Investigating the therapeutic potential of cellular FLICE-like inhibitory protein and TRAIL in preclinical models of breast cancer

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Table of Contents

Abstract

Apoptosis is an important process in normal mammary gland physiology and evasion of apoptosis has also been identified as a hallmark of cancer. In breast cancer cells apoptotic resistance is an acquired feature that can promote tumour growth and progression. Induction of apoptosis by the extrinsic death ligand TRAIL has been shown to be a promising clinical therapy targeting a number of different cancer cells whilst sparing normal cells. Unfortunately most breast cancers are inherently resistant to TRAIL treatment. Herein it is shown that by reducing the expression of the downstream TRAIL inhibitor c-FLIP, a range of different breast cancer subtypes can be sensitised to TRAIL treatment resulting in significant cancer cell death. Significantly, suppression of c-FLIP in combination with TRAIL (FLIPi/TRAIL) ablated the tumour-initiating breast cancer stem cell (bCSC) subset, as defined by mammosphere formation assay, within cell lines. This selective killing of bCSCs translated to reduced tumour initiation and metastasis in animal transplant models. However, continued culture of FLIPi/TRAIL treated cell lines in adherent conditions resulted in bCSC re-acquisition suggesting a phenotypic plasticity of non-bCSC cells. Re-acquired bCSCs also demonstrated sensitivity to repeated FLIPi/TRAIL treatment and maintaining reduced c-FLIP expression prevented bCSC re-acquisition. These results substantiate the importance of resistance to apoptosis in tumour initiation and metastasis and identify the targeting of c-FLIP proteins as a promising anti-cancer therapeutic approach.

Acquired resistance to existing mainstay therapies such as antiestrogens (AEs) (tamoxifen and Faslodex) and aromatase inhibitors (AIs) is an ongoing obstacle in treatment of a large number

of breast cancer patients. AE-resistant models of breast cancer and a multiple endocrine-resistant patient sample demonstrated hypersensitivity to TRAIL. This sensitivity was observed in both *in vitro* and *in vivo* models of AE resistance and cell death was prevalent in both bulk tumour cells and bCSCs. Sensitisation was not attributed to combination AE/TRAIL treatment suggesting cellular changes during the acquisition of AE resistance are responsible for TRAIL sensitivity in these models. Further investigation suggested that the mechanism of AE-resistant cell sensitivity to TRAIL was not dependant on functional estrogen receptor signalling and is most likely dependant on the AE agent that the cancer cells have acquired resistance to. Interestingly tamoxifen-resistant MCF-7 cells were shown to have reduced c-FLIP protein expression compared to parental cells, further supporting c-FLIP's potential in cancer therapy.

Recent success in the use non-MHC-restricted γδ T cells as a targeted immunotherapy in clinical trials has identified this therapeutic methodology as desirable. Here it is shown that TRAIL is readily expressed by this subset of T cells that also demonstrate cytotoxicity to breast cancer cell lines. Neither the secretion of TRAIL or surface expression of TRAIL appeared to contribute significantly towards γδ T cell cytotoxicity and the majority of breast cancer cell death induced by γδ T cells would seem to be perforin-mediated. The suppression of c-FLIP in target cells increased γδ T cell cytotoxicity but again not via TRAIL. Preliminary results also indicated that the bCSCs of some cell lines were exquisitely sensitive to γδ T cell treatment. In summary these results indicate that targeting c-FLIP and TRAIL can be therapeutically beneficial in a range of different breast cancer subtypes by certain therapeutic strategies.

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Declaration

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This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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List of Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
AE	Antiestrogen
AI	Aromatase inhibitors
ALDH1	Aldehyde dehydrogenase 1
AML	Acute myeloid leukaemia
AP-1	Activator protein 1
Apaf-1	Apoptosis protease activating factor 1
APC	Antigen presenting cell
bCCL	Breast cancer cell line
Bcl-2	B-cell lymphoma 2
BCMP	Breast cancer molecular pharmacology group
bCSC	Breast cancer stem cell
BLG	β-lactoglobulin
BMD	Bone marrow derived
c-FLIP	Cellular FLICE-like inhibitory protein
CAD	Caspase activated DNase
CD95	Fas receptor
CDK	Cyclin dependant kinase
CM	Conditioned medium
CMA	Concanamycin
CMF	Cyclophosphamide, Methotrexate, Fluorouracil
CRC	Colorectal carcinoma
CTB	CellTiter blue
CXCL7	Chemokine (C-X-C motif) ligand 7
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	Chemokine (C-X-C motif) receptor 4
DC	Dendritic cells
DcR	Decoy receptor
DED	Death effector domain
DISC	Death inducing signalling complex
DNA-PK	DNA-dependant protein kinase
DR4	Death receptor 4
DR5	Death receptor 5
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ErbB2	erythroblastic leukaemia viral oncogene homolog
ERK	Extracellular signal-regulated kinase

FEC	Fluorouracil, Epirubicin, Cyclophosphamide
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
FasL	Fas ligand
FASR	Faslodex resistant
FBS	Fetal bovine serum
FLIPi	c-FLIP inhibition
FLIPi/TRAIL	combination c-FLIP inhibiton + TRAIL treatment
FOXA1	Forkhead box protein A1
GRB7	Growth factor receptor-bound 7
Gsk3β	Glycogen synthase kinase 3 beta
HDACi	Histone deacetylase inhibor
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
Hh	Hedgehog
HIF-1	Hypoxia-inducible factor 1
HIF-2	Hypoxia-inducible factor 2
HLA-DR	Human leukocyte antigen DR receptor
HMB-PP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HMEC	Human mammary epithelial cells
IAPs	Inhibitors of apoptosis
IFN	Interferon
IkB	Inhibitors of NF-kB
IKK	Inhibitor of KappaB kinase
IL-2	Interleukin 2
IL-6	Interleukin 6
IPP	Isopentenyl pyrophosphate
ITCH (AIP4)	E3-itchy- ubiquitin ligase
JNK	c-jun N-terminal kinase
LTR	Long terminal repeat
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
Mcl-1	Induced-myeloid leukemia cell differentiation protein
MEKK1	MAP/ERK kinase kinase 1
MEP	Monoethylphosphate
MET	Mesenchymal-epithelial transition
MFU	Mammosphere forming units
MHC	Major histonecompatability complex
MKK7	MAP Kinase 7
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
mTOR	Mammalian target of rapamycin
NBP	Aminobisphosphonates

NF-kB	Nuclear factor binding to the intronic kappa-light-chain enhancer element in b cells
NHL	Non-hodgkins lymphoma
NOD/SCID	Nonobese diabetic severe combined immunodeficiency
OPG	Osteoprotegerin
PCR	Polymerase chain reaction
PI3k	Phosphatidylinositol 3-kinase
PR	Progesterone receptor
qRT-PCR	Quantitative reverse transcription PCR
RECIST	Response evaluation criteria in solid tumours
RIP	Receptor-interacting protein
RNAi	RNA interference
SAHA	Suberoylanilide hyroxamic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel elctrophoresis
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SP-1	Specificity protein 1
TAMR	Tamoxifen resistant
TCR	T cell receptor
TEA	Tocopherol ether-linked acetic acid
TGF-β	Transforming growth factor β
TIC	Tumour initiating cell
TIL	Tumour infiltrating lymphocyte
TIMP	Tissue inhibitors of metalloproteinases
TNFα	Tumour necrosis factor α
TRADD	TNF receptor type 1-associated death domain
TRAF	TNF receptor associated factor
TRAIL (APO-2L)	TNF-related apoptosis inducing ligand
USP8	Ubiquitin specific peptidase 8
WAP	Whey acidic protein
Wnt	Wingless-int
XL	MCF-7-X cells
ZOL	Zoledronate

Table of Figures

Table of Tables

CHAPTER 1:

General Introduction

1.1 Breast Cancer

1.1.1 Introduction

Development of cancer is a multistage process through which cells progress from a state of normalcy through a series of pre-malignant states into invasive cancers (Hanahan and Weinberg 2000). This process involves the accumulation and acquisition of oncogenic signals through genetic and epigenetic mutations in gene expression and/or function. Despite a vast array of cancer cell genotypes it has been suggested that the progression through to malignant growth is dictated by six essential physiological cell alterations: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). These six "hallmarks of cancer" are deemed essential in order to successfully overcome the intrinsic anticancer mechanisms that exist in every cell and tissue.

Breast cancer is the most prevalent form of cancer in women worldwide, in 2009 nearly 50,000 new diagnoses were made (Cancer research UK). This accounted for 31% of all new female cancers in the UK and although there have been significant advances in detection, therapy and surgical techniques over recent years, 50% of these sufferers will eventually die as a result of their disease (Caldas and Aparicio 2002). A large proportion of these deaths can be attributed to progression to metastasis and therapy resistance, however breast cancer is an inherently difficult disease to treat due to its biological complexity and both intra- and inter- tumour heterogeneity.

Tumours that originate from connective tissue are denoted as sarcomas and those that originate from epithelial tissue are known as carcinomas. Thus the majority of tumours arising in the breast are characterised as adenocarcinomas due to their origin within the glandular epithelia. These can be further subcategorised based on the different originating glandular epithelia within the breast that the tumour arises from. Initially neoplastic evolution commences in the form of flat epithelial atypia or hyperplasia of a normal cell that then progress to atypical hyperplasias that then become carcinoma in situ which may then progress to the final stage of invasive carcinoma such as lobular adenocarcinomas and ductal adenocarcinomas, the latter of which accounting for up to 75% of breast carcinomas (Bombonati and Sgroi 2010). This is representative of a normal cell that mutates and begins to grow uncontrollably with abnormal nuclear morphology until the lumen is almost completely constricted with the basement membrane remaining intact. Only when these cells evade the encapsulating basement membrane into the surrounding tissue can the tumour be described as invasive carcinoma (Wellings et al. 1975).

1.1.2 Heterogeneity of breast cancer

Gene expression profiling of breast cancer has recently identified the presence of multiple molecular subtypes of the disease. Microarrays performed characterised gene expression patterns in 65 samples of human breast tumours from 42 different individuals (Perou et al. 2000), revealing that the phenotypic diversity of breast tumours was associated with corresponding diversity in gene expression. These gene expression profiles were subsequently used to subdivide breast cancers into five different molecular groups: the oestrogen receptor (ER)-positive luminal A and luminal B tumours and the ER-negative

basal-like, ErbB2-positive and normal breast tumours (Perou et al. 2000; Sorlie et al. 2001). The luminal A and luminal B cancers over-express ER, oestrogen-responsive genes and genes that are characteristically expressed in luminal epithelial cells (Perou et al. 2000). The luminal A tumours often express the transcription factors, GATA-3 and FOXA1, and the progesterone receptor (PR) (Badve et al. 2007; Sorlie et al. 2003), whereas the molecular phenotype of luminal B tumours is not so well understood, they are however known to be PR-negative and express higher levels of the proliferation marker Ki67 and possibly the tyrosine kinase receptor ErbB2 (Cheang et al. 2009). As their name suggests the ErbB2-positive tumours over-express ErbB2 and other neighbouring genes such as growth factor receptor-bound protein-7 (*GRB7*); they commonly lack ER expression and generally have high levels of NF-κB activation (Bertucci et al. 2004; Biswas and Iglehart 2006; Sorlie et al. 2001). Basal-like tumours over-express a range of genes that characterise basal epithelial cells (Perou et al. 2000). These tumours are often also denoted 'triple-negative' due to their general lack of ER, PR and ErbB2 (Perou et al. 2000). Whilst the majority of basal-like tumours are triple-negative, this is not always the case as some basal-like tumours can express ErbB2 (Rouzier et al. 2005) and conversely some triple-negative tumours do not express basal markers (Tan et al. 2008; Tischkowitz et al. 2007). The normal breast-like tumours resemble normal breast tissue samples and over-express many genes that characterise non-epithelial mammary cells and lack luminal epithelial cell markers, but do have strong expression of basal epithelial genes (Perou et al. 2000; Sorlie et al. 2001). Notably, these different subtypes can be used to predict clinical outcome, luminal A cancers tend to have the best prognosis and ErbB2-positive and basal-like tumours are more aggressive with a

worse prognosis (Sorlie et al. 2003; Sotiriou et al. 2003). The most recently identified subtype of breast cancers is termed the claudin-low subtype. This group gains its name from the low expression of claudin genes that are involved in epithelial cell tight-tight junctions. These tumours are triple-negative and show similar gene expression profiles to the basal-like triple-negative tumours. Important features of the claudin-low tumours are that they lack cell-cell junction proteins such as E-cadherin and have stem cell-like features and features of EMT and are believed to be the subtype most similar to breast cancer stem cells (Perou 2010).

1.1.3 Estrogen receptor signalling and breast cancer

The estrogen receptor (ER) signalling pathway is a complex biological pathway that controls cell proliferation, apoptosis, invasion and angiogenesis (Curtis Hewitt et al. 2000; Warner et al. 1999) and expression of ER defines a major subtype of cancer. There are two types of ER, ERα and ERβ, with ERα being the main subtype that is expressed in human mammary epithelium. Upon binding, ER monomers dimerise, activate and translocate to the nucleus to bind to its responsive element in the target gene promoter stimulating gene transcription (McDonnell and Norris 2002; McKenna et al. 1999). ER has been shown to bind to the transcription factors activator protein-1 (AP-1) and specificity protein-1 (SP-1), at their specific sites on DNA, serving as coregulators of ER signalling (Kushner et al. 2000). Coregulators of ER signalling serve the important purpose of restricting the transcriptional activity of the receptor (Smith and O'Malley 2004). ER signalling can also be regulated by epidermal growth factor receptor (EGFR), HER2 and insulin-growth factor receptor (Schiff et al. 2004). These membrane receptor tyrosine kinases signal to

phosphorylate ER and its co-activators/repressors leading to its activation (Shou et al. 2004; Wu et al. 2005). Conversely ER signals can also suppress the expression of EGFR and HER2 (Newman et al. 2000; Yarden et al. 2001). ER signalling is frequently exploited by cancer cells to help drive tumour cell growth and as such 75% of all breast tumours diagnosed are ER+ve (Musgrove and Sutherland 2009). The discovery that the naturally produced hormone estrogen is capable of driving tumour growth led to the development of drugs specifically targeting ER signalling. Initially tamoxifen and fulvestrant (Faslodex) were developed to directly target estrogen receptors on tumour cell surface, this was then followed by the development of aromatase inhibitors that prevent the synthesis of circulating estrogen (Johnston and Cheung 2010; Jordan 2006; Taylor and Howell 1999). tamoxifen functions by competing for binding with estrogen at the ligand-binding domain on ER inducing a conformational change destabilising the AF-2 domain resulting in an overall stabilisation of receptor (Shiau et al. 1998). The other AE, Faslodex, prevents the translocation of ER from cytoplasm to the nucleus inducing cytoplasmic aggregation and and proteasomal degredation of receptors (Howell and Abram 2005). On the other hand, aromatase inhibitors, as their name suggests, inhibit the enzyme aromatase that is responsible for synthesis of estrogens from adrogenic substrates, specifically the synthesis of estrone from androstenedione and estradiol from testosterone (Chung and Carlson 2003). Typically the anti-estrogen (AE) tamoxifen is the first-line therapy for premenopausal breast cancer patients, however disease resistance to tamoxifen frequently occurs (Johnston 1997). However, aromatase inhibitors (AIs) are limited for use in postmenopausal women where the ovaries are no longer the primary source of estrogen production (Longcope et al.

1986). In premenopausal women aromatase inhibition leads to an increase in gonadotrophin secretion as a result of reduced estrogen feedback to the hypothalamus and pituitary resulting in an over-production of estrogen by the ovaries that can produce cystic hyperplasia (Sinha et al. 1998). Due to its distinct ER inhibitory mechanism Faslodex is a common second-line therapy for patients that have become resistant to tamoxifen or AI (Johnston and Cheung 2010). However, as with the other endocrine agents, tumour cells also frequently become resistant to Faslodex. The high incidence of resistance to endocrine therapies has encouraged the investigation into discovering novel therapeutics that can eliminate resistant tumours.

1.1.4 Breast cancer and metastatic progression

Treatment of primary breast cancers has improved drastically over the last decade; this can be mostly attributed to great advances in early detection and surgical techniques and disease-specific therapy approaches. Despite these advances in local and systemic therapies many primary tumours are not completely eradicated and develop to become invasive metastatic carcinoma. Metastasis is a complex process during which tumour cells must detach and evade the site of the primary tumour, invade the surrounding stromal tissue, migrate into and survive in the vasculature before extravasating and colonising at a distant secondary site (Figure 1.1) (Smith and Theodorescu 2009).

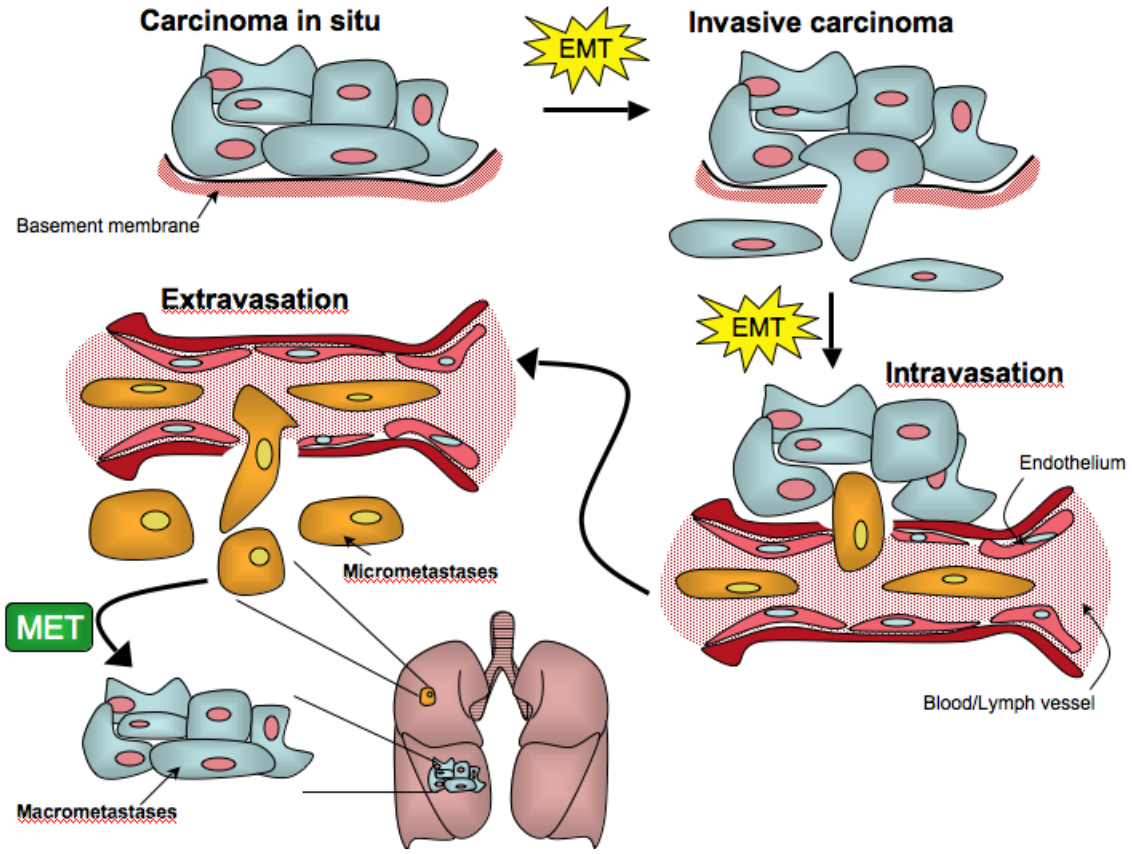


Figure 1.1: Stages of the metastatic cascade
Carcinoma *in situ* is maintained locally by surrounding basement membrane. Alterations such as EMT that increases invasive properties of cells encourage invasion through the basement membrane into the vasculature. Once in the bloodstream or lymphatic system, cells passively travel to secondary sites where single cells extravasate and establish micrometastases or undergo MET to form a new carcinoma (macrometastases).

For metastasis to take place there must be a suitable amount of vasculature available for the cells to invade, the process by which this vasculature is generated is termed angiogenesis. As the primary tumour begins to grow there is an increasing demand for oxygen and as a result the normal physiological conditions surrounding the tumour are disrupted. Consequently an imbalance between matrix metalloproteinases (MMPs), a family of enzymes that degrade the basement membrane, and their tissue inhibitors of metalloproteinases

(TIMPs) occurs. Concurrently the hypoxic environment during hyperplasia causes the accumulation of the hypoxia-inducible transcription factors HIF-1 and HIF-2. Under hypoxic conditions the HIF-1α subunit is no longer degraded and an increase in vascular endothelial growth factor is observed (Bos et al. 2001; Salceda and Caro 1997; Schneider and Miller 2005). The importance of angiogenesis extends beyond metastasis as it not only provides an avenue for cells to metastasise but additionally serves a purpose as a supply of oxygen and nutrients to the growing tumour. With suitable blood or lymph vasculature in close proximity, the primary tumour cells enter the lymphatic system or bloodstream and migrate toward secondary sites for colonisation stimulated by chemokine gradients. Common secondary sites such as lymph, liver, lung and bone marrow have an increased expression of the chemokine ligand CXCL12. This attracts the tumour cells with high expression levels of the chemokine receptor CXCR4 (Muller et al. 2001). Upon reaching their secondary site, tumour cells tether to the vasculature walls and chemoattractant gradients draw the cell into the new tissue. Once colonised the tumours may then divide and grow to become macrometastases. The importance of metastatic disease cannot be understated, despite an 88% 5-year survival rate of stage I breast cancers the 5-year survival rate of stage IV disease is still limited to just 15% (American cancer society). With the current statistics it is not surprising that much research focus is currently directed at the prevention and treatment of metastatic disease.

1.1.4.1 Epithelial-mesenchymal transition

The cellular process of epithelial to mesenchymal transition (EMT) is important throughout embryonic development for

organogenesis (Baum et al. 2008). Some of the key features of EMT include the loss of E-cadherin expression together with the loss of cell-cell adhesion proteins. As such, a cell that has undergone EMT would express mesenchymal markers such as N-cadherin, fibronectin and vimentin. A visible morphological change from cuboidal epithelial cells with apical-basal polarity to an appearance with an invasive leading and trailing edge asymmetry becomes evident (Christiansen and Rajasekaran 2006). Stimuli such as hypoxia, cell-stroma interactions and some intracellular signalling pathways, namely transforming growth factor β (TGF- β), NF-κB, hepatocyte growth factor (HGF), Wnt, Notch and Sonic Hedgehog (Hh) are capable of inducing EMT. These diverse stimuli converge in the expression of a group of EMT-inducers that orchestrate the EMT process. The EMT-inducers are a group of transcription factors including Twist, Snail, Slug, Zeb1, Zeb2 and FoxC2 (Polyak and Weinberg 2009).

The EMT program of cancer cells is similar to that observed in embryonic development but its activation is a result of aberrant stimuli. EMT has been associated with tumour progression and metastasis, creating cancer cells with more motile and invasive phenotype (Hugo et al. 2007; Yang and Weinberg 2008). This phenotype as a result of EMT is particularly important at the early stages of the metastatic cascade when cancer cells need to invade through the basement membrane into surrounding tissue and vasculature. Moreover, there is also evidence to indicate that EMT aids trans-differentiation of breast cancer cells to a stem cell-like phenotype (Mani et al. 2008; Morel et al. 2008). In addition to EMT, the reverse process of mesenchymal to epithelial transition (MET) has also been demonstrated for the colonization of cancer cells at secondary sites (Dykxhoorn et al. 2009). This supports observations

that metastases maintain a similar epithelial phenotype to its primary tumour. It also indicates that the mesenchymal cell state is transient in cancer cells and its maintenance can be influenced by the tumour micro-environment (May et al. 2011). Although aspects of the EMT program may be modulated in many cancers, these cancers may not appear as phenotypic EMT cells. Clinically, EMT cells are observed as spindle cell metaplasias, these cells have an EMT-like molecular make-up and correlate with poorer prognosis (Geyer et al. 2010). However, certain tumour subtypes do not commonly have metaplastic spindle cells despite their apparent EMT molecular make-up (Fulford et al. 2006). Thus defining EMT in breast cancer cells is still a difficult practice and may not be defined by a small collection of molecular attributes.

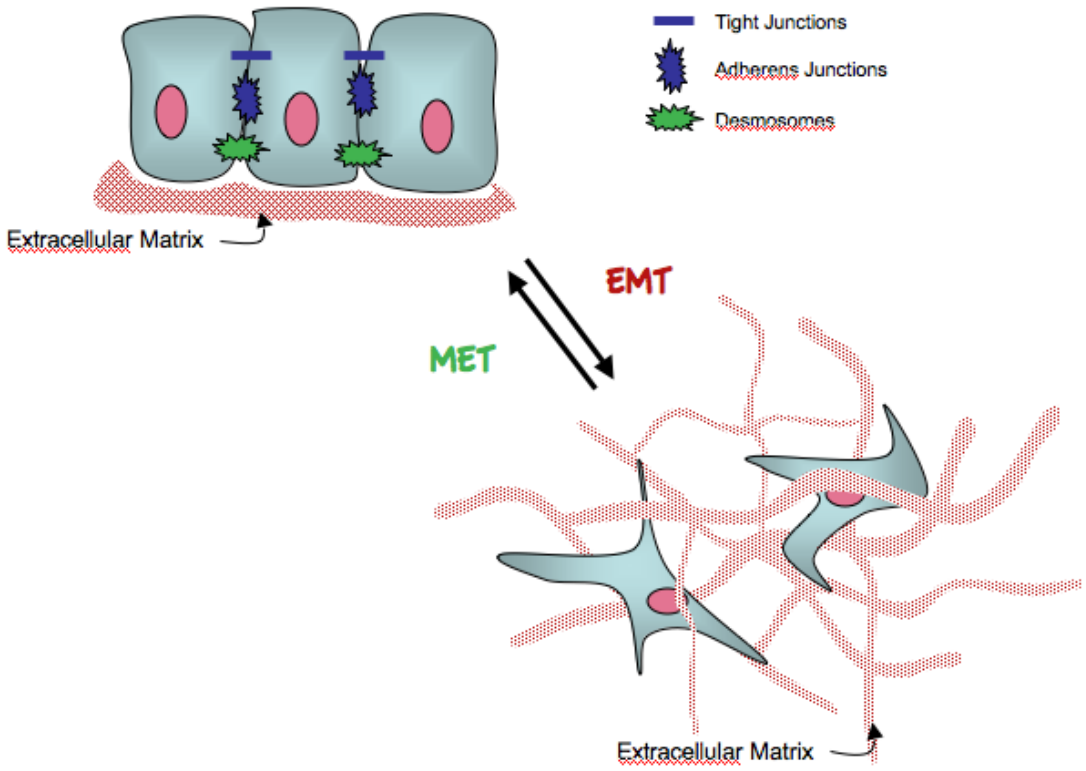


Figure 1.2: Features of epithelial-mesenchymal transition (EMT)
During EMT cells lose their cell-cell adhesion proteins and acquire a more invasive, more migratory, phenotype/behaviour. The reverse process of MET is also possible where cells revert back to their epithelial traits.

1.1.5 Experimental models of breast cancer

1.1.5.1 Breast cancer cell lines

Deregulation of normal cell processes such as genome instability, proliferation, apoptosis, motility and angiogenesis is necessary in the development of immortalised metastatic cells (Hanahan and Weinberg 2000). The use of immortalised breast cancer cell lines to provide insights to cellular physiology has been invaluable in advancing therapeutic research (Alimandi et al. 1995). As a heterogeneic disease, there have been over a thousand genes found to be deregulated in breast cancer and use of cell lines provides a means for investigating this heterogeneity *in vitro* (Fridlyand et al. 2006). Importantly, cell lines have been shown to display the same heterogeneity as primary tumours in terms of copy number and expression abnormalities (Neve et al. 2006). Furthermore, cell lines also cluster into basal-like and luminal expression subsets in a similar manner to primary tumours and show heterogeneous responses to targeted therapeutics mirroring observations in the clinic (Neve et al. 2006). Clearly there are distinct advantages for the use of cell lines, however, their relevance in disease is somewhat limited due to the lack of a surrounding microenvironment (stroma, vasculature etc.) that plays a major role in the development and progression of human disease (Place et al. 2011; Weaver et al. 1996).

1.1.5.2 Mouse models of breast cancer

The development of cancer mouse models has provided important insight into the development of breast cancer. Moreover, these models serve as preclinical platforms to verify efficacy of potential drug therapies. In some of the early models, mammary

lesions were induced by external factors such as the mouse mammary tumour virus (MMTV) and chemical carcinogens. Progress in transgenic technology then led to the development of a new generation of models where tumour suppressors can be knocked out, or onocogenes over-expressed, genetically. Apart from providing insight into the role of specific genes in breast carcinogenesis or progression, these models have also been useful in elucidating molecular pathways. The use of mammary gland-specific promoters such as MMTV-long terminal repeat (MMTVLTR), β-lactoglobulin (BLG) and whey acidic protein (WAP) gene promoters have allowed spatial control of transgene expression specifically in the mammary compartment. Despite these advances, there are still limitations to constitutively active promoters especially when a transgene of interest has unwanted effects throughout development. This problem has been addressed with the use of inducible transgenic systems. In addition, conditional gene expression models, such as the tetracycline-controlled system, have also proved useful in revealing mechanisms such as oncogene addiction. This is feasible with conditional systems because gene expression can be switched on or off in the presence or absence of its inducer (Allred and Medina 2008).

1.1.5.3 Transplantation models of breast cancer

An alternative approach to mouse modelling breast cancer has been the use of cell line transplantation. This can be performed either syngeneically where murine cells are transplanted into an appropriate host of the same background, termed allografts, or the transplant of human cancer cells into immunocompromised mice, termed xenografts. All of these approaches have their limitations and advantages (Vargo-Gogola and Rosen 2007). It has been hypothesized that tumour xenografts produce tumours that are more closely related

to breast cancers observed in humans in the clinic, since human cancer cell lines can be transplanted. However, the use of immunocompromised mice to establish xenografts neglects the contribution of certain components of the immune system to tumour progression. In general, using established transformed cancer cell lines for transplantation omits certain aspects related to tumour initiation. Equally, transplantation can be a way of rapidly elucidating questions pertaining to the latter, arguably more important, stages of breast cancer progression. Hence the selection of a particular model depends largely on the questions being addressed in the study because to date, none of the available models fully encompass all the features of breast cancer initiation and progression or indeed consistently predict clinical outcome (Vargo-Gogola and Rosen 2007).

1.1.6 Breast cancer stem cells

The classic models of carcinogenesis propose that cancer arises via a series of stochastic events whereby, given the right combination of mutations, any cell within an organ is able to be transformed and give rise to tumours containing cells that are all equally malignant (Nowell 1976). There is increasing evidence that now supports a fundamentally different model for describing cancer initiation, maintenance and progression. The 'cancer stem cell hypothesis' states that cancers originate from only stem or progenitor cells (Reya et al. 2001). Stem cells are defined by their ability to undergo both self-renewal and multi-lineage cell differentiation. The process of self-renewal can be either symmetrical or asymmetrical, either producing two daughter stem cells or one daughter stem cell and one cell capable of differentiating along one of a variety of lineages respectively. The cancer stem cell hypothesis states that deregulation of these normally

tightly controlled processes of self-renewal results in heterogeneous tumours that are driven by a small subset of cells referred to as cancer stem cells (CSCs) or tumour-initiating cells (TICs) (Wicha et al. 2006). Evidence supporting the existence of CSCs was first observed in acute myelogenous leukemia (AML) (Bonnet and Dick 1997). In this study, leukemic stem cells were identified by their ability to initiate human AML when transplanted into immunocompromised mice. This approach was then extended to demonstrate the existence of breast cancer stem cells. It was shown that, when grown in immunocompromised mice, only a small percentage of human breast cancer cells were capable of initiating tumours (Al-Hajj et al. 2003). Furthermore, this research also revealed that human breast cancers contain a small cellular population characterised by the expression of certain cell surface markers, which display stem cell-like properties. As few as 100 cells expressing these surface markers were able to initiate tumours when transplanted into immunocompromised mice. In accordance with the cancer stem cell hypothesis, the tumours formed in these mice had the same phenotypic heterogeneity as the initial tumour, suggesting they were driven by multipotent cells. Additionally, cells from these tumours were able to generate further new tumours after transplantation into new recipient mice, demonstrating the self-renewal ability of the cells. In contrast, transplantation of up to 20,000 cells lacking expression of these surface markers failed to initiate any tumours at all (Al-Hajj et al. 2003). However, recent studies have also shown that once the CSC subset has been depleted the remaining differentiated cells have the capability to re-acquire CSC properties, this is discussed in more depth later in this chapter. The identification of CSCs as the subset of cells responsible for tumour initiation and metastasis has driven research toward specifically targeting this subset. Traditional therapies targeting

bulk tumour cells may give significant regression; however, without targeting the CSC subset tumour relapse would seem inevitable (Figure 1.3). Thus CSC therapeutic intervention is now at the forefront of much cancer research.

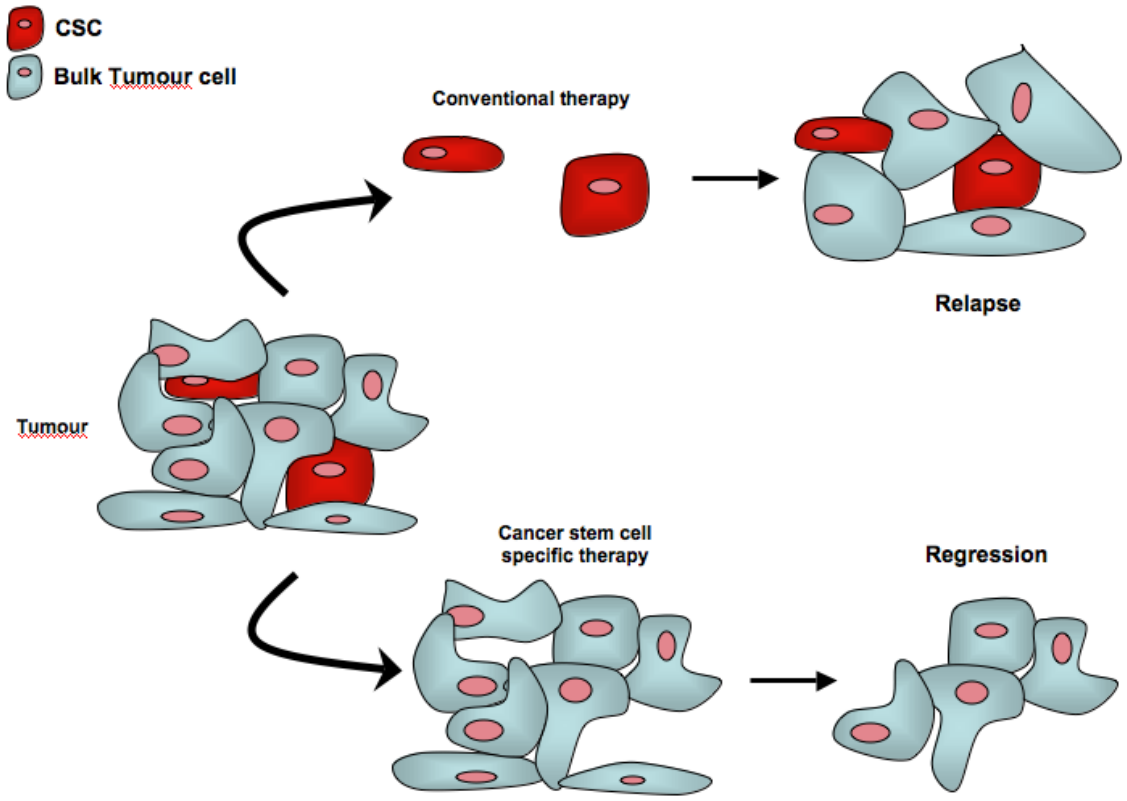


Figure 1.3: Therapeutic strategies of treating cancer
Conventional treatment can result in marked regression of a tumour but cancer stem cells remain as they are resistant to these therapeutic approaches and ultimately tumours relapse. CSC targeted therapies remove the cells responsible for tumour relapse and may have better long-term therapeutic benefit.

1.1.6.1 Functional identification of breast cancer stem cells

Advances in cell culture techniques have been important in the identification and study of both normal mammary and breast cancer stem cells. The *in vitro* study of mammary stem cells was developed along the lines of work from the neuronal field in which a cell culture assay, known as the neurosphere assay, was used to identify neural stem cells (Dontu et al. 2003a; Ponti et al. 2005). These cell culture conditions have since been adopted to aid the identification and study of both normal mammary and breast cancer stem cells (Dontu et al. 2003a). Plating normal human mammary epithelial cells in non-adherent, serum-free conditions with a specific set of growth factors results in the formation of spherical colonies termed mammospheres (Dontu et al. 2003a). Indeed these culture conditions mimic those which cells must survive *in vivo* to metastasise and these mammosphere colonies were shown to be enriched for cells that exhibit functional characteristics of stem/progenitor cells such as multipotency and self-renewal (Bhat-Nakshatri et al. 2010; Dontu et al. 2004; Fillmore and Kuperwasser 2008; Tao et al. 2011). The mammosphere-forming assay is now routinely used to identify key mediators of stem cell behaviour and maintenance in both human and rodent cell populations (Al-Hajj et al. 2003; Ponti et al. 2005).

The use of flow cytometry for isolating breast cancer stem cells on the basis of cell surface markers has also been crucial in studying these populations. Both limiting dilution transplant experiments and analysis of cell surface markers in mammosphere cultures helped identify the $CD44^{+ve}/CD24^{-ve}$ cell population as having human breast cancer stem cell attributes (Ginestier et al. 2007). An additional surface marker, aldehyde dehydrogenase 1 (ALDH1), an enzyme responsible for the oxidation of aldehydes to carboxylic acids, has also

recently been shown to be over-expressed in the human breast cancer stem cell population (Aktas et al. 2009; Charafe-Jauffret et al. 2009; Korkaya et al. 2008; Morimoto et al. 2009; Tanei et al. 2009). ALDH1 activity is detected by an enzymatic 'Aldefluor' assay resulting in fluorescence detectable by flow cytometry or immunohistochemistry and is now routinely used in the identification of this cell population (Hay 2005).

1.1.6.2 Interconversion between cancer stem and non-stem phenotypes.

Cellular phenotypic plasticity is a well-established phenomenon in developmental and embryonic stem cell research (Hay 1995; Thiery and Sleeman 2006). This describes the ability of cells to transition between differentiated and de-differentiated states and is best illustrated by the reversible process of epithelial to mesenchymal transition (EMT) in the embryo (Asiedu et al. 2011; Aslakson and Miller 1992; Chao et al. 2010; Charafe-Jauffret et al. 2009). EMT has long been implicated in breast tumour biology, as being a key step in the dissemination of tumour cells to distal sites during metastasis and has been more recently extended to include the reverse process of MET to explain the expansion of more differentiated cell progeny at the distal site (Mani et al. 2008; Morel et al. 2008). However, recently a conceptual link between EMT and CSCs (bCSCs) has been described (Scheel and Weinberg 2011) that implies the existence of functional plasticity between cancer stem and cancer non-stem populations. Thus like embryonic and adult stem cells, CSCs are not a static population, and that under permissive conditions, possibly dictated by the tumour microenvironment, cancer cells transition between stem-like and non-stem-like states (Drasin et al. 2011; Scheel and Weinberg 2011)

(Figure 1.4).

This has broad implications. It helps to explain how a minority population of CSCs can drive clonal tumour expansion through spontaneous mutations, thus successfully marrying current theories of CSC biology with models of tumour development (Scheel and Weinberg 2011). It is at the heart of tumour heterogeneity as described above; and at least conceptually, it provides an additional explanation for tumour relapse. The clinical implication of this is that even if all cancer stem cells were eliminated from a tumour by a selective cytotoxic agent (in itself an obstacle due to EMT dependent drug resistance, as described below), residual bulk tumour cells remaining in the tumour microenvironment have the capacity to de-differentiate to a CSC-like phenotype and consequently re-seed tumour growth (Polyak and Weinberg 2009). Future therapeutic strategies therefore will need to both eliminate CSCs and prevent their reacquisition from the residual tumour microenvironment (Figure 1.3).

The evidence for a link between an EMT-like process and the acquisition of stemness is compelling, particularly in breast cancer (reviewed in Polyak and (Mani et al. 2008; Morel et al. 2008)). Two independent studies from the Weinberg and Puisieux laboratories showed that cells that had undergone EMT possessed stem-like properties including increased tumour initiation and self-renewal capacity (Chaffer et al. 2011; Iliopoulos et al. 2011; Sarrio et al. 2012). Thus enforcement of an EMT through the ectopic expression of EMT mediators, Snail or Twist or with TGFβ1 treatment in immortalised human mammary epithelial cells (HMECs) promoted the formation of CSC-like cells from non-stem cells, a process which was augmented by oncogenic transformation. However, in these early studies, uninduced non-CSC-like cells failed to spontaneously generate a mesenchymal

CSC-like phenotype, suggesting a persistent unidirectionality in the stem-progenitor hierarchy that could only be overcome with direct intervention on EMT pathways – possibly mirroring EMT-like events during malignancy.

This unidirectional model of stem cell biology has since been challenged by a number of studies that demonstrate in normal, immortalised and neoplastic breast epithelial cells the spontaneous conversion of non-stem-like cells to a stem-like state (Chaffer et al. 2011; Iliopoulos et al. 2011). Thus non-stem-like breast epithelial cells isolated either from HMECs or basal cell lines (eg. MCF-10A) and cultured in isolation for prolonged periods re-acquired a subpopulation of cells with stem-like characteristics. The efficiency of this stem-like transition, however, was poor compared to the opposing conversion of mesenchymal-like stem cells to more differentiated luminal subtypes, providing a putative explanation for the small but stable proportion of stem cells found in tumours and in established cell cultures. The stem conversion rates ranged from 1% to 5% over 8-9 days of cell culture (Chaffer et al. 2011; Iliopoulos et al. 2011) and was dependent on the cellular context, with primary luminal HMECs and immortalised cells grown as 3D structures exhibiting marked reductions, or even reversals in the interconversion rates. Furthermore oncogenic transformation augmented the transition of non-stem cells to a stem-like phenotype and consequently increased the proportion of cancer cells with stem-like attributes in the transformed cell populations (Meyer et al. 2009). Breast cancer cell lines exhibit similar CSC plasticity, $CD44^{+ve}$ subpopulations derived from both luminal and mesenchymal breast cancer cell lines exhibited phenotypic plasticity both *in vitro* and *in vivo* (Sarrio et al. 2012). Interestingly therefore, unlike HMECs, the original phenotype of established breast cancer cell

populations had little bearing on the ability of non-CSCs to acquire stem-like characteristics.

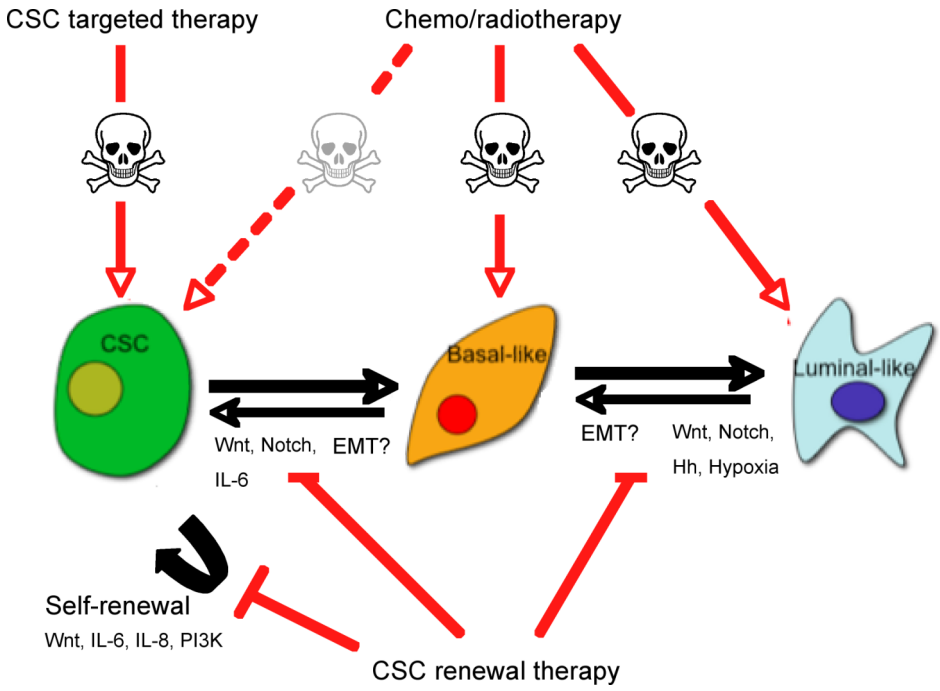


Figure 1.4: Phenotypic plasticity of cancer cells
Accumulating evidence now suggests that cell state is plastic whereby CSCs can differentiate into all other cellular subsets found in a tumour and differentiated cells and, under certain conditions, can also return to a CSC-like state. This plasticity appears to be under the influence of various signalling pathways including Notch, Wnt, Hh, hypoxia and interleukins.

These studies represent a paradigm shift in the way that we perceive the origin and behaviour of cancer stem cells. Yet a number of questions remain. For example, does the spontaneous interconversion of non-CSCs and cancer stem cells require an EMT, and if so do regulators of EMT initiate/drive this plasticity? Clues to the first of these questions were provided in a recent elegant study looking at epithelial and mesenchymal subpopulations within normal basal-like breast cell lines (Sarrio et al. 2012). A spontaneous, yet partial EMT was observed in the epithelial population resulting in two subpopulations of cells that exhibited distinct stem/progenitor

characteristics of invasive behaviour and self-renewal on the one hand and regenerative potential, ALDH1 activity and formation of differentiated 3D structures on the other (Meyer et al. 2009). Similarly the interconversion of $CD44^{+ve}$ subpopulations derived from breast cancer cell lines was shown to be dependent upon activin/nodal signalling (Yang and Weinberg 2008). These data suggest that EMT is integral to the plasticity of basal-like breast cells but is not necessarily the sole mediator of the stem/progenitor phenotype.

Whether these apparently spontaneous events occur in direct response to the upregulation of EMT regulators or in response to other locally derived factors has yet to be determined. However several lines of evidence suggest that the tumour microenvironment plays a key role in the modulation of plasticity. Cellular stress concomitant with the tumour microenvironment such as hypoxia, or arising as a direct result of anti-hormonal or chemotherapy may also impart selective pressures that result in CSC expansion (Santisteban et al. 2009). Moreover, immune mediated (CD8 T cell) induction of EMT in a mouse model of antigen-modified primary breast cancer lead to the enrichment of bCSCs in resultant tumour outgrowths (Liu et al. 2011). Bone marrow-derived (BMD) mesenchymal stem cells have also been demonstrated to traffic to the primary tumour site and expand the bCSC population through a cytokine loop involving CXCL7 and IL-6 (Iliopoulos et al. 2011). The relevance of this cytokine axis on bCSC plasticity was confirmed in genetically distinct breast cancer cell lines and primary tumours using a blocking antibody to IL-6 in conditioned culture medium (Bomken et al. 2010; McDermott and Wicha 2010). The influence of intra-tumour heterogeneity on bCSC plasticity may therefore also encompass BMD cells and the immune cell repertoire.

There are a number of other CSC-related signalling pathways

implicated in the maintenance/expansion of bCSCs, including Wnt, Notch, Hedgehog, p53, PI3K and HIF (reviewed in (Hardy et al. 2010)) but it remains to be established whether factors that influence self-renewal also direct phenotypic plasticity. Some of these pathways are known to induce an EMT, including EGF/Akt (Yook et al. 2006), Wnt (Leong et al. 2007), Notch (Kasper et al. 2009) and Hedgehog (Lopez-Tarruella and Martin 2009) and are therefore likely candidates for having such a role. More studies will be required using rigorous assays of plasticity to elucidate this.

The most recent evidence described above suggest that while phenotypic plasticity mirrors many aspects of EMT, blocking EMT may in itself be insufficient to prevent the acquisition of at least some stem/progenitor characteristics. Ultimately a better understanding of the signals that control the equilibrium between non-stem and stem-like compartments is required in order to prevent the reacquisition of bCSCs during therapy.

1.1.7 Treatment of breast cancer

The treatment of a newly diagnosed breast cancer patient generally depends on the extent of the disease and typically involves surgery in combination with post-operative radiotherapy. Adjuvant therapy in combination with chemotherapy regimens such as CMF (Cyclophosphamide, Methotrexate, Fluorouracil) or FEC (Fluorouracil, Epirubicin, Cyclophosphamide) in an attempt to reduce recurrence following surgery is also common (O'Shaughnessy 2005). In spite of treatment advances of newly diagnosed breast cancers, about 30% of patients still eventually develop metastatic disease that is generally incurable (reviewed in O'Shaughnessy 2005). The recent molecular identification and characterisation of distinct breast cancer subtypes

has allowed the development of more targeted therapies that are specifically directed towards certain biological features of individual tumours.

1.1.7.1 Target-based agents in breast cancer

Target-based therapies can be divided into three broad categories: subtype specific agents, signaling pathway inhibitors, and tumour microenvironment targeted drugs. The first target-based therapy developed for breast cancer was endocrine treatment for ER+ve subtypes of the disease, firstly with anti-estrogens such as tamoxifen and fulvestrant (Nicholson et al. 2003; Osborne et al. 1994) and then later with aromatase inhibitors (Nicholson and Johnston 2005). Since then, many agents that are active against the EGFR family receptors have also been developed. These include monoclonal antibodies such as trastuzumab, which bind the extracellular domain of the HER2 receptor (Carter et al. 1992) and small-molecule tyrosine kinase inhibitors that prevent their phosphorylation, such as lapatinib (Rusnak et al. 2001). When used in combination with chemotherapy, these agents have been shown to have considerable efficacy in treating certain subtypes of breast cancer that over-express certain EGFR family receptors (Gasparini et al. 2007; Geyer et al. 2006; Slamon et al. 2001). Additionally, agents targeting downstream signaling pathways of the EGFR family receptors (e.g. mTOR and Src) have been developed with limited success (Lombardo et al. 2004; Yeh et al. 2007; Yu et al. 2001). However, preclinical findings do suggest that they may be useful in certain breast cancer subtypes.

While responses to some of these treatments have been encouraging, resistance is still prevalent, and disease improvement can be short-lived. In particular, triple-negative cancers do not

respond to most targeted therapies due to their lack of molecular targets. Therefore, there is still a need to identify new or synergistic targets to treat all subtypes of the disease.

One such target may be the NF-κB family of transcription factors which have been shown to be activated downstream of a number of cell surface receptors. NF-κB plays a key role in many physiological processes, including innate and adaptive immune responses, inflammation and cell proliferation, death and motility. The potential of NF-κB as a therapeutic target in breast cancer has already been addressed in a number of reports in the literature (Aggarwal et al. 2005; Cao et al. 2007; Connelly et al. 2010; Helbig et al. 2003; Liu et al. 2010; Park et al. 2007). This has led to a great deal of interest in the development of signaling inhibitors of NF-κB.

Elucidation of signalling pathways that regulate self-renewal has become of prime importance for gaining further understanding of how deregulation of this process can lead to carcinogenesis. Recent evidence suggests that key oncogenic pathways such as those mediated by ErbB2, NOTCH, Akt, Wnt and NF-κB signalling regulate breast stem cell behaviour (Dontu et al. 2004; Korkaya et al. 2009; Korkaya et al. 2008; Liu et al. 2010; Takahashi-Yanaga and Kahn 2010; Wang et al. 2010) and as such may provide excellent targets for future breast cancer therapy.

1.1.8 Drug resistance in breast cancer

Despite significant advances, the success of many first-line cancer therapeutics can be limited as significant amount of patients will ultimately become resistant to them. Most patients with ER+ve breast cancer are treated with endocrine adjuvant therapy and ithis is also the most effective treatment for ER+ve metastatic breast cancer. The

success of endocrine therapy is limited, however, by *de novo* and acquired resistance of tumour cells in up to 30% of patients (EBCTCG, 2005). Tumours, especially advanced metastatic tumours, initially responsive to endocrine therapy will ultimately progress over time to the point where the endocrine agent is then driving tumour cell growth rather than inhibiting its proliferative ability (Nicholson et al. 2003; Osborne et al. 2003). In tamoxifen-resistant patients, subsequent Faslodex therapy can be beneficial but again the cells will become resistant to this anti-estrogen (AE) over time (Knowlden et al. 2003; McClelland et al. 2001; Nicholson and Johnston 2005). The mechanisms of AE resistance are complex and, in general, poorly understood. When retained, the ER signalling pathway interacts with growth signalling pathways that regulate cell survival and proliferation signalling resulting in the promotion of acquired resistance and relapse (Nicholson et al. 2003; Osborne et al. 2003). In particular the EGFR and ErbB2 pathways are believed to be particularly important in AE resistance (Knowlden et al. 2003; McClelland et al. 2001; Nicholson and Johnston 2005). Resistance is not limited to endocrine agents, resistance is also frequently observed in other forms of therapeutic intervention such as trastuzumab and as such many studies are now focussed on the underlying mechanisms of this resistance to aid in the development of further treatment options (Piccart 2008).

As the standard mode of treatment, the majority of breast cancer patients will receive some form of chemotherapy or radiotherapy during their treatment regime. However, there is increasing evidence to suggest that chemotherapy/radiotherapy treatment, even in patients showing significant response, does not target the heterogeneity of cells found within breast tumours, more specifically the CSC subset. As such, breast CSCs (bCSCs) are enriched in biopsies

taken from patients who received neoadjuvant chemotherapy (Li et al. 2008; Tanei et al. 2009; Yu et al. 2007b). Similarly, tumours engrafted into animals receiving radiation therapy were also found to have an enriched CSC compartment (Phillips et al. 2006; Zielske et al. 2011). The conclusion has been therefore that selective pressures predicated on the inherent resistance of bCSCs to chemotherapy and radiotherapy result in the skewed equilibrium of stem and non-stem cell populations *in vivo*. Several mechanisms have been ascribed to this resistance phenotype in bCSCs. ABC transporters, for example ABCG2, are upregulated in both stem and non-stem breast cancer cells (Patrawala et al. 2005). Signalling pathways such as Notch (Farnie and Clarke 2007) and Wnt (Yook et al. 2006) that, as already discussed, play a role in bCSC maintenance and potentially influence bCSC phenotypic conversion via EMT, are also associated with chemoresistance (Sajithlal et al. 2010) and radioresistance (Phillips et al. 2006; Woodward et al. 2007). Indeed there is direct evidence that EMT correlates with therapy resistance. Samples obtained from residual cancers (Creighton et al. 2009) or pleural effusions (Yu et al. 2007a) after therapy were found to have an increased expression of mesenchymal EMT markers. A similar effect has been observed *in vitro* where breast cancer cells that underwent EMT were resistant to two common chemotherapeutics vincristine and paclitaxel (Li et al. 2009). Whether this resistance is a case of selection of CSCs as a result of having undergone EMT or if the therapy itself induces an EMT thus pushing cells towards a more CSC-like phenotype remains to be elucidated.

Endocrine therapy under permissive conditions may potentiate the conversion of non-stem cancer cells to bCSCs (O'Brien et al. 2011). For example, ER-negative basal-like cell lines exhibit an increased CSC

subset (Fillmore and Kuperwasser 2008) while endocrine resistance in breast cancer cell lines predisposes to a more basal phenotype (Hiscox et al. 2006). This is supported by studies of primary breast cancers where ER+ve patients receiving aromatase inhibitors acquired mesenchymal-like tumour phenotypes with bCSC properties (Creighton et al. 2009). Conversely, in a study of 88 ER+ve primary breast cancer patients, 86% of the patients had disseminated tumour cells that were exclusively ER-ve (Fehm et al. 2008). There is also evidence that estrogen receptor signalling directly influences EMT and bCSCs, as the EMT modulators snail and slug are directly suppressed by ER signalling (Ye et al. 2008).

As outlined, there is potential therapeutic benefit in the targeting of bCSCs. Ideally the therapy must target both the tumour bulk and the CSCs due to the importance of cellular interactions within the microenvironment can result in phenotypic plasticity. Hirsch et al have recently demonstrated this concept by combining metformin, a diabetes drug that specifically kills CSCs by an unknown mechanism, with the chemotherapeutic drug doxorubicin resulted in long-term remission of MCF10A-ER-Src xenografts with no relapse up to 60 days later. However treatment with doxorubicin alone relapsed only 5 days after complete remission (Hirsch et al. 2009). Unfortunately such CSC targeted therapeutics may struggle to progress through phase II clinical trials given the current guidelines outlined by RECIST [response evaluation criteria in solid tumours] for progression to phase III (Eisenhauer et al. 2009). Objective response rates are traditionally used as the endpoint of phase II trials where tumour regression is seen as a predictive measure for prolonged survival. However in many cases there is weak correlation between tumour response and overall survival (Huff et al. 2006) and this may be due to the inability of such

therapies to target CSCs. The pitfalls of this model are clear in the circumstance of progressing CSC-specific agents. Whilst such agents may not give measurable shrinkage of the tumour itself, the overall effect on patient survival may be improved but not observed in the short duration of a specified phase II trial. Thus it has been suggested that the use of alternative endpoints evaluating CSC content may be more suitable for certain agents (Adjei et al. 2009; Huff et al. 2006).

1.1.8.1 Drug resistant models of breast cancer

Estrogen receptor positive (ER+ve) breast cancers account for up to 75% of all breast cancers diagnosed in the clinic. As such endocrine therapies targeting estrogen receptor or circulating estrogen have been shown to have much success in treating this cohort of patients. As previously stated, their success has been somewhat limited due to intrinsic and acquired resistance and tumours that initially respond will invariably progress (Musgrove and Sutherland 2009). To understand the mechanisms behind this resistance several groups have now developed anti-estrogen resistant preclinical models of the endocrine responsive, ER+ve, MCF-7 cell line (Berstein et al. 2003; McClelland et al. 2001). Resistant MCF-7 models to two commonly used anti-estrogen drugs, tamoxifen (TAMR) and Faslodex (FASR), have been developed order to help elucidate resistance mechanisms and identify new agents that may prove beneficial in the clinical setting. These cell lines were produced by long-term exposure of the MCF-7 cell line to the 4-hydroxy forms of both tamoxifen and Faslodex (Knowlden et al. 2003; McClelland et al. 2001). These *in vitro* models display a similar phenotype to that observed in patients, whereby the cells have an invasive phenotype and elevated EMT-like behaviour (Borley et al. 2008; Hiscox et al. 2006; Zhou et al. 2012).

Characterisation of the *in vitro* TAMR model revealed that they retained their ER and had elevated expression of both EGFR and HER2 when compared to their parental MCF-7 cells (Knowlden et al. 2003). As such treatment of the TAMR cells with the tyrosine kinase inhibitor gefitinib or trastuzumab resulted in decreased TAMR cell growth (Knowlden et al. 2003; Valabrega et al. 2007), supporting observations that EGFR/HER2 inhibition can be beneficial in endocrine-resistant patients in the clinic (Johnston 2008). During the development of the FASR cells, long-term exposure of the MCF-7 cells to Faslodex resulted in the complete loss of ER whilst still displaying an increased level of EGFR signalling (Nicholson et al. 2005). This increase in EGFR signalling is not limited to these MCF-7-derived models and is supported by studies undertaken by other groups in anti-estrogen-resistant models derived from alternative origins (Benz et al. 1992; Campbell et al. 2001; Coutts and Murphy 1998; Donovan et al. 2001; Kurokawa and Arteaga 2003; Kurokawa et al. 2000; Long et al. 1992). Studies have shown that this aberrant EGFR signalling drives anti-estrogen-resistant cell growth through the mitogen-activated protein kinase (MAPK) and phosphotidylinositol 3-kinase (PI3K)/Akt signalling pathways. Clinical tissue also exhibits this increased EGFR, HER2 and MAPK in sequential samples obtained from tamoxifen treated patients, validating the *in vitro* findings of other groups (Gee et al. 2001; Gee et al. 2005). It has been shown that ER can be reactivated by increased MAPK phosphorylation in these cells contributing to their continued growth in the absence of ER ligands (Bunone et al. 1996; Kato et al. 1995). Indeed TAMR cells, in line with their clinical counterparts, continue to express ER following the acquisition of resistance and appears to be functional, as demonstrated by the observation that tamoxifen-resistant cells will still

respond to Faslodex (Brunner et al. 1993; Chan et al. 2002; Lykkesfeldt et al. 1994; Robertson 1996). The AE Faslodex functions by degrading ER and thus FASR cell growth cannot be promoted by growth factor driven ER signalling. However, it has been noted that FASR cells demonstrate focally increased EGFR expression (Gee et al. 2004; Nicholson et al. 2005). Further analysis of Faslodex-resistant models elucidated a marked increase in the expression, nuclear translocation and transcriptional activity of NF-κB, this activity was also abrogated by treating these FASR cells with the IKK inhibitor parthenolide (Gee et al. 2006). Collectively these studies unveil the complex cell signalling that maintains cell growth in endocrine-resistant cells and identify potential therapeutic targets to overcome endocrine-resistant cell growth.

1.2 Mechanisms of apoptosis

There are two major pathways of apoptosis in mammals, namely, the extrinsic pathway that can be triggered through death receptors and the intrinsic pathway that is mitochondrial dependent. The extrinsic pathway involves stimulation of death receptors such as CD95, DR4, DR5 or TNFα receptor by their respective ligands, which then induces receptor oligomerization and recruitment of adaptor proteins with death domains. A series of proteolytic cascades involving caspases are integral to the apoptotic process. Caspases are cysteine proteases which cleave their respective substrates at aspartate residues (Vaux and Strasser 1996). They are synthesized as zymogens and their activation is dependant on various apoptosis regulators. Caspase-8, the initiator caspase of the extrinsic pathway, associates with the activated receptor and adaptor proteins forming a death

inducing signaling complex (DISC). This is followed by auto-activation of caspase-8 which sets off the caspase cascade resulting in the activation of the effector caspases (caspase-3, -6 and -7) (Ashkenazi and Dixit 1998). Alternatively, the intrinsic pathway is mediated by release of cytochrome c from the mitochondrion into the cytoplasm, which associates with apoptosis protease activating factor 1 (Apaf-1) and caspase-9 to form the 'apoptosome'. As with the DISC complex in the extrinsic pathway, the apoptosome then induces activation of caspase-9 and this leads to cleavage and activation of effector caspases, the point where both pathways converge (Shi 2002). Subsequently, other nucleases (e.g. caspase-activated DNase [CAD]) and proteases responsible for the terminal stages of apoptosis are then activated by the caspase cascade. Activation of caspase-8 via death receptor signalling can also lead to truncation of Bid and mitochondrial membrane permeation providing direct crosstalk between death receptor signalling and the intrinsic pathway in certain cell types (discussed later in 1.3.1.1.2) (Miller 1997). Caspases clearly play an important role in the apoptotic process and thus tight control of caspase activation is important as over-activation of caspases can lead to degenerative diseases whereas over-inhibition can promote tumourigenesis (Degterev et al. 2003).

It is the balance of pro-apoptotic and anti-apoptotic regulators in a cell that determines the sensitivity of cells to a particular death stimulus. At the death receptor level, pro-survival proteins such as cellular FLICE-like inhibitory protein (c-FLIP) antagonize the activation of caspase-8, however c-FLIP regulation of caspase-8 activation is extremely complex. Similarly, pro- and anti-apoptotic proteins of the B-cell lymphoma 2 (Bcl-2) family control mitochondrial permeation. In addition, inhibitor of apoptosis proteins (IAPs) can inhibit the effector

caspases and caspase-9 through direct binding (Degterev et al. 2003).

1.2.1 Evading apoptosis: A hallmark of cancer cells

Since the initial observation that decreasing apoptosis in cancer cells can contribute to aberrant tissue turnover in tumours, it is now widely accepted that the ability to evade apoptosis is a hallmark of cancer cells (Hanahan and Weinberg 2000; Kerr et al. 1972). The first evidence for a role of anti-apoptotic proteins in tumourigenesis was provided through expression of Bcl-2 in haematopoietic cells, where it can cooperate with c-Myc to promote neoplastic progression (Vaux et al. 1988). From these observations it was proposed that apoptosis regulators, which are normally pro-survival, can behave as oncogenes.

1.2.2 Apoptosis as a multistep barrier to metastasis

During the process of metastasis, there are multiple stages where cancer cells are exposed to death-inducing stimuli. The first of which is when tumour cells detach from surrounding cells in the tumour and/or the extracellular matrix (ECM). In normal mammary epithelial cells, following the loss of adhesion, anoikis or cell death is induced (Martin and Leder 2001; Streuli and Gilmore 1999). Furthermore, tumour cells have been shown to die when they come into contact with endothelial cells of vasculature during intravastion (Wyckoff et al. 2000). Once in the blood vessels circulating tumour cells are then subjected to mechanical stress due to shear forces and surveillance by immune cells (Kim et al. 2000; Weiss 1993). When metastatic tumour cells arrive at secondary sites they need to adapt to a foreign microenvironment and only a small percentage will actually result in micro-metastases (Wong et al. 2001). Collectively, these stresses suggest that metastasis is a difficult process and the ability to prevent apoptosis is imperative for successful formation of metastases.

Indeed overexpression of anti-apoptotic Bcl-2 family proteins in tumourigenic mammary epithelial cells have been shown to increase the metastatic potential, identiying apoptosis as a limiting factor in the metastatic cascade (Del Bufalo et al. 1997; Martin et al. 2004; Pinkas et al. 2004). These studies were performed using transplants in immunocompromised mice, which disregards the early stages of metastasis where cells must overcome anoikis and immune surveillance. Hence, the development and use of models that are capable of recapitulating all stages of metastasis will help provide additional insight into the role of apoptosis as a safeguard from metastasis and ways of reactivating apoptotic pathways in cancer cells (Mehlen and Puisieux 2006).

1.3 Instructive cell death as a putative cancer therapy

1.3.1 The TNF superfamily

Members of the tumour necrosis factor (TNF) superfamily of cytokines are key extracellular mediators of apoptosis (Ashkenazi 2002). TNFα, Fas and TRAIL are signalling pathways within the TNF superfamily that induce apoptosis in cells. Targeting the TNFα and Fas pathways were originally believed to be desirable for cancer treatment, however, clinical use of TNFα and FasL are severely limited by their toxicities (Aggarwal et al. 2006; Ogasawara et al. 1993). On the other hand TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in cancer cells but shows almost no toxicity to normal cells and this attribute has led to much interest in targeting TRAIL for cancer therapy (Ashkenazi et al. 1999).

1.3.1.1 TNF-related Apoptosis-inducing Ligand (TRAIL)

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL) or Apoptosis 2 ligand (APO-2L) was first identified due to its sequence homology to the extracellular domain of CD95 ligand (FasL) and tumour necrosis factor (Pitti et al. 1996; Wiley et al. 1995). TRAIL is a member of the TNF superfamily and is expressed in a wide range of tissues. The primary function of human TRAIL ligand is inducing extrinsic apoptosis in target cells; it performs this function by binding to its cognitive receptors found on the cell surface. As a naturally occurring ligand within the body, endogenous TRAIL is expressed on natural killer cells, macrophages, T-cells and dendritic cells and is believed to have an immune defense function destroying virus-infected and malignant cells (Duiker et al. 2006). Recently TRAIL has attracted much interest as one of the most promising anti-cancer molecules, despite being a membrane-bound ligand its extracellular region can be cleaved to produce a soluble recombinant form (Mariani et al. 1997). Furthermore, monoclonal antibodies (mAbs) directed against TRAIL agonistic receptors are currently being developed and investigated for their anti-tumour activity (Ashkenazi et al. 1999; Camidge et al. 2007; Kelley and Ashkenazi 2004).

1.3.1.1.1 TRAIL structure and signalling

Like other TNF cytokines TRAIL is a soluble homotrimeric molecule produced when the c terminus of the expressed type II transmembrane TRAIL protein is proteolytically processed. These homotrimers of TRAIL signal by cross-linking receptor molecules on the cell surface (Pitti et al. 1996). There are up to four surface receptors capable of binding TRAIL, two of which result in an induction of apoptosis whilst the remaining two, which maintain substantial sequence homology in the extracellular domain but lack the functional

death domain, serve as decoy receptors resulting in no subsequent signal transduction after binding (Ashkenazi 2002). Furthermore, TRAIL is also capable of binding to osteoprotegerin (OPG), a secreted TNF receptor homologue (Emery et al. 1998).

1.3.1.1.2 TRAIL induction of apoptosis

Apoptosis is initiated when TRAIL or agonistic monoclonal antibodies (mAbs) bind to DR4 (TRAIL-R1) and DR5 (TRAIL-R2) on the cell surface. Binding of homotrimeric TRAIL molecules induces the oligomerisation of DR4 or DR5 receptors resulting in homotypic interactions between their respective death domains in the carboxy terminus of the receptors that subsequently gives the recruitment of the adaptor protein Fas-associated death domain (FADD). Death effector domains (DED) of FADD then recruit membrane proximal caspase-8 and caspase-10. This multi-protein complex, designated the death inducing signalling complex (DISC), provides the molecular platform for initiator caspase auto-activation and sets off the proteolytic cascade of caspase-3, -6 and -7 cleavage and activation that follows, ultimately triggering the apoptotic process (Ashkenazi and Dixit 1999; Falschlehner et al. 2007).

Further to the apoptosis induction via the extrinsic pathway, TRAIL binding and DISC formation also engages the intrinsic apoptosis pathway by activation of Bcl-2 family members. The pro-apoptotic protein Bid is cleaved to activate its truncated form tBid, this in turn migrates to the mitochondria where it induces Bax and Bak activation and translocation to result in pore formation of the outer membrane of the mitochondria. Consequently, cytochrome C is released from within the mitochondria into the cytosol facilitating the interaction between apoptotic protease factor-1 (Apaf-1) and caspase-9 completing the

formation of the apoptosome complex and activation of caspase-9. The activation of the initiator caspase-9 in the apoptosome culminates in the activation of effector caspases and amplifies the apoptotic signal induced via the extrinsic pathway (Figure 1.6) (Chen et al. 2000; Gong et al. 1999). Although this process of amplification by intrinsic apoptosis occurs in all cells it is only required for activation of extrinsic apoptosis in type II cells, type I cells are capable of extrinsic apoptosis induction without this additional signal (Ozoren and El-Deiry 2002).

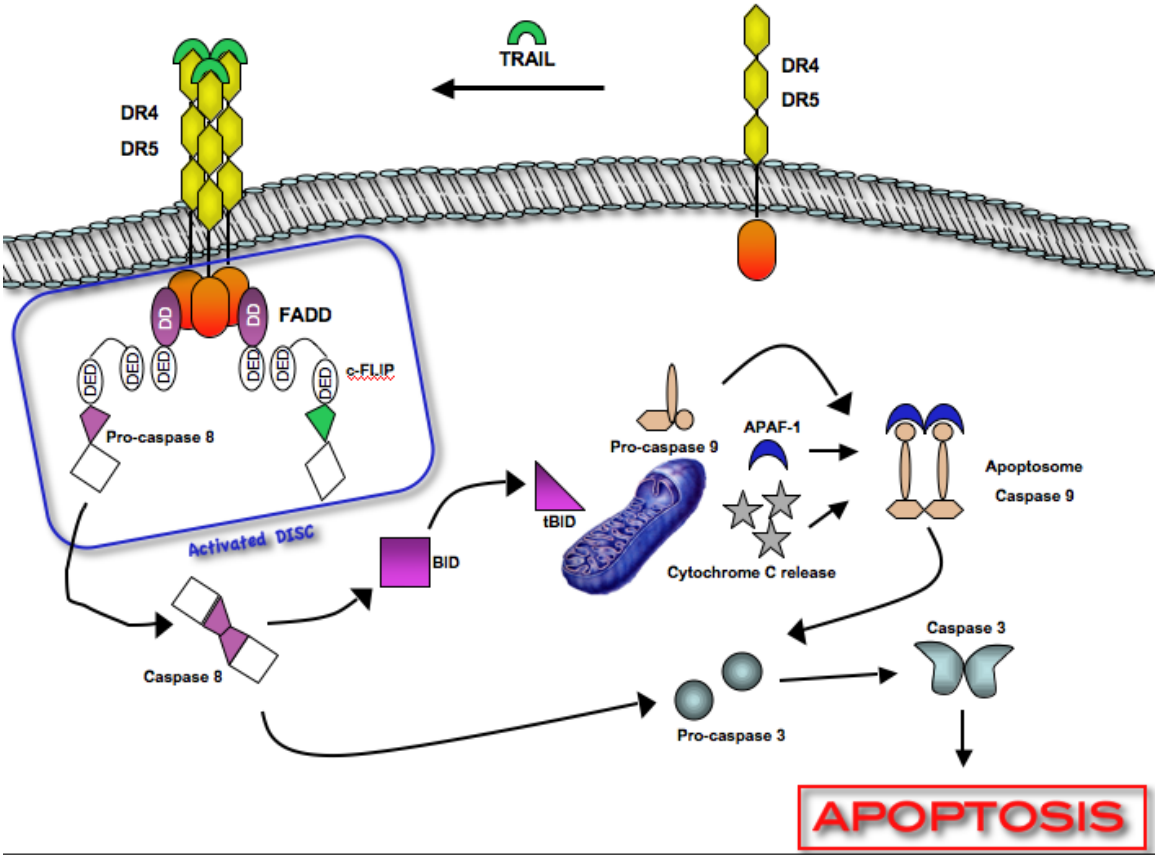


Figure 1.5: TRAIL signalling pathway
TRAIL binds to its cognitive surface receptors DR4 and DR5 that trimerize to initiate the formation of the DISC including of FADD, pro-caspase-8 and/or c-FLIP. Caspase-8 is activated and initiates a caspase cascade leading to apoptosis. Additionally, in type II cells, Bid is truncated activating the intrinsic apoptotic pathway. Cytochrome C is released and the apoptosome formed ultimately leading to apoptosis.

1.3.1.1.3 Non-apoptotic signalling of TRAIL

Classically TNF receptors binding to their respective ligand can result in the activation of the downstream NF-κB and MAPK signalling pathways having an effect on the subsequent transcription of particular genes. In general their signalling plays a role in regulating such processes as cell differentiation, survival and proliferation. It has been suggested that similar non-apoptotic roles may also exist for TRAIL signalling and that TRAIL is capable of activating NF-κB in the same manner as TNF (Harper et al. 2011). Here, receptor-interacting protein (RIP) is recruited to the TNF receptor complex via the death domain where it interacts with NF-κB essential modulator (NEMO)/IKKγ, thus recruiting IκB kinase α (IKKα) and IKKβ. This ultimately leads to the degredation of inhibitors of κB (IκB) by phosphorylation allowing NF-κB activation and signalling (Harper et al. 2001; MacFarlane 2003; Zandi et al. 1997). Additionally it has also been shown that in some instances DR4 and DR5 will recruit the adaptor molecule TNFR-associated death domain (TRADD) that in association with TRAF-2 is capable of activating the mitogen activating protein kinase (MAPK) pathway giving c-jun N-terminal kinase (JNK) activation (Hu et al. 1999). MAPK also activates extracellular signal-regulated kinase (ERK) that in turn can have either an anti-apoptotic or pro-apoptotic effect depending on the context (Borges et al. 2007; Frese et al. 2003; Tran et al. 2001).

1.3.1.1.4 Regulation of TRAIL and TRAIL signalling

As the platform for TRAIL signalling, death receptor expression can have an effect on determining cell sensitivity or resistance to TRAIL (Horak et al. 2005; Jin et al. 2004; Lee et al. 2001). DR4 and DR5 can be post-translationally regulated by glycosylation and palmitoylation modulating TRAIL signalling. It has been speculated

that O-glycosylation enzymes may serve as a useful biomarker in determining tumour sensitivity to TRAIL without altering cell response to other extrinsic or intrinsic stimuli (Wagner et al. 2007). Palmitoylation occurs in DR4, but not DR5, and appears to be important in its ability to form trimers in lipid rafts in the absence of ligand (Rossin et al. 2009). It is yet to be determined as to whether palmitoyl transferases have any direct effect on TRAIL-induced apoptosis. This is not the only example of death receptor localisation in lipid rafts as an important regulatory mechanism of TRAIL apoptosis (Uddin and Al-Kuraya 2011; Xiao et al. 2011; Xu et al. 2011). In one particular study it was shown that c-FLIP also mediates DISC formation in non-raft fractions of the cell membrane and selective knockdown of c-FLIP leads to the redistribution of DISC components to lipid rafts giving TRAIL-induced caspase-8-mediated apoptosis (Song et al. 2007). Functional death receptors may also be internalised by endocytosis and the absence of DR4 on tumour cell surface correlates with TRAIL resistance in colon and leukemia cancer cells (Cheng et al. 2006; Jin et al. 2004). Notably, endocytosis of DR4 and DR5 has also been demonstrated to effect breast cancer cell responsiveness to TRAIL (Zhang and Zhang 2008).

In addition to the functional TRAIL receptors DR4 and DR5, there are two decoy receptors DcR1 and DcR2 that lack the functional death domain preventing them from recruiting DISC components but compete with agonistic death receptors for TRAIL ligand binding (Degli-Esposti et al. 1997). Methylation of decoy receptor genes has been shown in certain tumour tissues, such as primary breast and lung cancers (Shivapurkar et al. 2004; van Noesel et al. 2002). However their role in TRAIL resistance remains controversial as DcR expression has been observed in primary gastrointestinal, lung and prostate

cancers (Aydin et al. 2007; Riccioni et al. 2005; Sheikh et al. 1999). Furthermore, there is a fifth TRAIL receptor, osteoprotegerin (OPG), a secreted TNF receptor family member that inhibits osteoclastogenesis. TRAIL binding to OPG results in no induction of apoptosis as OPG is a secreted factor, thus, OPG acts in a smilar fashion to DcRs by competing with agonistic death receptors for TRAIL binding. This inhibitory effect of OPG was confirmed when overexpression of OPG blocked TRAIL-induced apoptosis in Jurkat cells (Emery et al. 1998). Indeed OPG has been investigated as a prognostic marker in cancer and as such elevated levels of OPG have been correlated with poor prognosis (Brown et al. 2001; Ito et al. 2003; Mizutani et al. 2004). OPG has been shown to be expressed by tumour cells in a range of cancers including breast, prostate and myelomas both *in vitro* and *in vivo* and it has been suggested that this may contribute to TRAIL resistance (Holen et al. 2005; Nyambo et al. 2004; Shipman and Croucher 2003). Despite its TRAIL inhibitory effects, OPG has been shown to reduce bone metastases in an *in vivo* breast cancer model (Morony et al. 2001). Given the contradictory evidence surrounding OPG in malignancy further research must be undertaken before the therapeutic benefit of OPG can be determined.

Transcriptionally, the Myc oncogenic pathway has been shown to play a key role in the regulation of TRAIL sensitivity. It has been reported that Myc transcriptionally represses c-FLIP allowing caspase-8 to be activated at the DISC (Ricci et al. 2004). In addition to this c-FLIP repression, a marked increase in DR5 expression and increased coupled caspase-8 activation was also demonstrated (Wang et al. 2004). Moreover, TRAIL-dependant NF-κB-mediated transcription of Mcl-1 and cellular inhibitor of apoptosis protein 2 (cIAP2) genes was shown to be regulated by Myc, determining cell sensitivity to TRAIL

(Ricci et al. 2007). Targeting regulators of TRAIL signalling has clear implications for treatment, wherby altering their regulatory ability can have the potential to sensitise previously resistant cells to TRAIL treatment.

1.3.1.1.5 TRAIL and cancer

TRAIL is a particularly desirable target for cancer treatment as it induces apoptosis in cancer cells whilst sparing normal cells (Ashkenazi et al. 1999; Walczak et al. 1999). The exact mechanism of normal cell resistance to TRAIL is largely unknown, however, it has been shown that normal cells overexpress decoy receptors and that this may contribute to cell resistance to TRAIL (van Noesel et al. 2002). However, it is unlikely that this is the sole regulatory mechanism leading to normal cell resistance. Instead, it would seem more likely that a combination of intra- and extra-cellular factors act together to make normal cells resistant to TRAIL cytotoxicity (MacFarlane 2003). Additionally, many types of cancer cells also demonstrate resistance to TRAIL, for example only the mesenchymal triple-negative breast cancer cell lines respond to TRAIL *in vitro* (Rahman et al. 2009a). Nonetheless, the specificity demonstrated by TRAIL in preclinical studies to certain types of cancer has lead to the development of proapoptotic receptor agonists that target DR4 and DR5. Recombinant human (rh) APO2L/TRAIL is a soluble protein based on the naturally existing ligand capable of binding to the extrinsic death receptors DR4 and DR5. In addition to soluble rhTRAIL, monoclonal antibodies directed to activate DR4 and DR5 have also been developed. Many phase I safety studies of TRAIL monotherapies in patients with advanced solid tumours have now been completed (Camidge 2008; Herbst et al. 2006; Hotte et al. 2008; Wakelee et al. 2009). In line

with *in vitro* studies, these agents were well tolerated and in most cases did not reach a maximum tolerated dose. Single agent treatment of patients with refractory disease has shown modest antitumour activity, 3 responses out of 40 treated patients were observed in a phase II clinical trial of mapatumumab in follicular non-hodgkins lymphoma (NHL) and no responses reported in treatment-refractory NSCLC and colorectal cancer (CRC) patients (Greco et al. 2008; Trarbach et al. 2010). Results from these early clinical trials suggest that the majority of human tumour cells are actually resistant to TRAIL therapies (Thorburn et al. 2008), contradicting many observations in transformed cell lines. As such, methods of sensitising tumours to TRAIL killing are underway. Combination therapies with chemo- and radio-therapeutics and targeted agents have provided much more promising results in preclinical models (Frew et al. 2008; Jin et al. 2008; Jin et al. 2007; Keane et al. 1999; Shrader et al. 2007). The addition of TRAIL to either single agent cytotoxics (gemcitabine, doxorubicin and permetrexed), cytotoxic combinations (FOLFIRI, carboplatin/paclitaxel and cisplatin/gemcitabine), targeted agents (rituximab, panitumumab, bortezomib and vorinostat) displayed no significant increase in normal cell apoptosis in phase I studies and ongoing phase II trials of some of these combinations will help evaluate their clinical efficacy (Kindler et al. 2012; Leong et al. 2009; Mom et al. 2009; Niyazi et al. 2009; Rosevear et al. 2010; Smith et al. 2007; Soria et al. 2010; Wu et al. 2007).

TRAIL also plays a physiological role in tumour immunosurveillance suggesting it may act as a natural cancer killer (Smyth et al. 2003). It is not expressed on the surface of resting peripheral blood T cells but when the T cells are activated, a marked increase in expression of TRAIL is observed in the presence of

interferon (IFN) (Kayagaki et al. 1999). Furthermore, bone marrow-derived dendritic cells also express surface TRAIL (Lu et al. 2002). In both these instances it was demonstrated that TRAIL contributes to tumour cytotoxicity displayed by these cells further supporting a natural cancer killer role of TRAIL in the immune system.

1.4 Cellular FLICE-Like Inhibitory Protein (c-FLIP)

Cellular FLICE-Like Inhibitory Protein (c-FLIP) was first identified independently by a number of groups in 1997, as a cellular homologue to viral FLIPs. A number of c-FLIP mRNA splice variants exist but at present only three proteins have been isolated, c-FLIP short (c-$FLIP_s$), long (c-$FLIP_L$) and Raji (c-$FLIP_R$). Both c$FLIP_L$ and c-$FLIP_S$ have been implicated as regulating a number of signalling pathways involved in cell survival and apoptosis, whereas the functional role of the c$FLIP_R$ isoform has not been extensively studied and is yet to be uncovered. The c-$FLIP_L$ and c-$FLIP_S$ isoforms have been shown to be overexpressed in a number of cancer cells and their downregulation using siRNA or non-specific compounds can successfully render previously resistant tumours sensitive to treatment with apoptotic cytokines and chemotherapy. Further study into this function of c-FLIP will help to understand the implications of its specific inhibition. Moreover, the study of signalling pathways in which c-FLIP has been implicated has the potential to lead to the identification of alternative targets for anti-cancer therapy.

1.4.1 c-FLIP structure and function

The c-FLIP isoforms, especially c-$FLIP_L$, conform structurally to the pro-apoptotic caspases -8 and -10 and are also found in close

proximity on the genome suggesting they may have evolved from gene duplication. c-FLIP$_L$ is a 55kDa protein made up of two death effector domains (DEDs) and a c-terminal caspase-like domain. The caspase-like domain is catalytically inactive as a result of various amino acid substitutions, especially the critical cysteine residue found in catalytic domain which has been been substituted by a tyrosine residue. Cleavage sites at Asp 376 or Asp 198 on c-FLIP$_L$ produce the proteolytic variant 43kDa (p43c-FLIP$_L$) and 22kDa (p22c-FLIP$_L$) proteins respectively. c-FLIP$_S$ is a 26kDa isoform which is similar in structure to c-FLIP$_L$ but lacks the caspase-like domain. c-FLIP$_R$ is a 24kDa protein similar to c-FLIP$_S$ but contains a shorter C terminus. All three isoforms are capable of interacting with the adaptor protein FADD through their DED at the DISC.

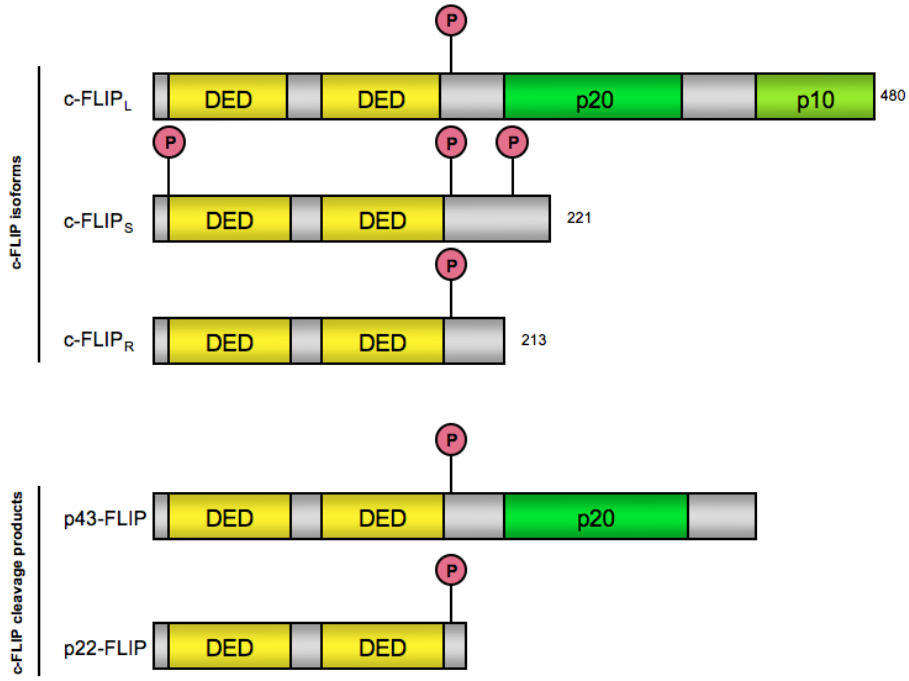


Figure 1.6: Structure of c-FLIP isoforms
Death effector domains (DED) are conserved across all isoforms of c-FLIP, however, the p20 and p10 domains are only present in the c-FLIP$_L$ isoform. Additionally c-FLIP$_S$ has multiple phosphorylation sites not found on other isoforms.

Although c-FLIP has been implicated in development, cell proliferation and drug resistance, the primary function of c-FLIP protein is as an apoptosis regulator. The exact mechanism by which c-FLIP regulates apoptosis still remains controversial, although it does seem clear that regulatory roles differ between the distinct isoforms. For example, it has been demonstrated that c-FLIP$_S$ directly inhibits TRAIL-induced DISC formation by interaction with the adaptor molecule FADD preventing the activation of caspase-8 (Kataoka et al. 2000; Safa et al. 2008; Shirley and Micheau 2010). The role of c-FLIP$_L$ is somewhat more complicated, high expression of this isoform has been shown to inhibit Fas-induced caspase-8 activation whilst low expression appears to activate caspase-8 and caspase-10 (Boatright et al. 2004; Dohrman et al. 2005; Micheau et al. 2002). Some studies have established mechanisms by which c-FLIP$_L$ regulates caspase-8 activation. Inducing c-FLIP overexpression in cell lines has been shown to more efficiently process caspase-8 to its p41/p43 form with the production of p18 being consistently blocked by p43-c-FLIP$_L$, p43-caspase-8 is then retained at the DISC preventing caspase-8 activation (Scaffidi et al. 1999). Additionally, higher levels of c-FLIP$_L$ may also directly compete with caspase-8 for downstream substrates. In the situation where c-FLIP$_L$ is expressed at low levels it is suggested that it is more efficiently processed into its proteolytically active p43 form that can activate procaspase-8. In this instance the heterodimerisation of p43-c-FLIP with procaspase-8 can result in a conformational change at the active site making the heterodimer enzymatically active with almost equal substrate specificity as caspase-8 homodimers (Boatright et al. 2004; Yu et al. 2009).

Meanwhile c-FLIP$_S$ has only been demonstrated to be anti-

apoptotic; not only does it directly prevent caspase-8 activation at the DISC by competition but it also inhibits the cleavage of c-FLIP$_L$ to the p43 form capable of procaspase-8 auto-activation (Kirchhoff et al. 2000; Krueger et al. 2001). Interestingly, the overexpression of both isoforms of c-FLIP has more of an inhibitory effect on FasL and TNFα than either isoform alone in BJAB cells (Krueger et al. 2001). However, in the same cells, overexpression of c-FLIP$_S$ only demonstrated more of an inhibitory effect against the administration of TRAIL. Conversely, in Jurkat and melanoma cells c-FLIP$_L$ appears to be more effective at blocking apoptosis than c-FLIP$_S$ (Irmler et al. 1997). Collectively, the variable ways in which c-FLIP isoforms regulate apoptosis in different cell types suggest that its apoptosis regulatory mechanism is cell-dependent.

1.4.2 c-FLIP and intracellular signalling pathways

In addition to regulating apoptosis, c-FLIP has also been shown to interact with a series of intracellular signalling pathways that contribute to proliferation, cell survival, motility, carcinogenesis and epithelial-mesenchymal transition. Recent studies have shown that c-FLIP both induces and is regulated by NF-κB, enhancing proliferation signals in Jurkat cells (Kataoka et al. 2000). These studies showed that increased recruitment of RIP, TRAF1 and TRAF2 to the CD95 DISC activates NF-κB and ERK signalling pathways independent of caspase signalling (Kataoka et al. 2000). Cleavage of c-FLIP$_L$ to p43-c-FLIP efficiently recruits TRAF2 and RIP1 to the DISC, leading to NF-κB activation (Fang et al. 2004; Kataoka et al. 2000; Kataoka and Tschopp 2004). This was shown to occur in a concentration-dependant manner, whereby at high levels of c-FLIP the DISC is already occupied and c-FLIP activation of NF-κB does not occur (Kreuz et al. 2004).

Additionally, the heterodimer formed between c-FLIP and procaspase-8 produces a NH_2-terminal fragment of c-FLIP that binds directly to the IKK complex also activating NF-κB (Golks et al. 2006). Further studies demonstrated that TNFα signalling induced proteasomal degradation of c-FLIP by c-jun kinase (JNK) activation that in turn increases the turnover of NF-κB- induced c-FLIP. Phosphorylation of JNK activates E3 ubiquitin ligase Itch (ITCH) which specifically ubiquitinates c-FLIP leading to its degradation, thus antagonising NF-κB (Chang et al. 2006). In contrast, c-$FLIP_L$ inhibits the activation of NF-κB and the degradation of IKappa B alpha (IκBα) by TRAIL in human keratinocytes, resulting in a significant decrease in proinflammatory cytokine IL-8. In this instance, NF-κB inhibition by c-FLIP was TRAIL-specific as treatment with TNFα resulted in NF-κB activation regardless of c-FLIP levels (Wachter et al. 2004). Not only does c-FLIP regulate NF-κB but NF-κB is also a key regulator of c-FLIP expression (Kreuz et al. 2001; Micheau et al. 2001). Studies showed that activation of NF-κB results in upregulation of c-FLIP increasing resistance to Fas ligand (FasL) and TNF, whilst also showing that preventing NF-κB activation, by blocking TNF signalling or mutating Ikappa B/removing IKappa B kinase gamma expression, prevented c-FLIP mRNA upregulation in Jurkat cells (Kreuz et al. 2001). These conflicting results demonstrate both pro- and anti-apoptotic functions in the relationship between c-FLIP and NF-κB and further investigation into this complex relationship may help identify novel methods to regulate both molecules.

The serine-threonine kinase Akt is an important transducer of cell survival signals and regulator of proteins involved in apoptosis signaling. A recent study has shown that Akt interacts with c-$FLIP_L$ through its caspase-like domain and that c-$FLIP_L$ modulates Gsk3β, enhancing the anti-apoptotic function of Akt (Quintavalle et al. 2010).

In this study it was also elucidated that c-FLIP$_L$ modulation of Gsk3β induced cancer cell resistance to TRAIL (Quintavalle et al. 2010). Similarly, inhibition of DNA-PK, decreasing Akt and Bad phosphorylation, resulted in increased sensitivity to TRAIL (Kim et al. 2009). Here an increased expression of both TRAIL death receptors DR4 and DR5 was observed together with a decrease in c-FLIP expression, providing further evidence of an Akt/c-FLIP relationship. Further links between Akt and c-FLIP have been made where Akt signals lead to c-FLIP ubiquitination and degradation sensitizing glioblastoma cells to TRAIL. Atrophin-interacting protein 4 (AIP4) and ubiquitin-specific protein 8 (USP8) are both downstream targets of Akt and both also regulate the ubiquitination of c-FLIP (Panner et al. 2010; Panner et al. 2009).

Another regulator of c-FLIP ubiquitination is itchy E3 ligase (ITCH). α-tocopherol ether linked acetic acid analogue (α-TEA) induces ER stress-dependant activation of c-jun kinase (JNK) activating ITCH-dependant ubiquitination, and subsequent degradation, of c-FLIP giving effective sensitisation of breast cancer cells to TRAIL (Tiwary et al. 2010; Yu et al. 2010b). A study performed on liver cells added further evidence of c-FLIP regulation by JNK. In this study TNFα signalling increased JNK activation and ITCH-dependant degradation of c-FLIP (Chang et al. 2006). Furthermore, it was noted that chemotherapeutic treatment of target cells results in the downregulation of c-FLIP (Sanchez-Perez et al. 2009; Stagni et al. 2010). This reduction was shown to be a result of increased proteasomal degradation that could be inhibited by the proteasome inhibitor MG-132. It was also revealed that chemotherapy treated breast cancer cells had sustained JNK phosphorylation, inhibition of which using SP600125 reversed c-FLIP$_L$ degradation and reduced

apoptosis (Sanchez-Perez et al. 2009). Interestingly, the relationship between c-FLIP and JNK has also been shown to work vice versa. c-$FLIP_L$ can directly interact with MAP kinase activator 7 (MKK7), a JNK activator, in a TNFα-dependant manner inhibiting MKK7 interactions with MAP/ERK kinase kinase 1 (MEKK1) suggesting that c-$FLIP_L$ may also selectively repress JNK activation (Nakajima et al. 2006).

A number of studies have provided evidence that c-FLIP also functions within the classical Wnt signalling pathway. Wnt signalling mediates proliferation, differentiation and stem cell characteristics (Sethi and Vidal-Puig 2010). In brief, the binding of Wnts to frizzled receptors on the cell surface recruits Axin and leads to the dissociation of the degradation complex. This degredation complex is normally made up of Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3 (GSK3) and degrades β-catenin. The dissociation of the degradation complex by Wnt binding prevents the degradation of β-catenin leading to its accumulation in the cytosol. The accumulation of β-catenin in the cytosol is then followed by its translocation to the nucleus where it activates a number of transcription factors (Sethi and Vidal-Puig 2010). The Wnt pathway was found to be significantly activated, following Wnt3a stimulation, in cell lines overexpressing c-$FLIP_L$ but not in those overexpressing c-$FLIP_S$ or with wild-type c-FLIP (Naito et al. 2004). This elevated Wnt signalling was shown to be a result of c-FLIP increasing β-catenin ubiquitination (Naito et al. 2004). Later studies then revealed that c-FLIP itself accumulates and aggregates in the cells and disrupts the ubiquitin-proteasome system (Ishioka et al. 2007). Additional studies also unveiled a potential role for c-$FLIP_L$ in nuclear translocation of β-catenin. Potential nuclear localisation and nuclear exportation signals were identified at the c-terminal of c-FLIP protein (Katayama et al. 2010). Mutating these

signals prevented this translocation and resulted in the localisation of c-FLIP solely in the cytoplasm or nucleus dependant on the mutant form. Interestingly, abrogating this translocation had no significant effect on extrinsic signalling but did prevent the enhanced Wnt signalling by Wnt3a previously observed. The accumulation of β-catenin was also completely abolished in the localisation mutants, collectively suggesting that c-FLIP may have a function as a co-transporter of β-catenin (Katayama et al. 2010). In the same manner, suppression of c-$FLIP_L$ in A459 cells was associated with decreased Wnt signalling (Naito et al. 2004).

Collectively, all these studies clearly demonstrate that c-FLIP does not solely function as an extrinsic anti-apoptosis factor but is also a regulator of many different intracellular signalling pathways that may effect cell proliferation, differentiation and function.

1.4.3 c-FLIP and cancer

Expression of c-FLIP variants decreases apoptosis induced by death ligands and anticancer agents, thus their overexpression in cancer cells may cause resistance to anticancer drugs (Safa et al. 2008). As a result, drugs targeting c-FLIP are very desirable as not only do they have the potential capability of inducing cell death but can also lower the threshold of cancer cell apoptosis, allowing a reduction in subsequent drug doses, decreasing systemic toxicities of agents such as chemotherapeutics. In its own right, reduction of c-FLIP has been demonstrated to induce cell death without the need of combination therapy. Injection of liposomal c-FLIP-specific siRNA into MCF-7 xenografts destroyed neoplastic cells whilst having no effect on the normal stromal and fibroblastic cells (Day et al. 2009). This *in vivo* observation was consistent with the *in vitro* results seen in a previous

study, siRNA knockdown of c-FLIP induced a DR5, FADD, caspase-8 and caspase-9 dependant cell death in MCF-7 cells (Day et al. 2008). Similar results have also been observed in lung (Sharp et al. 2005), colorectal (Wilson et al. 2007) and prostate (Zhang et al. 2004) cancer cells. Transient transfection experiments using a c-FLIP targeted antisense phosphorothioate oligonucleotide (AS PTO) demonstrated significant caspase-8 activation and apoptosis following the downregulation of c-FLIP (Logan et al. 2010). This effect was observed in non-small cell lung carcinoma (NSCLC), colorectal and prostate cancer cells but not normal cells. Similarly, this method of c-FLIP interference sensitised NSCLC cells to both TRAIL and chemotherapy (Logan et al. 2010). The *in vivo* results seen from these RNAi-based therapeutic interventions of c-FLIP warrant further preclinical development, however, siRNA design, delivery and stability are still difficulties that must be overcome before RNAi-based therapies are truly feasible as a clinical intervention.

Besides inhibiting c-FLIP proteins directly, alternative methods, including degradation and transcriptional regulation of c-FLIP, may also have potential for c-FLIP intervention. As previously discussed, chemotherapeutic agents have been demonstrated to reduce c-FLIP expression in multitude of cancer cells (Chatterjee et al. 2001; El-Zawahry et al. 2005; Kinoshita et al. 2000; Logan et al. 2010; Longley et al. 2006). In many of these studies, cancer cell pre-treatment with chemotherapy has proven to be a successful method of sensitisation to extrinsic cell death ligands. Histone deacetylase inhibitors are another set of compounds that have displayed similar properties of malignant cell growth inhibition and sensitisation to further therapy (Bangert et al. 2012; Seo et al. 2011; Yerbes and Lopez-Rivas 2010). Specifically, the HDACi, suberoylanilide hydroxamic acid (SAHA, vorinostat), has

been demonstrated on numerous occasions to inhibit c-FLIP proteins (Bijangi-Vishehsaraei et al. 2010; Carlisi et al. 2009; Ellis and Pili 2010; Shankar et al. 2009; Yerbes and Lopez-Rivas 2010). The use of HDACis that suppress c-FLIP in combination with extrinsic death ligands have also proven to be successful sensitisers in different types of cancer, in fact, SAHA or droxinostat treatment successfully sensitised previously resistant breast cancer cells to TRAIL (Bijangi-Vishehsaraei et al. 2010; Shankar et al. 2009). One particular study identified degradation of c-FLIP isoforms by ubiquitination following SAHA treatment, although this degradation was found to be independent of ITCH/AIP4 (Yerbes and Lopez-Rivas 2010). Other agents also lead to the degradation of c-FLIP and several of these agents successfully act as sensitisers to TRAIL and other extrinsic apoptosis death signals (Jeon et al. 2005; Olsson et al. 2001; Perez et al. 2010; Sayers et al. 2003; Schimmer et al. 2006; Seki et al. 2010; Shanker et al. 2008; Tiwary et al. 2010; Tobinai 2007).

Clearly this evidence makes c-FLIP an ideal target for disruption using small molecule inhibitors. Unfortunately, to date there has not been a c-FLIP specific inhibitor developed since it has such significant structural similarity to caspase-8. Due to this structural similarity, small molecules that inhibit c-FLIP may also hinder caspase-8 function too, providing no apoptotic benefit. Specific drug design to c-FLIP is an ongoing area of research both for individual and combined therapy of many different types of cancer.

1.5 T Cell immunity and the surveillance of cancer

T cells are immune cells that move through tissues in search for their corresponding major histone compatibility complex (MHC)-peptides that specifically activate their cell surface T cell receptors (TCRs). Typically these TCRs are made up of variable α and β protein chain heterodimers (~95%) expressed as part of a complex with the invariant CD3 chain molecules (Janeway and Bottomly 1994). $\alpha\beta$ T cells respond to a variety of different signals that alert them to threatening pathogens and cancer. $\alpha\beta$ T cells are activated at tumour cells by tumour-associated antigens presented by antigen presenting cells (APCs) and dendritic cells (DCs). However, once $\alpha\beta$ T cells are activated they are capable of directly recognising these tumour antigens themselves. Further evidence of T cell recognition of tumours is demonstrated by the isolation of tumour-infiltrating lymphocytes, whereby tumours are commonly enriched for T cells, that can then be isolated, specific for the tumour in which they are found (Ahmadzadeh et al. 2009). Unfortunately, neoplastic cells are particularly difficult to recognise for many T cells as they frequently downregulate their MHC molecules helping tumour cells evade classical T cell-dependant immune responses (Bubenik 2004; Restifo et al. 1996). This led to much interest in a small subset of T cells that are not MHC-restricted, the $\gamma\delta$ T cells.

1.5.1 $\gamma\delta$ T cells

$\gamma\delta$ T cells are a small subset of cells that account for approximately 1-5% of circulating T cells in the blood. The Vγ9Vδ2 T cells account for up to 90% of the $\gamma\delta$ T cell population and herein is the specific gene pair that will be referred to in the generalised term '$\gamma\delta$ T

cells' (Hayday 2000). Although the effector functions of both γδ T cells and αβ T cells are extremely similar to each other, producing the various cytokines such as TNFα and IFNγ, the immune system has maintained these two separate types of TCR as they seem to recognise different ligands, thus providing additional immunosurveillance (Girardi et al. 2001; Hayday 2000). Additionally, selective recognition of bacterial metabolites and viral antigens by γδ T cells identifies a non-redundant role for γδ T cells. γδ T cells are unique in that they are not MHC-restricted and show low dependence on co-stimulation and, in line with this, lack expression of CD4 and CD8 (Hayday 2000).

1.5.1.1 γδ T cell activation

Early studies showed that γδ T cells are activated by a range of bacteria and parasites (Hayday 2000) by non-peptidic phosphorylated intermediates (phosphoantigens) of the non-mevalonate pathway of bacterial isoprenoid biosynthesis (Viey et al. 2005). In contrast, eukaryotic cells use the mevalonate pathway for isoprenoid biosynthesis producing isopentenyl pyrophosphate (IPP), which has also been suggested to activate γδ T cells. However, non-transformed cells are incapable of producing high enough concentrations of IPP to be recognised by γδ T cells, whereas some tumour cells are (Girardi et al. 2001; Gober et al. 2003). There is now substantial evidence that this class of compounds contains multiple members and the natural phosphoantigen with the highest bioactivity is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the 2-C-methyl-D-erythritol (MEP) pathway utilised by eubacteria and protozoa but not eukaryotes (Thedrez et al. 2007). HMB-PP also has the ability to induce the formation of high-density TCR nanoclusters on γδ T cell surface further increasing the activation properties of HMB-PP

stimulation (Chen et al. 2008). Aminobisphosphonates (NBPs) can alter intracellular levels of IPP by inhibiting the IPP-processing enzyme farnesyl pyrophosphate synthase leading to IPP accumulation that in turn activates γδ T cells, inducing them to differentiate toward a more IFNγ producing effector memory phenotype (Bonneville and Scotet 2006; Dieli et al. 2007; Eberl et al. 2002; Gober et al. 2003; Kunzmann et al. 2000). Due to their apoptosis-inducing properties in osteoclasts, NBPs such as zoledronate and ibandronate have been clinically developed and approved for therapeutic treatment of osteoporosis and bone metastasis (Body et al. 2003; Costa 2007).

1.5.1.2 γδ t cells and cancer

γδ T cells have been consistently isolated from TILs in a number of different cancers including colorectal, breast, ovarian, and renal cell carcinoma (Corvaisier et al. 2005; Groh et al. 1999; Viey et al. 2005; Xu et al. 2007). Isolating γδ T cell lines from these TILs display potent antitumour activity not only to their autologous tumour but also to closely related tumour cells. Importantly these γδ T cells maintain a tumour specificity and exhibit almost no effect on non-transformed cells (Corvaisier et al. 2005; Viey et al. 2005). Subsequently, the therapeutic potential of γδ T cells was explored in a variety of cancers using different activating compounds in an array of *in vitro* and *in vivo* models (Correia et al. 2009; Naoe et al. 2010). HMB-PP in combination with IL-2 activation of γδ T cells was shown to induce MEK/ERK and PI-3K/Akt-dependant anti-tumour properties in leukaemia cells (Correia et al. 2009). Further investigation into the *in vivo* activation and cytotoxicity of γδ T cells stimulated by HMB-PP will help support its transition into clinical application. The use of NBPs to activate γδ T cells has clear advantages over other methods due to the inherent anti-

tumour effects of zoledronate as a monotherapy, whereby metastasis and tumour cell invasion can be reduced and cell death increased in tumour cells, most notably, breast cancer cells (Body et al. 2003; Boissier et al. 2000; Boissier et al. 1997; Lowe et al. 2005; van der Pluijm et al. 1996; Verdijk et al. 2007). Furthermore, combining zoledronate with the frequently used endocrine therapies in premenopausal women significantly increased disease-free survival of patients compared to placebo (Gnant et al. 2009). These anti-cancer properties of zoledronate together with the fact that zoledronate is already approved for clinical use meant it attracted much attention to determine any additional benefits that can be exploited as an activator of γδ T cells. Preclinical models of various cancers showed that *in vivo* and *ex vivo* zoledronate activated γδ T cells show potent tumour cell cytotoxicity (Beck et al. 2010; Kondo et al. 2008; Meraviglia et al. 2010; Saitoh et al. 2008; Siegers et al. 2011). Furthermore, the use of zoledronate has now been extended to clinically activate and expand γδ T cells *ex vivo* for adoptive transfer therapy in some forms of cancer (Bennouna et al. 2008; Dieli et al. 2007; Kobayashi et al. 2011; Kunzmann et al. 2000; Nicol et al. 2011). Further investigation of zoledronate's potential as a γδ T cell activator and anti-cancer agent in breast cancer is currently ongoing and elucidating the mechanisms of cell death induced by activated γδ T cells may help to identify further cooperative treatments for clinical translation.

1.6 Aims of this study

Research has identified that there are specific cohorts of patients that require novel therapeutic intervention techniques. Those diagnosed with later stage disease and those that have become resistant to existing therapies, with the prevention of metastasis the key to controlling disease progression in these patients. With this in mind, using cell lines as *in vitro* and *in vivo* models that represent the heterogeneic nature of breast cancers, the aims of this study were to:

1) Determine if TRAIL has potential as a treatment for breast cancer

2) Assess the extent of any treatment that proves successful on the inter- and intra-tumour heterogeneity that exists between breast cancers

3) Uncover alternative delivery methods of TRAIL to breast tumours.

CHAPTER 2:

Materials and methods

2.1 Animal experiments

All procedures involving the use of animals were carried out according to the institutional guidelines in compliance with UK Home Office Regulations (Animals [scientific procedures] Act 1986)

2.1.1 Animals

The generation of c-FLIP transgenic mice has been previously described (Zhang and He 2005). These animals were obtained from the Zhang lab (Duke University, NC, US) and crossed with mice expressing Cre under the control of β-lactoglobulin milk gene (BLG-cre) (Selbert, Bentley et al., 1998) on a BL6 background. These animals were then in-bred creating the cohorts of mice BLG-cre/c-FLIP$^{fl/fl}$, BLG-cre/c-FLIP$^{fl/+}$, BLG-cre/c-FLIP^{WT}.

For immunocomprised animal transplantation experiments Balbc/SCID mice were obtained from Charles River Laboratories (Wilmington, US) or Athymic Nude (Hsd:Athymic Nude-Foxn1^{nu}) mice were obtained from Harlan Laboratories (Indianapolis, US). Animals were acquired at six to eight weeks of age and maintained in individually ventilated cages (Allentown Inc. NJ, US) with a 12hr day/night cycle. Mice received a Teklad global 19% protein extruded rodent diet (Harlan Laboratories) and water *ad libitum*. All food, drink, saw dust and water bottles were sterilised by autoclaving prior to use. All procedures and animal husbandry was performed within a laminar flow hood (Allentown).

2.1.2 Genotyping

All animals were genotyped using polymerase chain reaction (PCR) using DNA extracted from tail or ear biopsies after weaning at 4 weeks of age.

2.1.2.1 DNA extraction

Prior to DNA isolation ear and tail biopsies obtained were stored in 1.5ml eppendorf tubes at -20°C. DNA was then extracted by the addition of 0.5ml lysis buffer (100mM Tris-HCl; pH8.5 [Sigma], 5mM EDTA [Fisher Scientific], 0.2% w/v sodium dodecyl sulphate [SDS; Sigma], 200mM sodium chloride [Fisher Scientific] and 5μl proteinase K [Roche]) to each biopsy and incubated overnight at 55°C. Samples were mixed thoroughly and centrifuged at 13,000rpm for 10 minutes. Supernatants were transferred to a clean eppendorf containg 0.5ml of isopropanol (Fisher Scientific) and left for 5 minutes to allow DNA to precipitate. Samples were centrifuged for 5 minutes to pellet the DNA that was subsequently washed in 70% ethanol (Fisher Scientific) and then dried at 55°C before being re-suspended in 100μl of RNAse and DNAse free H_2O at 55°C for 1 hour. This DNA was then used for the PCR protocols outlined in Table 2.2

2.1.2.2 Generic PCR protocol

Primers were purchased from Sigma-Genosys and were either designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) or using previously published sequences (Table 2.3). In silico PCR was then used to verify all sequences checking for any mis-priming (http://genome.csdb.cn/cgi-bin/hgPcr).

In general, 2μl of DNA extract or DNAse/RNAse free water (Sigma) were loaded into 0.2ml PCR 8-strip tubes (StarLabs) and 23μl

of PCR mastermix, as described in Table 2.1 added to each reaction. Gentle pipetting homogeneously mixed the reactions and the tubes sealed using 8-strip PCR caps (StarLabs). Tubes were briefly spun in a Technico Mini Centrifuge (Technico) prior to being run in an iCycler PCR machine (BioRad) according to the cycling programs detailed in Table 2.2.

2.1.2.3 Gel electrophoresis

After completion of the PCR reactions, DNA products were visualised by gel electrophoresis. An appropriate DNA marker (e.g. EasyLadder 1 [Bioline]) and all samples were loaded onto a 3% agarose gel (made by dissolving 0.5g agarose tablets in 1x Tris-Borate-EDTA [TBE] Buffer [Sigma] containing 0.006% [v/v] SafeView Nucleic Acid Stain [NBS Biologicals]). Gels were run in 1x TBE buffer at 150V for 20 minutes and PCR products visualised under UV-light using a GelDoc trans-illuminator (BioRad).

PCR Reaction component	FLIP/Flox	BLG-Cre
Crude DNA	2μl	2μl
PCR-grade Water (Sigma)	15μl	32.2μl
GoTaq Buffer (Promega)	5μl	10μl
Magnesium Chloride (25mM; Promega)	2μl	5μl
dNTPs (25mM; dATP, dCTP, dGTP, dTTP)	0.4μl	0.4μl
Forward Primer (Sigma Genosys, 100μM)	0.1μl	0.1μl
Reverse Primer (Sigma Genosys, 100μM)	0.1μl	0.1μl
GoTaq DNA Polymerase (Promega)	0.5μl	0.2μl
Final Volume	25μl	50μl

Table 2.1: PCR reaction components

PCR Step	BLG-Cre		
	No. Cycles	Temp (°C)	Time
Initial denaturation	1	94	3 min
Denature	30	92	30 sec
Anneal		95	30 sec
Extension		55	1 min
Final extension	1	72	5 min
Hold	1	4	∞

PCR Step	FLIP/Flox		
	No. Cycles	Temp (°C)	Time
Initial denaturation	1	95	2 min
Denature	10	95	30 sec
Anneal		76 (-2 every cycle)	30 sec
Extension	1	72	35 sec
Denature	28	95	30 sec
Anneal		66	30 sec
Extension		72	35 sec
Hold	1	4	∞

Table 2.2: PCR cycling conditions

Target	Forward primer sequence (5'-3')	Reverse primer sequence (3'-5')	Product size
FLIP Flox/WT	AGCTGATGCATGAGCCTGAGC	GTTCATACTGACCTCTCAAGACCATG	Flox – 520bp WT – 340bp
BLG-Cre	TGACCGTACACCAAAATTTG	CTATCACTTTGTCCCCGTTA	1000bp

Table 2.3: Genotyping primer sequences

2.1.3 Experimental procedures involving animals

2.1.3.1 Orthotopic cell transplants

Prior to transplantation, cells were prepared as single cell suspensions. Cells were washed in serum-free L-15 media (Invitrogen, Paisley, UK), then serum-free Joklik's media before another wash in serum-free L-15. Cells were then passed through a 40um cell strainer (BD Biosciences, Oxford, UK) and kept on ice until transplantation. For BT474 and MDA-MB-231 cell transplants, 8-12 week old Athymic Nude and NOD/SCID/Balbc mice were used respectively. Before surgery, mice were anaesthetized using a vaporizer to deliver 5% isoflurane (Abbot, Maidenhead, UK) with oxygen at a flow rate of 0.8 l/min and nitrous oxide at a flow rate of 0.4l/min in an induction chamber. Isofluorane was subsequently delivered at 2.5% through an anaesthetic mask to maintain the animal unconscious throughout surgical procedures. Consequently, a small patch of fur that is dorsal to the fourth abdominal mammary gland was removed and cleaned with Betadine (povidone-iodine) surgical scrub. Then, a small superficial incision was made to expose the abdominal mammary gland and cells were injected directly into the mammary fat pad with a Hamilton or insulin needle syringe (BD Micro-Fine). The lymph nodes in the fourth abdominal gland were used to orientate the point of injections, where cells were transplanted dorsal to the lymph nodes. Wounds were then sealed with Vetbond Tissue Adhesive (3M). Animals were then allowed to recover in a temperature regulated chamber at 30^{o}C for 15 minutes.

2.1.3.2 Tumour monitoring and measurements

Mice transplanted orthotopically with breast cancer cells were inspected at least twice weekly for tumours via palpation. The growth kinetics of tumours were documented by measuring the diameter of tumours using digital calipers (Fisher Scientific, Loughborough, UK). The size of tumours were calculated as volume in mm3 using the formula; Volume = (Length X [Width x 2])/2.

2.2 Tissue sampling and processing

2.2.1 Removal and fixation of tissues

At appropriate experimental endpoints, animals were culled by cervical dislocation prior to necropsy.

2.2.1.1 Mammary tissues for gestational studies

Animals used for gestational studies were mated and animals sacrificed midway through gestation. Before extraction of mammary tissues, the lymph nodes in the fourth inguinal glands were excised and discarded. The left abdominal gland was then removed and fixed in 4% formalin for histological analysis. For protein and RNA analysis, the remaining abdominal and inguinal glands were snap frozen and stored at -80^{o}C.

2.2.1.2 Tissues for tumour studies

Mice bearing tumours were excised when reaching their defined scientific endpoint (often either due to reaching maximal tumour size of 4000mm^3 or showing signs of morbidity). Upon necropsy, the left abdominal mammary gland, liver, lung and a portion of tumour from experimental mice were fixed in 4% formalin for histological analysis.

The remainder of tumour and normal mammary gland tissues were snap frozen in liquid nitrogen and stored at -80^{o}C until required for molecular analysis.

2.2.2 Tissue processing and sectioning for histology

After fixing tissues in 4% formaldehyde for 4-8 hours, tissue processing and sectioning was carried out by the Histology Unit in Cardiff School of Biosciences.

2.2.2.1 Dehydration of tissues

Paraffin emdedded sections were prepared by the School of Biosciences Histology Unit. With the aid of a Leica TP1050 automatic processor in the Histology Unit, tissues were dehydrated in a series of solvents; 70% ethanol for 1 hour, 95% ethanol for 1 hour, 100% ethanol for 1.5 hours twice, 100% ethanol for 2 hours, xylene for 1 hour twice and paraffin for 2 hours twice. Following that, tissues were embedded in paraffin wax for sectioning into 5μm thick slices with the aid of a Leica RM2135 microtome cutter. Tissue sections were then immobilized onto poly-L-lysine (PLL) coated slides (Thermo Fisher, Loughborough, UK) and heated at 58^{o}C for 24 hours. Slides were then stained with H&E or used for immunohistochemistry (IHC).

2.3 Histological analysis of tissue sections

2.3.1 Dewaxing and rehydration

Paraffin was removed and tissues were rehydrated by incubating slides in a series of solvents; 100% ethanol for 2 minutes twice, 95% ethanol for 2 minutes, 70% ethanol for 2 minutes and a rinse in distilled water.

2.3.2 Haematoxylin and eosin (H&E) staining

Slides were stained with H&E after dewaxing and rehydration. Initially by staining in Meyer's Haemalum (Thermo Fisher, Loughborough, UK) for 5 minutes, followed by a wash under running tap water for 5 minutes. Subsequently, slides were counterstained in 1% aqueous Eosin (Thermo Fisher, Loughborough, UK) for 5 minutes before being washed twice for 15 seconds with tap water. Finally, slides were dehydrated, cleared and mounted as described in section 2.3.3.4.

2.3.3 Visualisation and quantification of H&E stained sections

Visualisation and quantification of all histological sections were carried out using an Olympus BX41 Light Microscope (Olympus). All digital photos taken of sections were done so using a Colorview III (5 megapixel, Soft Imaging Systems) camera using Analysis software package (Version 3.2, build 831, Soft Imaging Systems)

2.3.3.1 Analysis of lung metastases

Photos were taken of all visible lung metastases and the average area of these metastases calculated using measurements taken by Image J software.

2.4 Cell culture maintenance and procedures

2.4.1 Experimental cell lines

The human MDA-MB-231, MCF-7, SKBR3, XL, TAMR and FASR cell lines used in this study were a gift from Dr. Julia Gee (Breast Cancer Molecular Pharmacology (BCMP) group, Dept. of Pharmacy,

Cardiff University). The human BT474 cell line was purchased from ATCC (http://www.atcc.org). The human MDA-MB-231-luc-D3H2LN cell line was purchased from Caliper Life Siences (Hopkinton, MA, US). The mouse 4T1 cell line (Aslakson and Miller 1992) was obtained from Dr. Robin Anderson (University of Melbourne). Mouse N202.1A cells were a kind gift from Dr. Pier-Luigi Lollini (Sezione di Cancerologia, Italy). The EPH4 cell line was obtained from Dr. Christine Watson (Cambridge University). The MCF10A cell line was a kind gift from Dr. Hiscox of the BCMP group, Dep. Pharmacology, Cardiff University.

2.4.1.1 MDA-MB-231

MDA-MB-231 is a highly metastatic, human basal epithelial cell line isolated from the pleural effusion of an adenocarcinoma. These cells are 'triple negative' as they lack oestrogen, progesterone and HER2 receptors (Neve, Chin et al., 2006) whereas they strongly over-express EGFR.

2.4.1.2 MCF-7

The MCF-7 is an oestrogen dependant, poorly metastatic, human luminal epithelial cell line isolated from the pleural effusion of an adenocarcinoma. These cells are oestrogen and progesterone receptor positive but do not over-express HER2 receptor (Neve, Chin et al., 2006)

2.4.1.3 SKBR3

SKBR3 is a poorly metastatic human luminal epithelial cell line derived from a pleural effusion of an adenocarcinoma. These cells are oestrogen and progesterone receptor negative but over-express the HER2 receptor and have low levels of EGFR expression (Neve, Chin et

al., 2006)

2.4.1.4 XL, TAMR, FASR

The XL, TAMR and FASR cell lines are sub-clone derivatives of the MCF-7 cell line. The XL cell line was cultured long-term in the absence of any estrogen or steroid such that the cells no longer require these for maintenance. The TAMR and FASR cell lines are acquired endocrine therapy resistant models of the MCF-7 cell line to tamoxifen and Faslodex respectively. Whilst TAMR cell lines retain their estrogen receptors the FASR cell line is completely estrogen receptor negative in culture.

2.4.1.5 MDA-MB-231-luc-D3H2LN

The MDA-MB-231-luc-D3H2LN cell line was derived from the parental MDA-MB-231 cell line and isolated from a spontaneous lymph node metastasis of orthotopically mammary transplanted MDA-MB-231-luc-D3H1 cell line. The D3H2LN cell line shows enhanced tumour take and metastatic potential by comparison to it's parental lines.

2.4.1.6 EPH4

The EPH4 cell line is a spontaneously immortalized non-transformed murine mammary epithelial cell line.

2.4.1.7 N202.1A

N202.1A is a murine tumour cell line isolated and immortalized from tumour driven by MMTV/Neu transgene on an FvB background that over-express the ErbB2 oncogene (Nanni et al., 2000)

2.4.1.8 4T1

The 4T1 cell line is an aggressive metastatic murine cell line isolated and immortalized from a spontaneous tumour arising in a wild-type Balb/c mouse.

2.4.1.9 MCF10A

The MCF10A cell line is a human luminal epithelial non-transformed, non-tumourigenic cell line isolated and immortalized from fibrocystic breast tissue.

2.4.2 Maintenance of cell lines

The 4T1, MCF-7, BT474, MDA-MB-231, MDA-MB-231-luc-D3H2LN and SKBR3 cell lines were cultured in RPMI medium (Invitrogen, Paisley, UK) with 10% v/v fetal bovine serum (FBS; Sigma, Dorset, UK), 2mM L-glutamine (Invitrogen) and 50 units/ml penicillin-streptomycin (Invitrogen). TAMR and FASR cells were cultured in RPMI (phenol red free) with 5% charcoal stripped FBS (Sigma), 2mM L-glutamine, 50 units/ml penicillin-streptomycin and $1x10^{-7}$ 4-OH tamoxifen or Faslodex. Eph4 and N202.1A cells were cultured in DMEM medium (Invitrogen) that is supplemented with 2mM L-glutamine and 50units/ml penicillin-streptomycin. FBS was supplemented into Eph4 and N202.1A cell media at 10% and 15% v/v respectively. The MCF10A cell line was cultured in a 1:1 mix of DMEM/F12 (Invitrogen) medium supplemented with 5% v/v horse serum (Sigma), 50units/ml of penicillin-streptomycin, 100ng/ml cholera toxin (Sigma), 20ng/ml epidermal growth factor (EGF, Sigma), 0.5mg/ml hydrocortisone and 10μg/ml insulin.

Cells were maintained in a sterile, humidified 37°C incubator and CO_2 levels were kept at 5%. All cell lines were routinely cultured in

T25 tissue culture flasks (Nunc, Leics, UK). When cells reached a confluency of 80-90%, they were passaged on at a split ratio of 1:6-1:12 every two days or at appropriate times. Cell passaging was carried out by completely removing used medium, followed by a rinse with PBS. This was followed by the addition of 2mls of 0.05% Trypsin/EDTA (Invitrogen, Paisley, UK) to each flask and left to incubate at 37^{o}C for 5-10 minutes. Following this incubation period, cells were checked under the microscope to ensure that all cells had detached and were then diluted with culture medium according to appropriate splitting ratios. All cell lines were not maintained for any more than 30 recorded passages.

2.4.3 Cell counting

To aid the seeding of cells in assays, cells were counted using a haemocytometer counting chamber (Hawksley, Lancing, UK). For this, cells were detached with trypsin/EDTA as described in Section 2.5.2. Once detached cells were transferred to a 15ml Falcon tube (BD Biosciences) and re-suspended 10mls of growth media. Cells were then pelleted by centrifugation at 1100rpm for 5 minutes and resuspended in a known appropriate volume of growth media. 10μl of cell suspension was loaded into the counting chamber of the haemocytometer and the number of cells in four 1mm^2 squares was quantified. The four counts were averaged and multiplied by the conversion factor of 1x10^4 to give the number of cells/ml.

2.4.4 Long-term cell storage

In order to maintain a low passage number for the experimental cell lines, cells were frozen and cryo-stored in liquid nitrogen. For that, a confluent T75 flask (Nunc, Leics, UK) of each cell line was detached

and resuspended in 10mls of culture medium in a 15ml falcon tube. Cells were then pelleted by centrifugation at 1100rpm for 5 minutes. The pellet was re-suspended in freezing medium (culture medium with 10% v/v dimethyl sulfoxide [DMSO; Sigma, Dorset, UK]) and 1ml aliqouts placed in 1.5ml cryo-tubes (Nunc, Leics, UK). The tubes were then placed in a container containing isopropanol to facilitate gradual freezing at -80^{o}C overnight. After that, cell aliquots were transferred into the liquid nitrogen storage container. For retrieval of cells from cryo-storage, cells were quickly defrosted at 37^{o}C in a waterbath, resuspended in 10mls of complete growth culture medium in a 15ml falcon tube and pelleted by centrifugation at 1100rpm for 5 minutes. The resulting pellet was then resuspended in 7mls of culture medium and cultured in T25 flasks.

2.4.5 TRAIL treatment of target cells

Cells were treated with soluble human recombinant TRAIL (SuperKillerTRAIL, Enzo Life Sciences) at a concentration of 20ng/ml for 18 hours at 37^{o}C in 5% CO_2. For mouse target cells, soluble mouse recombinant TRAIL (Enzo Life Sciences) was added at a concentration of 100ng/ml for 18 hours.

2.4.6 Transient siRNA transfection

STEALTH small interfering RNA (siRNA) duplexes targeting an irrelevant scrambled control, c-FLIP, c-FLIP$_S$ and c-FLIP$_L$ RNA were purchased from Invitrogen (Table 2.4). Each siRNA was custom designed to target the entire c-FLIP gene (mouse and human) or the c-FLIP-short and c-FLIP-long splice forms (human only). Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturers protocols to give a final siRNA

concentration of 10nM. Volumes and concentrations of each of the reagents used are outlined in Table 2.5. In brief, the appropriate amount of siRNA was diluted in additive free Opti-MEM 1 culture media (Invitrogen) and added to the desired well in the culture dish. An appropriate amount of Lipofectamine RNAiMAX was then added to each well containing siRNA. This was then mixed and left at room temperature for 20 minutes to allow siRNA-Lipofectamine complexes to form. Meanwhile, cells were diluted in complete growth media free of antibiotics to a density that would give 30-50% confluency 24 hours post-seeding (Table 2.6). Following the 20minute incubation of siRNA-Lipofectamine, cells were gently added such that the final concentration of siRNA in each well was 10nM. Cells were then incubated at 37^{o}C in 5% CO_2. The optimal transfection period for each cell line was optimized to give maximum gene knockdown.

siRNA	Target sequence (5'-3')	Final Conc	Source
c-FLIP	GGAUAAAUCUGAUGUGUCCUCAUUA	10nM	Invitrogen
c-FLIP 2	GAGUGAGGCGAUUUGACCUGCUCAA	10nM	Invitrogen
c-FLIP-long	CCCUAGGAAUCUGCCUGAUAAUCGA	10nM	Invitrogen
c-FLIP-short	CCCAGGCUACGUGUCAUAUAUACGA	10nM	Invitrogen
Scrambled Control	GGACUAAUAGUUGUGCUCCAAUUUA	10nM	Invitrogen

Table 2.4: siRNA reagents and concentrations

Tissue Culture Vessel	Relative Surface Area	Volume of cell plating medium	Volume of OptiMEM 1 dilution medium	SiRNA (pmol)	Final SiRNA concentration (nM)	Lipofectamine RNAiMAX
96-well	0.2	100µl	20µl	1.2	10	0.2
48-well	0.4	200µl	40µl	2.4	10	0.4
24-well	1	500µl	100µl	6	10	1
6-well	5	2.5ml	500µl	30	10	5

Table 2.5: Volumes and concentrations for siRNA transfections

Tissue Culture vessel	No. of cells seeded for siRNA transfection			
	MDA-MB-231	**MCF-7**	**BT474**	**SKBR3**
96-well	6×10^3	8×10^3	1.5×10^4	1×10^4
48-well	1.2×10^4	1.6×10^4	3×10^4	2×10^4
12-well	3×10^4	3.5×10^4	8×10^4	5×10^4
6-well	1.5×10^5	1.5×10^5	2.5×10^5	2×10^5

Table 2.6: Cell line seeding densities for siRNA transfection

2.4.7 Lentiviral transduction of cell lines

Prior to transduction with lentivirus, MCF-7 and MDA-MB-231 cell lines were seeded in 24-well plates at a density of 100,000 cells per ml to give 30-50% confluency after 24 hours. One day later the complete culture media was removed from the cells and 1ml of fresh complete media containing 7μg/ml Polybrene (Millipore, MA, US) was added to the wells. Lentivirus was then added to each well in a range of volumes (0, 1, 2, 5, 10 and 20μl). Following 24 hours of lentiviral infection the culture medium was removed and replaced with fresh complete culture media without polybrene to allow cells to expand.

2.4.7.1 Generation of cell lines deficient of c-FLIP

In order to generate cell lines deficient of c-FLIP gene products, MCF-7 and MDA-MB-231 cells were transduced with lentivirus encoding shRNA against the c-FLIP gene (Open Biosystems, CO, US). MCF-7 and MDA-MB-231 cells were transduced with 2 shRNA sequences (Fwd 5'-

3',GATCTCCGGGGATAAATCTGATGTGTCCTCATTACTCGAGTAATGAGGAC ACATCAGATTTATCCTTTTTA; Rev 5'-3',AGCTTAAAAAGGATAAATCTGAT GTGTCCTCATTACTCGAGTAATGAGGACACATCAGATTTATCCCCGGA) targeting the c-FLIP gene and cells were then cultured in 4μg/ml (MDA-MB-231) or 2μg/ml (MCF-7) puromycin to select for stably transduced cells. Cells transduced with lentivirus containing the empty vector pLKO1 (Open Biosystems, CO, US) were used as a control. The levels of knockdown achieved by different shRNA sequences were compared with cells transduced with non-target empty vector (NT) shRNA by western blot and qRT-PCR.

2.4.8 CellTiter Blue (CTB) viability assays

The CellTiter Blue assay (Promega, Southampton, UK) provides a homogenous, fluorometric method for the number of viable cells present. This reagent measures metabolic capacity of cells using resazurin as an indicator dye. Viable cells have the ability to convert resazurin into resofurin, a highly fluorescent derivative. Any non-viable cells will lack the metabolic capability to make this conversion and thus fail to produce a fluorescent signal.

Before use, CellTiter Blue reagent was thawed to room temperature. 20μl of CellTiter Blue reagent was added per 100μl of culture media and incubated at 37^{o}C with 5% CO_2 for 1 hour. The resulting fluorescence was then measured by setting excitation/emission wavelengths to 560/590nm on a Fluostar Optima plate reader (BMG Labtech, Bucks, UK).

2.4.9 Tryphan blue exclusion cell viability counts

In order to estimate the number of cells present in a culture, cells were lifted from plates by 0.05% trpsin/EDTA incubation at 37oC for 5-10 minutes. The cells were then resuspended in a known volume of culture media. Tryphan blue dye was then added to 10ul of cell suspension at a 1:1 ratio and this was loaded onto a haemocytometer cell counting chamber. Only viable cells without blue stain were counted and the number of cells in a ml of suspension can be calculated by the average number of cells per square multiplied by $2x10^4$.

2.4.9.1 Proliferation assay

For proliferation assays, cells were seeded at a density of 10,000 cells/well in 6-well plates (Nunc, Leics, UK). For each cell line, nine wells were seeded to represent triplicates of three timepoints. After 24 hours, cells from triplicate wells for the first time point were trypsinized and individually counted as described in Section 2.4.3. The same was done for each cell line at 48 hours and 72 hours post-seeding. The average cell counts for respective cell lines were then plotted on a log scale, normalized against the number of cells present at 24 hours.

2.4.10 Flow cytometry

Transfected cells were removed from tissue culture plates using 1mM EDTA (Sigma) and centrifuged at 1100rpm for 5 minutes. The pellet was washed twice in Fluorescence activated cell sorting (FACS) buffer (PBS supplemented with 2% v/v FBS). Cells were resuspended at a density of $4x10^6$ cells /ml and 100µl of this suspension was aliquoted into the appropriate wells of a 96 well plate before

centrifugation at 1100rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 100µl of the fluorescently conjugated primary antibody (Table 2.6) and left on ice for 30 minutes in the dark. Cells were then centrifuged and the primary antibody removed. The pellet was washed twice in FACS buffer before being filtered through a 40µm cell strainer (BD Biosciences) into a FACS collection tube (BD Biosciences) to ensure a single cell suspension. FACS was performed on a FACS Canto I or Canto II Flow Cytometer (BD Biosciences) and analysis of results was performed using a FlowJo software package. Gates were set to exclude >99% of cells labelled with isoform-matched control antibodies conjugated with the corresponding fluorochromes.

2.4.10.1 Aldefluor assay

The aldefluor assay (Stemcell Technologies, Grenoble, France) is a method to identify stem cells on the basis of their high aldehyde dehydrogenase (ALDH) activity. A fluorescent aldefluor reagent diffuses into cells and is a substrate for ALDH. The amount of fluorescent ALDH reaction product is directly proportional to the ALDH activity in cells. Cells with high expression of ALDH are recognised by comparing the fluorescence of test cells with that of a control sample containing the specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB).

Aldefluor reagents were prepared according to manufacturer's instructions. Transfected cells were removed from tissue culture plates using 1mM EDTA and centrifuged at 1100rpm for 5 minutes. The cells were washed twice in FACS buffer by resuspension and centrifugation. Cells were suspended in 1ml of aldefluor assay buffer and counted. The samples were adjusted to a concentration of $1x10^6$ cells/ml with

aldefluor assay buffer. A 'control' and a 'test' tube were prepared for each sample to be tested and 1ml of the cell suspension was placed into each 'test' tube. 5µl of DEAB reagent was added to the control tube and recapped immediately before 5µl of activated aldefluor substrate was added per ml of test suspension in the 'test' tube. The suspension was mixed and 0.5ml was immediately transferred to the 'control' tube containing the DEAB substrate. The 'test' and 'control' tubes were incubated for 45 minutes at 37^{o}C. The tubes were then centrifuged at 1100rpm for 5 minutes, the pellets resuspended in aldefluor buffer and placed on ice. The fluorescence of cells was measured in the green fluorescence channel of a FACS Canto flow cytometer (BD Biosciences). Analysis was performed using FlowJo software. Gates were set to exclude >99% of DEAB control cells

Target	ErbB2	ERα	Tubulin	c-FLIP (NF6)	c-FLIP (5D8)	DR5	DR4
Primary Ab source	AbCam	CST	AbCam	Enzo	Santa Cruz	CST	AbCam
Catalogue No.	Ab2428	8644	Ab6160	ALX-804-428-C050	sc-136160	3696	Ab8414
Primary Ab Raised in	Rabbit (pAb)	Rabbir (mAb)	Rat (mAb)	Mouse (pAb)	Mouse (pAb)	Rabbit (pAb)	Rabbit (pAb)
Primary antibody dilution	1:200 in 5% w/v BSA	1:500 in 5% w/v BSA	1:10000 in 5% w/v BSA	1:200 in 5% w/v BSA	1:200 in 5% w/v BSA	1:500 in 5% w/v BSA	1:500 in 5% w/v BSA
Secondary Ab	HRP-anti Rabbit	HRP-anti Rabbit	HRP-anti Rat	HRP-anti Mouse	HRP-anti Mouse	HRP-anti Rabbit	HRP-anti Rabbit
Secondary Ab conditions	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA	1:2000 in 5% w/v BSA
Detection reagents	ECL	ECL	ECL	ECLprime	ECLprime	ECLprime	ECLprime
Protein size	185 kDa	66 kDa	50 kDa	55 kda / 25 kDa	55 kDa	40 kDa	57 kDa

Table 2.9: Western antibodies and conditions
CST- Cell signalling technologies, pAb – polyclonal antibody, mAb – monoclonal antibody,

2.4.11 Mammosphere assays

Mammosphere assays can be used for the *in vitro* propagation of mammary epithelial cells in an undifferentiated state in non-adherent cell culture conditions. The resulting mammosphere colonies are enriched for mammary stem/early progenitor cells that are capable of self-renewal and form new colonies upon disaggregation and re-seeding. This assay is therefore suitable for the functional identification of this cell population.

Cells were removed from tissue culture plates using 0.25% w/v Trypsin/EDTA and centrifuged at 1100rpm for 5 minutes. Cells were then resuspended in mammosphere culture medium (serum-free epithelial growth medium [MEBM, Lonza], supplemented with B27 [invitrogen], 20ng/ml Epidermal Growth Factor [EGF, Sigma], 5mg/ml Insulin [Sigma], 0.0008% v/v β-mercaptoethanol [Sigma] and 1mg/ml hydrocortisone [Sigma]) and disaggregated into single cell suspensions by mechanical agitation. Cells were counted and seeded into ultra-low attachment plates (Corning) at density of 4000 cells/ml (unless otherwise stated) before being incubated for 7 days at 37^{o}C and 5% CO_2 to allow the formation of mammosphere colonies. After this incubation period, the numbers of mammospheres per well were counted. In order to determine the self-renewal capacity of cells, mammospheres were collected by gentle centrifugation at 1100rpm and mechanically and enzymatically dissociated into single cells in 0.25% w/v Trypsin/EDTA at 37^{o}C. Cells were centrifuged at 1100rpm before being counted and re-seeded into ultra-low attachment plates at a density of 4000 cells/ml. Cells were then incubated for 7 days at 37^{o}C and 5% CO_2 after which time the numbers of mammospheres per well were counted.

2.4.12 γδ T cell isolation and stimulation from healthy human blood donors

40mls of blood was removed from healthy human donors and added to 10ml of heparin/EDTA to prevent clotting. 30ml of this blood was then gently pippetted onto 20mls of Ficoll lymphoprep and then spun in a centrifuge for 20 minutes at 1800rpm with 0 deceleration at 4°C. The interface, between Ficoll and plasma, of peripheral blood mononuclear cells (PBMCs) was collected and washed 3 times in MACS buffer, collecting cells by centrifugation at 1100rpm for 8 minutes between washes. From PBMCs γδ T cells/monocytes were purified using 2 methods. To isolate pure unstimulated populations, γδ T cells/monocytes were isolated from PBMCs using MACs γδ T cell/CD28 magnetic purification kit (Miltenyi Biotec) and a 1:1 ratio of γδ T cells:monocytes used at stimulation. Alternatively PBMCs were cutured in media containing the desired stimulatory compound for 2 weeks until the population of γδ T cells was larger than 85% of the total cell population. In either case cells were stimulated using HMB-PP, zoledronate (Novartis) or ibandronate (Boniva).

2.4.13 ELISA

To determine secreted concentrations of TRAIL and perforin, supernatants collected from culturing cells were collected and compared to standard concentrations of TRAIL and Perforin. ELISA kits for TRAIL and Perforin were obtained from R&D systems (DTRL00) and MabTech (3465-1H-20) respectively and performed according to the manufacturers instructions.

2.4.14 Heterotypic cell death assay

siRNA treated cells were treated with 0.25% trypsin for 10 mins, washed and stained with PKH67 or PKH26 cell linker kits according to the manufacturers protocol (Sigma). PKH67+ve FLIPi cells and PKH26+ve SCi cells were mixed 1:1 and cultured overnight with or without TRAIL or γδ T cells and subsequently resuspended in 4μl (1 in 10 dilution of stock solution) of 1:10 fixable near-IR live/dead stain (Invitrogen) and incubated for 15 mins at 4°C in the dark. Cells were then washed twice in PBS and analysed by flow cytometry. Gates were then set for PKH staining versus live/dead staining using a FACS Canto (Becton Dickinson) using untreated and unstained control cells.

2.4.15 Annexin-V assay

siRNA treated cells were treated with 0.25% trypsin for 10 mins, washed and stained with Annexin-V (APC) (eBioscience) for 15 mins at 4°C in the dark. Cells were then washed twice in PBS and analysed by flow cytometry using a FACS Canto.

2.5 Protein analysis by western blotting

2.5.1 Protein extraction from cells

Media was completely aspirated from cell culture flasks and the cells were washed twice in ice cold PBS. A further 12ml of PBS was added to the flask and cells were scraped using a cell scraper (Nunc) and transferred into 15ml falcon tubes. The cells were centrifuged at 1100rpm for 5 minutes and the supernatant removed. Cell pellets were either stored at -80°C until required or immediately lysed by the addition of 100-300μl of RIPA buffer (150mM sodium chloride *Fisher Scientific+, 1% v/v Nonidet-P40 [Roche], 0.5% w/v sodium

deoxycholate [Sigma], 0.1% w/v sodium dodecyl sulphate [SDS; Sigma], 50mM Tris [Sigma], pH8) containing protease (Complete mini protease inhibitor tablets [Roche]) and phosphatase inhibitors (1mM sodium orthovanadate [Sigma], 10mM sodium fluoride [Fluka Biochemika], 10mM sodium pyrophosphate [Sigma]). The cell pellet was passed through a 23G needle 10 times to ensure complete cell lysis and incubated on ice for 30 minutes. Lysates were centrifuged at 10,000rpm for 15 minutes at 4^{o}C to pellet cell debris and the supernatant was aliquoted into fresh tubes and snap frozen in liquid nitrogen for storage at -80^{o}C until required.

2.5.2 Determination of protein concentrations

Protein concentrations were analysed using the BCA protein Assay kit (Pierce) according to manufacturer's instructions. Protein samples were first diluted by 1:5 and 1:10 in RIPA Buffer (20mM Tris-HCL [pH 7.5], 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NF-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na_3VO_4, 1μg/ml leupeptin) and 12.5µl of each diluted sample was added to a 96 well round bottomed plate (Nunc) in duplicate. BCA protein assay reagent A was added to BCA protein assay reagent B in a ratio of 50:1 and 100µl of this mix was added to each sample. Standard samples were also generated by diluting 2mg/ml BSA in PBS to produce 4 known protein concentrations (2mg/ml, 1mg/ml, 0.5mg/ml, 0.25mg/ml) to which 100µl of BCA reagent mix was added. Samples were mixed well and incubated at 37^{o}C for 30 minutes to allow a purple colour to develop. Colour intensity of each sample was measured on a nanodrop spectrophotometer (ND-1000; Labtech International) at 562nm. The relative concentration of protein in each sample was extrapolated from

the standard curve generated using the standard samples.

2.5.3 Western analysis

2.5.3.1 Preparation of protein samples

After protein concentrations were determined, 70µg of protein were diluted in RIPA buffer to produce a final volume of 10µl and 2.5µl of 5X laemmli buffer (0.125M Tris-HCL pH6.8, 4% w/v SDS, 40% v/v glycerol, 0.1% w/v bromophenol blue [Sigma], 6% v/v beta-mercaptoethanol [Sigma] in ddH20) was added to each sample. Just before loading, the samples were heated to 95^{o}C for 5 minutes to denature the proteins.

2.5.3.2 Casting of polyacrylamide gels

All Mini western SDS-PAGE gels were set in Mini-Protean III (Bio-Rad) gel casting apparatus. Resolving gel mixtures composed of varying concentrations of Acrylamide/Bis (depending on target protein size) were prepared (Table 2.7), poured and left to set for 30 minutes. Once set, the 4% stacking gel mixture was prepared and poured over the resolving gel (Table 2.7). A clean 10 or 15-well comb was inserted immediately into the apparatus and the gel was left to set for 30 minutes. Once set, the combs were removed and the wells rinsed with ddH20.

Component	8% Resolving Gel (x2)	10% Resolving Gel (x2)	12% Resolving Gel (x2)	4% Stacking Gel (x2)
Molecular Weight of Protein	40-150kDa	25-100kDa	15-75kDa	
ddH_2O	4.7ml	4.1ml	3.4ml	6.1ml
30% Acrylamide/Bis (Sigma)	2.7ml	3.3ml	4.0ml	1.3ml
1.5M Tris-HCl, pH8.8	2.5ml	2.5ml	2.5ml	
0.5M Tris-HCl, pH6.8				2.5ml
10% w/v SDS	100µl	100µl	100µl	100µl
10% Ammonium Persulphate (Sigma)	50µl	50µl	50µl	50µl
TEMED	5µl	5µl	5µl	10µl

Table 2.7: Composition of polyacrylamide gels

2.5.3.3 Gel electrophoresis

Gels were placed in the Mini-Protean III (Bio-Rad) electrophoresis tank and immersed in 1 x Tris-Glycine running buffer (Table 2.8). Protein molecular weight marker (PageRuler Plus; Fermentas) was loaded into the first lane of each gel and prepared protein samples were loaded into the appropriate remaining wells. The samples were resolved down SDS-PAGE gels for approximately 45-60 minutes at 150V until the dye front reached the bottom of the gel.

2.5.3.4 Transfer of proteins to PVDF membranes

After separation of protein, gels were carefully removed from glass plates and soaked in 1 x Tris-Glycine transfer buffer (Table 2.8) for 10 minutes. Immobilon-P, Polyvinylidene difluoride (PVDF; Millipore) membrane was cut to size, pre-soaked in methanol (Fisher Scientific) for 10 seconds and washed in ddH20. The stacking gel was then cut away and discarded and the resolving gel was placed onto the membrane in a standard wet electroblotting system (BioRad). Air bubbles were carefully rolled out after the addition of each layer. An ice block was inserted into the transfer apparatus and proteins were transferred by electroblotting in 1 x Tris-Glycine transfer buffer (25mM Tris, 192mM glycine, pH8.3) at 100V for 1 hour. Blotting apparatus was then dismantled and membranes were washed for 5 minutes in PBS/T.

2.5.3.5 Probing of membranes

Following transfer, membranes were blocked in milk blocking solution (Table 2.8) under agitation for 1 hour at room temperature and then incubated in 5mls of primary antibody (Table 2.9) at 4^{o}C on a roller mixer (Stuart, Merton, UK) overnight. The following morning the membrane was washed (3 x 5 minutes) in PBS/T before being incubated in 5mls of the appropriate horseradish-peroxidase (HRP)-conjugated secondary antibody (Table 2.9) for 1 hour at room temperature on a roller mixer. Following this incubation, membranes were washed 3 x 5 minutes in PBS/T prior to protein detection by enhanced chemiluminesence.

2.5.3.6 Visualisation of protein bands

The ECLprime reagent kit (GE Healthcare) was used to visualise the immobilised protein bands conjugated to HRP-labelled secondary antibodies according to manufacturer's instructions. ECL solutions were mixed well and 2mls were immediately distributed evenly across the membrane and incubated in the dark for 1 minute (ECLprime). After removal of excess reagent, the membrane was placed in a light-proof cassette and exposed to light sensitive films (Amersham Hyperfilm ECL; GE Healthcare) under safelight conditions for varying lengths of time to give a range of exposure intensities. All films were developed on an automatic film processor (Xograph Compact X4 automatic X-ray film processor). Processed films were then realigned with the original membrane and the target protein was identified by comparison to the molecular weight marker.

2.5.3.7 Stripping and re-probing of membranes

In order to study more than one protein band from the same set of samples, membranes were stripped of their original antibodies and re-probed with a new one. The membrane was washed in PBS/T (3 x 5 minutes) and incubated in stripping buffer (Table 2.8) at 55^{o}C for 30 minutes with gentle agitation. The stripping buffer was removed and the membrane was rinsed 5 times in dH_20 before being incubated in PBS/T for 30 minutes at room temperature. After incubation for a further hour in milk blocking solution, the membrane was re-probed with the desired primary and secondary antibodies. Protein bands were then visualised using ECLprime reagent as described in section 2.5.3.6.

2.5.3.8 Quantification of protein bands

In some cases protein bands were semi-quantified. The intensity of protein bands was measured using a GelDoc (BioRad) and normalised to the band intensity of their corresponding loading control.

Solution	Composition
10 x Electrophoresis Buffer	30.3g Tris base (Sigma), 144.4g Glycine (Sigma), Upto 1L dH20
1 x Tris-Glycine SDS-PAGE Running Buffer (1L)	890ml dH_20, 100ml 10 x Electrophoresis Buffer, 10ml 10% w/v SDS
1 xTris-Glycine Transfer Buffer	700ml dH_20, 100ml 10 x Electrophoresis Buffer, 200ml Methanol
Milk Blocking Solution	5% w/v non-fat milk powder (Marvel) in PBS/T
Stripping Buffer	62.5mM Tris-Hcl (pH6.8), 2% w/v SDS, 100mM 2-beta-mercaptoethanol

Table 2.8: Composition of solutions used in western analysis

2.6 Caspase assays

Caspase-Glo 8 assay (Promega) is a luminescent assay that measures the activity of caspase-8 providing a measurable luminogenic substrate. Addition of the Caspase-Glo reagent to the sample results in cell lysis followed by caspase cleavage of the substrate and a resulting luminescent signal which is proportional to the amount of caspase activity present in the sample.

2.6.1 Determination of caspase activity in cells

Transfected or treated cells were removed from tissue culture plates using 0.25% w/v Trypsin/EDTA and centrifuged at 1100rpm for 5 minutes before being resuspended in complete growth medium. Cells were counted and resuspended at a density of $2x10^5$ cells/ml and 100µl of this suspension was plated into appropriate wells of a black-walled 96-well plate. At the same time, 100µl of complete media alone was plated into triplicate wells to serve as a 'blank' control. The plate was incubated at 37°C and 5% CO_2 for an appropriate amount of time (as indicated in the results sections). After incubation, Caspase-Glo reagents were prepared according to manufacturer's instructions, and plates were removed from the incubator and allowed to equilibrate to room temperature. To each well containing cells or 'blank' controls, 100µl of Caspase-Glo reagent was added. The plate was then gently agitated on a plate shaker at 300-500rpm for 30 seconds before being incubated at room temperature 1 hour. The luminescence of each sample was then measured on a FLUOstar Optima plate reader and the 'blank' control value was subtracted from all other values.

2.7 RNA analysis

2.7.1 Isolation of RNA

All bench work surfaces and equipment were treated with RNAseZAP (Ambion) before use and RNAse free H20 (Sigma) was used throughout.

Purification of RNA from cells was achieved using the RNeasy Mini Kit according to manufacturer's instructions. Concentration and quality of RNA was quantified on a NanoDrop spectrophotometer.

2.7.2 Reverse transcription

Reverse transcription was performed using RevertAID Premium Reverse Transcriptase (Fermentas). cDNA was synthesised from 500 – 1000ng RNA samples diluted in 12.5µl of RNase free H_20. A master reaction mix containing Random Primers and dNTPs (Table 2.10) was prepared in a nuclease free tube on ice and 2µl was added to each sample to make a total volume of 14.5µl. Samples were then mixed gently, centrifuged and heated to 65^oC for 5 minutes before being returned immediately to ice. A second reaction mix containing the reverse transcriptase (Table 2.10) was prepared and 5.5µl was added to each sample to make a final volume of 20µl. Samples were then incubated for 10 minutes at 25^oC, 30 minutes at 50^oC and 5 minutes at 85^oC before being returned to ice. For each experiment, controls were prepared in exactly the same way but with reaction mix lacking the reverse transcriptase enzyme. Samples were either used immediately or stored -70^oC until required.

Reaction Mix 1 Component	**Volume Per Reaction**
dNTPs (10mM; dATP, dCTP, dGTP, dTTP; Promega)	1µl
Random Primers (500ug/ml [Promega])	1ul
Reaction Mix 2 Component	**Volume Per Reaction**
5xRT Buffer (Fermentas)	4µl
RNasin Plus (40u/µl; Promega)	0.5µl
RevertAID Premium Reverse Transcriptase (200u/µl; Fermentas)	1µl

Table 2.10: Reaction mix components for reverse transcription

2.7.3 Quantitative real-time polymerase chain reaction (qRT-PCR) & semi-quantitative PCR

2.7.3.1 Primer design

All primer sets were designed across exon boundaries using the Primer3 web-based program http://frodo.wi.mit.edu/primer3/. To check for mis-priming, all sequences were verified by performing in silico PCR (http://genome.csdb.cn/cgi-bin/hgPcr). All primers were purchased from Sigma-Genosys and sequences can be found in Table 2.12.

2.7.3.2 qRT-PCR

QRT-PCR was performed using Step One Plus Real-time PCR System (Applied Biosystems), in conjunction with StepOne (v2.1; Applied Biosystems) software. Each reaction was performed in triplicate and a minimum of 3 separate biological samples were analysed. One housekeeping gene (β-*Actin*) was always run as a reference.

An arbitrary standard curve was generated by pooling 5µl of cDNA from each sample to make a neat cDNA control which was then diluted 1:5 and 1:25 with PCR-grade water to make three cDNA standards. Appropriate volumes (Table 2.11) of cDNA test samples, cDNA standards or PCR-grade H_20 (Sigma) control were loaded into separate wells of a 96 well reaction plate (MicroAmp Fast Optical 0.1ml; Applied Biosystems).

A PCR mastermix containing all other reaction components was then prepared according to Table 2.11. Mastermix (22.5µl) was added to each well and gently pipetted to ensure homogeneous mixing of cDNA and reaction mix. The PCR plate was then sealed with appropriate caps (MicroAmp Optical 8-capstrip; Applied Biosystems) before being loaded into the Realtime PCR machine.

All reactions were run under the same cycling conditions of initial denaturation at 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (with a plate read after each cycle). A melting curve (95°C for 15 seconds, 60°C for 1 minute [optics off], 60°C to 95°C at 0.2°C increments every 15 seconds [optics on]) was constructed at the end of the experiment and data was collected with. Step one plus software.

Target	Species	Fwd Primer Seq (5'-3')	Rev Primer Seq (3'-5')
c-FLIP	Human	TGATGGCAGAGATTGGTGAG	GATTTAGACCAACGGGGTCT
β-actin	Human	CCCAGCACAATGAAGATCAA	CAGGTGGAAGGTCGTCTACA

Table 2.11: qRT-PCR primer sequences

2.7.3.3 Analysis of qRT-PCR data

Data was initially examined using Step One Plus software. Melting curves were analysed to ensure that only one peak at the expected melting temperature was observed and that the H_20 control samples produced no product. Ct values for standards were plotted against relative amounts (neat cDNA = 1, 1:5 dilution = 0.2, 1:25 dilution = 0.04) to generate a standard curve for each primer set. Relative mRNA expression in each sample was extrapolated by plotting Ct values on the standard curve. The differences between the amounts amplified in samples for a target gene and corresponding samples amplifying a reference gene was then calculated to get a normalised relative expression value. Generally, an average expression value across all biological replicates was taken and values were normalised back to the control samples.

Reaction Component	QRT-PCR
cDNA Template	2.5µl
PCR grade Water	12.65µl
Go Taq PCR Buffer	5µl
Magnesium Chloride (25mM)	2.5µl
dNTPs (10mM; dATP, dCTP, dGTP, dTTP)	0.5µl
Forward Primer (10µM)	0.25µl
Reverse Primer (10µM)	0.25µl
Go Taq DNA Polymerase	0.1µl
Sybr Green (Invitrogen)	1.25µl
Total Reaction Volume	25µl

Table 2.12: qRT-PCR reaction components

2.8 Statistical analysis

2.8.1 Kaplan-Meier survival analysis

All tumour-free survival curves and statistical analysis of tumour-free survival times were performed using the Kaplan-Meier method with the aid of MedCalc statistical analysis software (Version 11.4.3.0, www.medcalc.org/).

2.8.2 Kolmogorov-Smirnov analysis

Differences in the distribution of data to determine whether data was parametric or non-parametric were tested for using the Kolmogorov-Smirnov test. This test was performed using the Minitab statistics package (Version 14.20).

2.8.3 Mann-Whitney U-test

The Mann-Whitney U-test was used to determine statistical differences between non-parametric data sets. This was performed using the Minitab statistics package (Version 14.20).

2.8.4 Student's t-test

The Student's T-test was used to determine statistical differences between normally distributed data sets and between data sets with sample sizes of n=3. This test was performed using Excel 2007 software.

2.8.5 Chi-Squared test

The Chi-Squared test was used to determine statistical differences between observed frequencies and expected frequencies of data.

CHAPTER 3:

Sensitising Breast Cancer Cells to TRAIL Treatment

3.1 Introduction

The realisation that breast cancer is a heterogenous disease has lead to great advancements in the way that we treat the disease, resulting in more targeted therapeutic strategies that have improved survival rates in discrete disease subgroups (Badve and Nakshatri 2009). This is demonstrated by the treatment of ER-positive and HER2-positive breast cancers by agents such as tamoxifen and Herceptin respectively (Dean-Colomb and Esteva 2008; Yamashita 2008). However, despite these advances tumours still frequently relapse due to their innate or acquired resistance to therapeutic insult. The complexity of therapy due to inter-tumour heterogeneity is compounded further with the existence of intra-tumour heterogeneity that is defined by number of different cell types that can found within each tumour. Functionally one of the most important of these subsets of cells found within a breast tumour is the breast cancer stem cell (bCSC). It is these cells that are at the root of innate therapy resistance and provide the source for new tumour growth (Al-Hajj et al. 2003; Dontu et al. 2003a; Visvader and Lindeman 2008; Zhou et al. 2009). Consequently, there is considerable interest in the targeting of this cell subset with cytotoxic agents across a broad range of breast cancer subtypes to benefit as wide a patient group as possible.

TRAIL is a promising anti-cancer agent that exhibits tumour specificity with almost no effect on normal cell populations in a wide range of different tissues. Currently, clinical trials are ongoing using TRAIL for treatment of colorectal cancer, non-small cell lung carcinoma and non-Hodgkins lymphoma (Ashkenazi et al. 1999; Kruyt 2008). However, its therapeutic potential in breast cancer is limited with most subtypes of disease displaying resistance (Kendrick et al. 2007;

Rahman et al. 2009b). Additionally, it has been documented that CSC subsets are also resistant to TRAIL therapy and thus patients receiving TRAIL therapy are still likely to incur relapse of disease (Capper et al. 2009; Szegezdi et al. 2009). As a result, a prime focus of many studies has been to elucidate sensitising agents to TRAIL treatment and whilst this has been performed to varying degrees of success, most undermine the key attribute of TRAIL treatment that is the relative tumour specificity of the agent (Cuello et al. 2001; Keane et al. 1999; Ortiz-Ferron et al. 2008; Palacios et al. 2006; Sanchez-Perez et al. 2009). Common to many of these studies is the demonstration that the endogenous inhibitor of death receptor signalling c-FLIP is downregulated during the sensitisation process (Bijangi-Vishehsaraei et al. 2010; Ortiz-Ferron et al. 2008; Palacios et al. 2006; Sanchez-Perez et al. 2009).

Whilst it is clear that c-FLIP, as a regulator of TRAIL instructive cell death, plays an important role in cancer cell resistance, the direct suppression of c-FLIP as a means of sensitisation to TRAIL has not been fully investigated.

In this chapter the potential of c-FLIP suppression to sensitise a broad range of breast cancer cell subtypes to TRAIL treatment was investigated with important focus on its capability in preventing disease relapse whilst maintaining tumour specificity of treatment.

3.2 Results

3.2.1 Breast cancer cell line sensitivity to TRAIL is not determined by c-FLIP mRNA expression levels

Breast cancer cell line resistance to TRAIL has been previously documented (Rahman et al. 2009a). However, it is important to ascertain the specific sensitivity of cell lines 'in-house' as differing sources, culture conditions and passage time of cell lines may change their response to certain cytotoxic agents.

Here a panel of breast cancer cell lines representing a broad spectrum of disease was selected on the basis of their estrogen and herceptin receptor expression (Figure 3.1A) and their response to TRAIL ligand (Figure 3.1B) assessed using assays detailing cell viability, apoptosis and cell death. Additionally, mRNA was extracted from culturing cells and c-FLIP expression measured in each of the cell lines to determine the correlation between c-FLIP mRNA levels and TRAIL sensitivity.

Using the selected four breast cancer cell lines representing all combinations of ERα and HER2 expression, only the basal ER-negative/HER2-negative MDA-MB-231 cell line gave a significant increase in apoptosis and cell death with a conferring reduction in cell viability (Figure 3.1, $p<0.01$) when treated with TRAIL. The remaining luminal-like cell lines MCF-7 (ER-positive/HER2-negative), SKBR3 (ER-negative/HER2-positive) and BT474 (ER-positive/HER2-positive) displayed resistance to TRAIL treatment with no significant increases in cell death or apoptosis and no significant reduction in cell viability (Figure 3.1). The functional concentration of TRAIL capable of giving the maximum amount of caspase-8 activation was identified as 20ng/ml by exposing the sensitive MDA-MB-231 cell line to increasing

TRAIL concentrations (Figure 3.1C). Interestingly, c-FLIP mRNA levels did not determine sensitivity to TRAIL. SKBR3 cells expressed significantly less c-FLIP mRNA but still remained resistant to TRAIL treatment (Figure 3.1D).

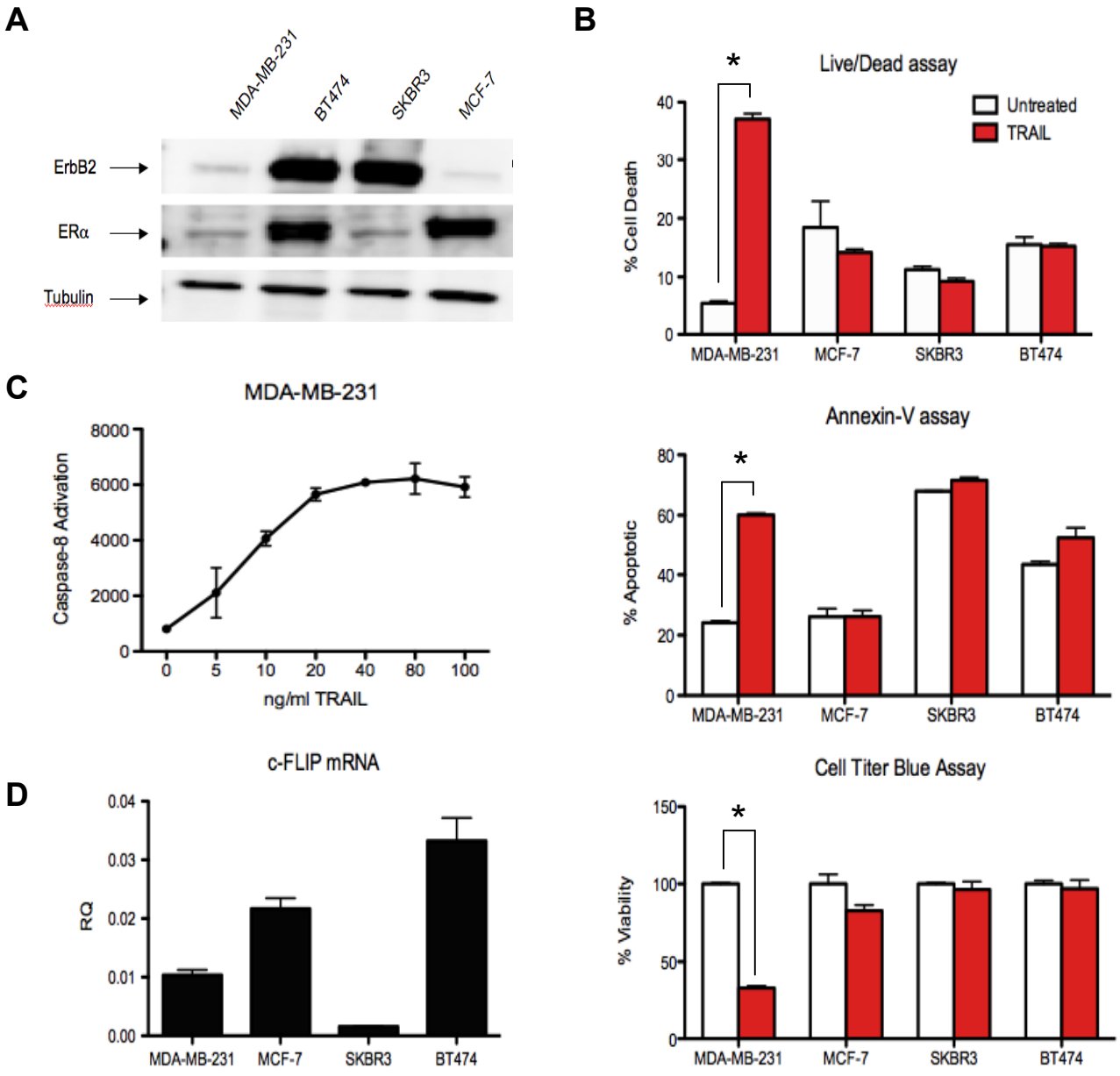


Figure 3.2.1: Most breast cancer subtypes are resistant to TRAIL treatment. A) Western blot indicating breast cancer cell line protein levels of ErbB2 and ERα. Tubulin expression was used to determine equal loading. B) Cell lines were treated with 20ng/ml TRAIL for 18 hours and cell death, apoptosis and viability of breast cancer cell lines was measured by FACS (Live/dead assay and Annexin-V) or fluorescence (CTB assay) * $p<0.01$. C) MDA-MB-231 cells were treated with increasing concentrations of TRAIL and caspase-8 activation (CaspaseGlo assay) was measured. D) c-FLIP mRNA expression in BCCLs as measured by qRT-PCR relative to β-actin. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.2 Optimisation of c-FLIP siRNA knockdown in breast cancer cell lines

To investigate the contribution of c-FLIP in breast cancer cell line resistance to TRAIL a number of *in vitro* experiments were performed. In these experiments a siRNA approach was adopted to efficiently suppress c-FLIP.

Initially the c-FLIP siRNA transfection technique was optimised on the basis of the manufacturer's protocol. Cells were plated into transfection medium containing 10nM of either c-FLIP (entire transcript, FLIPi) or scrambled control siRNA (SCi) for 24-72 hours. Triplicate wells of cells were then harvested at defined timepoints of 24, 48 or 72 hours and cDNA prepared for qRT-PCR analysis with all results being normalised to a control internal housekeeping gene, β-actin. Subsequently, protein levels of c-FLIP$_L$ and c-FLIP$_S$ were analysed by western blot in order to confirm the c-FLIP knockdown post-transcriptionally.

In the MDA-MB-231 and MCF-7 cell lines the optimum transfection period was identified at 48 hours. At this timepoint c-FLIP transcript levels had reduced to 27% and 19.6% of their constitutive controls respectively. In the SKBR3 and BT474 cell lines, the optimum transfection period was identified as 72 hours, during which time c-FLIP transcript levels had reduced to 19.3% and 4% of their constitutive controls respectively. Although a reduction in c-FLIP transcript levels could be observed at other transfection period timepoints these were the timepoints at which the greatest knockdown could be achieved and thus for all subsequent siRNA experiments the MDA-MB-231 and MCF-7 cell lines were transfected for 48 hours and the SKBR3 and BT474 cell lines were transfected for 72 hours. Additionally, c-FLIP transcript levels were routinely checked across all

siRNA experiments to ensure a greater than 75% knockdown was being achieved.

Western blotting of protein extracted from scrambled control siRNA (SCi) and c-FLIP siRNA (FLIPi) treated cells showed that both the long and short isoforms of c-FLIP had been significantly reduced in all cell lines. Unfortunately after numerous attempts the suppression of c-FLIP$_S$ could not be confirmed at the protein level in the MCF-7 cell line treated with either SCi or FLIPi.

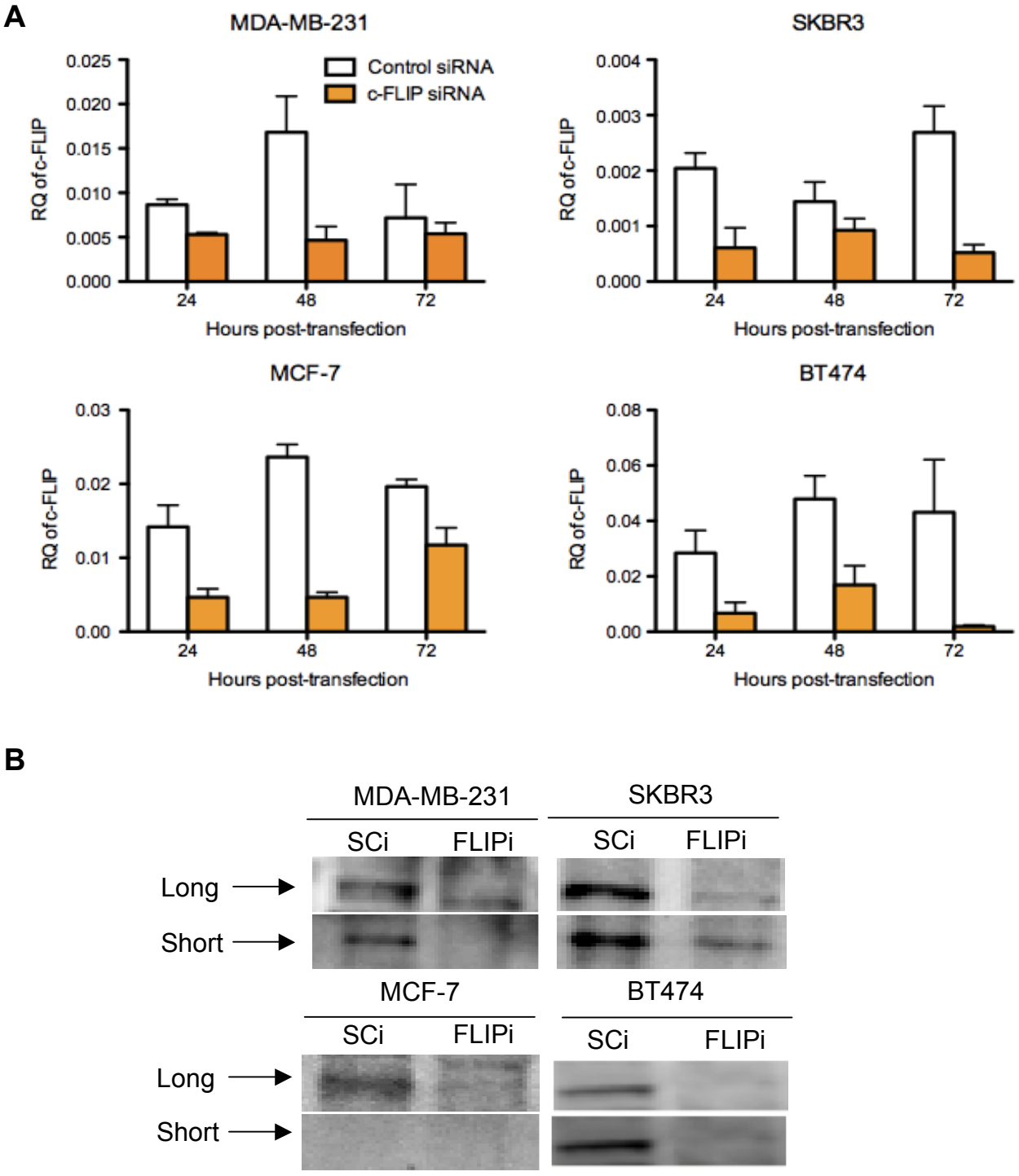


Figure 3.2.2: c-FLIP siRNA transfection results in significant reduction of c-FLIP mRNA and proteins. A) mRNA expression of c-FLIP in BCCLs following control or c-FLIP siRNA transfection, for different lengths of time, relative to β-actin. Data shown is representative of 3 experiments performed in triplicate ±SEM. B) c-FLIP short and long isoform protein levels in BCCLs following SCi of FLIPi treatment for 48 hours (MCF-7 and MDA-MB-231) or 72 hours (BT474 and SKBR3).

3.2.3 c-FLIP deficiency does not sensitise normal mouse cells to TRAIL

It has been reported that TRAIL preferentially targets tumour cells over normal cells (Ashkenazi 2002; Ashkenazi et al. 1999; Lawrence et al. 2001). To determine if the targeted inhibition of c-FLIP exhibited similar specificity for tumour cells, mammary epithelial cell viability was assessed in non-tumourgenic c-FLIP-deficient mouse mammary glands, non-tumourgenic murine cell lines and in the non-tumourgenic human breast cell line MCF-10A. c-FLIP was conditionally deleted from mammary epithelial cells of juvenile mice by crossing the BLG-Cre transgene (Selbert et al. 1998a, b) into the c-FLIP$^{fl/fl}$ line (Zhang and He 2005), and the mammary epithelial compartment subsequently assessed in adult virgin and pregnant animals.

Mammary epithelial morphogenesis and cell number in BLG-Cre/c-FLIP$^{fl/fl}$ mammary glands was indistinguishable from wild-type controls, while isolated primary epithelial cells from both genetic backgrounds exhibited comparable cell viability either in the presence or absence of TRAIL *in vitro* (Figure 3.2.3A, B). Furthermore, inhibition of c-FLIP (FLIPi) using murine specific siRNA had no effect on a non-tumourgenic murine cell line's response to TRAIL but significantly reduced viability in the tumourgenic, ErbB2 overexpressing, N202.1A cell line (Figure 3.2.3C, $p<0.00001$). Similarly, in the human non-tumourgenic breast cell line, MCF-10A, cell viability was unaffected by c-FLIP inhibition (FLIPi) alone, nor did it increase sensitivity to TRAIL, although treatment with TRAIL induced a significant cell death response (Figure 3.2.3D, $p= 0.0307$). This confirms previous reports of partial TRAIL sensitivity in human transformed cell lines (Keane et al. 1999). These data indicate that

the targeted inhibition of c-FLIP may exhibit tumour specific effects, similar to those observed with TRAIL, in other cancer types.

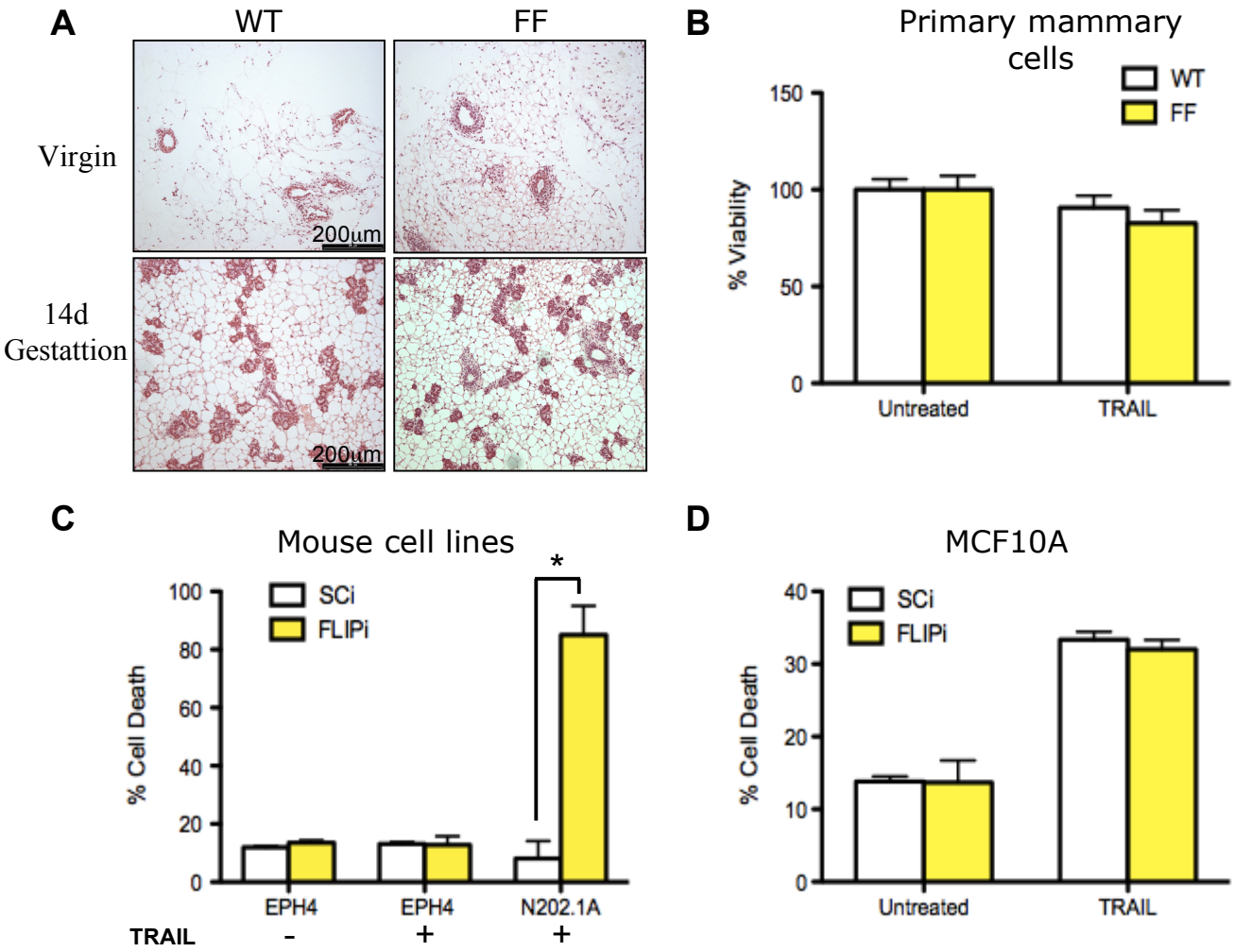


Figure 3.2.3: c-FLIP knockdown has no significant effect on normal mouse mammary epithelial cells and normal transformed cell lines. A) Representative H+E stained sections of the 4th inguinal mammary glands excised from c-FLIP$^{+/+}$ (WT) and c-FLIP$^{fl/fl}$ (FF) mice. B) Viability of primary mammary epithelial cells isolated from WT or FF mammary glands following TRAIL treatment as measured by CTB assay. C) Cell death of normal (EPH4) and cancer (N202.1A) mouse mammary cell lines following SCi, FLIPi treatment alone or in combination with TRAIL as measured by fixable live/dead assay * $p<0.01$. D) MCF10A cell death following treatment with SCi or FLIPi alone or in combination with TRAIL. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.4 c-FLIP knockdown increases basal apoptosis levels in breast cancer cell lines via caspase-8 and/or caspase-10 activation.

As one of the key regulators of TRAIL induced apoptosis, c-FLIP acts by competing with caspase-8 to bind to FADD at the DISC. When c-FLIP is bound caspase-8 is unable to be activated and set off the caspase cascade that ultimately results in the onset of apoptosis. Despite c-FLIP having no apparent effect on the morphology or cell death of normal human and mouse cells it may inhibit the base rate of apoptosis in tumour cells. To investigate the effect of c-FLIP removal on tumour cell apoptosis and caspase activation, cells were treated with control or c-FLIP siRNA and incubated in the presence or absence of the cell permeable, non-reversible caspase inhibitors IETD (caspase-8), LEHD (caspase-9) and AEVD (caspase-10) and apoptosis assessed by Annexin-V staining.

In each of the cell lines tested, c-FLIP knockdown (FLIPi) increased the base level of apoptosis between 10% and 15% over their corresponding SCi treated controls (Figure 3.2.4A). For the MDA-MB-231 and BT474 cell lines, this increase in apoptosis from c-FLIP knockdown could be inhibited and returned to control base levels by the caspase-10 inhibitor, AEVD. The caspase-8 inhibitor, IETD, could prevent the increased apoptosis from c-FLIP knockdown observed in the MCF-7 and SKBR3 cell lines, although the caspase-9 and -10 inhibitors also provided a similar inhibitory effect on the SKBR3 cells. These data suggest that c-FLIP regulates the base level of apoptosis seen in breast tumour cells by inhibiting the activation of capase-8, -9 and -10 in a cell type dependant manner (Figure 3.2.4B).

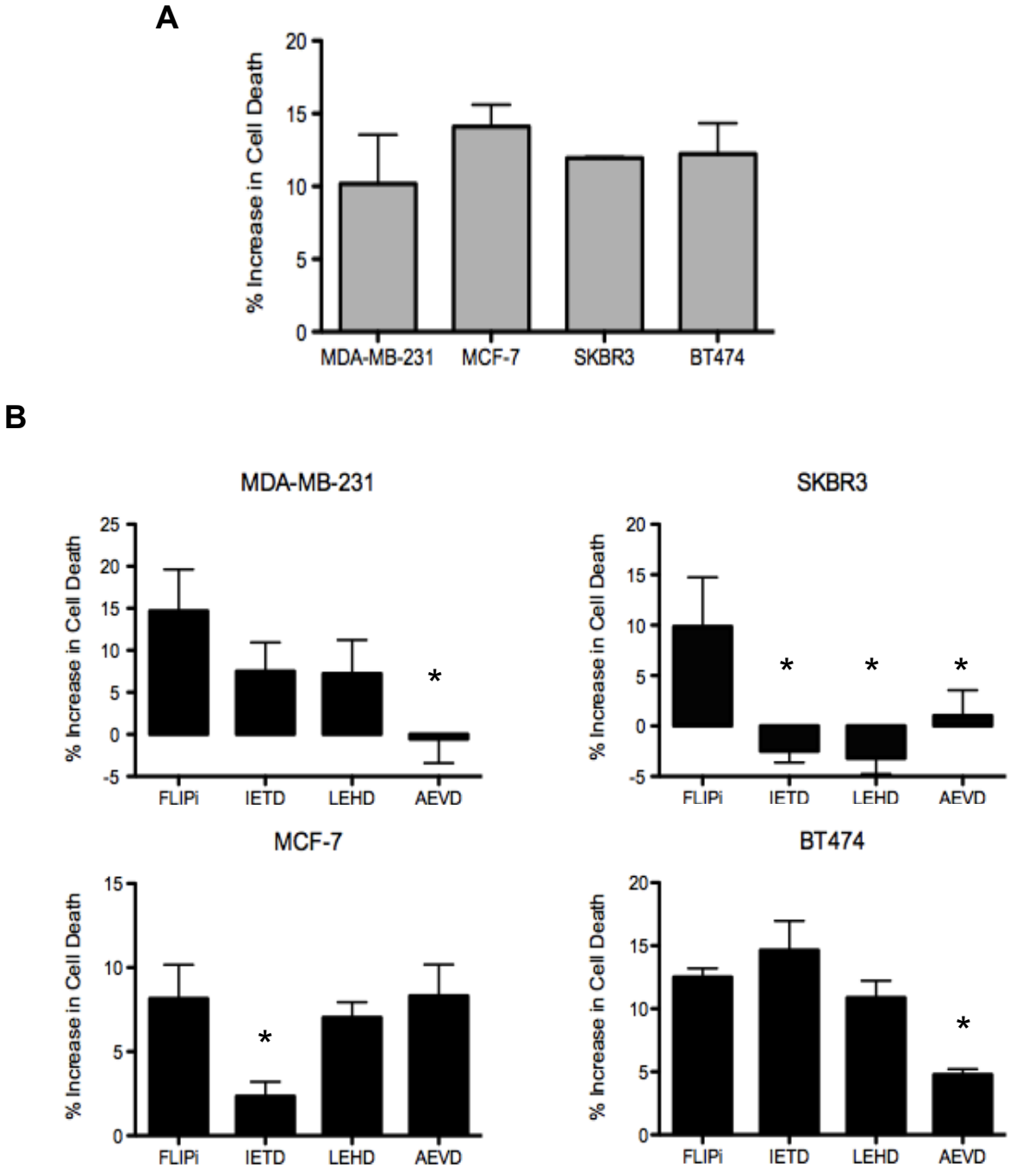


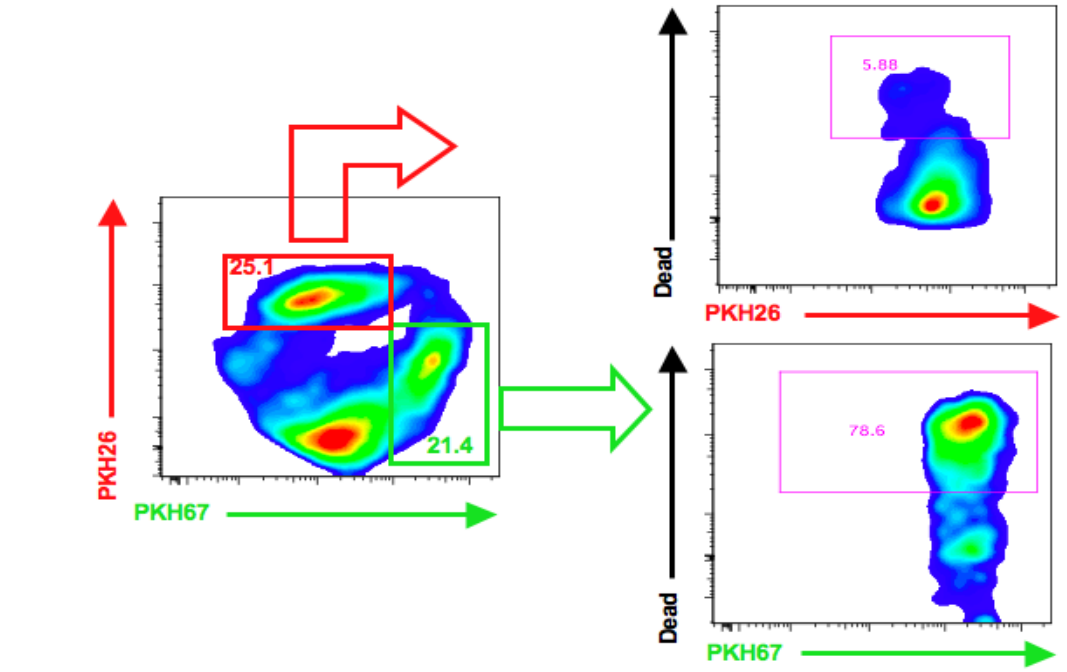
Figure 3.2.4: c-FLIP knockdown induces a caspase dependant cell death in BCCLs. A) BCCL cell death following FLIPi treatment, normalised to SCi cell death as measured by fixable live/dead assay. B) Breast cancer cell death following FLIPi treatment normalised to SCi treated control cell death, in the presence or absence of IETD, LEHD and AEVD-FMK caspase inhibitors, * $p<0.05$. Data shown is representative of 3 experiments performed in triplicate ±SEM.

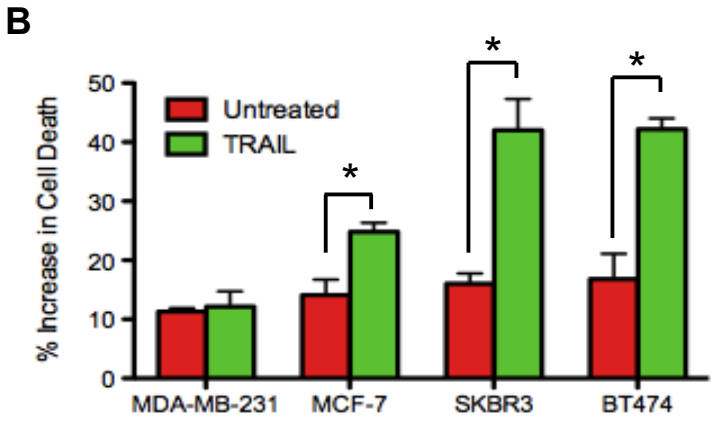
3.2.5 Suppression of c-FLIP (FLIPi) sensitized breast cancer cell lines to TRAIL induced apoptosis irrespective of hormone receptor status

Whilst most breast cancers are resistant to TRAIL induced apoptosis, it has recently been reported, and confirmed here, that mesenchymal breast cancer cell lines that lack hormone receptors (HER2 and ERα) partially respond to TRAIL treatment (Rahman et al. 2009a). This is a clinically important subgroup of breast cancer, yet it represents only 15-20% of the breast cancer patient population. In order to establish the extent to which c-FLIP might broaden the specificity of TRAIL-induced cytotoxicity, the relative sensitivity of different breast cancer subtypes to the combined effects of c-FLIP inhibition and TRAIL treatment was directly compared. To ensure treatment conditions between FLIPi and SCi cells were consistent the cell's membranes were stained with PKH67 and PKH26 respectively, mixed at an equal ratio and treated in the same well as outlined in the 'heterotypic culture' materials and methods section. The FLIPi and SCi cell populations were then separated and analysed for cell death as represented in Figure 3.2.5A (unstained control Appendix I).

When c-FLIP inhibition (FLIPi) was combined with TRAIL administration, a significant TRAIL-dependent kill over their corresponding SCi cell death was observed for all of the breast cancer cell lines tested (Figure 3.2.5B, *green bars*, $p<0.01$). This demonstrated a marked sensitization to TRAIL in resistant cell lines, but no more than an additive effect of FLIPi in the TRAIL-sensitive MDA-MB-231 cell line ($p=0.22$). Thus FLIPi/TRAIL treatment significantly reduced breast cancer cell viability irrespective of hormone receptor status. Despite this sensitization to TRAIL, between 8% (MDA-MB-231) and 33% (MCF-7) of the cell populations survived

the combined (TRAIL/FLIPi) treatment (Figure 3.2.5C), which suggested a differential response to this apoptotic insult by these heterogeneous cell populations.





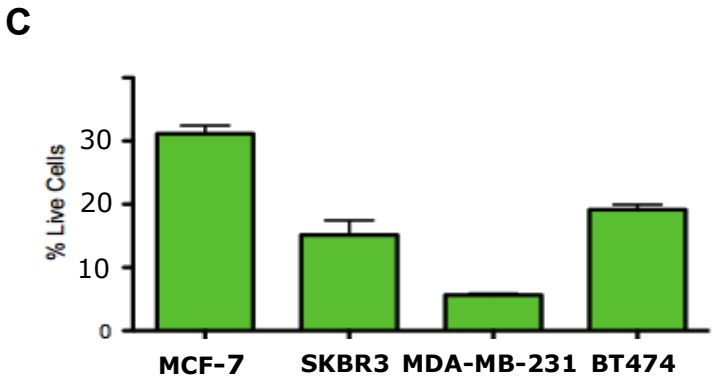


Figure 3.2.5: c-FLIP knockdown sensitises resistant breast cancer cell lines to TRAIL A) Schematic demonstrating the heterotypic culture approach. PKH26 stained SCi cells and PKH67 stained FLIPi cells were cultured in the same well and treated with or without 20ng/ml TRAIL for 18 hours before assessing cell death of each of these populations based on their plasma membrane stains by fixable live/dead assay. B) Percentage cell death of FLIPi cells untreated or treated with TRAIL, normalised to their respective SCi controls (i.e. red = percentage cell death increase as a result of FLIPi over SCi cell death and green = percentage increase in cell death as a result of FLIPi/TRAIL over SCi/TRAIL treatment) * $p<0.01$. C) Percentage of live breast cancer cells following FLIPi/TRAIL treatment. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.6 FLIPi sensitises breast cancer stem cells (bCSCs) to TRAIL

Breast tumours and breast cancer cell lines, contain a small sub-population (up to 2%) of tumour-initiating (cancer stem) cells (Dontu et al. 2003a). These cells have been shown to be resistant to existing chemotherapeutic agents (Liu and Wicha 2010). To establish whether the cells surviving the FLIPi/TRAIL treatment within each cell line (Figure 3.2.5C) included a resistant sub-population of breast cancer stem cells (bCSCs), the functional mammosphere formation assay was used as previously described (Charafe-Jauffret et al. 2009; Dontu et al. 2003a; Harrison et al. 2010). Each of the cell lines was subjected to c-FLIP siRNA prior to transfer of viable cells to non-adherent low serum conditions, whereupon cells were treated with TRAIL.

Each of the untreated cell lines formed mammospheres of distinct size and morphology with the ER+ve lines, BT474 and MCF-7, forming the largest, most uniform colonies (Figure 3.2.6A) and the ER-ve lines, SKBR3 and MDA-MB-231 forming loose, irregular colonies, as previously demonstrated (Harrison et al. 2010). Suppression of c-FLIP alone had no discernable effect on mammosphere integrity while TRAIL treatment alone partially impaired MCF-7 and MDA-MB-231 mammosphere morphology. Combined treatment however severely disrupted mammosphere formation in all four cell lines. This was confirmed by quantitation of mammosphere forming units (MFUs) in short-term culture and serial passage (Figure 3.2.6B), whereby all self-renewing MFUs were deleted from the cell populations.

The frequency of mammosphere forming cells in the untreated cell lines ranged from 0.4% to 1.4% of the total cell populations. SKBR3 and MCF-7 MFUs were partially sensitive to TRAIL induced

anoikis, as less than a quarter of the mammospheres formed in the presence of TRAIL alone during the first passage compared to untreated controls (Figure 3.2.6B, Passage 1). Similarly SKBR3, but not MCF-7, MFUs were significantly depleted with FLIPi treatment alone whilst MDA-MB-231 and BT474 MFUs were completely resistant to either FLIPi or TRAIL treament alone. In all cases however sensitivity to anoikis was dramatically enhanced with combined treatment. From starting populations of 12,000 cells, no mammospheres survived in MDA-MB-231 and BT474 cultures, while 2 and 1 loose-forming colonies, respectively, were evident in SKBR3 and MCF-7 cells.

Serial passaging of mammospheres in the absence of TRAIL and/or FLIPi revealed enrichment of MFUs in all cell cultures except those pre-treated with both TRAIL and FLIPi (Figure 3.2.6B, *Passage 2*). MFU enrichment is indicative of stem cell self-renewal due to symmetric cell division (Dontu et al. 2003a). The complete loss of mammospheres from FLIPi/TRAIL treated cultures in subsequent passages suggests that the few surviving cancer-initiating cells from 18 hours combined treatment were severely compromised and unable to undergo additional symmetric cell divisions.

This complete ablation of functional MFUs represents a preferential sensitisation of bCSCs to TRAIL compared to the rest of the tumour cell population.

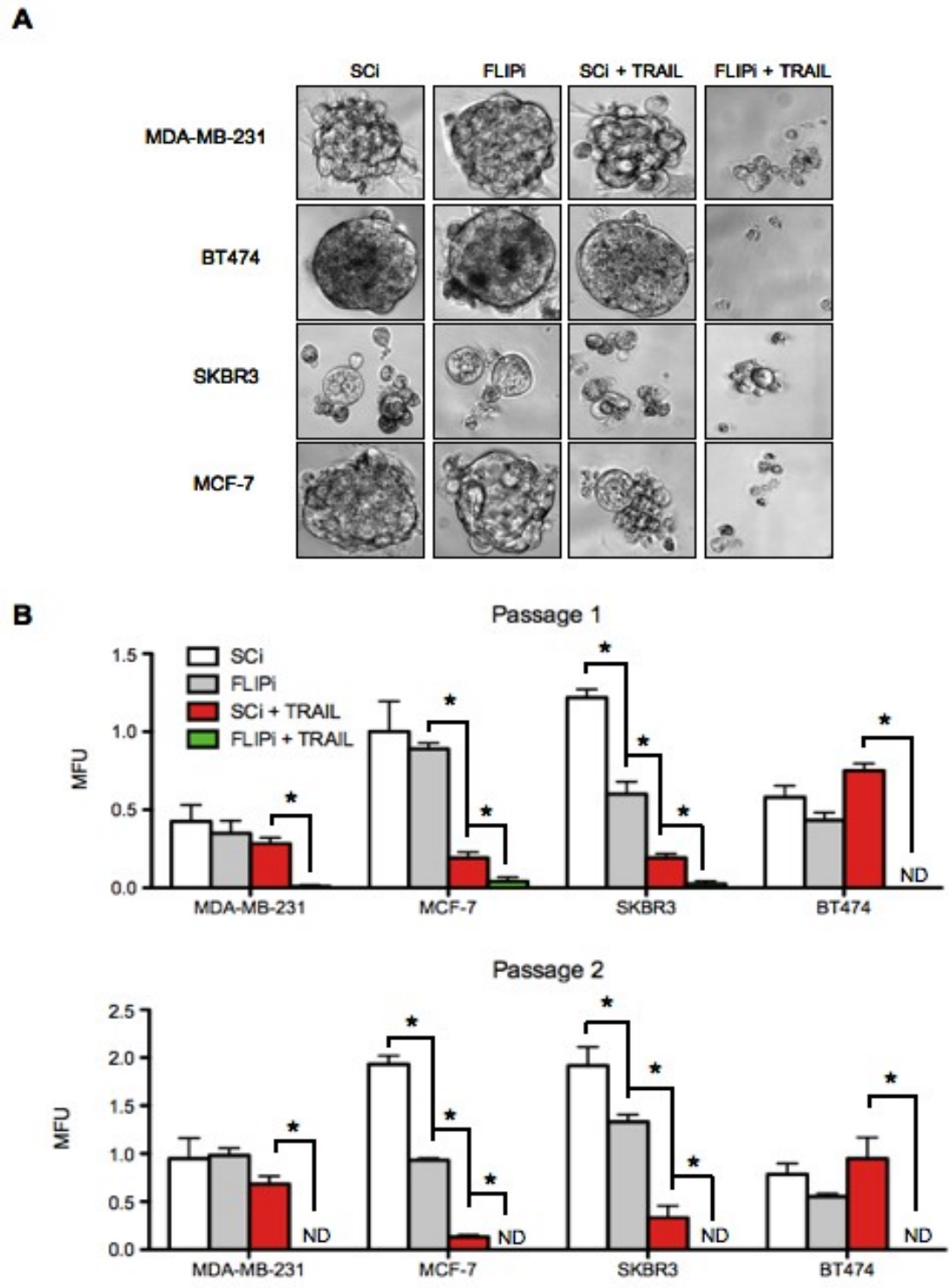


Figure 3.2.6: combined FLIPi/TRAIL treatment ablates the self-renewing cell population in BCCLs. A) Representative phase contrast images of SCi or FLIPi BCCL mammosphere morphology with or without 20ng/ml TRAIL treatment. B) BCCLs were treated with SCi or FLIPi and plated into mammosphere culture conditions in the presence or absence of 20ng/ml TRAIL. Percentage MFU from total cells plated was calculated following 7 days in culture (Passage 1). Spheres from Passage 1 were dissociated by trypsin and re-plated in mammosphere culture conditions for Passage 2 and %MFU re-calculated, * $p<0.01$. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.7 Confirmation of c-FLIP knockdown sensitisation

In order to eliminate the possibility of any off-target effects of the siRNA a second oligo was designed to confirm the observed results. BT474 cells were transfected with this alternate siRNA oligo designed to suppress both c-FLIP$_L$ and c-FLIP$_s$ transcripts (knockdown confirmed by qRT-PCR and western blot, data not shown) and the sensitisation of both bulk tumour cells and CSC's to TRAIL assessed under the same experimental protocol as Figure 3.2.6.

The alternate siRNA sequence sensitised both the bulk and cancer stem cells to TRAIL in an almost identical manner to previous experiments. A significant increase in cell death In the FLIPi/TRAIL treated cells was observed over its corresponding SCi control (Figure 3.2.7A, $p=0.0075$). Furthermore, the BT474 mammosphere forming capability was only marginally lower to that observed previously whilst the combined FLIPi/TRAIL treatment successfully abolished the anoikis resistant cancer stem cells with no mammospheres forming from the total 12,000 cells seeded (Figure 3.2.7B and C, $p<0.00001$).

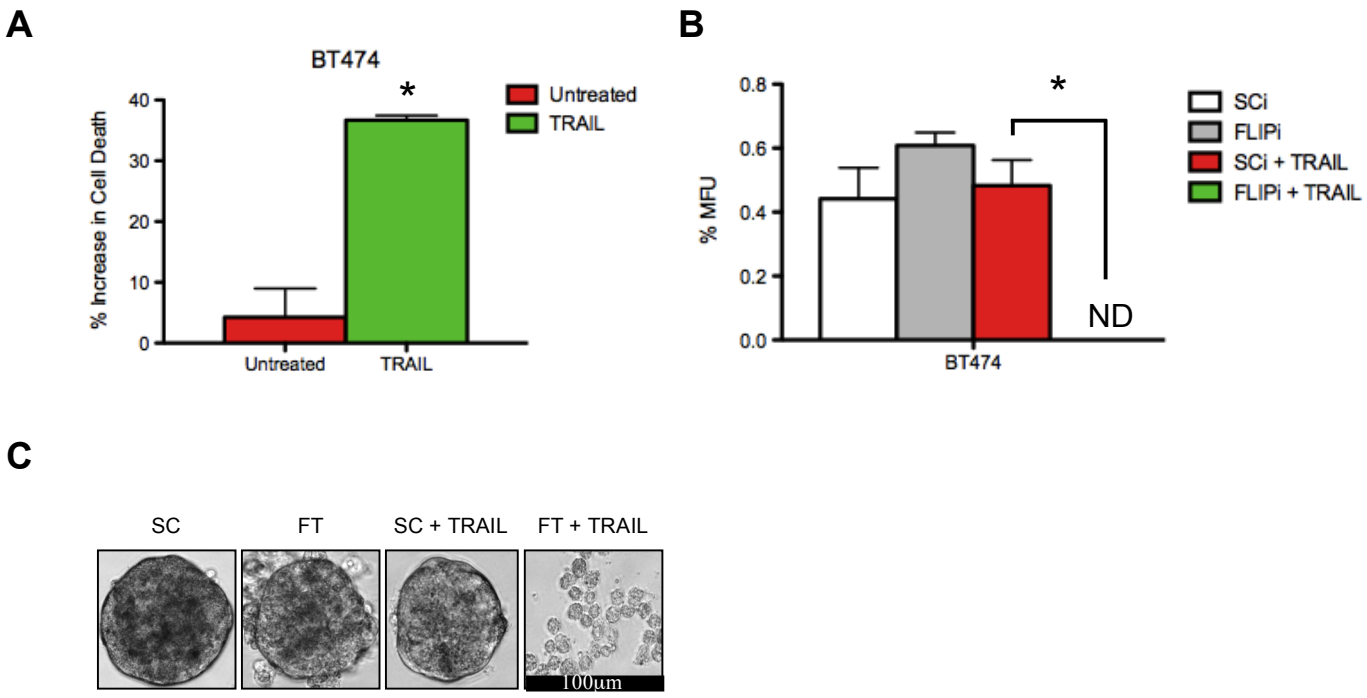


Figure 3.2.7: c-FLIP knockdown using a different siRNA oligo confirms previous sensitisation observed A) Increase in BT474 cell death following FLIPi (red) or FLIPi/TRAIL (green) treatment over SCi and SCi/TRAIL cell death respectively, * $p<0.01$. B) Percentage MFU following SCi or FLIPi treatment alone or in combination with 20ng/ml TRAIL for 18 hour, * $p<0.01$. C) Representative phase contrast images of mammospheres formed in B. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.8 c-FLIP knockdown reduces the ALDH1 expressing cancer cell population

Aldehyde dehydrogenase 1 (ALDH1) is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes and has been shown to be particularly active in the stem/progenitor cell population of both the normal human mammary gland and mammary carcinomas (Ginestier et al. 2007). Moreover, it has been detailed that overexpression of HER2 leads to an increase in stem/progenitor cell population as characterised by both the mammosphere and ALDH1 assays (Korkaya et al. 2008). Thus the enzymatic activity of ALDH1 presents itself as a suitable surrogate marker for the bCSCs, especially in tumour cells that overexpress HER2.

The HER2 overexpressing cell lines SKBR3 and BT474 were treated with FLIPi/TRAIL and knockdown was confirmed by qRT-PCR. The population of cells with high ALDH1 activity was assessed using the aldefluor assay as described in materials and methods. Briefly, this assay functions by determining the ability of cells to convert the ALDH1 substrate, BAAA, into the bright fluorescent product, BAA-, that is retained in cells expressing high levels of ALDH1 and is subsequently detected by flow cytometry.

As an ALDH1 enzyme inhibitor, DEAB treated cells were used as a negative control such that a gate can be set for less than 1.5% positive cells. Using this gate a population of ALDH1-high (+ve) was identifiable in both the SKBR3 and BT474 cell lines (Figure 3.2.8A), with both the SCi controls averaging approximately 20% ALDH1+ve cell populations (Figure 3.2.8B). This positive population was significantly reduced in the surviving cells following FLIPi/TRAIL treatment of the SKBR3 and BT474 cell lines (SKBR3, $p=0.027$; BT474, $p=0.037$). In these cells the ALDH1+ve population was

reduced to 2.1% and 4.7% in the SKBR3 and BT474 cell lines respectively, supporting the previous observation in the functional mammosphere assay.

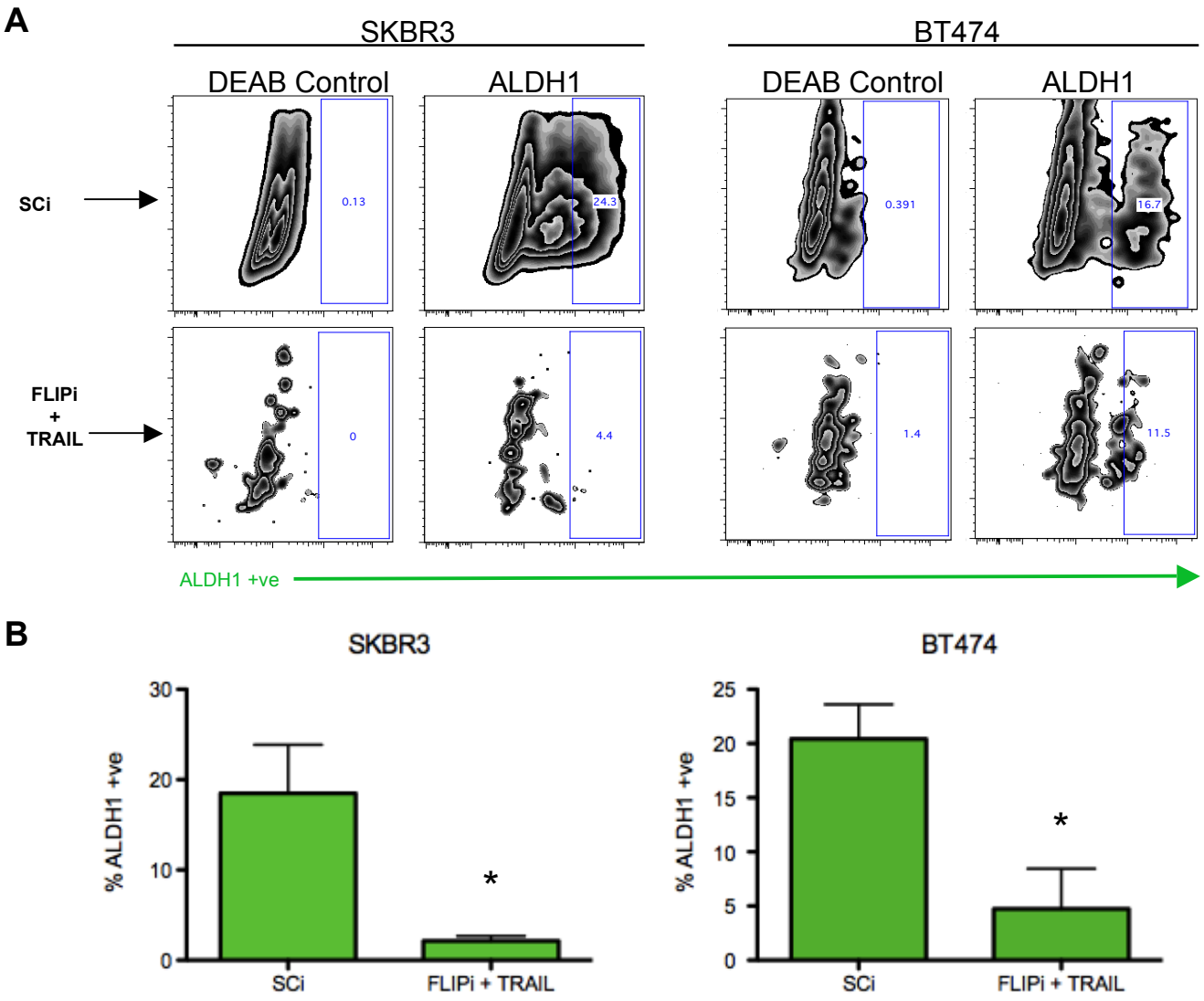


Figure 3.2.8: FLIPi/TRAIL treatment reduces the ALDH1 expressing population in BCCLs. A) Representative FACS plots of ALDH1 expression in the surviving cell populations of SCi or FLIPi/TRAIL treated BT474 and SKBR3 cell lines. B) Percentage of ALDH1+ve cells in BT474 and SKBR3 surviving cell populations after SCi or FLIPi/TRAIL treatment, * $p<0.05$. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.9 c-FLIP suppression sensitises bCSCs to TRAIL under adherent conditions

The mammosphere formation assay primarily tests the ability of cells to resist anoikis, which is an important characteristic of tumour-initiating (cancer stem) cells. As c-FLIP has previously been reported to be an inhibitor of anoikis in other tumour cell types (Mawji et al. 2007), it was necessary to test whether the MFU sensitization to TRAIL was dependent on the additional stresses imparted by the non-adherent conditions. Each of the cell lines was subjected to c-FLIP siRNA and then incubated with TRAIL for 18 hours in adherent culture, as previously performed in the viability assays (Figure 3.2.5B). Viable cells were subsequently washed and plated in non-adherent, mammosphere culture for 7 days, in the absence of TRAIL, and the number of mammospheres counted.

The self-renewing capacity of MFUs was once again abolished in MCF7, MDA-MB-231 and BT474 cell lines, although SKBR3 cells exhibited a residual MFU self-renewal capacity. Thus while some cell lines exhibited reduced sensitivity to combined treatment when maintained in adherent conditions, all continued to display a significant reduction in CSCs.

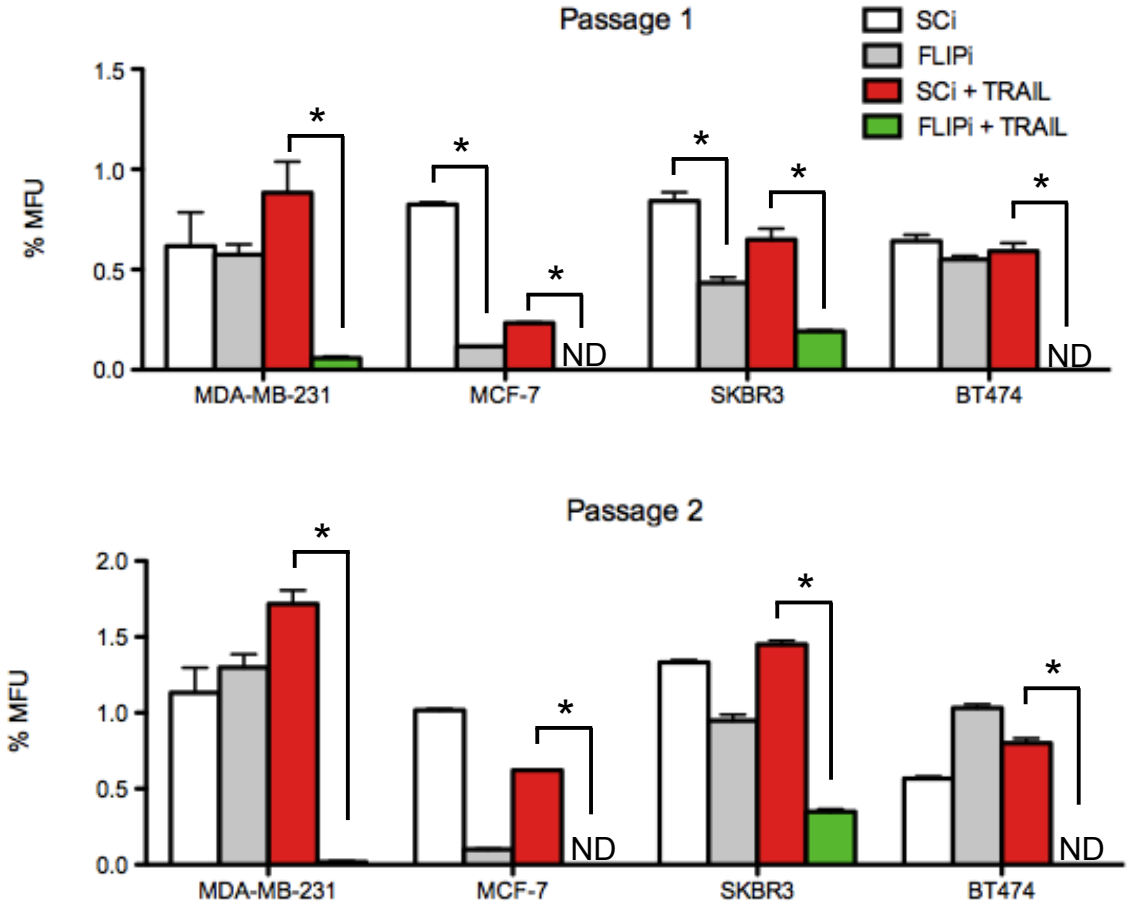


Figure 3.2.9: c-FLIP knockdown sensitises bCSCs to TRAIL under adherent conditions. BCCLs were treated with SCi or FLIPi alone or in combination with 20ng/ml of TRAIL for 18 hours. Surviving cells were then plated into mammosphere culture conditions (Passage 1) and % MFU calculated from the total number of cells plated. Spheres from Passage 1 were dissociated by trypsin and cells re-plated in mammosphere culture conditions for Passage 2 and %MFU re-calculated, * $p<0.03$. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.10 c-FLIP suppression senisitises bCSCs to sub-toxic TRAIL concentrations

Although the bulk tumour cell populations of the luminal-like cell lines are resistant to TRAIL it was noted that the mammosphere forming potential of SKBR3 and MCF-7 cell lines was slightly abrogated when treated with TRAIL alone. To determine the contribution of TRAIL alone in the combined FLIPi/TRAIL treatment of bCSCs, a lower non-toxic dose of TRAIL was used in the mammosphere assay.

Suppression of c-FLIP also sensitized cancer stem cells to sub-toxic levels of TRAIL (Figure 3.2.9). TRAIL concentrations were reduced from 20ng/ml to 1ng/ml, levels that failed to activate a cell death response in the TRAIL-sensitive MDA-MB-231 cell line (Figure 3.2.1C), and mammosphere cultures were performed as described above. TRAIL addition alone had reduced effects on mammosphere integrity, yet combined treatment abrogated MFUs in BT474, SKBR3 and MDA-MB-231 cell cultures, as previously observed with higher concentrations of TRAIL. The poorest responding cells to combined treatment, MCF-7 (Figure 3.2.6B), developed self-renewing MFUs at a very low frequency (1/12,000 cells seeded) in reduced TRAIL conditions (Figure 3.2.9).

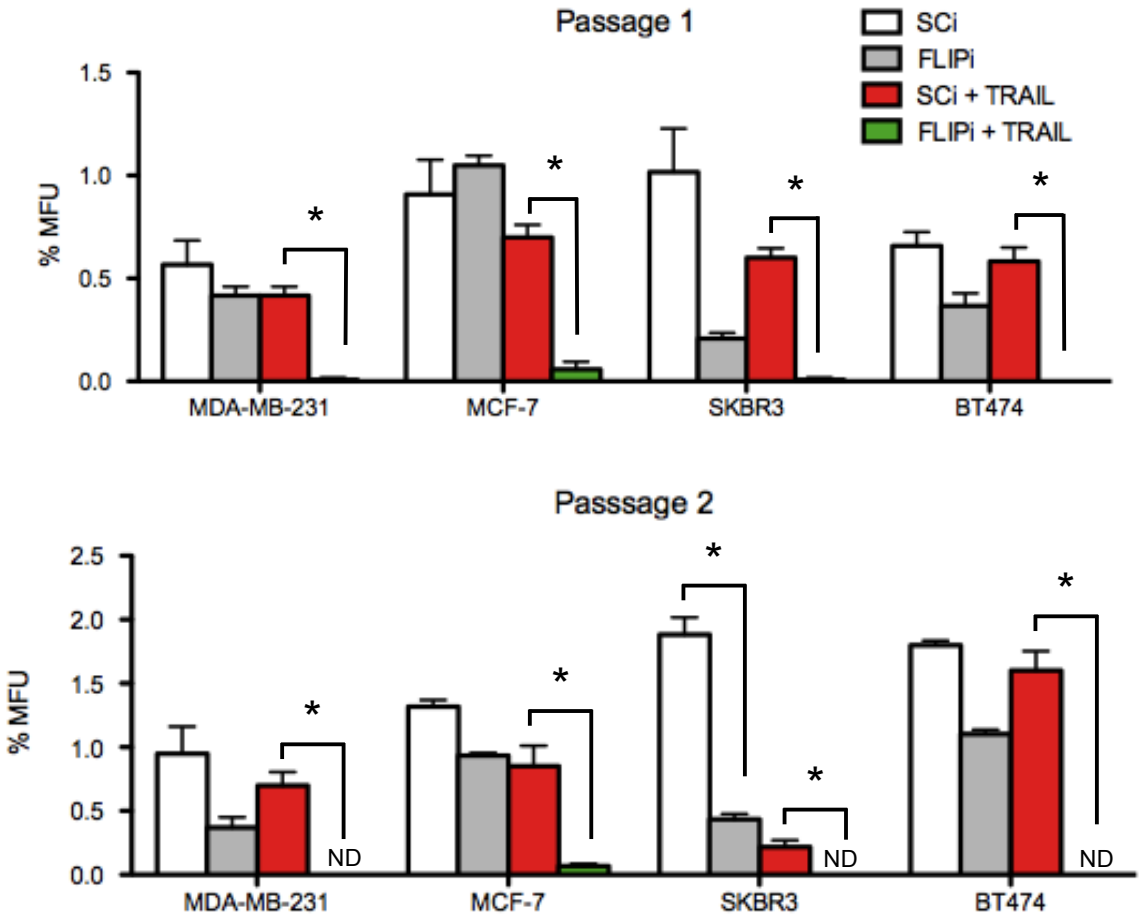


Figure 3.2.10: c-FLIP knockdown sensitises bCSCs to sub-toxic TRAIL concentrations. BCCLs were treated with SCi or FLIPi alone or in combination with 1ng/ml of TRAIL for 18 hours. Surviving cells were then plated into mammosphere culture conditions (Passage 1) and % MFU calculated from the total number of cells plated. Spheres from Passage 1 were dissociated by trypsin and cells re-plated in mammosphere culture conditions for Passage 2 and %MFU re-calculated, * $p<0.01$. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.11 Suppression of the c-FLIP$_L$ isoform is responsible for bCSC sensitisation to TRAIL

In order to address which c-FLIP isoform was responsible for the ablation of the self-renewing activity of the cancer stem cell population, siRNA sequences specific for cFLIP-short (c-FLIP$_S$) and c-FLIP-long (c-FLIP$_L$) transcripts were used prior to TRAIL treatment and mammosphere assay.

In BT474 cells, silencing of c-FLIP$_L$, but not c-FLIP$_S$, followed by TRAIL treatment mimicked the effects previously observed when suppressing the entire c-FLIP transcript with no mammospheres forming from the total 12,000 cells seeded (Figure 3.2.11B). Interestingly, the silencing of c-FLIP$_S$ actually increased the mammosphere forming units, opening the possibility that c-FLIP$_S$ may play some role in the maintenance of cancer stem cells in the BT474 cells (Figure 3.2.11A, *Passage 1*). In the MDA-MB-231 cells the combined knockdown of c-FLIP$_L$ and TRAIL treatment significantly reduced the mammosphere forming capability of the cells compared to SCi control or either treatment alone. In addition, c-FLIP$_S$ silencing combined with TRAIL treatment also resulted in a significant reduction of mammosphere forming units (Figure 3.2.11A, *Passage 1*). Importantly, however, mammospheres forming after c-FLIP$_L$ were not capable of self-renewal with the same proportion of mammosphere forming units in passage 2 being counted (Figure 3.2.11A, *Passage 2*). Contrastingly, this was not the case for c-FLIP$_S$/TRAIL combined treatment. In this instance, the number of mammosphere forming units increased from 0.3% to 2% showing a significant capability to self-renew. Taken together these data indicate it is the c-FLIP$_L$ isoform that is responsible for determining bCSC sensitivity to TRAIL.

To investigate which isoform of c-FLIP was responsible for sensitising bulk tumour cells, the BT474 cell line was treated with isoform specific siRNA and treated with TRAIL before cell death was assessed. Whilst suppressing the c-FLIP$_S$, but not c-FLIP$_L$, isoform of c-FLIP gave a significant increase in cell death in combination with TRAIL (Figure 3.2.11C: Short, p=0.005; Long, p=0.133), this sensitization was not of the same scale as previously observed when suppressing the entire c-FLIP transcript (Figure 3.2.5B). This may suggest that both c-FLIP$_S$ and c-FLIP$_L$ both contribute to bulk tumour sensitization to TRAIL.

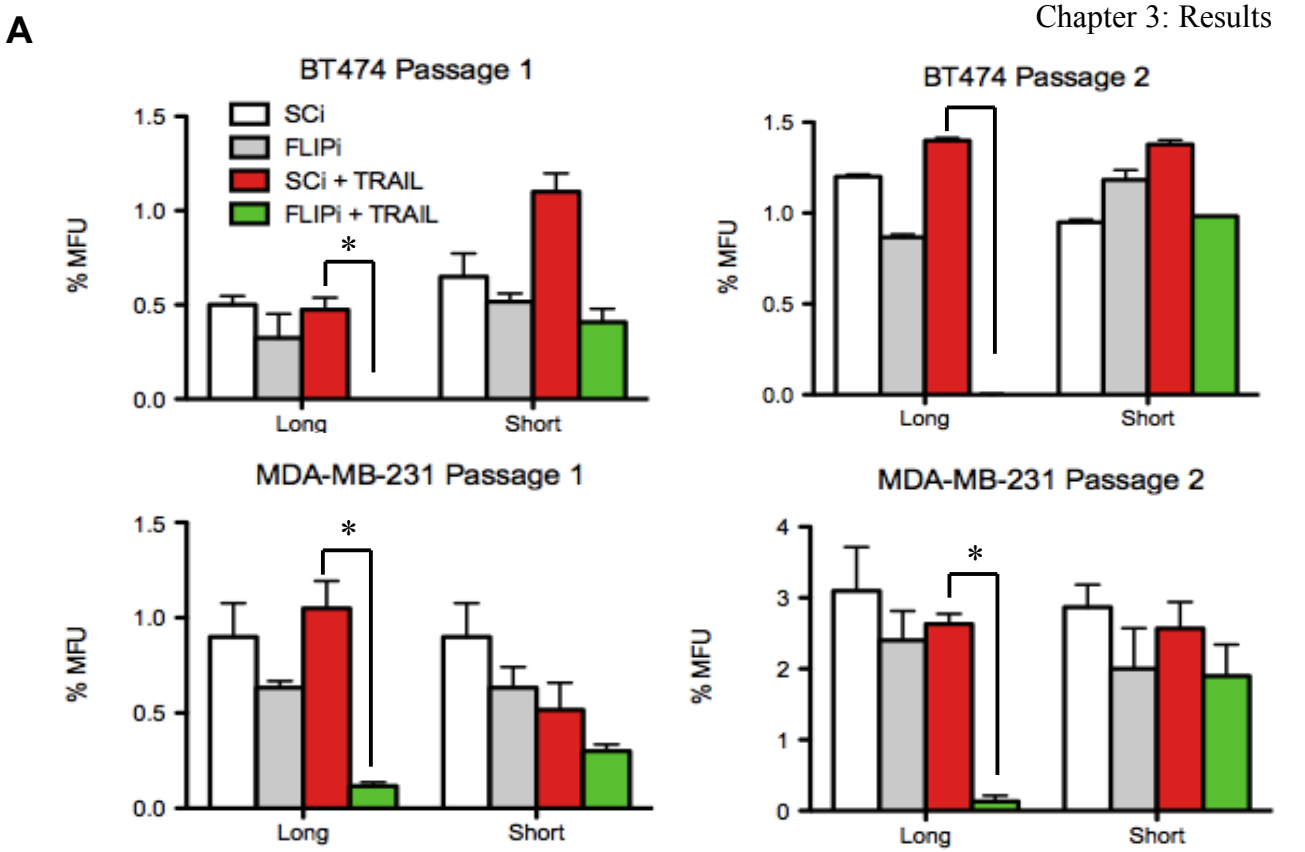


Figure 3.2.11: c-FLIP$_L$ but not c-FLIP$_S$ knockdown sensitises bCSCs to TRAIL.

A) BCCLs were treated with SCi or FLIPi, specific for the long and short isoforms, alone or in combination with 20ng/ml of TRAIL for 18 hours. Live cells were then plated into mammosphere culture conditions (Passage 1) and % MFU calculated from the total number of cells plated. Spheres from Passage 1 were dissociated by trypsin and cells re-plated in mammosphere culture conditions for Passage 2 and %MFU re-calculated. B) Representative phase contrast images of BT474 mammospheres formed in A. C) BT474 cells were treated with c-FLIP-long and -short siRNA and treated with 20ng/ml TRAIL for 18 hours. Increased cell death of FLIPi and FLIPi/TRAILtreated BT474 cells, over SCi and SCi/TRAIL treated BT474 celss respectively, was then assessed by live/dead FACS. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.12 Tumour initiation and metastasis formation are severely compromised following combined FLIPi/TRAIL treatment

BT474 cells formed the most well defined mammospheres *in vitro* and thus were used to further characterise previous observations *in vivo*. In order to confirm that the loss of MFUs was consistent with a reduction in tumour initiating capacity, adherent cultures of BT474 cells were treated with c-FLIP siRNA and 10^6 viable cells orthotopically transplanted into the 4^{th} inguinal mammary glands of severe combined immune-deficient (SCID) mice in the presence or absence of TRAIL (Figure 3.2.12A + B). Injected cells were also cultured under mammosphere conditions to assess the mammosphere forming capability of the transplanted cells *in vitro*. The occurrence of palpable tumours was then monitored for up to 16 weeks after transplantation.

Tumours arose at the site of transplantation within 8 weeks (Figure 3.2.12C) of surgery in all mice transplanted with either untreated BT474 or FLIPi-treated BT474 cells, while 3 out of 5 mice with TRAIL-treated BT474 transplants acquired tumours in the same time-frame. However, 4 out of 5 transplants co-treated with FLIPi and TRAIL failed to acquire tumours within 16 weeks of surgery (Figure 3.2.12A + C). Tumour growth and histology (Figure 3.2.12B) was unaffected in all conditions. This residual tumour initiating capacity following combined treatment occurred despite complete loss of self-renewing mammosphere forming potential *in vitro* (Figure 3.2.12D).

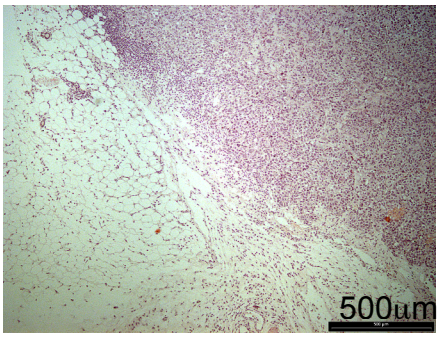
Cancer stem cells are thought to be responsible for the seeding of new tumour growth at distal sights, which is central to the progression of metastatic disease – the major cause of mortality in breast cancer patients. An established *in vivo* model of breast cancer metastasis, intravenous transplantation of MDA-MB-231 cells, was used to determine the effect of FLIPi/TRAIL on disease progression.

Adherent cultures of MDA-MB-231 cells were treated with c-FLIP siRNA (or control) and 10^6 viable cells transplanted intravenously into SCID mice in the presence or absence of TRAIL (Figure 3.2.12E). After 6 weeks, the number of lung metastases was determined by dissection and serial section of lung tissues from recipient mice (Figure 3.2.12F). An average of 23 secondary tumours per mouse were found in animals transplanted with control cells, compared to an average of 0.4 tumours (a total of 2 micrometastases from 5 mice) in transplants subjected to FLIPi and TRAIL. This represented a 98% reduction in tumour burden and a significant sensitization to TRAIL-mediated suppression of metastatic disease (Figure 3.2.12E, p=0.0154). Significant reductions, up to 95%, were also observed in animals transplanted with FLIPi (FLIPi, p=0.028) or TRAIL (SCi + TRAIL, p=0.028) treated cells, however neither single treatment was as effective as FLIPi/TRAIL treatment in the prevention of metastasis.

A

Treatment	Tumours/Cohort
SCi	4/4
FLIPi	3/5
SCi + TRAIL	5/5
FLIPi + TRAIL	1/5

B



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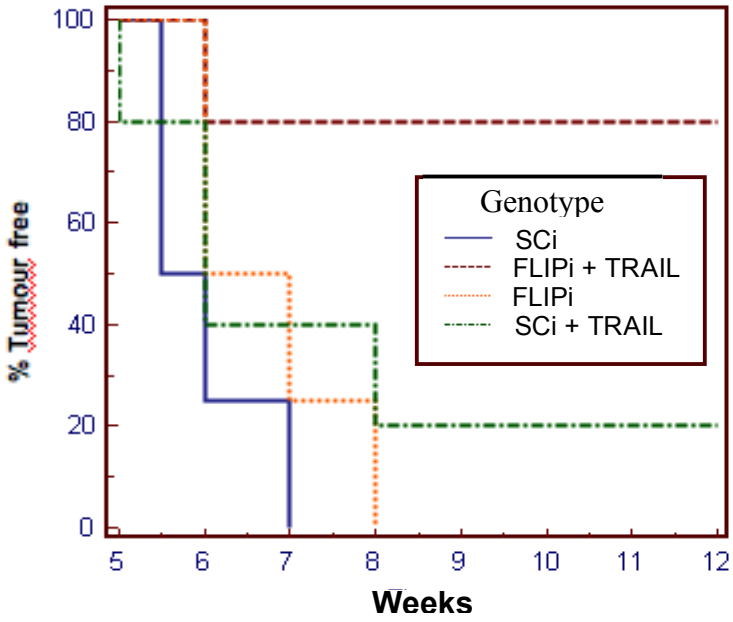

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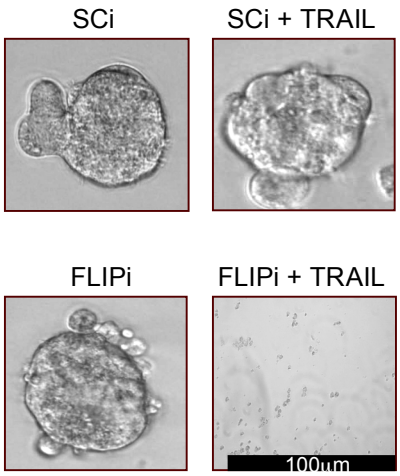

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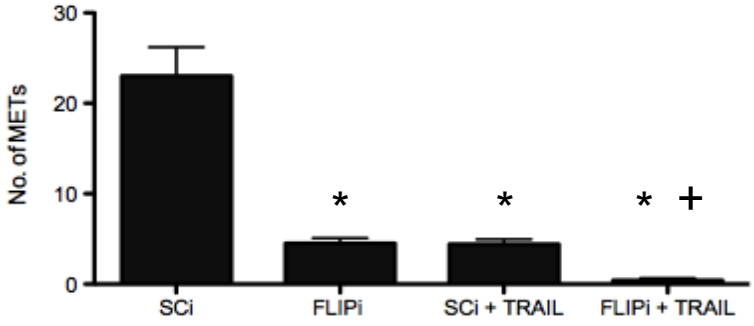

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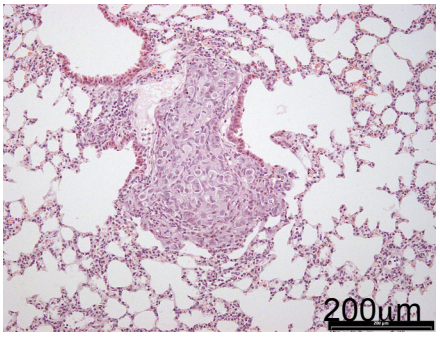

Figure 3.2.12: FLIPi/TRAIL treatment reduces tumour initiation and metastasis in animal models. A) 1x10^7 SCi or FLIPi BT474 cells were xenografted, with or without 20ng/ml TRAIL, into the 4th inguinal mammary gland of athymic nu/nu mice. Tumour penetrance was then determined by the number of tumours formed per cohort. B) Representative image of H+E stained tumour and adjacent mammary gland excised from a SCi tumour-bearing animal. C) Kaplan-Meier survival curve of xenografted tumours in A. D) Representative phase contrast images of mammospheres formed from excess cells not transplanted in A. E) 1x10^6 MDA-MB-231 cells were treated, as in A, and injected into SCID mice tail veins. Lungs from these animals were excised 6 weeks post-injection and metastases observed in H+E 50µm serial sections were counted, * $p<0.03$ compared to SCi, $^+$ $p<0.05$ compared to SCi + TRAIL. F) Representative H+E stained metastasis and surrounding lung tissue excised from SCi transplanted MDA-MB-231 cells. Data shown is representative of 3 experiments performed in triplicate ±SEM

3.2.13 Re-acquired stem cells remain sensitive to repeat combined FLIPi/TRAIL treatment.

Despite no MFUs forming from the residual transplanted cells when cultured *in vitro* (Figure 3.2.12D), a tumour-initiating capacity was still observed in 1 out of 5 FLIPi/TRAIL treated BT474 *in vivo* transplants (Figure 3.2.12A). In order to determine whether this tumour-initiating potential was re-acquired from the surviving (post-treatment) cell population, FLIPi/TRAIL treated cultures - with no residual mammosphere forming ability (Figure 3.2.13, *Treatment 1*) - were maintained in adherent culture for four weeks then transferred to mammosphere culture or re-treated with TRAIL/FLIPi (Figure 3.2.13A, *Treatment 2*).

The surviving population slowly re-populated under the adherent culture conditions (Figure 3.2.13B) and re-acquired an equivalent proportion of mammospheres to the original untreated population (compare SCi samples, Figure 3.2.13A, *Treatments 1 and 2*). However, this subset of self-renewing cells was still exquisitely sensitive to FLIPi/TRAIL, as combined treatment of the re-established adherent cultures once again eradicated MFUs from the cell population (Figure 3.2.13A, *Treatment 2*). Identical results were generated using the MDA-MB-231 cells, however in this instance the re-acquisition of MFUs was quicker taking only 2 weeks for the population to return.

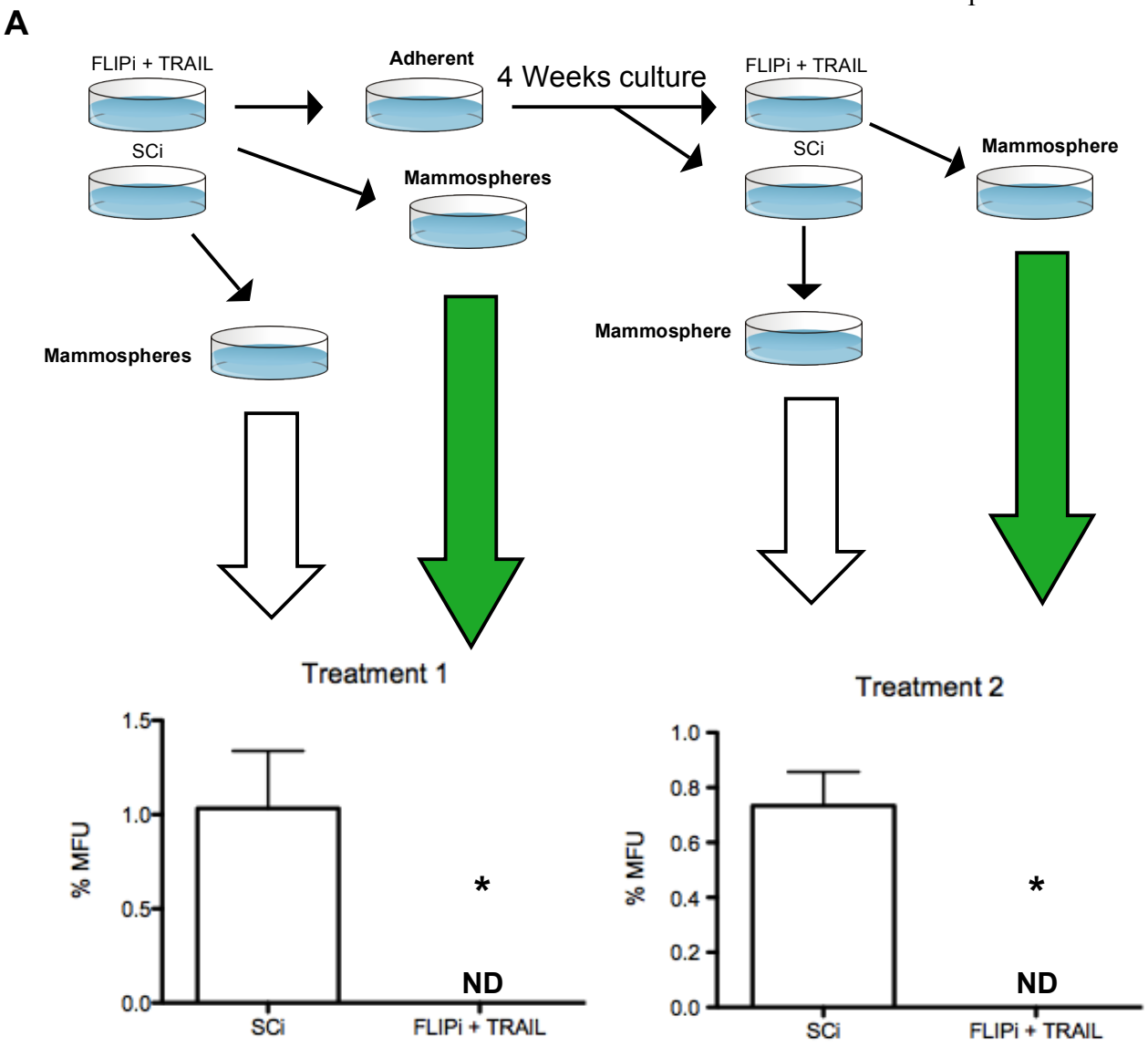

Figure 3.1.13: Re-acquired bCSCs remain sensitive to repeated FLIPi/TRAIL treatment. A) BT474 cells were treated with SCi or FLIPi/TRAIL and % MFU calculated. Surviving cells were then also plated into normal adherent culture conditions for 4 weeks prior to a second SCi or FLIPi/TRAIL treatment. Treated cells were then once again plated in mammosphere forming conditions and %MFU calculated, * $p<0.01$. B) FLIPi/TRAIL treated BT474 adherent cell growth during the 4 week incubation period in A. Data shown is representative of 3 experiments performed in triplicate ±SEM.

3.2.14 Maintaining c-FLIP suppression prevents the re-acquisition of MFUs in breast cancer cell lines

Repetitive treatment of FLIPi/TRAIL upon tumour relapse may prove useful therapeutic approach to treating breast cancer, however, a far more robust therapeutic strategy has and always will be the prevention of tumour relapse in the first place. Hitherto we have demonstrated an important role for the anti-apoptotic factor c-FLIP in regulating death receptor-induced apoptosis but it is also clear that c-FLIP has alternative functions in regulating other signalling pathways within the cell beyond its anti-apoptotic property. As such, it has been shown to regulate NF-κB (Fang et al. 2004; Kataoka et al. 2000; Kataoka and Tschopp 2004), Wnt (Katayama et al. 2010; Naito et al. 2004) and Akt (Quintavalle et al. 2010) signalling, each of which have themselves been demonstrated to regulate cancer stem cell maintenance (Hinohara et al. 2012; Korkaya et al. 2009; Lu and Carson 2011; Shostak and Chariot 2011; Takahashi-Yanaga and Kahn 2010).

To investigate the role of c-FLIP in cancer stem cell maintenance a lentiviral vector containing c-FLIP shRNA was used to stably suppress c-FLIP long-term in breast cancer cell lines and the mammosphere re-acquisition potential of cells surviving FLIPi/TRAIL treatment was assessed in the same manner as, and in tandem with, the previous siRNA methodology (Figure 3.2.13A).

Cells infected with c-FLIP shRNA (shFLIPi) successfully suppressed both c-FLIP mRNA and protein levels in the MCF-7 cell line (Figure 3.2.14A). The shFLIPi cells maintained a similar number of MFUs to the FLIPi treated cells and the mammospheres formed had the same morphology and also self-renewed in a similar proportion (Figure

3.2.14B, *white bars*). Furthermore, these cells responded to TRAIL treatment in a manner consistent with cells receiving FLIPi/TRAIL treatment, with no MFUs with self-renewal capacity forming (Figure 3.2.14B, *green bars*). Conversely, when the surviving population of both FLIPi/TRAIL and shFLIPi/TRAIL treated MCF-7 cells was re-plated, as in Figure 3.2.13A, only the FLIPi/TRAIL treated cells were capable of mammosphere re-acquisition and adherent cell growth (Figure 3.2.14C). After 2 weeks of culture the shFLIPi/TRAIL treated cells did not proliferate in adherence and subsequent mammosphere assays demonstrated no MFUs were re-acquired. These results were then replicated using a separate c-FLIP shRNA sequence at a different viral titre in the MCF-7 cells (data not shown). These data suggest that c-FLIP may regulate the re-acquisition of bCSCs following their ablation from the tumour bulk and thus may play some part in tumour cell plasticity.

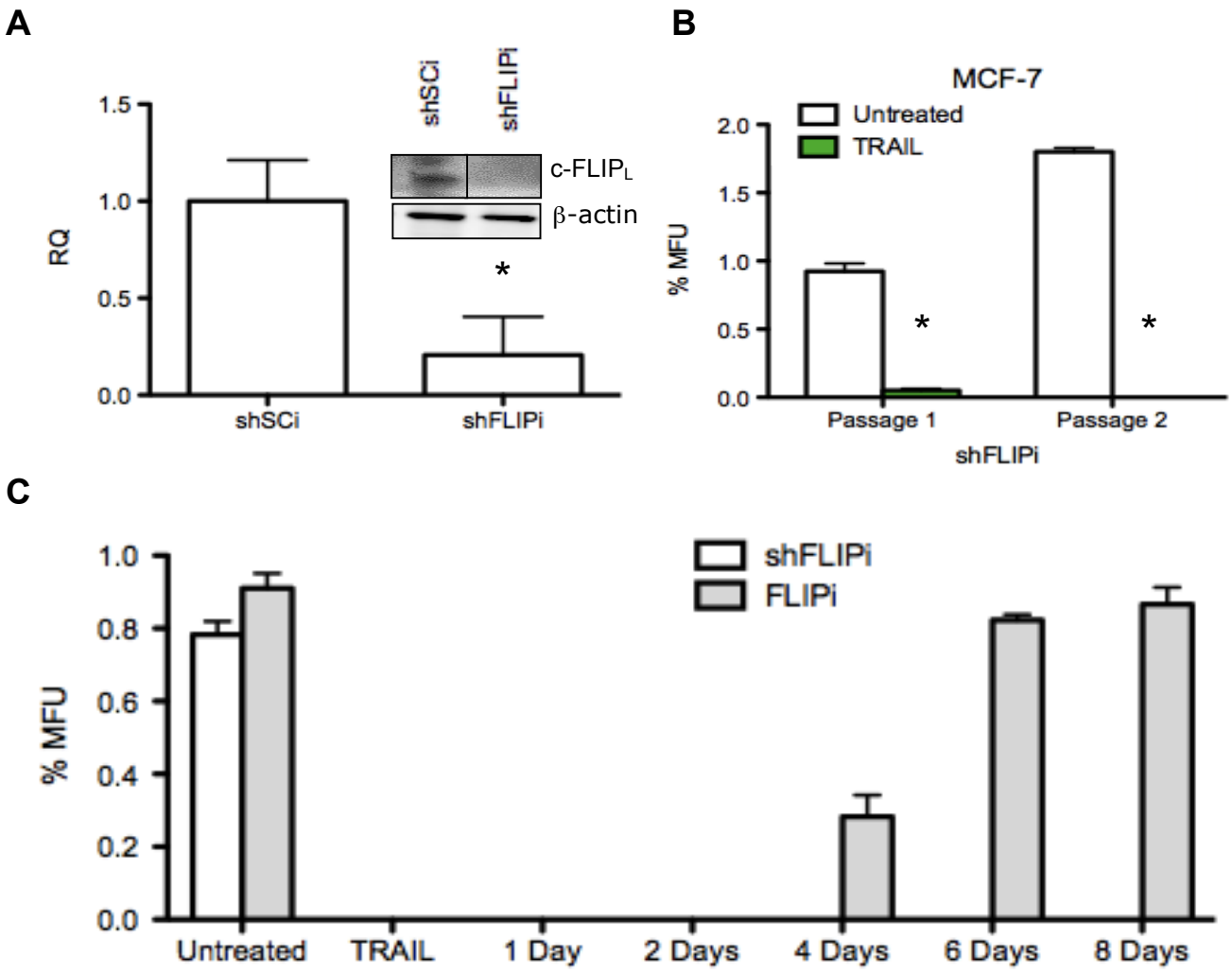

Figure 3.2.14: Maintaining suppression of c-FLIP prevents re-acquisition of bCSCs. A) c-FLIP mRNA expression and protein levels in shFLIPi MCF-7 cells. B) Percentage MFU in the live cell population of untreated and 20ng/ml TRAIL treated (18 hours) shFLIPi cells, * $p<0.01$. C) Surviving FLIPi/TRAIL and shFLIPi/TRAIL cell populations were cultured in adherent conditions and checked for re-acquistion of MFUs at regular intervals. Data shown is representative of 3 experiments performed in triplicate ±SEM

3.3 Discussion

Tumour heterogeneity is a major obstacle to therapy. Recent insights into the hierarchical organisation of tumour cell populations highlight the potential importance of targeting the minority tumour-initiating (cancer stem) cell population associated with cancers in order to radically improve patient outcome. However, cancer stem cells have been shown to be inherently resistant to chemotherapeutic challenge. TRAIL receptor agonists have shown much promise as a cancer therapeutic, exerting a cytotoxic effect on cancer cells whilst sparing normal cells (Ashkenazi et al. 1999; Kruyt 2008). Hitherto it has been demonstrated that the majority of breast cancer subtypes display resistance to TRAIL agonists and cell death is only achieved when used in combination other agents (Dyer et al. 2007; Rahman et al. 2009a). Previous studies have indicated that c-FLIP expression levels correlate with resistance/sensitivity to TRAIL-induced cell death (Frew et al. 2008; Jonsson et al. 2003; Katayama et al. 2010; Palacios et al. 2006; Shankar et al. 2009). The main role for c-FLIP is as a direct repressor of caspase-8 activation, however it has also been shown to regulate cell fate via alternate signalling pathways such as Akt, NFκB and Wnt (Kataoka et al. 2000; Naito et al. 2004; Quintavalle et al. 2010). Despite all this evidence, no studies have previously been performed to fully evaluate the efficacy of suppressing c-FLIP protein levels as a sensitization method for TRAIL on a broad range of both inter- and intra-tumorally heterogeneic breast cancer subtypes and on metastatic disease. The aim of this chapter was to address this by assessing FLIPi/TRAIL treatment both *in vitro* and *in vivo* with particular focus on its efficiency to target both tumour heterogeneity and metastasis.

3.3.1 c-FLIP knockdown selectively sensitises bCSCs to TRAIL mediated cell death without effecting normal epithelium

Before investigation of c-FLIP suppression as a broad sensitising agent to TRAIL in breast cancer, it was important to first assess the effect of c-FLIP suppression both individually and in combination with TRAIL on normal epithelium. The apoptosis observed following c-FLIP inhibition is, like TRAIL, a phenomenon that is cancer-specific. At present this observation has not been extended beyond non-tumourgenic cell lines, it will be imperative to assess the effects of FLIPi on normal cells both *in vivo* and in normal primary human cells before identifying FLIPi as a viable therapeutic strategy. Analysis of non-transformed mammary tissues from c-FLIP-deficient mice indicated that the absence of c-FLIP was not detrimental to normal tissue and did not sensitise normal tissue cells to TRAIL induced apoptosis (Figure 3.2.3). Currently there is no available means (in Cardiff) for testing normal human epithelial cell susceptibility to FLIPi/TRAIL, thus it has not been established whether normal stem cells of the breast are affected by either intervention. However, the non-tumourgenic transformed human epithelial cell line MCF10A also showed this same resistance to FLIPi/TRAIL treatment despite being a TRAIL-sensitive cell line. Interestingly, neural progenitor cells are resistant to TRAIL in a c-FLIP independent manner (Peng et al. 2005) and future investigation whether murine mammary stem cells are similarly refractory is of great interest.

Significantly, mouse breast cancer cell lines did show sensitivity to TRAIL when treated with c-FLIP siRNA (FLIPi) and TRAIL as demonstrated by the ErbB2 positive N202.1A cell line (Figure 3.2.3). This sensitisation effect was then subsequently demonstrated in a panel of human bCCLs representing different subtypes of disease

(Figure 3.2.5). Interestingly, knockdown of c-FLIP alone resulted in a 10-15% increase in apoptosis that was shown to be a caspase-dependant process (Figure 3.2.4). This result was consistent with a previous study that has shown FLIPi as an anti-tumour monotherapy both *in vitro* and *in vivo* (Day et al. 2008; Day et al. 2009). These results both confirm and broaden the scope for c-FLIP as a therapeutic target in breast cancer.

Recent studies demonstrating the importance of CSCs in cancer recurrence and metastatic progression identify them as a key target cell population in new therapy design. Here it is established both *in vitro* and *in vivo* that FLIPi selectively sensitizes this population to TRAIL-mediated killing independent of hormone status (Figure 3.2.6). This was demonstrated when combined FLIPi/TRAIL treatment resulted in the complete ablation of self-renewing MFUs and reduction in ALDH1 expressing population, both of which are assays frequently used for *in vitro* identification of bCSCs. More specifically, the suppression of c-$FLIP_L$ was responsible for the sensitisation of both the bulk tumour cells and the bCSCs (Figure 3.2.11). This supports other studies that have also revealed that c-$FLIP_L$ isoform plays an important role in sensitivity to TRAIL (Rosato et al. 2007; Yerbes et al. 2012). Confirmation of this bCSC ablation was demonstrated by transplanting *ex vivo* FLIPi/TRAIL treated cells into mice. The marked reduction in both tumour initiation and metastasis that followed showed the real therapeutic potential FLIPi/TRAIL treatment (Figure 3.2.12). Importantly, these results were acquired by a single treatment of TRAIL co-injected with the FLIPi cells. TRAIL sensitization has not previously been described in solid tumour stem cells. Thus, these results are the first demonstration of TRAIL-mediated loss of functional stem cell activity in a solid tumour cell type and the first indication

that CSC activity is directly influenced by c-FLIP. It did however raise further questions as to the therapeutic efficiency of the combined treatment, as one out of five xenografts still managed to form a primary tumour and two lung metastases were observed in the FLIPi/TRAIL tail vein injection experiments. The residual tumourgenicity observed *in vivo* was recapitulated *in vitro* by continued culture of the FLIPi/TRAIL surviving cell population in adherent culture condition. Following weeks of culture under these conditions, the MFU population returned (Figure 3.2.13). Crucially, however, Figure 3.2.13 shows that the newly acquired MFU activity remained responsive to re-administration of FLIPi/TRAIL. A similar sensitivity to repeat treatments has previously been observed for the Akt inhibitor perifosine, in a xenograft model of Sum159 cells (Korkaya et al. 2009). These observations have important implications for the future prevention of disease relapse in the clinical setting as they demonstrate that the tumourgenic cell population may be targeted without selecting for resistant cells. Given the inferences that c-FLIP may also play a role in signalling pathways regulating CSC population maintenance, it was satisfying to discover that the long-term suppression of c-FLIP prevented this re-acquisition of the bCSC population. This may have cost implications by indicating that repetitive combined FLIPi/TRAIL treatment may not be necessary but instead FLIPi must be maintained following a complete FLIPi/TRAIL regime.

3.3.2 Therapeutic implications of combined FLIPi/TRAIL treatment

It has been suggested that tumour cells in their natural context do not necessarily exhibit the sensitivity to TRAIL monotherapy as

observed *in vitro*, implying that a combined therapy would be required to re-sensitize to TRAIL (Dyer et al. 2007). RNAi was used to demonstrate the proof of principle that suppression of c-FLIP expression is sufficient to sensitize breast cancer cells to TRAIL. In light of this, a key future objective is to establish whether long-term suppression of c-FLIP *in vivo*, following the cessation of TRAIL treatment, might help prevent the recurrence of tumours as demonstrated *in vitro* (Figure 3.2.14). Despite limitations in drug design due to structural homology between c-FLIP and caspases, agents with broad specificity for c-FLIP have been described, each with anti-tumour properties (Bijangi-Vishehsaraei et al. 2010; Frew et al. 2008; Hyer et al. 2005; Lee et al. 2009; Palacios et al. 2006; Tazzari et al. 2008). It remains to be determined if these agents exhibit selective targeting of cancer stem cells and whether this too can be recapitulated *in vivo* in the absence of off-target effects.

The breadth of the breast tumour cell types affected here raises the question of the potential ubiquity of FLIPi/TRAIL treatment in targeting other cancer types *in vivo*. Of the few studies that have addressed the sensitivity of cancer stem cells to TRAIL (Kruyt 2008), the majority, including medulloblastoma (Yu et al. 2010a), glioblastoma (Capper et al. 2009) and lymphoma (Zobalova et al. 2008) -derived stem cells, are resistant, with the exception of colorectal cancer cell lines in which a FACS sorted side-population was shown to be TRAIL responsive (Sussman et al. 2007). Sensitization of cancer stem cells to TRAIL has only previously been demonstrated in haematological cancers, including AML (Tazzari et al. 2008) and T cell lymphoma cells (Zobalova et al. 2008), both of which have implicated, but not functionally proven, a role for c-FLIP in the process. Other mechanisms for targeting breast cancer stem cells have been

described. Notably, a recent study demonstrated reduced stem cell activity in response to Notch1 or Notch4 suppression using the same breast cancer cell lines described here (Harrison et al. 2010), which supports the use of gamma-secretase inhibitors in clinical trials (Liu and Wicha 2010). The Akt/Wnt pathway inhibitor, perifosine, reduces breast cancer stem cell numbers (Korkaya et al. 2009) and incidentally is responsible for the reduction in c-FLIP levels in AML stem cells (Tazzari et al. 2008). Furthermore, it has been suggested that breast cancer stem cells may selectively express HER2 (Magnifico et al. 2009) and that inhibition of this pathway could have beneficial consequences for breast cancer patients with both HER2-positive and HER2-negative disease (Korkaya et al. 2008; Liu and Wicha 2010). As significant responses of CSCs to combined FLIPi/TRAIL were seen, independent of HER2 receptor status, it will be of interest in the future to establish whether primary human tumour stem cell populations are equally susceptible and whether this is due to amplification of a DISC-related mechanism.

3.4 Summary

In summary, work detailed in this chapter has demonstrated that c-FLIP suppression acts as a sensitiser to TRAIL in a broad range of breast cancer subtypes but more importantly, c-FLIP suppression in combination with TRAIL selectively ablates the self-renewing bCSC population *in vitro* in all different subtypes of disease investigated whilst also successfully reduced tumour initiation and metastasis *in vivo*. Significantly, this treatment combination appears to have almost no effect on the normal cell populations tested making it a very attractive potential therapy for development into the clinical setting.

CHAPTER 4:

Evaluating TRAIL as a Potential Therapy in Endocrine Therapy Acquired Resistant Breast Cancer

4.1 Introduction

Breast cancer is well recognised as a hormone-dependant tumour and with nearly 70% of breast tumours expressing estrogen receptor (ER) it is clear that estrogen itself plays a vital role in tumour development and progression (Anderson et al. 2002). This was first confirmed by George Beatson in 1896 where studies showed that oopherectomy gave tumour remission in women with metastatic breast cancer by depriving tumours of circulating estrogen (Beatson 1896). This indicated that endocrine therapy would be beneficial to patients with tumours being driven by estrogen as it provides anti-tumour properties without the adverse side effects connected with chemotherapeutic intervention (Pritchard et al. 1997; Robertson 1996). As such, the selective ER modulator tamoxifen has become the standard anti-estrogen therapy over the last 25-30 years giving improved survival and quality of life in early and advanced ER+ve disease sufferers (EBCTCG 1998; Jaiyesimi et al. 1995). Since the discovery of tamoxifen, further endocrine agents have been developed to either directly target estrogen production itself, for example aromatase inhibitors (AIs) (Osborne 1999) (Johnston and Cheung 2010), or degrade ER, such as fulvestrant (Wakeling et al. 1991). These agents abrogate ER signalling in a different manner to tamoxifen and have also been shown to be clinically successful, with AIs now the gold standard endocrine agent in ER+ve postmenopausal women (Buzdar 2003). Collectively, these agents have dramatically improved the outcome and quality of life of ER-positive breast cancer sufferers by providing a relatively non-toxic anti-tumour therapy.

Unfortunately, one major pitfall of endocrine therapy is that a large proportion of patients are intrinsically resistant or acquire

resistance to this form of treatment following initial responsiveness and in some cases the endocrine agent itself may even begin to drive tumour growth and progression (Musgrove and Sutherland 2009; Schiff et al. 2003). For example, most patients with metastatic disease, and up to 40% of ER+ve patients, receiving adjuvant tamoxifen will acquire resistance to its inhibitory effects on breast cancer cell proliferation (Musgrove and Sutherland 2009; Schiff et al. 2003). Unfortunately, resistance also equates with tumour aggressiveness and poorer prognosis (Hiscox et al. 2006). Acquired resistance is therefore a hurdle that must be overcome to maintain the beneficial effects of endocrine therapy.

It is suggested ER expression remains a stable phenotype for many acquired resistance tumours that initially responded to endocrine agents (Staka et al. 2005) and this is further corroborated by data showing that a proportion of acquired resistance tumours remain sensitive to additional endocrine treatment, however, as a therapeutic avenue this too has its limitations as response to additional endocrine therapies declines over time and tumours act more and more independent of ER (Johnston et al. 1995; Kuukasjarvi et al. 1996; Osborne et al. 1994). A further cohort is also believed to lose ER entirely during endocrine treatment. Recent model systems studies have also elucidated that endocrine agents upregulate various growth factor signalling pathways whilst the cells are still responding. Subsequently, these growth factors have been attributed to promoting tumour growth once cells have acquired resistance (Arpino et al. 2008; Beeram et al. 2007; Nicholson et al. 2004b). Thus many attempts to overcome endocrine resistance by targeting these signalling pathways have been made alongside endocrine agents (Johnston et al. 2008). Whilst there have been great advances in understanding and

treatment of endocrine resistant tumours, it still remains a difficult hurdle to overcome in the management and prevention of long-term disease since many AE-resistant patients are also resistant to anti-growth factor treatment.

In this chapter the efficacy of TRAIL as a novel anti-tumour agent for endocrine resistant breast cancer was investigated. Using *in vitro* models developed by the breast cancer molecular pharmacology group to mimic acquired endocrine resistance, where ER-positive cell lines (e.g. MCF-7) are cultured long-term (up to 2 years) in low dose 4-hydroxy-tamoxifen or Faslodex and following initial responses eventually become resistant, the effectiveness of TRAIL treatment on these acquired resistance cell lines was then addressed *in vitro* and in *in vivo* transplantation models.

4.2 Results

4.2.1 Acquired endocrine-resistant MCF-7-derived cell lines are hypersensitive to TRAIL induced cell death

A great advantage of combined FLIPi/TRAIL treatment in the context of breast cancer is the breadth of response seen across models representing many different disease subtypes. Importantly a marked response was seen in the reduction of metastasis (Figure 3.2.12), targeting the key process responsible for fatality. Another important therapeutic group with poor prognosis is endocrine therapy-resistant patients. These patients no longer respond to typical adjuvant first-line therapies, show an increased rate of metastasis and there is a great need for, and hence much research in the development of, novel therapeutic strategies in the adjuvant and advanced setting for these patients.

Using MCF-7-derived acquired endocrine resistant cell lines, the efficacy of TRAIL as a potential remedy was investigated. Treating long-term tamoxifen and Faslodex acquired resistant MCF-7 cell lines (TAMR and FASR respectively) with TRAIL resulted in a significant reduction in cell viability and an increase in cell death contrasting the parental MCF-7 model (Figure 4.2.1: TAMR, $p<0.001$; FASR, $p=0.001$). TAMR cells responded particularly well with very few surviving cells visible by microscopy following treatment (Figure 4.2.1C). Furthermore both TAMR and FASR cells gave significant cell death at a ten times lower concentration of TRAIL than TRAIL-sensitive breast cancer cell line MDA-MB-231 (Figure 3.2.1C: TAMR, $p=0.001$; FASR, $p=0.002$), indicating that endocrine resistant cell lines display a hypersensitivity to TRAIL (Figure 4.2.1D).

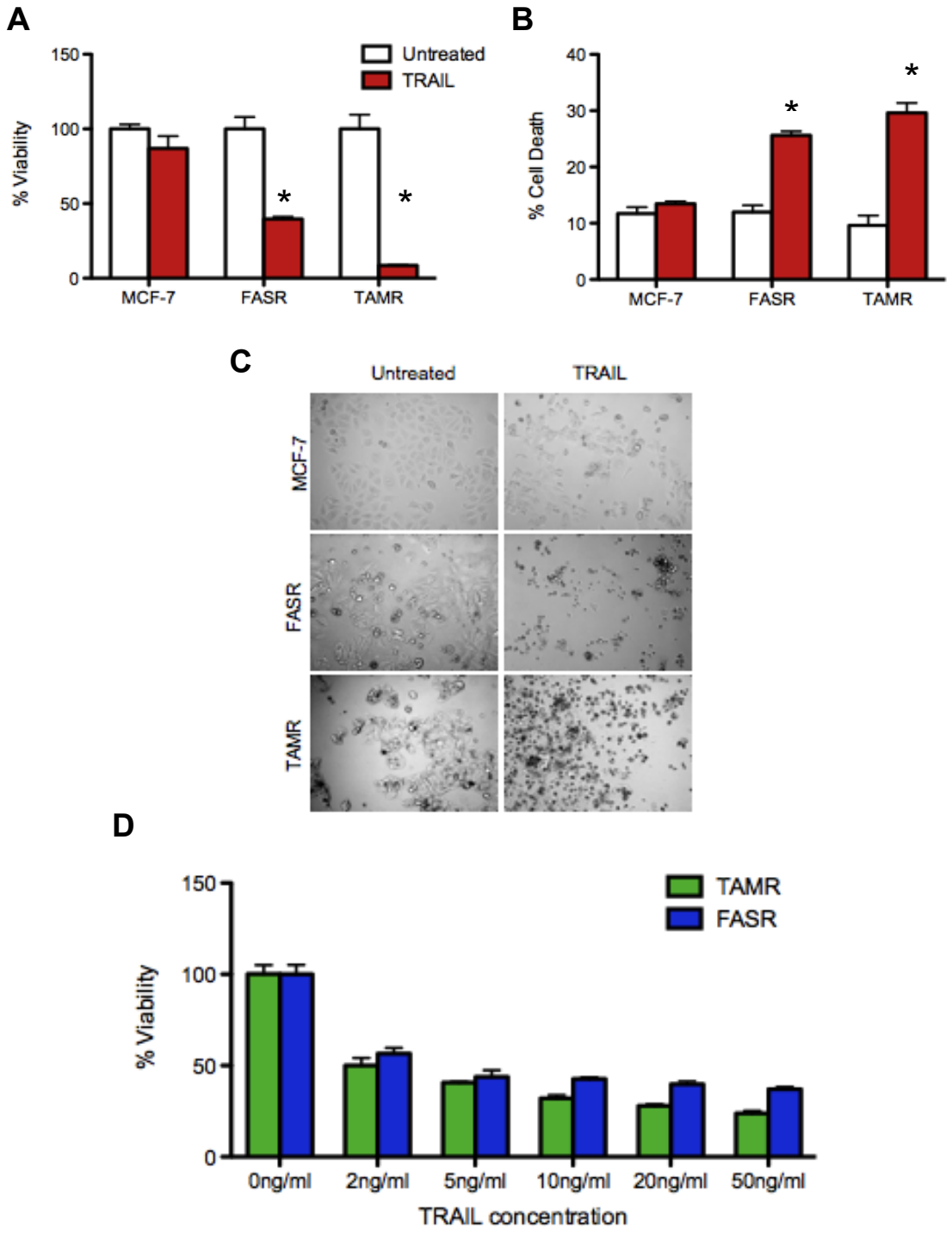

Figure 4.2.1: MCF-7-derived AE resistant cell lines are hypersensitive to TRAIL A) Cell lines were incubated with 20ng/ml soluble TRAIL for 18 hours and cell viability assessed by cellTiter Blue viability assay,* $p<0.01$. B) Cell lines were incubated for 18 hours +/-20ng/ml TRAIL, and assessed by FACS for live/dead cells as described in methods, * $p<0.01$. C) Phase contrast images of cell lines following TRAIL treatment. D) TAMR and FASR cell lines were treated with increasing concentrations of TRAIL for 18 hours and viability assessed by cellTiter Blue assay.

4.2.2 TRAIL treatment of acquired endocrine resistant cell line targets bCSC population

As previously stated, within each tumour there exists different subsets of tumour cells, with one of the most functionally important being the cancer stem cell. This small subset of cells have been shown to be particularly resistant to radio- and chemo-therapeutic challenge (Phillips et al. 2006; Tanei et al. 2009) and as already shown targeting this subset has profound effects on tumour initiation and metastatic capability of cells (Chapter 3). Therefore for TRAIL to maintain a significant long-term preventative effect the CSCs must be targeted.

The effect of TRAIL treatment on stem-like activity in the endocrine resistant cell lines was analysed by treating TAMR and FASR cells in mammosphere culture conditions and, additionally, by treating adherent cultures of TAMR and FASR cells prior to plating the surviving cell population into mammosphere culture conditions in the absence of TRAIL.

Both TAMR and FASR cell lines were capable of forming mammospheres similar in morphology and size to their parental MCF-7 cell line (Figure 4.2.2C). A small difference was observed in the FASR mammospheres that appeared to form more of a lumen in the centre of their spheres, which is normally occupied by cells in the MCF-7 spheres (Figure 4.2.2C). These hollow spheres more closely resemble the morphology seen in primary breast cancer mammospheres, however, the reason for this is unknown and further research may provide further insight into FASR cell behaviour. Spheres formed from untreated endocrine-resistant TAMR and FASR cells showed that they contained a larger population of MFUs in passage 1 than their parental MCF-7 cells, signifying that these cell lines may contain a larger pool of anoikis-resistant cells. A single residual mammosphere was observed

in the TRAIL treated FASR cells (Figure 4.2.2C), however, there was no increase in MFU formation from this mammosphere in passage 2 (Figure 4.2.2A). No MFUs were identifiable in the TRAIL treated TAMR cells. Surviving cell populations from adherent treated cells also failed to form any MFUs for both FASR and TAMR cells (Figure 4.2.2B) which contrasted the more modest reduction in MFU forming capability of MCF-7 cells (Figure 3.2.10), albeit mammospheres which maintained the capacity to self-renew (Figure 4.2.2B, *passage 2*).

These data show that both bulk tumour cells and CSCs are hypersensitive to TRAIL, implying long-term benefits and providing further support for TRAIL as a treatment for endocrine-resistant tumours.

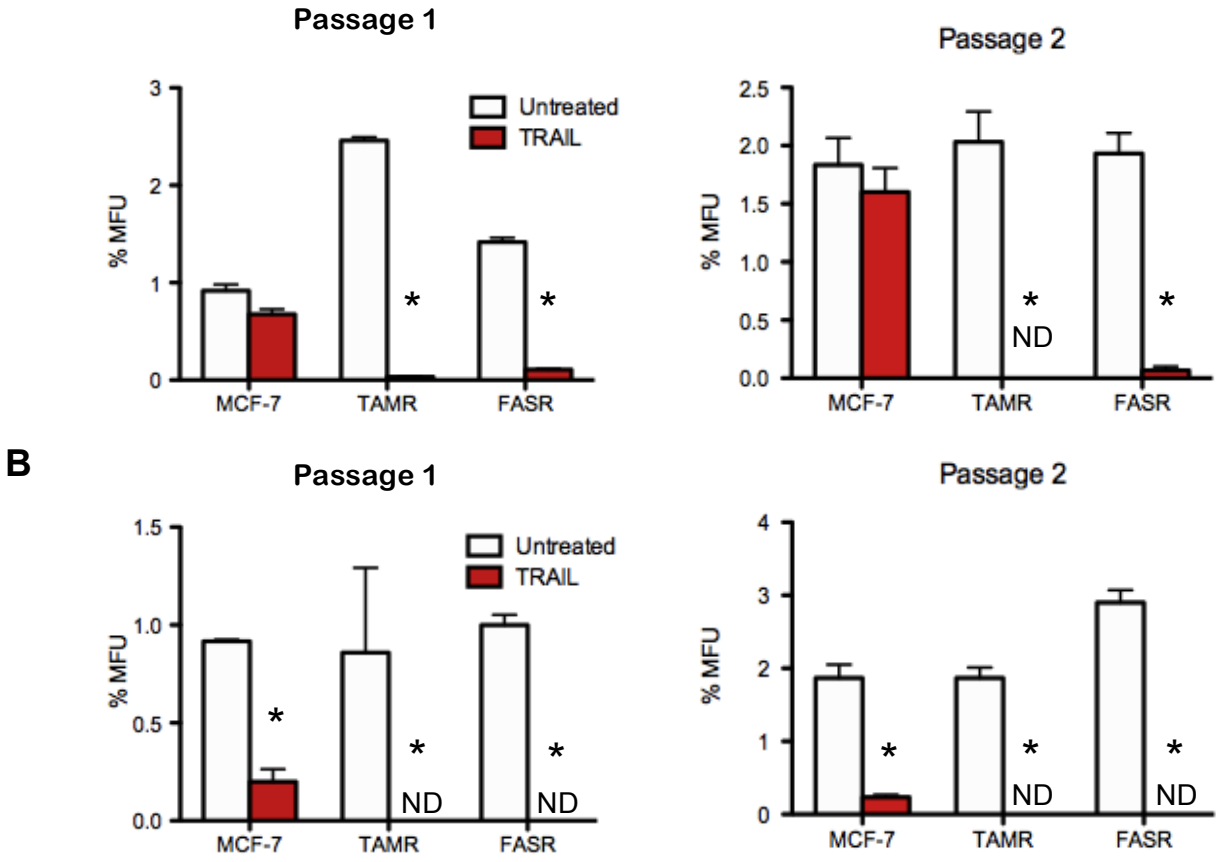

Figure 4.2.2: bCSCs of AE resistant MCF-7 cell lines are also hypersensitive to TRAIL A) Cell lines were plated in low-serum non-adherent (mammosphere) culture conditions in the presence or absence of 20ng/ml TRAIL at a density of 4,000 cells per well (20,000 cells/ml) (ND = Not Detected). Mammospheres from 3 replicate wells per condition were counted following 7 days culture (Passage 1). Mammospheres were dissociated using trypsin, passaged at a density of 2,000 cells/well (10,000 cells/ml) in the absence of TRAIL and counted after 7 days culture (Passage 2). B) Cell lines were treated in adherent conditions with 20ng/ml TRAIL and then surviving cells plated in mammosphere conditions, as described in (A). As in (A), graphs represent the percentage of mammospheres formed following 7 days culture (Passage 1) and 7 days after trypsinisation and re-seeding (Passage 2). C) Representative phase/contrast images of mammospheres formed in (A). Data shown is representative of 3 experiments performed in triplicate ±SEM, * $p<0.05$.

4.2.3 TAMR and FASR hypersensitivity to TRAIL is a result of acquired resistance

As most breast cancers display resistance to TRAIL-induced cell death many attempts have been made to sensitise these resistant cells by combination therapies, some of which have been successful, including histone deacetylase inhibitors (HDACs) and cyclin dependant kinase (CDK) inhibitors (Ortiz-Ferron et al. 2008; Palacios et al. 2006; Shankar et al. 2009). To date there has been no evidence to suggest that either tamoxifen or Faslodex act as sensitising agents for TRAIL.

In order to determine if the hypersensitivity to TRAIL observed in the TAMR and FASR cell lines was a result of sensitisation by combination treatment with endocrine agents or changes to the cells as a consequence of long-term acquisition of endocrine resistance, the parental MCF-7 cells were treated with TRAIL in combination with tamoxifen and Faslodex respectively. Additionally MCF-7 cells were treated short-term with tamoxifen and Faslodex for 7 days, prior to TRAIL treatment, a standard timeframe for ER blockade. Furthermore, tamoxifen was also withdrawn from TAMR cell cultures for 1 week (TAMR-W), prior to TRAIL challenge, in order to determine if the response to TRAIL was due to irreversible cellular changes from long-term acquired resistance.

Treating MCF-7 cells for 18 hours with either endocrine agent alone or in combination with TRAIL resulted in no significant reduction of cell viability (Figure 4.2.3A), confirming the sensitivity of the TAMR and FASR cells to TRAIL as a long-term acquired resistance phenotype. Furthermore, treating MCF-7 cells with tamoxifen and Faslodex for 7 days prior to TRAIL treatment had no significant effect on cell viability suggesting that only long-term acquired resistance precipitated sensitivity to TRAIL (Figure 4.2.3B). Moreover, removing the influence

of tamoxifen on cell growth (TAMR-W) prior to TRAIL treatment also had no effect on the sensitivity of TAMR cells to TRAIL with an almost identical profile of sensitivity to a range of TRAIL concentrations being portrayed between the TAMR and TAMR-W cells (Figure 4.2.3C). This suggests that the TAMR sensitivity to TRAIL was due to irreversible cellular changes resulting from the prolonged effects of tamoxifen treatment. Some small differences in TRAIL sensitivity were observed at lower doses so, to confirm that this was not a specific effect on the CSCs, surviving cells were plated into mammosphere conditions where both TAMR and TAMR-W untreated MFUs were both identical in size and equally targeted by TRAIL treatment (Figure 4.2.3D).

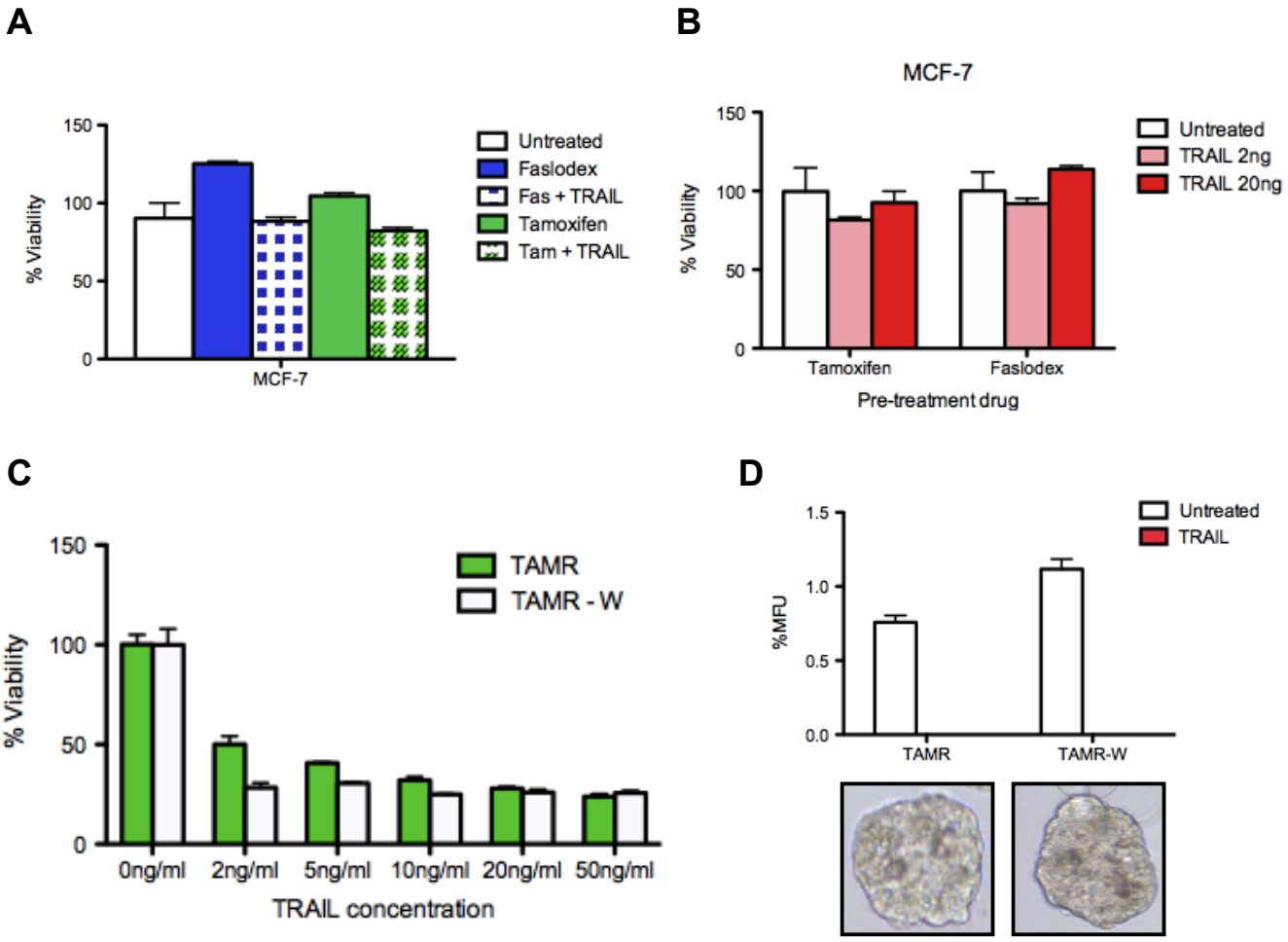

Figure 4.2.3: AEs do not sensitise MCF-7 cell lines to TRAIL A) MCF-7 cells were treated with Faslodex or tamoxifen alone or in combination with 20ng/ml TRAIL for 18 hours and cell viability assessed by cellTiter Blue assay. B) MCF-7 cells were treated for 7 days with either tamoxifen or Faslodex prior to 20ng/ml TRAIL treatment for 18 hours and cell viability assessed by cellTiter Blue assay. C) tamoxifen was withdrawn from culturing TAMR cells for 7 days (TAMR-W) and treated with increasing concentrations of TRAIL for 18 hours and cell viability assessed by cellTiter Blue assay. D) TAMR and TAMR-W cells were treated as in Figure 4.2.2A and %MFU calculated. Representative phase contrast images of mammospheres formed in untreated TAMR and TAMR-W cells. Data shown is representative of 3 experiments performed in triplicate ±SEM

4.2.4 Acquired tamoxifen-resistant EFM19 cells display similar hypersensitivity to TRAIL as MCF-7-derived cell lines

Although both TAMR and FASR cells showed hypersensitivity to TRAIL treatment both these endocrine resistant cell lines were derived from the hormone responsive ER+ve parental MCF-7 cell line. To ensure the observed hypersensitivity was not a cell line specific effect of acquired resistance an EFM19-derived tamoxifen-resistant line (~1 year tamoxifen treated), developed in the same manner as the TAMR cells by the BCMP group from ER+ve, hormone responsive, EFM19 cells (Westley and May 1988), was cultured in the presence of TRAIL.

The sensitivity to TRAIL of the parental EFM19 cell line has not been previously reported. EFM19 cells demonstrated some sensitivity to TRAIL, even at a low dosage of 5ng/ml, reducing cell viability by 40%. Cell viability was reduced by up to 60% when treated at 20ng/ml TRAIL. However, the acquired tamoxifen-resistant derivative of the EFM19 cell line (EFM-TAMR) again showed increased sensitivity to TRAIL over their parental counterpart with 40% more cell death at 5ng/ml TRAIL and viability being reduced down to as low as 10% when treated at 20ng/ml (Figure 4.2.4A). From phase contrast microscopy pictures following TRAIL treatment it is clear that 20ng/ml treatment of EFM19-TAMR cells leaves almost no detectable surviving cells whereas the parental EFM19 cells appear to still have a population of adherent surviving cells (Figure 4.2.4B). This increased cell death over the parental cell line confirms the hypersensitivity of tamoxifen-resistant cells in multiple cell lines.

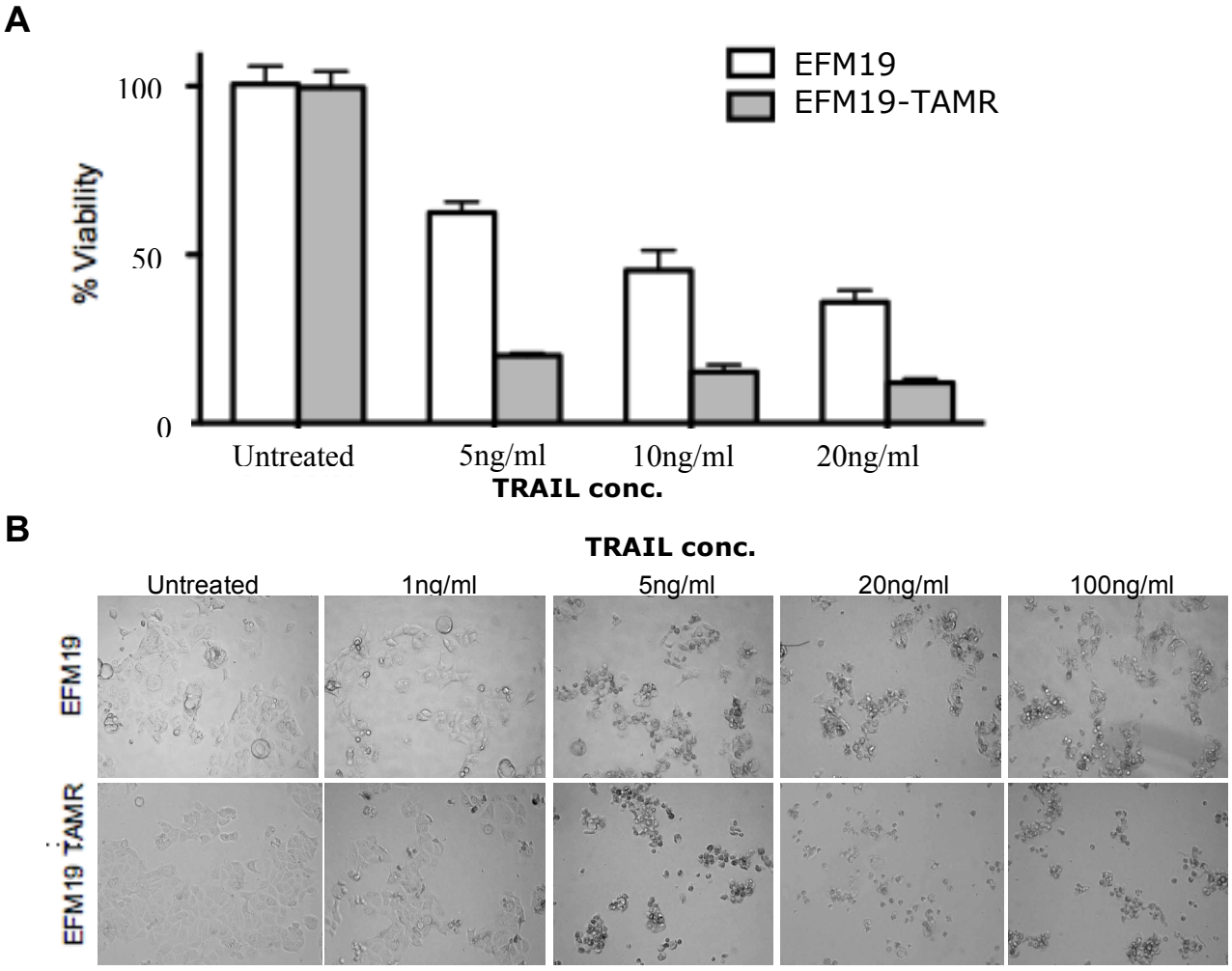

Figure 4.2.4: EFM19-derived AE resistant cell lines are more sensitive to TRAIL than parental EFM19 cells. A) Cell lines were incubated with 20ng/ml soluble TRAIL for 18 hours and cell viability assessed by cellTiter Blue viability assay, * $p<0.01$ compared to EFM 5ng/ml, $^{+}p<0.01$ compared to EFM untreated. B) Representative phase contrast images of cell lines after treatment outlined in (A). Data shown is representative of 3 experiments performed in triplicate ±SEM

4.2.5 TRAIL hypersensitivity of TAMR and FASR cells is not a result of estrogen deprivation.

During long-term culture and acquisition of resistance of MCF-7 cells to tamoxifen or Faslodex the cells lose their reliance on estrogen for cell growth. It is possible therefore that this loss of estrogen dependence may be responsible for the sensitivity to TRAIL observed in the TAMR and FASR cell lines. To determine if lack of estrogen-dependent signalling is responsible for TAMR/FASR hypersensitivity to TRAIL a third MCF-7 derived model of estrogen deprivation was used. These cells (MCF-7-XL (Staka et al. 2005)) were developed by up to 2 years maintenance in the absence of estrogen and hence their cell growth no longer relies on estrogen-induced signalling. The MCF-7-XL cells were treated with TRAIL in both adherent and mammosphere culture conditions to ascertain their sensitivity.

Treating the XL cells with TRAIL under adherent culture conditions gave no significant reduction in cell viability with the cells demonstrating almost identical resistance to TRAIL as their parental MCF-7 cells (Figure 4.2.5A). This effect was then mimicked in the EFM19 estrogen deprivation model, where EFM19-XL cells were also resistant to TRAIL (Figure 4.2.5F). Previously it was shown that tamoxifen or Faslodex as a combination therapy with TRAIL, did not cause any significant cell death in MCF-7 cells (Figure 4.2.3A) and it was explored if this was also true for such AE ER blockade in XL cells. The XL cells were treated with each endocrine drug alone and also in combination with TRAIL. The resulting response of the XL cells was the same as the MCF-7 cells with no significant cell death observed by combination endocrine/TRAIL treatment (Figure 4.2.5B). In the context of bCSCs, the XL cells appear to have a larger mammosphere

forming population, similar to the TAMR and FASR cells (Figure 4.2.5C, *white bars*). Interestingly this increase in MFUs was not consistent in passage 2 where the percentage of MCF-7 MFUs increased more than that of the XL cells suggesting the XL cells have a reduced self-renewal capacity. Previous data showed that MCF-7 mammosphere formation capability was reduced by treatment of adherent cultures with TRAIL, although MFUs with self-renewal capacity did still form. The XL cells on the other hand did not demonstrate any reduction in mammosphere forming capacity when treated with TRAIL under adherent conditions, indicating that the XL MFUs are more resistant to TRAIL (Figure 4.2.5D). However, the mammospheres formed by the XL cells showed no difference in morphology or size in comparison to their parental MCF-7 cells (Figure 4.2.5D, 4.2.5E). Interestingly, the estrogen-deprived EFM19-XL cells also demonstrated increased resistance to TRAIL compared to their parental EFM19 cells, further supporting the TRAIL resistance of the MCF-7-derived XL cell line.

Collectively these data confirm that ER signalling is not a determinant for sensitivity to TRAIL in MCF-7-derived acquired AE-resistant models. XL cells still maintain their ER signalling, in the absence of estrogen, in a similar fashion to TAMR cells, however, the FASR cells are an ubiquitously ER-ve cell line and TRAIL response between each of these cell lines differs greatly. This indicates that the sensitivity to TRAIL demonstrated in the TAMR and FASR cell lines is a result of the prolonged exposure, and acquisition of resistance, to anti-estrogens and is not dependant on ER signalling.

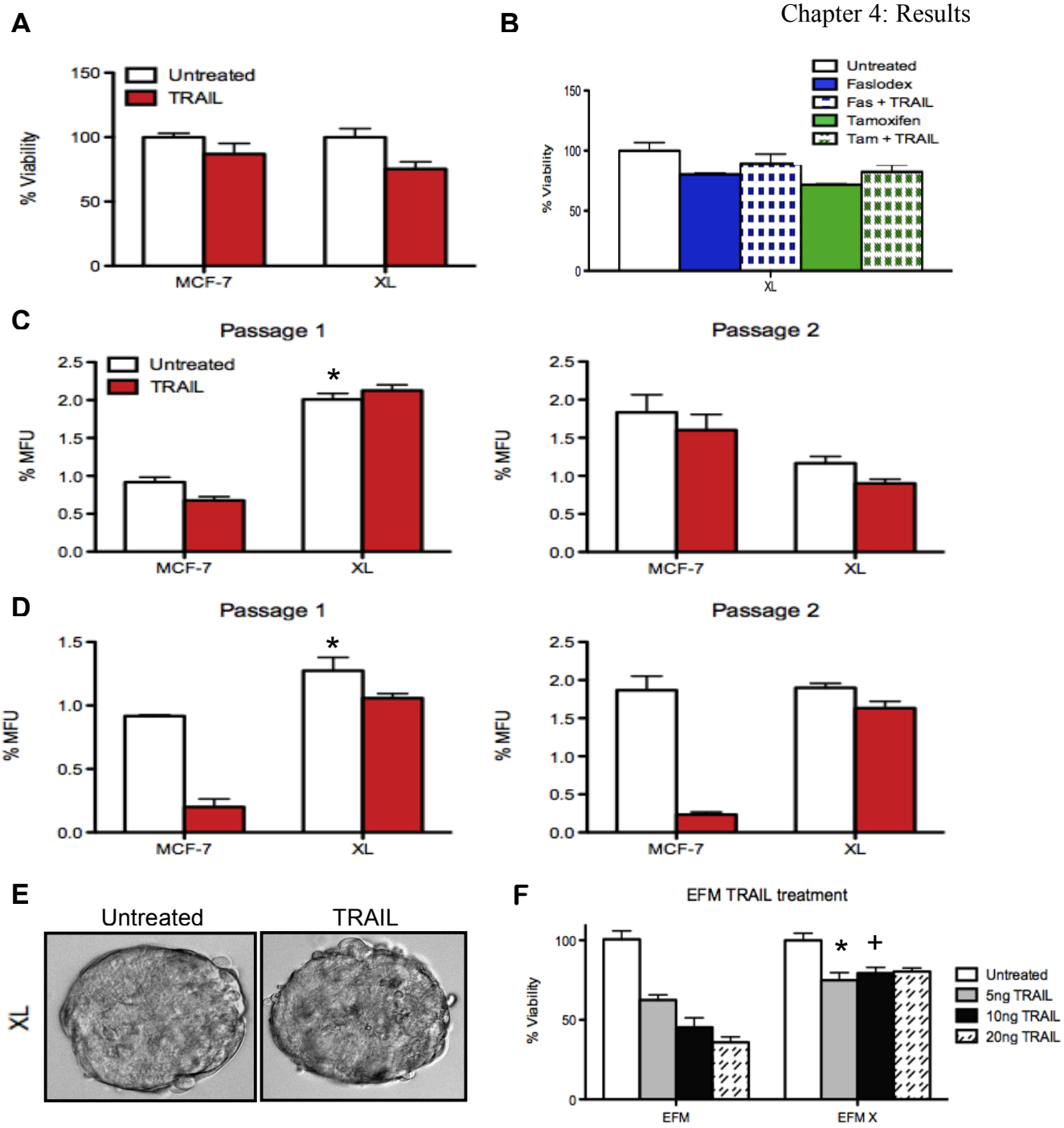

Figure 4.2.5: Estrogen deprived breast cancer cell lines are resistant to TRAIL A) Cell lines were treated with TRAIL and cell viability assessed by CTB viability assay. B) XL cells were treated with Faslodex or tamoxifen alone or in combination with TRAIL and cell viability assessed by CTB assay. C) Cell lines were plated in mammosphere culture conditions in the presence or absence of 20ng/ml TRAIL. Mammospheres from 3 replicate wells per condition were counted following 7 days culture (Passage 1). Mammospheres were dissociated using trypsin and passaged in the absence of TRAIL and counted after 7 days culture (Passage 2), * $p<0.05$ compared to MCF-7 untreated. D) Cell lines were treated with 20ng/ml TRAIL and then plated in mammosphere conditions, as described in (C) and MFUs calculated as in (C) E) Representative phase contrast images of mammospheres formed in (C). F) Representative phase contrast images of mammospheres formed in untreated and TRAIL treated XL cells. F) EFM19 cell lines were incubated with increasing concentrations of TRAIL for 18 hours and cell viability assessed by CTB viability assay, * $p<0.05$ compared to EFM-X untreated, $^{+}$ $p<0.01$ compared to EFM 10ng/ml. Data shown is representative of 3 experiments performed in triplicate ±SEM.

4.2.6 TAMR and FASR cells maintain similar levels of death receptor expression as parental MCF-7 cells

There is some evidence to suggest that DR4 and DR5 cell surface expression may contribute to TRAIL sensitivity in some cancer cells (Mitsiades et al. 2001; van Geelen et al. 2011; Wu 2009) and as previously stated (Chapter 3), c-FLIP is clearly an important regulator of TRAIL-induced cell death. The expression of c-FLIP, DR4 and DR5 proteins and cell surface expression of DR4 and DR5 were assessed by western blot and FACS analysis in TAMR FASR and XL cells to uncover any contribution each may have in the hypersensitivity to TRAIL observed in AE-resistant models.

Flow cytometry showed that MCF-7, TAMR and FASR cells all had similar expression of DR5 on their cell surface with 60-80% of the cells expressing equivalent levels of surface DR5 (Figure 4.2.6B). Positive staining was gated on the basis of corresponding isoform controls. Average expression levels of DR5 in each cell line as determined by mean antibody labelling were also similar (Figure 4.2.6A). While mean expression levels between cell lines were also similar for DR4, the relative proportion of MCF-7 and TAMR cells expressing surface DR4 was significantly less than that for FASR cells (5% compared to 20%, $p=0.004$). Total protein levels of DR5, as determined by pooled sample western blot, were also similar across most the cell lines with just the FASR cells having increased amount of DR5 protein (Figure 4.2.6C). DR4 expression was confirmed to be very low in the MCF-7 and TAMR relative to the FASR and XL cells. This data suggests that death receptor expression is not determinant of AE-resistant cell sensitivity to TRAIL. As shown in the previous chapter, reducing levels of c-FLIP protein can sensitise cells to TRAIL-mediated cell death so it was

interesting to discover that the most TRAIL-sensitive MCF-7-derived cell line expressed much lower levels of both short and long c-FLIP protein (Figure 4.2.6C). This was then confirmed using a different c-FLIP antibody against only the c-FLIP-long isoform, also showing no expression in the stable knockdown MCF-7 shFLIPi cell line (Figure 4.2.6D). Contrastingly c-FLIP protein levels in the FASR cells remained comparatively high, thus c-FLIP protein expression is not the sole determining factor in endocrine-resistant breast cancer sensitivity to TRAIL. Co-operatively these data suggest that TRAIL hypersensitivity of endocrine-resistant cell lines is dependant on the endocrine agent used and does not correlate with one specific mechanism of sensitisation as neither DR4, DR5 nor c-FLIP were consistently altered across all endocrine-resistant cell lines.

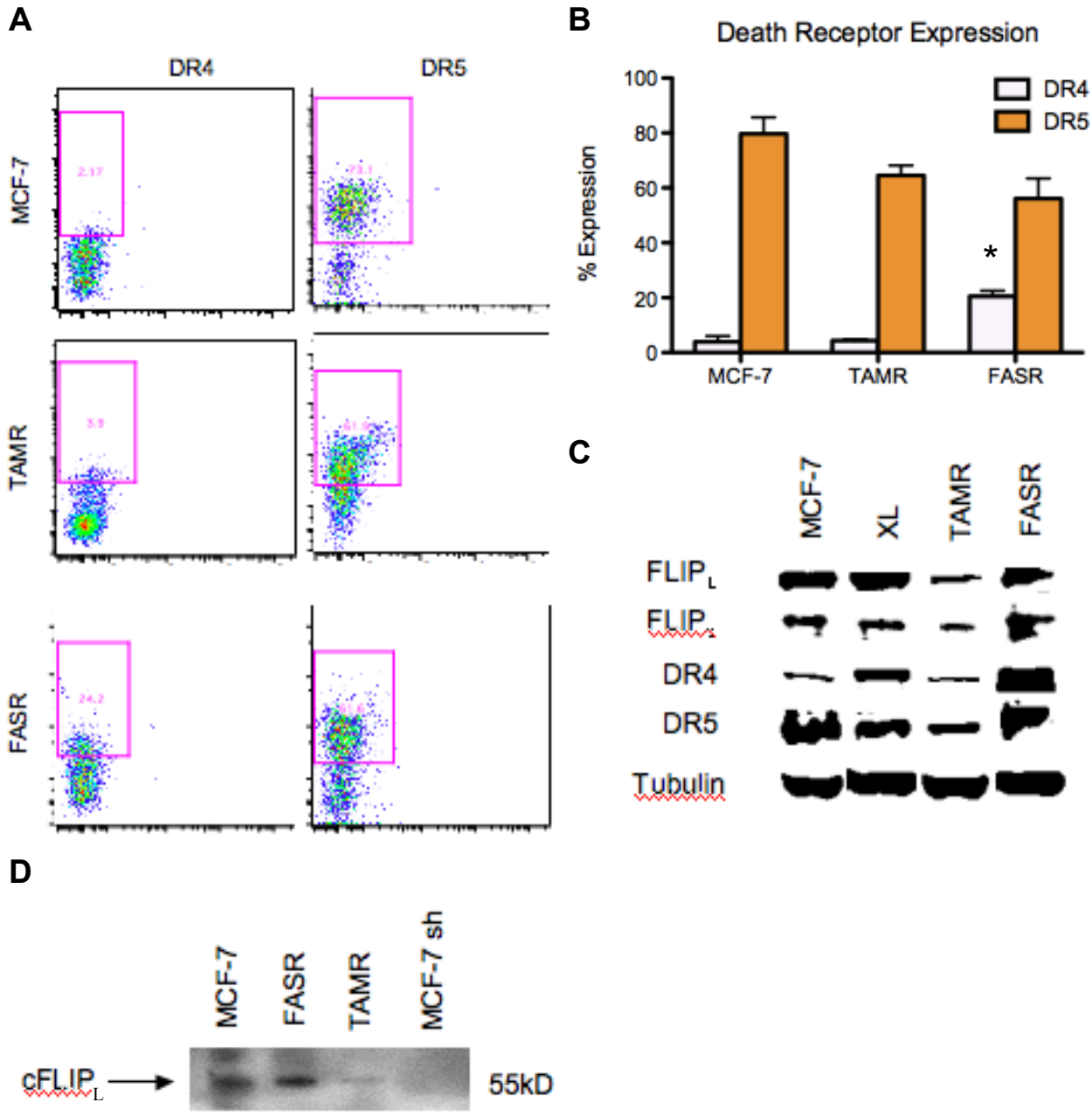

Figure 4.2.6: Endocrine resistant cell lines differentially express regulators of TRAIL signalling. A) Representative FACS plots of cell lines stained with conjugated antibodies to surface death receptors DR4 and DR5. Data shown is representative of experiments performed in triplicate ±SEM B) Average percentage expression of cell surface death receptors on cell lines, * $p<0.01$. C) Western blot analysis of DR4, DR5 and c-FLIP isoform (NF6 antibody) total protein levels in cell lines. D) c-FLIP$_L$ (5D8 antibody) protein levels in cell lines. Western data is representative of single experiments.

4.2.7 Systematic TRAIL administration eliminates TAMR and FASR tumours *in vivo*

Hypersensitivity of AE-resistant cells to TRAIL identifies it as a promising treatment for patients who are no longer responding to these agents in the clinic. To test TRAIL's therapeutic potential in a preclinical setting NOD-SCID mice were transplanted with 10^7 MCF-7, TAMR or FASR tumour cells and the orthotopic tumours allowed to reach 10mm in diameter before receiving 4 daily injections of 0.4mg of TRAIL. This treatment regime was then repeated 10 days later and tumour size monitored weekly.

Tumour initiation for both MCF-7 and TAMR was 100%, with all transplantations resulting in tumour growth. Hitherto an *in vivo* model for the BCMP group FASR cells has not been previously described; here 2 out of 8 transplants successfully resulted in tumour initiation. Following the two TRAIL treatments described, the MCF-7 cells demonstrated a small but significant regression in tumour growth. Following this small regression, MCF-7 tumours then continued to grow at the same rate as the untreated cohort of animals (Figure 4.2.7). This response was consistent with the *in vitro* findings for MCF-7 cells where a small reduction in overall viability and MFU integrity was observed (Figure 4.2.2B). Treatment of the TAMR cells with TRAIL resulted in complete regression of the tumours and no subsequent re-growth was observed for up to 14 weeks following TRAIL treatment (Figure 4.2.7). Again this regression correlated with the *in vitro* data where TAMR cells were killed by TRAIL treatment in adherent conditions. Additionally the maintenance of this complete regression was consistent with the ablation of MFUs witnessed *in vitro* (Figure 4.2.2). Similar effects were seen in FASR transplants where complete regression was observed in the tumour treated with TRAIL, however

this tumour then relapsed 6 weeks later. Importantly the resulting tumour, following relapse, remained sensitive to subsequent TRAIL dosages, completely regressing the tumour following two additional TRAIL dose regimes administered 4 weeks apart (Figure 4.2.7). Taken together these data support the *in vitro* findings in this chapter and indicate that systematic TRAIL administration, either singly or repetitively, may prove beneficial as a treatment for endocrine-resistant patients.

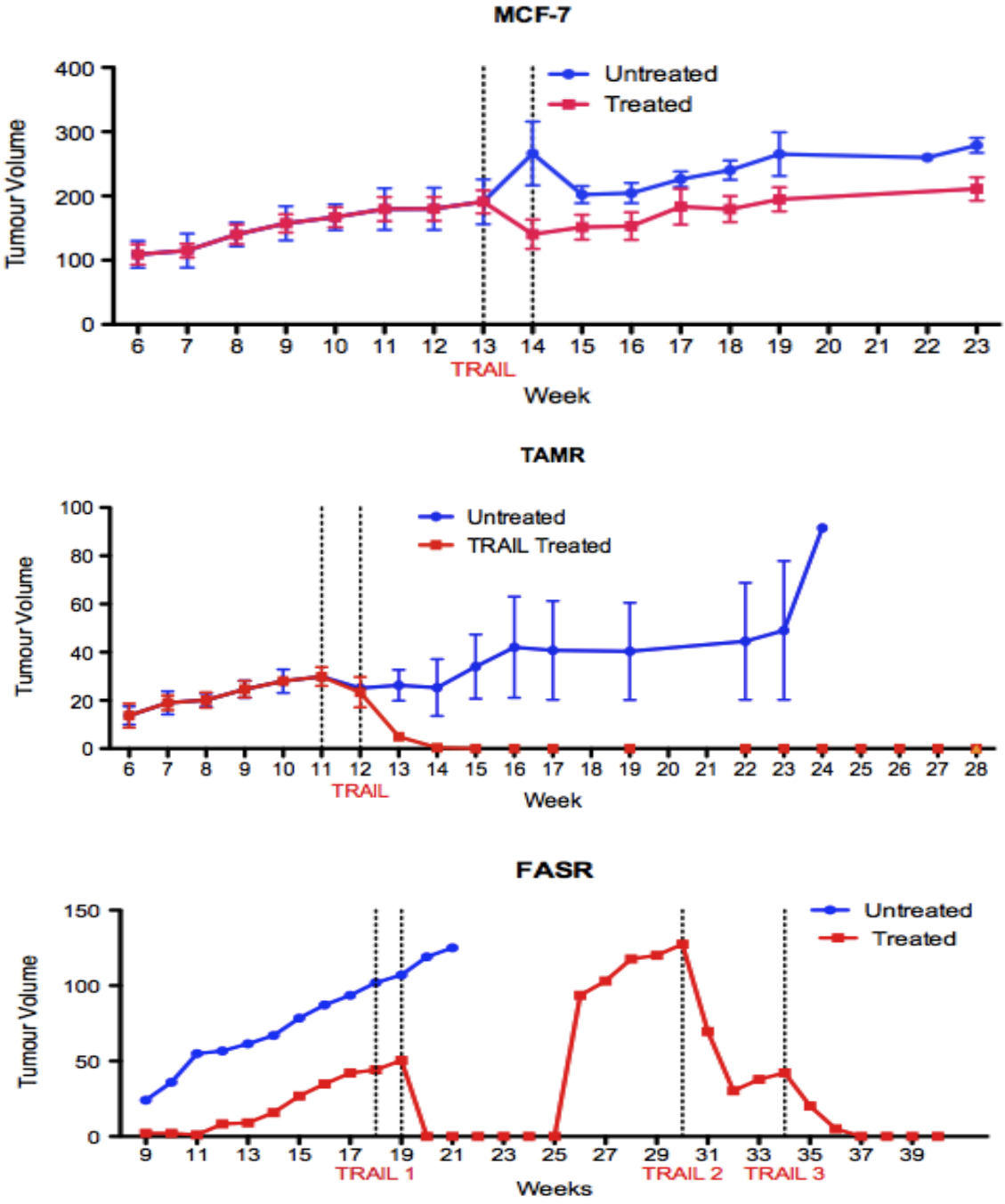

Figure 4.2.7: Systemic TRAIL treatment completely regresses AE resistant tumours in vivo. 1x10^7 MCF-7, TAMR and FASR cells were xenografted into the 4th inguinal mammary glands of Balb/c NOD/SCID and tumour growth monitored weekly. Once tumours had established animals were given 4 daily injections of 0.4mg of TRAIL or PBS (untreated). 10 days later this dose was repeated a second time. Further 4 daily doses of TRAIL were administered to FASR animals upon disease relapse. MCF-7 animals were pre-implanted with a 90-day release 0.75mg 17β-estradiol pellet. TAMR animals were pre-implanted with 90-day release Tamoxifen (Free base) pellet.

4.2.8 Cells surviving TRAIL treatment are capable of re-acquiring MFUs in FASR but not TAMR cells

Transplantation experiments of FASR cells showed that despite complete tumour remission, after a period of time without TRAIL treatment the tumours would re-grow. This was in spite of the complete abrogation of mammospheres with self-renewal capacity observed *in vitro* (Figure 4.2.2). In an attempt to recapitulate this tumour relapse *in vitro*, post TRAIL treated surviving cells were replated following complete removal of MFUs, as previously described in chapter 3 (section 3.2.13).

TRAIL treatment successfully removed all MFUs from TAMR and FASR cell populations (Figure 4.2.8A) and residual surviving cells were plated into adherent culture conditions. Following 2 weeks culture, replacing culture media when necessary, cells were once again plated in mammosphere conditions. The FASR cells re-acquired their mammosphere forming capacity back to an almost identical percentage as their untreated counterparts (Figure 4.2.9B). Interestingly the TAMR cells were unable to re-acquire any MFUs following their time in adherent culture (Figure 4.2.8B). Taken together these data support previous observations (Figure 3.2.13) that tumour cell populations containing no MFUs are capable of re-acquiring stem-like characteristics if cultured for a period of time in adherent conditions. Additionally these data indicate that resistance to specific endocrine agents may alter their cellular plasticity. Extrapolation of this data from the *in vitro* to *in vivo* context suggests that targeting the CSCs in tamoxifen-resistant patients may have long-term beneficial effects.

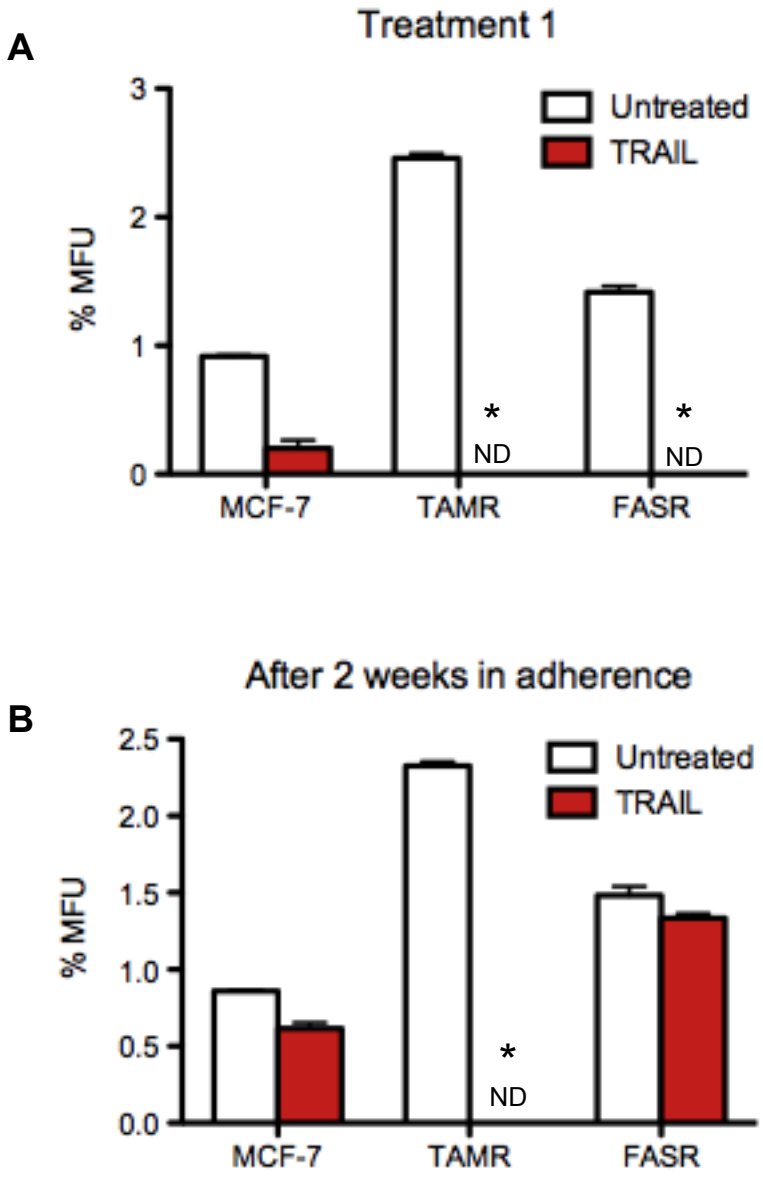

Figure 4.2.8: FASR but not TAMR cells are capable of re-acquiring MFUs following TRAIL treatment. A) Cell lines were treated with 100ng/ml TRAIL for 18 hours and live cells plated into mammosphere culture conditions as previously described. B) Live cells from (A) were also plated back into adherent culture conditions for 2 weeks. Following 2 weeks culture, MFU re-acquisition was then assessed by plating these cells in mammosphere conditions and MFU calculated as previously described. Data shown is representative of 3 experiments performed in triplicate ±SEM

4.2.9 TRAIL reduces viability of a multiple endocrine drug resistant patient sample

Having seen clear benefits of TRAIL treatment in preclinical AE-resistant cell models both *in vitro* and *in vivo*, the efficacy of TRAIL-induced cell death on clinical samples would confirm it as a prime candidate for therapy in this patient cohort. The clinical potential of TRAIL was assessed by collecting breast cancer cells from ascites in a patient with advanced resistant disease having received multiple endocrine, including AEs, and chemotherapy agents (Appendix II) and were plated into adherent culture conditions for 72 hours prior to treatment with TRAIL.

Patient sample BB3RC50 showed a significant reduction in cell viability when treated with TRAIL (Figure 4.2.9A, p=0.0425) and the amount of cell death determined by phase contrast microscopy of the adherent monolayers was visibly increased (Figure 4.2.9B). Furthermore, when cell death was analysed by flow cytometry using a live/dead fixable stain a relatively small, yet significant, increase in cell death was observed (Figure 4.2.9C, p=0.0219). The difference in the magnitude of cell death between assays may be related to the loss of adhesion and metabolic activity occurring more rapidly than the compromisation of cell membrane. Experiments over a timecourse and including annexin-V staining may confirm the TRAIL cytotoxicity observed. Notwithstanding these data support the hypothesis, based on *in vitro* cell lines, that TRAIL may be a beneficial treatment for AE-resistant patients.

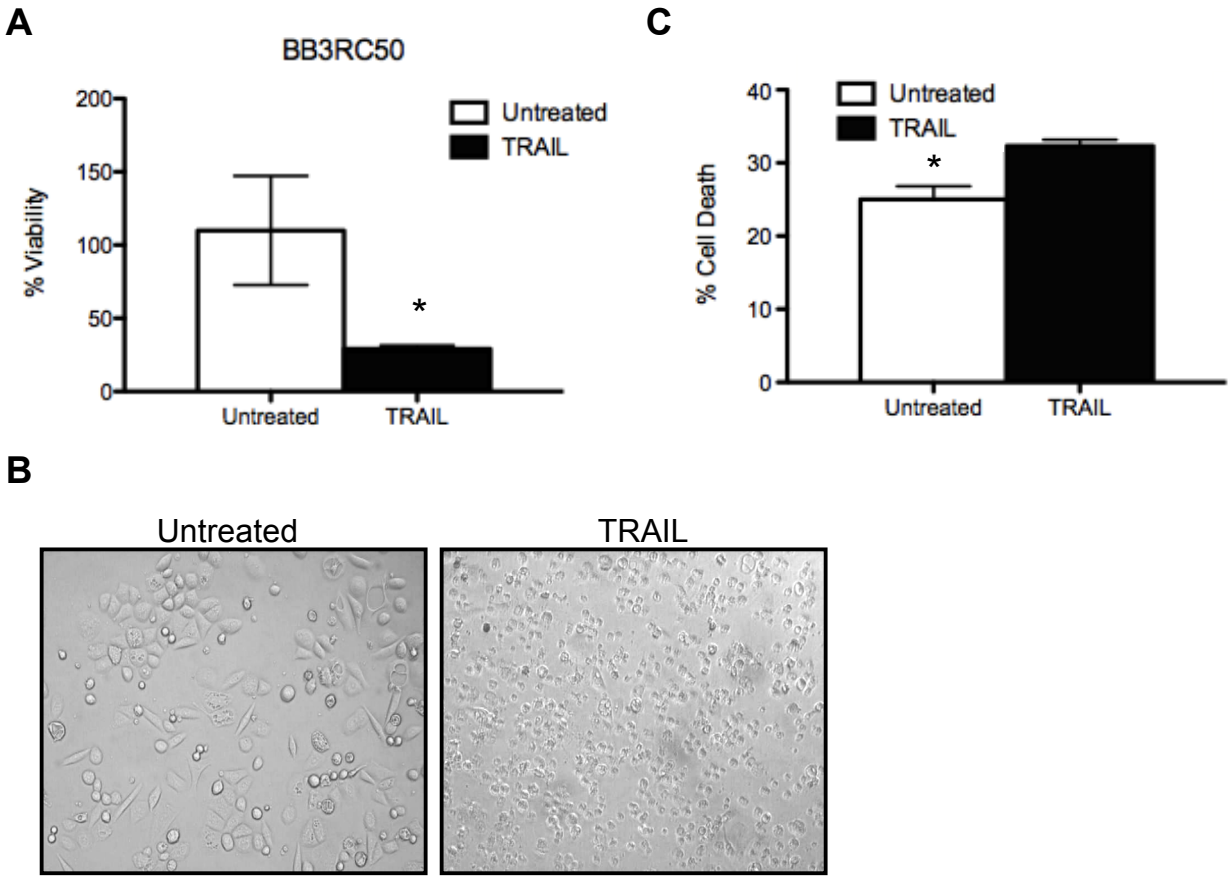

Figure 4.2.9: TRAIL reduces the viability of a multiple endocrine drug resistant patient sample. A) Primary human breast cancer cells collected from ascites were plated in adherent conditions for 48 hours prior to treatment with 100ng/ml TRAIL for 18 hours and cell viability assessed by cellTiter Blue assay. B) Representative phase contrast images of cells treated in (A), * $p<0.05$. C) Cells were treated as in (A) and assessed for live/dead cells by FACS, * $p<0.03$. Data shown is representative of single experiments performed in triplicate ±SEM

4.3 Discussion

Despite recent advances in the treatment of breast cancer, disease recurrence remains a major obstacle to curative therapy. Currently around 40% of all initially responsive breast cancer patients relapse, with 60-70% of these recurrences being distant metastases (Gerber et al. 2010; Normanno et al. 2005). There are many reasons for this disease recurrence following resistance, including under-treatment of disease, local sites of tumour growth missed during surgery or the prior development of micrometastases (Gerber et al. 2010). In addition to these therapeutic deficiencies, intrinsic properties of breast cancer stem cells (bCSCs) predispose to tumour relapse during conventional therapy (Dontu et al. 2003b; Polyak and Weinberg 2009). Consequently there is much interest in identifying new therapeutic strategies to overcome resistance of tumour cells and more specifically CSCs in breast and other cancers.

Estrogen receptor-positive breast cancer represents over 70% of all breast cancers diagnosed worldwide (www.breastcancer.org), this led to the discovery of various agents to inhibit estrogen signaling in cancer cells such as tamoxifen, Faslodex (Fulvestrant) and AIs. Endocrine agents then became the standard treatment for ER+ve breast cancer and indeed they provide much benefit to these patients (Davies et al. 2011; Perey et al. 2007). Unfortunately, a large percentage of patients will eventually become resistant to these anti-estrogen therapies and additionally to second-line anti-hormone therapies resulting in anti-estrogen or multihormone-resistant phenotypes (Henderson and Canellos 1980; Yano et al. 1992). Clinical relapse on these agents has been linked to a more aggressive phenotype and increased metastastic capacity of the tumour cells

(Hiscox et al. 2006). Further research then elucidated that these tumours may have increased growth factor signaling, in some instances promoted by endocrine agents, and although this upregulation is acquired during the drug responsive phase of treatment, it is not until the drug resistant phase that they actually promote tumour growth and metastasis (Arpino et al. 2008; Nicholson et al. 2004b). It is suspected that cross-talk from signaling pathways such as EGFR, HER2, HER3 and IGF1R with ER signaling contribute to endocrine resistance and even sustain tumour cell growth through their independent pathway activation (Britton et al. 2006; Hutcheson et al. 2003; Knowlden et al. 2003; Kurokawa and Arteaga 2001; Parisot et al. 1999). Furthermore, it has been hypothesized that within ER+ve breast cancers there may be a small population of ER-ve bCSCs that would not respond to endocrine therapies, these cells would then remain and maintain the capacity for tumour cells to grow and metastasise (O'Brien et al. 2011). Additionally bCSCs have been described as having a basal/mesenchymal phenotype similar to that seen in cells having undergone EMT (Hiscox et al. 2006), coincidentally ER signaling can also negatively regulate key factors in the EMT process (Dhasarathy et al. 2007). Together this provides some evidence that CSCs may play a role in endocrine resistance and the progression of endocrine-resistant tumours. The aim of this chapter was to extend the potential of TRAIL treatment, on bulk tumour and cancer stem cells, elucidated in chapter 3, to endocrine-resistant breast cancer models.

4.3.1 Endocrine resistant breast cancers are hypersensitive to TRAIL treatment

The FLIPi/TRAIL treatment has thus far shown promise across a broad range of breast cancer subtypes, however, one key subtype of breast cancer patients are those who have received therapy but have become resistant to it. As ER+ve breast cancer is the most common form of breast cancer the endocrine-resistant subset was investigated, using MCF-7-derived cell lines that have acquired resistance to the common endocrine drugs tamoxifen (TAMR) and Faslodex (FASR). Surprisingly the TAMR and FASR cell lines displayed significant sensitivity to TRAIL treatment alone, without the need for c-FLIP inhibition, despite their parental MCF-7 cells showing no sensitivity to TRAIL (Figure 4.2.1). Further investigation revealed that this sensitivity was not a result of endocrine agents working cooperatively with TRAIL as a sensitising agent but that the sensitivity was a result of acquired resistance to prolonged endocrine agent treatment. Furthermore this sensitivity was observed at very low concentrations of TRAIL that previous 'sensitive' cell lines would not respond to. This sensitivity was true for both the bulk tumour cells and also the bCSC subset such that no self-renewing cells remained in TAMR and FASR cell lines following TRAIL treatment. Notably the TAMR and FASR cell lines both appear to have a larger stem cell pool (Figure 4.2.2A) than the MCF-7 cells. It has been demonstrated that the TAMR (Kurokawa and Arteaga 2001) have increased EGFR signalling including detectable HER2 whilst other research has also shown that HER2 regulates the stem cell pool in cancer cells (Korkaya et al. 2008). Estrogen deprivation resistance has also been shown to increase growth factor signalling (Nicholson et al. 2004b; Staka et al. 2005), here estrogen deprivation represented *in vitro* by the XL cells also shows an increase

in the stem cell pool (Figure 4.2.5C), further corroborating with the endocrine-resistant models. Interestingly sensitivity to TRAIL was not a consequence of cell dependance on estrogen signalling, as XL cell resistance to TRAIL confirms. Nor could the sensitivity be attributed to the combination of either tamoxifen or Faslodex with TRAIL, irrespective of their dependence on estrogen (Figure 4.2.5). Significantly, estrogen ablation therapy using aromatase inhibitors (AIs) is frequently used in postmenopausal women. Patients that become resistant to AIs may have some phenotypic similarities to the XL cells and as a consequence may also be resistant to TRAIL in a similar manner. Further investigation into TRAIL sensitivity of AI-resistant breast cancer would be appreciably aided by the development of *in vitro* (including clinical *ex vivo*) and *in vivo* models. Collectively these data then suggest that the AE-resistant-dependent sensitivity to TRAIL is mediated through an as-of-yet undetermined mechanism associated with long-term acquired resistance to AEs.

4.3.2 Acquired resistance to endocrine agents may alter regulation of c-FLIP

In an attempt to uncover the mechanism by which endocrine-resistant cells become sensitive to TRAIL, death receptor and c-FLIP protein levels were assessed by western blot. Western data indicates TAMR cells have reduced c-FLIP protein levels compared to FASR cells and their parental MCF-7s (Figure 4.2.6C). As demonstrated in chapter 3, c-FLIP reduction gives very significant sensitisation of bulk tumour cells and bCSCs to TRAIL correlating with the extremely sensitive TAMR cells. Importantly Figure 3.2.14 suggested that c-FLIP may also be important for cellular plasticity in re-acquisition of stem cells after ablation. Here the TAMR cells were not capable of re-acquiring MFUs

after TRAIL treatment and culture in adherence (Figure 4.2.8) whereas the FASR cells, that have comparatively higher c-FLIP protein levels, did, with MFUs returning to original levels after just 1 week in adherent culture. This was consistent with the observations of tumour kinetics *in vivo* where the FASR tumour treated with TRAIL re-grew 5 weeks following complete tumour regression while in contrast TAMR tumours treated with TRAIL did not re-grow, even 14 weeks after complete regression (Figure 4.2.7). In this set of *in vivo* experiments TRAIL was administered systemically over 4 days. Although the results from the *in vivo* experiments were very promising, in the clinical setting a more systematic TRAIL regime may prove more therapeutically beneficial. Additionally, FASR *in vivo* results are based on n=1 as many of the transplants did not initiate a tumour. FASR cells *in vitro* become ubiquitously ER-ve (Nicholson et al. 2004a; Nicholson et al. 2004b), however this was not observed *in vivo*. Nonetheless this is the first time that a human Faslodex-resistant cell line has been described *in vivo* and additional transplants are currently ongoing within the lab to develop a better *in vivo* model of Faslodex resistance by maintaining Faslodex levels with injections.

In contrast to the c-FLIP protein expression data presented here, mRNA expression data (Affymetrix microarray) collected on TAMR and FASR cells indicated that c-FLIP transcript levels were actually slightly elevated in the TAMR and FASR cells (BCMP group, School of Pharmacy, Appendix IV). This suggests that c-FLIP in the TAMR cells may be regulated post-transcriptionally and the reduction in c-FLIP protein may be responsible for TAMR hypersensitivity to TRAIL. Previous studies have shown c-myc as a major regulator of c-FLIP protein (Ricci et al. 2004) and a key determinant in sensitivity to TRAIL, however, TAMR and FASR cells showed no increase in c-myc

transcript levels (BCMP group, School of Pharmacy, affymetrix data not shown) The mechanism underlying the decline in c-FLIP levels in TAMR cells remains undetermined; a hypothesis of this mechanism is discussed in chapter 6.

4.4 Summary

To summarize, work in this chapter suggests that TRAIL may prove to be a beneficial treatment for AE-resistant disease by killing both bulk tumour cells and bCSCs in long-term endocrine-resistant tumour cells. Furthermore in TAMR cells c-FLIP was once again implicated as a key regulator in this increased sensitivity to TRAIL, however, there was no correlation between c-FLIP expression and TRAIL sensitivity. Therefore endocrine-resistant cell line sensitivity to TRAIL is dependant on the agent to which the cells have become resistant to. Cell line models of endocrine resistance, such as TAMR and FASR, can be criticised on the basis that they have been developed from a single parental ER+ve cell line; attempts to address this have been made within this thesis by the inclusion of the EFM19 cell line and its tamoxifen-resistant counterpart, however, development and use of additional cell line models together with primary endocrine-resistant patient samples will help to uncover new therapeutic methods to overcome resistance. However, the data in this chapter extends and supports that of chapter 3, identifying TRAIL as a potential therapy in a broad range of breast cancer subtypes. Without the need for additional targeting of c-FLIP (and the associated complications of identifying pharmacological c-FLIP inhibitors) and in light of the fact that TRAIL is already progressing in clinical trials, TRAIL would seem more likely to be of immediate benefit to endocrine-

resistant patients, if confirmed *in vivo*. This represents a large cohort of patients, as AEs are still the mainstay therapy in premenopausal women and the understanding of TRAIL resistance in estrogen-deprived disease may also help to reveal future therapeutic strategies.

CHAPTER 5:

TRAIL's Role in γδ T cell Cytotoxicity to Tumour

5.1 Introduction

The human immune system has evolved in order to provide the body with its own internal defence mechanism that responds to foreign agents or stressed tissue that occurs on a daily basis. The immune system can be separated into two major subdivisions, the innate immune system and the adaptive immune system. In brief, the innate immune system is capable of a rapid response against foreign agents that gain access into the body and the adaptive immune system comprises of T and B lymphocytes, with antigen-specific receptors that recognise threats and produce a slower immune response but also expand and form memory cells capable of recognising and responding to the same antigen upon its next encounter.

There is evidence to show that the immune system naturally controls tumours (Smyth et al. 2006) and there are many strategies for immunotherapy, most of which target the adaptive responses of B cells and MHC-restricted $\alpha\beta$ $CD8^+$ T cells (Chhabra 2011). However sustainable responses are not common and as of yet immunotherapy of tumours is not fully established. One common escape mechanism used by tumour cells is the downregulation of MHC antigens (Bubenik 2003). There is a small subset of T cells carrying the $\gamma\delta$ T cell receptor (TCR) that make up approximately 1-5% of $CD3^+$ T cells which are not MHC-restricted and thus most $\gamma\delta$ T cells do not express CD4 or CD8. In a healthy individual, up to 90% of $\gamma\delta$ T cells typically use the TCR pair $V\gamma9V\delta2$ expressing the $V\delta2$ gene that pairs with the $V\gamma9$ chain (Hayday 2000). Originally $\gamma\delta$ T cells were shown to be activated by bacteria and parasites by non-peptidic phosphorylated intermediates of the non-melavonate pathway of bacterial isoprenoid biosynthesis such as (E)-4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMB-PP) and in

mammalian cells by phosphoantigens produced by the mevalonate pathway for isoprenoid biosynthesis such as isopentenyl pyrophosphate (IPP) (Viey et al. 2005). These intermediates stimulate the lytic activity of the cells whilst they also expand and release pro-inflammatory cytokines. Importantly, certain models have shown that non-transformed cells are incapable of producing high enough physiological concentrations of IPP to activate γδ T cells whereas tumour cells are (Gober et al. 2003). Furthermore synthetic aminobisphosphonate compounds, used in the treatment of osteoporosis and metastatic bone disease, are also capable of activating γδ T cells as they inhibit the IPP-processing enzyme farnesyl pyrophosphate synthase resulting in IPP accumulation (Kunzmann et al. 2000).

The relative specificity of cytotoxic activity of γδ T cells towards tumour cells and the potential use of already licensed aminobisphosphonates as their activators sparked interest in γδ T cells as a potential immunotherapy for cancer. The presence of γδ T cells in tumour infiltrating lymphocytes (TILs) (Chen et al. 2001) suggested these cells also home to tumour sites and prompted further investigation into their anti-tumour efficacy and indeed these cells are capable of inducing cell death in a broad range of tumours (Bank et al. 1993; Corvaisier et al. 2005; Groh et al. 1999; Maeurer et al. 1996). Additionally, the cytotoxic effects of γδ T cell immunotherapy have been shown in a range of cancers both *in vitro* and *in vivo* (Bank et al. 1993; Kabelitz et al. 2004; Liu et al. 2005). Correlations between patient survival and absolute number of blood circulating γδ T cells further support the evidence for γδ T cells and their anti-tumour activity.

Recently a study demonstrated that activated γδ T cells express TRAIL (Dieli et al. 2007), microarray studies within the Eberl group then supported this finding by identifying TRAIL as a gene that is differentially regulated between activated γδ T cells and activated $CD8^+$ cells (unpublished data). These studies suggest that TRAIL may contribute to the tumour cell cytotoxicity effects of γδ T cells. Therefore it is possible that γδ T cells express TRAIL at tumour sites in order to specifically target them for cell death without harming normal cells in the surrounding tissue.

In this chapter the ability of activated γδ T cells to produce TRAIL and their use as a targeted immunotherapy for breast cancer was addressed.

5.2 Results

5.2.1 Activation and expansion of human γδ T cells from healthy human donors.

As with many lymphocyte subsets for the γδ T cells to perform their cytotoxic function they must first be stimulated to do so. It is well accepted that one key activator is HMB-PP, produced during bacterial isoprenoid biosynthesis (Viey et al. 2005). Additionally, aminobisphosphonates (NBPs) have been shown to activate γδ T cells, these provide further therapeutic benefits and are already approved drugs in the clinic, thus NBPs are more desirable activators of the γδ T cells. To compare the activation capabilities of aminobisphosphonates to the natural stimulator HMB-PP, blood was drawn from healthy donors and γδ T cells were isolated as described in the Materials and Methods. The activation of γδ T cells was then assessed by flow cytometry for surface marker expression indicative of T cell activation.

As the natural stimulator of γδ T cells, HMB-PP stimulated 100% of the cells to express CD25 at concentrations as low as 10nM (Figure 5.2.1A). This level of expression could also be achieved using the aminobisphosphonates zoledronate and ibandronate at a concentration of 10μM but was only capable of achieving 80% CD25 expression. The weaker stimulation ability of aminobisphosphonates was then confirmed using a second marker for activation, HLA-DR; again a 1000-fold increase in zoledronate and ibandronate concentration was required to achieve similar levels of HLA-DR to HMB-PP stimulated γδ T cells (Figure 5.2.1B). Once stimulated, γδ T cells produced common T cell cytokines and cytolytic proteins. Increases in expression of both interferon-γ (IFNγ) and perforin were detected when γδ T cells became activated (Figure 5.2.1C), both of which are important for γδ T cell

cytotoxic function. Taken together these data successfully replicate that of other researchers in the field confirming that γδ T cells can be activated by aminobisphosphonates in order to carry out their cytotoxic functions.

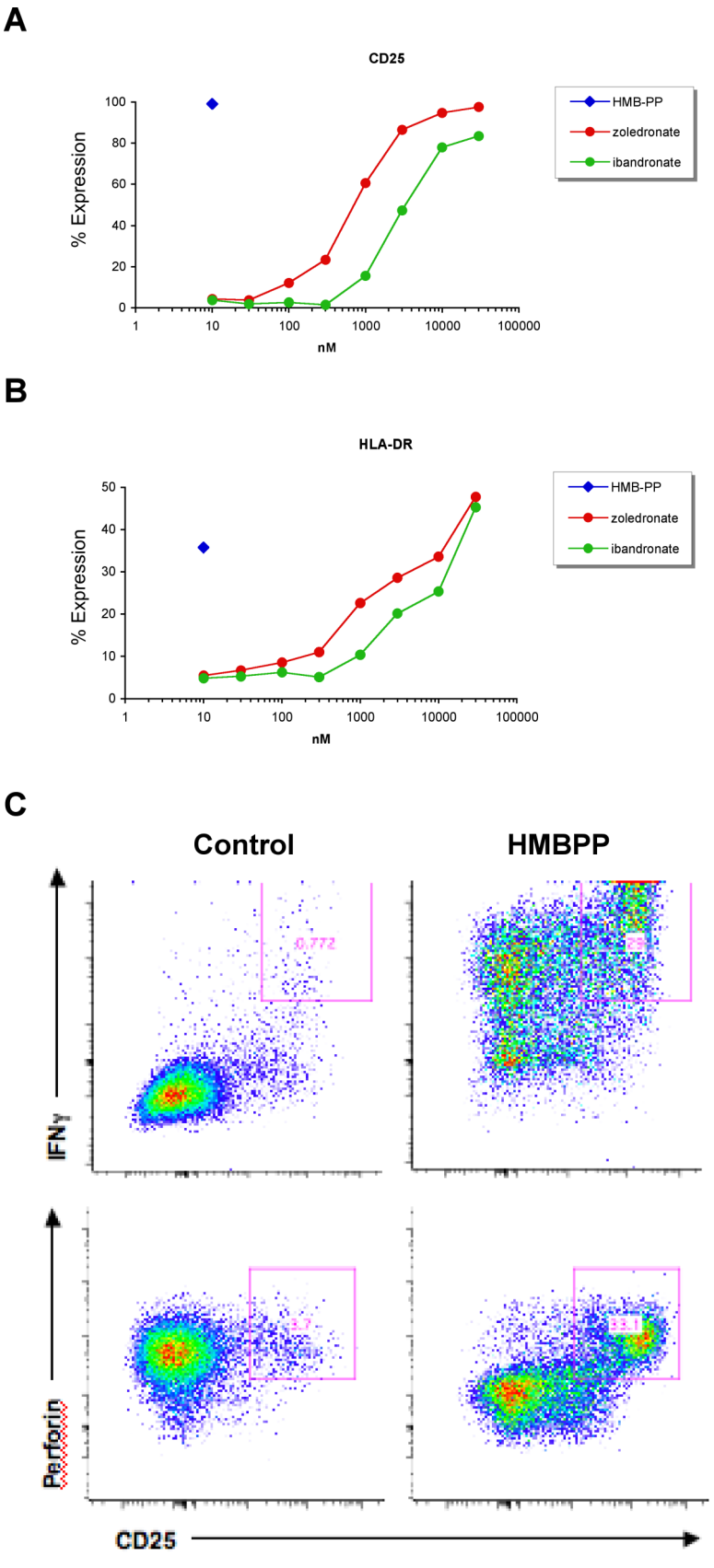

Figure 5.2.1: γδ T cells can be activated by HMB-PP and NBPs. γδ T cells isolated from healthy blood donors were cultured with increasing concentrations of HMB-PP zoledronate or ibandronate for 7 days and A) CD25 B) HLA-DR measured by flow cytometry. C) Representative FACS plots comparing unactivated and 10nM HMB-PP activated intracellular γδ T cell expression of IFNγ and perforin. Results are representative of 3 experiments performed in triplicate, using cells from 2 separate donors.

5.2.2 Activated γδ T cells have increased surface and intracellular TRAIL and secrete it into the surrounding supernatant

As previously mentioned, a recent study revealed TRAIL as being differentially expressed by activated Vγ9+ve cells in response to zoledronate (Dieli et al. 2007). To better understand this increase in TRAIL expression flow cytometry was used to assess intracellular and surface TRAIL expression in resting and activated γδ T cells. Additionally, TRAIL secretion was analysed by removing culture supernatants from resting and activated γδ T cells and measuring TRAIL and perforin by ELISA.

Activating γδ T cells using HMB-PP resulted in increased intracellular TRAIL expression, where almost all $CD25^+$ cells also expressed surface TRAIL compared to the resting γδ T cells in which less than 1% of the cells expressed TRAIL (Figure 5.2.2A). This TRAIL expression could also be achieved when activating γδ T cells using aminobisphosphonates but as previously demonstrated, much higher concentrations were required to achieve the same level of expression seen in HMB-PP activated γδ T cells (Figure 5.2.2B). Similarly, surface TRAIL levels also increased upon stimulation with HMB-PP (Figure 5.2.2C). Finally, culturing γδ T cells in HMB-PP or zoledronate increased the amount of TRAIL secreted into the culture supernatant (Figure 5.2.2D). However, this increase was only pico-molar, a far higher concentration than is typically required to induce cell death in TRAIL sensitive cells. Interestingly the amount of perforin secreted by activated γδ T cells was far greater, with concentrations of up to 40ng/ml being achieved when stimulated with HMB-PP. Collectively these data demonstrate that activating γδ T cells results in increased

TRAIL production in 3 different ways; intracellularly, on the cell surface and secretion.

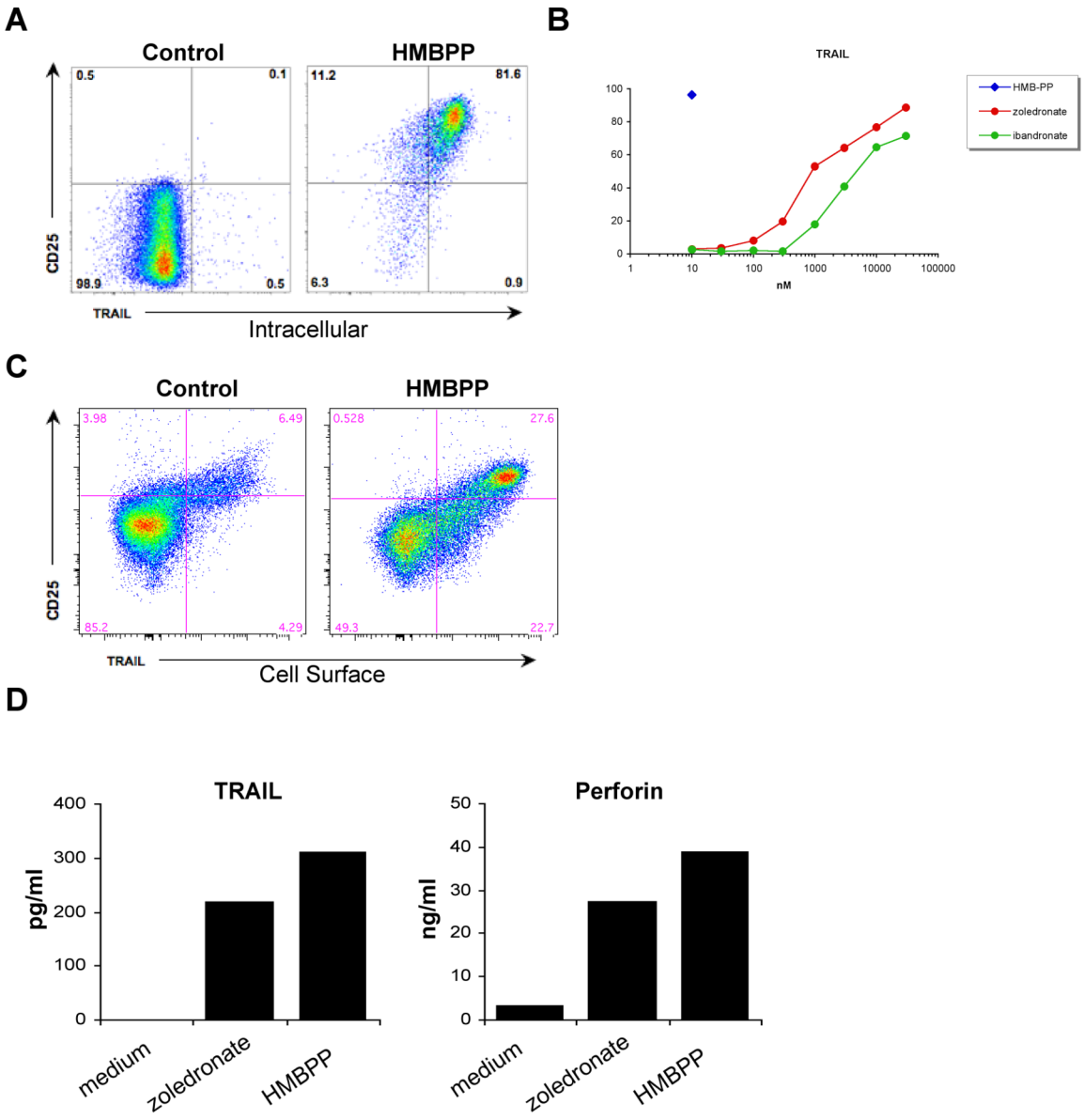

Figure 5.2.2: Activated γδ T cells express TRAIL and secrete perforin and TRAIL. *A*) Representative FACS plot of intracellular CD25 and TRAIL expression in unactivated and 10nM HMB-PP activated γδ T cells, isolated from healthy donors, permeabilised by brefeldin. B) Intracellular TRAIL expression in γδ T cells at increasing concentrations of HMB-PP, zoledronate and ibandronate. C) Representative cell surface expression of TRAIL in unactivated and 10nM HMB-PP activated γδ T cells. D) Unactivated (Media) and activated (either by 10nM HMB-PP or 100μM zoledronate) γδ T cell supernatant concentration of TRAIL and perforin. Results shown are representative of 2 individual experiments using cells from 2 independent donors.

5.2.3 Activated γδ T cell supernatants are cytotoxic to breast cancer cell lines

The cytotoxic effects of both TRAIL and perforin are well known and thus the secretion of these factors into the surrounding environment of the γδ T cells may contribute to tumour cell killing. The cytotoxicity of activated γδ T cell supernatants was assessed by adding them to breast cancer cell lines. Blocking antibodies/compounds targeted to TRAIL (RIK-2), perforin (concanamycin [CMA]) and caspase-8 (C8i) were then used to assess their contribution to any cell death observed.

Initially culture of breast cancer cell lines in activated γδ T cell supernatant (conditioned media) was performed. This resulted in significant reductions in cell viability in each of the cell lines irrespective of ER or HER2 status (Figure 5.2.3A, $p<0.05$). Using the MDA-MB-231 cell line for further investigation, it was clear that this cell death was, at least in part, through an apoptotic mechanism resulting in caspase-3/7 activation (Figure 5.2.3B). Significantly this apoptosis was also activated using conditioned medium (CM) from zoledronate-activated γδ T cells, in fact zoledronate CM resulted in similar levels of caspase-3/7 activation as the HMB-PP-activated supernatants. The reduction in viability seen in the MDA-MB-231 cells following CM treatment (Figure 5.2.3A) was then shown to be predominantly perforin mediated, as blocking perforin action in the CM using CMA returned cell viability back to that of cells cultured in media from unactivated cells. Perforin is capable of inducing cell death both using caspases or independently of caspases (Voskoboinik et al. 2006), blocking caspase-8 in activated γδ T cell CM, using IETD-FMK, also resulted in the recovery of cell viability back to control levels (Figure

5.2.3C) suggesting that the apoptosis initiated in these cells was caspase-8 mediated. It should be noted however that this family of caspase inhibitors exhibit some cross-reactivity with other caspases, and therefore it is possible that other caspase pathways may be involved in this process. A series of controls were also used to confirm that the perforin blocking compound CMA was not inhibiting any apoptosis induced by TRAIL. Here, the efficient blocking of TRAIL by the RIK-2 antibody, but not by CMA, was observed (Figure 5.2.3D). Additionally, a small amount of caspase-3/7 activation was observed from unactivated supernatants compared to cells cultured in normal complete media, signifying a resting secretion of apoptosis-inducing factors by γδ T cells. Together these results indicate that activated CM induces cell death in breast cancer cells predominantly by a perforin and/or a caspase-8-mediated apoptosis and TRAIL may only contribute in small part to this cell death, consistent with the significant difference in concentrations observed in activated γδ T cell supernatants in Figure 5.2.2D.

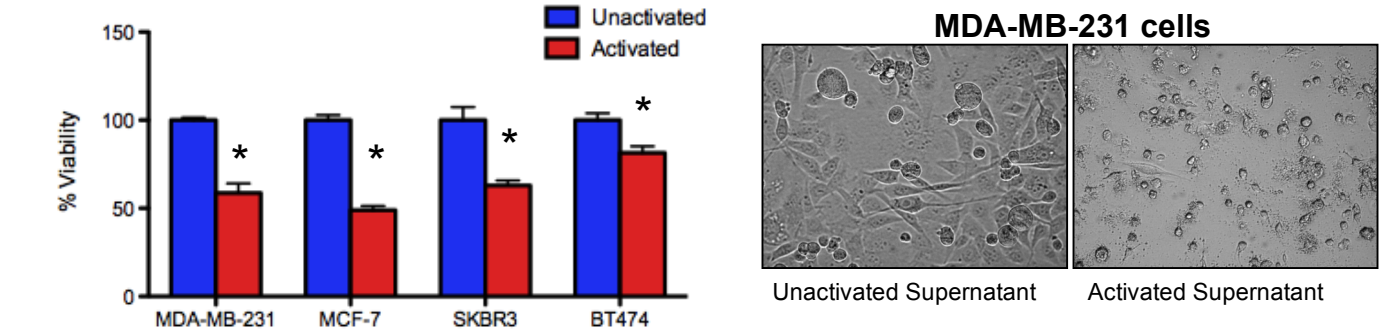

Figure 5.2.3: Supernatants from activated γδ T cells are cytotoxic to BCCLs. A) Breast cancer cell line viability, as measured by CTB assay, after treatment with unactivated or 10nM HMB-PP activated γδ T cell CM and representative phase contrast image of the visible cell death in MDA-MB-231 cells, * $p<0.05$. B) Caspase-3/7 activity in MDA-MB-231 cells following treatment with γδ T cell, activated with either 10nM HMB-PP or 100μM zoledronate, supernatants. C) MDA-MB-231 cell viability following 10nM HMB-PP activated γδ T cell supernatant pre-treated with blocking compounds, * $p<0.01$. D) Caspase-3/7 activation in MDA-MB-231 cells treated by unactivated γδ T cell CM, media or TRAIL in the presence RIK-2 or CMA blocking compounds. Data shown from 3 independently performed experiments performed in triplicate ± SEM using cells from 2 individual donors.

5.2.4 Co-culturing γδ T cells with breast cancer cell lines

Secreted factors from activated γδ T cell cultures appear to induce apoptosis in breast cancer cells but much of T cell function is mediated through cell to cell contact (Janeway 2001). Thus activated γδ T cells were co-cultured together with bCCLs at different effector:target cell ratios and cell death assessed in the MDA-MB-231 and MCF-7 cell lines by flow cytometry.

A significant increase in cell death was observed for both the MDA-MB-231 and MCF-7 cell lines when cultured at an effector:target cell ratio of 1:1 with γδ T cells (Figure 5.2.4A). Not surprisingly this cell death induced by the γδ T cells increased as effector:target cell ratio increased, with up to 45% cell death being observed in both cell lines at the 25:1 ratio (Figure 5.2.4A, *Donor 1*). Using γδ T cells taken from a different donor a similar cell death profile was observed in the MCF-7 cell line and, whilst still a significant cell death, a lesser effect was seen in the MDA-MB-231 cell line (Figure 5.2.4B, *Donor 2*). Although it seems γδ T cells from different sources are consistently cytotoxic to breast cancer cells, this implies different γδ T cell subsets between patients may display different cytotoxic efficiency on different subtypes of breast cancer. The contribution of TRAIL, perforin and caspase-8 activation to the co-culture induced cell death was then assessed by the use of different blocking antibodies/compounds. The results demonstrate that little to none of the killing of the TRAIL-sensitive MDA-MB-231 cell line was actually induced by TRAIL itself (Figure 5.2.4B), despite the significant expression of TRAIL on γδ T cell surface after activation (Figure 5.2.2). It appears that the majority of cell death observed in these co-cultures is a result of perforin expression/production as blocking perforin with CMA resulted in a significant reduction of cell death in the 5:1 and 25:1 effector:target

ratios (Figure 5.2.4C). Notably, inhibiting caspase-8 at these ratios also significantly reduced cancer cell death, suggesting caspase-8 activation is important in the γδ T cell induction of cancer cell death.

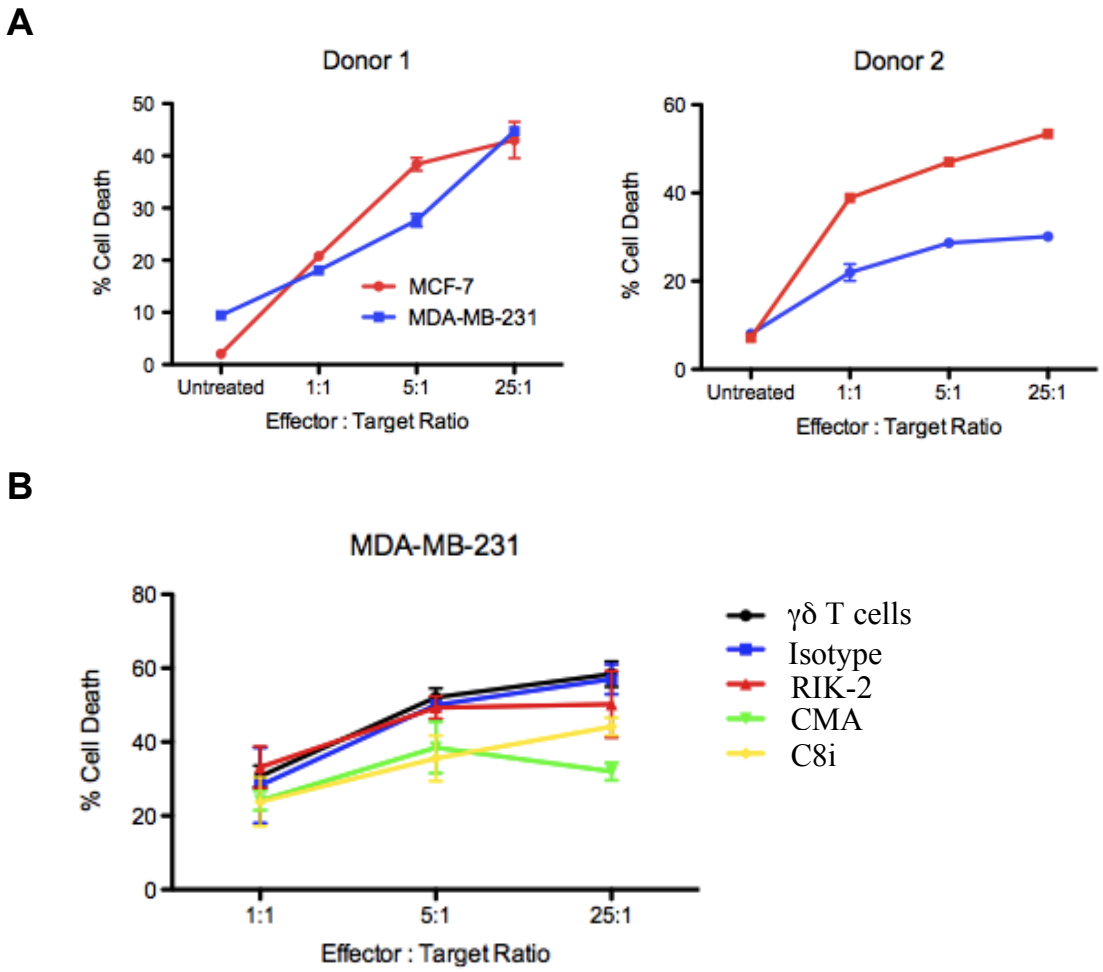

Figure 5.2.4: Activated γδ T cells are cytotoxic to BCCLs in co-cultures
A) Percentage cell death, as measured by fixable live/dead assay, of MDA-MB-231 and MCF-7 cell lines co-cultured for 18 hours with expanded γδ T cells at increasing effector:target ratios from two separate donors. B) Percentage cell death of MDA-MB-231 cells, as measured by fixable live/dead assay, after co-culture with expanded γδ T cells in the presence of blocking compounds. Data shown is representative of triplicate experiments ± SEM.

4.2.5 c-FLIP suppression increases γδ T cell induced cell death

As demonstrated in previous chapters and many other published studies, c-FLIP is a key regulator of TRAIL cell death and a major determinant of cancer cell sensitivity to TRAIL (Lee et al. 2006; Palacios et al. 2006; Ricci et al. 2004). Hitherto it has been demonstrated that TRAIL secretion and expression by activated γδ T cells plays a very minor role in their cytotoxic effects on bCCLs (Figure 5.2.3 + 5.2.4). Thus the approach adopted in Chapter 3 was adopted in an attempt to increase TRAIL's participation in γδ T cell cytotoxicity by suppressing c-FLIP in cancer cells using siRNA (FLIPi) and then co-culturing these cells with γδ T cells at different effector:target ratios. As in Figure 3.2.5A, MDA-MB-231 FLIPi and SCi cells were stained with PKH26 and PKH67, mixed at a 1:1 ratio and then γδ T cells added to the culture at 1:1, 5:1 and 25:1 effector:total target cell ratios for 4 hours (as shown in appendix III) and cell death of each cell population then assessed by fixable live/dead assay.

The knockdown of c-FLIP in FLIPi cells when cultured with γδ T cells resulted in a significant increase in cancer cell death compared to the cancer cell death of the SCi cells (Figure 5.2.5A). Successful sensitisation of the MDA-MB-231 cells was demonstrated by treating SCi and FLIPi cells with TRAIL (Figure 5.2.5A, *bar graph*), however, over the 4 hours treatment, FLIPi gave no increase in cell death indicating the increase in cell death observed in co-cultures with γδ T cells is due solely to their cytotoxic effect on the cells. This increased cytotoxic effect was then duplicated using γδ T cells from a second donor (Figure 5.2.5B, *red and blue lines*). In the same experiment γδ T cells were also pre-incubated with RIK-2, CMA and caspase-8 inhibitor to determine the contribution of TRAIL, perforin and caspase-8 mediated apoptosis respectively. As previously demonstrated in Figure

5.4.4 blocking TRAIL had no significant effect on γδ T cell killing of MDA-MB-231 cells (Figure 5.2.5B, *Blue and green lines*). TRAIL blocking also had no effect on the increased cell death seen in the FLIPi cells (Figure 5.2.5B, *red and orange lines*) signifying the increase observed was not due to a sensitisation to TRAIL. Similar results were obtained when using CMA to block perforin, a reduction in γδ T cell killing was observed in the SCi cells, however, CMA did not block the additional death seen in the FLIPi cells. This suggested that the additional death incurred from loss of c-FLIP expression was not an increase in perforin-mediated killing from the γδ T cells, nor from a TRAIL-mediated kill. Interestingly inhibiting caspase-8 was capable of reducing the additional cell death in the FLIPi cells, thus the additional cell death in the FLIPi cells may be via a TRAIL-independent caspase-8-mediated apoptotic signal (Figure 5.2.5B).

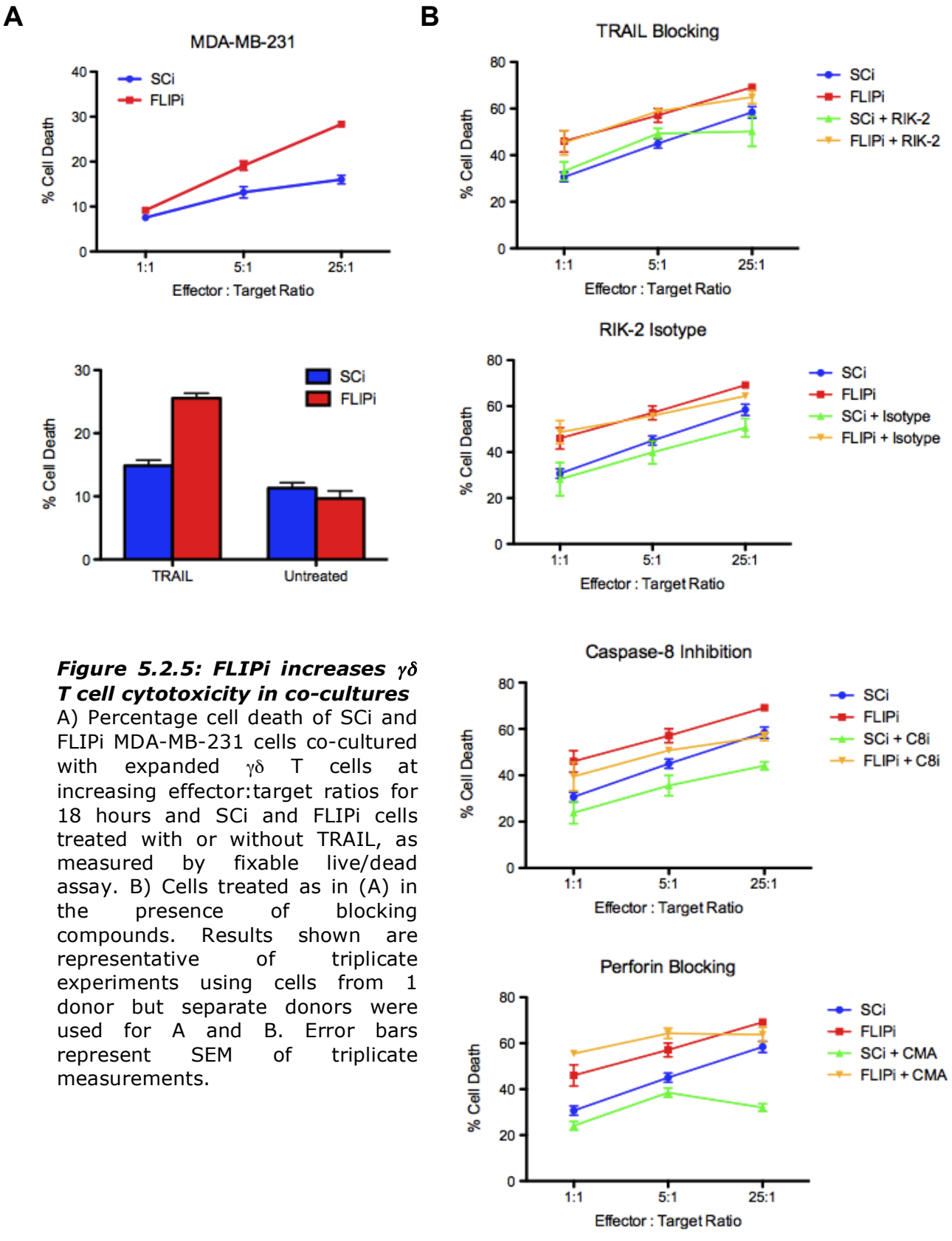

Figure 5.2.5: FLIPi increases γδ T cell cytotoxicity in co-cultures
A) Percentage cell death of SCi and FLIPi MDA-MB-231 cells co-cultured with expanded γδ T cells at increasing effector:target ratios for 18 hours and SCi and FLIPi cells treated with or without TRAIL, as measured by fixable live/dead assay. B) Cells treated as in (A) in the presence of blocking compounds. Results shown are representative of triplicate experiments using cells from 1 donor but separate donors were used for A and B. Error bars represent SEM of triplicate measurements.

5.2.6 γδ T cells target the bCSC subset

A common theme throughout this study has been the importance of targeting the bCSC subset in therapeutic approaches as it can have significant implications in long-term disease management (Charafe-Jauffret et al. 2009; Ginestier et al. 2007; Pardal et al. 2003; Phillips et al. 2006). Accordingly the cytotoxic effect of γδ T cells on the bCSC subset was assessed using the mammosphere assay to determine the MFU capacity of cancer cells following co-culture with γδ T cells.

MCF-7, TAMR, FASR and MDA-231 cells were co-cultured with γδ T cells and the surviving cancer cell population then plated into non-adherent low serum conditions. The MDA-MB-231 cells maintained their MFU capacity irrespective of the effector:target ratio used in treatment (Figure 5.2.6). Contrastingly the MFU capacity of MCF-7 cells was halved after co-culturing with γδ T cells and the MFU capacity of the endocrine-resistant cell lines TAMR and FASR was completely abolished following treatment at a 25:1 effector:target ratio (Figure 5.2.6).

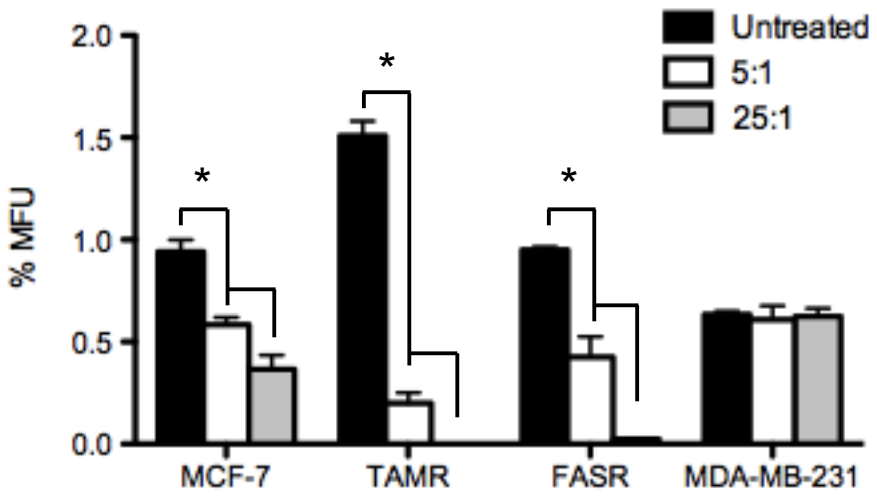

Figure 5.2.6: Activated γδ T cells are cytotoxic to the MFU population of some breast cancer cell lines. Breast cancer cell lines were treated with expanded γδ T cells at different effector:target ratios and the surviving cancer cell population seeded into mammosphere culture conditions for 7 days and MFUs counted. Results shown are from a single experiment performed in triplicate using cells from a single donor, * $p<0.05$.

5.3 Discussion

The capacity of the immune system for specificity makes it an appealing therapeutic option for cancer. This was first demonstrated by the clinical success of mAbs in the treatment of breast cancer and lymphomas. This brought about the interest in T cell-based immunotherapy with their capability to recognise peptides derived from protein in any cellular compartment. There are now several clinical studies supporting immunotherapy as a realistic therapeutic approach and thus development and application of immunotherapy has attracted much attention over the last decade. Augmentation of immune response can be achieved *in vivo* by stimulatory compounds and *ex vivo* expansion of effector cells followed by adoptive transfer. The end result of each is a population of activated effector cells that recognise a target population of cells for destruction (reviewed in (Restifo et al. 2012)) of course, *ex vivo* stimulation of effector cells will ultimately result in behavioural differences in the cells as the *in vivo* environment could never be accurately recapitulated *in vitro*. Despite these differences each of these two modalities have their benefits and show some promise as a methodology for cancer treatment.

One particular subset of T cells that has shown promise as an immunotherapy for many different types of cancer is the γδ T cells (Beck et al. 2010; Chargui et al. 2010; Dieli et al. 2007; Gomes et al. 2010; Todaro et al. 2009). These T cells appear to expand and respond to cellular antigens frequently displayed on cells that have undergone malignant transformation, especially those of epithelial origin. Additionally, γδ T cells have been documented as being present amongst tumour-infiltrating lymphocytes (TILs) (Kabelitz et al. 2007). The aim of this chapter was to build on existing studies that have

demonstrated the cancer cell cytotoxicity of γδ T cells whilst also determining the contribution of TRAIL and other cytotoxic factors to this killing.

5.3.1 γδ T cells are cytotoxic to breast cancer cells

Culturing γδ T cells with breast cancer cells has previously been demonstrated to induce cancer cell death, however, much focus of that research was surrounding the antigens recognised by the γδ T cells and their activation. Data presented here supports these observations and elucidates the contribution of different cytotoxic molecules to the well-documented cancer cell cytotoxicity of γδ T cells. Primarily, the activation and expansion of γδ T cells was shown to result in the increased expression of TRAIL, IFNγ and perforin all of which are known to contribute to cancer cell death. IFNγ itself is a key cytokine in anti-tumour immune responses; the immunostimulatory function of IFNγ helps augment the correct immune response at the site of the tumour (Restifo et al. 2012). As shown in Figure 5.4.3, once γδ T cells are activated they secrete soluble factors such as TRAIL and perforin. In breast cancer it would seem that the majority of the apoptosis induced by γδ T cells is being induced by perforin (Figure 5.2.3C). Not surprisingly this is consistent with the fact that the activated γδ T cells secreted far higher concentrations of perforin than TRAIL. Whilst inhibiting caspase-8 with the IETD-FMK compound also resulted in blocking γδ T cell supernatant cancer cell death. Although it should be noted that this compound is also capable of inhibiting granzyme B. Accordingly, it's inhibitory effect may be most likely explained through its role in preventing perforin-mediated killing, however, this does not mean the contribution of caspase-8 mediated cell death should be ignored, even though TRAIL appears to contribute little to the

supernatant cell death, effects of other death receptors, such as CD95, can not be discounted.

Despite the low concentrations of secreted TRAIL from activated γδ T cells, the close proximity of these cells to their target cancer cells allows both cell contact with TRAIL expressed on the cell surface and a higher local concentration of TRAIL in the surrounding microenvironment. In spite of this, TRAIL appeared to play no significant role in cancer cell death induced in co-cultures with γδ T cells (Figure 5.2.4B). Again the predominant killing mechanism seemed to be perforin-mediated. In chapter 3 it was demonstrated that suppression of c-FLIP protein gave significant sensitisation of breast cancer cell lines to TRAIL, both in bulk tumour cells and bCSCs. Therefore c-FLIP protein levels were also suppressed in MDA-MB-231 cells in attempt to sensitise the cancer cells to TRAIL being delivered via γδ T cells. Although a significant sensitisation to γδ T cell killing was observed this was not as a result of TRAIL sensitisation (Figure 5.2.5B). Even though CMA inhibited the γδ T cell killing of SCi cells it was not capable of blocking the increased killing observed in FLIPi cells. Interestingly the only blocking agent capable of preventing the increased cell death in the FLIPi cells was the caspase-8 inhibitor. This may suggest that FLIPi sensitised the cancer cells to an alternative caspase-8 mediated cell death such as CD95. However, further work is required to confirm exactly what is responsible for the increased cell death detected in the FLIPi cells.

Significantly, the γδ T cells could also be activated using aminobisphosphonates (NBPs). The concentration of zoledronate (ZOL) and ibandronate required for achieving similar stimulation as using HMB-PP is much higher, however there are several advantages of using NBPs as a stimulatory compound. NBPs inhibit bone resorption

by osteoclasts and are a common treatment for bone-loss disorders in postmenopausal osteoporosis (Delmas 2002) and cancer treatment induced bone loss (Clezardin 2011). In breast cancers a common site of metastasis is bone, consequently there is currently clinical trials being performed to assess the advantage of ZOL treatment in preventing bone metastasis in patients with advanced breast cancer. The results in this chapter appear to show TRAIL as a small/non-contributor to activated γδ T cell induced cell death, however these assays were performed using HMB-PP stimulated γδ T cells. A recent study has shown that treatment of breast cancer cells with ZOL increased the TRAIL:OPG ratio in MDA-MB-231 cells (Rachner et al. 2009). Thus such a pre-treatment may increase γδ T cell killing, not only by increasing TRAIL expression but also chemotaxis of γδ T cells to the cancer cells (Benzaid et al. 2011).

5.3.2 Potential of γδ T cells as a therapy for breast cancer

Ex vivo expansion and stimulation of γδ T cells for adoptive transfer therapy has previously been demonstrated in mice (Beck et al. 2010). Here *ex vivo* expanded and labelled γδ T cells were well tolerated by the recipient mice and readily localized to tumour sites, supporting previous observations of tumour homing in prostate cancer (Liu et al. 2008). Stimulating γδ T cells *ex vivo* allows the use of compounds that may otherwise produce harmful side effects if given systemically, additionally, γδ T cells may be labelled as described in Beck et al. 2009 to follow their chemotaxis and potentially aid tumour tracing. Thus the potential for *ex vivo* expanded γδ T cell therapy may be a realistic target in human disease. There have now been a number reports of adoptively transferred γδ T cells in humans to treat various forms of cancer (Bennouna et al. 2008; Kobayashi et al. 2011;

Kobayashi et al. 2007; Sakamoto et al. 2011). Overall these studies demonstrated adoptive transfer of *ex vivo* expanded γδ T cells for systemic infusion is well tolerated but that objective responses are uncommon. In general the cohort size for these studies was small and thus further investigation is still required determine if adoptive transfer γδ T cell immunotherapy is a realistic therapeutic modality.

In spite of TRAIL's seemingly minimal contribution to γδ T cell killing of breast cancer cells it is interesting that breast cancer cell line MFU forming capacity following γδ T cell co-culture closely replicated that seen by treating these cell lines directly with TRAIL. As shown in chapter 4, TAMR and FASR MFUs are hypersensitive to TRAIL treatment with a partial response being observed in the MCF-7 cells. One explanation could be that although TRAIL production by γδ T cells is not important in bulk tumour cell killing it may be contributing to the reduction in MFU capacity of these cell lines, however these results are very preliminary and further assessment of TRAIL's contribution is required.

The importance of using currently approved drugs for disease treatment cannot be understated, in 2003 a survey by DiMasi et al revealed that the average cost of developing a drug to the point of marketing is approximately 802 million dollars (DiMasi et al. 2003). Using ZOL as a stimulatory compound for γδ T cells is therefore extremely desirable as the patent for Zometa (zoledronic acid [Novartis]) expires in March 2013 and will ultimately result in a reduction in cost. Future prospects could even incorporate a combination of FLIPi, TRAIL and ZOL for breast cancer, attacking breast cancer cells from multiple angles whilst hopefully maintaining relatively minimal side effects.

5.4 Summary

In summary, activating γδ T cells may be a beneficial therapeutic approach in breast cancer sufferers due to their apparent cytotoxic effect on, and recognition of, cancer cells. Stimulation of these cells using aminobisphosphonates may also have multiple benefits for patients. Although it would seem TRAIL contributes little to these cytotoxic effects and the majority of cancer cell death is being induced via a perforin-mediated mechanism, it is possible that TRAIL's contribution is more significant in the reduction of bCSCs. Further work should focus on the γδ T cell killing on the bCSC subset and the investigation of γδ T cell adoptive transfer as a potential therapeutic approach for breast cancer patients.

CHAPTER 6:

General Discussion

6.1 General discussion

Over the past decade many advances have been made in breast cancer disease management. Significant improvements in survival rates clearly represent these advances with 90% of stage I, 70% of stage II, 50% of stage III and 13% of stage IV patients surviving at least five years following diagnosis (Cancer Research UK). Much of this can be attributed to improvements in surgical techniques and equipment. Subsequent therapy following surgery has remained fairly consistent with the main therapeutic interventions being Herceptin (trastuzumab), anti-hormones (tamoxifen, fulvestrant and aromatase inhibitors) and chemo/radio-therapy. Each of these therapies represents an important leap forward in breast cancer treatment, however, there are also pitfalls for each of these modalities including *de novo* resistance, acquired resistance and off-target toxicity. Herceptin has been shown to potentially have severe side effects on the heart, many patients develop resistance to endocrine therapies, and many patients see little to no clinical benefit whatsoever from radio- or chemo-therapy whilst experiencing the severe side effects that accompany the treatment (Goldhirsch et al. 2005; Mauri et al. 2005; Shimizu et al. 2007). Recently many studies focussing on the breast cancer stem cell subset have elucidated it as being responsible for tumour initiation and seeding of metastasis. Furthermore bCSCs have been shown to be particularly resistant to therapeutic, in particular radio- and chemo-therapy, challenge and thus remain following treatment, resulting in disease relapse (Eyler and Rich 2008; Li et al. 2008). TRAIL is a naturally existing death-inducing ligand that displays tumour specificity with almost no side effects on normal cells (Ashkenazi et al. 1999). However, TRAIL is currently not a suitable

therapy for breast cancer as most breast cancer cells are resistant to TRAIL treatment (Rahman et al. 2009a).

Clearly there are specific cohorts of patients that require novel therapeutic intervention techniques including those diagnosed with later stage disease and those that have become resistant to existing therapies. The key to controlling disease progression in these patients is prevention of metastasis. With this in mind, using cell lines as *in vitro* and *in vivo* models that represent the heterogeneic nature of breast cancers, the aims of this study were to (1) determine if TRAIL has potential as a treatment for breast cancer (2) Assess the extent of any treatment that proves successful on the inter- and intra-tumour heterogeneity that exists between breast cancers (3) uncover alternative delivery methods of TRAIL to breast tumours.

6.1.1 Sensitising breast cancer cells to TRAIL

Previous studies have demonstrated a correlation between the regulation of c-FLIP expression and sensitivity to TRAIL. In one of these studies it was also shown that reducing c-FLIP expression using siRNA successfully sensitised certain breast cancer cell lines to TRAIL. However, the extent of this effect on the heterogeneity within breast cancer was not fully elucidated in this study (Palacios et al. 2010). This thesis revealed that suppressing c-FLIP by siRNA sensitises a range of breast cancer cell types to TRAIL whilst maintaining the tumour cell specificity of the treatment. The relative specificity of c-FLIP suppression in sensitising only tumour cells to TRAIL was limited to differences between normal mouse epithelial cells and cancer cell lines and thus was not fully investigated in this thesis. The fact that knocking out c-FLIP in animals is embryonic lethal suggests that it does play a key role at least in development, more specifically in

vascularisation (Varfolomeev et al. 1998; Yeh et al. 1998; Yeh et al. 2000; Zhang et al. 1998). In chapter 3 of this thesis c-FLIP was conditionally deleted in luminal epithelial cells of the normal mouse mammary gland without sensitising them to additional TRAIL treatment, in support of this data the non-tumourgenic Eph4 normal mouse mammary epithelial cell line also did not respond to TRAIL following c-FLIP suppression. However, for clinical translation of combined FLIPi/TRAIL treatment a drug suppressing c-FLIP would need to be produced and evaluated for systemic toxicity in animal models. Such a drug would not specifically inhibit c-FLIP in mammary tissue and at present the effects of FLIPi/TRAIL on other normal tissues are unknown.

There are currently compounds that are capable of providing some c-FLIP suppression, however, these compounds are not c-FLIP-specific inhibitors and come with a host of additional side effects. Despite this, the results observed in Figures 3.2.6, 3.2.12 and 3.2.13 suggest that combining these compounds with TRAIL may be a valid therapeutic approach for targeting the bCSC subset. Whilst targeting bCSCs has been demonstrated in chapter 3 (Figure 3.2.12) and in other studies to drastically reduce tumour initiation and metastasis, cells outside the bCSC pool display cellular plasticity and acquire CSC attributes (Figure 3.2.13). Thus tumour relapse even following the ablation of the bCSCs would seem inevitable. Although subsequent bCSCs still remained sensitive to repeat FLIPi/TRAIL treatment (Figure 3.2.13), from a clinical perspective it could be hypothesised that a continuous relapse/treatment cycle is only going to increase the likelihood of eventual resistance and disease progression. As a consequence a more pertinent therapeutic strategy would be to prevent this cellular plasticity displayed by surviving non-CSCs as

demonstrated by maintaining suppressed c-FLIP levels (Figure 3.2.14). Interestingly, c-FLIP has been shown to interact with various important signalling pathways outside of death receptor apoptosis. Interactions have been demonstrated with Akt, NF-κB and Wnt suggesting that c-FLIP is not limited solely to its anti-apoptotic function but also cell survival, proliferation, differentiation and cell fate (Figure 6.1.1). Furthermore Akt, NF-κB and Wnt have all been implicated in cancer stem cell maintenance (Korkaya et al. 2009; Shostak and Chariot 2011; Takahashi-Yanaga and Kahn 2010). In many cases, changing these signalling pathways also results in a reduction, but not the elimination, of MFUs. It is plausible, therefore, that by maintaining suppressed c-FLIP expression each of these signalling pathways is being regulated to cooperatively prevent cellular plasticity. Additional experiments using activators of each of these signalling pathways (such as the Wnt activator 6-bromoindirubin-3'-oxime [BIO] and the Akt activator 2-deoxyglucose [2-DG]) will help reveal the importance of their regulation by c-FLIP in cellular plasticity.

Further characterisation of these observations in preclinical models will be key in realising the potential translation of FLIPi/TRAIL to the clinical setting. Using the bicistronic KRAB doxycycline inducible c-FLIP construct currently being developed in the lab would allow the switching on and off of c-FLIP expression *in vivo*. Thus tumour cells containing this construct could be transplanted into animals and following tumour establishment, c-FLIP could be suppressed and TRAIL subsequently administered systemically. This approach could also be applied to primary tissue collected from breast cancer patients, cancerous and normal, *in vitro* and *in vivo*. These models would help determine normal human cell and stem cell response to FLIPi/TRAIL and better mimic the clinical treatment regime, although more

selectively and may provide important insight into FLIPi/TRAIL as a therapeutic strategy.

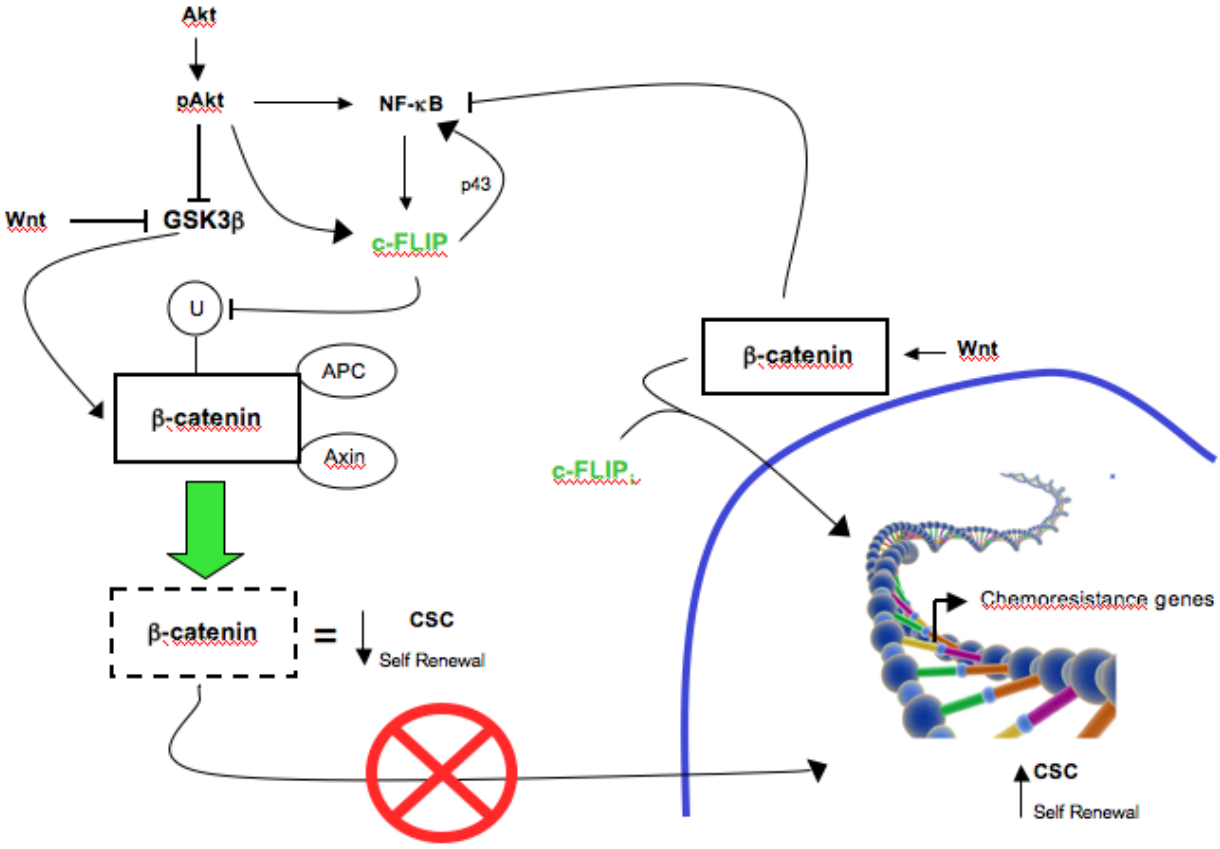

Figure 6.1.1 – proposed c-FLIP regulation of CSCs by non-apoptotic intracellular signalling pathways.

Breast cancer is a heterogeneous disease and as such the efficacy of many current therapies is limited to specific subtypes of disease. It is therefore encouraging to see the breadth of response seen to FLIPi/TRAIL on disease subtypes. Although existing anti-estrogen agents can give significant positive clinical responses in ER+ve breast cancer, acquired resistance to existing treatments limits the timeframe these agents are efficient for. Some response to

alternate endocrine agents can be seen in anti-estrogen-resistant disease but ultimately in many cases further resistance occurs. As a result there is a desire to identify novel therapies to which these resistant cancers may respond. It was hypothesised that the effects of FLIPi/TRAIL treatment could be extended to endocrine-resistant breast cancer. Early observations revealed that the TAMR and FASR cells were hypersensitive to TRAIL treatment alone without the need for FLIPi (Figure 4.2.1). Further investigation revealed that this hypersensitivity was also seen in the self-renewing mammosphere forming population (Figure 4.2.2). This had significant implications during the *in vivo* treatments where complete regression of the TAMR and FASR cells was seen following systemic TRAIL treatment (Figure 4.2.7). Importantly, in these experiments TRAIL was administered by intraperitoneal injection rather than co-injection with the tumour cells, as in chapter 3 (Figure 3.2.12), suggesting FLIPi/TRAIL as a combination treatment for breast cancer may also be successful using systemic injections. Furthermore the response seen in the human primary tumour cells tested in Figure 4.2.10 also suggest that TRAIL treatment of primary cancer cells may also result in significant cell death. Of course further experimentation would be required to confirm such observations in these preclinical models of anti-estrogen-resistant breast cancer. Advances are now being made at Cardiff University to obtain consecutive primary human tissue samples from endocrine-resistant patients and the *ex vivo* use of this tissue in culture and in transplantation models will help to provide further insight. However, what would seem clear from the data collected thus far is that the hypersensitivity observed was a result of the long-term acquired resistance independent of ER status and not a sensitisation through combination treatment. Interestingly the most sensitive anti-estrogen-

resistant cell line, the TAMR cells, but not the FASR cells, showed decreased c-FLIP protein levels. This may be the reason TAMR cells show sensitivity to TRAIL. This is not the first time drug resistant cells have been shown to display increased sensitivity to TRAIL (Kim et al. 2011; Mitsiades et al. 2001). In a study of multi-drug resistant MCF-7 (MCF-7-MDR) cells, c-myc was upregulated and c-myc has also been shown to regulate c-FLIP expression, although c-FLIP's role in MCF-7-MDR cell sensitivity to TRAIL was not addressed (Kim et al. 2011). Significantly, TAMR cells were incapable of MFU re-acquisition following TRAIL treatment and the reduced c-FLIP protein levels in TAMR cells (Figure 4.2.6) correlate with results seen in Figure 3.2.14 where low c-FLIP protein levels prevent the re-acquisition of MFUs. Conversely the FASR cell line, where c-FLIP levels were not reduced, was capable of re-acquiring MFUs. Further investigation of c-FLIP's role in cellular plasticity may help to uncover additional benefits of c-FLIP inhibition in breast cancer.

Affymetrix data collected from the Gee lab (School of Pharmacology, Cardiff University) (Appendix IV) revealed no reduction in c-FLIP transcript levels in either of the TAMR or FASR cells compared to their parental MCF-7 cells signifying c-FLIP protein levels may be being regulated by a post-transcriptional mechanism (Appendix IV). Previous studies have shown that c-FLIP$_L$ can be proteasomally degraded by Itchy E3-ligase (ITCH) following TNFα activation of JNK (Chang et al. 2006). A hypothesis was formed on this basis of c-FLIP post-transcriptional regulation in TAMR cells and is summarised in Figure 6.1.2. Consultation of the Affymetrix data supported this hypothesis showing that ITCH, TNFα and MEKK1 are overexpressed in TAMR cells but not in FASR cells and JNK expression was elevated in both TAMR and FASR cells (Appendix IV). This has lead to the

formulation of the hypothesis that c-FLIP protein may be regulated through TNFα signalling that activates JNK, via MEKK1 activation, which in turn ubiquitinates c-FLIP through ITCH leading to its degradation as depicted in Figure 6.1.2.

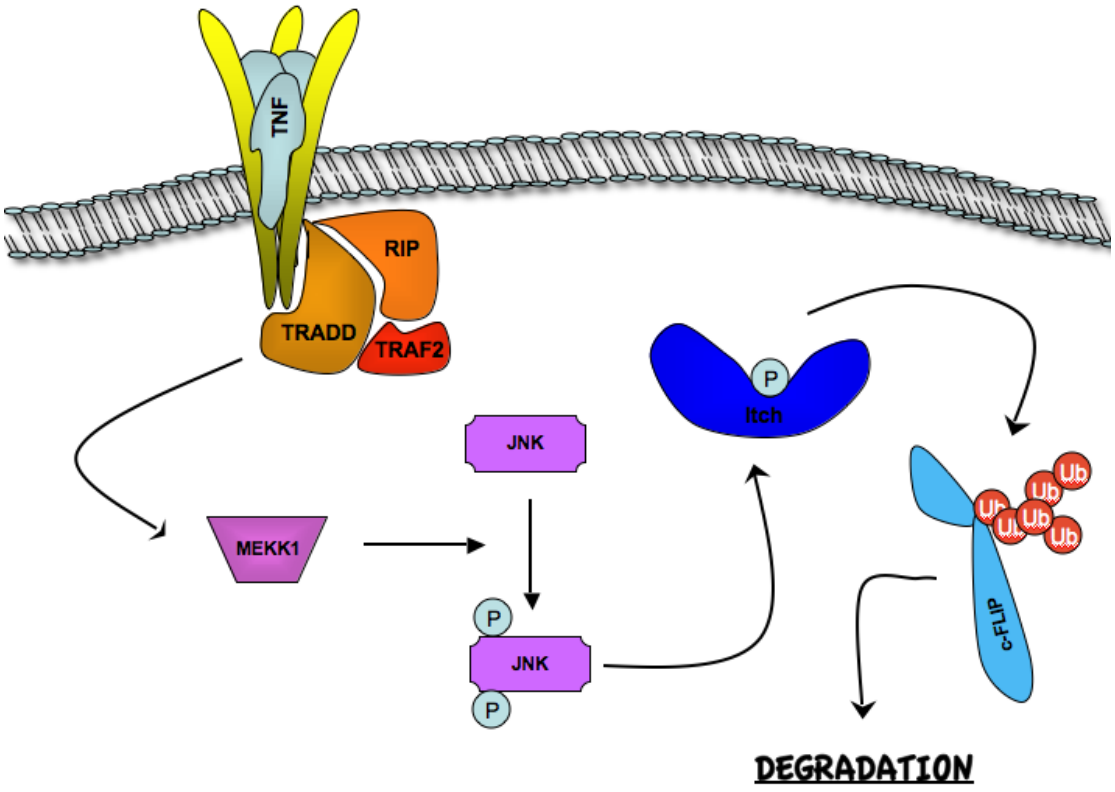

Figure 6.1.2: Hypothesised post-transcriptional regulation of c-FLIP in TAMR cells. TNFα signalling results in MEKK1 mediated phosphorylation and subsequent activation of JNK. This activates Itch that ubiquitinated c-FLIP protein targeting it for degradation.

Although FASR cells also have increased JNK mRNA expression, the comparably low levels of TNFα and MEKK1 could mean there is no observable increase in JNK activation. Additionally the FASR cells show a small decrease in ITCH expression, again inhibiting the hypothesised pathway from degrading c-FLIP in these cells. At this point this regulatory mechanism is speculation and does not explain FASR sensitivity to TRAIL. This is a testable hypothesis as future work will

first focus on confirming the relative levels of phosphorylated and native forms of these constituent proteins, and if consistent with the model of regulation, the siRNA (or pharmacological) inhibition of each component of the pathway will be performed to functionally verify its role in c-FLIP degradation. FASR cells did show an increase in DR4 expression (Figure 4.2.6), a mechanism that has been previously reported to increase TRAIL sensitivity (Gomez-Benito et al. 2007) and additionally DR4 has now been shown to be the functional TRAIL receptor in primary breast cancer samples (Twiddy D 2010). Although at this point the contribution of this increased expression of DR4 to FASR TRAIL sensitivity is unclear. The contribution of increased DR4 expression in FASR cells to TRAIL sensitivity can be clarified in future experiments using siRNA to knockdown DR4 expression or antibodies to specifically block its binding to TRAIL. Future experiments will therefore be focussed on further characterisation of TAMR and FASR hypersensitivity.

Uncovering the mechanism of AE-resistant cancer cell sensitivity to TRAIL and further extending this work to more models of endocrine resistance including additional primary samples from multi-drug-resistant patients will be key in further evaluating TRAIL's potential in the clinical setting.

6.1.2 Using the immune system to deliver TRAIL to tumour cells

The capacity of the immune system for specificity makes it an appealing therapeutic option for cancer. This was first demonstrated by the clinical success of mAbs in the treatment of breast cancer and lymphomas. This brought about the interest in T cell-based immunotherapy with their capability to recognise peptides derived

from protein in any cellular compartment. There are now several clinical studies supporting immunotherapy as a realistic therapeutic approach and thus development and application of immunotherapy has attracted much attention over the last decade. Augmentation of immune response can be achieved *in vivo* by stimulatory compounds and *ex vivo* expansion of effector cells followed by adoptive transfer. The end result of each is a population of activated effector cells that recognise a target population of cells for destruction (reviewed in (Restifo et al. 2012)). Of course *in vivo* and *ex vivo* stimulation of effector cells will ultimately result in behavioural differences in the cells as the *in vivo* environment can not be accurately recapitulated *in vitro*. Despite these differences each of these two modalities have their benefits and show some promise as a methodology for cancer treatment.

Adoptive transfer of γδ T cells is in the early stages of development and phase I clinical trials for treating renal and lung cancer are currently ongoing (Bennouna et al. 2008; Kobayashi et al. 2011; Sakamoto et al. 2011). As of yet there are no active trials for breast cancer treatment with adoptively transferred γδ T cells although there is strong evidence of their efficiency in killing breast cancer cells in preclinical models and additionally γδ T cells have also been identified in breast tumour infiltrating lymphocytes (Beck et al. 2010; Capietto et al. 2011; Guo et al. 2005; Meraviglia et al. 2010). In another study where prostate cancer patients were treated with ZOL and IL-2, a transition of γδ T cells toward an activated effector-memory-like state was observed in conjunction with maintained serum TRAIL levels. This correlated with reduced prostate-specific antigen and improved clinical outcome (Dieli et al. 2007). Furthermore TRAIL has also been indicated to partly contribute to activated γδ T cell killing

of chronic myelogenous leukaemia (D'Asaro et al. 2010). Data shown in Chapter 5 collectively suggests that breast cancer killing by activated γδ T cells is predominantly a TRAIL-independent cytotoxicity. Similar results have also been seen in γδ T cell treatment of solid tumour cells, where the killing observed was also predominantly perforin-mediated (Mattarollo et al. 2007). This is not to discount any role TRAIL may have in the cytoxicity that was observed in bCSC subset (Figure 5.2.6). In fact the cytotoxicity of activated γδ T cells on the cancer stem cell subset has not been previously addressed.

Importantly the γδ T cells could also be activated using zoledronate, producing similar expression of cytotoxic factors (Figures 5.2.1, 5.2.2, 5.2.3). Zoledronate shows great promise in facilitating *ex vivo* expansion of γδ T cells from cancer patients for adoptive transfer (Kondo et al. 2008). Zoledronate is generally non-toxic (Rosen et al. 2001; Rosen et al. 2004) maintaining the tumour cell specificity of the γδ T cells by limiting off-target effects. Coincidentally, zoledronate has also been demonstrated to improve endocrine therapy response in premenopausal breast cancer patients (Delea et al. 2010; Gnant et al. 2009). Although the mechanism of this combination treatment benefit was not elucidated in the study, this combination therapy would allow the delivery of TRAIL to tumours by activation of γδ T cells. The treatment regime was performed over three years during which time recurrence events were significantly reduced by combination treatment. Although individual contribution of immune responses, TRAIL and disease resistance were not investigated in this study, it is possible that in this timeframe some patients may have acquired resistance to the endocrine agent resulting in cellular changes that may sensitise the tumour cells to γδ T cell treatment, similar to the effect observed in the bCSCs of the TAMR and FASR cells shown in

Figure 5.2.6. At present this is highly speculative and there are many other possible explanations for the cytotoxicity that was observed in Figure 5.2.6. The contribution of perforin and Fas to bCSC cell death in TAMR and FASR cells was not explored due to time constraints and cannot be ignored as possible inducers of cell death in these cells. Future work will focus on elucidating any bCSC cytotoxicity of activated γδ T cells alone or also in combination with FLIPi. It is possible that FLIPi can sensitise bCSCs to activated γδ T cell delivery of TRAIL. Furthermore it may be of interest to investigate activated γδ T cell delivery of TRAIL to AE-resistant tumours. Importantly the efficiency of activated γδ T cell cytotoxicity to breast cancer cells must be assessed in preclinical models including primary cancer cells and *in vivo* studies.

The work performed in this thesis collectively identifies potential therapies that may prove effective in combination, or in some cases individually, in the treatment of breast cancer by overcoming disease resistance and preventing distant metastasis.

6.1.3 Future perspectives

Work in this thesis has touched upon creating and exploiting certain signalling pathways of tumour cells across the breadth of breast cancer sub-types. Existing therapies for breast cancer are generally sub-type specific and resistance is prevalent to these treatments resulting in the need for new or adjuvant therapies. On the basis of the data shown here, further investigation into the therapeutic efficacy of c-FLIP inhibition to sensitise breast tumours to TRAIL treatment is warranted. The feasibility of designing drugs to target c-FLIP is at present unknown. It is clear from the homology c-FLIP

shares with caspase-8 that drug development will not be without difficulty. In addition it is also currently unclear as to what the overall effect on normal tissue homeostasis would be in response to systemic c-FLIP inhibition. Evaluation of toxicity of such compounds in both *in vitro* and *in vivo* models will help provide insight into the potential use of c-FLIP inhibition in future treatment of breast cancer.

Data presented in Chapter 4 of this thesis supports the potential use of TRAIL as an adjuvant therapy in patients who have become resistant to anti-estrogens. Continuation of this work will be primarily focussed on the completion of *in vivo* studies of AE-resistant cell line sensitivity to TRAIL and the treatment of additional primary samples obtained from AE-resistant patients. It may also prove beneficial to correlate primary sample response with c-FLIP expression potentially identifying it as a biomarker for AE-resistant patient response to TRAIL.

The use of adoptively transferred *ex vivo* expanded γδ T cells as a therapeutic strategy for cancer patients is currently ongoing. Work performed in this thesis identifies c-FLIP inhibition as a potential sensitizer of breast cancer cells and further supports this therapeutic approach. Investigating CSC targeting and preclinical *in vivo* modelling of combined FLIPi/ activated γδ T cell treatment will help to elucidate its potential benefit to breast cancer patients.

In all cases, each one of these therapeutic approaches is particularly desirable in light of the tumour specificity implied by the data collected hitherto.

APPENDICES

Appendix I – Heterotypic cultures unstained control

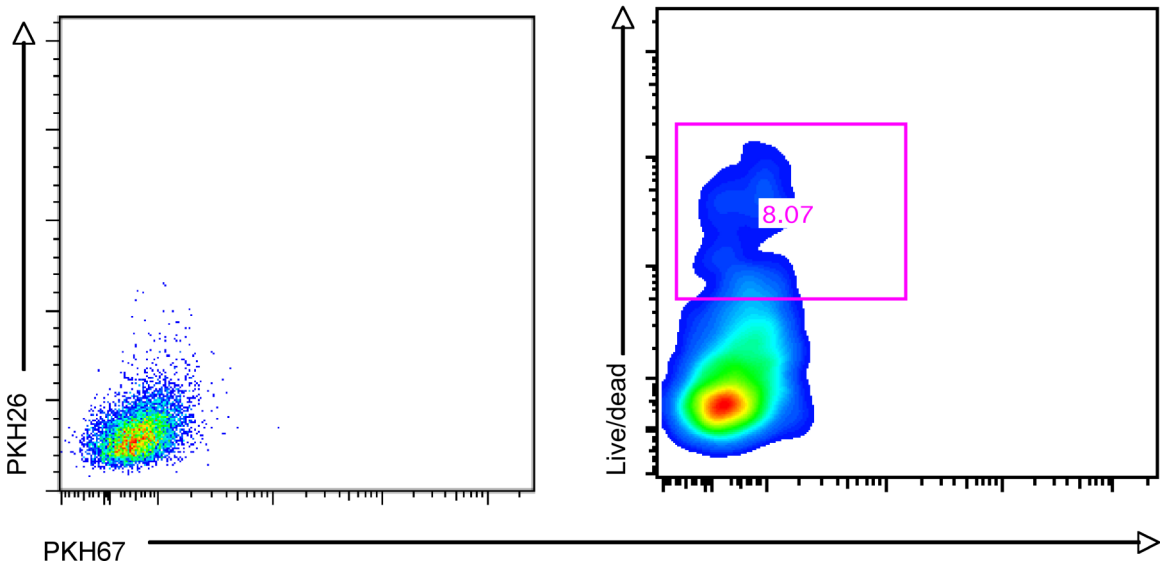

Figure 7.1: Unstained control. Untreated breast cancer cells were stained with only fixable live/dead stain before analysis for background fluorescence in APC and FITC channels and background cell death.

Appendix II – Human primary sample details

2004: Right WLE (wide local excision), Axilar node biopsy, 3/5 LN (Lymph node) positive

Patient Details	Collected Material	Chemotherapy /Radiotherapy	Date	Endocrine Therapy	Date	Patient Status
BB3RC50 **ER +++** **PR +++** **HER2 -ve**		**8 x FEC60**	**2005**	**Tamoxifen**	**2005**	**Bone, Liver, Ovarian Mets 2008**
		Paclitaxel	**02/04/08-23/06/08**			**PR** **PD**
		Capecitabine	**08/04/09-01/08/09**	**Anastrazole**	**08/04/09-01/08/09**	
		Vinorelbine	**08/2009-03/2010**			**SD**
				Exemestrane	**04/2010-06/2011**	**SD**
	Ascites 01/2012			**Fulvestrant**	**07/2011-092011**	**PD** **Died 03/2012**

Table 7.1 – Full details of primary human sample. ER- estrogen receptor, PR- progesterone receptor, PR (patient status) – partial remission, PD – progressive disease, SD – stable disease.

Appendix III – γδ T cell treatment of heterotypic cultures

γδ T cell

Control siRNA (PKH 67 labelled) Tumour Cells

c-FLIP siRNA (PKH 26 labelled) Tumour Cells

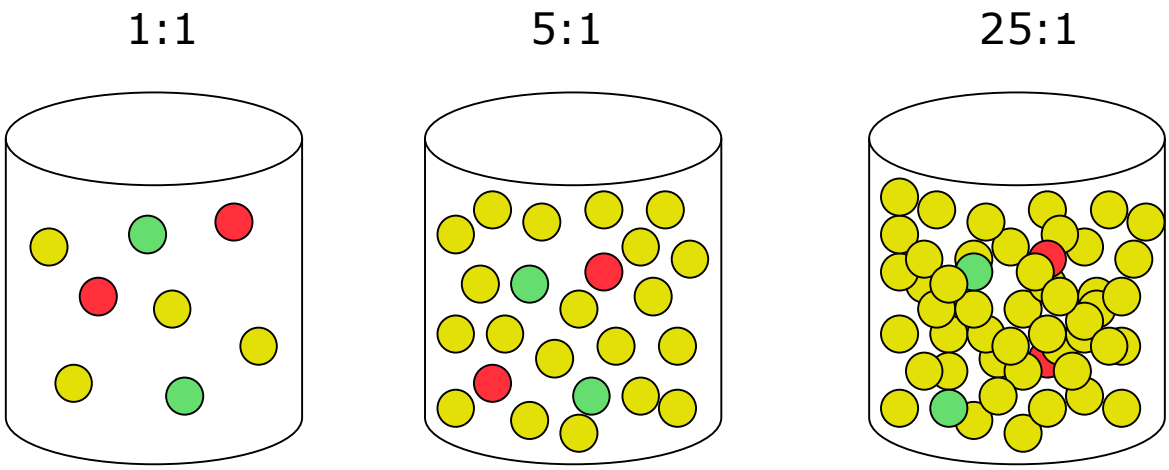

Figure 7.2: Illustration of γδ T cell treatment of heterotypic SCi and FLIPi cultures.

Appendix IV– Miccroarray heatmaps of gene expression

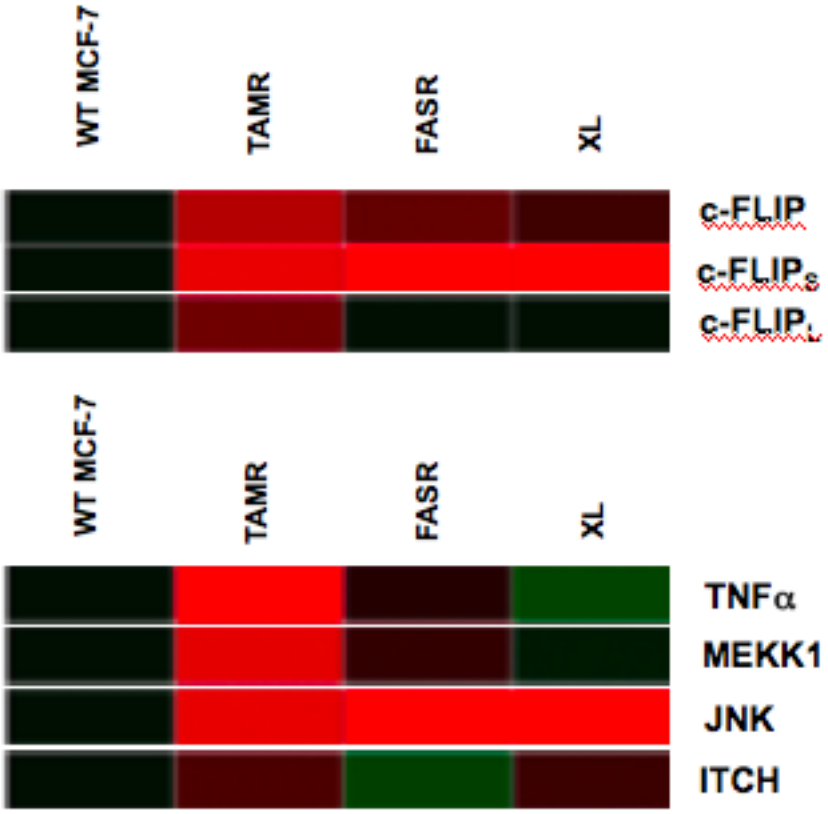

Figure 7.3: Microarray heatmaps. Gene expression of TAMR, FASR and XL cells compared to parental WT MCF-7 cells. Red = upregulation Green = downregulation.

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