR-155 (Figure 4.6 top row). Furthermore, the same experiment was performed using two further cell lines: MDA-MB-157 cells (Figure 4.6 middle row), which had also demonstrated CAF-dependent chemoresistance (see Fig 3.4), and MDA-MB-468 cells (Figure 4.6 bottom row), which had not shown chemoprotection from CAFs (see Figure 3.3).

All 3 reporter genes (OAS1, MX1 and miR-155) demonstrated CAF-induced up-regulation in the two cell lines that were previously protected from chemotherapy by CAF1s (MDA-MB-231 and MDA-MB-157) (Figure 4.6 top and middle row), with expression of these endogenous reporter genes further increased by epirubicin treatment. However, epirubicin treatment was not itself sufficient to induce interferon signaling in the absence of CAFs. In contrast, MDA-MB-468 showed no sign of CAF-dependent activation of interferon signaling (Figure 4.6 bottow row), which can be observed by the much lower

levels of expression compared to MDA-MB-231 and MDA-MB-157 cells under all conditions (note the very different y-axis scales between Figure 4.6 top row and bottom row). To conclude, CAF-dependent protection from epirubicin appeared to correlate with CAF-dependent up-regulation of the interferon pathway.

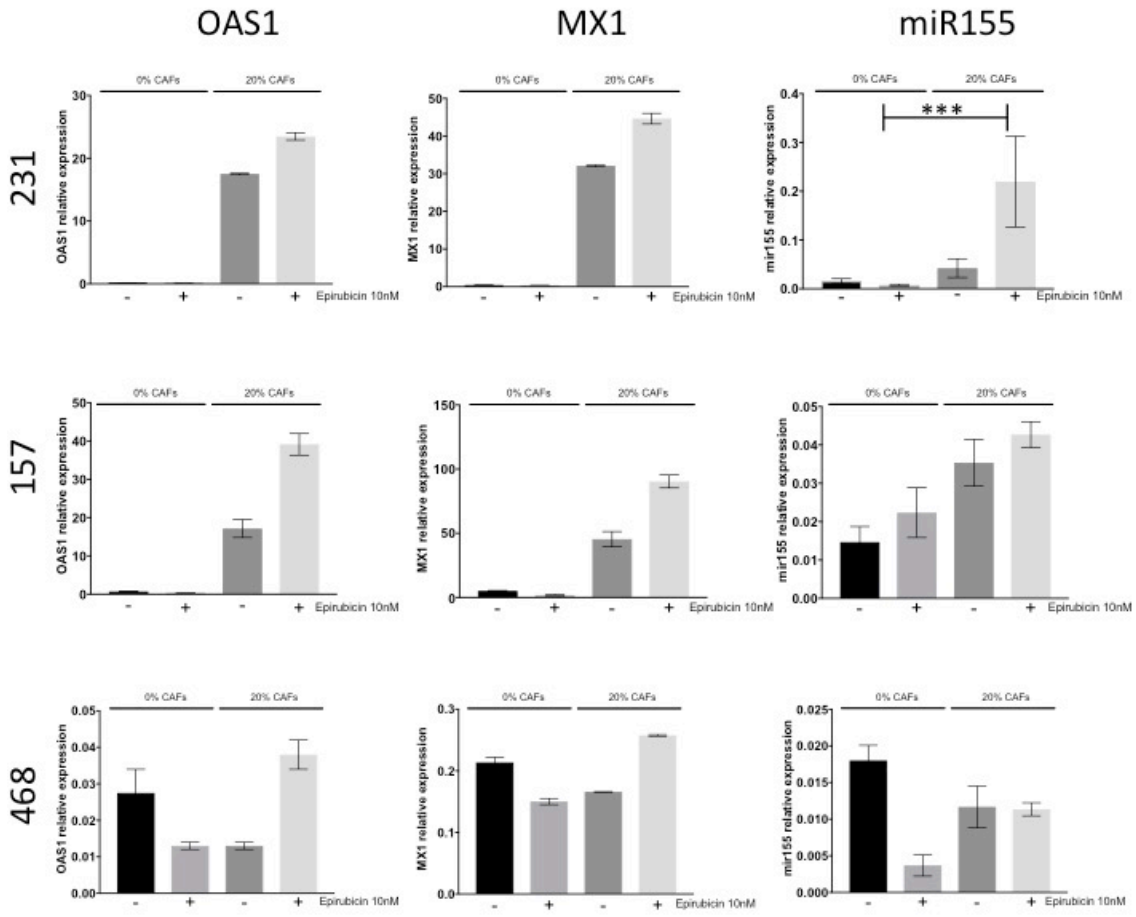


Figure 4.6: Interferon response genes are up-regulated in MDA-MB-231 and MDA-MB-157 cells by CAF1s, but not in MDA-MB-468 cells
MDA-MB-231-GFP/luc, -468-GFP and -157 cells were cultured on their own or in combination with 20% CAF1s with and without 10nM epirubicin treatment. Cultures were then sorted by FACS to give pure epithelial populations. RNA was then extracted from the epithelial cells and qPCR used to determine relative expression of interferon response genes OAS1, MX1 and miR-155. mRNA qPCRs were normalised to ACTB and miR-155 to RNU48. Data represent the mean of technical triplicates (+/- SD) from one biological experiment apart from miR-155 analysis in MDA-MB-231 cells, which is from 3 biological experiments (+/- SE). Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value ***<0.001.

4.3.7 Recombinant IFNs are sufficient to induce chemoresistance in MDA-MB-231 and MDA-MB-157 cells, but not MDA-MB-468s

Having demonstrated that interferon activation correlated with CAF-induced chemoprotection, my next aim was to identify if an interferon signal was *sufficient* to induce this chemoresistance in TNBC cell lines in the absence of CAFs. In order to do this, recombinant IFN-α and IFN-γ, representing interferons that signal through the Type I or Type II receptors respectively, were added in a range of concentrations to mono-cultures of, initially, only MDA-MB-231-GFP/luc cells for 24h. Cells were then treated with 10nM epirubicin for 24h in the continued presence of recombinant IFNs. Clonogenic survival was assessed (Figure 4.7).

The first finding to note was that recombinant IFN-α or recombinant IFN-γ increased the plating efficiency of the cells in a dose-dependent manner in the absence of epirubicin, which was significant for IFN-γ (Figure 4.7 “Colony counts” data, $p<0.01$). This observation was similar to the co-culture experiments, where the presence of CAFs in cultures enhanced the clonogenicity of these cells (for example, see Figure 3.3). As previously, in order to determine effects on chemoresistance, colony counts after epirubicin treatment were normalized to their respective untreated values (Figure 4.7 “Relative to no chemo” data). This revealed a significant dose-dependent increase ($p<0.0001$) in survival after epirubicin treatment in MDA-MB-231 cells resulting from treatment with either recombinant IFN-α or IFN-γ.

Having determined that recombinant IFN-α and IFN-γ separately were sufficient to induce chemoresistance in MDA-MB-231 cells, I next investigated whether IFN-α and IFN-γ in combination would have an additive effect. In addition, I expanded the experiment into MDA-MB-157 and MDA-MB-468 cells (Figure 4.8). Again IFN-α or IFN-γ significantly increased clonogenic plating efficiency in both MDA-MB-231 and MDA-MB-157 (Figure 4.8 “Colony counts” data, $p<0.05$), which was further increased by the combination of IFN-α and IFN-γ. However, IFNs had no significant influence on MDA-MB-468 cells in this assay (Figure 4.8). It should be noted that MDA-MB-468 cells are the cell line that was

previously found to be un-responsive to the presence of CAFs in this assay in terms of chemo-response (see Figure 3.3) and to CAFs in terms of up-regulation of IFN-signaling (Figure 4.6)

As previously, chemotherapy-specific effects were determined by normalization. Introduction of IFN-α or IFN-γ on their own provided significant protection for MDA-MB-231 and MDA-MB-157 cells from epirubicin, which was further increased by the combination of IFN-α and IFN-γ (Figure 4.8 “Relative to no chemo, $p<0.0001$, $p<0.01$). However, there was no significant change in chemo-response in MDA-MB-468 cells (Figure 4.8 “Relative to no chemo”).

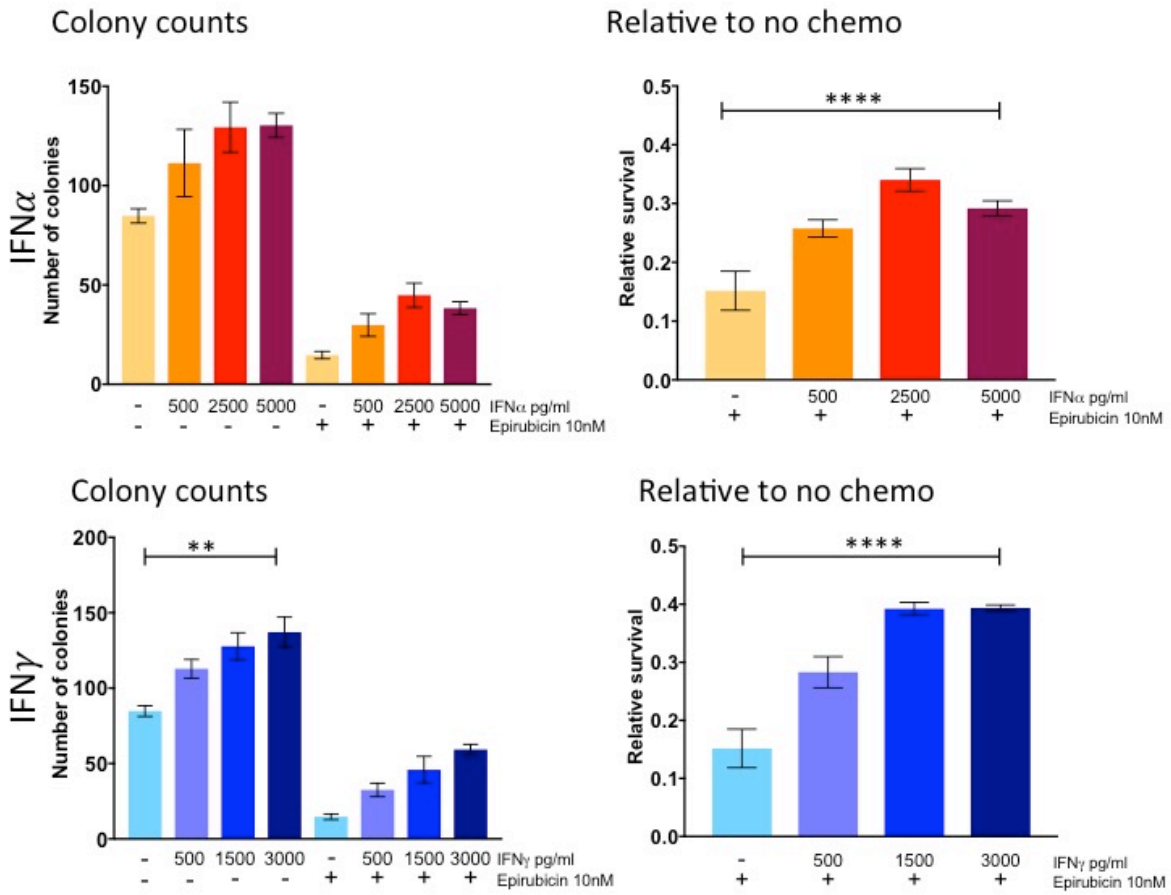


Figure 4.7: Recombinant IFNs are sufficient to stimulate chemoresistance in MDA-MB-231

MDA-MB-231-GFP/luc cells were cultured with IFN-α or IFN-γ separately at 3 different doses for 24h (500, 2500, 5000pg/ml and 500, 1500, 3000pg/ml respectively). Cells were then treated with 10nM epirubicin hydrochloride for 24h along with re-addition of fresh IFNs. Cells were then treated to determine clongenic survival as previously. Data are normalised to the no treatment value for each IFN condition and represent the means (+/- SE) of 3 independent experiments. One-way ANOVA was performed on no treatment data from colony counts and normalised treated data with significant differences between no IFN and doses of IFN calculated. P value ** <0.01 and **** <0.0001

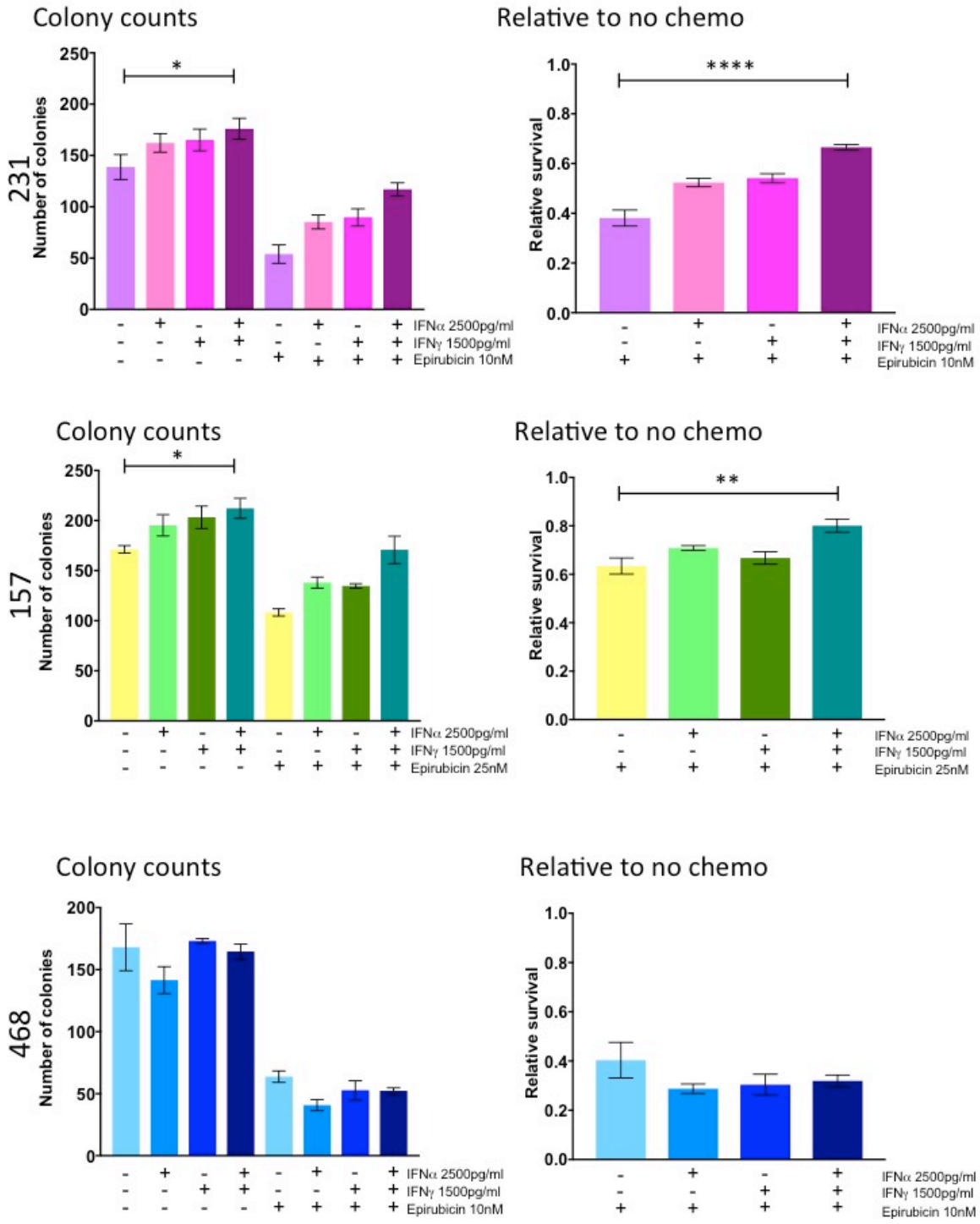


Figure 4.8: Recombinant IFNs are sufficient to stimulate chemoresistance in MDA-MB-231 and MDA-MB-157 cells but not MDA-MB-468 cells

MDA-MB-231-GFP/luc, -468-GFP and -157 cells were cultured with IFN-α or IFN-γ separately or combination for 24h (2500pg/ml and 1500pg/ml resepectively). Cells were then treated with 10nM or 25nM epirubicin hydrochloride for 24h with IFNs re-added. Cells were then treated to determine clongenic survival as previously. Data are normalised to the no treatment value for each IFN condition and represent the means (+/- SE) of 2 independent experiments. One-way ANOVA was performed on no treatment data from colony counts and normalised treated data with significant differences between no IFN and doses of IFN calculated. P value * <0.05, ** <0.01 and **** <0.0001

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In an effort to understand why MDA-MB-468 cells failed to be protected by either CAF1 cells or recombinant IFNs, I used qPCR for the interferon reporter genes OAS1 and MX1 to assess whether the MDA-MB-468 cell line was capable of activating the interferon signaling pathway in response to an appropriate signal. MDA-MB-468 cells were treated with recombinant IFN-α or IFN-γ, or both, with or without epirubicin exactly as previously. RNA was then extracted and qPCR was performed (Figure 4.9). OAS1 and MX1 expression levels were not stimulated by treatment with recombinant IFNs suggesting that this cell line is simply unable to respond to an IFN stimulus.

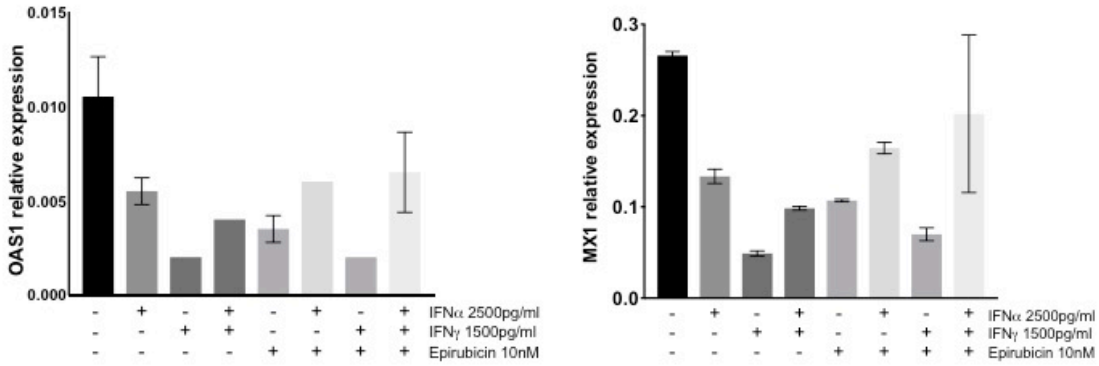


Figure 4.9: MDA-MB-468 are unable to activate interferon signaling in the presence of recombinant IFNs
MDA-MB-468-GFP cells were cultured IFN-α or IFN-γ separately or combination for 24h (2500pg/ml and 1500pg/ml resepectively) with and without 10nM epirubicin treatment for 24h. RNA was then extracted from the epithelial cells and equal amounts of RNA were used for qPCR to determine relative expression of IFN-target genes OAS1 (left plot) and MX1 (right plot) that were normalised to ACTB. Data represent the mean (+/- SD) for n=1

4.3.8 CAF1s, but not NF1s, activate interferon signaling in co-culture with MDA-MB-231 cells

Both NFs and CAFs have vast secretomes, so I next wanted to identify whether activation of interferon signaling in co-cultured epithelial cells was a generic fibroblast function or if this attribute was specific to CAFs. In order to test this, I transfected interferon signaling luciferase reporter constructs into MDA-MB-231 cells and co-cultured these cells with either NF1 or CAF1 cells. As previously mentioned in the introduction (section 1.13.1 and 1.13.2), the interferon signaling pathway acts through two different transcription factor binding sites - the ISRE (Type I signaling) or the GAS element (Type I and II signaling) – and separate reporters for each were used. Following transfection with the reporters, MDA-MB-231 cells were either cultured alone, or in co-culture with increasing proportions of NF1s or CAF1s for 24h and 48h without treatment or with 10nM epirubicin treatment for 24h. Luciferase assays were performed to assess ISRE or GAS activity within the epithelial cells (Figure 4.10).

Interferon signaling activity at the ISRE and GAS elements was relatively low in MDA-MB-231 cells cultured alone at all timepoints, either without or with epirubicin treatment. Co-culture with normal fibroblasts had no significant influence on these basal levels. However, co-culture with CAFs produced a dramatic activation of both reporters, which increased with increasing proportion of CAFs.

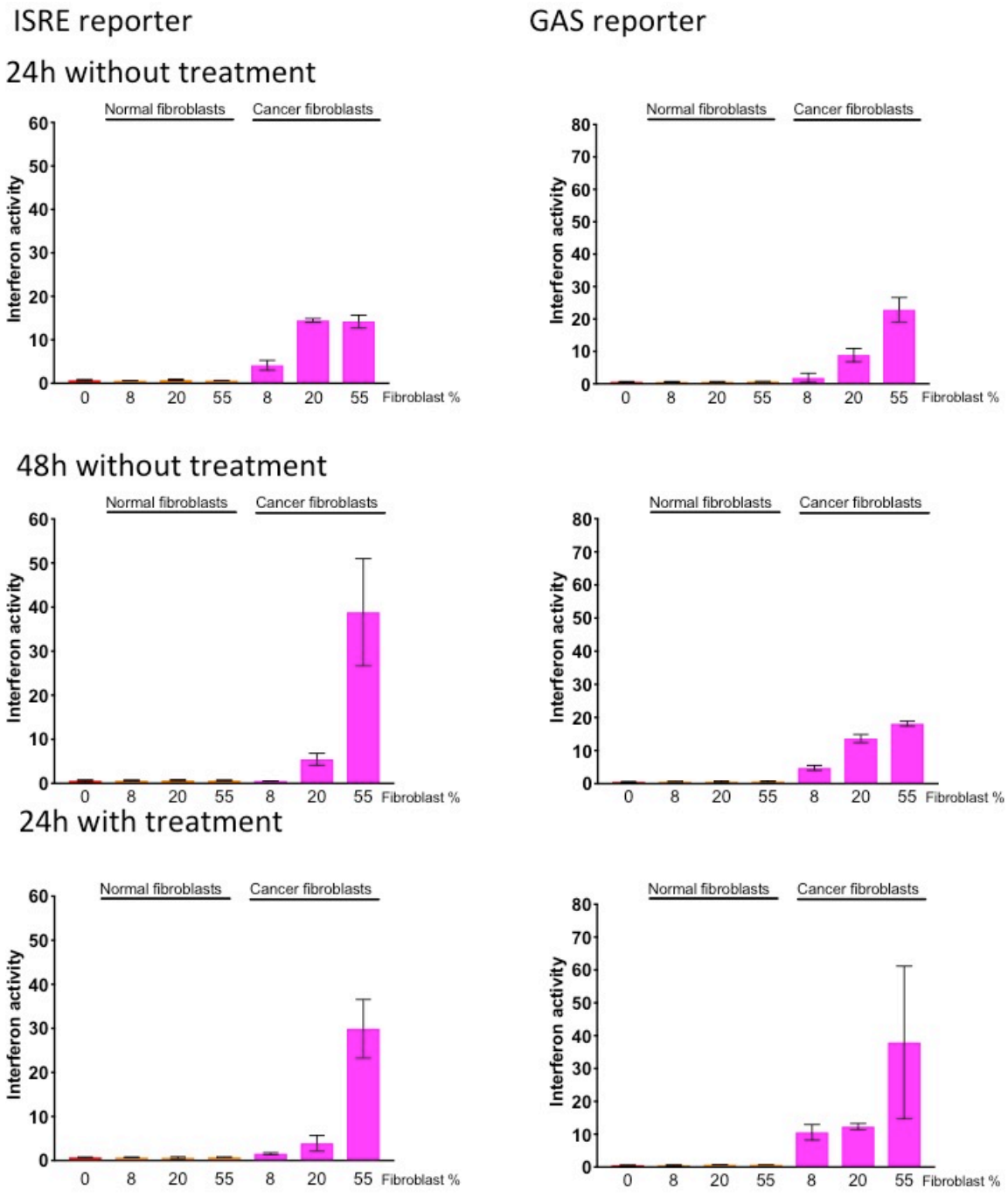


Figure 4.10: CAF1s activate ISRE and GAS activity in co-cultured MDA-MB-231 cells

MDA-MB-231 cells were transfected with ISRE or GAS reporter plasmids driving firefly luciferase expression, and a control plasmid (pRL-TK) with the HSV thymidine kinase promoter driving renilla luciferase expression. Transfected MDA-MB-231 cells were then cultured on their own or with different proportions of immortalised NF1s or CAF1s for 24h and 48h without treatment or with 10nM epirubicin treatment for 24h. Dual luciferase assays were performed, with firelfy readings normalised to renilla readings. Data represent the mean (+/- SD) for triplicate wells, for 1 biological replicate.

4.3.9 IFN-β is secreted by CAF1s, and not NFs, following co-culture with MDA-MB-231 cells, but not in CAF1 mono-cultures

Based on data in this chapter so far, my hypothesis was that CAFs, but not NFs, release interferon(s) as a paracrine signal, which stimulates interferon signaling in co-cultured breast cancer cells that are capable of responding to this signal (ie MDA-MB-231 and MDA-MB-157 cells, but not MDA-MB-468 cells). My next aim was to identify which interferon, of the many different subtypes (see section 1.11), might be responsible for this signaling and, therefore, for the subsequent chemoprotection observed in MDA-MB-231 and MDA-MB-157 cells.

Initially, ELISAs were used to attempt to quantify the levels of IFN-α or IFN-β. Media was taken from NF1s and CAF1s alone, MDA-MB-231 cells alone, and from 20% co-cultures of MDA-MB-231 with NF1 or CAF1s. All these cultures were treated with or without epirubicin. However, following analysis, levels of both IFN-α and IFN-β proved to be below the levels of accurate detection for this assay.

As an alternative, expression of IFN-α2, IFN-β1 and IFN-γ was assessed by qPCR in mono-cultures of immortalized NF1s, CAF1s and MDA-MB-231-GFP/luc cells as well as CAF1s and MDA-MB-231-GFP/luc cells isolated from 20% CAF1 co-cultures by FACS. In each case, these mono- or co-cultures were also treated with or without epirubicin.

Levels of IFN-α2 and IFN-γ were below the level of reliable detection and therefore expression data were not available for these interferons. However, data for IFN-β1 were more informative. There was little expression of IFN-β in NF1 mono-cultures with or without epirubicin treatment (Figure 4.11A left panel). IFN-β expression was similarly low in mono-cultures of CAF1s with and without epirubicin, However, when CAF1s were cultured with MDA-MB-231 cells, the expression of IFN-β in the CAF1s increased slightly without epirubicin, but very substantially with epirubicin treatment (Figure 4.11A, left panel). IFN-β1 was consistently not detected in mono-cultures of MDA-MB-231 cells or in

MDA-MB-231 cells sorted from the co-cultures with CAF1s (Figure 4.11A, right panel).

The same experiment described above was also carried out using a matched pair of primary NFs and CAFs taken from a TNBC patient (pNF and pCAF). Similarly to the result using the immortalised fibroblasts, IFN-β1 expression was only detected in the pCAFs after they were co-cultured with MDA-MB-231 cells (Figure 4.11B, left panel), and was not detected in the MDA-MB-231 cells (Figure 4.11B, right panel).

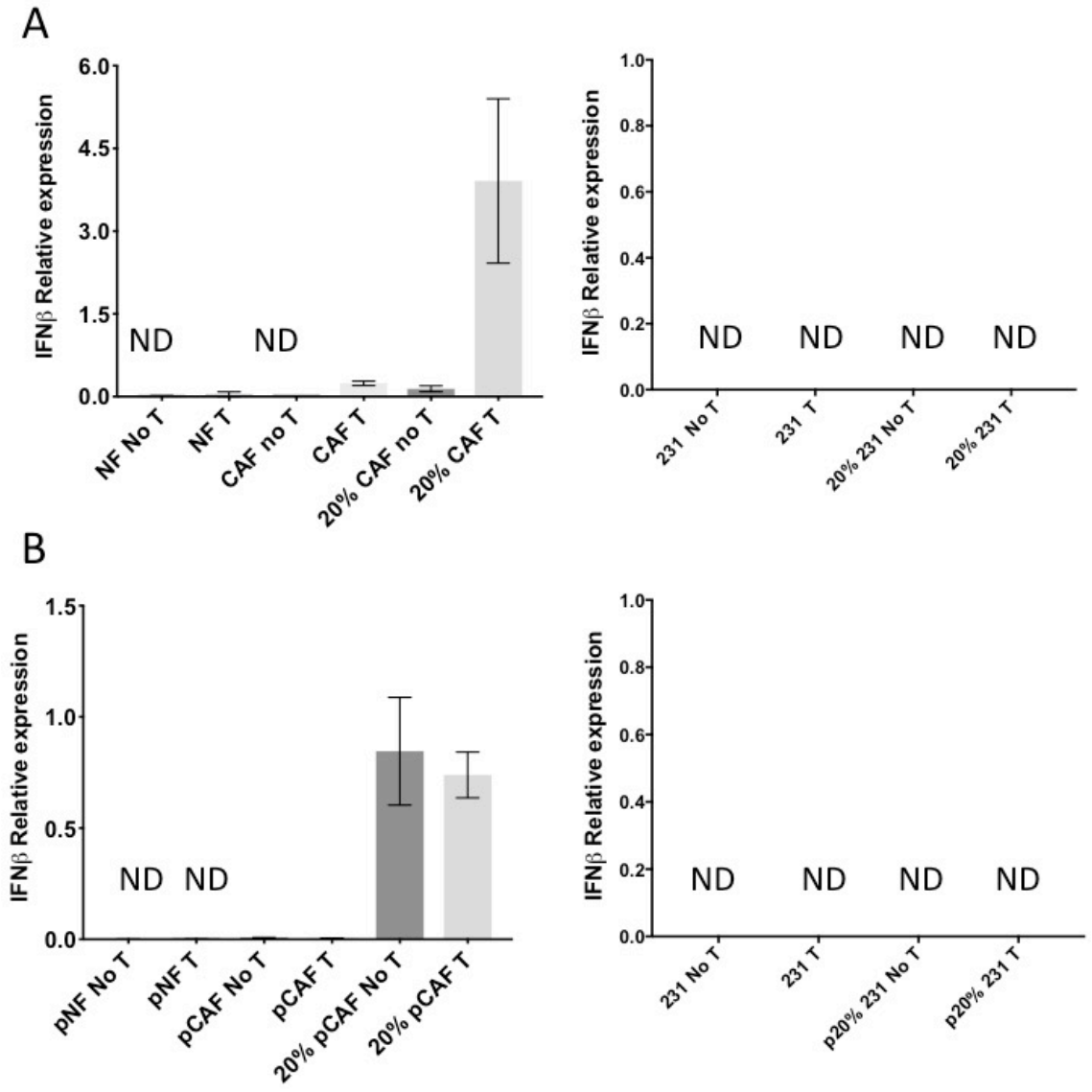


Figure 4.11: IFN-β1 expression in CAF1s is up-regulated in the presence of MDA-MB-231 cells

Immortalised CAF1 (A left), or primary CAF (B left) were cultured on their own or with MDA-MB-231-GFP/luc cells (20% fibroblasts; 80% epithelial cells; "20%") for 24h. MDA-MB-231-GFP/luc cells (A and B right) were also cultured alone for 24h. Cultures were then either untreated (No T) or were treated with 10nM epirubicin (T). All cultures were treated for fluorescence-activated cell sorting, allowing separation of fibroblasts and MDA-MB-231-GFP/luc cells from the co-cultures on the basis of GFP expression. RNA was then extracted from mono- and co-cultured cells, and qPCR used to determine expression of IFN-β1 relative to ACTB. Left plots represent expression in fibroblast populations from mono-cultures or co-cultures (denoted "20%"), while right plots represent expression in the MDA-MB-231 cells (mono-culture or "20%" co-culture). Data represent the mean of duplicate culture wells (+/- SD) for 1 biological replicate. ND denotes non detected values.

4.4 Discussion

The main aim in this chapter was to determine the potential molecular mechanisms responsible for the CAF-induced chemo-protection of TNBC cells that were observed in chapter 3. In order to achieve this, differences in expression profiles for both mRNAs and miRNAs were identified between MDA-MB-231-GFP/luc cells treated with epirubicin alone, or MDA-MB-231-GFP/luc cells treated in the presence of CAFs.

4.4.1 The choice of expression profiling methodology

A key consideration for expression profiling work, was the choice of expression profiling technology to be used, with the basic choice being between RNA sequencing (Stadler et al, 2009) or expression microarrays (Ness, 2007).

RNA sequencing is a relatively recent advance in gene expression profiling technology (Wang et al, 2009). At a very basic level, RNA is fragmented and each individual fragment is then sequenced. Sequence reads are then aligned to a reference genome, and expression of individual genes is estimated from the number of reads aligning to the gene (Kukurba and Montgomery, 2015). RNA sequencing can therefore provide analysis that is high coverage in terms of proportion of the transcriptome detected, high sensitivity (if sufficient read depth is available to detect rare transcripts) and high resolution in terms of splicing level information, in comparison to other gene expression profiling technologies (Hrdlickova et al, 2017). However, analysis of RNA-sequencing is known to involve complex bioinformatics and as yet standardized and generally accepted protocols for analysis have not been developed. In addition, RNA-sequencing can also be extremely expensive (Nagalakshmi et al, 2010). It was for these reasons that RNA sequencing was not used in this chapter for gene expression analysis.

Expression microarrays, on the other hand, are a well-established technology with reliable and relatively easy experimental and analysis pipelines. The technology enables the analysis of the RNA levels of thousands of genes simultaneously (Li et al, 2002), although a key point is that targets are pre-defined and limited, while RNA-sequencing has the potential to identify transcripts that were not previously known. The basic technology is that arrays contain single-stranded nucleic-acid probes that hybridise to target gene products and allow fluorescent detection proportional to expression (Kumar et al, 2012). Analysis of microarray data is relatively simple and user friendly with compatible software available for analysis with data exported into excel for further analysis (Cooper, 2001).

Microarrays have a long successful history of use in breast cancer research as demonstrated by their use in identifying differentially expressed genes between normal breast tissue and breast cancer (Perou et al, 1999) as well as identifying particular genes that predict pathological complete response and disease free survival in breast cancer patients who have different subtypes and stages of cancer (Perou et al, 2000, Okuma et al, 2016). In addition, gene expression analysis has also been undertaken in stromal cells, as opposed to the breast cancer cells. One study used microarrays to identify stromal gene expression changes in breast cancer progression, which could separate breast cancer patients into distinct groups displaying different clinical outcomes (Planche et al, 2011), with another study highlighting that molecular composition of tumour stroma in breast cancer was important for breast cancer prognosis (Winslow et al, 2016). Therefore, I was confident that the use of microarray technology would be suitable for identification of CAF-induced expression changes in my experiments.

In addition, analysis of miRNA expression levels was a further aim of this chapter. This can also be achieved using a wide-range of technologies, including RNA-sequencing, hybridization arrays, and qPCR arrays. qPCR arrays were chosen to determine miRNA expression as qPCR arrays are reliable with high sensitivity as well as being relatively easy experimentally. In addition, qPCR arrays could be carried out within the institute rather than being

outsourced. Use of qPCR arrays, again, had a successful relevant history in previous literature. MiRNA expression profiling identified up-regulated miRNAs in breast cancer patients by isolating circulating miRNAs. MiRNA expression was found to be higher in early stages of breast cancer, which could be useful for early detection of breast cancer. In addition, miRNA expression was slightly higher in TNBC and HER2 patients compared to ER positive patients (Hamam et al, 2016). The use of qPCR array was therefore a viable option for miRNA screening in this chapter.

4.4.2 miR-155: an oncomiR, as well as potential role in chemoresistance?

CAF-induced up-regulation of miR-155 in epithelial cancer cells was highlighted in this chapter, with the up-regulation implicated as an interferon-related mediator of chemoresistance.

The role of miR-155 in the CAF compartment has been explored previously. Expression in CAFs is of clear relevance to my findings, as it has been reported that miR-155 can be transfered between cell types in exosomes (Santos et al, 2018), and therefore the CAFs could be the source of the up-regulated expression of miR-155 in breast cancer cells. MiR-155 has been shown to be up-regulated in ovarian CAFs when compared to matched NFs (Mitra et al, 2012). Furthermore, miR-155 was required for the conversion of NFs to CAFs in the presence of ovarian tumour cells (Mitra et al, 2012). However, up-regulation of miR-155 has not been observed in breast cancer CAFs when compared to NFs (Schoepp et al, 2017). The cell-to-cell transfer of miR-155 was actually reported in the context of breast cancer cells, with miR-155 apparently transferred from cancer stem-like cells and treatment resistant MCF-7 cells to treatment sensitive MCF-7 breast cancer cells in culture (Santos et al, 2018) via exosomes. However, transfer of miR-155 from CAFs has not been reported.

In the context of clinical breast cancer samples, miR-155 has been shown to be significantly up-regulated in breast cancer and is associated with tumour subtype (Mattiske et al, 2012). When comparing 76 breast cancer samples to 10 normal breast samples, miR-155 was one of the most consistently up-

regulated miRNAs in the cancer samples (Iorio et al, 2005). Another study identifying differential expression of miRNAs in specific tumour subtypes, identified up-regulation of miR-155 in only HER2+ breast cancer in comparison to other breast cancer subtypes (Tsai et al, 2018). Although these are excellent examples of miR-155 up-regulation, it is important to note that these studies used tumour samples comprising both cancer cells, and cancer stroma, therefore miR-155 expression could be present in either the CAFs or breast cancer cells or both.

In my experiments (section 4.3.6), three TNBC cell lines were cultured on their own as well as in co-culture with CAFs. When grown on their own, relative expression levels of miR-155 were low (figure 4.6), however in this instance there is no comparison to any normal breast line to assess whether this level is "up-regulated" as would be compatible with the studies using cancer samples containing mixed cell types. Up-regulation is clearly observed in the presence of CAFs for two breast cancer cell lines (MDA-MB-231 and MDA-MB-157) but, again, miR-155 levels were not compared to levels of miR-155 in normal breast cancer cells with CAFs, so it is difficult to conclude where CAF induced up-regulation of miR-155 fits in with what is already known. Expression of miR-155 was determined in cell lines, in this chapter, instead of breast samples. Expression of miRNAs has also been studied in multiple breast cancer cell lines. One study has shown molecular subtype specific differential expression of miR-155, with relatively high expression of miR-155-3p in the normal-like/claudin-low cell lines (MDA-MB-231 and -157) that were estrogen receptor negative. More interestingly, miR-155-5p expression was high in basal-like estrogen receptor negative (MDA-MB-468) cell lines (Riaz et al, 2013), High expression levels of miR-155-5p in basal cell lines contradicts with my observations in section 4.3.6 and figure 4.6, in which I show higher expression levels of miR-155-5p in MDA-MB-231 and MDA-MB-157 cells in comparison to MDA-MB-468 cells. The changes in miR-155 expression observed in section 4.3.6 and figure 4.6, clearly highlight the importance of CAFs in inducing expression of miR-155-5p.

The functional role of miR-155 has previously been explored and interestingly miR-155 plays a role in interferon signaling. Not only is miR-155-5p transcription increased by interferon signaling, but miR-155-5p also plays an important role in the signaling cascade of Type I interferon signaling (Forster et al, 2015). Normally SOCS1 prevents the formation of the ISGF3 complex containing the STAT1/STAT2 heterodimer and IRF9, leading to inhibition of interferon signaling (Piganis et al, 2011). However, miR-155-5p has been shown to bind to the 3'UTR of SOCS1 and therefore enables the formation of the ISGF3 complex of STAT1/2 and IRF9 (Jiang et al, 2010). The identification of CAF-induced miR-155-5p up-regulation as well as up-regulation of interferon signaling (Table 4.3) fits with the known role of miR-155-5p in the interferon signaling pathway. Increased levels of miR-155-5p will therefore drive increased interferon signaling. Additionally, the activation of interferon signaling could be the stimulus that leads to CAF-induced enhanced expression of miR-155-5p, a hypothesis supported by the observation that miR-155-5p was not induced by CAFs in the one cell line that also did not activate interferon signaling (MDA-MB-468 cells; Figure 4.6).

In this chapter, the function of miR-155 was studied in terms of miR-155 effect on survival and growth of cells (sections 4.3.3 and 4.3.4) in order to identify the functional importance of increased miR-155 expression. It has already been shown that miR-155 is involved in many key cellular pathways that potentially promote cancer development or progression, including apoptosis, proliferation and epithelial-mesenchymal transition (Liu et al, 2015). MiR-155 also been linked to cellular functions in MCF-7 cells, with miR-155 promoting proliferation (Martin et al, 2014). MAPK signaling is also a target of miR-155. MiR-155 up-regulates p38 and enhances MAPK signaling to promote phosphorylation of ERK1/2 and cell proliferation (Martin et al, 2014). My data do not support a role for miR-155 in proliferation in the cell lines tested (MDA-MB-231 and MDA-MB-157; Figure 4.4 and 4.5), although it is important to note that the experiments were not designed to test this hypothesis, rather to investigate any potential role in chemo-response.

Importantly, miR-155's role in chemoresistance was investigated, because of the CAF-induced increase of miR-155 expression (section 4.3.2) in TNBC cells following epirubicin treatment. Interestingly miR-155 has previously been implicated as a potential marker of chemoresistance in TNBC (Ouyang et al, 2014). It is thought that miR-155 can lead to drug resistance in multiple different ways. MiR-155 has been shown to bind directly to and repress the function of FOXO3a, which is involved in processes such as cell death and cell cycle arrest (Dijkers et al, 2000). p27, an inhibitor of cell cycle progression, is a downstream target of FOXO3a and the presence of miR-155 therefore prevents activation and function of p27. p27 normally leads to the arrest of cells in G_0/G_1 (Chiarle et al, 2001). Presence of miR-155 and inhibition of FOXO3a and subsequently decreased levels p27 have been shown to be associated with docetaxel resistance in breast cancer cell lines (Brown et al, 2004). Here, miR-155 demonstrated a trend towards contributing to chemoresistance, although the findings were formally not statistically significant. MiR-155 mimics increased chemoresistance and miR-155 inhibitors decreased chemoresistance in both MDA-MB-231 and MDA-MB-157 cells (Figure 4.4 and 4.5). This trend was both consistent with the published literature above, and with my hypothesis that miR-155 would provide chemo-protection, based on its CAF-induced up-regulation in the context of CAF-induced chemo-protection. The function of miR-155 in chemoresistance is studied further in chapter 5.

4.4.3 miR-27a

The second miRNA that was identified as up-regulated in breast cancer cells by the presence of CAFs was miR-27a (section 4.3.2). CAF-induced up-regulation of miR-27a in epithelial cells has not previously been reported, however, the roles of miR-27a in CAFs themselves have been investigated. MiR-27a has previously been shown to induce the transition of NFs to the CAF phenotype, which in turn induced chemoresistance in esophageal cancer cells (Tanaka et al, 2015). However, up-regulation of miR-27a was not observed in the esophageal cancer cells nor did transfection with miR-27a confer chemoresistance in these cells, therefore this observation may be unrelated to my findings.

The functional role of miR-27a has been studied in TNBC. MiR-27a has been shown to have prognostic value in breast cancer patients, with high expression of miR-27a associated with poorer survival (Tang et al., 2012) and has been shown to part of a four miRNA signature specific to TNBC (Gasparini et al, 2014). However, my aim was to identify potential mediators of chemoresistance, and in this regard miR-27a was not promising since it was up-regulated in chemoresistant cancer cells (those co-cultured with CAFs, section 4.3.2), yet miR-27a over-expression or knock-down was not associated with significant or consistent changes in survival after chemotherapy treatment (Figure 4.4). By contrast, miR-27a inhibition in ER+ breast cancer cells has previously been shown to increase the sensitivity of the cells to cisplatin treatment (Zhou et al, 2016). MiR-27a is a positive regulator of multidrug resistance 1 (MDR1) / P-glycoprotein drug pump and has been shown to increase expression of MDR1 (Zhu et al, 2008). Both of these studies highlight that miR-27a can impact on chemoresistance, although this does not appear to be the case in my assays.

4.5 Conclusions and aspects of future work

It is evident from data presented in this chapter, that the presence of CAFs can up-regulate activity of the interferon signaling pathway and, related to this, expression of miR-155. It is also clear that interferons alone are sufficient to induce chemoresistance in representative cell lines of the claudin-low TNBC subtype. In my next chapter, I explore whether it is possible to block this influence of CAFs in order to inhibit the chemoprotective effect of CAFs.

Chapter 5 – Inhibition of interferon signaling reduces fibroblast-induced chemoresistance in triple negative breast cancer

5.1 Abstract

In chapter 4, I showed that interferon signaling was activated in selected TNBC cell lines by the presence of CAFs, and that activation was sufficient to induce relative chemoresistance. My next hypothesis was that inhibition of interferon signaling would improve the efficacy of the chemotherapy agent epirubicin in TNBC cell lines co-cultured with CAFs. This could raise the possibility that this combination treatment could be effective in patients with tumours containing high proportions of CAFs. Antibodies directed against interferon receptors that inhibit their activation, a JAK/STAT small molecule inhibitor, or miR-155 inhibitors were used to inhibit CAF-induced interferon activation.

CAF-induced chemoresistance in MDA-MB-231 and/or MDA-MB-157 was significantly reduced by the addition of interferon blocking antibodies directed against Type I or Type II receptors. qPCR was used to confirm that interferon signaling was indeed down-regulated. Similarly, the use of the JAK/STAT inhibitor ruxolitinib reduced CAF-induced chemoresistance in MDA-MB-231 and MDA-MB-157 cells. Chemoresistance induced by both immortalized and primary CAFs was sensitive to these inhibitions. MiR-155 inhibition also reduced CAF-induced chemoresistance in MDA-MB-231 cells.

I concluded that the use of interferon blocking antibodies, a JAK/STAT inhibitor, or miR-155 inhibitors can all synergise with epirubicin treatment by inhibiting CAF-induced chemoresistance.

5.2 Introduction

Interferon activation in TNBC cells is sufficient to drive increased resistance to the chemotherapeutic epirubicin (Chapter 4). My next interest was to examine whether inhibition of this activation could reduce CAF-induced protection from chemotherapy and could therefore present a potential therapeutic approach to sensitize cells to chemotherapy.

Interferon signaling is classically known to be involved in antiviral responses, however more recently roles of interferons in cancer have been studied. An example is the observation that activation of the interferon signaling pathway is associated with myeloproliferative disorders and hematological cancers. Enhanced JAK-STAT activities, down-stream mediators of interferon signals, have been identified in acute T-cell lymphocytic leukemia, acute B-cell lymphocytic leukemia and acute myeloid leukemia (Furumoto and Gadina, 2013). Therefore, inhibitors of these signaling intermediates have been developed as potential cancer therapeutics. Ruxolitinib is a leading JAK-STAT inhibitor and inhibitor of interferon signaling (Harrison et al, 2012). Ruxolitinib is a targeted therapy that selectively binds to non-receptor janus kinases (JAK), therefore inhibiting JAK1 and JAK2 activation and signaling. Ruxolitinib is currently FDA approved for treatment of myelofibrosis and polycythaemia vera (Yi et al, 2015). As ruxolitinib is already in clinical use, it could potentially be used in breast cancer patients in the near term. Ruxolitnib is therefore an ideal inhibitor for me to use in combination with chemotherapy to assess whether inhibition of interferon signaling improves chemotherapy response.

I also identified miR-155 as a potential regulator of chemoresistance, through the screen of changes in miRNA expression completed in Chapter 4. MiR-155 has previously been found to be over-expressed in a large number of solid cancers, including colon, breast, pancreatic, thyroid, cervical and lung cancers (Faraoni et al, 2009). MiR-155 has an established role as a positive regulator of interferon signaling, through targeting the protein SOCS, which normally functions to down-regulate interferon signaling (Forster et al, 2012). MiR-155 is itself also a down-stream target of interferon signaling, with activation of the

pathway leading to the transcription of further miR-155; this represents a positive feedback loop (Jiang et al, 2010). Inhibition of miR-155 function therefore provides a further strategy to inhibit interferon signaling.

An interesting aspect of the CAF-induced chemoprotection I have observed is that this may be a paracrine activity, with CAFs releasing signals (presumably interferons themselves) that are received by the epithelial cancer cells. This means that use of antibodies that block the ability of interferons to act on their receptors provides another strategy to inhibit activation of the pathway. Different blocking antibody strategies are available: those that bind to interferons themselves or those that bind to the receptors to prevent interferon binding. One study has shown successful blocking of IFN-α and IFN-β by targeting them directly using monoclonal antibodies and by targeting the IFNAR2 receptor chain. Blocking of IFN-γ directly and the IFNGR receptor chain also successfully neutralized IFN-γ activity (Moll et al, 2008).

In this chapter, I aimed to inhibit interferon signaling in breast cancer epithelial cells, using multiple different strategies as outlined above, and determine whether this reduced CAF-induced chemoresistance.

5.3 Results

5.3.1 Type I and/or Type II interferon blocking antibodies individually block CAF1-induced chemoresistance in TNBC cells but the combination of both antibodies has no increased effect

My first aim was to assess whether antibodies capable of blocking activation of interferon receptors would inhibit CAF-induced chemoresistance.

To achieve this, I adapted my standard colony formation assay protocol and introduced Type I and Type II receptor blocking antibodies. MDA-MB-231-GFP/luc cells were cultured either on their own or with 20% CAF1 cells with either an isotype control antibody or blocking antibody added. Blocking antibodies for either Type I or Type II signaling were used either individually or in combination. Cultures were incubated for 24h and then treated with 10nM epirubicin for 24h, with the antibodies re-added to ensure inhibition of the pathway was maintained. As before, the cultures were then sorted to purify the GFP-expressing epithelial cells with equal numbers of cells plated at a low seeding density and cultured to allow individual colonies to be counted (Figure 5.1).

As seen previously and as expected, the results in Figure 5.1 consistently demonstrate that epirubicin treatment significantly reduced cell survival, and that co-culture with CAF1s both significantly increased seeding efficiency and induced significant chemoresistance ($p<0.05$). However, the key results in this experiment are the comparison of survival after epirubicin treatment in cells treated with either control or IFN-blocking antibodies. In CAF1 co-cultures both the Type I blocking antibody (Figure 5.1A, right plot) and the Type II blocking antibody (Figure 5.1B, right plot) caused significant reductions in survival after epirubicin treatment ($p<0.05$), meaning that the CAF-dependent protection was completely inhibited. Surprisingly, however, the combination of the two blocking antibodies did not significantly inhibit the CAF-dependent protection (Figure 5.1C, right plot, p value 0.486).

It was also evident from these data that each of the Type I and Type II blocking antibodies also inhibited the CAF-dependent increase in seeding density (Figure 5.1, compare control with blocking antibody for epithelial + CAFs without epirubicin in the colony count data set [left plot]), indicating that interferon signaling is also required for this effect of CAFs that is unrelated to chemo-response.

It was also important to confirm that the blocking antibodies were indeed inhibiting interferon signaling as expected. Therefore, in addition to the colony forming assays, qPCR analysis was performed on the remaining cells that had been sorted but were not required for colony plating to assess levels of interferon pathway activity using the interferon stimulated genes OAS1 and MX1 as indicators of activity (Figure 5.2). As previously seen and expected, co-culture with CAFs led to activation of interferon signaling with epirubicin treatment enhancing this activation ($p<0.05$). The blocking antibodies, either individually (Figure 5.2A and B) or in combination (Figure 5.2C), consistently and significantly reduced ($p<0.05$) this activation, although levels were not reduced to basal.

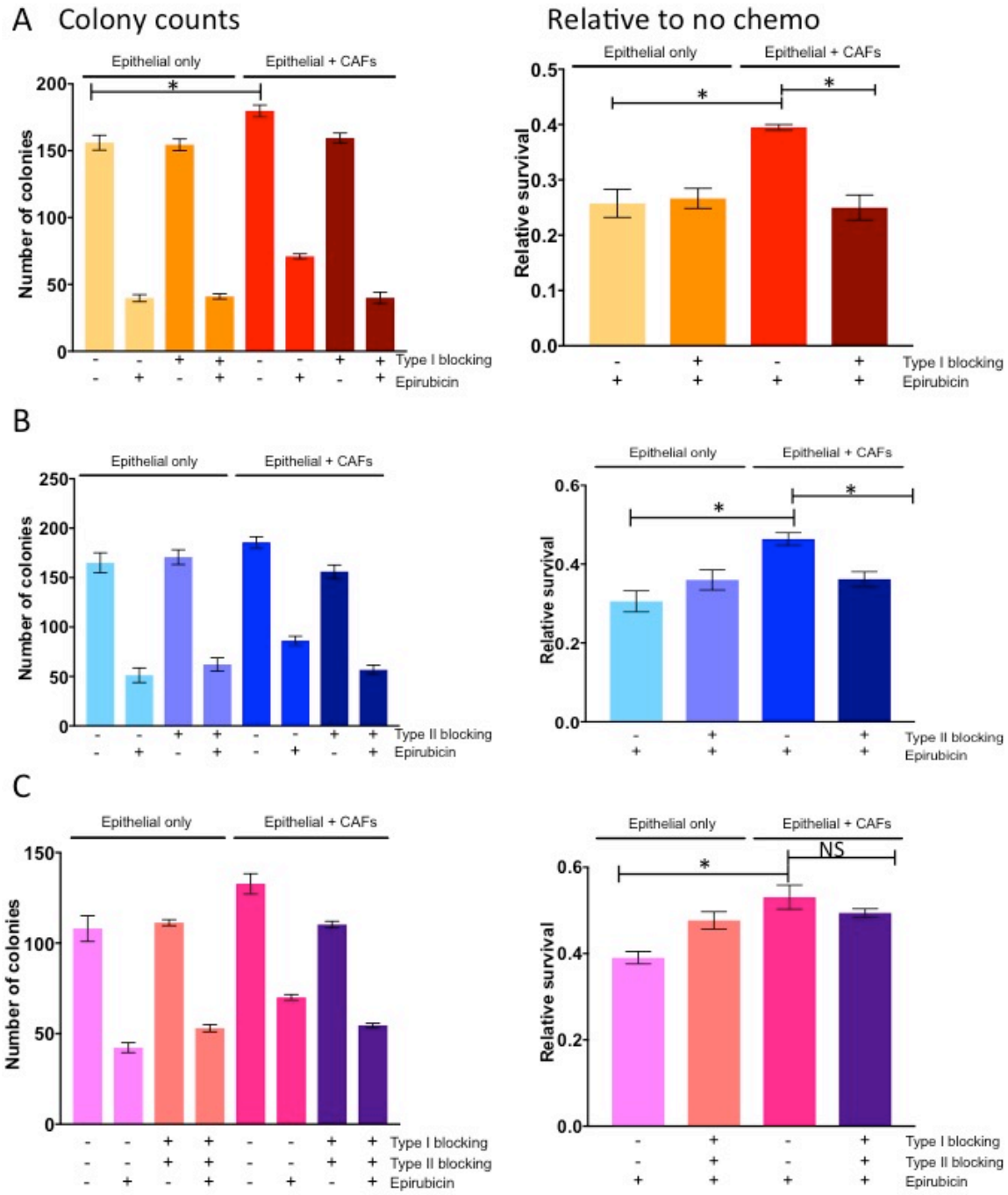


Figure 5.1: Blocking Type I and Type II interferon signaling prevents CAF1-induced chemoresistance in MDA-MB-231 cells

Cultures of MDA-MB-231-GFP/luc cells were established in mono-culture or co-culture with 20% immortalised breast CAF1s. Type I interferon signaling (1µg/ml) or Type II interferon signaling (5µg/ml) blocking antibodies were either added separately (A and B) or in combination (C) or appropriate isotype controls were added. Cultures were treated with 10nM epirubicin or untreated for 24h with antibodies re-added. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), and represent the means (+/- SE) of 3 independent experimental repeats. Two-tailed Mann-Whitney U tests were carried out and selected significant differences are shown. P values * <0.05.

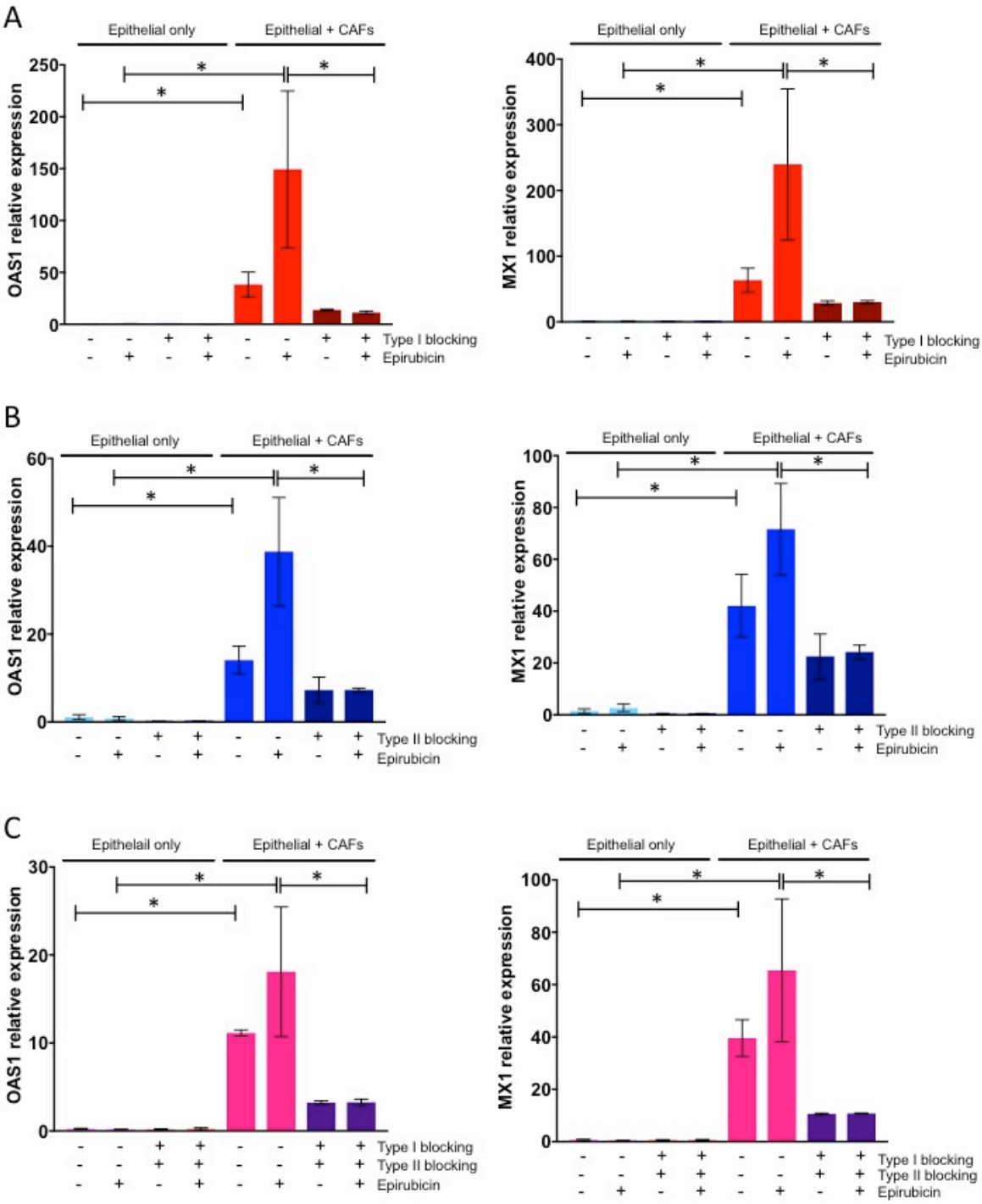


Figure 5.2: Interferon neutralizing antibodies successfully blocked CAF1-dependent interferon activation in MDA-MB-231 cells when treated with epirubicin

Cultures of MDA-MB-231-GFP/luc cells were established in mono-culture or co-culture with 20% immortalised breast CAF1s. Type I interferon signaling (1µg/ml) or Type II interferon signaling (5µg/ml) blocking antibodies were either added separately (A and B) or in combination (C) or appropriate isotype controls were added. Cultures were treated with 10nM epirubicin or untreated for 24h with antibodies readded. Cultures were then separated into pure epithelial populations by FACS. RNA was extracted from the epithelial cells and equal amounts of RNA were used for qPCR to determine relative expression of IFN-target genes OAS1 (left plots) and MX1 (right plots). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

5.3.2 Type I, but not Type II, interferon blocking antibodies block CAF1-induced chemoresistance in MDA-MB-157 cells

Next, the same entire experimental approach of clonogenic assays and qPCR pathway activity assessment was repeated using the alternative TNBC cells line MDA-MB-157, in which I had also observed CAF-induced chemoprotection (Chapter 3). Clonogenic survival data are presented in Figure 5.3, while matched qPCR data to assess interferon pathway activity are presented in Figure 5.4.

As previously, epirubicin treatment reduced survival, while co-culture with CAFs afforded chemoprotection in the absence of blocking antibodies ($p<0.05$ and $p<0.01$). In accordance with the findings in MDA-MB-231 cells, the Type I blocking antibody significantly ($p<0.01$) and completely inhibited this protection in MDA-MB-157 (Figure 5.3A, right panel). However, in contrast to findings in MDA-MB-231 cells, the Type II blocking antibody had no effect on CAF-dependent protection (Figure 5.3B, right panel, p value 0.486). Analysis of interferon pathway activity by qPCR confirmed that the Type I blocking antibody inhibited interferon signaling (Figure 5.4A, $p<0.01$), as expected. However, it was evident that in this cell line the Type II blocking antibody failed to inhibit the CAF-dependent activation of the interferon pathway (Fig 5.4B, p values 0.486 and >1), which provided an explanation for why this antibody had also had no functional effect on the CAF-dependent chemoprotection (Figure 5.3B, right panel).

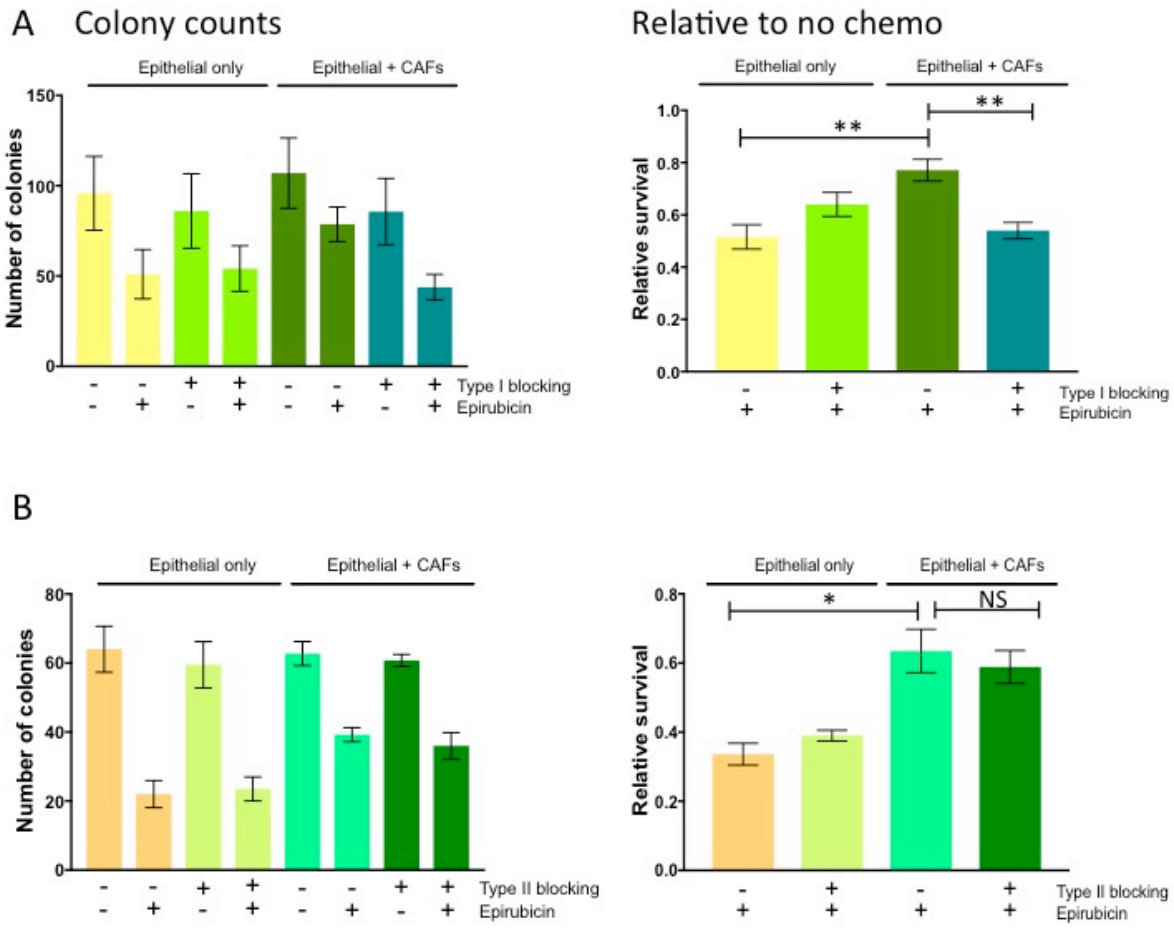


Figure 5.3: Type I but not Type II interferon neutralization blocks immortalised CAF1 induced chemoresistance in MDA-MB-157 cells

Cultures of MDA-MB-157 cells were established in mono-culture or co-culture with 20% immortalised breast GFP-CAF1s. Type I interferon signaling (1µg/ml) or Type II interferon signaling (5µg/ml) neutralising antibodies were added separately (A and B) or appropriate isotype controls were added. Cultures were treated with 10nM epirubicin or untreated for 24h with antibodies readded. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), and represent the means (+/- SE) of 3 independent experimental repeats. Two-tailed Mann-Whitney U tests were carried out and selected significant differences are shown. P values * <0.05.

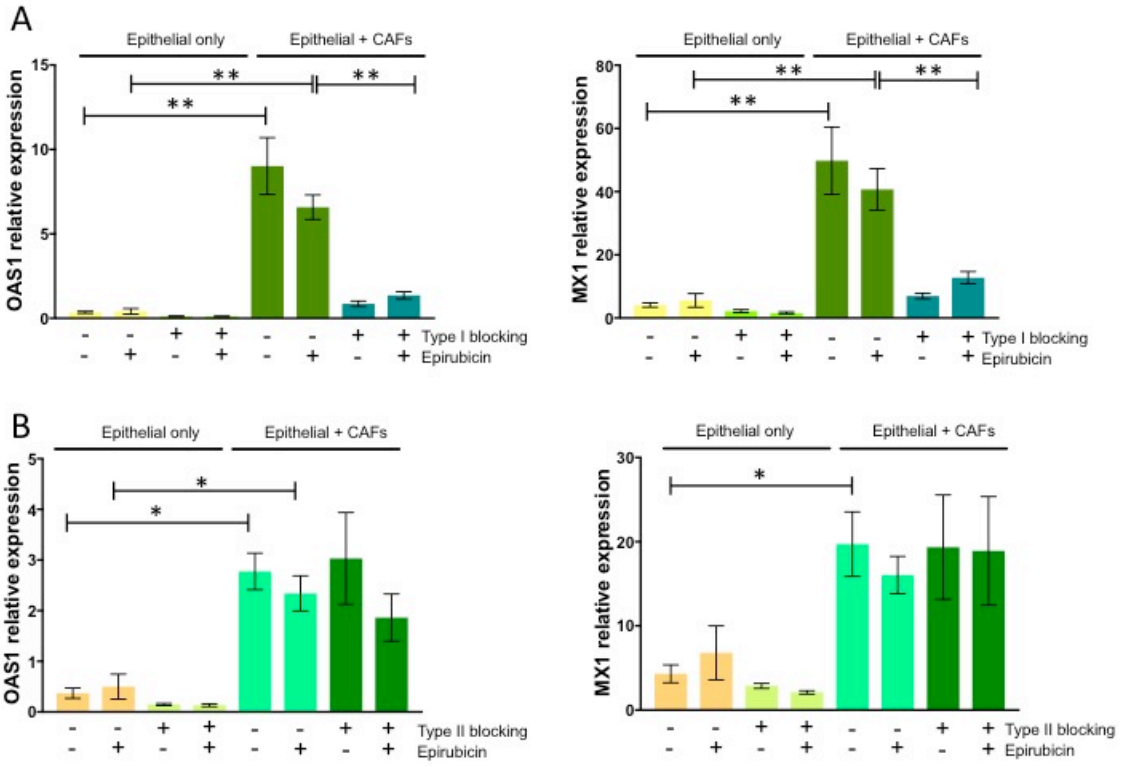


Figure 5.4: Type I but not Type II interferon neutralizing antibodies block CAF1-dependent interferon activation in MDA-MB-157 cells

Cultures of MDA-MB-157 cells were established in mono-culture or co-culture with 20% immortalised breast GFP-CAFs. Type I interferon signaling (1µg/ml) or Type II interferon signaling (5µg/ml) blocking antibodies were either added separately (A and B) or appropriate isotype controls were added. Cultures were treated with 10nM epirubicin or untreated for 24h with antibodies readded. Cultures were then separated into pure epithelial populations by FACS. RNA was extracted from the epithelial cells and equal amounts of RNA were used for qPCR to determine relative expression of IFN-target genes OAS1 (left plots) and MX1 (right plots). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

5.3.3 Type I interferon blocking antibodies block pCAF-induced chemoresistance in MDA-MB-231 cells

The same approach was then taken to examine whether interferon blocking antibodies could block chemoresistance induced by *primary* CAFs (pCAFs) derived from a TNBC patient. These pCAFs have already been shown to induce chemoprotection of MDA-MB-231-GFP/luc cells (Figure 3.5). Clonogenic assays and qPCR assessment were performed as above in section 5.3.1 and 5.3.2, in this case with the Type I interferon blocking antibody, pCAFs and MDA-MB-231 cells (Figure 5.5).

As previously, epirubicin treatment reduced survival, while co-culture with pCAFs afforded chemoprotection in MDA-MB-231 cells in the absence of blocking antibody ($p<0.05$). In accordance with the findings in MDA-MB-231 and MDA-MB-157 cells using immortalized CAF1s, the Type I blocking antibody significantly ($p<0.05$) and completely inhibited pCAF-induced protection in MDA-MB-231 (Figure 5.5A, right panel). Analysis of interferon pathway activity by qPCR confirmed that the Type I blocking antibody inhibited the CAF-induced interferon signaling as expected, with reduced induction of OAS1 and MX1 (Figure 5.5B, p values 0.029 and 0.343 respectively).

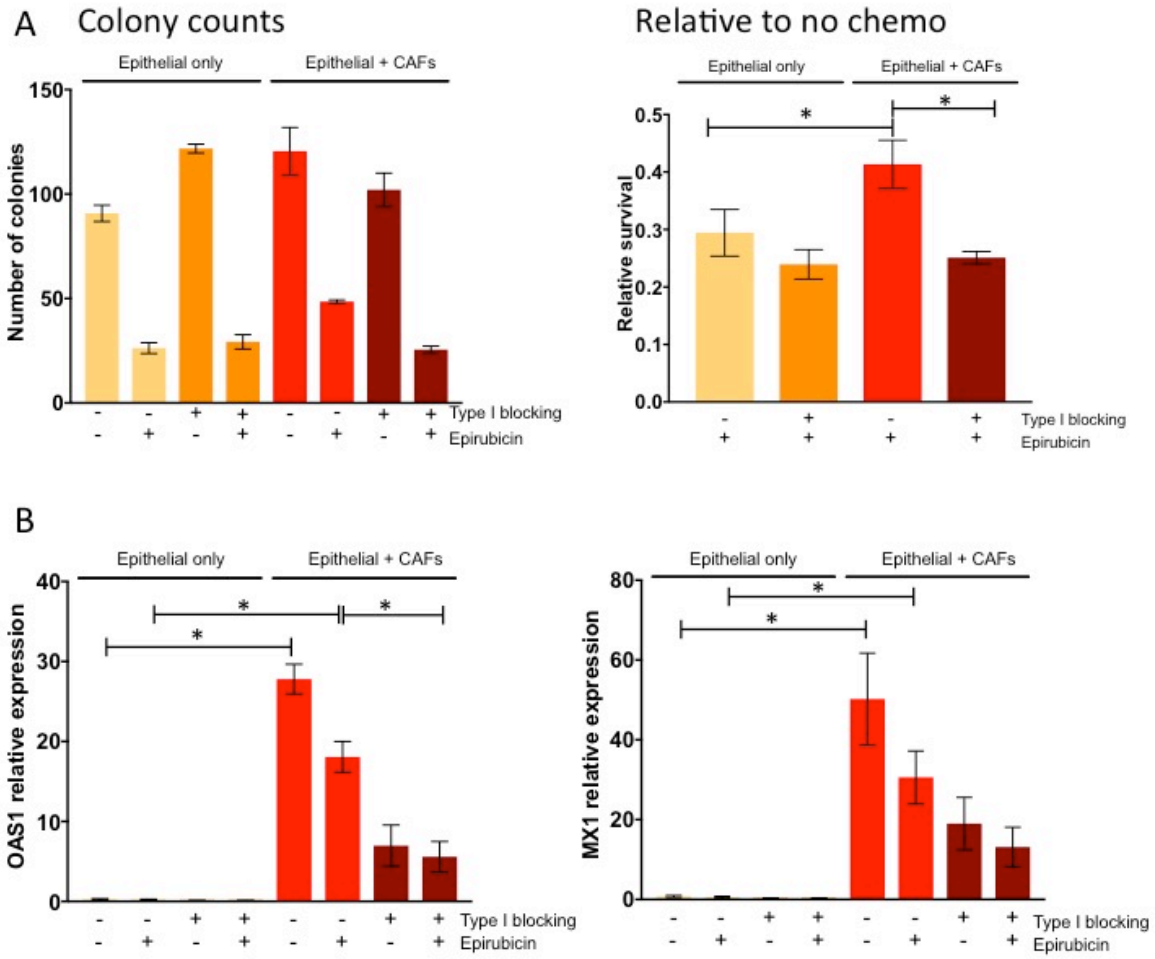


Figure 5.5: Type I interferon neutralization blocks pCAF-induced chemoresistance, and pCAF-induced interferon activation in MDA-MB-231 cells
Cultures of MDA-MB-231-GFP/luc cells were established in mono-culture or co-culture with 20% pCAFs. Type I interferon signaling (1µg/ml) blocking antibody was added or appropriate isotype controls were added (A). Cultures were treated with 10nM epirubicin or untreated for 24h with antibodies readded. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), RNA was also extracted from the epithelial cells and equal amounts of RNA were used for qPCR (B) to determine relative expression of IFN-target genes OAS1 (left plot) and MX1 (right plot). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

5.3.4 The JAK-STAT inhibitor ruxolitinib reduces CAF1-induced chemoresistance in MDA-MB-231 and MDA-MB-157 cells

Blocking antibodies successfully inhibited CAF-dependent protection above, however, the process for moving these blocking antibodies forward towards assessing their effect in cancer patients is likely to be prohibitively slow and expensive. Therefore, I searched for an alternative that might potentially make it into clinic more quickly if appropriate. A JAK-STAT inhibitor, ruxolitinib, was chosen as it has been shown to have high efficacy for inhibiting interferon signaling via binding to JAK2 (Thomas et al, 2015). Ruxolitinib is already available within the NHS and is used for treatment of myelofibrosis.

Experiments were performed broadly as above to assess colony formation in MDA-MB-231 or MDA-MB-157 cells after mono-culture or co-culture with CAF1s, and after treatment with epirubcin and/or ruxolitinib. qPCR was again used to assess interferon pathway activity. In this case, mono-cultures or co-cultures with CAF1s were pre-treated for 24h with either vehicle control or 10nM ruxolitinib and new vehicle or ruxolitinib was added with epirubicin treatment for 24h. Colony forming potential of the epithelial cells was then determined as previously (Figures 5.6, MDA-MB-231 cells, and 5.7, MDA-MB-157 cells).

As previously, epirubicin reduced survival in both MDA-MB-231 and MDA-MB-157 cells, and the presence of CAF1 cells induced relative chemoresistance (p values 0.067 and $p<0.01$ respectively). More interestingly, ruxolitinib significantly inhibited CAF-induced protection in MDA-MB-157 (Figure 5.7A, right panel, $p<0.01$), and a trend towards the same result was seen in MDA-MB-231 cells, although this inhibition was not significant (Figure 5.6A, right panel, p value 0.114). Analysis of interferon pathway activity by qPCR highlighted a trend that ruxolitinib inhibited CAF-induced interferon signaling in both MDA-MB-231 and MDA-MB-157 cells, as indicated by reduced levels of OAS1 and MX1, although this was not significant (Figure 5.6B and 5.7B, 231 p values 0.132 and 0.240 respectively, 157 p values 0.589 and 0.394 respectively).

It was also evident from these data that inhibition of interferon signaling, in this case with ruxolitnib, inhibited the ability of CAF1 cells to enhance the clonogenic plating efficiency of MDA-MB-231 and MDA-MB-157 cells in the absence of epirubicin (Figure 5.6A and 5.7A).

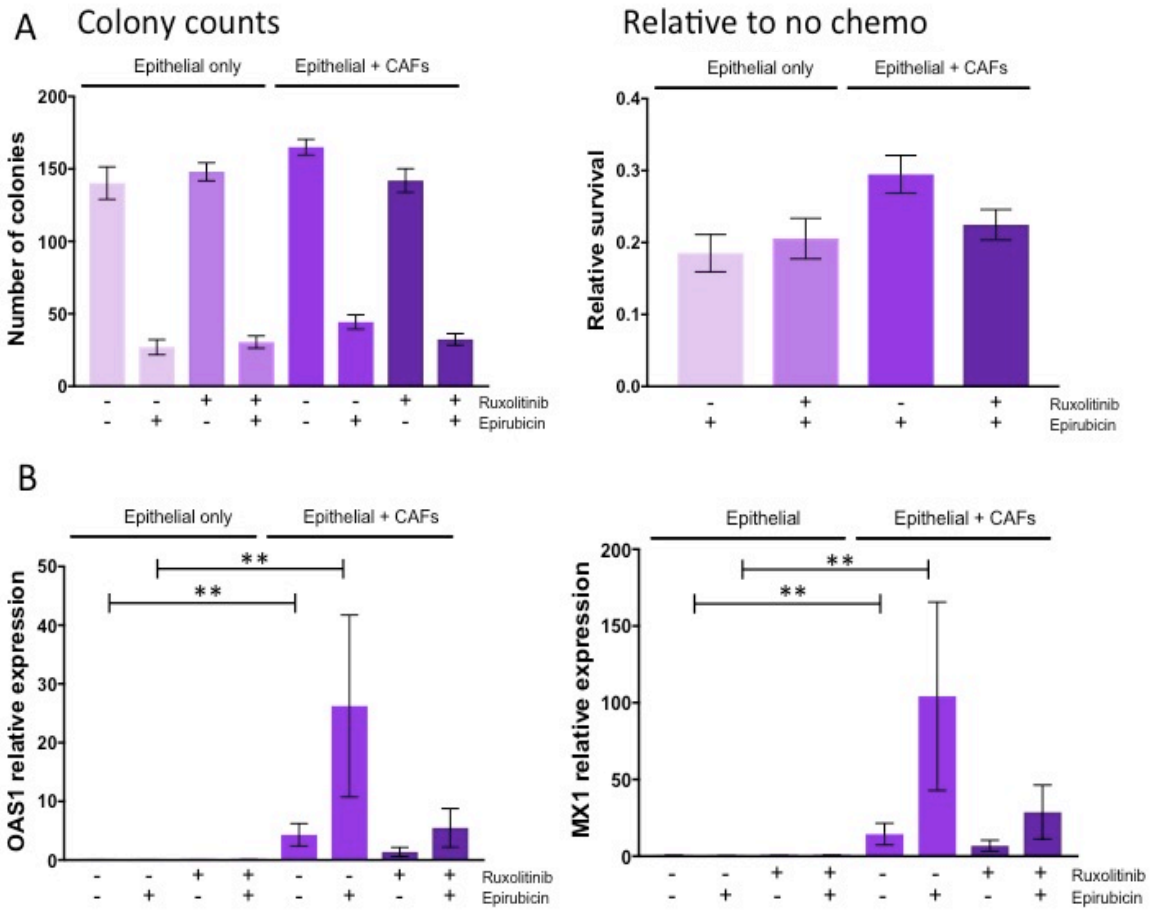


Figure 5.6: Ruxolitinib reduces, although not significantly, CAF1-induced chemoresistance in MDA-MB-231 cells

Cultures of MDA-MB-231-GFP/luc cells were established in mono-culture or co-culture with 20% immortalised breast CAF1s. Ruxolitinib was added to cultures at 10nM or vehicle control (A). Cultures were treated with 10nM epirubicin or untreated for 24h with ruxolitinib/vehicle readded. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), RNA was also extracted from the epithelial cells and equal amounts of RNA were used for qPCR (B) to determine relative expression of IFN-target genes OAS1 (left plot) and MX1 (right plot). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

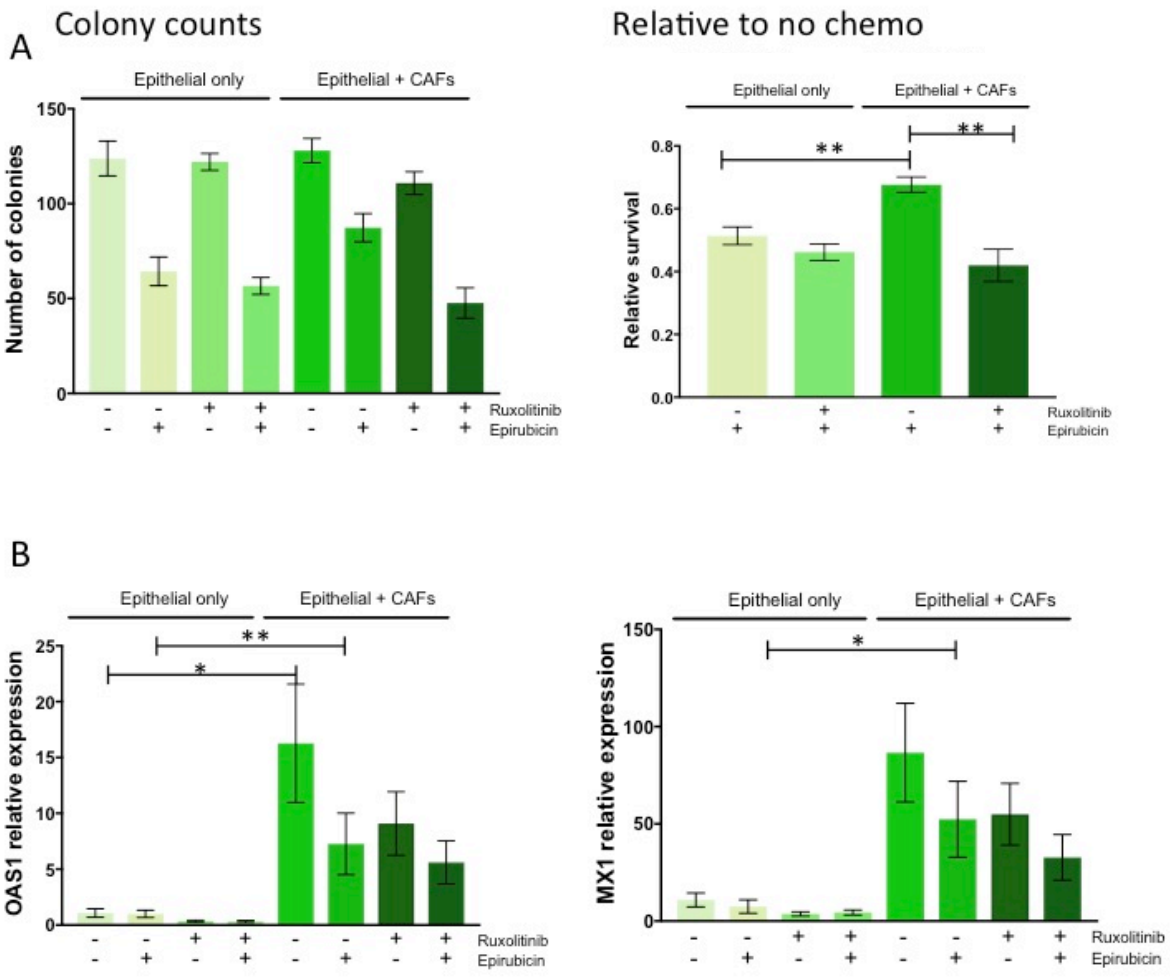


Figure 5.7: Ruxolitinib significantly reduces CAF1-induced chemoresistance in MDA-MB-157 cells

Cultures of MDA-MB-157 cells were established in mono-culture or co-culture with 20% immortalised breast GFP-CAF1s. Ruxolitinib was added to cultures at 10nM or vehicle control (A). Cultures were treated with 10nM epirubicin or untreated for 24h with ruxolitinib/vehicle readded. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), RNA was also extracted from the epithelial cells and equal amounts of RNA were used for qPCR (B) to determine relative expression of IFN-target genes OAS1 (left plot) and MX1 (right plot). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

5.3.5 Ruxolitinib inhibits pCAF-induced chemoresistance in MDA-MB-231 cells

The same experiment as in section 5.3.4 was also performed using pCAFs derived from a TNBC, and MDA-MB-231-GFP/luc cells (Figure 5.8). Ruxolitinib significantly inhibited pCAF-induced chemoresistance in MDA-MB-231 cells (Figure 5.8A, right panel, $p<0.05$), and also inhibited pCAF-induced interferon signaling, although this was not significant (Figure 5.8B, p values 0.200 and 0.200 respectively).

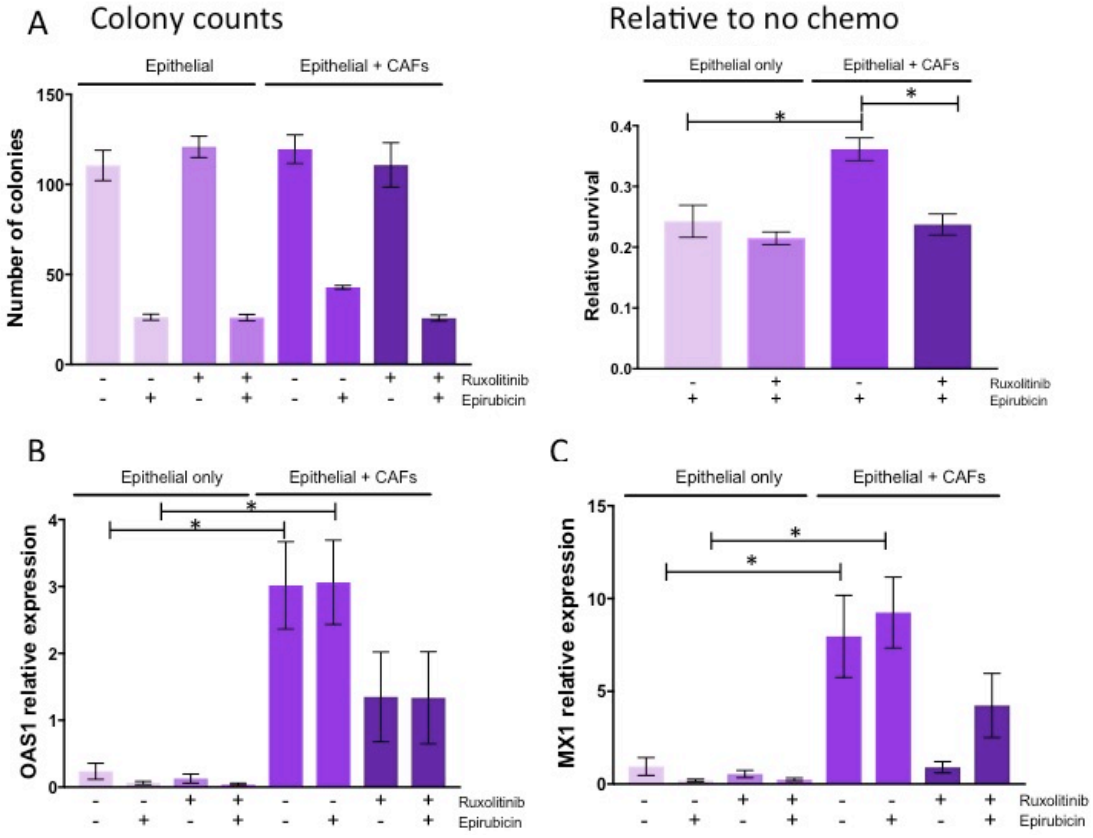


Figure 5.8: Ruxolitinib significantly inhibits pCAF-induced chemoresistance in MDA-MB-231

Cultures of MDA-MB-231-GFP/luc cells were established in mono-culture or co-culture with 20% primary breast CAFs. Ruxolitinib was added to cultures at 10nM or vehicle control (A). Cultures were treated with 10nM epirubicin or untreated for 24h with ruxolitinib/vehicle readded. Cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. Plates were then stained with crystal violet and individual colonies were counted. Data are shown as numbers of colonies (left plots), or as counts normalised to the no treatment value for each individual fibroblast proportion (right plots), RNA was also extracted from the epithelial cells and equal amounts of RNA were used for qPCR (B) to determine relative expression of IFN-target genes OAS1 (left plot) and MX1 (right plot). qPCRs were normalised to ACTB. Data represent the mean(+/- SE) for n=3. Two tailed Mann-Whitney U tests were carried out and significant differences shown. P value *<0.05.

5.3.6 MiR-155 inhibition reduces CAF1-induced chemoresistance of MDA-MB-231 cells

Inhibition of miR-155 is another potential mechanism of inhibiting CAF-induced interferon activity and thereby CAF-induced chemoresistance, based on its known role as a positive regulator of interferon signaling, and my previous observations that miR-155 was up-regulated by CAFs (section 4.3.2). Furthermore, I previously demonstrated that manipulation of miR-155 levels showed a trend to alter sensitivity of MDA-MB-231 and MDA-MB-157 cells to epirubicin treatment in the absence of CAFs (section 4.3.3/4).

A slightly adapted version of the clonogenic assay that has previously been used was carried out to determine whether inhibiting miR-155 in epithelial cells could inhibit CAF1-induced effects. Prior to seeding MDA-MB-231-GFP/luc cells in mono-culture or co-culture with CAF1 cells, MDA-MB-231-GFP/luc cells were transfected with either control inhibitor miRNAs or targeted miR-155 inhibitors. The protocol for the previous experiments was then followed for both assessment of clonogenic potential, and use of qPCR to monitor interferon pathway activity. In this case, additionally, qPCR was also used to monitor miR-155 expression.

Inhibition of miR-155 in the MDA-MB-231 cells significantly inhibited CAF1-induced chemoresistance in the MDA-MB-231 cells when compared to control transfected cells (Figure 5.9A, right panel, $p<0.05$). qPCR analysis confirmed the expected CAF-induced up-regulation of miR-155, particularly when treated with epirubicin ($p<0.0001$), and the successful inhibition of miR-155 expression by the specific miR inhibitor (Figure 5.9B, $p<0.05$). Although not significant, qPCR also suggested that miR-155 inhibition in epirubicin-treated cultures reduced CAF-induced interferon signaling activation, as demonstrated by reduced levels of OAS1 and MX1 (Figure 5.9C p values 0.393 and 0.588 respectively).

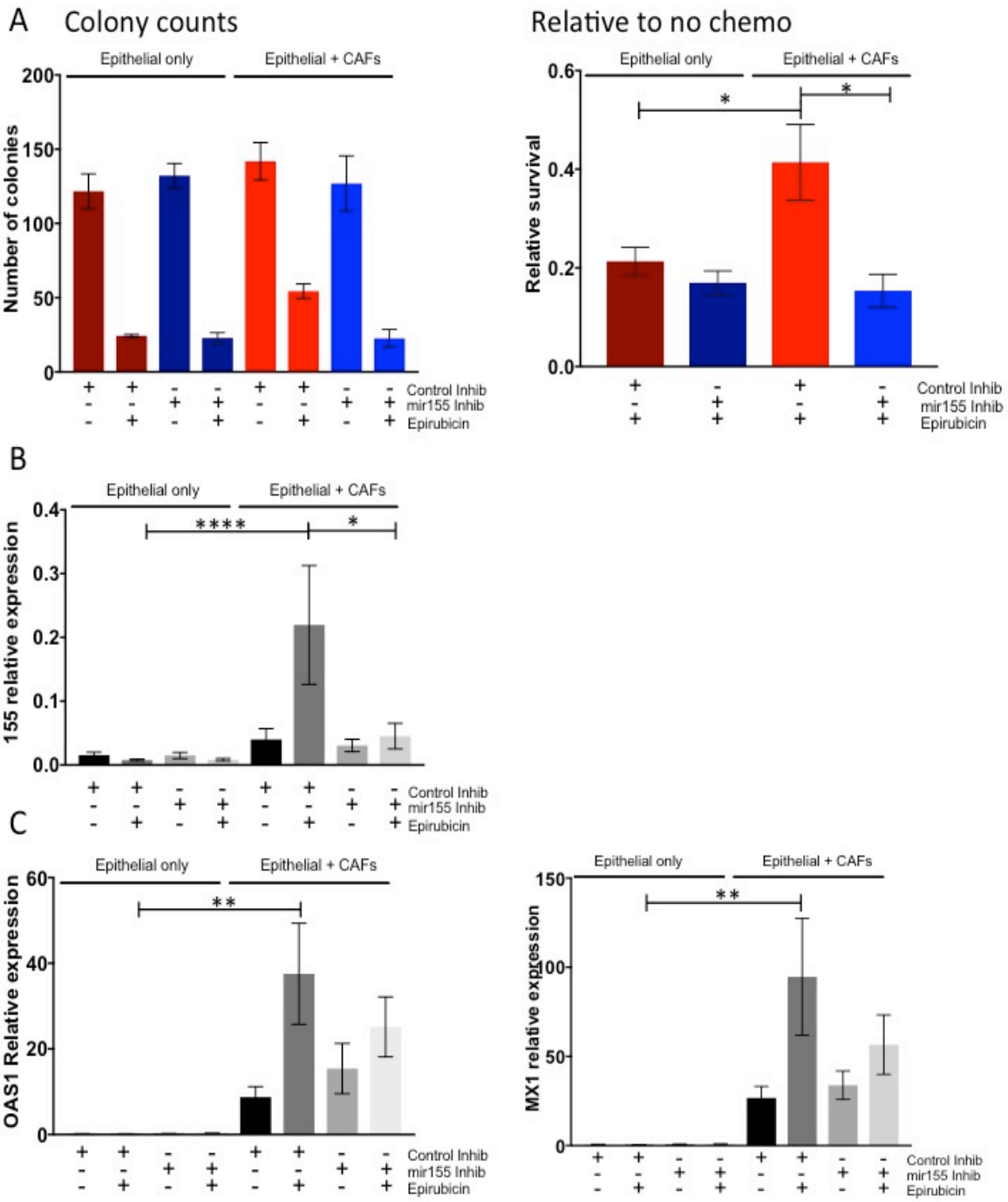


Figure 5.9: MiR-155 inhibition significantly inhibits CAF1-induced chemoresistance in MDA-MB-231 cells

MDA-MB-231-GFP/luc were transfected with control or miR155 inhibitor. Transfected MDA-MB-231-GFP/luc cells were plated in mono-culture or co-culture with 20% immortalised breast CAF1s. MDA-MB-231-GFP/luc had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Co-cultures were treated with 10nM epirubicin or untreated for 24h. Co-cultures were then separated into pure epithelial populations by FACS. 500 epithelial cells were then seeded at low density and left for 14 days for colonies to form. RNA was extracted from remaining cells. Plates were then stained with crystal violet and individual colonies were counted. Data are normalised to the no treatment value for each individual fibroblast proportion and represent the means (+/- SE) of 3 independent experimental repeats (A). RNA was extracted from the MDA-MB-231 cells and relative expression of miR-155 (B) or OAS1 and MX1 (C) determined by qPCRs. Data represent means (+/- SE) of 3

independent experimental repeats. Two-tailed Mann-Whitney U tests were carried out with any significant differences shown between no treatment 0% to 20% fibroblasts, treated 0% to 20% fibroblasts and treated control 20% to miR155 20%. P values * <0.05, **<0.01 and ****<0.0001.

5.4 Discussion

The aims of the work in this chapter were to identify potential mechanisms to inhibit CAF-induced interferon signaling and to determine if this would reduce CAF-induced chemoresistance in TNBC cells.

5.4.1 Use of antibodies for inhibition of interferon signaling

Antibodies provide successful current, and promising future therapeutic possibilities through their ability to bind selectively to cancer cells or to soluble targets, and thereby influence cancer cell function. Use of blocking antibodies to inhibit the interferon signaling pathway could therefore be a viable option in preventing interferon activation and chemoresistance. This could be done by either blocking soluble interferon ligands or by blocking interferon receptors. The use of blocking antibodies to block soluble ligands represents a viable option as evidenced by the fact that an antibody drug has been successful in sequestering VEGF-A. Bevacizumab, which binds and sequesters VEGF-A, inhibits angiogenesis, which in turn inhibits tumour growth and proliferation (Ferrara et al, 2004). *In vitro,* blocking of soluble interferons has also been successful in targeting soluble IFN-β (Hosein et al, 2015). Although soluble ligand blocking can be extremely effective and could have been used to block soluble interferons, this approach was not used because of the diversity of the interferon ligands, which might require multiple different antibodies.

Antibodies that bind to and block surface receptors have proved succesful in the treatment of cancer, and this was the approach I chose to use in this chapter. Blocking antibodies act by neutralizing the effects of oncogenic pathways and are extremely effective treatment options in breast cancer with various monoclonal antibodies currently being used in the clinic (Simpson and Caballero, 2014). Cetuximab is used in breast cancer treatment to reduce

activation of the EGFR pathway by binding to the extracellular domain of EGFR. Binding of cetuximab therefore prevents EGF binding to its receptor and transduction of the signaling cascade. In addition, cetuximab can lead to receptor internalization and degradation of EGFR and down-regulation of EGFR expression (Herbst et al, 2002). Both trastuzumab and pertuzumab are targeted treatments available for HER positive breast cancer patients. Trastuzumab acts by binding to the extracellular domain of HER2 receptors with high affinity (Vu et al, 2012). Pertuzumab binds to the extracellular dimerization domain II of HER2 receptors with pertuzumab and trastuzumab able to work in syngery with one another as they bind to different epitopes (Franklin et al, 2004). In addition, pertuzumab and trastuzumab can be given in combination with docetaxel (Baselga et al, 2010). The current use of blocking antibodies targeting cell surface receptors as effective breast cancer therapeutics was a determining factor for their use in this chapter to block interferon signaling.

A survey of relevant literature identified 2 previously published studies in which IFN blocking antibodies were used in the context of breast cancer, one of which even involved the presence of CAFs in co-culture with breast cancer cells. Therefore, these studies are highly-related to work covered within this chapter. Blocking antibodies have been used to bind to the Type I interferon receptor (IFNAR1, clone MMHAR2) to prevent activation of the intra-cellular signaling cascade in ER-positive aromatase inhibitor resistant breast cancer cells (Choi et al, 2015). The Type I blocking antibody clone used in this study is the same clone that I have used this chapter, although it is interesting to note that these authors used western blot analysis of expression of the ISG IFITM1 (the protein on which their study was focused) to confirm interferon signaling blockade, rather than my qPCR analysis of OAS1 and MX1. In their study, the Type I blocking antibody or IFITM1 knockdown both separately reduced cell proliferation and sensitized the resistant cells to estrogen deprivation (Choi et al, 2015). Type I blocking antibodies targeted to soluble IFN-β have also been used in the context of CAFs and breast cancer. Using gene expression profiling, a subgroup of interferon positive CAFs, which expressed ISGs and released IFN-β, were identified. Indirect co-culture of the interferon positive CAFs with MCF-7 cells promoted MCF-7 cell growth, with the blocking antibody inhibiting

this effect. However, confirmation of decreased interferon signaling in the target MCF-7 cells was not performed (Hosein et al, 2015). Targetting of soluble IFN-β differs from targeting the Type I receptor, as above and in my work, and – critically - does not target potential roles of other Type I interferons.

In the studies highlighted above by Choi et al and Hosein et al, the authors did not attempt to block both Type I and Type II signaling in combination, and therefore my work with combining antibodies to block both pathways appears to be unique in the context of breast cancer assays in tissue culture. Interestingly, the combination of both Type I and II did not have additive effects in terms of inhibiting chemoresistance (Figure 5.1), and curiously appeared to work less well in combination than individually. It is plausible that the addition of both antibodies caused some steric hindrance of the binding of each antibody to its target receptor. Therefore, this combination was not explored further. Furthermore, CAF-induced activation of interferon signaling and CAF-induced chemo-protection in MDA-MB-157 was not inhibited by Type II blocking approaches (Figure 5.4) suggesting that Type I signaling may be the dominant influence. However, it is possible that the dose of the Type II neutralization antibody was not enough in this cell line, although it proved sufficient in MDA-MB-231 cells, or it may be that the Type I activation is so dominant in the MDA-MB-157 cell line that the contribution of Type II is relatively unimportant. It is worth highlighting that MDA-MB-157 cells certainly can respond to Type II interferons, as the presence of recombinant IFN-γ in section 4.3.5 and Figure 4.5 caused a (slight) increase in chemoresistance when used on its own and significantly increased chemoresistance in combination with IFN-α.

Based on *in vitro* data from this chapter, use of Type I neutralizing antibodies in combination with epirubicin has potential as a treatment option for TNBC patients with high proportions of CAFs within their tumours, as a method for reducing CAF-induced chemoresistance and therefore making chemotherapy more effective. However, a concern would be that use of IFN blocking antibodies could potentially inhibit tumour suppressing immune cell responses, given the immune cell roles of interferons previously highlighted in section 1.14.1. Nevertheless, interferon blockade during chemotherapy, when the

immune system is suppressed in any event, remains worthy of further exploration.

5.4.2 Use of small molecules for inhibition of interferon signaling

As well as the use of antibodies to inhibit interferon function at the level of ligand or receptor, small molecule inhibitors of the intra-cellular JAK/STAT cascade have also been developed. The main mechanism that these drugs employ is inhibition of the JAKs; some JAK inhibitors have selective potency for both the predominant JAKs (JAK1 and JAK2), whereas other drugs only target one particular JAK (JAK1, 2 or 3). One JAK inhibitor, Tofacitinib, can target all 3 JAKs (JAK1, 2 and 3) (Thomas et al, 2015).

JAK inhibitors are undergoing clinical trials to treat a variety of different diseases, with some inhibitors approved for clinical use in selected diseases. Tofacitinib, a JAK1, 2 and 3 inhibitor, is used to treat patients with rheumatoid arthritis, skin conditions including psoriasis, and irritable bowel syndrome (Miklossy et al, 2013). A further JAK inhibitor, ruxolitinib, has been approved for use myelofibrosis, but of more relevance to my work, ruxolitinib is also being trialled for use in treatment of different forms of leukemia: acute myeloid, chronic myeloid and acute lymphocytic leukemia (Furumoto et al, 2013). However, the use of JAK inhibitors in solid tumours is less advanced. Different tumours have been implicated as potential targets of JAK inhibitors, as a result of STAT up-regulation being associated with recurrences and reduced overall survival in NSCLC, prostate cancer and melanoma (Xu et al, 2014, Messina et al, 2008, Mirtti et al, 2013), but JAK inhibitors have not yet been used in these solid tumours.

Clinical trials have been carried out using JAK inhibitors in patients with metastatic breast cancer. Patients, in this clinical trial, had been heavily pre-treated with up to 48% having received 3 or more regimens of chemotherapy in the metastatic setting (Stover et al, 2018). The use of Ruxolitinib, a JAK1/2 selective inhibitor, is currently being explored as a potential treatment option for these patients due to high expression levels of STAT3 in metastatic disease.

Although up-regulation of STAT3 was observed in metastatic TNBC patients, no responses were observed in 21 out of 23 patients following ruxolitinib treatment despite on-target activity. The use of ruxolitinib as a single agent to improve treatment options for metastatic TNBC patients, therefore, did not meet the primary efficacy endpoint in this study (Stover et al, 2018).

Interestingly, ruxolitinib showed potential in my experiments, which are also in the context of TNBC, albeit with major differences in essentially all other ways. In section 5.3.4 and 5.3.5, ruxolitinib reduced CAF-induced chemoresistance in both MDA-MB-231 and MDA-MB-157 cells. These data support the idea that ruxolitinib may have some efficacy in combination with epirubicin, by reducing CAF-induced chemoresistance. The clinical trial carried out in metastatic breast cancer highlighted above used ruxolitinb as a single agent and it seems until only recently that studies have been reported regarding clinical use in combination with cytotoxic chemotherapy. Ruxolitinib has been used in combination with capecitabine in HER2 negative breast cancer, with an overall response rate of 28.9% for the combination in comparison to 13.7% in the capecitabine alone group (O'Shaughnessy et al, 2018); this appears to support the combination of ruxolitinib and traditional cytotoxics. Clinical trials of ruxolitinib in breast cancer are ongoing with ruxolitinib used in combination with trastuzumab in metastatic HER2 positive breast cancer, without the use of cytoxic chemotherapy (Cigler et al, 2018), and in stage III triple negative inflammatory breast cancer in combination with paclitaxel followed by doxorubicin/cyclophosphamide. The results of these studies are still being collated (Overmoyer et al, 2018).

5.4.3 MiRNAs or miRNA inhibitors (specifically miR-155 inhibitor) as viable therapeutic options.

In recent years, work has been carried out to develop RNAs, and particularly miRNAs, as potential cancer therapeutics (Mehrgou and Akouchekian, 2017). The advantages of this approach are the low cost of synthesis of miRNAs as well as their small molecular size and the ease of design. MiRNA therapeutics act by either mimicking tumour suppressor miRNAs or by inhibiting oncogenic

miRNAs. Due to the ease of production of miRNA therapeutics, the use of miRNAs and miRNA inhibitors are a potential therapeutic option (Yeung and Jeang, 2011), although they have not yet reached routine clinical practice. This is of potential interest here, since I have identified inhibitors of miR-155 as a potential therapeutic in combination with epirubicin (Figure 5.9).

Various different miRNAs are being tested *in vitro* and *in vivo* for their potential for clinical use. For example in breast cancer, both miR-132 and miR-10b have oncogenic properties aiding in cancer progression, therefore inhibitors of miRNAs have been tested *in vivo* to try to target these miRNAs and prevent their function (Anand et al, 2010). Anti-miR-21 has also been investigated as a potential treatment option in human breast cancer. Anti-miR-21 in MCF-7 and MDA-MB-231 cells *in vitro* and *in vivo* inhibited growth as well as migration of breast cancer cell (Yan et al, 2011). I investigated the results of inhibiting miR-155 (section 4.3.3/4 and 5.3.6), and inhibition of this specific miRNA has been previously investigated in the context of breast cancer. MiR-155 antisense oligonucleotides (mechanistically the same as the miR-155 inhibitor used in this thesis) were shown to reduce the viability of MDA-MB-157 cells *in vitro*, and to reduce cell growth of xenograft tumours (Zheng et al, 2013), although this function was not linked to interferon activity, as in my work. This study, and my own, do support the concept that inhibition of miR-155 could have therapeutic value in the context of breast cancer.

Due to the ease of production of miRNA therapeutics, the main area for work and a key difficulty with miR-based therapeutics is the delivery method. *In vitro* use of miRNA therapeutics is relatively simple with the ease of transfection into cells. However, the ability to target miRNA therapeutic entry into only cancer cells in cancer patients (without targeting normal cells) is problematic. Neverthesless, one miRNA-based therapeutic, miravirsen - an inhibitor of miR-122, is currently in clinical trials (van der Rhee et al, 2016). Miravirsen is designed to work without specific targeting in the belief that it will be taken up sufficiently by cancer cells for efficacy when delivered systemically, without excess toxicity from non-target tissues. While miRNA therapeutics clearly have

potential - and specifically targeting miR-155 in the context of this work - efficient delivery remains a barrier to future development.

5.5 Conclusion and aspects of future work

It is clear that interferon signaling can be inhibited in multiple different ways, from targeting the receptor to prevent interferon binding and activating signaling, to targeting the JAK/STAT pathway to inhibit the intra-cellular signaling cascade, or inhibiting formation of the correct transcriptional complex by inhibiting miR-155. Inhibition of interferon signaling using any of these mechanisms can reduce CAF-induced chemoresistance in TNBC cells, at least *in vitro*. To identify if these inhibitory mechanisms could be used in combination with chemotherapeutic agents as a possible treatment option for patients, *in vivo* studies will be required.

Chapter 6 - What are the cellular mechanisms of cancer-associated fibroblast-induced chemoresistance in breast cancer cells?

6.1 Abstract

CAF-induced chemoresistance in TNBC was identified in previous chapters. In this chapter, I have attempted to investigate the direct effectors that drive this chemoresistance down-stream of interferon signaling. I have also explored the nature of the CAF-induced increase in clonogenicity observed in the absence of chemotherapy.

The first potential mechanism of CAF-induced chemoresistance to be tested was altered drug loading in the epithelial cells. Using fluorescent-assessment of epirubicin loading, it was shown – surprisingly – that CAFs-induced *increased* drug uptake in MDA-MB-231 cells, rather the decrease that would be expected if this were the mechanism of protection. The second potential mechanism of CAF-induced chemoresistance to be tested was alteration of the cell cycle in the epithelial cells. CAFs did not induce significant changes in the proportions of MDA-MB-231 cells in each phase of the cell cycle, although there was a trend for CAFs to induce reduced S-phase representation, which is potentially compatible with this as a resistance mechanism. Finally, the CAF-induced increase in clonogenicity was assessed; this was shown to be associated with an increase in anoikis resistance in both MDA-MB-231 and MDA-MB-157 cells.

I concluded that CAF-induced changes in epithelial cell cycle profiles, or in sensitivity to apoptotic stimuli remain plausible potential mechanisms by which CAFs induce chemoresistance in epithelial breast cancer cells.

6.2 Introduction

I have shown in previous chapters that CAFs induce chemoresistance in TNBC cells, probably through paracrine signaling, leading to up-regulation of the interferon signaling pathway. However, the molecular mechanisms that allow activated interferon signaling to result in chemoresistance are not understood.

Interferon signaling results in activation of the JAK/STAT signaling cascade as highlighted in section 1.12.1 and 1.12.2. It seems reasonable to assume that the mediators of the chemoresistance seen in my experiments must be down-stream of JAK activation. A number of studies have investigated the down-stream targets of interferon and JAK activation in various cell types. One of the major sets of kinases that have been shown to induce IFN stimulated responses are the MAP kinases. p38 MAP kinases have been shown to be activated by the Type I IFN receptor (Uddin,1999). ERK is another MAP kinase family member activated by IFNs, although ERK appears to be activated by both Type I and Type II IFN receptors. MEK/ERK activation is driven by JAK1 activation in response to IFNs. JAK1 can activate Raf1 to enable downstream effectors of the MEK/ERK pathway (Platanias, 2005). Jnk kinase, a further member of the MAP kinase family, has also been reportedly activated by both Type I and Type II IFNs to mediate IFN responses (Zhao et al, 2011). Another alternative downstream effector of IFN signaling is the mTOR pathway. Activation of both Type I and II receptors led to activation of upstream effectors of mTOR, which have been identified as PI3K and Akt (Kaur et al, 2008, Kaur et al, 2008).

All of these potentially activated signaling pathways remain substantially up-stream of the actual effector molecules or functions that could directly lead to chemoresistance. Multiple different direct chemoresistance mechanisms have been explored previously to gain increased mechanistic understanding, and in efforts to use these insights to bypass their potential influences to make chemotherapy treatment more effective (O'Reilly et al, 2015). One such mechanism that has been explored is the role of the xenobiotic membrane transporters of the ATP-binding cassette (ABC) transporter family. The main

ABC transporters that have been investigated in TNBC are multi-drug resistant protein 1 (MRP1, ABCC1), breast cancer resistance protein (BCRP, ABCG2) and P-glycoprotein (MDR1, ABCB1) (Kovalev et al, 2013). High expression and activity of these transporters has been shown to contribute to chemoresistance in TNBC by reducing intra-cellular levels of the chemotherapeutic agents. BCRP, in particular, has been shown to reduce intra-cellular levels of doxorubicin in TNBC cells (Chen et al, 2014), while MDR1 acts on various different classes of chemotherapeutics, including paclitaxel, doxorubicin and epirubicin (Wind et al, 2011).

Another direct effector mechanism of chemoresistance in cancers is increased resistance to apoptosis. In a large number of cancers, apoptosis signaling is dysregulated leading to evasion of cell death and enhanced survival of cancer cells. It has been shown in multiple different types of cancer such as pancreatic, ovarian and lung that there is up-regulation of anti-apoptotic factors (Mohammed et al, 2015). For example, over-expression of Bcl-2, or other family members such as Bcl-xl or Mcl-1 has been shown to be associated with chemoresistance in cancers (Azmi et al, 2009, Quin et al, 2011).

Finally, another potential mechanism of chemoresistance is the induction of changes to cell cycle. A large number of chemotherapeutic drugs' main mechanism of action occurs during specific phases of the cell cycle, for example at mitosis (taxanes) or during S phase (anthracyclines) (Hassan et al, 2010). Therefore, the proportion of cells in each cell cycle phase, and the overall proliferation rate of cells, can be key factors in determining chemotherapeutic function. Studies in early breast cancer have shown patients with high proliferation rates (determined by high Ki67 expression) responded better to NAC and achieved a pCR of 40% in comparison to lower proliferation rates (determined by low Ki67 expression), where pCR was 19% with patients more resistant to chemotherapy (Alba et al, 2015).

Therefore, based on some of the acknowledged chemoresistance mechanisms, in this chapter I have investigated potential mechanisms that could be directly

responsible for the CAF-induced chemoresistance I have seen in selected TNBC cell lines.

6.3 Results

6.3.1 CAF1 cells increase intra-cellular epirubicin levels in co-cultured MDA-MB-231 cells

A possible mechanism of CAF-induced chemoresistance is reduced drug uptake by the epithelial cells, perhaps mediated by enhanced expression or activity of xenobiotic transporters such as the ABC transporters. In order to examine this, drug loading assays were performed taking advantage of the fact that epirubicin is itself fluorescent.

MDA-MB-231-GFP/luc cells were either mono-cultured or co-cultured with 20% CAF1 cells for 24h as previously. As a further control CAF1 cells were also mono-cultured. Cultures were treated with epirubicin for 24h and then analysed by flow cytometry to quantify intra-cellular epirubicin loading (Figure 6.1).

Surprisingly, CAF1s induced MDA-MB-231 cells to contain significantly more epirubicin compared to the levels in MDA-MB-231 mono-culture (compare dark blue bars in Figure 6.1, $p<0.01$). Conversely, uptake of epirubicin appeared to decrease in the CAF1s from the co-culture with MDA-MB-231 cells, compared to CAF mono-cultures (compare light blue bars in Figure 6.1), although this difference was not significant (p value 0.224). I concluded that CAFs were not protecting TNBC cells from epirubicin by reducing the loading of the drug into the cells. In fact, quite the reverse appeared to be happening – CAFs induced additional drug loading in the cancer cells.

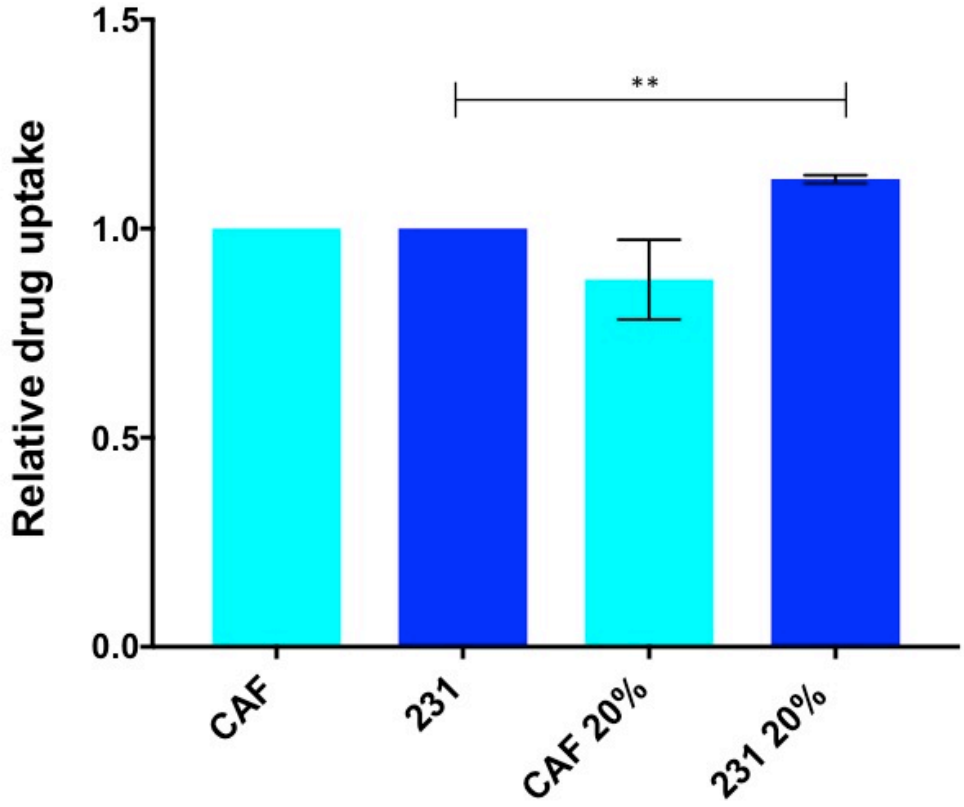


Figure 6.1: CAF1s enhance intra-cellular epirubicin levels in MDA-MB-231
Mono-cultures of MDA-MB-231-GFP/luc cells or CAF1 cells, and co-cultures of MDA-MB-231-GFP/luc cells and 20% CAF1 cells were established. Cultures were treated with 1μM epirubicin for 24h. Cells were then analysed by flow cytometry, gating on viable cells (using FSC v SSC) and on MDA-MB-231-GFP/luc cells or CAF1 cells separately (on the basis of GFP expression). Epirubicin loading was determined by red fluorescence as the median of BL-3 fluorescence. Co-culture values (denoted "20%") were normalised to the matched mono-cultures values. Data represent the means (+/- SE) of 3 independent biological replicates. Two-tailed Mann-Whitney U tests were carried out and significant differences to corresponding cell type are shown. P values **<0.01.

6.3.2 CAF1s may induce changes in cell cycle progression

CAFs are known to influence growth and metastatic properties of cancer cells (see section 1.9). Therefore, it was possible that the presence of CAFs was having an impact on the cell cycle of the co-cultured TNBC epithelial cells. A decrease in epithelial proliferation might provide protection from chemotherapy, since cytotoxic chemotherapy agents target proliferating cells. It should, however, be noted that the reported influence of CAFs on epithelial proliferation is generally that CAFs stimulate increases in proliferation rather than the reverse (Verghese et al, 2013). In order to examine this, cell cycle profiling of mono- or co-cultures of MDA-MB-231 and CAF1 cells was undertaken.

MDA-MB-231-GFP/luc cells were either mono-cultured or again co-cultured with CAF1 cells, as usual, and treated with epirubicin. After 24h, cells were treated with a fluorescent dye to allow quantification of cellular DNA and therefore measurement of the proportion of cells in G1, S or G2 phase of the cell cycle. Cell cycle profiles were determined by flow-cytometry in the GFP positive MDA-MB-231 cells. Following the production of the cell cycle profile, the proportion of cells in each stage of the cell cycle was determined using ModFit cell cycle software, and proportions of cells in each stage were determined (Figure 6.2).

MDA-MB-231 in co-culture with 20% CAF1 cells were compared with those in mono-culture; there was an increase in the co-culture of the proportion of cells in G0/G1 and in G2, and a corresponding decrease in the proportion of cells in S phase (compare the red bars to the matched purple bars in Figure 6.2), although none of these differences were statistically significant (p values 0.400, 0.200 and 0.200 respectively). I concluded that although CAF1 cells did not induce significant changes in epithelial cell cycle profiles, a potential trend for cell cycle changes was apparent.

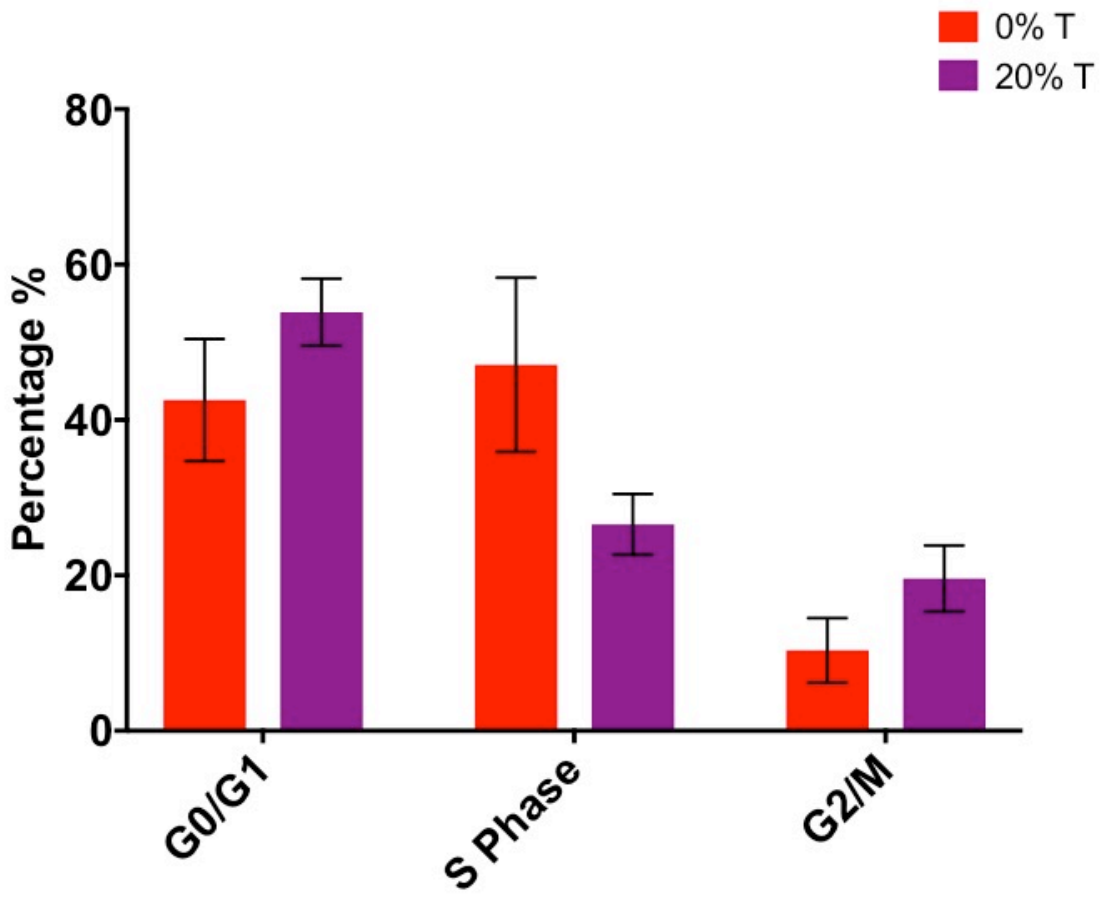


Figure 6.2: CAF1s do not induce significant differences in epithelial cell cycle profile

Mono-cultures of MDA-MB-231-GFP/luc cells and co-cultures of MDA-MB-231-GFP/luc cells and 20% CAF1 cells were established. Cultures were treated with 10nM epirubicin for 24h. Dye cycle violet was then added to cultures for 30 min prior to flow cytometry. Cells were then analysed by flow cytometry gating on viable cells (using FSC v SSC) followed by gating on dyecycle loaded viable cells (VL1 vs FSC). Singlet Dyecycle violet postive cells were then gated (VL1-H vs VL1-A) followed by identification of Dyecycle violet MDA-MB-231-GFP/luc cells in a population with CAF1 (VL1 vs BL1). VL1 was then counted to produce a cell cycle profile. Using ModFit software the percentage of cells in the different stages of the cell cycle was determined. Data represent the means (+/- SE) of 3 independent experimental repeats. Two-tailed Mann-Whitney U tests were carried out with no significant differences observed between 0% and 20% cultures

6.3.3 CAF1s significantly increase anoikis resistance in MDA-MB-231 and MDA-MB-157

In Chapter 3, I found that co-culture of the TNBC cell lines MDA-MB-231 or MDA-MB-157 with CAFs enhanced their replating efficiency after cell sorting (and in the absence of chemotherapy agents) (section 3.2.2; Figure 3.3 and 3.4). One hypothesis based on this observation was that the CAFs may induce resistance to the pro-apoptotic effects of detachment from the substrate. Apoptosis induced by detachment from the substrate – something to which most epithelial cells are susceptible – is referred to as anoikis (Chiarugi et al, 2008). CAF-induced anoikis resistance would be of interest since it would explain the enhanced plating efficiency mentioned above, but it could also potentially explain the chemoresistance if anoikis resistance were a reflection of a more general resistance to apoptosis, since it is well known that chemotherapeutics kill cells in part by inducing apoptosis (Kaufmann et al, 2000). Therefore, I carried out assays to test whether CAFs were indeed inducing anoikis-resistance in co-cultured TNBC cancer cells, using an established anoikis assay.

MDA-MB-231-GFP/luc or MDA-MB-157 cells were cultured alone or with increasing proportions of breast fibroblasts. In the case of MDA-MB-231-GFP/luc cells, either NF1s or CAF1s were used, while for MDA-MB-157 only GFP-CAF1s were used. Cultures were incubated for 48h. Epithelial cells were then purified by FACS on the basis of GFP expression. As previously, even cultures without fibroblasts (ie epithelial cells only) were sorted, in order to allow for properly-controlled comparisons to be made with epithelial cells sorted from the co-cultures. Standard numbers of collected epithelial cells were then suspended in medium in non-adherent plates for 36h. MTT assays were performed to quantify cells remaining viable under these conditions (Figure 6.3).

Co-culture with CAF1s induced dose-dependent and significant increases in survival in both MDA-MB-231 ($p<0.05$) and MDA-MB-157 ($p<0.05$) cells (Figure 6.3 B and C). By contrast, when MDA-MB-231 cells were co-cultured with NF1s,

there was no increase in survival. I concluded that CAFs, but not NFs, can induce anoikis-resistance in TNBC cell lines.

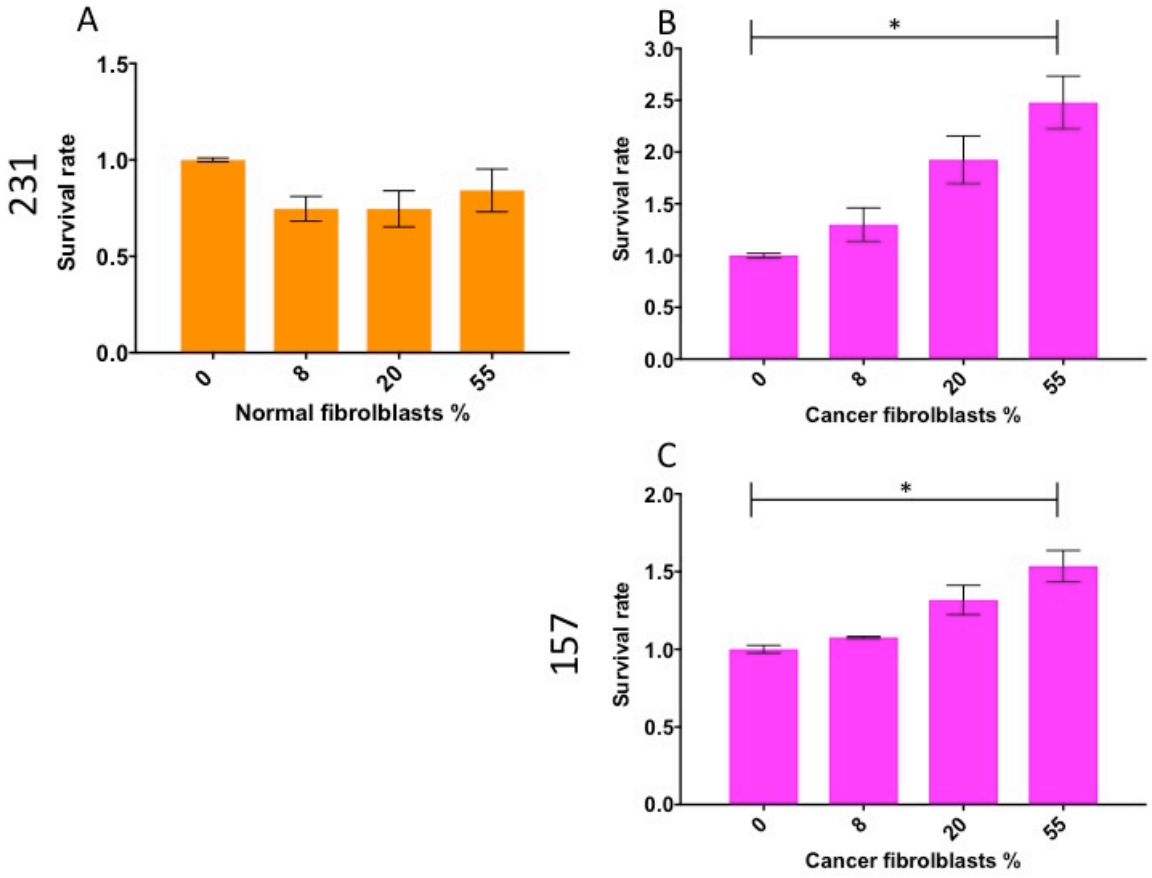


Figure 6.3: CAF1s enhance anoikis resistance in MDA-MB-231 and -157 cells
Co-cultures of MDA-MB-231-GFP/luc (A and B) or -157 (C) cells were established with various proportions of either immortalised normal breast fibroblasts (NF1) or immortalised breast CAFs (CAF1). Either fibroblasts or epithelial cells had been labelled with GFP, so the cell types could be differentiated by flow-cytometry. Co-cultures were cultured for 48h with a media change after 24h. Co-cultures were then separated into pure epithelial populations by FACS. 150,000 epithelial cells were then seeded on a low attachment plates for 36h and cells remaining viable after this were quantified using MTT assays. Survival is expressed relative to the epithelial mono-culture (0%) from each experimental repeat. Data represent the means (+/- SE) of 3 independent biological repeats. Linear regression analysis was carried out on no treatment for colony counts and normalised treated data with significant differences in the overall trend across the fibroblast proportions calculated. P values * <0.05

6.4 Discussion

The aims of the work in this chapter were to identify potential effector mechanisms of CAF-induced chemoresistance.

6.4.1 Anoikis resistance

In chapter 3 (section 3.3.2), I identified that co-culture with CAFs induced increases in epithelial plating efficiency. During this experiment, the cells remain in suspension for an extended period of time prior to replating for the clonogenic assays. Epithelial cells in suspension can respond by undergoing programmed cell death, which is termed anoikis in this context.

Anoikis occurs when epithelial cells become detached from the extracellular matrix (ECM), and it acts as a defense system to prevent any aberrant growth of cells in the absence of adhesion as well as preventing detached cells from colonizing inappropriate sites (Frisch and Francis, 1994). Commonly, when cells remain attached to the ECM, they are protected from anoikis, which is driven by anti-apoptotic and pro-survival signals from integrin signaling (Frisch and Ruoslahti, 1997). Even during migration of cells, integrin attachment to focal contacts can again activate signaling to produce pro-survival signals to prevent anoikis (Baum et al, 2008). Anoikis resistance is thought to be an essential pre-requisite for cancer progression and metastasis to distant organs for the formation of secondary tumours (Kim et al, 2011). It is thought that anoikis resistance in cancer cells can be achieved through a number of different mechanisms. Integrins can be dysregulated by expression changes occurring in cancer cells, for example more metastatic breast cancer cell lines (MDA-MB-435 and MDA-MB-231) expressed higher levels of integrin β5 or integrin β3 (Taherian et al, 2011). Increased expression of α6 integrin is also related to poorer survival of breast cancer patients (Friedrichs et al, 1995). Increased expression of integrins is functionally important to enable enhanced integrin signaling and activation of MEK and FAK for survival. Increased expression of integrins can also enable breast cancers to adapt to their metastatic site and enable colonization of secondary tumours (Guo et al, 2004). Another potential

mechanism that cancer cells have exploited to gain anoikis resistance is EMT, as it is thought that anoikis resistance is associated with cancer cells gaining a mesenchymal phenotype (Cao et al, 2016). A stimulus such as the presence of stromal cells, oncogene activation through epigenetic changes, or hypoxia can trigger EMT induction. Stromal cells, in particular CAFs, can induce EMT in breast cancer cells. Breast cancer MCF-7 cells cultured with CAFs showed increased expression of vimentin and lower expression of E-cadherin, which are known expression changes that occur in EMT (Soon et al, 2013). CAF-conditioned media can also transform breast cancer cell lines (MCF-7, T47D and MDA-MB-231) into a more aggressive and invasive phenotype through secretion of TGF-β from the CAFs, something that can be reversed by blocking TGF-β signaling (Yu et al, 2014). EMT induction in cancer cells leads to expression of pro-survival and anti-apoptotic genes to enable anoikis resistance (Chiarugi et el, 2008).

Finally, another mechanism involved in anoikis resistance is constitutive activation of signaling pathways involved in anti-apoptotic pathways, particularly the PI3K/Akt pathway (Davies et al, 1999). Constitutive activation of the PI3K/Akt pathway can occur in multiple different ways, such as overexpression of receptor tyrosine kinase receptors, constitutive activation of Ras (which is common in many cancers), or loss of PTEN expression (which is common in TNBC) (Tokunaga et al, 2008). Activation of PI3K and the conversion of PIP_2 to PIP_3 enables activation of Akt, which is enhanced by the loss of PTEN (which would otherwise convert PIP_3 back to PIP_2) (Vitolo et al, 2009). Activation of Akt leads to decreases in expression of pro-apoptotic Bid, Bim, Bad and Bax, and increases in expression of anti-apoptotic genes Bcl-2 and IKK. Src kinases have also been implicated in anoikis resistance. Sustained activation of Src kinases has been shown to phosphorylate focal adhesion kinase (FAK), which recruits and activates PI3K and thereby the anti-apoptotic pathway described above leading to inhibition of Bad and reduced caspase-2, -3, -8 and -9 function (Bouchard et al., 2007). Finally, growth factor signaling and activation of MAPK signaling leads to enhancement of cell survival as MAPK signaling induces degradation of the pro-apoptotic protein Bim (Reginato et al, 2003). Importantly, CAFs have also been shown to be involved in anoikis resistance. One study

has shown that CAFs can secrete elevated levels of insulin-like growth factor binding proteins (IGFBP) that are necessary for inhibition of anoikis in breast cancer cells, through stabilization of the anti-apoptotic protein Mcl-1 (Weigel et al, 2014), although in my experiments this may not be relevant since the anoikis resistance I see appears to be dependent on interferon signaling.

Interestingly, miRNAs have also been implicated in anoikis resistance as they have been shown to control expression of pro-apoptotic genes in breast cancer. For example miR-19, miR-21, miR-27, miR-29, miR-155, miR-181, miR-206, miR-221/222 and miR-375 can target and inhibit the function of pro-apoptotic genes such as Bim, Bax and Puma (Halytskiy, 2017).

From experiments carried out in Chapter 6, there was no direct data concerning whether CAFs led to expression/activity changes in any of these anoikis resistant pathways highlighted above. However, from gene level microarray data produced in Chapter 4, pathways known to be involved in anoikis resistance were not identified as significantly altered by CAFs in the presence of epirubicin. The use of IFN blocking antibodies in Chapter 5 reduced interferon-induction and halted the anoikis phenotype observed in colony count data sets (change in plating efficiency). Therefore, it is likely that interferons are up stream of CAF-induced anoikis resistance rather than other secretory molecules such as IGFBP. Interestingly from the miRNA screen in chapter 4, miR-155 was upregulated in the presence of CAFs. MiR-155 has already been implicated as a mediator of anoikis resistance by inhibiting the function of pro-apoptotic genes (Bim, Bax, Puma and caspase-3) discussed previously, and could therefore play a role in the CAF-induced anoikis resistance in TNBC observed in this chapter. In addition, given miR-155's role in enhancing interferon signaling, it is plausible that interferon signaling and its downstream targets (PI3K pathway), as well as the direct targets of miR-155, could therefore be the best candidate mediators of anoikis resistance.

From this chapter, it is clear that CAF-induced activation of interferon signaling can induce anoikis resistance in TNBC cells. It is plausible that clinically, if patients present with a higher proportion of CAFs in the primary tumour, cells

could become anoikis resistant and therefore more likely to survive in the circulation and metastasize to form secondary tumours.

6.4.2 Cell cycle changes and chemoresistance

My data suggest that CAFs do not induce significant changes in epithelial cell cycle profiles (section 6.3.2). However, these data contain some variance between individual repeats (n=3) and it is tempting to speculate that if the experiment could be made more reproducible, then significant findings may be revealed. The reason to discuss cell cycle arrest and chemoresistance in detail is that the results displayed a trend for reduced proliferation as a potential mechanism for chemoresistance.

Interestingly, the role of CAFs on the cell cycle has been investigated in cancer cells previously. As highlighted in section 1.10, CAFs can enhance the growth of breast cancer cells. The opposite influence has, however, been seen in other contexts. For examples, dermal fibroblasts have been shown to induce cell cycle arrest (in G0/G1), and block epithelial-mesenchymal transition in melanoma (Zhou et al, 2016) while CAFs in pancreatic cancer repressed cell growth in clonogenic assays and reduced the malignancy of pancreatic cells (Ding et al, 2018). However, CAF-induced cell cycle arrest has not directly been implied in breast cancer cells.

In cell cycle analysis performed in section 6.3.2, the cells were also treated with epirubicin, as has been standard in my chemoresistance studies. Previous studies have shown epirubicin itself induced cell cycle arrest occurring in G0/G1. Surprisingly, cell cycle arrest resulting from treatment with a number of agents all of which act in totally different ways, namely - quercetin, tamoxifen and epirubicin, has been attributed to changes in the expression levels of the same single molecule - p21. p21 is a CDK-inhibitor and can inhibit all stages of the cell cycle from inhibiting cyclin E–CDK2 and cyclin D-CDK4/6 activation in G1 to inhibiting Cdc2 activation in G2 (Karimian et al, 2016). Increased p21 levels have been shown to inhibit proliferation of breast cancer cells (Xiong et al, 2016), with down-regulation of p21 shown to remove tamoxifen induced cell

cycle arrest (Cariou et al, 2000). Critically, Type I and Type II interferons have been shown to regulate expression of p21 (Sangfelt et al, 2000). Type I interferons have been shown to induce cell cycle arrest in G0/G1. Increased levels of p21 were identified following IFN treatment (Subramaniam et al, 1997). More recently, Type I IFNs have been found to activate the p21 gene promoter and subsequent expression of p21. Both IFN-α2b and IFN-β were able to enable p21 expression, with p21 down-regulation reversing cell cycle arrest (Katayama et al, 2007). Type II IFN-γ can also induce p21 expression in MCF-7, however in this study p21 expression was apparently not detectable in MDA-MB-231 cells (Gooch et al, 2000).

Therefore, three observations can be brought together to suggest a mechanistic model. Firstly, interferons have previously been shown to induce cell cycle arrest, as described above. Secondly, I have shown CAFs to induce interferon-dependent chemoresistance in co-cultured epithelial cells (Chapter 5). Thirdly, my data suggest that CAFs may induce reduced proliferation in these co-cultured epithelial cells (albeit this was not significant; Figure 6.2), which would – if true – potentially lead to chemoresistance. Thus, a plausible mechanistic pathway would be that CAFs induce chemo-protective cell cycle changes in co-cultured epithelial cells, via interferon signaling.

Chapter 7- Discussion

The aims of the work in this thesis were to identify if CAFs play a role in chemoresistance in TNBC, to determine the pathways responsible for CAF-mediated chemoresistance and inhibit these as potential therapeutic options to improve chemotherapy response, and finally to investigate the effector mechanisms that enable CAF-induced chemoresistance.

7.1 CAF-induced therapy resistance in breast cancer

CAFs are known to enhance breast cancer progression, as highlighted in section 1.10. However, here, the primary goal was understanding whether CAFs are also involved in chemoresistance in breast cancer, focusing in particular on TNBC.

CAFs have previously been implicated in chemoresistance pathways in cancer, however in breast cancer, CAFs' role in chemoresistance is still poorly understood, with only a few mediators of chemoresistance identified. Breast CAFs have previously been shown to modulate tamoxifen resistance in ER-positive breast cancers, through activation of the PI3K and MAPK pathways (Shekhar et al, 2007). Co-culture experiments involving human skin fibroblasts and the ER-positive breast cancer line MCF-7 cells led to the identification of fibroblast-induced resistance to tamoxifen and fulvestrant in the MCF-7 cells (Martinez-Outschoorn et al, 2011). Although co-culture of fibroblasts and ER-positive breast cancer cells induced therapy resistance, the use of human skin fibroblast and not breast CAFs is a weakness in this work, whereas in this thesis both normal breast fibroblasts and breast CAFs are used.

CAF-secreted soluble factors have also been implicated in therapy-resistance in TNBC, the subtype on which I have focused. HGF, present in conditioned media from reduction mammoplasty fibroblasts, activated Met signaling to promote resistance in SUM102 and SUM149 TNBC cells to the EGFR inhibitor gefitinib (Mueller et al, 2012). Although this is a prime example of fibroblast driven resistance in TNBC, both the cell lines used and the class of drug are

dissimilar to my work, therefore there is no reason to expect the resistance pathways to be similar.

However, two studies have been published that are similar to my work in some main respects, in that they have used similar techniques (cell lines, *in vitro*, in co-culture), cell lines (MDA-MB-231, MDA-MB-468 and MDA-MB-157 cells) and therapeutic agents (use of cytotoxic chemotherapy, albeit not the same drug) as in my investigations. Importantly, they have also made highly-related findings about the role of CAFs in therapy resistance. Therefore, I have described below their findings in some detail, and have then compared these systematically with my own (below, and Table 7.1).

One study showed that CXCL12, a chemokine often regarded as a hallmark of the CAF phenotype, can enable paclitaxel resistance in MDA-MB-231/CAF co-culture spheroids in comparison to MDA-MB-231/normal human mammary fibroblasts. The resistance involved activation of MAPK and PI3K pathways, down-stream of the CXCR4 receptor (Ham et al, 2018). Only one TNBC cell line was used in this study (MDA-MB-231), but the normal breast fibroblasts and CAFs used were extracted from breast cancer patients, representing an advance on studies using unrelated sources of fibroblasts. In order to inhibit CXCR4 receptor signaling and remove chemoresistance, an inhibitor of CXRC4 was used. In the second study, MRC5 normal human fibroblasts (isolated from lung tissue) were grown in co-culture with TNBC cell lines MDA-MB-231, MDA-MB-468 and MDA-MB-157 and cells were treated with radiation and the gamma-secretase inhibitor DAPT, or cisplatin chemotherapy. This showed that cell death from these therapies was significantly reduced in co-cultures with MRC5 in comparison to mono-cultures of TNBC cell lines (Boelens et al, 2014). Critically, this study also found interferon signaling to be involved in therapy resistance – this is discussed in detail in section 7.2. In addition, this study highlighted that MDA-MB-468 cells were not protected by MRC5 fibroblasts, as I found in my own work. However, clinically practicable methods of inhibiting the protection were not used, with only siRNA used to confirm dependence on the relevant pathways.

The similarities and differences between these two studies and my own work are summarised in Table 7.1. Some comparative strengths of my work are that are I used a range of TNBC cell lines (unlike Ham et al) paired with both primary and immortalised breast NFs and CAFs (unlike Boelens et al), a range of therapies (different chemotherapeutic drug classes) that are relevant to current chemotherapy treatments for primary breast cancer (unlike either study). I also investigated the potential for blocking the influence of fibroblasts using a range of approaches (blocking antibodies, JAK/STAT inhibitor and miR-155 inhibitor), some of which could potentially be used in patients (unlike either study to an extent, and certainly unlike Boelens et al). Work covered in this thesis therefore provides a comprehensive analsysis of the role of CAFs in TNBC in chemoresistance and approaches to block CAF-induced chemoresistance.

A further point for comparison is the assays used to assess chemo-response. I measured the ability of fibroblasts to impact on chemoresistance in both short-term chemo-survival assays and long-term clonogenic assays (section 3.3.1 and 3.3.2). Ham et al assessed chemoresistance by quantifying GFP expression levels in TNBC cells 6 days following treatment, representing a measure between the time-scale of my two assays. By contrast, Boelens et al analysed cell death in the short-term rather than cell survival. The clonogenic assay I have used in particular may be more relevant than other assays in a cancer setting, as this may reflect the ability of the surviving cancer cells following treatment, to regrow and form clinical recurrences. In any event, these studies used a single assay, while my use of two independent assays may provide a more in depth profile of CAFs' role in TNBC chemoresistance.

	Ham et al (2018)	Boelens et al (2014)	Thesis
1. Cell lines used	Triple negative cell line - MDA-MB-231 Fibroblasts- Normal breast fibroblasts and breast CAFs extracted from patients	Triple negative cell lines - MDA-MB-231, -468 and -157 Fibroblasts- MRC5 normal human fibroblasts isolated from lung	Triple negative cell lines - MDA-MB-231, -468 and -157 Fibroblasts- Immortalised and primary breast normal fibroblasts and breast CAFs
2. Therapies investigated	Paclitaxel chemotherapy	Radiation and cisplatin chemotherapy	Epirubin and docetaxel chemotherapy
3. Techniques used	Co-culture spheroids quantifying GFP expression after 6 days	Co-culture cell death quantified	Co-culture short-term survival assay and clonogenic assay
4. Pathway responsible	CXCL12 and CXCR4 signaling	Exosomal transfer of RIG-1 to induce interferon stimulated genes	Interferon signaling
5. Inhibition of pathway leads to sensitization	YES- inhibitor of CXCR4 signaling	YES- siRNA	YES- interferon blocking antibodies, JAK/STAT inhibitor and miR-155 inhibitor

Table 7.1: Key features of Ham et al (2018) and Boelens et al (2014) compared to work carried out in this thesis

In the clinical setting, the evidence for CAFs having these roles in therapy resistance is disappointingly poor – since very few studies have quantified CAFs in clinical samples. There has been no published data identifying that an increase in chemoresistance in breast cancer is attributed to increasing numbers of CAFs. A large number of studies identify simply the presence of CAFs but not whether a difference in the number of CAFs has an impact. In most cases, this is ascertained by using tumour stroma ratio as tumour stroma contains CAFs. Comparisons have been made in 10-year disease-free survival between patients with invasive breast tumours with higher than 50% tumour stroma ratio, or lower; the former group had much worse outcomes (17% 10-year disease-free survival) in comparison to the latter (87%) (Vangangelt et al, 2018). However, in inflammatory breast cancer, the tumour stroma ratio has no impact on prognosis for patients (Downey et al, 2015). Therefore, tumour stroma ratio can play different roles in different breast cancer subtypes. Importantly, in TNBC, patients displaying higher than 50% tumour stroma had a poorer prognosis when compared to patients with less than 50% tumour stroma (Moorman et al, 2012). Therefore, this evidence could be extrapolated to support the idea that the presence of large numbers of CAFs correlates with relative chemoresistance, in agreement with my *in vitro* study.

7.2 Interferons as mediators of chemoresistance in TNBC

The interferon signaling pathway was identified as up-regulated in TNBC cells by the presence of CAFs during chemotherapy treatment, with up-regulation of various components of the signaling pathway itself (for example, STAT2 and IFR9) as well as of multiple interferon stimulated genes (ISGs), such as OAS1/2 and MX1/2 (section 4.3.5). The prototypical role for activation of the interferon pathway is in immune responses. Viral induction of interferon release enables interferon to bind to their respective Type I or Type II receptors and activation JAK/STAT signaling. Activation of JAK/STAT signaling and binding of respective STAT complexes to either ISRE (Type I) or GAS (Type II) elements, enables transcription of multiple ISGs that have antiviral, anti-proliferative and immune-modulatory functions as highlighted in section 1.13 (Tsuno et al, 2009).

A number of related studies have been published that implicate interferon signaling as a factor in chemoresistance in breast cancer. Of most relevance, is work from the Minn group, as already cited above, which identified fibroblasts as an activator of interferon signaling in some TNBC cell lines, leading to therapy resistance (Weichselbaum et al, 2008, Boelens et al, 2014, Benci et al, 2016). However, these papers define that this is a result of exosomal transfer of RNA (largely non-coding RNA), which activates the latter stages of the interferon signaling pathway. From the genes that were up-regulated in the data set in section 4.3.5, there were increased levels of ISGs but also components of the signaling pathway itself which indicates that the resistance seen is potentially driven by interferon release from CAFs driving the very start of the pathway. Furthermore, up-regulation of IFN-β in CAFs in the presence of TNBC cells and chemotherapy was observed (Figure 4.11). The difference between IFN-driven activation or exosomal RNA-driven activation is critical, as only the first method of activation could be blocked therapeutically using antibodies targeting the IFN receptors. In my work, the activation of interferon signaling was blocked by inhibition of receptor activation (Figure 5.2 and 5.4). This suggest that in this thesis, in which I have used breast CAFs rather than the unrelated lung fibroblast line MRC5, paracrine secretion of interferons and activation via classical receptor-mediated pathways predominate, rather than exosomal transfer of RNA.

In addition, 2 further studies have been carried out that implicate interferon activation in chemo-response. Firstly, an interferon-related gene signature has been predictive for chemotherapy response in breast cancer. Using 34 cancer cell lines from the NCI60 panel, an IFN-related DNA damage resistance signature (IRDS) was associated with resistance to chemotherapy and/or radiation. The IRDS signature was then analysed in clinical samples following adjuvant therapy in a range of tumours. In breast cancer, a seven gene-pair classifier successfully predicted efficacy of adjuvant chemotherapy. The IRDS included the interferon-related genes ISG15, STAT1 and IFITM1 (Weichselbaum et al, 2008). A further paper has highlighted that activation of STAT1 predicts poor response to chemotherapy in breast cancer. This was determined by analyzing expression profiles in residual tumour cells in chemo-

responder patient derived xenografts following antracycline and cyclophosphamide treatment (Legrier et al, 2015). Both of these studies implicate IFN-γ as the main pathway involved in chemoresistance in breast cancer given the dependence of up-regulation of STAT1. However, work in this thesis points more towards activation of the Type I interferon pathway as up-regulation of STAT2 and IRF9 in section 4.3.5 was observed, which is specific to the Type I interferon pathway, as well as the fact that IFN-β was up-regulated when CAFs and MDA-MB-231 cells were combined (Figure 4.11) and blocking antibodies against Type I signaling worked more consistently in both MDA-MB-231 and -157 TNBC cells (Figures 5.1 and 5.3).

There are also other data in the literature that provide support for the idea that stromal fibroblasts can produce interferons, and that epithelial cells can potentially respond to such signals. Both murine and human normal fibroblasts have been shown to produce IFN-β when cultured with the bacterial species *Shigella flexneri* or *Escherichia coli* (Hess et al, 1987). Secretion of IFN-β has also been observed previously in CAFs (Slaney et al, 2013). In addition, studies have shown that normal human skin fibroblasts produce IFN-β when grown on collagen membranes blended with fibronectin and vitronectin, indictating fibroblast attachment to the ECM can enable secretion of IFN-β (Higuchi et al, 2002). The identification of the specific interferon responsible for CAF-induced chemoresistance was required and therefore investigated as there is no literature determining a direct role for a particular CAF-secreted interferon in chemoresistance. Interestingly, in section 4.3.9, interferons were not normally produced in either the fibroblasts or epithelial cells. Basal levels of IFN-α and IFN-γ were undetermined in both CAFs and MDA-MB-231 cells, with basal levels of IFN-β in both NFs and CAFs undetectable or low and basal levels undetermined in MDA-MB-231. Surprisingly, IFN-β levels only increased in CAFs when co-cultured with MDA-MB-231 cells, with IFN-β expression remaining undetermined in MDA-MB-231 component. Induction of expression of IFN-β in CAFs *only* in the presence of TNBC cells is a key finding that has not previously been observed, and represents complex cross-talk between the two cell types, where the TNBC cells induce IFN-β expression in the CAFs in order for the TNBC cells then to respond to this paracrine signal.

In addition, there has been increasing evidence that epithelial cells can respond to interferons. IFN-γ has been shown to increase MUC1 expression in haematopoietic and epithelial cancer cells (Reddy et al, 2003) and breast cancer cells also have the ability to secrete interferons (Wan et al, 2012). The ability of breast cancer epithelial cells to respond to interferons is evident throughout much of my data set, with CAFs inducing interferon signaling activation (Figure 4.6), and breast cancer cells responding to recombinant interferons in terms of both activation of the interferon signaling pathway, and induction of chemoresistance (Figure 4.7 and 4.8).

Interestingly, IFN driven chemoresistance contradicts some of the previous literature cited. IFN-β has been shown to repress cancer stem cell properties in TNBC and can also reduce the aggressive nature of breast cancers by reducing migration and metastasis (Doherty et al, 2017). However, data in this thesis suggests that activation of the interferon signaling occurs through secretion of IFN-β from CAFs (Figure 4.11) with both Type I and Type II interferons sufficient for enhancing tumorigenesis and protecting TNBC cells from chemotherapy (Figure 4.7 and 4.8).

It is also worth commenting on the studies using fibroblast conditioned medium in section 3.3.5, which has been previously discussed in detail in section 3.4.2. However, this requires further discussion here, given results in the following chapters. Conditioned media from CAFs did not confer chemoresistance on TNBC cells (section 3.3.5) casting doubt that a secreted factor was responsible for chemoresistance. Another plausible explanation for this was the requirement of cross-talk between TNBC cells and CAFs. From section 4.3.9 and figure 4.11, it is clear that cross-talk between breast cancer cells and CAFs is required for chemoresistance to occur given CAF secretion of IFN-β was only up-regulated *in the presence MDA-MB-231 cells*. This finding explains why conditioned media experiments did not enable protection in TNBC cells. To further improve the conditioned media experiment, cross-talk between the 2 cell lines could be taken into account by using conditioned media from CAF/TNBC co-cultures, or by using transwell approaches.

7.3 Claudin-low TNBC phenotypes in CAF-induced chemoresistance

As described in section 1.4.2, there are multiple different TNBC subtypes. In order to cover some of this diversity, three different TNBC cell lines were used in my experiments. Both MDA-MB-231 and MDA-MB-157 are characterized as claudin-low with negative ER, PR and HER2 status, high Ki67 and low expression of claudin-3, -4 and -7. MDA-MB-468 are classified as ER, PR and HER2 negative, but EGFR positive, high Ki67 and with cytokeratin 5/6 expression, thereby defining a basal subtype (Grigoriadias et al, 2012, Neve et al, 2006).

Interestingly, in section 3.3.1 and 3.3.2, differing responses could be observed between the claudin-low cell lines (MDA-MB-231 and MDA-MB-157) and the basal cell line (MDA-MB-468). CAFs induced consistent chemoresistance in the claudin-low triple negative phenotype but not in the basal triple negative phenotype. Here, I therefore highlight a novel role of CAF-induced chemoresistance in claudin-low TNBC to epirubicin and docetaxel, which is extremely relevant in the treatment of primary TNBC. In addition, up-regulation of ISGs OAS1 and MX1 (section 4.3.6) and the ability to respond to recombinant interferons and confer chemoresistance (section 4.3.7) were attributed only to the claudin-low TNBC subtype, and not the basal subtype (Figures 4.7, 4.9 and 4.10). ISG expression analysis has previously been carried in all 3 cell lines used here (MDA-MB-231, -157 and -468) when either in mono-culture or when cultured with MRC5 fibroblasts in the absence of treatment (Boelens et al, 2014). Work in this thesis is therefore further confirmation of the ability of the claudin-low TNBC subtype to be able to respond to and activate interferon signaling, while the single representative of the basal TNBC subtype was not able to.

Although CAF induced chemoresistance to epirubicin and docetaxel in the claudin-low subtype in comparison to the basal subtype is a novel finding, the two subtypes have been compared in many other respects previously, including clinical response to treatments, disease free survival and overall survival. In one

study, when comparing pCR of claudin-low and basal subtypes, claudin-low tumours had a 39% pCR whereas basal had a higher pCR 73%, which was not deemed significant (p value=0.08) (Prat et al, 2010). However, another study showed the pCR of claudin-low and basal at 32% and 33% respectively (Sabatier et al, 2014), therefore a firm conclusion as to whether the pCR rates differ remains impossible. When looking at 5-year disease free survival, claudin-low TNBC has higher disease free survival at 79.3% with basal TNBC at 73.1%, which was, again, not significantly different (p=0.40) (Dias et al, 2017). Another study had similar findings with disease free survival of 67% and 60% respectively, which was not significant (Sabatier et al, 2014). 5-year overall survival of claudin-low TNBC patients was also higher in at 89.7% compared to basal TNBC at 82.6% (Dias et al, 2017). Curiously, some of these differences in survival – if they are to be believed - are not what one would predict when extrapolating from my data that suggest that the claudin-low cancers may have an extra chemoresistance mechanism not available for the basal cancers. Based on data in section 3.3.1 and 3.3.2, predictions could be made that claudin-low breast cancer patients would have worse overall survival when compared to basal breast cancer patients as a result of this extra chemoresistance, while the reverse is actually seen clinically. It is also important to remember that this extra chemoresistance mechanism in claudin-low TNBC is CAF dependent (section 3.3.1 and 3.3.2), therefore will not be relevant to all claudin-low TNBCs.

7.2 Limitations

Although this work is promising, there is further scope to build on these data sets. A major limitation is the lack of translation of these findings into *in vivo* models. 2 key findings need to be tested - to back up these findings: firstly, a demonstration that CAFs induce chemoresistance in TNBC tumours; secondly, a demonstration that blocking CAF-induced interferon activation, using interferon blocking antibodies or small molecules, halts CAF-induced chemoresistance and thereby increases chemotherapy responses without major side-effects.

In addition, it is necessary to support the pathways proposed here using evidence from clinical breast cancer samples. Assessments could be made of whether tumours with higher proportions of CAFs show evidence of activation of interferon signaling in the cancer cells, and whether this correlates with relatively poor responses to chemotherapy or cancer survival.

7.3 Final conclusions

A novel role for CAFs has been identified in some TNBC cell lines. CAFs, but not NFs, induce chemoresistance in claudin-low TNBC lines. This appears to be through the paracrine release of IFN-β from the CAFs, which drives interferon signaling in the epithelial cells and chemoresistance, perhaps associated with reduced proliferation of the epithelial cells. In addition, CAFs can also induce anoikis resistance in claudin-low TNBC cells, likely through the interferon signaling pathway also. Both observations have potential clinical implications. First, CAF-induced chemoresistance could be reversed through blocking the interferon signaling pathway during chemotherapy, thereby improving chemotherapy responses. Secondly, CAF-induced anoikis-resistance could be targeted similarly to reduce the chances of metastasis, by reducing the survival of the cancer cells in the circulation. Further work is required to take advantage of these potential therapeutic directions.

Appendix

Target	Average Fold Change	Target	Average Fold Change
OAS1	30.21	VCAN	3.99
MX1	28.32	USP41	3.86
SPARC	26.53	XAF1	3.83
GREM1	25.35	BGN	3.65
GREM1	23.75	CXCL12	3.65
GREM1	23.26	ANPEP	3.61
POSTN	22.29	IFITM1	3.56
MX2	14.19	LDB2	3.49
COL1A2	13.88	CDH13	3.41
NID1	13.27	STAT2	3.4
MMP2	12.83	DPYSL3	3.36
IFI6	10.29	HIST1H4E	3.31
IFI44L	9.22	EPB41L3	3.31
IFI27	8.73	THBS2	3.26
COL1A1	8.73	MYH10	3.19
CLDN11	7.93	LYPD1	2.99
THY1	7.69	KIAA1549L	2.95
OAS2	7.66	EHD3	2.9
RAB3B	7.54	TAGLN	2.87
MFAP5	7.52	DPT	2.79
COL6A3	7.49	CCBE1	2.74
USP18	7.01	C19orf66	2.73
SPOCK1	6.89	WNT5A	2.7
RGS4	6.5	ASIC1	2.69
ITGA11	6.44	XIST	2.67
IRF9	6.16	PARP9	2.64
TERT	5.59	GPNMB	2.59
CCL2	5.59	AEBP1	2.58
TCF4	5.14	COL5A2	2.55
FGF2	5.11	TAP1	2.52
CDH6	5.06	PLAGL1	2.5
DCN	4.97	LOC353194	2.5
GREM1	4.75	SLC16A1	2.41
ADAM12	4.72	TRANK1	2.41
TLE4	4.71	LBH	2.41
SULF1	4.7	MSC	2.41
LGALS9	4.68	TGFBI	2.35
SEMA5A	4.65	FKBP10	2.35
TBX3	4.63	PTGFRN	2.3
CDH2	4.45	SYT11	2.29
KRT14	4.4	FYCO1	2.28
TRIM22	4.3	C16orf45	2.28
TMEM47	4.29	NUAK1	2.26
PRRX1	4.25	SYNC	2.22
COL3A1	4.15	PARP12	2.21

Target	Average Fold Change
RTBDN	2.2
OLFML3	2.19
BVES	2.19
MTA3	2.19
PTGS2	2.17
SNX33	2.17
MYH16	2.15
FLI1	2.08
SIGLEC14	2.08
TNFRSF9	2.06
BNC2	2.06
MRC2	2.06
NLRC5	2.05
SHROOM2	2.05
RSAD2	2.04
APOL1	2.03
ARHGAP30	2.02
ASPHD2	2.02
AL445199.1	-2.07
RP11-252K23.2	-2.08
MTND4P32	-2.1
AC107218.3	-2.17
U3	-2.22
RNA5SP405	-2.41
AC093642.4	-2.57

Appendix Table 1: Full list of gene expression changes between 0% and 20% cultures following Affymetrix analysis

MiRNAs present in 0% but not in 20%
miR-let-7f
miR-32
miR-452

MiRNAs present in 20% but not in 0%
miR-27a
miR-31
miR-130b
miR-145
miR-146b
miR-152
miR-185
miR-196b
miR-199a-3p
miR-424
miR-433
miR-485-3p
miR-483
miR-510
miR-539
miR-551b
miR-674
miR-708

Appendix table 2: List of miRNAs differentially expressed that are present in 0% cultures but not 20% and miRNAs present in 20% cultures but not 0%.

MiRNAs decrease from 0 to 20%
miR-22
miR-25
miR-106b
miR-125a-3p
miR-193b
miR-210
miR-218
miR-365
miR-532
miR-652-
miR-671-3p

MiRNAs increase from 0 to 20%
miR-34a
miR-98
miR-132
miR-139-5p
miR-200c
miR-324-3p
miR-324-5p
miR-155
miR-411
miR-422a
miR-454
miR-455-3p
miR-489

Appendix table 3: List of miRNAs differentially expressed that are decreased from 0% to 20% and miRNAs that are increased from 0% to 20%

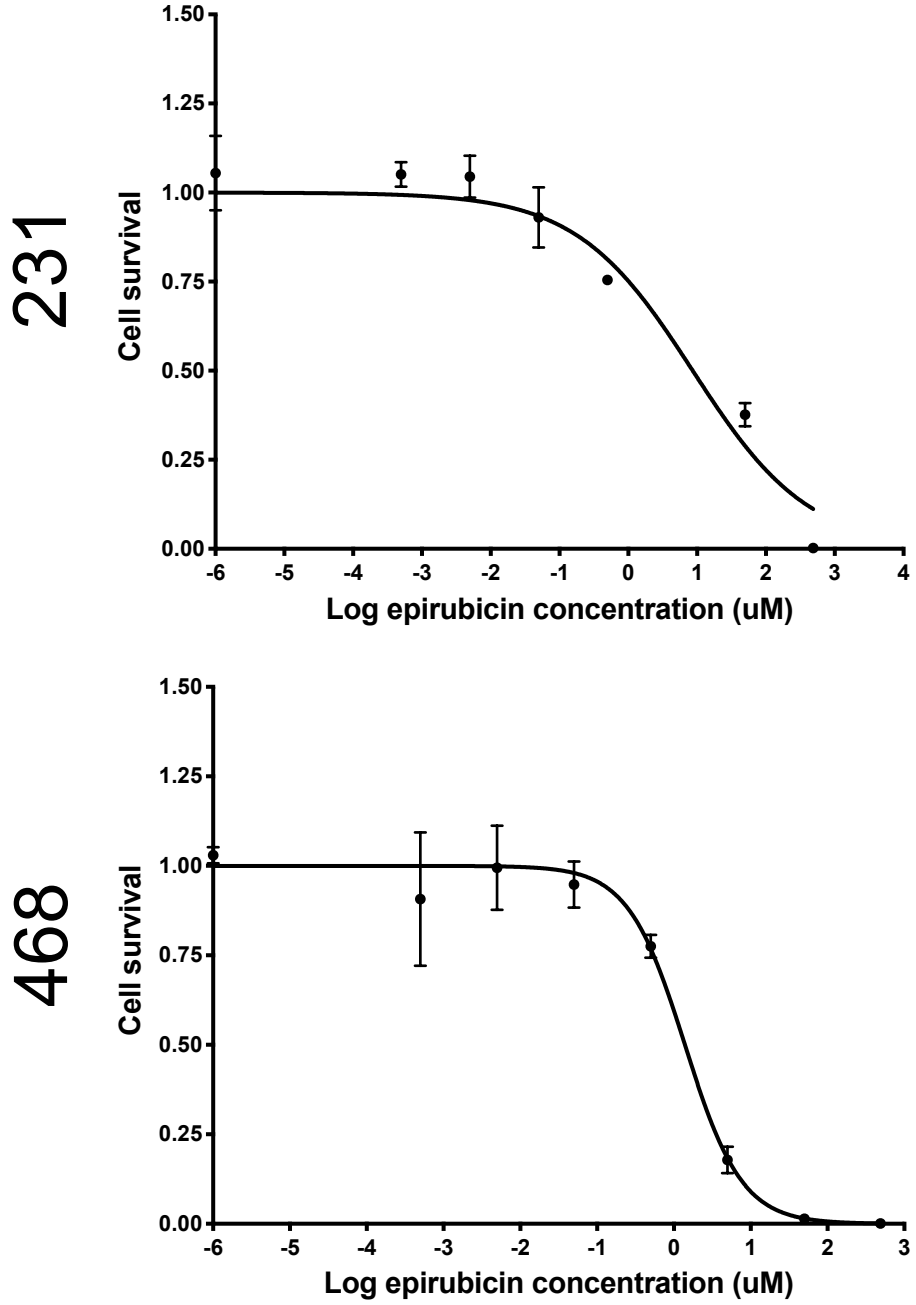
Target	Average Fold Change
COL3A1	4.15
COL5A2	2.55
COL6A3	7.49
DPT	2.79
SPARC	26.53
SPOCK1	6.89
WNT5A	2.7
SEMA5A	4.65
DCN	4.97
ADAM12	4.72
BGN	3.65
LGALS9	4.68
TGFBI	2.35
POSTN	22.29
MFAP5	7.52
THBS2	3.26
AEBP1	2.58
SULF1	4.7
CCBE1	2.74
VCAN	3.99
FGF2	5.11
NID1	13.27
CCL2	5.59
GREM1	25.53
MMP2	12.83
CXCL12	3.65
COL1A1	8.73
COL1A2	13.88

Appendix table 4: List of extracellular matrix genes up-regulated from 0% to 20% cultures from Affymetrix analysis

Pathway	P Value	Genes from input	Genes from annotation
Interferon alpha/beta signaling	1.38E-14	12	69
Ensemble of genes encoding core ECM	1.26E-12	17	275
Ensemble of genes encoding ECM and ECM associated proteins	1.45E-11	28	1028
Interferon signaling	3.29E-11	14	202
ECM organisation	5.45E-09	14	298
Cytokine signaling in immune system	8.42E-09	21	763
Genes encoding collagen proteins	8.62E-06	5	44
Defective CHST14 causes ED, musculocontractual type	8.77E-07	3	7
Defective CHST3 causes SEDCJD	8.77E-07	3	7
Defective CHSY1 causes TPBS	8.77E-07	3	7
Collagen chain trimerization	1.20E-05	5	47
Binding and uptake of ligands by scavenger receptors	1.48E-05	5	49
Genes encoding structural ECM glycoproteins	3.60E-05	8	196
Assembly of collagen fibrils and other multimeric structures	4.00E-05	5	60
Dermatan sulphate biosynthesis	4.06E-05	3	11
Validated transcriptional targets of AP1 family members Fra1 and Fra2	6.33E-05	4	34
Genes encoding proteoglycans	7.11E-05	4	35
Collagen biosynthesis and modifying enzymes	8.43E-05	5	70
CS/DS degradation	8.84E-05	3	14
Influenza A	1.99E-04	7	173
Regulation of Wnt-mediated beta catenin signaling and target gene transcription	1.50E-04	5	79
ECM-receptor interaction	1.79E-04	5	82
Diseases of glycosylation	2.24E-04	5	86
Defective B3GAT3 causes JDSSDHD	2.29E-04	3	19

Defective B4GALT7 causes EDS, progeroid type	2.29E-04	3	19
Defective B3GALT6 causes EDSP2 and SEMDJL1	2.29E-04	3	19
Protein digestion and absorption	2.77E-04	5	90
Scavenging by class A receptors	3.13E-04	3	21
Collagen formation	3.23E-04	5	93
Chondroitin sulphate biogenesis	3.60E-04	3	22
AGE-RAGE signaling pathway in diabetic complications	4.31E-04	5	99
ECM proteoglycans	4.82E-04	4	57
Diseases associated with glycosaminoglycan metabolism	5.97E-04	3	26
A tetrasaccharide linker sequence is required for GAG synthesis	5.97E-04	3	26
Cell-cell junction organisation	6.65E-04	4	62
Integrin signaling pathway	7.05E-04	6	167
Interleukin-4 and 13 signaling	8.21E-04	5	114
Adherens junction interactions	1.01E-03	3	31
Syndecan-4 mediated signaling events	1.01E-03	3	31
Herpes simplex infection	1.20E-03	6	185
Type I (alpha/beta IFN) pathway	1.43E-03	2	9
ISG15 antiviral mechanism	1.50E-03	4	77
Antiviral mechanism by IFN-stimulated genes	1.50E-03	4	77
Hepatitis C	1.53E-03	5	131
Measles	1.69E-03	5	134
Cell junction organisation	2.55E-03	4	89
Interferon gamma signaling	3.11E-03	4	94

Appendix Table 5: List of dysregulated pathways taken from ToppGene pathway analysis. Top hits were then used to create Table 4.4.



Appendix Figure 1: Dose response curves used to calculate IC10, IC50 and IC75 epirubicin values for MDA-MB-231 and -468 cells

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