The Role of cFLIP in Breast Cancer Stem Cells

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Abstract

Breast tumours have intrinsic heterogeneity. The cancer stem cell hypothesis is currently challenging the notion that all cancer cells are equally malignant, suggesting that it is important to evaluate the efficacy of potential anti-cancer drugs by their ability to target the stem-like population. TRAIL is a cytotoxic agent the efficacy of which has been limited by a lack of patient stratification in clinical trials (Lemke 2014). In pre-clinical studies TRAIL has shown specificity towards mesenchymal-like breast cancer cell lines (Rahman *et al.* 2009)

We show here that TRAIL is able to target the tumoursphere-forming population of four out of six breast cancer cell lines, including two epithelial-like lines, the bulk population of which is TRAIL-resistant. Furthermore, TRAIL also reduced the tumour-initiating capacity of the MCF-7 line. In addition, we have also investigated a paracrine mechanism of sensitising breast cancer cell lines to TRAIL. We have shown that a soluble factor produced by MDA-MB-231 cells, fibroblasts, and cancer-associated fibroblasts (CAFs) can sensitise both MCF-7 cells and SKBR3 tumoursphere-forming cells to TRAIL.

Our data shows that cytoplasmic levels of Cellular FLICE-Like Inhibitory Protein (cFLIP) – a naturally occurring inhibitor of TRAIL's cell toxicity effects- are lower in TRAIL-sensitive cells and suggest that tumoursphere populations are TRAIL-sensitive due to the re-localisation of cFLIP to the nucleus. We believe cFLIP is nuclear in stem-like cells due to a role as a promoter of the Wnt pathway. We have shown that inhibition of cFLIP by siRNA resulted in a reduction in both beta-catenin protein levels and Wnt-target gene transcription in both the MCF-7 and MDA-MB-231 breast cancer cell lines. We have also demonstrated a novel role for cFLIP as a promoter of bCSC maintenance. We have found that inhibition of cFLIP by shRNA decreased the self-renewal of tumoursphere-forming cells and also reduced colony formation.

As TRAIL alone does not completely eradicate tumoursphere-forming or tumour-initiating cells in any breast cancer cell line, we believe our data are evidence of bCSC heterogeneity existing in terms of susceptibility to TRAIL. We propose a model of phenotypic heterogeneity within breast cancer cell lines and bCSCs whereby there exist two populations of cells which can be distinguished based on TRAIL susceptibility correlating with the known distinction of epithelial-like or mesenchymal-like status and our novel observation of cFLIP localisation. While these findings are currently restricted to cell lines, if confirmed in primary breast cancer cells, the clinical implication of our model is that although TRAIL alone is a potential therapy, a much more effective therapeutic strategy would be to also inhibit cFLIP, the consequences of which would not just be a sensitisation to TRAIL but also a reduction in Wnt signalling, and potentially a reduction in bCSC self-renewal and proliferation.

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Declaration

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

ALDH1 Aldehyde dehydrogenase 1

ATM Ataxia-telengiectasia-mutated kinase

APC Antigen presenting cell

bCCL Breast cancer cell line

Bcl-2 B-cell lymphoma 2

bCSC Breast cancer stem cell

BMD Bone marrow derived

c-FLIP Cellular FLICE-like inhibitory protein

CD95 Fas receptor

CD133 Prominin 1

CM Conditioned medium

CMA Concanamycin A

CNA Copy number abberations

CSC cancer stem cell

CXCL7 Chemokine (C-X-C motif) ligand 7

CXCL12 Chemokine (C-X-C motif) ligand 12

CXCR4 Chemokine (C-X-C motif) receptor 4

DcR Decoy receptor

DED Death effector domain

DISC Death inducing signalling complex

DR4 Death receptor 4

DR5 Death receptor 5

ECM Extracellular matrix

EEA Early endosome marker

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

FACS Fluorescence activated cell sorting

FADD Fas associated death domain

FasL Fas ligand

FBS Fetal bovine serum

FEC Fluorouracil, Epirubicin, Cyclophosphamide

FLIPi c-FLIP inhibition

FLIPi/TRAIL combination c-FLIP inhibiton + TRAIL treatment

Gsk3Beta Glycogen synthase kinase 3 beta

HDAC Histone deacetylase

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

HMEC Human mammary epithelial cells

HRG Heregulin

IAPs Inhibitors of apoptosis

IFN Interferon

IGF Insulin-like growth factor

IkB Inhibitors of NF-kB

IKK Inhibitor of KappaB kinase

IL-6 Interleukin 6

IL-8 Interleukin 8

JNK c-jun N-terminal kinase

LAMP1 Lysosomal marker

mAb Monoclonal antibody

MAPK Mitogen-activated protein kinase

MaSC Mammary stem cell

MEKK1 MAP/ERK kinase kinase 1

MET Mesenchymal-epithelial transition

MFU Mammosphere forming units

MMP Matrix metalloproteinase

MMTV Mouse mammary tumour virus

mTOR Mammalian target of rapamycin

NF-kB Nuclear factor binding to the intronic kappa-lightchain enhancer element in b cells

NHL Non-hodgkins lymphoma

NOD/SCID Nonobese diabetic severe combined Immunodeficiency

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PI3k Phosphatidylinositol 3-kinase

PR Progesterone receptor

qRT-PCR Quantitative reverse transcription PCR

RIP Receptor-interacting protein

RNAi RNA interference

SAHA Suberoylanilide hyroxamic acid

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel

SNP Single nucleotide polymorphism

elctrophoresis

shRNA Short hairpin RNA

siRNA Small interfering RNA

TFU Tumoursphere-forming unit

TGF-Beta Transforming growth factor Beta

TNF-alhpa Tumour necrosis factor alpha

TIC Tumour initiating cell

TIMP Tissue inhibitors of metalloproteinases

TNFalpha Tumour necrosis factor alpha

TRADD TNF receptor type 1-associated death domain

TRAF TNF receptor associated factor

TRAIL (APO-2L) TNF-related apoptosis inducing ligand

Wnt Wingless-int

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Chapter 1:
General Introduction

Chapter 1 Introduction

1.1 Breast Cancer

1.1.1 Introduction

Cancer remains one of the most feared diagnoses, accounting for 162,000 deaths in the UK in 2012 alone (Cancer Research UK, http://www.cancerresearchuk.org/cancer-info/cancerstats/mortality/all-cancers-combined/newpagetemp). Breast cancer is the most common cancer and second most leading cause of cancer death in women. It is estimated that 1 in 8 women will be diagnosed with breast cancer in their lifetime. In the UK, approximately 50,000 women and 350 men are diagnosed with breast cancer every year, and 12,000 women and 80 men die of breast cancer every year (Breast Cancer Campaign, http://www.breastcancercampaign.org/about-breast-cancer/breast-cancer-statistics).

A tumour is a large mass of cells which occurs as the result of extensive proliferation, and may be benign or malignant (cancerous). A malignant tumour is defined not only by its ability to proliferate independently of normal cellular control and protection mechanisms, but also by its ability to invade surrounding tissues and metastasise (spread) to other sites in the body to initiate new tumour growth. Cancer is classified according to the organ in which it arises, e.g. breast cancer. A breast tumour itself is only life-threatening when it metastasises to essential organs such as the bone, lungs, liver or brain. The presence of a tumour in any one of these organs will impair its function and, if left untreated, ultimately will cause the death of the individual.

Eight hallmarks of cancerous cells have been proposed: These comprise; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction (Hanahan and Weinberg, 2000 and 2013).

1.1.2 Possible Causes of Breast Cancer

Despite a few examples (notably the well-characterised relationship between smoking and lung cancer), it is most often impossible to trace a cancer from cause to effect due to the duration and complexity of tumourigenesis. However, there are some examples of factors which are known to increase an individual's risk of developing breast cancer.

1.1.2.1 Hereditary Breast Cancer

The presence of a mutated gene in germ-line cells, for example the p53 gene in Li-fraumenni syndrome, or the BRCA1 and 2 genes, can increase an individual's risk of developing a number of types of cancer including breast cancer. Mutations in the tumour suppressor genes BRCA1 and 2 predispose to breast, ovarian, and prostate cancer. Approximately one in 400 to one in 800 of the population is thought to carry these mutations depending on ethnicity. BRCA mutations have been estimated to increase an individual's risk of developing breast cancer by 57-90% (Petrucelli *et al.* 2010). The wide ranging

penetrance of BRCA mutations may be indicative of the involvement of other factors in disease instigation.

1.1.2.2 Breast Cancer Risk Factors

A number of factors have been implicated in the development of breast cancer including oral contraceptives, hormone replacement therapy, late age at first childbirth, obesity, alcohol consumption, and a sedentary lifestyle, whereas breastfeeding is considered protective. Some of these factors, notably obesity, are thought to contribute to breast cancer risk by causing an increase in endogenous oestrogen production. Others, including oral contraceptives and hormone replacement therapy, may increase risk by providing an additional source of oestrogen (http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/riskfactors/breast-cancer-risk-factors).

1.1.3 Genetics of Breast Cancer

Risk factors only enhance the susceptibility of an individual to developing breast cancer and in any individual case of the disease the actual cause cannot be determined. Despite this, it is understood that the instigation of tumourigenesis requires the mutation of genes involved in cell cycling, growth, and proliferation. The complexity of cellular protection mechanisms suggests that many de-regulating events must take place in the process of malignant transformation. The genes involved are divided into two categories; oncogenes and tumour suppressor genes.

1.1.3.1 Oncogenes

Oncogenes were first discovered in viral genomes but were found to originate from the genome of a cellular organism. The oncogenes had been incorporated into the genome of an ancestor of the Rous sarcoma virus producing an evolutionary advantage to the virus as it induced the host's cells and by extension the viral DNA to replicate (Pierotti, Holland-Frei *et al.* Cancer Medicine, 6th Ed.). Oncogenes result from gain-of-function mutations in proto-oncogenes the normal function of which is to promote cellular growth, proliferation, or survival, therefore their over-activation causes excessive and inappropriate growth. Proto-oncogenes can encode for any protein involved in a growth signalling pathway e.g. growth factors, receptors, DNA-binding transcription factors or cell cycle proteins. There are relatively few examples of oncogenes in breast cancer compared to other cancers. The most notable breast cancer oncogene is the human epidermal growth factor receptor 2 (Her-2) which activates proliferation pathways and is amplified in approximately 20-30% of primary breast cancers. Others include mammalian target of rapamycin (mTOR), cyclins, and also c-myc; a transcriptional regulator which is amplified in 15-25% of breast cancers (Osborne *et al.* 2004). Protein products of oncogenes are useful targets of drugs designed to target cancer cells, such as Her-2 by Herceptin.

1.1.3.2 Tumour Suppressor Genes

The functional opposite of oncogenes, tumour suppressors are involved in growth arrest and oncogene deactivation. Tumour suppressor genes become involved in cancer development due to loss-of-function mutations in the normal gene. Knudson's two-hit hypothesis states that whereas in theory only one copy of an oncogene is required to induce uncontrolled proliferation of a cell, two functional copies of a tumour suppressor gene must be lost within a cell in order to have a comparable affect. An individual that inherits only one functional copy of a tumour suppressor gene is more disposed to cancer than another individual with two functional copies; only one mutagenic "hit" would be required to instigate cellular transformation (Knudson 1971).

The tumour suppressor p53 is one of the most genes found mutated in cancers including breast cancer. The wild-type protein functions to regulate cell cycle genes, apoptosis, and DNA repair mechanisms. A reduction in functional p53 is associated with oestrogen receptor negative breast cancer and a poor prognosis (Thor *et al.* 1992). Other examples of tumour suppressors in breast cancer include p27 which encodes a protein involved in halting the cell cycle, and the BRCA1 and 2 genes which are involved in the repair of double-stranded DNA breaks (Osborne *et al.* 2004).

1.1.4 Tumourigenesis

Following the instigation of transformation, another complex series of events must take place to form a breast tumour.

1.1.4.1 Normal Mammary Gland Structure

In order to better understand the process of tumourigenesis within the human breast it is first necessary to examine briefly the structure of the normal mammary gland. The human mammary gland consists of two major tissue types; parenchyma and stroma. The parenchyma consists of a network of milk ducts which branch out from the nipple and end in terminal ductal lobule units (TDLUs) (Figure 1.1A). The ducts are made up of an inner "luminal" layer of epithelial cells and an outer "basal" layer of myoepithelial cells, surrounded by the basement membrane (BM) (Figure 1.1 B). During lactation, luminal cells of the TDLUs produce milk which is secreted along the ducts, aided by the contractile myoepithelial cells. The stroma surrounding the lobules and ducts is made up of extracellular matrix (ECM), associated cells such as fibroblasts and immune cells, and adipose tissue which provides support for parenchyma and contains the lipid store which can be turned into milk (Figure 1.1A-C) (Visvader 2009, Polyak 2010).

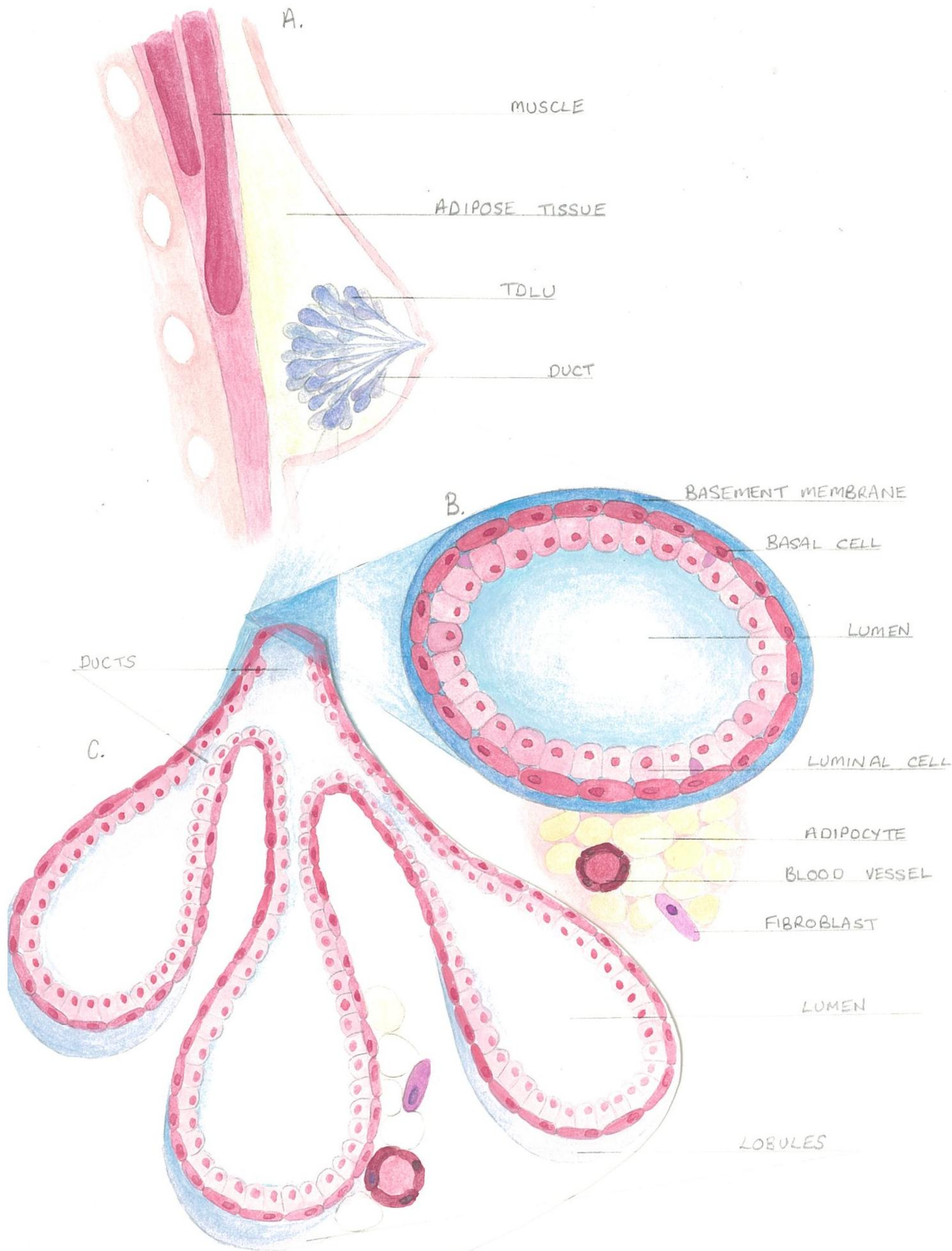


Figure 1.1: Structure of the Human Mammary Gland: **A** The human mammary gland consists of a network of branching ducts ending in terminal ductal lobule units. **B** Transverse section of a duct; luminal and basal cells are indicated **C** Structure of the terminal ductal lobule unit

1.1.4.2 Mammary Tumourigenesis

The process of tumourigenesis in breast cancer is not as well understood as in other cancers such as colorectal, but can be simplified into a number of stages. The most common form of breast cancer, invasive ductal carcinoma (IDC) is thought to develop from a flat epithelial atypia (FEA), to an atypical ductal hyperplasia, to a benign ductal carcinoma *in situ* (DCIS) which as the name suggests sits in the lumen of a duct. DCIS may progress by increased growth and invasion of the surrounding tissue to become IDC (Figure 1.2). IDC can then progress to a metastatic stage by dissemination of tumour cells. The molecular mechanisms underlying tumour progression are not well understood although it has been associated with epithelial-to-mesenchymal transition (EMT) (Section 1.1.5) (Polyak 2008, Bombonati and Sgroi 2011).

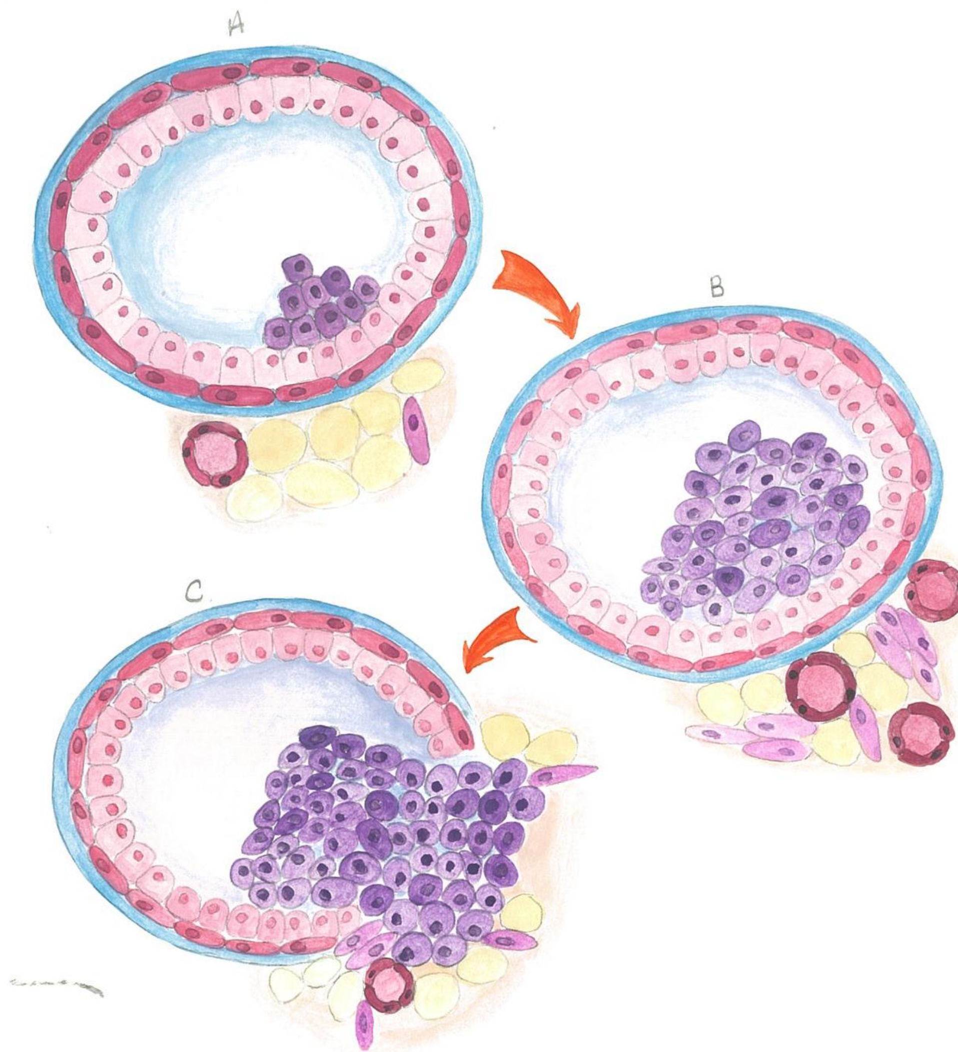


Figure 1.2: Mammary Tumourigenesis: Breast tumours are thought to progress from (**A)** an atypical lesion situated in the lumen of a duct to (**B)** ductal carcinoma *in situ* (DCIS) to (**C)** Invasive ductal carninoma (IDC).

1.1.5 Metastasis

At some point during tumourigenesis, malignant cells can detach from the tumour and seed at distant sites to form secondary metastases. A primary breast tumour, being not in an organ essential for life, is only a life-threatening disease by its ability to spread to more vital areas, notably the bone, lungs, liver, and brain. Therefore the study of the biology of secondary metastases deserves at least as much if not more attention as that of the primary breast tumour. The incidence of secondary metastases in breast cancer patients is different depending on clinical subtype (Section 1.1.8): approximately 10-15% of patients with more aggressive breast cancers such as Her-2 positive or triple-negative disease are highly likely to develop secondary tumours within three years of diagnosis, whereas patients with oestrogen receptor (ER) positive disease may experience recurrence in the form of secondary metastases more than 10 years after remission of the primary tumour (Colzani *et al.* 2014).

The process of metastasis can be simplified into five stages (Figure 1.3);

1. Invasion: The invasion of surrounding breast tissue by tumour cells. Invasion requires an increase in motility possibly via EMT, and the ability to disintegrate extracellular matrix.
2. Intravasation: The process by which invasive tumour cells enter lymphatic or blood vessels through the endothelial cell junctions.
3. Survival in Circulating Blood: An ability to survive anoikis; the form of apoptosis induced by cellular detachment and a property attributed to cancer stem cells (Section 1.2).
4. Extravasation: The process by which invasive tumour cells exit lymphatic or blood vessels through the endothelial cell junctions.
5. Colonisation of a foreign tissue: The invasion and propagation of cancer cells in a distant organ.

It is very common in breast cancer to have a long (greater than three years) period of "metastatic latency" as defined as the time between initial diagnosis and detection of metastasis. This latency is thought to be due to the time between the seeding of a cancer cell in a foreign organ and formation of secondary tumour at that site. An alternative explanation for metastatic latency may be the positive effect of treatment; chemotherapy may be able to target some cells thus conferring a selective advantage to those with low proliferative capacity which therefore take longer to form detectable metastases (Nguyen *et al.* 2009, Weigelt *et al.* 2005).

The difficulty in treating metastatic breast cancer is that secondary metastases develop a genotype and phenotype often highly distinct from that of the primary tumour (Kuukasjarvi *et al.* 1997). This supports the expectation that secondary tumours evolve separately under different selective pressures to the primary tumour. Previous treatments can also generate a selective environment in which therapy-resistant secondary tumours often arise. Her-2-positive breast cancer patients treated with the Her-2 antibody trastuzumab (Herceptin) are more likely to experience brain metastases. This has been attributed to the brain environment as being protective in terms of the blood brain barrier shielding disseminated cancer cells from Herceptin, however the increased incidence of brain tumours compared with individuals not treated with Herceptin suggests that other factors could be involved (Weil 2005).

Despite the distinctive nature of secondary tumours, the predilection of breast cancer for certain sites; bone, lung, liver, and brain, suggests that the genotype or phenotype of the primary tumour does in

some part determine the secondary site. In addition, the site of secondary metastases may also be specific for breast tumour subtypes; for example ER-positive disease has been found to be more likely to colonise bone (James *et al.* 2003). Predilection of tumour cells for certain sites could be influenced by molecular interactions between cancer cells and certain organ cells; for example the ability of pulmonary vasculature to bind the metadherin found over-expressed on breast cancer cells (Brown and Ruoslahti 2004). Alternatively, certain environments may be more permissive to colonisation than others; for example less extravasion is required in metastasis to bone marrow (Minn *et al.* 2005).

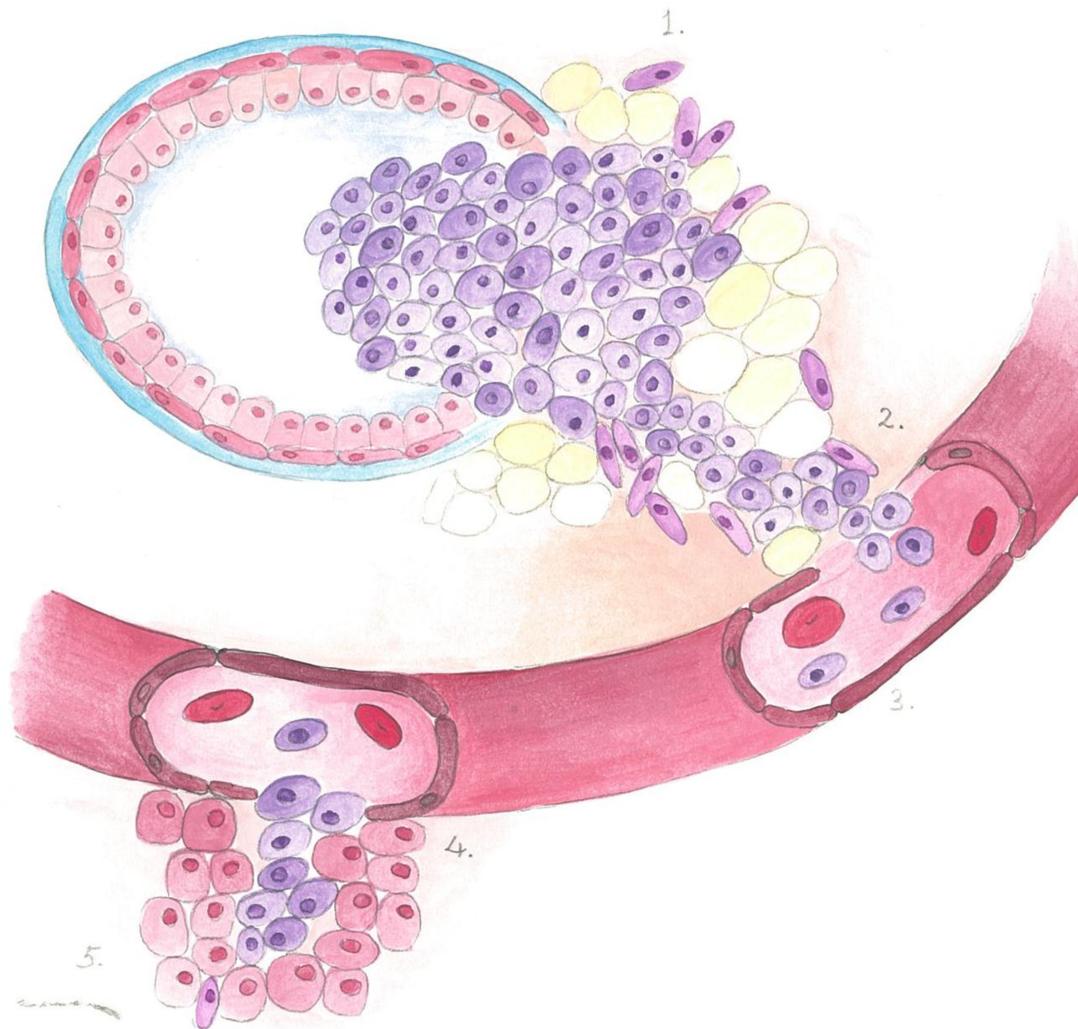


Figure 1.3: Depicting the five stages of breast cancer metastasis: **1. Invasion:** Tumour cells leave the lumen of a duct and invade surrounding tissue **2. Intravasation:** Tumour cells enter lymphatic or blood vessels **3. Survival in circulation:** Cancer cells disseminate throughout the body in the lymphatic system or bloodstream **4. Extravasation:** Cancer cells exit blood vessels into surrounding tissue **5. Colonisation:** Cancer cells invade and propagate in foreign tissue.

1.1.6 EMT in Breast Cancer Progression

"Epithelial" and "mesenchymal" define cell types distinguishable on the basis of their morphology and organisation: epithelial cells have a relatively more regular shape due to apical-basal polarity and generally are compacted into epithelial sheets, whereas mesenchymal cells are less constrained both in terms of morphology and localisation. Epithelial cells are thought to be more evolutionarily primitive than mesenchymal cells; primitive species such as the sponges consist simply of two layers of epithelial sheets, whereas the development of metazoans involves several stages in which the generation of mesenchymal cells is required for the creation of vital organs such as heart and muscle. There are three types of EMT: Type I EMT occurs during metazoan development where epithelial cells undergo a morphological transition from an organised structure to cells with migratory capacity that are held together loosely in ECM. Resulting mesenchymal cells have front-back end polarity and their cytokeratin based intermediate filament network has been replaced with vimentin and actin stress fibre formation. These changes result in a more spindle-like cell or fibroblast-like morphology and allow the cells to move to where they are required in the embryo in order to participate in organogenesis (Thiery 2002, Drasin 2011). Type II EMT occurs as a result of injury during wound healing and can also occur inappropriately in response to the presence of myofibroblasts during chronic inflammation. EMT which occurs during carcinogenesis is referred to as Type III. Types II and III are generally partial transformations where EMT only occurs in cells of the leading edge and can be incomplete even in an individual cell (Drasin 2011).

Given the flexibility that EMT offers to development and wound healing it is not hard to see why it would be an attractive process for carcinoma cells to sabotage for their own devices. Carcinomas including breast IDC lose epithelial characteristics during tumour progression (Thiery 2002). EMT is thought to be responsible for both the transition from DCIS to IDC and the generation of metastatic disease, as both these steps require an invasive and migratory ability (Figure 1.3). For example, the (epithelial-like) MCF-7 breast cancer cell line metastasises poorly in mouse models but is able to form more distant metastases after having undergone EMT (Micalizzi *et al.* 2009). Furthermore, EMT is able to generate more breast cancer cells which have the properties of stem cells (Mani *et al.* 2008, Morel *et al.* 2008). This has major implications for the progression of disease due to their malignant traits (and will be discussed in detail in Section 1.2). In contrast, a mesenchymal –to-epithelial transition (MET) can also occur in cancer and is thought to be necessary for the colonisation of distal sites following metastasis. Co-culture of the (mesenchymal-like) MDA-MB-231 breast cancer cell line with hepatocytes restored an epithelial morphology by decreasing methylation of the E-cadherin gene (CDH1), implicating a partial MET-like process in breast cancer cell colonisation of the liver (Chao *et al.* 2010). Induction of EMT has also been implicated in breast tumour recurrence (Moody *et al.* 2005). Taken together these studies are evidence for EMT as a promoter of tumour progression and a mechanism by which tumours can obtain more aggressive characteristics.

The major player in EMT is E-cadherin. E-cadherin is a protein which functions in adherens junctions to maintain epithelial cell contacts. E-cadherin associated with an adherens junction is also linked via α or β-catenin to actin microfilaments in the cytoskeleton which function to maintain the rigid morphology of an epithelial cell (Kemler 1993). Loss of E-cadherin correlates with the acquisition of

mesenchymal-like characteristics; disruption of contacts by anti-E-cadherin antibodies is sufficient to induce EMT in MDCK (Madin-Darby Canine Kidney) epithelial cells (Imhof *et al.* 1983). In some cases during EMT, E-cadherin is replaced by N-cadherin which forms weaker cell contacts and therefore is associated with more mesenchymal-like cells (Kim *et al.* 2000). E-cadherin can be transcriptionally repressed by transcription factors including Snail, Slug and Twist which bind to the promoter of the E-cadherin (CDH1) gene and silence its expression (Drasin 2011 and references therein). Snail is thought to play an important role in EMT-mediated breast cancer progression; in cases of breast cancer, low E-cadherin correlates with high Snail production which in turn is indicative of high grade tumours with poor prognoses (Blanco *et al.* 2002). EMT can also be regulated post-transcriptionally by microRNAs (Gregory *et al.* 2008).

There is much interest in trying to block EMT in cancer as it has the potential to lead to prevention of disease progression and metastasis; the cause of mortality in breast cancer patients. Whilst it is not easy to study an oncogenic EMT in real-time *in vivo*, the process of EMT can be followed in cell lines derived from carcinomas in culture. These models have made it possible to identify the molecular mechanisms underlying an oncogenic EMT. The induction and maintenance of an oncogenic EMT requires the inappropriate activation of those signalling pathways involved in a developmental EMT including Wnt, PI3K/Akt, Notch, Transforming growth factor-beta (TGF-β) and Hedgehog signalling (Drasin 2011). Importantly, loss of E-cadherin may increase cell proliferation and further promote EMT via its connection to β-catenin and the Wnt pathway. Whilst membrane-bound, E-cadherin is bound to actin filaments via β-catenin. Upon loss of E-cadherin, β-catenin is more freely available to participate in the Wnt signalling pathway. In the absence of a Wnt ligand, β-catenin is phosphorylated in the cytosol by a degradation complex, the main components of which include Axin, APC (adenomatous polyposis coli) and GSK3β (glycogen synthase kinase-3 beta). The binding of Wnt ligands to Frizzled receptors recruits Dishevelled (Dvl) and results in the dissociation of the degradation complex and inhibition of β-catenin phosphorylation. This allows β-catenin to accumulate in the cytosol from where it can translocate to the nucleus to activate a number of transcription factors (Figure 1.2.3). Nuclear β-catenin can correlate with EMT in some cases such as in colorectal cancer and has been used as an EMT marker (Morali *et al.* 2001). Additionally, Wnt signalling can also promote EMT via transcription of genes associated with EMT (Wu *et al.* 2012). In breast cancer, Wnt ligands can also act via Axin to stabilise both Beta-catenin and Snail, thereby leading to induction of EMT (Yook *et al.* 2006).

Many extracellular growth factors have also been implicated in EMT in breast cancer including; insulin-like growth factor (IGF), transforming growth factor beta (TGFβ), and matrix metalloproteases (MMPs) (Walsh and Damjanovski 2011, Kalluri and Zeisburg 2006, Thiery 2002). This raises the possibility that the tumour environment may be complicit in tumour progression via the instigation of EMT.

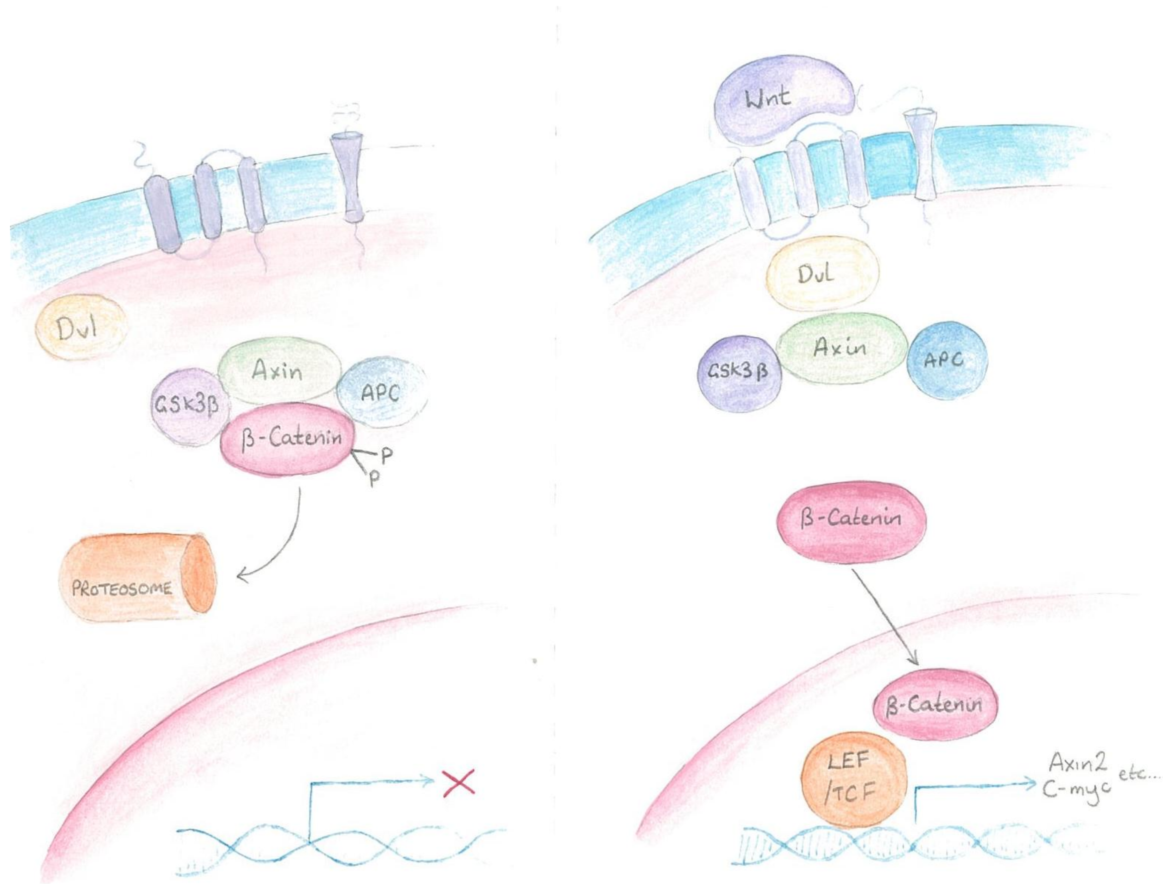


Figure 1.4: The canonical Wnt pathway: **A Inactive state:** In the absence of a Wnt signal, β-catenin is held by a destruction complex in the cytosol **B Active state:** Upon ligand binding, the frizzled receptor recruits Disheveled (Dvl), resulting in the release of β-catenin from the destruction complex. β-catenin is then free to move into the nucleus and complex with the TCF and LEF transcription factors to promote Wnt-target gene expression

1.1.7 Influence of Tumour Microenvironment

The significant contribution of the tumour environment to cancer progression and metastasis was proposed as early as 1889 when Dr Stephen Paget published the "Seed and Soil" hypothesis. He suggested that the cancer cell as the "seed" required an appropriate and permissive environment "soil" in which to implant and grow, without which metastasis would not be possible (Paget 1889, Fidler 2003). Indeed it is unfeasible for a tumour to metastasise without at least the presence of responsive endothelial cells in the stroma. Tumour-associated stroma is markedly different to normal mammary stroma and consistent with its involvement in EMT, its composition more resembles that which is observed during a wound-healing response (Brown *et al.* 1991). A number of different cell types have been observed to associate with breast tumours including cancer-associated fibroblasts (CAFs), myofibroblasts, immune cells including macrophages and T lymphocytes, adipocytes, endothelial cells, and bone-marrow derived mesenchymal stem cells (BM-MSCs) (Arendt *et al.* 2010, Karnoub *et al.* 2007). The amount of stroma is also altered in breast cancer and can in fact make up greater than 90% of tumour in certain cases (Bissell *et al.* 1996,

Dvorak 1986). ECM is also modified in tumour stroma with an increase in collagens, fibrins, and proteoglycans including fibronectin, again similar to that seen during a wound healing response (Bissell *et al.* 1996, Yeo *et al.* 1991). The presence of certain cell types including CAFs and BM-MSCs within the tumour environment have been shown to have tumour-promoting effects. For example, CAFs have been shown to promote EMT in breast cancer cells via TGFbeta signalling, and BM-MSCs have been shown to promote the metastasis of breast cancer cells to the lung (Karnoub *et al.* 2007, Yu *et al.* 2014).

These findings raise the questions as to the degree of importance of the stroma in breast tumourigenesis; what comes first, the activated stroma or transforming mutations? While it is generally accepted that the tumour is generated before the environment is altered, there is some evidence to the contrary: Mice generate to over-express the ECM-degrading protein Stromyelysin-1 (expressed in the mammary gland) underwent branching before pregnancy, involution during pregnancy and developed tumours at three to four months of age (Sympson *et al.* 1995). In addition, hepatocyte growth factor (HGF) and TGFbeta-expressing fibroblasts injected into cleared mammary fat pads were able to promote the generation of tumours, whereas normal fibroblasts only allowed normal outgrowths (Kupperwasser *et al.* 2004). Conversely, signals from embryonic mammary mesenchyme are able to induce differentiation in and reduce the malignancy of breast tumour cells (DeCosse *et al.* 1973). This suggests that stromal modifications do have the capacity to significantly affect the progression and even instigation of tumourigenesis. However this process does also operate in reverse: tumour cells can generate CAFs via down-regulation of caveolin-1 (Martinez-Outshorn *et al.* 2010). As there is evidence for both possibilities, it may be the case that either breast cancer subtypes differ in terms of the contribution of the stroma, or more likely that both the tumour and environment evolve in parallel, in a symbiotic manner, during the process of tumourigenesis.

The interactions between tumour and stromal cells are known to be mediated in part by cytokine networks (Karagianis *et al.* 2010). Expression of genes involved in cytokine and paracrine networks are altered between normal and tumour-associated stroma, for example MMPs are up-regulated in CAFs over normal fibroblasts (Allinen *et al.* 2004). Many of these changes are thought to be epigenetic and may be instigated by the tumour or by other factors such as prolonged inflammation (Hu *et al.* 2005). Tumour-stroma interactions may have arisen from the abrogation of normal cellular cross-talk occurring in the mammary gland at specific stages of remodelling such as development or wound healing. The ability to respond to stromal signals is not confined to breast tumour cells; normal mammary stroma is able to reprogram non-mammary epithelia to perform the function of mammary epithelial cells (Booth *et al.* 2011). In mouse mammary gland development, the primary fibroblastic mesenchyme induces the expression of milk proteins in epithelial cells, whereas adipocyte mesenchyme is required for structural development (Sakakura *et al.* 1976 and 1982). Post-embryonic development relies upon responses to secreted hormones and cytokines in mammary epithelia and stroma such as locally produced insulin-like growth factor (IGF1), TGFbeta, oestrogen, and growth hormone (GH) (Kleinberg *et al.* 2000, Arendt *et al.* 2011).

Tumours have long been termed "wounds that do not heal" and there are many similarities between wound-activated stroma and tumour stroma including ECM remodelling, secretion of growth

factors, angiogenesis, activation of fibroblasts, and invasion or migration (Dvorak 1986). A wound healing response can be initiated by infiltration of BMD-MSCs which are also observed in tumour stroma, and genetic profiles of tumour associated stromal cells reveal the up-regulation of genes involved in wound healing for example ECM remodelling enzymes (Allinen *et al.* 2004). The marked similarities between stromal interactions during development, wound healing, and tumourigenesis suggest that crosstalk may involve the instigation or maintenance of EMT in each case.

1.1.8 Subtypes

Due to the sheer complexity of tumourigenesis and metastasis, it is not surprising that breast cancer is a highly heterogeneous disease; a breast tumour can differ widely between individuals at both the cellular and molecular level. In order to aid treatment, many attempts have been made to classify breast tumours based on pathology and molecular characteristics. There is sufficient intra-tumour heterogeneity to ensure that sub-classifying breast cancer will always be an over-simplification but it is hoped that these subtypes will continue to be refined with further research.

1.1.8.1 Histopathological

Breast cancers are traditionally classified based on differences in morphology and organisation. Breast carcinomas are thought to originate in the terminal ductal lobule units, and can be first broadly classified as non-invasive (carcinoma *in situ*) or invasive. These tumours can be further defined as ductal or lobular. Ductal carcinoma *in situ* (DCIS) is more common than lobular carcinoma *in situ* (LCIS), and can be further classified by its structural organisation into Comedo, Cribiform, Micropapillary, Papillary and Solid subtypes (Malhotra *et al.* 2010). Invasive Carcinomas are also sub-classified according to origin as Ductal, Lobular, Medullary, Neuroendocrine, Tubular, Apocrine, Metaplastic, Mucinous, Inflammatory, Comedo, Adenoid Cystic, and Micropapillary types. Invasive Ductal Carcinoma (IDC) is the most common form of invasive carcinoma accounting for 75% of cases, and Invasive Lobular Carcinoma (ILC) accounts for 10% (Li *et al.* 2005, Bertos and Park 2011). IDC can also be further classified based on levels of differentiation into 3 grades, I being well differentiated, II moderately differentiated and III poorly differentiated. Whilst this form of sub-classification does often reflect prognosis, it has not been able to improve the development of targeted therapeutics.

1.1.8.2 Hormone Receptor expression

Breast cancer was first defined at the molecular level in terms of the presence or absence of the oestrogen receptor. Today breast cancer is classified based on the presence or absence of three hormone receptors: the oestrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor (Her-2). These receptors define three common subtypes of breast cancer: ER^+, $Her\text{-}2^+$ and triple-negative. These groups have different prognoses: ER^+ tumours have the best prognosis with higher overall survival rates, whilst triple-negative tumours have the poorest prognosis (Nishimura and Arima 2008). Sub-classification based on hormone receptor expression has enabled the identification of patients able to respond to treatments targeting these receptors and as such has increased survival rates

significantly (Smith *et al.* 2007). However, due to intra-tumoural heterogeneity, the affect of these treatments is variable (Stecklein *et al.* 2012). An Her2$^+$ tumour is defined as a tumour where greater than 30% of cells express the Her-2 receptor. This may explain why some tumours are refractory to treatment or become resistant over time. In addition, sufficient differences may exist between the primary tumour and secondary metastases to render treatment ineffective when relapse occurs (Arslan *et al.* 2011).

1.1.8.3 Molecular

In a seminal work by Perou and Sorlie, breast cancer was subdivided into 5 types based on gene expression analysis by microarrays which have been confirmed as: Luminal A, Luminal B, Her-2-amplified, Basal-like and Normal breast-like (Perou and Sorlie 2000, Perou and Sorlie 2001, Sorlie *et al.* 2003). A sixth subtype was identified later and has been described as claudin-low (Prat *et al.* 2010). This stratification has enabled more accurate predictions of patient survival. However, it is not feasible to determine the entire gene expression profile of a patient's tumour due to cost of microarray analysis. In order to overcome this problem, the gene expression profile has been reduced to a signature profile of the most predictive 50 genes (termed PAM50), the expression of which may be analysed by qPCR. This method is capable of classifying tumours according to the six molecular subtypes and is able to predict relapse more accurately (Prat *et al.* 2014). Another study has subdivided breast cancer into 10 subtypes: Using breast cancer samples from over 2000 patients, tumours were classified at the genetic level according to copy number aberrations (CNAs) and single nucleotide polymorphisms (SNPs). This revealed 10 distinct subtypes with distinct clinical outcomes, and also identified a number of previously unstudied genomic regions which are likely to contain drivers of tumourigenesis. This study has not yet translated into treatment, but studies such as this facilitate the identification of novel therapeutic targets and bring us closer to the possibilities of personalised medicine (Curtis *et al.* 2012).

1.1.9 Treatment

The ability to sub-classify breast tumours has enabled clinicians to give more accurate prognoses and better identification of tailored treatments most likely to improve patient survival. The five-year survival rate of patients increased up to 90% between 1987 and 2007, largely due to the development of targeted therapeutics such as Tamoxifen and Herceptin (Siegal *et al.* 2012).

An initial diagnosis of breast cancer is obtained following a triple-assessment comprising a clinical exam, an ultrasound scan, and a biopsy. Histological analysis of biopsy specimens determines whether any identified lesion is benign or malignant. A malignant tumour is then graded from one to three on the basis of mitotic activity, and analysed for the expression of the hormone receptors ER, PR and Her-2. Patients with high-grade tumours are given further ultrasound scans of the lymph nodes. All this information is taken into account to "stage" the tumour from one to four on the basis of tumour size, lymph node disease, and presence or absence of metastases. Course of action for each patient is decided at a multidisciplinary meeting and outcomes can range from no immediate action for benign lesions, surgery and chemotherapy for invasive tumours, or if metastases are present, to chemotherapy without surgery. If the tumour is able to be resected with surgery, the patient may receive neo-adjuvant chemotherapy in

order to first shrink the tumour. The most common form of neoadjuvant chemotherapy is FEC, a combination of fluorouracil, epirubicin and cyclophosphamide, the side effects of which include alopecia, sickness and diarrhoea, mouth ulcers, skin rashes, and low bone marrow white blood cell count. Her-2$^+$ or triple-negative subtypes of tumours are more likely to respond to neo-adjuvant chemotherapy than ER$^+$ tumours. Those patients with tumours which are unresponsive following surgery and those which presented with metastatic disease are treated with a range of drugs depending on the hormone receptor subtype of the disease (Senkus *et al.* 2013).

1.1.9.1 Oestrogen Receptor Positive

Those patients with tumours which are classified as ER$^+$ are prescribed anti-oestrogen (endocrine) therapies such as Tamoxifen or Fulvestrant which target the oestrogen receptor directly, or Aromatase inhibitors which work by targeting oestrogen receptor signalling (Jordan and Brodie 2007). Endocrine therapies are the first-line treatment for ER$^+$ tumours especially if the patient has a Luminal A type tumour or bone metastases for which anti-endocrine therapies can be effective; some patients can remain on tamoxifen for up to seven years. Despite the effectiveness of ER-targeted therapies, 50% of patients who relapse will have secondary tumours that are completely refractory to treatment, and the remainder will develop resistance over time (Ring and Dowsett 2004). The progesterone receptor is prognostic but does not necessarily predict response to endocrine therapies (Mackay 2011)

ER$^+$ disease is also associated with over-activation of the PI3K/PTEN/Akt/mTOR (mammalian target of rapamycin) signalling network which contributes to cell proliferation in cancer. Agents which target these pathways have been developed for breast cancer including mTOR inhibitors (Rapalogs). However, Rapalogs have been shown to elicit the activation of a feedback mechanism via the IGF receptor to compensate for the loss of mTOR signalling and restore cell survival. For this reason, the Rapalog Ridaforolimus has been trialled in combination with a tyrosine kinase inhibitor targeting the Insulin-like growth factor receptor (Dalotuzumab) for ER$^+$ disease with some success (Di Cosimo *et al.* 2010).

1.1.9.2 Her-2 Positive

Her-2 is a tyrosine kinase receptor involved in a cell proliferation. In advanced breast cancer 100 or more copies of its gene may be present, elevating the number of Her-2 receptors to up to two million. Patients diagnosed with Her-2 positive breast tumours are prescribed monoclonal antibodies such as Tratsuzumab and Lapatanib which are tyrosine kinase inhibitors that work by antagonistically binding to the ERBB2 receptor to inhibit the growth of cancer cells (Slamon *et al.* 2001 Duffy 2005). Tratsuzumab has also been developed which is covalently bound to a chemotherapeutic emtansine which derives from the antimicrotubule agent maytansine. This form of treatment (T-DM1) is designed to target Tratsuzumab directly to the tumour and has shown considerable efficacy in phase II clinical trials with minimal side effects (Krop and Winer 2014).

1.1.9.3 BRCA1/2-Mutated Breast Cancers

An understanding of the DNA repair pathway malfunctions implicated in BRCA mutated cancers has led to the development of a number of drugs targeting this pathway. The therapeutic strategy employed in these cases relies on the basis that any damage induced in the tumour cell cannot be repaired due to the loss of BRCA function inhibiting the double-strand break (DSB) repair pathway. In this manner, the therapy would be selective for the tumour cells, and should therefore have minimal side effects (Boulton 2006). One approach is the inhibition of the enzyme Polyadenosine diphosphate ADP Ribose Polymerase (PARP) of which PARP1 is the most common. This enzyme normally functions in the base excision repair pathway to repair ssDNA breaks before they lead to DSBs at replication forks. In BRCA mutated tumour cells the repair pathway for DSBs is non-functional; therefore inhibition of PARP1 results in the accumulation of DSBs in tumour cells that can not be repaired (de Bono *et al.* 2009). This in theory should become lethal to the tumour and clinical trials of the PARP-inhibitor, Olaparib, for BRCA-mutated breast cancers have been successful at reducing tumour burden (Lee *et al.* 2014). An alternative approach is the use of Cisplatin; a drug which induces cross-links in DNA strands usually repaired by homologous recombination, therefore selecting for tumour cells in the same manner as a PARP inhibitor (Turner and Tutt 2012). Despite the success of initial drug trials, development of insensitivity to these drugs, particularly Cisplatin, has been observed. Breast cancers have been shown to acquire secondary mutations that restore BRCA function, rendering tumours insensitive to treatment (Lord and Ashworth 2013).

1.1.9.4 Triple Negative Breast Cancer

Triple negative breast cancers with a poor response following surgery, can only be treated with chemotherapy. Chemotherapy attempts to selectively kill cancer cells by exploiting the fact that as proliferating cells they are more susceptible to DNA damage than normal healthy cells. However this has the obvious disadvantage of also effecting normal healthy cells which are proliferating such as hair follicles, and stomach and intestinal cells, and is the reason that adverse side effects such as alopecia and nausea are usually associated with traditional cancer treatment. Patients are most often prescribed a combination of anthracyclanes and taxanes which is considered the most effective regimen and is estimated to have decreased breast cancer mortality by a third (Senkus *et al.* 2013).

1.2 Breast Cancer Stem Cells

1.2.1 Cancer Stem Cells

The efficacy of current cancer therapeutics, despite being tailored to disease subtypes, may be impeded by the intrinsic heterogeneity of a breast tumour. A single tumour can itself be made up of multiple cell types which may respond to drugs differently. The cancer stem cell hypothesis proposes that certain sub-populations of cells may be more tumourigenic than others, and therefore the efficacy of a potential anti-cancer drug should be evaluated, at least in part, by its ability to target these more malignant populations. An in-depth understanding of breast cancer stem-like populations is necessary in order to identify and develop effective therapeutic strategies capable of targeting cancer stem cells.

1.2.1.1 Models of Tumourigenesis and the Cancer Stem Cell Hypothesis

The cancer stem cell hypothesis first arose as a result of the noted similarities between normal stem cells and tumourigenic cells: Both have the ability to self-renew and to generate the heterogeneous cell populations observed in tissues and tumours respectively. These similarities together with the rarity of stem cells in adult tissues are what led to the notion that there may be only a subset of cancer cells within a tumour with tumourigenic capacity (Reya *et al.* 2001). This theory is in accordance with the cell-of-origin model of tumourigenesis which is one of two theories proposed to account for the generation of heterogeneity observed within tumours such as those of the breast (Figure 1.5) (Campbell and Polyak 2007). The cell-of-origin model states that intra-tumoural diversity derives from a single cell sitting at the top of a cellular hierarchy similar to that observed in the normal mammary epithelium. The cell-of-origin is the putative "cancer stem cell" (CSC) which has either innately, or following the acquisition of transforming mutations, the ability to divide both symmetrically to self-renew, and asymmetrically to generate differentiated cells. Alternatively, it was also proposed that some cancers may arise in a non-hierarchal manner, whereby transforming genetic lesions occur to multiple cells during the process of tumourigenesis. This model of clonal evolution was put forward initially as an opponent of the cell-of-origin hypothesis; however the two need not be mutually exclusive. It is possible that the phenotypic heterogeneity of a tumour may arise as a result of both the phenotype of a cell-of-origin i.e. the CSC, the type of mutagenic "hits" incurred, and that conferred by evolution.

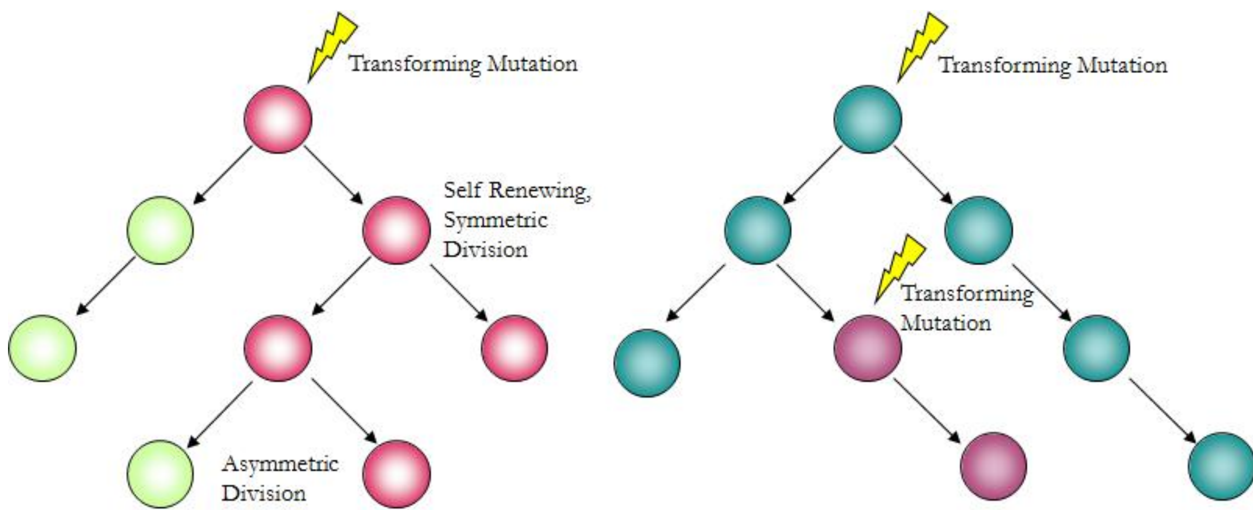


Figure 1.5: *Models of Tumourigenesis:* **A The cell-of-origin hypothesis:** Transforming mutations occur to a single cell which acquires or de-regulates stem-like characteristics, allowing it to divide symmetrically to self-renew or asymmetrically to generate differentiated cells in order to propagate and form a tumour. **B The clonal expansion hypothesis:** transforming mutations occur to a number of cells during the process of tumourigenesis

1.2.1.2 Discovery

Evidence in support of the cell-of-origin hypothesis was generated by the discovery of putative CSCs first in acute myeloid leukaemia by Bonnet and Dick in 1997, who by virtue of the cell surface markers $CD34^+/CD38^-$, isolated a population of cells with enhanced tumourigenic capacity as demonstrated by transplantation into NOD/SCID mice (Bonnet and Dick 1997). This was first recapitulated in a solid malignancy by the discovery of a subpopulation with 50-fold enhanced tumourigenic potential in patient samples of breast cancer. Al hajj *et al.* identified this population by the isolation of cells with the surface marker profile of $CD44^+/CD24^-/ESA^-$/lin- (lin^- referring to negative for several lineage markers and therefore considered undifferentiated) (Al Hajj *et al.* 2003). Tumourigenic populations have since been discovered in brain, colon and pancreatic cancers to name a few (Singh *et al.* 2003, O-Brien *et al.* 2007, Ricci-Vitiani *et al.* 2007, Hermann *et al.* 2007). Despite this progress, the existence of the cancer stem cell still remains controversial, a problem which its supporters may attribute to its complex and plastic nature.

1.2.1.3 Definitions

A normal stem cell is defined by its ability to both self-renew and to generate differentiated cells. Therefore a cancer stem cell also should be defined as such, possibly adding the properties of extensive proliferation and the ability to tolerate foreign niches (Reya *et al.* 2001, Greaves 2010). However, as it is not possible to isolate cells based purely on this premise, a number of phenotypic properties have also been associated with stemness in cancer (Table 1.1). Despite the persistence of the term "cancer stem cell", it is often apparent that these stem-like characteristics do not necessarily occur within the same sub-

population, either between or within breast tumours. Furthermore, observation of CSCs requires intervention on their environment which could alter significantly their biological properties and capabilities. Therefore, for the sake of accuracy, a stem-like cell can only be defined as far as the assay used to identify it (Table 1.1). A tumour cell with any of the known stem-like properties outlined below can be considered to be responsible for sustaining tumourigenesis and is consequently a detrimental and targetable aspect of malignancy. In addition to the heterogeneity of CSCs, there is a mounting body of evidence to suggest that these populations are dynamic; both stem and non-stem-like populations may be capable of inter or intra-conversion in response to environmental cues (Chaffer *et al.* 2011, Gupta *et al.* 2010).

Table 1.1: Properties attributed to cancer stem cells

Proposed Stem-like Characteristics	Corresponding Assays
Tumour initiation/maintenance of tumour growth	*In vivo* transplantation
The capacity for symmetrical division (self-renewal)	*In vitro* by the tumoursphere assay (Dontu 2003) *In vivo* by serial transplantation
The capacity for asymmetrical division or fate (differentiation)	The generation of heterogenous colonies *in vitro*/tumours *in vivo*
Resistance to anoikis	*In vitro* tumoursphere assay *In vivo* anaysis of circulating tumour cells
Metastasis Formation (Combination of anoikis-resistance and tumour formation)	*In vivo* metastasis models e.g. tail-vein or intra-cardiac injection
Drug resistance	*In vivo*/ *in vitro* cell survival following drug treatment

1.2.2 Cell-of-Origin of Breast Cancer

1.2.2.1 Normal Mammary Stem cells

An understanding of the cellular organisation of the human mammary gland is necessary in order to address the question of the tumour cell-of-origin.

Mammary stem cells (MaSCs) have been defined experimentally by their ability to reconstitute an entire mammary gland when transplanted into the cleared mammary fat pad of mice. Initial studies showed that mammary repopulating potential exists within every structure of the mouse mammary gland whether ductal/lobule etc, and therefore unlike in other organs such as intestinal crypts, MaSCs are not restricted to a single location (Visvader 2009). Experiments using sorted mouse mammary cells revealed that cell populations with certain surface marker profiles are enriched for mammary gland initiating potential; notably the $Lin^{+}/CD29^{hi}/CD24^{+}/CD49f^{hi}/Sca1^{+}$ population of the mouse mammary gland was highly enriched for MaSCs (Shackleton *et al.* 2006, Stingl *et al.* 2006) In addition, a single, genetically labelled $Lin^{+}/CD29^{hi}/CD24^{+}$ cell was able to fully recapitulate the mouse mammary gland *in vivo* (Shackleton *et al.* 2006). The majority of cells within these sorted populations appeared to be cycling, however Shackleton *et al.* identified a label-retaining population which it is postulated may be activated at puberty or pregnancy. The $Lin^{+}/CD29^{hi}/CD24^{+}/CD49f^{hi}/Sca1^{+}$ marker profile also isolates

basal/myoepithelial cells and it is not currently possible to distinguish these two populations in the mouse mammary gland experimentally, which may be evidence of the basal/myoepithelial nature of mouse MaSCs.

The limitation of mammary repopulating studies is that cells are analysed in a foreign environment. Lineage tracing experiments allow observation of the *in situ* development of the mouse mammary gland. In one study, labelling of (Keratin 14^{+}) myoepithelial cells during embryogenesis revealed that these cells gave rise to both luminal and myoepithelial progeny in the adult gland. However, induction of YFP expression in $K14^{+}$ cells during puberty labelled only myoepithelial cells, and at no point throughout puberty labelled luminal cells. This suggested that $K14^{+}$ cells do not differentiate into luminal progeny in the adult gland. Similarly, expression of YFP in (Keratin 8^{+}) luminal cells during embryogenesis showed that $K8^{+}$ cells could give rise to both luminal and alveolar cells in the adult mouse. However, induction of YFP expression in $K8^{+}$ cells during puberty was only able to mark the luminal lineage (Van Keymeulen *et al.* 2011). In contrast to these findings, a later study has provided evidence for the existence of bi-potent stem cells within the adult mouse mammary gland. This study used Elf5 and Keratin 5 as markers of luminal and basal cells respectively, in combination with an inducible confetti reporter construct. Induction of the confetti reporter was instigated during and after puberty. Analysis of clonal populations marked by the confetti reporter revealed that Elf5 marked a progenitor lineage with limited clonal expansion that only contributed to the luminal layer of the adult gland. However, keratin 5 marked a stem-like population which gave rise to large clonal populations containing cells of both the basal and luminal lineages. This study suggests that a bi-potent basal-like stem cell exists within the adult mouse mammary gland (Rios *et al.* 2014). The discrepancy between the two studies could be explained by differences in the marker proteins used; keratin 5 may be expressed by true stem cells and keratin 14 by a progenitor population. However, as the human gland is structurally different, the implications for human biology are unclear.

Analysis of prospective human MaSCs has only made possible by the "humanisation" of the mouse mammary fat pad, by injection of human fibroblasts into the cleared mouse gland (Kupperwasser 2004). This generates a more supportive environment for human mammary cells. Using this assay, Ginestier *et al.* found that the $ALDH^{+}$ population, (luminal/epithelial cells) of human mammary cells were enriched for mammary repopulating potential (Ginestier *et al.* 2007). Lim *et al.* subsequently showed that only the $Lin^{-}/CD49f^{hi}/ESA^{-}$ population had mammary repopulating potential. In contrast to the ALDH study, this subpopulation was of the basal/myoepithelial lineage, and had (albeit limited) self-renewing potential. Lim *et al.* also identified four subpopulations corresponding to cells of different lineages, thought to describe the organisation of the human mammary gland: Lin^{-} cells were divided into four populations based on the surface expression of ESA (epithelial-specific antigen) and CD49. High ESA and CD49 marked cells of the luminal and basal lineages respectively. The population negative for both these markers contained the highest proportion of fibroblasts and $ALDH^{+}$ cells. Committed luminal progenitors can be isolated by the marker profile $ESA^{+}CD49f^{+}MUC1^{+}CD24^{+}CD133^{+}Thy1^{-}CD10^{-}$ (Lim *et al.* 2000). These experiments suggest that the human mammary gland is organised in a hierarchical manner whereby different lineages derive from a single multipotent stem cell (Figure 1.6).

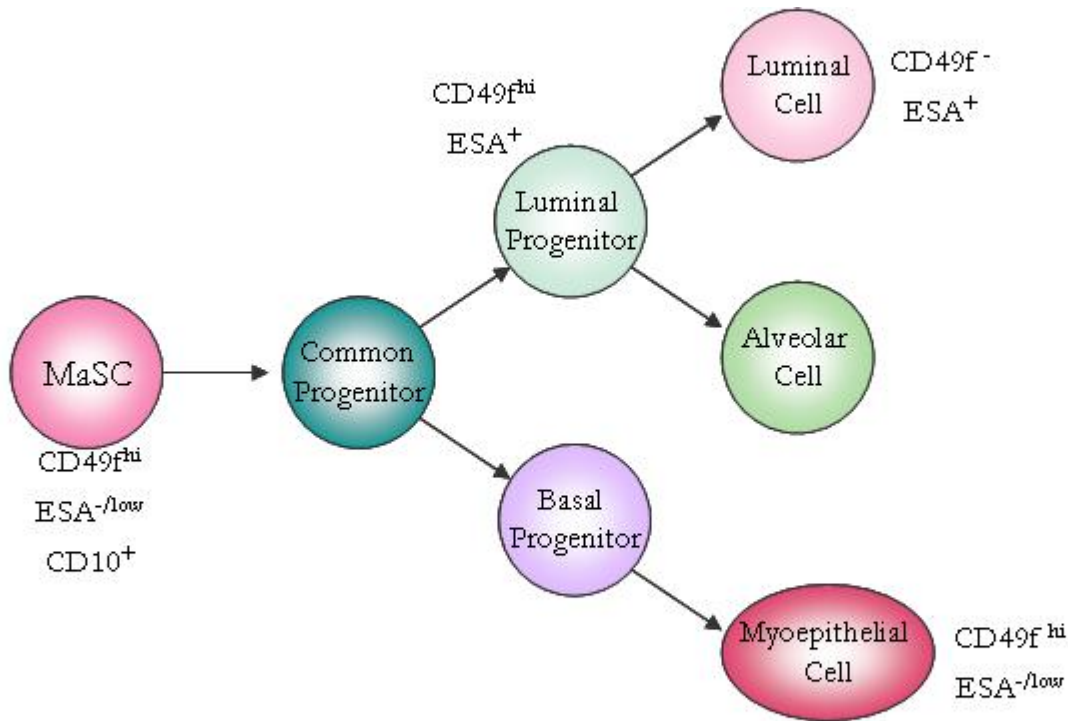


Figure 1.6: Normal Mammary Cellular Heirarchy: CD49f^{hi}/ESA$^{-/low}$ enriches for basal/myoepithelial cells and cells capable of repopulating an entire mammary gland (MaSCs). CD49f^{hi}/ESA^{+} defines luminal cells progenitors, and CD49f^{-}/ESA^{+} defines commited luminal/epithelial cells.

1.2.2.2 The tumour cell-of-origin; is cancer a stem cell disease?

Later studies have attempted to identify the cell-of-origin of breast cancers from among these profiles (Figure 1.6). Oncogenic transformation of the ESA positive subset of primary mammary epithelial cells gave rise to both ER-positive and negative tumours (Keller *et al.* 2010). Oncogenic transformation of the CD10^{+} subset, reflective of the stem-like CD49f^{hi}/ESA^{-} population, gave rise to more aggressive tumours reminiscent of the rare claudin-low subtype (Keller 2010). These studies suggest that breast tumours can arise from stem, progenitor and committed cells, but that most breast cancers do not arise from MaSCs. This is supported by another study showing that BRCA1 mutated tumours arise from luminal progenitors, despite their basal phenotype (Molyneux *et al.* 2010).

The tumour cell-of-origin is often considered synonymous with CSC and although this may not necessarily continue to be the case throughout tumourigenesis, it is by definition the first CSC. Although these aforementioned studies suggest that most tumours do not arise from MaSCs, they are limited by the oncogenic transformations used. As the phenotype of the resulting tumour is likely to arise from a combination of both the phenotype of the cell-of-origin, the oncogenic "hits" aquired, and the selective pressures of different tumour environments, these studies do not rule out the possibility of identifying breast tumours which arise from MaSCs. It has been suggested that the quiescent nature and hence long lifespan of a normal mammary stem cell, together with its existing stem-like properties, make it more likely than a progenitor or differentiated cell to acquire a phenotype necessary for tumourigenesis (Reya *et al.* 2001).

Regulation of self-renewal in normal stem cells is already known as an important method of tumour suppression (Clevers 2005, Morrison and Kimble 2006). In a study of the self-renewal capacity of normal and cancerous cells, Cicalese *et al.* found a greater number of stem-like cells occurring within tumours of an ERBB2 mouse model compared to the normal murine mammary gland. Using mammosphere assays in combination with mammary fad pad transplantation, the group showed that five stem-like cells (mammary gland-initiating) occurred per tumoursphere, compared to one per normal mammosphere. In addition tumoursphere number increased with passage whereas mammospheres derived from normal cells eventually depleted after extensive passaging; they lost self-renewal potential. Using the same approach they were able to calculate stem cells to occur at approximately 1 in 30,000 normal cells compared to 1 in 4000 tumour cells. Furthermore the group used time-lapse microscopy to observe the nature of the initial cell division upon mammary fat pad transplantation. In the normal cells, symmetric division was observed in 20% of the cells seeded in contrast to symmetric division in 78% of tumour cells seeded. The number of stem-like cells, as defined by ability to initiate mammary gland formation, was higher in p53 null mice compared to wild-type controls and increased with time. Restoration of p53 in this model by Nutlin 3 was able to reverse the effect (Cicalese *et al.* 2009). This study highlights marked differences in normal and cancer stem cells, in particular, differences in proliferative potential and stem cell number suggest that unlike normal stem cells, bCSCs do not appear to be quiescent in nature. Although this study does not prove that tumours arise from stem cells, taken together, these findings strongly implicate de-regulation of self-renewing divisions in tumourigenesis. Interestingly, symmetric divisions are in part controlled by the orientation of mitotic spindles and centrosomes, processes which also govern the generation of aneuploidy observed in many cancers (Morrison and Kimble 2006).

1.2.3 bCSC Heterogeneity

1.2.3.1 Inter-tumour heterogeneity: cell surface marker profiling

The identification of targetable stem-like features relies on the ability to isolate and study stem-like cell populations. The identification of signature “stem-like” cell-surface protein expression profiles has made it possible to enrich for breast cancer cells with increased tumourigenic and self-renewal capacity. However, the heterogenous nature of breast cancer makes the existence of a universal stem cell surface marker profile unlikely. Breast cancer has been divided into different subtypes based on tumour cell type; basal-like, Her-2-amplified, normal breast like, luminal subtype A and luminal subtype B, and the more recently identified claudin-low subtype (Perou *et al.* 2000, Prat *et al.* 2010). To date, a number of different marker profiles have been used to isolate stem-like cells from breast cancers, each with varying degrees of specificity across the molecular subtypes (Table 1.2).

The first identification of bCSCs was achieved by the isolation of cells with the marker profile of $CD44^{+}/CD24^{-}/ESA^{+}/Lin^{-}$. Both CD44 and 24 are cell-surface adhesion molecules whilst ESA is a marker of epithelial cells; all three correlate with a negative prognosis (Al Hajj *et al.* 2003). Despite the initial impact of these findings, subsequent studies have shown that the $CD44^{+}/CD24^{-}/ESA^{+}/Lin^{-}$ profile does not achieve the same results when applied to all breast tumours, but instead is particular to basal-like

breast cancer; e.g. in one study the profile was only identified in 31% of 240 samples where it correlated with basal subtypes (Honeth *et al.* 2008). Honeth *et al.* analysed expression of CD44 and CD24 across a range of breast tumour samples by immunohistochemistry. The stem-like phenotype of $CD44^+/24^-$ correlated strongly with low Her-2 expression and elevated expression of EGFR (epithelial growth factor receptor) and was predominantly found in the basal-like/Her-2$^-$ subgroup, as defined by gene expression analysis of tumour samples. The $CD44^+/24^-$ phenotype was under-represented in Her-2 positive tumours which instead had a strong association with $CD44^-/24^+$ expression. In addition 94% of BRCA null and all medullary tumours expressed the $CD44^+/24^-$ phenotype (Honeth *et al.* 2008).

In contrast to the findings of Al Haij, Meyer *et al.* have since demonstrated that in the $CD44^+$ population, both CD24 negative and positive cells of ER^- tumours were tumourigenic with a few as 250 cells. In addition, Meyer *et al.* identified a novel set of markers for ER^- tumours. Further characterisation of the $CD44^+$ population by surface protein expression revealed that highly tumourigenic subsets could be isolated by the profile $CD44^+/CD49f^{hi}/CD133/2^{hi}$; markers associated with normal mammary stem cells (Meyer *et al.* 2010). Cariati *et al.* also analysed the normal mammary stem cell marker CD49f as a potential marker of breast cancer stem cells in the (ER^+) MCF-7 cell line. An MCF-7 population derived from tumourspheres was found to have a greater proportion of $CD49f^{hi}$ expressing cells than the total population; 72.65% vs. 23.28%. In addition, knockdown of CD49f completely prevented tumoursphere formation and tumour initiation *in vivo* (Cariati *et al.* 2008).

Aldehyde dehydrogenase (ALDH) was identified as a marker of tumourigenicity by Ginestier *et al.*. ALDH is an enzyme which detoxifies aldehydes such as retinol, and was studied because of its previous associations with leukaemic stem cells (Yoshida *et al.* 1998). In this study it was found that 3-10% of the cell population of each of four tumour samples (three triple negative and one Her-2$^-$) was positive for ALDH. When isolated, $ALDH^+$ cells were tumourigenic when as few as 500 cells were transplanted whereas $ALDH^-$ cells did not produce tumours. Although limited growth was observed with 50000 $ALDH^-$ cells, resulting tumours could not be serially passaged, suggesting that tumour initiation may have been instigated by a progenitor population (Ginestier *et al.* 2007). $ALDH^+$ populations have since been identified in 23 out of 33 cell lines where, as consistent with the $CD44^+/CD24^-/ESA^+/Lin^-$ profile, it correlated with those lines of a basal-like nature, for example as few as 100 ALDH positive cells of the basal-like SUM159 cell line were required to generate tumours (Charaffe-Jauffret 2009).

CD133 or prominin 1 is a transmembrane glycoprotein which has been associated with CSCs in both haematological and solid tumours (Mizrak *et al.* 2008). CD133 has more recently been found to be associated with bCSCs of tumours in a BRCA1 deficient mouse model (Wright *et al.* 2008). Wright *et al.* used five of these tumours to generate 16 cell lines for cell surface marker expression analysis. Cell lines derived from one of these tumours exhibited a notable $CD133^+$ population and as few as 50 of these $CD133^+$ cells were able to form tumours *in vivo*. $CD133^+$ cells were shown to have stem-like properties comparable to the $CD44^+/24^-$ populations isolated from other $BRCA^-$ tumour cell lines, including similar gene expression profiles, although significantly no overlap between the $CD44^+/24^-$ and $CD133^+$ populations was observed despite all tumours occurring in BRCA-null mice. Similarities in gene expression included over-expression of seven stem-cell related genes including Notch1, CD44, and

ALDH1, although there were some notable differences such as the basal marker keratin5 which was 17fold higher in $CD133^+$ subsets compared to CD44/24 cells (Wright 2008).

The range of marker profiles currently used to enrich for stem like characteristics across tumours could be an indication that a universal bCSC marker profile has not yet been identified. However it is more likely that the bCSC population mirrors that which is observed amongst all breast cancer cells and is heterogeneous in nature. Further studies of bCSC marker profiles, perhaps those incorporating gene expression analyses, will undoubtedly increase our understanding of heterogeneity and may even make it possible to classify breast tumours based on the distribution of bCSC characteristics.

<u>Table 1.2:</u> Markers used to isolate breast cancer cell populations enriched for tumourigenic capacity:

Marker	Target	Cell numbers required for tumour formation *in vivo*	Reference
$CD44^+/CD24^-/ESA^+$	Human breast cancers: Basal-like, claudin-low, $Her2^-$, $BRCA^-$ and medullary tumours	200	Al-Haij 2003, Honeth 2008
	Cell lines	Not tested	
$CD44^+/CD49f^{hi}/CD133/2^{hi}$	ER^- tumours	250	Hwang-Verslues 2009
$CD49f^+$	MCF-7 cell line	Not tested	Meyer 2010
$CD133^+$	$BRCA^-$ mouse tumours	50	Wright 2008
$ALDH^+$	Human breast cancers; Triple negative, Her-2 negative	500	Ginestier 2007 Charrafe-Jauffret 2009
	Cell lines	Not tested	
$CD44^+/CD24^-/ESA^+$ $ALDH^+$	Human breast cancers	20	Ginestier 2007
$PROCR^+/ESA^+$	MDA-MB-231 cell line	100	Hwang-Verslues 2009

1.2.3.2 Intra-tumour heterogeneity

The cell-of-origin model of tumourigenesis assumes that CSCs are a rare population at the top of a cellular hierarchy, but this concept is now being challenged. In addition to the bCSC heterogeneity observed between breast tumours, there is some evidence to suggest that there exists more than one population of stem-like cells within a single tumour or cell line. Intra-tumour CSC populations have come to light by investigating different aspects of stem-like behaviour within a single tumour which by our current definitions do not completely overlap. Intra-tumour heterogeneity is most easily observed by cell-surface marker profile analysis due to the ability to analyse both potential populations simultaneously in the same environment. With a sequential analysis it is not possible to determine whether two stem-like populations exist or whether plasticity has occurred; i.e. in the time it takes to perform transplantation assays, a stem-like cell could have derived from a non-stem.

Although intra-tumour diversity in stem-like cells has been observed in other cancers (Biddle *et al.* 2011), evidence for intra-tumour CSC heterogeneity in breast cancer has only arisen from a few papers including the aforementioned study of cell surface markers by Ginestier *et al.* In the four samples investigated, the $ALDH^{+}$ population only overlapped with the $CD44^{+}/CD24^{-}/ESA^{+}/Lin^{-}$ profile by 0.1-1.2%. Only 500 $ALDH^{+}$ cells were required to form a tumour *in vivo*, but the $CD44^{+}/CD24^{-}/ESA^{+}/ALDH^{+}$ population required only 20 cells to produce a tumour (Ginestier *et al.* 2008). On the basis of these findings, two inter-converting stem-like subpopulations have been proposed, each possessing distinct stem-like features (Figure 1.7). In a recent paper, Liu *et al.* have related bCSC markers to normal mammary stem cell markers and found that the EMT-like $CD44^{+}/CD24^{-}$ bCSC population as defined by Al Haij resides within the $ESA^{-}CD49f+$ population, and $ALDH^{+}$ within the $ESA^{+}CD49f^{-}$ population, the latter being considered more epithelial-like. On this basis, bCSCs have been divided into more luminal or MET-like and more basal or EMT-like. Furthermore, there is evidence to suggest that these populations reside at different areas or niches of the tumour: the MET-like at the centre and the EMT-like at the invasive front (Liu *et al.* 2014). The possible dynamics of this system are discussed in more detail in section 1.2.4.2.

Further evidence which may support this theory comes from a study of rat mammary tumours by Zucci *et al.* Three clonal subpopulations were isolated from a rat mammary tumour; stem-like, mesenchymal-like and epithelial-like. Both the stem-like and epithelial-like cells, although morphologically distinct, were able to initiate tumour growth *in vivo* and to form tumourspheres, however the epithelial-like cells were incapable of both serial-transplantation and self-renewal. This is another example of a cell population exhibiting some but not all stem-like attributes. The lack of self-renewal capacity in this population led the authors to describe it as a progenitor-like but nonetheless tumourigenic population. In addition, the epithelial-like population expressed a cell-surface expression signature comparable to that observed in the core of the tumour, whereas the stem-like population was comparable to the tumour invasive front, thus echoing the findings of Liu *et al.* as mentioned above (Zucci *et al.* 2008).

1.2.4 bCSC Plasticity

1.2.4.1 Plasticity between non-stem breast cancer cells and bCSCs

Plasticity is a feature of cancer cells; inter-conversion is known to occur via EMT and MET (Drasin 2011). A certain degree of plasticity may also exist within a tumour cell population which allows inter-conversion between CSC and non-CSC states when driven by selective pressures (including therapy) or clonal evolution. Indeed, CSCs within a tumour may be a different entity at instigation of tumourigenesis compared to diagnosis or following therapy (Greaves 2010). Plasticity between non-CSCs and CSCs could easily be confused with a potential heterogeneity of stem-like populations and therefore cannot simply be observed by an alteration of stem-like characteristics in the total population. To study plasticity, subpopulations of cancer cells must be assayed separately. If a certain stem-like characteristic were to appear in a population previously devoid of such an attribute, only then would it be possible to conclude that plasticity has occurred.

An analysis of the plastic potential of human mammary epithelial cells (HMECs) was carried out in this manner by Chaffer *et al.* This study found evidence of plasticity occurring in both normal and cancerous cell populations. Non-transformed HMECs were considered first and the cell population first divided by the isolation of a sub-population of cells found floating in the media of HMEC cultures. The floating cells (designated HME-Flopc) were found to contain a greater proportion of stem-like cells than the adherent population as determined by the expression of the stem cell markers $CD44^{+}/CD24^{-}/ESA^{+}$, (2.5% compared to 0.2%). Clonal flopc populations were subsequently fractionated into $CD44^{lo}$ and $CD44^{hi}$ subsets. Re-assaying for marker profile following adherent culture revealed $CD44^{hi}$ cells in the $CD44^{lo}$ fraction. Contamination of the population with $CD44^{hi}$ cells was ruled out due to the lack of a significant difference in proliferation between the two populations. In addition, even in the presence of a high proportion of labelled $CD44^{hi}$ cells, $CD44^{hi}$ cells could still be generated from unlabelled $CD44^{lo}$ cells. These findings are significant as they are evidence of plasticity, the *de novo* generation of stem cells, occurring in normal cell populations *in vitro*.

To analyse the oncogenic counterparts of flopc cells, oncogenic transformation was induced by introduction of the SV40ER and H-Ras oncogenes. Transformation of flopc cells resulted in five-fold more efficient conversion of $CD44^{lo}$ cells to $CD44^{hi}$ than had been observed in the untransformed populations. This was also found to be true *in vivo*; $CD44^{lo}$ cells injected into mice formed tumours with up to 16% $CD44^{hi}$ cells (Chaffer *et al.* 2011). This study was highly significant as the first to definitively demonstrate plasticity upwards in the established hierarchy of both normal and neoplastic cell populations, and to demonstrate an increase in plasticity occurring in transformed cells over normal cells. These findings have important implications for breast cancer treatment as they suggest that targeting only the stem-like population of cancer might not be enough to eradicate a tumour of its metastatic potential, as stem-like cells could then re-generate from the non-stem population. Furthermore, when taken together with the Cicalese paper (Cicalese *et al.* 2009) which showed that stem cell self-renewal occurred more often in cancer than in normal tissues, it is possible that an increase in stem cell number may be a defining feature, even a hallmark, of breast tumours.

Plasticity was also observed in a similar analysis of the breast cancer cell lines SUM149 and SUM159 (Gupta *et al.* 2011). Both lines were fractionated into subpopulations based on the marker profile identified by Al haij: luminal: $CD44^{-}/CD24^{+}/ESA^{H}$, basal: $CD44^{+}/CD24^{-}/ESA^{-}$, and stem-like; $CD44^{+}/CD24^{-}/ESA^{+}$. These subtypes were confirmed by gene expression analysis; no functional assay was used. All isolated populations were able to transition back to a heterogeneous population which recapitulated the proportions of each subset observed in unsorted cells. As with the Chaffer study, these data show that a subpopulation of cancer cells not expressing stem cell markers is able to generate this population *de novo*. It was not likely that any stem-like component remaining within the basal or luminal populations increased via enhanced self-renewal due to no difference in proliferation being observed and it was estimated that any minority surviving population would require a division rate three times greater than that of embryonic stem cells in order to achieve this. A mathematical model was generated to describe state transitions and make predictions about luminal or basal cell transitioning to a stem-like state. These predictions were corroborated subsequently by *in vivo* experiments; luminal or basal subpopulations

were able to form tumours thus suggesting that plasticity is occurring within these populations. However it cannot be ruled out that a tumourigenic subset without a stem-cell profile remained within the purified populations; the $CD44^{+}/CD24^{-}/ESA^{+}$ phenotype only enriches for tumourigenic cells and it is often still possible to generate tumours from the stem-like negative population (Al Hajj *et al.* 2003). Furthermore, inhibition of TBX3 (a gene involved in the regulation of differentiation) by shRNA was able to perturb plasticity by decreasing the probability of luminal to basal transitions in SUM159 cells and increasing the probability of basal-to-luminal transitions in SUM149 cells (Gupta *et al.* 2011). Although not able to prevent conversion to a stem-like state, this demonstrates a useful tool for the identification of genes involved in the process of plasticity as potential therapeutic targets.

A number of studies have since indicated the importance of an EMT-like process in the conversion of cells to a CSC state. EMT is a process of cell transition which occurs primarily during embryogenesis, but can also take place during wound healing and tumourigenesis (Drasin 2011 and Section 1.1.6). In tumourigenesis EMT has been implicated in metastasis of disseminated cancer cells, which are thought to require stem-like characteristics such as anoikis resistance. This correlation led to the notion that EMT may be responsible for imparting CSC traits. Mani *et al.* investigated this possibility by induction of EMT in HMECS by over-expression of Twist, a transcription factor known to be important in EMT. In addition to the expected down-regulation of epithelial markers and up-regulation of mesenchymal markers such as N-cadherin, cells acquired a stem-like phenotype and formed over 30-fold greater number of spheres than wild-type cells. Induced EMT in Her-2neu immortalised HMLEs resulted in a 10-fold increase in spheres and 2-fold increase in tumour-initiating cells (Mani *et al.* 2008). These data strongly implicate a role for EMT in plasticity, however it cannot be distinguished from this study whether induction of EMT has increased the self-renewal of existing bCSCs or plasticity towards a stem-like phenotype. The lack of difference between proliferation rates between subpopulations supports the latter possibility, however an analysis of subpopulations would be more conclusive. Morel *et al.* supported this line of investigation by focussing on $CD24^{-}$ cells which were completely absent from HMECS. Transformation by the Ras oncogene resulted in the generation of a $CD24^{-}$ population which increased gradually to become 65% of the population. This phenotype was consistent with stem-like cancer cells; $CD24^{-}$ cells could form tumourspheres and tumours *in vivo* whereas $CD24^{+}$ cells could not. Furthermore $CD24^{-}$ cells could be generated from $CD24^{+}$ cells. Enrichment in $CD24^{-}$ cells following transformation coincided with morphological changes and alteration of gene expression profiles consistent with EMT. Experimentally induced EMT by TGFβ also resulted in the appearance of $CD24^{-}$ cells and an increase in the expression of mesenchymal markers. TGFβ also accelerated transformation by Ras (Morel *et al.* 2008). Similar observations linking plasticity and EMT have been made in cell lines (Blick *et al.* 2010). More recently, Yang *et al.* have visualised plasticity between non-stem cancer cells and bCSCs occurring *in situ* in MCF-7 cells and shown that this process could be enhanced by the induction of EMT or perturbed by its inhibition (Yang *et al.* 2012).

The link between EMT and the formation of cancer stem cells has now been recognised but the molecular mechanisms underlying this plasticity have yet to be firmly established. To date only a few groups have addressed this issue. A number of molecular mechanisms which potentially underlie this stem

cell and EMT plasticity have been identified including the p130Cas/Cyclooxygenase-2 axis and Brd4 (Bisaro *et al.* 2012, Alsarraj *et al.* 2011). Meyer *et al.* have also demonstrated that non-invasive epithelial-like $CD44^{+}/CD24^{+}$ cells of breast cancer cell lines can generate invasive, mesenchymal-like $CD44^{+}/CD24^{-}$ cells in culture and following transplantation *in vivo.* Importantly, inhibition of TGFβ receptors by the compound SB431542, prevented the generation of heterogeneous populations from $CD44^{+}/CD24^{+}$ cells which could only then generate vimentin-low progeny. This study revealed the importance of TGFβ signalling in plasticity which highlights a potential therapeutic target (Meyer 2010).

Evidence for the regulation of stem cell and EMT plasticity has come from investigations into the role of microRNAs in this process. MicroRNAs are untranslated RNAs which regulate the expression of multiple coding regions of the genome and have been implicated in the regulation of many cellular processes including stem cell division and have also been implicated in carcinogenesis (Kato and Slack 2008). Inhibition of the tumour suppressor p53 by shRNA induced EMT and increased the percentage of $CD44^{+}/CD24^{-}$ cells in breast cancer cell lines. Over-expression of miR-200c was able to rescue the phenotype of p53shRNA cells. Conversely, the induction of EMT by TGF-β was reversed by over-expression of p53 which also reduced the percentage of $CD44^{+}/CD24^{-}$. These data are evidence of the important relationship between p53 and miRNAs in the regulation of the conversion of non-stem breast cancer cells to bCSCs (Chang *et al.* 2011).

The aforementioned studies all used cell surface marker profiling to isolate stem-like populations and whilst this has proved an effective method to identify plasticity within tumours the evidence is mostly limited to a single stem-like attribute. Lineage tracing experiments have recently been used to track cancer cell growth in both skin and mammary tumours and have produced the first *in vivo* evidence of CSCs as defined by tumour initiation (Driessens *et al.* 2012, Zomer *et al.* 2012). In order to observe plasticity *in vivo* Zomer *et al.* induced random expression of one of four colours in all tumour cells of the PyMT mouse model of spontaneous tumour formation. Using this system and by imaging at various stages during tumour progression it was possible to identify clonal populations by single colour expression which either expanded at a later stage of tumour formation or regressed, thereby implying loss or gain of the bCSC property of tumour initiation (Zomer *et al.* 2012). The regulation of this observed plasticity was not investigated in this study. To further expand our knowledge of plasticity and to investigate the underlying molecular mechanisms, it may be advantageous to develop functional plasticity assays; i.e. the elimination and reacquisition of a single functional stem-like attribute such as tumour initiation as mentioned above (Zomer *et al.* 2012) or tumoursphere formation as proposed by Piggott *et al.* (Piggott *et al.* 2011).

1.2.4.2 Plasticity between subpopulations of bCSCs

Adding yet another layer of complexity to the issue of plasticity is the notion that there may exist within a tumour more than one distinct cancer stem-like state between which cells can transition, possibly depending on the influence of external cues. Evidence for multiple stem-like states can only come from studies which further purify the CSC component into putative subpopulations.

As mentioned previously, two putative subpopulations of bCSCs have been identified in breast tumour samples based on the two marker profiles of ALDH^{+} and CD44^{+}/CD24^{-}/ESA^{+} respectively (Ginestier *et al.* 2008). In a recent study it was suggested that these two populations are present in distinct compartments of the normal mammary gland hierarchy and exist in EMT-like (ALDH^{+}), and MET-like CD44^{+}/CD24^{-}/ESA^{+} states. This study showed that inter-conversion between stem-like states can occur via EMT-like or MET-like processes. There is some evidence to suggest that inter-conversion is regulated by the expression of a number of microRNAs. The MET-like CSC population exhibits increased levels of mi93 which when over-expressed is capable of increasing this population further. Consequently, inhibition of mi93 is able to induce EMT in isolated populations of ALDH^{+} SUM159 cells. In contrast, up-regulation of mi100 or 221 decreases the ALDH^{+} population and increases CD44^{+}/CD24^{-}/ESA^{+} cells (Liu *et al.* 2014). Whilst evidence for intra-bCSC plasticity is still lacking, this group has demonstrated convincing support for this hypothesis which may explain a previous discrepancy in breast tumourigenesis; why EMT is known to increase metastasis but metastases are often as epithelial-like as the primary tumour (Kowalski *et al.* 2003, Liu *et al.* 2012).

Further support for this theory comes from an investigation into the effect of hypoxia on tumour initiating cells. The authors used the SK3rd cell line which had been derived from the SKBR3 parental cell line. Compared to the SKBR3 line, SK3rd cells have a higher proportion of tumoursphere-forming and CD44^{+}/CD24^{-} cells, and an enhanced capacity for metastasis formation. Surprisingly, the SK3rd cell line had only a small increase in invasive ability compared to SKBR3 cells. However, culture in hypoxic conditions was found to confer the property of invasiveness on the SK3rd cells but had no effect on the SKBR3 line. This effect was mediated by membrane type 1 matrix metallo-protease (Mt1-MMP), the inhibition of which was found to reduce invasiveness under hypoxic conditions. Hypoxia was not able to affect the expression of Mt1-MMP, but instead altered is localisation to the cell surface. Elevated levels of surface Mt1-MMP, although associated with enhanced invasive ability, correlated with a decrease in the bCSC marker CD44, and also disrupted tumoursphere formation. Hypoxia or elevated surface Mt1-MMP did not alter the tumourigenic capacity of the TICs but the volume of lung metastases was increased. In addition the non-invasive bCSCs in this model were found to be mesenchymal-like in comparison to the SKBR3 parental line: Twist1 was elevated in the SK3rd cells and was found to be capable of regulating MT1-MMP expression via miR10b. These findings suggest that normoxic, non-invasive bCSCs are EMT-like and anoikis-resistant, whereas hypoxic, invasive CSCs are epithelial-like but more anoikis-sensitive. However as hypoxia is not able to regulate MT1-MMP expression, the relevance of EMT in this model remains to be firmly established. In addition, the authors did not report on the epithelial or mesenchymal status of the SK3rd cells under hypoxic conditions (Li *et al.* 2012). These data imply the existence of two distinct stem-like populations which can inter-convert in response to oxygen levels in the tumour environment. However the findings are in contrast to those of Liu *et al.* which suggest that the MET-like bCSCs reside at the centre of the tumour (hypoxic) and the EMT-like bCSCs at the invasive front (normoxic) (Liu *et al.* 2014).

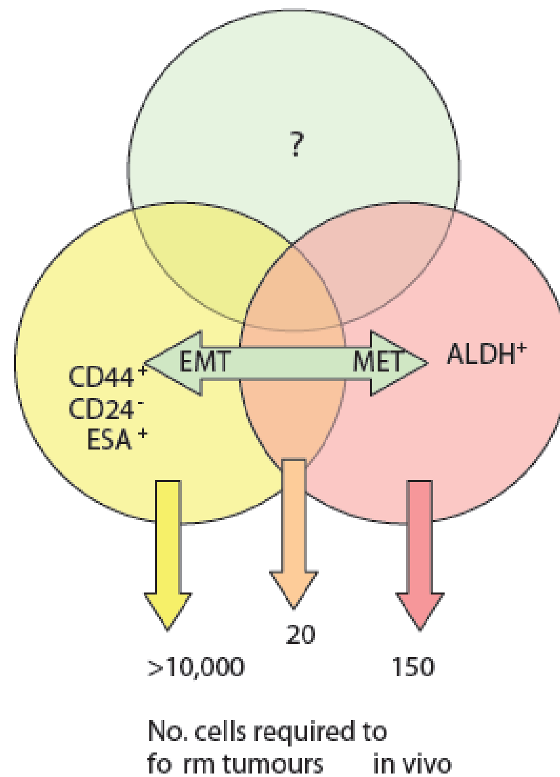


Figure 1.7: *Intra-tumour heterogeneity and plasticity of stem-like populations:* Two or more inter-converting stem-like populations may exist within breast tumours. Further investigation may reveal yet more subpopulations of bCSCs.

1.2.5 Implications for Treatment

1.2.5.1 Therapy Resistance

Despite the complexity of CSCs, it is clear that stem-like properties contribute the most detrimental aspects of malignancy to a tumour and therefore are highly desirable targets of anti-cancer drugs. However, bCSCs are resistant to most of the most commonly prescribed breast cancer therapies and in fact, by preferentially targeting non-stem cancer cells, most traditional approaches actually increase the proportion of bCSCs in a cancer cell population. For example, in a murine breast cancer cell line, just one week of treatment with paclitaxel and epirubicin resulted in the majority of surviving cells expressing the bCSC marker profile $CD44^{+}/CD24^{-}$ (Li *et al.* 2008). Similar findings have been reported with docetaxol (Creighton *et al.* 2009) and cisplatin for BRCA positive breast cancers (Shafee *et al.* 2008). Resistance is not restricted to standard chemotherapies; in ER^{+} breast tumours, the selective agents Tamoxifen and Fulvestrant could not target the bCSC population, which despite the phenotype of the majority of the tumour cells, itself was ER^{-}. This may be a possible explanation for the failure of these agents to prevent tumour relapse in many instances (Kabos *et al.* 2011). A number of mechanisms by which bCSCs avoid drugs have been postulated: In the case of chemotherapy, it is thought that the quiescent nature of at least some of the bCSC population prevents targeting by chemotherapy agents that inhibit cell proliferation (Reya *et al.* 2001). Furthermore, bCSCs have been associated with increased

expression of anti-apoptotic genes such as Bcl2 which may inhibit cell death, and also ATP-binding cassette transporters which could function to promote drug efflux (Dean *et al.* 2005). bCSCs have also been found to be more resistant to radiotherapy than non-stem cancer cells, possibly via the activation of the ATM kinase, a protein involved in the repair of DNA damage (Yin *et al.* 2011).

At present, the resistance of stem-like cells to commonly used therapeutic strategies poses a major problem for the successful treatment of breast cancer patients. The ability of bCSCs to survive treatments which otherwise shrink tumour growth suggests that they may be responsible for tumour relapse following therapy.

1.2.6.2 Therapeutic Strategies

The aforementioned studies are evidence of the fact that agents which do not target the bCSC subset of a breast tumour are unlikely to be successful as they result in an increase in the proportion of bCSCs in the remaining tumour (Figure 1.2.4 A). In theory, an increased proportion of CSCs increases the likelihood of tumour re-growth and metastasis, and this is supported by a study which found that an increased proportion of bCSCs reflected a poor prognosis (Gong *et al.* 2010). Evidence for the existence of CSCs and their resistance to chemotherapy led to the dandelion hypothesis. This uses the analogy of a tumour as a weed; unless you can remove the root, it will re-grow and re-seed elsewhere. This theory suggests that in order to prevent tumour re-growth and metastasis a cancer therapy must target the CSCs (Figure 1.2.4 B). However, the clinical implications of CSC plasticity are that even if you can eradicate the bCSC population, non-stem cancer cells can become CSCs and cause tumour relapse. Therefore an improved strategy would be to target both the CSC and the non-stem populations in order to prevent the reacquisition of stem-like characteristics by plasticity and consequently prevent tumour relapse following therapy (Figure 1.2.4 C).

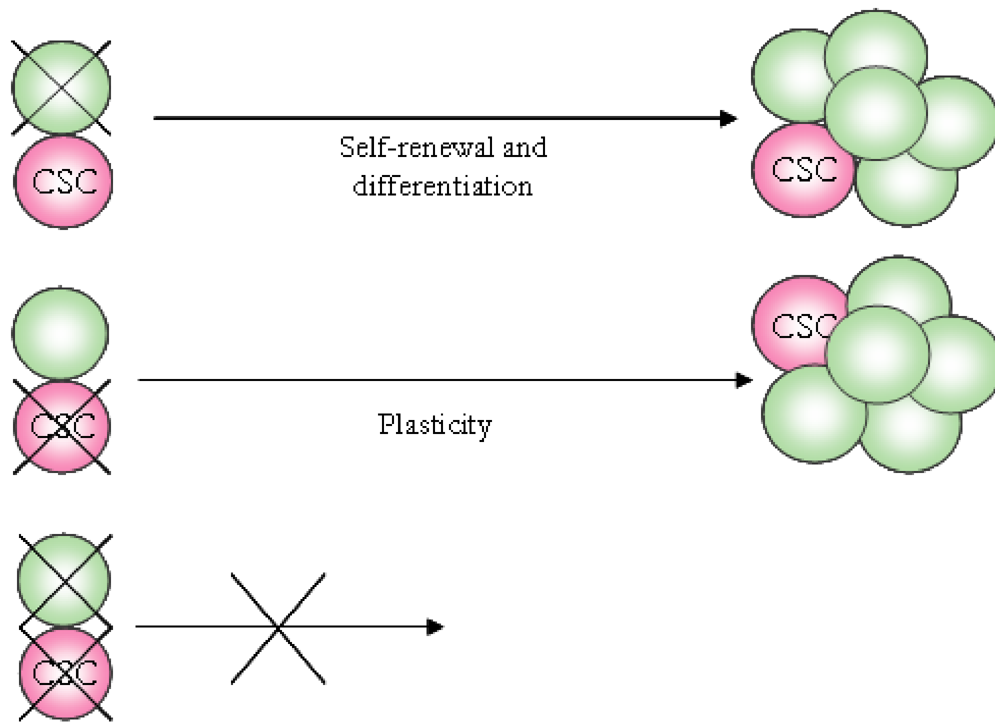


Figure 1.8: Treatment Strategies: Targeting either the (**A)** non-stem or (**B)** stem cell subset of a tumour will likely result in tumour re-growth via differentiation and plasticity respectively, whereas targeting of both cell types (**C)** in theory is more likely to prevent tumour re-growth.

1.2.6.3 Targeting bCSCs

Many agents are known to de-bulk tumours but due to the resistance of bCSCs to most existing therapies, relatively few agents have been shown to be effective at targeting the bCSC population. Hence there is great interest in the development of novel therapeutics designed to target bCSCs. A number of potential avenues have been explored including targeting CSC markers: An antibody to the CSC marker CD44 decreased the growth and recurrence of xenografts consistent with the depletion of bCSCs (Marangoni *et al.* 2009) and CD8+ T cells targeting ALDH did the same (Visus *et al.* 2011). There is also much interest in targeting pathways associated with bCSCs. A number of molecular pathways have been highlighted as important for the regulation and maintenance of bCSC-like traits. Unsurprisingly due to their association with EMT, some of the pathways are developmental and also de-regulated in an oncogenic EMT (Section 1.1.4.3). Inhibition of Pi3K or Wnt signalling have both been successful at reducing tumoursphere formation (Korkaya *et al.* 2009, Lamb *et al.* 2013).

A few existing agents are able to target bCSCs including the antibiotic Salinomycin (Gupta *et al.* 2009) and the anti-diabetic drug Metformin. Metformin is a drug routinely prescribed to patients with type 2 diabetes mellitus. Interestingly, hyperinsulinaemia, insulin resistance and diabetes are all associated with increased breast cancer risk (Wolf *et al.* 2005). The first indications of the potential of Metformin as a breast cancer drug came from large studies that identified a decreased breast tumour incidence and relapse of breast tumours following therapy in diabetes patients prescribed Metformin (Evans *et al.* 2005). In pre-clinical studies, Metformin decreased the proliferation and inhibited the growth of xenografts by 55% (Sahra *et al.* 2008). A more recent study has shown that Metformin selectively targets bCSCs: At doses low

enough to not harm non-stem cancer cells, Metformin reduced both tumoursphere and colony formation, and reduced tumour initiation *in vivo*. Significantly, Metformin also inhibited the oncogenic transformation of the MCF10A breast epithelial cell line by Src kinase (Hirsch *et al.* 2009). The ability of Metformin to prevent oncogenic transformation may explain the reduced incidence of breast cancer in diabetes patients taking the drug (Evans *et al.* 2005). This study also showed that in combination, Metformin and the chemotherapeutic Doxorubicin were more effective than either alone at preventing the re-growth of tumour xenografts (Hirsch *et al.* 2009). This is an example of a therapeutic strategy designed to target both bCSCs and non-stem cancer cells having a greater efficacy than targeting either alone. Consistent with a prevention of plasticity, Metformin and Doxorubicin also prevented tumour relapse by at least 60 days (Hirsch *et al.* 2009). Metformin is thought to target non-stem cancer cells via insulin reduction and activation of AMPK to prevent cell division (Gonzalez-Angulo and Meric Bernstom 2010). However, more recent findings suggest that Metform may reduce bCSCs via an ability to inhibit TGFβ signalling and a transcriptional EMT (Vasquez-Martin *et al.* 2010). Taken together with the finding that Metformin can prevent tumour relapse in a xenograft model, its ability to prevent EMT suggests that Metformin may inhibit bCSC plasticity, but this possibility has not been tested directly.

More recently Herceptin was shown to be able to target the bCSC population of ER^+ ($Her\text{-}2^-$) breast cancer cell lines and tumours. Surprisingly, the bCSC population was found to express Her-2 which was required for its role in Notch signalling. Administration of Tratsuzumab reduced both tumoursphere formation and the proportion of $ALDH^+$ cells. Furthermore, Tratsuzumab inhibited tumour initiation and prevented the recurrence of tumours when used in conjunction with the chemotherapeutic agent Docetaxol. This is another example of an adjuvant therapy whereby a chemotherapeutic agent is used to de-bulk the tumour of non-stem cells and a targeted agent used to kill the bCSCs. As each drug was less effective alone, this suggests that only in combination there is a prevention of plasticity (Ithimakin *et al.* 2013). Furthermore, when taken together with the finding that Tamoxifen cannot target bCSCs of ER^+ tumours (Kabos *et al.* 2011), these studies suggest that Herceptin may be a better therapy for ER^+ breast tumours than Tamoxifen.

Finally, we have found that inhibition of the anti-apoptotic cellular FLICE-Like Inhibitory Protein (cFLIP) selectively and completely sensitised the tumoursphere-forming cells of four breast cancer cell lines to the cytotoxic agent Tumour-necrosis-factor Receptor Apoptosis Inducing Ligand (TRAIL). Inhibition of cFLIP also sensitised the majority of the non-stem cells to TRAIL (Piggott *et al.* 2011). This suggests that the combination of cFLIP inhibition plus TRAIL has enormous potential for efficacy in the treatment of breast cancer. As such, TRAIL and cFLIP will be explored in more detail in the subsequent section.

1.3 TRAIL as a Therapy for Breast Cancer

1.3.1 Rationale

The ability to evade apoptosis is one of the proposed hallmarks of cancerous cells (Hanahan and Weinberg 2000). Therefore, one strategy for targeting cancer cells is to activate apoptosis either by initiating redundant latent apoptosis mechanisms within cancer cells, or by re-sensitising them to the apoptosis they have overcome during the process of tumourigenesis. Apoptosis is a form of programmed cell death that can occur via one of two pathways: Extracellular stimuli can induce apoptosis by activating the "extrinsic" apoptosis pathway, or alternatively, apoptosis can be controlled within the cell by Bcl2 proteins which can activate the "intrinsic" or "mitochondrial" apoptosis pathway (Johnstone *et al.* 2008). In order to identify mediators of apoptosis in the mammary gland, microarray profiling of the mouse mammary gland was carried out during pregnancy, lactation and involution. Following lactation, the mammary gland undergoes a distinct remodelling process called involution which involves a high level of cell death as the cells no longer required for lactation are programmed to die (i.e. undergo apoptosis). The screen identified a number of proteins up-regulated during involution that function to induce apoptosis. This included members of the tumour-necrosis-factor-alpha (TNFα) superfamily, a group of related ligands which, as well as other roles, function to activate the extrinsic apoptosis pathway. Among these, the array identified TRAIL as a potential candidate for inducing apoptosis in the mammary gland (Clarkson and Wayland 2000).

TRAIL is a soluble cytokine endogenously manufactured by cells of the immune system. TRAIL functions to activate a number of pathways including the extrinsic apoptosis pathway in target cells thus inducing caspase-mediated cell death. In some cases, this can also result in activation of the intrinsic mitochondrial apoptosis pathway. Conversely, TRAIL can also activate the NFkB, MAPK and JNK pathways to promote cell survival (Johnstone *et al.* 2008). TRAIL was discovered by virtue of its homology to another death ligand CD95 or FasL which also activates the extrinsic pathway. Initial reports of the ability of TRAIL to target and induce apoptosis preferentially in cancerous cells led to its recombinant production as an anti-cancer agent. Recombinant human TRAIL is non-toxic has been clinically trialled for a number of cancers (Section 1.2.4).

1.3.2 Structure of TRAIL

Tumour Necrosis Factor alpha-Related Apoptosis Inducing Ligand (TRAIL) is a member of the TNF superfamily and a type II transmembrane protein which is proteolytically processed to form a soluble ligand. As a homotrimer, TRAIL can bind to any of four transmembrane receptors two of which are functional (DR4 and 5), and two of which are decoy receptors (TRAILR3 and R4) involved in TRAIL-resistance of normal but not cancerous cells (Griffiths *et al.* 1998). TRAIL receptors are made up of an extracellular cysteine-rich domain (CRD) and an intracellular "death" domain. TRAIL binding to DR4 or 5 induces oligomerisation of the receptor which allows its intracellular death domain to function as a platform for downstream signalling (Hymowitz *et al.* 1999) (Figure 1.9).

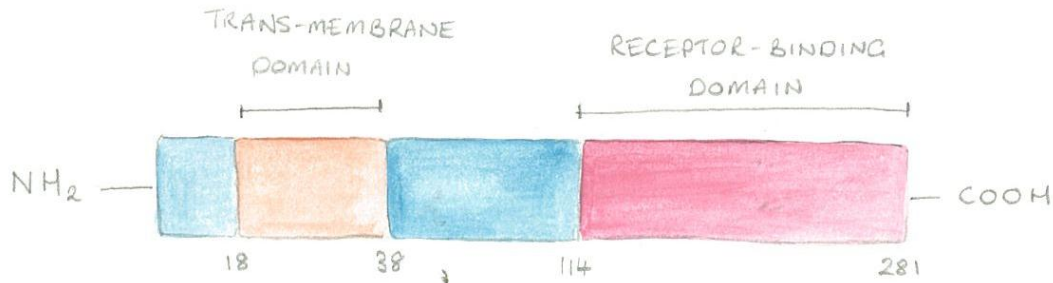


Figure 1.9: Structure of TRAIL: TRAIL is produced as a transmembrane protein which is cleaved to become a soluble ligand. TRAIL recognises any of four receptors via its receptor-binding domain.

1.3.3 TRAIL function

To activate the extrinsic apoptotic pathway, TRAIL binds to one of the two functional death receptors (DR4 or 5) at the cell surface. Ligand binding induces a conformational change in the receptor which allows the death domain (DD) of the receptor to recruit and activate the adaptor protein Fas-associated death domain (FADD) to its carboxyl terminal resulting in the formation of the death-inducing signal complex (DISC). The death effecter domain (DED) of FADD in turn recruits the zymogen procaspase 8. Procaspase 8 can be cleaved to a p18 subunit which can then activate the "executioner" caspases responsible for inducing apoptosis, most commonly caspase 3. The protein "Bcl2 homology domain 3 Interacting Domain" (BID) can also be activated by caspase 8 and functions to initiate the mitochondrial apoptotic pathway (Johnstone 2008) (Figure 1.10).

TRAIL can also activate a number of cell survival pathways such as the NF-κB, MAPK, and JNK pathways. Whether TRAIL induces cell death or survival depends on the components of the DISC complex. It is not yet clear which proteins are involved in the activation of these alternative pathways, however they are thought to include FADD, TRADD caspase 8, RPA-interacting protein (RIP), cFLIP, and inhibitor of apoptosis proteins 1 and 2 (IAP1/2) (Johnstone 2008). This suggests that the composition of the DISC can regulate the susceptibility of a cell to TRAIL.

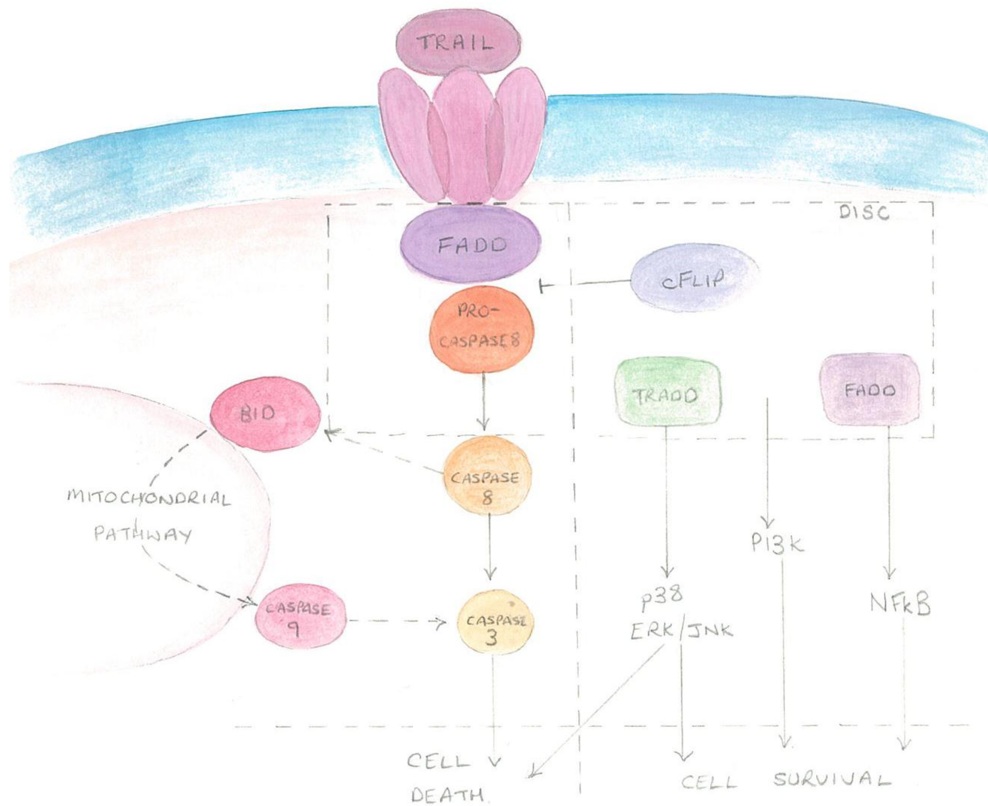


Figure 1.10: TRAIL-activated Pathways: TRAIL binds the functional death receptors DR4 or DR5 at the cell surface inducing receptor trimerisation and resulting in the recruitment FADD and associated proteins in a complex known as the Death Inducing Signalling Complex (DISC). TRAIL binding can result in cell death or survival depending on the composition of the DISC.

1.3.4 TRAIL and Cancer

TRAIL is produced predominantly by natural killer (NK) cells, T cells, monocytes, and neutrophils of the immune system, and is thought to function as part of our immune defence system by being a natural cytotoxic to tumour but not normal cells (Cassatella 2008, Takeda *et al.* 2001). Evidence for its role as a tumour suppressor first came from *in vivo* studies in which NK cell-mediated cytotoxicity towards tumour xenografts was found to be dependant partly on the production of TRAIL (Takeda *et al.* 2001). Furthermore, TRAIL-deficient mice have an increased incidence of spontaneous tumour formation (Zerafa *et al.* 2005). In humans, mutations in the TRAIL pathway components such as DR5 and caspase 8 have been found in breast tumour cells (Shin *et al.* 2001, MacPherson *et al.* 2004).

The specificity of TRAIL for inducing apoptosis in cancerous cells but not effecting normal cells suggested that it had potential as a therapeutic agent. Recombinant human TRAIL first entered clinical trials for lymphoma and lung cancer, and as expected from a recombinant human protein exhibited no toxicity in lymphoma patients (Herbst *et al.* 2010). However, despite the initial promise of *in vitro* studies, TRAIL has not performed as well as hoped in phase II and III clinical trials. These studies have had some partial responses, mainly when administered in combination with chemotherapy (Lemke *et al.* 2014, Table 1.3).

Activating antibodies to TRAIL receptors; Mapatumumab (DR4) and Lexatumumab (DR5) have also been trialled, again with mixed outcomes. Overall, both were found to be more toxic than TRAIL itself possibly because TRAIL is specific for tumour cells, and may be sequestered by the decoy receptors of normal cells whereas activating antibodies are not (Griffiths *et al.* 1998, Johnstone *et al.* 2008). Incidences of hepatotoxicity did occur in patients with liver disease but the rarity of occurrence suggests that TRAIL receptor antibodies are still safe compared to chemotherapy (Johnstone *et al.* 2008).

TRAIL has not been trialled clinically for breast cancer but pre-clinical studies suggest that TRAIL is cytotoxic specifically towards mesenchymal-like breast cancer cell lines. However, the majority of breast cancer cell lines are resistant to TRAIL (Rahman *et al.* 2009).

Table 1.3: Clinical Trials for rhTRAIL (AMG-591)

Combination	Phase	Cancer	n	Safety	Efficacy	Reference
-	I	Advanced cancers	71	Safe	Two patients with metastatic chondrosarcoma had partial responses. 33 patients had stable disease for longer than 6 months.	Herbst 2010
-	IA	Advanced Cancer and Lymphoma	39	Safe	None reported	Ling 2010
-	IA	CRC, sarcoma, NSCLC	31	Safe	1 partial response, 5 stable disease	Pan 2007
+ Chemotherapy + Bevacizumab	I	Colorectal	23	Safe	13 partial responses	Wainberg 2013
	I	Colorectal	27	Safe	6 partial responses	Kasubhai 2012
	I	Lung	24	Safe	1 complete response, 13 partial responses	Soria 2012
	II	Lung	213	Safe	No responses	Soria 2011
+ Chemotherapy + Cetuximab	I	Colorectal	30	Safe	-	Yee 2009
+ Rituximab	I	Lymphoma	7	Safe	2 complete responses, 1 partial response	Yee 2007
	II	Lymphoma	48	Safe	No responses	Belada 2010

1.3.5 TRAIL Resistance

It is clear that as patient responses vary, for TRAIL to be a useful therapy there is a need to be able to identify those patients likely to respond and sensitise those likely to not. This requires further investigation into TRAIL resistance mechanisms in cancer cells.

The apoptotic pathways activated by TRAIL can be inhibited at a number of levels and several of their constituents have been implicated in TRAIL resistance in cancer cells. At the receptor level, polymorphisms, reduced expression and increased receptor internalisation have all been observed to correlate with TRAIL resistance (Johnstone *et al.* 2008).

Mutations in DR4 and DR5 have been associated with metastatic breast cancer. Out of 34 metastatic specimens, three examples of DR4 mutations and four of DR5 mutations were identified whereas no receptor mutations were found in any of the 23 non-metastatic specimens (Shin *et al.* 2001). All DR4 mutations were mis-sense point mutations occurring in the DD domain. Two of the DR5 mutations also occurred within the DD, the other two in the flanking regions. These mutations in both DR4 and 5 were shown to prevent apoptosis in MDA-MB-231 cells (Shin *et al.* 2001).

TRAIL is detectable in a wide variety of normal tissues (Spierings *et al.* 2004). The resistance of normal cells to TRAIL is thought to be mediated at least in part by the elevated expression of decoy receptors (Griffiths *et al.* 1998). In theory, the increased expression of decoy receptors on a cell surface also has the potential to serve as a mechanism of TRAIL resistance in cancer cells, but this has not yet been observed. Instead, differential expression of the functional receptors DR4 and DR5 has been shown in some cases to mediate TRAIL susceptibility of cancer cells. For example the geranylgeranyltransferase I (GGTase I) inhibitor GGTI-298 has been shown to increase TRAIL-induced apoptosis in lung cancer cells via the upregulation of DR5 but not DR4. This study observed that silencing of DR4 allowed apoptosis induced by GGTI-298 and TRAIL to occur, whereas DR5-silencing prevented apoptosis (Chen *et al.* 2010). TRAIL resistance may also be conferred by down-regulation or internalisation of both receptors (Kim *et al.* 2000, Zhang and Zhang 2008). The converse is also true; TRAIL sensitivity can be enhanced via the up-regulation of both DR4 and DR5 (Zhu *et al.* 2010).

Other known mechanisms of TRAIL resistance include the down-regulation of apoptosis inhibitory molecules (IAPs) and cellular Flice-Like Inhibitory Protein (cFLIP). Due to its relevance to the current project, cFLIP will be discussed in greater detail below.

1.3.6 cFLIP

1.3.6.1 Introduction

Cellular Flice-like Inhibitory Protein (cFLIP) was first identified independently by a number of groups in 1997, as a cellular homologue to viral FLIPs (Irmler *et al.* 1997, Goltsev *et al.* 1997, Hu *et al.* 1997, Inohara *et al.* 1997). A number of cFLIP mRNA splice variants exist but so far only three of these have been isolated as proteins and are designated cFLIP short (S), long (L) and Raji (R). The role of cFLIP R has not been extensively studied but both cFLIP L and S have been implicated in a number of signalling pathways involved in the mediation of cell survival and apoptosis. In addition, both cFLIP L and S have been found to be over-expressed in a number of cancers and their inhibition successfully renders

previously resistant tumours sensitive to treatment by the recombinant cytokine TRAIL (Medema *et al.* 1999, Siegmand *et al.* 2002).

1.3.6.2 Structure

All three cFLIP isoforms, but especially cFLIP L, are structurally similar to the pro-apoptotic caspases 8 and 10 which together with their close proximity on the genome suggests they may have evolved by gene duplication (Irmler *et al.* 1997). cFLIP L is a 55kDa protein containing two death effector domains (DEDs) and a C-terminal caspase-like domain (Irmler *et al.* 1997). The caspase-like domain is catalytically inactive due to a cysteine to tyrosine substitution in the region corresponding to the active site. cFLIP L can be cleaved at Asp 376 or Asp 198 to produce products of 43kDa (p43cFLIPL) and 22kDa (p22cFLIPL) respectively. cFLIP S is a 26kDa isoform which contains both DEDs but is without the caspase-like domain of cFLIP L (Kreuger *et al.* 2001). cFLIP R is a 24kDa protein similar in structure to cFLIP S but shortened at the C terminus (Golks *et al.* 2005) (Figure 1.11).

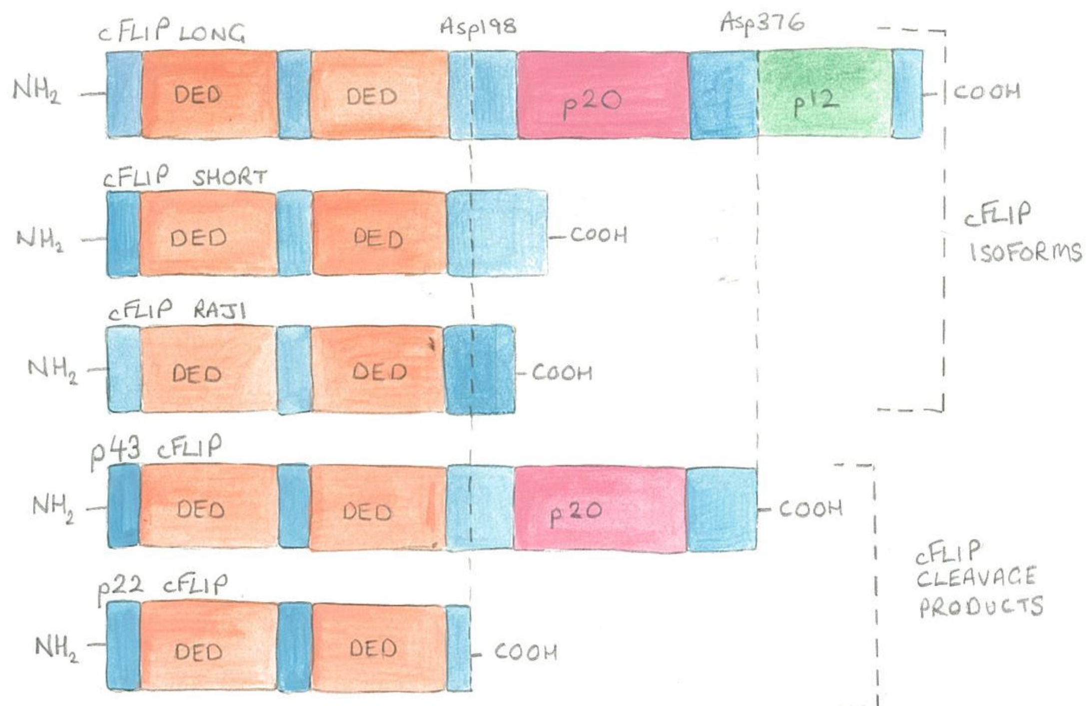


Figure 1.11: Structure of cFLIP isoforms and the cleavage products of cFLIP L: Three functional cFLIP isoforms have been described; cFLIP Long, short and Raji. Two functional cleavages products of cFLIP L are known; p43 cFLIP and p22 cFLIP.

1.3.6.3 Function

1.3.6.3.1 cFLIP in the Extrinsic Apoptosis Pathway

Both cFLIP L and S are regarded widely as inhibitors of the extrinsic apoptosis pathway due to the fact that their inhibition sensitises to TRAIL-mediated apoptosis (Seigmand *et al.* 2001) and their over-expression results in a resistance to death-receptor activating ligands such as FasL or TRAIL (Scaffidi *et al.*

1999, Pennarun *et al.* 2010). However, in a number of cases the over-expression of cFLIP L has had a pro-apoptotic effect. These conflicting results have led to much confusion over its mechanism of action and as such this process is still not fully understood. The current explanation for the apparent dual role of cFLIP L is that its level of expression in relation to that of procaspase 8 determines the outcome of death receptor activation (Figure 1.12) (Wachter *et al.* 2004, Yu *et al.* 2009). At levels lower than procaspase 8, cFLIP is efficiently processed to its p43 form, which promotes apoptosis by increasing the proteolytic activity of procaspase 8. Understanding of the activation process has been greatly advanced by a study of Yu *et al.* (Yu *et al.* 2009). Using an uncleavable mutant form of the protease domain of procaspase 8 they demonstrated that proteolytic activity is only acquired following heterodimerisation with cFLIP L which is then cleaved by procaspase 8 to its p43 form. Procaspase 8 activity was found to increase in the presence of p43 compared to unprocessed cFLIP due to greater binding efficiency. Heterodimerisation with p43 induces a conformational change in the active site of procaspase 8 which enhances its ability to process natural substrates downstream of cFLIP such as the apoptotic activator procaspase 3 (Yu *et al.* 2009). Although providing a much needed explanation of the pro-apoptotic function of cFLIP L, this study is limited by its use of mutant forms of cFLIP and procaspase 8. As these both lacked DEDs their affinity for each other was so low that the active site inhibitor Ac-IETD-cho was required to produce a stable association between the two proteins. These observations are therefore yet to be made in a physiological context.

cFLIP appears to switch to an anti-apoptotic role when present at higher levels than procaspase 8. The anti-apoptotic situation has been studied by inducing the over-expression of cFLIP in cell lines. Under these conditions, receptor triggering results in the cleavage of both cFLIP and caspase 8 to their p43 and p41/43 forms respectively. However production of the p18 subunit of caspase 8 is consistently blocked by elevated cFLIP L levels. To examine the roles of full length and p43 cFLIP L, Kreuger *et al.* separately over-expressed a mutant form of uncleavable cFLIP L and a recombinant p43 form. The full length cFLIP L allowed procaspase 8 cleavage to its p41/43 form but this step was inhibited by the overexpression of p43 cFLIP L. Both prevented the appearance of p18 caspase 8. The over-expression of the uncleavable mutant cFLIP L, as with the wild type resulted in the occurrence of only p43/41 caspase 8 at the DISC (Kreuger *et al.* 2001). Similar observations have been made by other groups (Kataoka *et al.* 2000, Wachter *et al.* 2004). On the basis of these findings a number of anti-apoptotic scenarios can be postulated: 1. Elevated cFLIP levels are able to more efficiently process caspase 8 to its p41/43 form and as this is retained at the DISC, less caspase 8 is present in the cytosol to activate downstream effector caspases. 2. At higher levels, cFLIP L may also be able to compete with FADD or downstream substrates for caspase 8, thus preventing apoptosis. 3. With caspase 8 levels being lower than cFLIP levels, cFLIP may be less efficiently cleaved to its p43 form and therefore less able to enhance the proteolytic activity of procaspase 8 (Figure 2.12).

Unlike cFLIP L, only an anti-apoptotic role in the extrinsic pathway has ever been attributed to cFLIP S but its mechanism of inhibition differs from that of cFLIP L. The overexpression of cFLIP S was found to be sufficient to prevent the generation of all caspase 8 cleavage products including p41/43 (Kreiger *et al.* 2001). In addition, over-expression of cFLIP S significantly reduced the cleavage of cFLIP L

to its p43 form (Kirchoff *et al.* 2000, Kreuger *et al.* 2001). These results suggest that cFLIP S may act on both cFLIP L and procaspase 8 in the same manner to prevent cleavage. Alternatively, the effect of cFLIP S on cFLIP L may occur via its inhibition of caspase 8. Kreuger *et al.* also observed that the over-expression of both cFLIP L and S in BJAB cells resulted in greater inhibition of apoptosis than each form alone, indicating an additive effect of cFLIP isoforms. In the case of FasL or TNFα-mediated apoptosis, this was not biased towards the effect of a single isoform but when administered with TRAIL, a greater reduction in apoptosis was in fact observed in BJAB cells over-expressing cFLIP S (Kreuger *et al.* 2001). This is contrary to the findings of Irmler *et al.* which showed that cFLIP L was more effective than cFLIP S at inhibiting apoptosis in Jurkat T cells and melanoma cells (Irmler *et al.* 1997). This suggests that the relative contribution of cFLIP L and S to the extrinsic pathway may depend on other molecular factors and cell types.

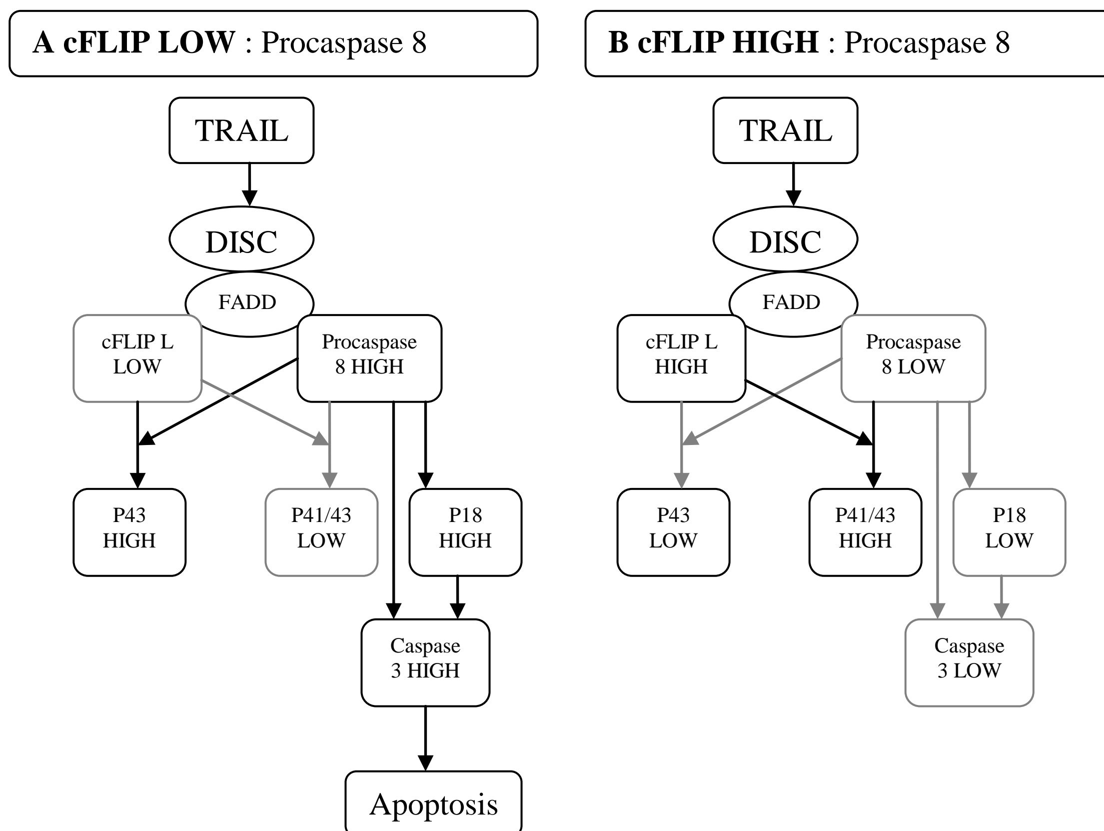


Figure 1.12: Model of cFLIP L action in the extrinsic pathway: **A** When cFLIP levels are lower than caspase 8, cFLIP is efficiently cleaved by procaspase 8 to its p43 form which complexes with and activates procaspase 8. This allows caspase 8 to activate downstream caspase 3 which results in apoptosis. The efficient processing of cFLIP prevents the cleavage of caspase 8 to its p41/43 product and prevents the inhibition of caspase 8 cleavage to its p18 product. **B** When cFLIP levels are higher than procaspase 8, procaspase 8 is efficiently cleaved by cFLIP to its p41/43 product which is retained at the DISC preventing further signalling (see text for details).

1.3.6.3.2 cFLIP and NFϰB

NFϰB is a transcription factor responsible for the up-regulation of a number of anti-apoptotic genes and therefore acts as an inhibitor of cell death. The activation of NFϰB requires the formation of a protein complex at the DISC including TNF Receptor Associated Factors (TRAFs) and Receptor Interacting Protein (RIP). Complex formation leads to the degradation of IϰBα followed by NFϰB translocation to the nucleus (Kataoka *et al.* 2000). In order to investigate the relationship between cFLIP and NFϰB, Kataoka *et al.* over-expressed cFLIP in T cells. NFϰB was found to be activated by both over-expressed cFLIP L and S, although the effect of cFLIP S appeared to be much weaker. cFLIP L was also shown to be capable of interacting with TRAFs 1-3 and RIP, and the over-expression of FLIP L resulted in their increased incorporation into the DISC. In addition, in wild-type Raji B cells the efficient incorporation of endogenous cFLIP L p43 into the DISC, which is indicative of low cFLIP L, correlated with a decrease in RIP recruitment (Kataoka *et al.* 2000). These results are evidence for an anti-apoptotic role of cFLIP as an activator of the NFϰB pathway. A later study by the same group reported that the p43 form of cFLIP L is also capable of activating the NFϰB pathway in 293T cells in the presence of procaspase 8 or p41/43 caspase 8. It was found that heterodimers of p43 cFLIP L and caspase 8 directly interacted with TRAF2 whereas TRAF2 only weakly interacted with full length cFLIP L (Kataoka *et al.* 2004). However the implication of this study that p43/caspase 8 heterodimers activate the NFϰB pathway is contrary to their pro-apoptotic role suggested by the findings of Yu *et al.* which are outlined above. They also conflict with the previous finding by the same group that the presence of p43 at the DISC correlated with low RIP recruitment and therefore reduced NFϰB activity (Kataoka *et al.* 2000). In contrast, cFLIP L inhibited the activation of NFϰB and the degradation of IϰBα by TRAIL in human keratinocytes (Wachter *et al.* 2004). As with the extrinsic apoptosis pathway, cFLIP L has been associated with both pro and anti-apoptotic effects via its mediation of NFϰB. The apparently conflicting results obtained by the aforementioned studies do not appear to have been reconciled and therefore the relationship between cFLIP and NFϰB requires further investigation.

1.3.3.4.3 cFLIP and Mitogen-Activated Protein Kinases (MAPKs)

There are three forms of MAPK: extracellular receptor kinase (ERK), c-Jun-N-Terminal kinase (JNK) and p38 MAPK. MAPKs are involved in a very complex array of signalling cascades that effect many cellular processes including cell survival and apoptosis. Although not fully understood, both JNK and p38 activation is generally considered to promote apoptosis whereas ERK activation results in cell survival and proliferation (Grambhila 2003, Lee 2006). cFLIP has been shown to interact with these pathways to promote ERK function but inhibit p38MAPK, and therefore act as a promoter of cell survival: Overexpression of cFLIP L or S in T cells and in hepatocarcinoma cell lines resulted in activation of ERK (Kataoka *et al.* 2000, Lee *et al.* 2006).

High levels of cFLIP have also been shown to inhibit the phosphorylation and activation of p38 MAPK (Grambhila *et al.* 2003). This is thought to occur independently of death receptor signalling as the inhibition of the extrinsic pathway by both death receptor blocking antibodies and the pan-caspase inhibitor Z-VAD-fmk had no effect on p38 MAPK phosphorylation in hepatocarcinoma cells. A direct

effect of cFLIP on p38 MAPK is further supported by the co-immunoprecipitation of cFLIP L with unphosphorylated p38 (Grambhila *et al.* 2003).

1.3.3.4.4 cFLIP as a Molecular Switch

A role for cFLIP in mediating between the extrinsic apoptosis, NFkB and MAPK pathways has recently been explored by Song *et al.* When investigating the molecular mechanisms involved in TRAIL resistance in NSCLC cells they observed that FADD and caspase 8 were only recruited to the DISC in non-raft fractions of these cells following TRAIL treatment, whereas they appeared in the lipid raft fractions of TRAIL-sensitive cells. NFkB and ERK activation as a result of TRAIL treatment in resistant cells was also not disrupted by cholesterol inhibition, suggesting that the components of non-rafts are responsible for their activation. cFLIP inhibition by shRNA in these resistant cells resulted in the redistribution of DISC formation to the lipid rafts and sensitised cells to apoptosis by TRAIL. In addition, inhibition of RIP had the same effect. In RIP inhibited cells, both cFLIP isoforms were redistributed among both non rafts and lipid rafts and cFLIP L was cleaved to its p43 product (Song 2007). These results are evidence of cFLIP acting as a molecular switch, whereby high levels of cFLIP relocate DISC formation from lipid rafts and switch cell fate from apoptosis via the extrinsic pathway to NFkB and ERK survival signalling.

1.3.3.4.5 cFLIP and Akt

Akt is a serine-threonine kinase involved in many pathways which promote cell survival including the Wnt pathway (Sethi and Vidal-Puig 2010, Quintaville *et al.* 2010). AKT interacts directly with the caspase-like domain of cFLIP. Over-expression of cFLIP in HeLa cells had no effect on the phosphoryation of Akt itself, but did correlate with decreased phosphorylation of the Akt substrate GSK3β. Mutant cFLIP proteins, not able to interact with Akt, did not have any effect on GSK3β phosphorylation. In addition, TRAIL resistance induced by cFLIP overexpression could also be counteracted by the GSK3β inhibitor lithium chloride, suggesting that the protective effect of cFLIP in the TRAIL pathway requires active GSK3β (Quintaville *et al.* 2010). GSK3β is also thought to be able to control the cell cycle via p27 (Wang *et al.* 2008). Overexpression of cFLIP in HEK293 and HeLa cells also reduced p27 levels (Quintaville *et al.* 2010). Interestingly, low amounts of p27 have also been associated with TRAIL resistance (Garofalo *et al.* 2008) suggesting that cFLIP may be the link between these two factors.

1.3.3.4.6 cFLIP and Wnt Signalling

A number of studies have found evidence to suggest that cFLIP also functions within the canonical Wnt pathway (Naito *et al.* 2004). This signalling pathway mediates a number of cell fates including cell proliferation, differentiation, and the determination of stem cell characteristics (Sethi and Vidal-Puig 2010) (Section 1.1.6).

Wnt-target gene expression was found to be increased significantly following over-expression of cFLIP L but not cFLIP S (Naito *et al.* 2004). In addition, shRNA-induced inhibition of cFLIP L resulted

in a significant decrease in Wnt-target gene expression in A459 cells. Enhanced expression of cFLIP L was also associated with an increase in the amount of cytosolic β-catenin, the degradation of which is mediated by GSK3, but in this case and in contrast to the AkT study (Quintaville *et al.* 2010), cFLIP levels appeared to have no effect on GSK3 itself. Instead, high cFLIP levels resulted in a significant reduction of β-catenin ubiquitylation (Naito *et al.* 2004). A later study by the same group reported that cFLIP accumulates and forms aggregates within cells which cause the disruption of the ubiquitin-proteasome system (Ishioka *et al.* 2007). In addition to being a possible explanation for the inhibition of β-catenin ubiquitylation, this finding may also reveal an additional pathological consequence of the cFLIP L over-expression observed in cancer cells (Ishioka *et al.* 2007, Fenglin *et al.* 2014).

The most recent study by this group found that cFLIP itself is capable of translocating to the nucleus (Katayama *et al.* 2010). This was revealed by a strong nuclear signal following immunostaining of myc-tagged cFLIP. Biochemical fractionation found endogenous cFLIP present in both the nuclear and cytoplasmic fraction of lung carcinoma cells, whereas cFLIP S was present predominantly in the cytoplasm. To determine whether cFLIP moves between these two compartments, the nuclear transporter CRM1 was inhibited using the compound leptomycin B. This resulted in cFLIP appearing only in the nuclear fraction. Potential nuclear localisation signals (NLS) and nuclear export signals (NES) were identified at the C-terminal of the cFLIP protein. Mutation of the NLS and NES sequences resulted in cFLIP localisation to the cytoplasm and nucleus respectively. Unexpectedly, Wnt-target gene expression was not induced in the mutants compared to a substantial increase observed in cells transfected with wild-type cFLIP L. Cytosolic accumulation of β-catenin, and β-catenin-induced gene expression was also completely abolished in the NLS and NES mutants but increased in the wild-type transfectants. It was concluded that mutation of the NLS and NES sequences abrogated the ability cFLIP to promote Wnt signalling, and therefore did not reflect the true functions of cytoplasmic and nuclear cFLIP. Subsequently, immunoprecipitation was used to demonstrate that cFLIP associates with a reporter plasmid of Wnt-target gene expression, showing that cFLIP does indeed promote Wnt signalling when nuclear (Katayama *et al.* 2010).

Taken together these results show that cFLIP promotes Wnt-signalling via the inhibition of β-catenin degradation and the promotion of Wnt-target gene expression.

1.3.6.4 Regulation of cFLIP

1.3.6.4.1 Transcriptional Regulation of cFLIP

In accordance with its function as an anti-apoptotic protein when expressed at high levels, a number of anti-apoptotic factors have been found to upregulate the expression of cFLIP. These include Gli2 (glioma factor 2), NFκB, and stem cell factor (SCF) (Kreuz *et al.* 2001, Micheau *et al.* 2001, Chung *et al.* 2002, Kump *et al.* 2008). Gli2 is a zinc finger transcription factor up-regulated and activated by the sonic hedgehog (Shh) pathway. Abberrant Shh signalling has been implicated in the development of a number of cancers including basal cell carcinoma and breast cancer (Kump *et al.* 2008). Kump *et al.* Showed that the up-regulation of Gli2 led to cFLIP over-expression and the resistance of HaCat cells to TRAIL-mediated apoptosis. On further investigation, they identified two Gli2 binding sites in the cFLIP

promoter region. The specificity of Gli2 for these sites was confirmed by the absence of reporter gene expression following the introduction of site-directed mutations (Kump *et al.* 2008). This study demonstrates that Gli2 is directly involved in the control of cFLIP expression and reveal how increased Shh signalling could lead to enhanced cFLIP expression. Like Gli2, cFLIP is also known to be over-expressed in a number of cancers (Fenglin 2014) but these findings suggest a role for cFLIP over-expression during tumourigenesis.

The expression of cFLIP can also be controlled by vascular endothelial growth factor (VEGF) via PI3K and Akt signalling (Suhara *et al.* 2001, Panka *et al.* 2001) Inhibition of PI3K by wortmannin in endothelial cells prevented an increase in cFLIP expression levels by VEGF and also sensitised these previously Fas-resistant cells to Fas-mediated apoptosis (Suahra *et al.* 2001). Inhibition of PI3K in cancer cell lines including the MCF-7 breast cancer cell line, also resulted in a decrease in cFLIP mRNA and protein. However, cFLIP expression was dependant on Akt activity, suggesting that control of cFLIP expression occurs downstream of PI3K in this pathway (Panka *et al.* 2001). As inhibition of PIK also correlates with a loss of stem cell self-renewal in breast cancer cells (Korkaya *et al.* 2009) it would be interesting to explore a possible relationship between self-renewal and cFLIP.

1.3.6.4.2 Post-Translational Regulation of cFLIP

The fact that cFLIP inhibition results in TRAIL sensitivity has led to a number of chemotherapeutics being studied for their ability to down-regulate cFLIP. Stagni *et al.* reported that the drug Neocarzinostatin causes DNA damage leading to a decrease in the protein levels of cFLIP. This inhibition was reversed by the addition of the proteasome inhibitor MG132, suggesting that cFLIP levels were reduced by proteosomal degradation in this case. However, inhibition of proteasome activity resulted in an increase in the levels of cFLIP S (Stagni *et al.* 2010).

Microtubule-interfering agents including nocodazole or taxol are also able to sensitise breast tumour cells to TRAIL-induced apoptosis by reducing cFLIP levels (Sanchez-Perez *et al.* 2010). Sanchez-Perez *et al.* reported that culture of MDA-MB-231 and BT474 breast cancer cells with nocodazole or taxol caused sustained phosphorylation of JNK leading to a loss of cFLIP. The addition of the JNK inhibitor SP600125 or the proteasome inhibitor MG-132 was then able to reverse the loss of cFLIP L and S seen in these cells and dramatically reduce the level of apoptosis, suggesting that nocodazole and taxol also reduce cFLIP levels via proteosomal degradation (Sanchez-Perez *et al.* 2010).

These results show that chemotherapeutic agents initiate the proteasomal degradation of cFLIP.

1.3.3.6 cFLIP in Cancer

cFLIP expression is elevated in many cancers including prostate, colorectal and breast cancer (Hernandez *et al.* 2001, Zhang *et al.* 2004, Fenglin *et al.* 2014). In breast cancer, elevated expression of cFLIP correlates with a more aggressive tumour and a poor prognosis (Fenglin *et al.* 2014). Over-expression of cFLIP also correlates with resistance to apoptosis induced by TRAIL and chemotherapeutic agents (Seigmand *et al.* 1999). Evidence for cFLIP over-expression in breast cancer promotes the use of a cFLIP inhibitor in combination with TRAIL as a therapeutic strategy. The homology of cFLIP with the pro-apoptotic caspase 8 has prevented the development of specific small molecule cFLIP inhibitors and therefore combination studies rely on the use of chemotherapeutic agents that reduce cFLIP or impair its function indirectly. Many agents are known to target cFLIP, notably HDAC inhibitors such as Vorinostat, however most exhibit some degree of toxicity (Safa and Pollock 2011).

As mentioned before, TRAIL either alone or in combination with other drugs has not been trialled for breast cancer. However, pre-clinical studies have shown that inhibition of cFLIP sensitises breast cancer cells, including stem-like cells, to TRAIL both *in vitro* and *in vivo* (Seigmand *et al.* 1999, Frew *et al.* 2001, Piggott *et al.* 2011).

1.4 Aims and Objectives

Although the disappointing results of clinical trials have decreased interest in TRAIL as a monotherapy we propose that as some patients do respond, patient stratification may improve its success significantly and given that TRAIL is available now, could lead to rapid utilisation in the clinic. This would rely on the ability to identify those patients most likely to respond favourably to TRAIL. Although TRAIL has not entered clinical trials for breast cancer, pre-clinical studies show a distinct relationship between mesenchymal-like breast cancer cell lines and TRAIL sensitivity, and therefore offer the possibility of patient stratification were a clinical trial to take place (Rahman *et al.* 2009). However, the mechanism of TRAIL sensitivity in mesenchymal-like breast cancer cell lines is not understood.

We have shown previously that inhibition of cFLIP can sensitise tumoursphere-forming cells to TRAIL. However it is unclear whether TRAIL alone can target the stem-like fraction. Therefore further investigation is required in order to understand the differences between TRAIL resistant and sensitive breast cancer cells and to determine whether TRAIL alone can target bCSCs. The correlation between bCSCs and EMT (Mani *et al.* 2008, Morel *et al.* 2008) together with the specificity of TRAIL for mesenchymal-like cells (Rahman *et al.* 2009), suggests that TRAIL may be able to target at least the EMT-like subset of bCSCs. Therefore we tested the hypotheses that:

1. In addition to differences between cell lines (Rahman 2009), subpopulations of cells within breast cancer cell lines also respond differently to TRAIL.
2. That cFLIP levels are responsible, at least in part, for these differential responses
3. That the phenotypic state of the cell determines its sensitivity to TRAIL.

In addition, we have found that when cultured in the presence of TRAIL-sensitive cells, the labelled TRAIL-resistant MCF-7 breast cancer cell line develops sensitivity to TRAIL (unpublished data). There is much interest in the identification of novel methods of sensitising cells to TRAIL, therefore we aimed to investigate the underlying mechanism of this system in the hope of identifying a potential therapeutic strategy. We tested the hypothesis that:

1. A soluble factor produced by TRAIL-sensitive cells sensitises resistant cells to TRAIL.

Our previous findings identified a strategy for sensitising breast cancer stem cells to TRAIL by sensitisation to apoptosis via the inhibition of cFLIP (Piggott *et al.* 2011). Although the mechanism of action of cFLIPi/TRAIL is known, the specificity of this combination towards the bCSC-like population is not understood. As well as an inhibitor of apoptosis, cFLIP also has a number of other roles in promotion of cell survival and proliferation including an ability to promote the Wnt signalling pathway (Naito *et al.* 2004, Katayama *et al.* 2010). Wnt signalling is known to be important in bCSCs and its inhibition can decrease tumoursphere formation (Lamb *et al.* 2013). This suggests that via the Wnt pathway, cFLIP inhibition alone may abrogate bCSC function. Therefore we aimed to investigate a potential non-apoptotic role for cFLIP in the promotion of Wnt signalling and to determine if this had an effect on bCSC biology. We tested hypotheses that:

1. cFLIP promotes Wnt signalling in breast cancer cell lines

2. Abrogation of cFLIP impairs bCSC maintenance and self-renewal via inhibition of Wnt-signalling

We have shown previously that RNAi inhibition of cFLIP sensitises four breast cancer cell lines to treatment with recombinant TRAIL . Although this sensitisation was a partial effect, the combination treatment resulted in a complete ablation of the tumoursphere-forming population of all four breast cancer cell lines. This was accompanied by a reduction in tumour initiation in an orthotopic xenograft model of 80% and in a metastasis model by 90% (Piggott *et al.* 2011). While the xenograft effect is profound, the residual tumour initiation capacity of 10-20% is in contrast with the complete ablation of functional bCSC activity exhibited in the tumoursphere assay *in vitro*. There are two possible explanations for the discrepancy between tumoursphere formation and tumour initiation: The first is that tumoursphere formation is not indicative of, but merely an enrichment for tumour-initiating cells. Alternatively, plasticity (the conversion of non-stem cells to bCSCs) may have occurred to initiate tumour growth. This possibility is supported by the fact that if the cells surviving FLIPi/TRAIL treatment were left to recover, a subpopulation of cells were able to reacquire a bCSC phenotype in proportion to the original untreated population. This may be evidence of a functional plasticity occurring between non-stem cancer cells and bCSCs, and a possible explanation for the tumour take observed in 1/5 treated samples. bCSC plasticity is a major obstacle to any therapeutic strategy aimed at targeting bCSCs. cFLIPi/TRAIL treatment has endowed us with a unique model in which to study functional plasticity *in vitro*. We aimed to investigate the molecular mechanisms underlying the bCSC plasticity observed following FLIPi/TRAIL treatment with the aim of identifying inhibitors of bCSC plasticity. As bCSCs return as cFLIP expression returns, we also set out to determine whether a permanent or prolonged cFLIP inhibition prevents the re-acquisition of bCSCs in this model. We tested the hypotheses that:

1. Following FLIPi/TRAIL, surviving non-stem cancer cells can reacquire a bCSC-like phenotype
2. Inhibition of cFLIP inhibits plasticity

Chapter 2: Materials and Methods

Chapter 2 Materials and Methods

2.1 DNA

2.1.1 DNA Constructs

The pcDNA3.1cFLIPL overexpression vector, containing the full length coding sequence of the long form of human c-FLIP (accession number NM_003879) was a kind gift from Dr. Naito (Tokyo University, Japan). The MissioncFLIPSh vector was a kind gift from Dr. Ladislav Andera (Prague). The FOPFlash and TOPFlash reporter plasmids were kind gifts from Dr. Ken Ewan, Cardiff University.

Table 2.1: Constructs used in this study

Construct	Expression System	Antibiotic selection 1	Antibiotic selection 2	Reference
pcDNA3.1	Over-expression plasmid (Empty)	Ampicillin	Neomycin	Naito 2004
pcDNA3.1cFLIPL	Over-expression plasmid (cFLIP)	Ampicillin	Neomycin	
MissionSH cFLIP	SH Lentiviral	Ampicillin	Puromycin	-
FOPFlash	Luciferase Reporter Plasmid	Ampicillin	-	Molenaar 1996
TOPFlash	Luciferase Reporter Plasmid	Ampicillin	-	
LacZ	Reporter Plasmid	Ampicillin	-	-

2.1.2 Plasmid Propagation

2.1.2.1 Transformation into E.coli

OneShotStbl3 cells (Invitrogen) were thawed on ice. To 50 µl cells, 1 µl of DNA to be transformed was added and incubated on ice for 30 mins. Cells were heat-shocked at 42˚C in a water-bath for 45 s followed by 2min incubation on ice during which cells take up the DNA. To each transformation, 250 µl SOC media (Invitrogen) was added and the cells incubated at 37˚C for 1 h with shaking at 225 rpm. 100 µl of this culture was then spread on an agar plate containing the appropriate antibiotic which was incubated, overnight at 37˚C.

Colonies were selected by inoculation of a pipette tip which was then placed into 2 ml of LB-Broth containing the appropriate antibiotic (ampicillin), and incubated at 37˚C for 12-16 h with shaking at 225 rpm.

For a midi or maxi-culture of colonies, the 2 ml starter culture was used to inoculate a 100 or 200 ml volume of LB-Broth containing the appropriate antibiotic (ampicillin), and incubated at 37˚C for 12-16 h with shaking at 225 rpm before DNA extraction.

2.1.2.2 Preparation of glycerol stocks

For long-term storage of transformed cells, 850 µl of bacterial starter or maxi-cultures was mixed with 150 µl glycerol to obtain a 15% glycerol solution which was stored at -80˚C. Future propagation was performed using glycerol stock culture diluted 1:1000 in LB-broth containing appropriate antibiotic.

2.1.2.3 DNA Extraction

DNA extraction was carried out using mini, midi or endotoxin-free maxi-prep DNA extraction kits (Qiagen) according to the manufacturer's instructions: overnight cultures were pelleted by centrifugation at 10,000 rpm for 3 mins for a 1 ml starter culture or 3,000 rpm for 15 mins for a 50-100 ml preparation. The resulting cell pellet was then re-suspended in buffer P1, to which buffers P2 and N3 were added to lyse the bacterial cells. This was then centrifuged at 13,000 rpm for 10 mins and the resulting supernatant transferred to a DNA-binding spin column (Qiagen). This was then centrifuged for 1 min at 13,000 rpm, and all subsequent centrifugation steps were carried out at this speed. The DNA column was then washed with buffers PB and PE, and then centrifuged whilst empty to remove any remaining ethanol before the DNA was eluted: 30 µl buffer EB or TE was added to the spin column and incubated for 1min at room temperature before the column was centrifuged and the eluate collected in a micro-centrifuge tube. DNA content was measured using a Nanodrop (GE healthcare, UK).

2.1.2.4 Sequencing of cFLIP

DNA was diluted to 50-100 ng/µl in 15 µl, and primers were diluted to 2 pMol. All primers were custom designed and then produced by Sigma. All sequencing was carried out by BIOSI sequencing core, Cardiff University. Sequencing primers for cFLIP were designed against the human cFLIP sequence (accession number NM_003879) (Table 2.2 and Appendix 2).

Table 2.2: Sequencing primers

Primer Name/Target	Primer Sequence
cFLIP 1	5'-GGCAATGAGACAGATTCT-3'
cFLIP 2	5'-TTGTGTGTGTCCTGGTGAGCCGAG-3'
cFLIP 3	5'-TATGTGGTGTCCAGAGGGCCAGCTG-3'
cFLIP 4	5'-CTGCTGGAGCAGTCTCACAG-3'
cFLIP 5	5'-AATATTATGTCTGGCTGCAG-3'

2.1.3 Site Directed Mutagenesis of cFLIP

Site directed mutagenesis was performed on the pcDNA3.1cFLIP construct, using the QuickChange kit (Stratagene) according to the manufacturer's instructions:

2.1.3.1 Primer Design

Mutagenic primers were custom designed following the recommended guidelines (Stratagene). Primers were designed to contain the desired mutations in the middle of the primer with 10-15 bases of correct sequence on either side. The following considerations were adhered to; primers were between 25-45 bp, with a melting temperature of 78˚C or greater, a GC content of at least 40%, and terminated in on or more G or C bases. Three primers were designed; firstly to mutate the shRNA targeting sequence in cFLIP to allow for the possibility of expression in a cell line without endogenous cFLIP, and also to mutate the nuclear export and localisation at the C-terminus of the protein according to Katayama et al 2010 (Table 2.3) (Appendix 1).

Table 2.3: Mutagenic primers:

Target Mutation	Primer
Sh-targeting sequence	3′-GGACGAGTCCTTGGGCGTGGAGCAAAGTCTGATGTCTCACGACTACCG-5′ 5′-CCTGCTCAGGAACCCGCACCTCGTTTCAGACTACAGAGTGCTGATGGC-3′
NES LL439AA	3′-CTGTTCTTTCTTTTGCGGGTCGGCGCCTAGAAGTGTAACTTGAGTTACC-5′ 5′-GACAAGAAAGAAAACGCCCAGCCGCGGATCTTCACATTGAACTCAATGG-3′
NLS RKR435LIL	3′-GGTCTTTGACTCTGTTCTTGATTATGAGGGTGAGGACCTAGAAGTGTCG-5′ 5′-CCAGAAACTGAGACAAGAACTAATACTCCCACTCCTGGATCTTCACAGC-3′

2.1.3.2 PCR

For each mutagenesis reaction to be carried out, 50 ng of double-stranded DNA template (pcDNA3.1cFLIPL) was diluted in 5 µl of reaction buffer to which 125 ng of each of the mutagenic forward and reverse primers were added together with 1µl of dNTP mix and 1 µl Pfu DNA polymerase (NEB). The reaction was made up to 50 µl in dH_2O. The PCR reaction was then carried out on a PCR machine (Biorad) as shown in table 2.4:

Table 2.4:

<table>
<tr><th>Step</th><th>Temperature</th><th>Time</th><th>Cycle</th></tr>
<tr><td>Denaturation</td><td>95˚C</td><td>30 s</td><td>1</td></tr>
<tr><td>Denaturation</td><td>95˚C</td><td>30 s</td><td rowspan="3">12</td></tr>
<tr><td>Annealing</td><td>55˚C</td><td>1 min</td></tr>
<tr><td>Extension</td><td>68˚C</td><td>6.5 min (2 min/kb of plasmid length)</td></tr>
<tr><td>Final Extension</td><td>68˚C</td><td>10 min</td><td>1</td></tr>
<tr><td>Incubation</td><td>4˚C</td><td>-</td><td>-</td></tr>
</table>

2.1.3.3 Template Strand Digestion

Following PCR, template strands were digested by the addition of 1 µl DpnI restriction enzyme for 1 h at 37˚C. DNA was used immediately to transform competent *E.coli* XL1-blue cells (Stratagene) and the remaining DNA stored at -20˚C. (For transformation and DNA extraction protocol see section 2.1.2.1 and 2.1.2.3). Mutations were confirmed by sequencing (Section 2.1.2.4, and Appendix 2)

2.2 Cell Culture and Cell-based Assays

2.2.1 Cell Culture

2.2.1.1 Cell Lines

The human MCF-7 breast cancer cell line was a kind gift from Dr Julia Gee, Cardiff University. The MCF-7, MDA-MB-231 (ATCC, UK), MDA-MB-468 (CLS, Germany), MDA-MB-436 (CLS), ZR75-1 (CLS), SKBR3 (ATCC) and BT474 (ATCC) lines were maintained in RPMI 1640 (Invitrogen) medium supplemented with 10% foetal bovine serum (FBS) (Invitrogen), and 1% penicillin-streptomycin and L-glutamine mix (Invitrogen). Cells were maintained at 37˚C in 5% CO_2. The SUM159 line was a kind gift from Dr. Robert Clarke (Manchester University, UK), and was maintained in DMEM-F12 base medium supplemented with 10% FBS, hydrocortisone (Sigma) and insulin (Sigma) (Table 2.5). Cells were maintained at 37˚C in 5% CO_2 in 7ml culture medium for a T25 (25 cm^2) flask or 14 ml culture medium for a T75 (75 cm^2) cell culture flask (Nunc, Fisher), and passaged routinely every 3-4 days at a ratio depending on the cell line (Table 2.5 and Section 2.2.1.2).

Table 2.5: Cell lines used in this study

Cell Line	**Base media** (All supplemented with 10%FBS and 1% Penicillin/streptomycin L-glutamine mix)	**Splitting ratio for confluent cells**	**Clinical Subtype**
MCF-7	RPMI (Invitrogen)	1:8	ER positive
MDA-MB-231	RPMI	1:10	Triple negative
MDA-MB-468	RPMI	1:8	Triple-negative
MDA-MB-436	RPMI	1:8	Triple-negative
ZR-75-1	RPMI	1:6	ER positive
SKBR3	RPMI	1:6	Her-2 amplified
BT474	RPMI	1:8	ER positive/ Her-2 amplified
SUM 159	DMEM-F12 (Invitrogen)	1:10	Triple-negative

2.2.1.2 Splitting Cells

Cells were split at appropriate ratio when 80-100% confluent (see Table 2.5). Culture medium was first removed from the tissue culture flask and replaced with trypsin-EDTA (Invitrogen); 2 ml for T25, 4 ml for T80, and incubated at 37˚C for ~5-10 mins until all cells had rounded up and lifted off the flask surface. Trypsin was inactivated by addition of at least 10 times the volume of cell culture medium

containing foetal bovine serum (Invitrogen). Cells were split at the appropriate ratio (Table 2.5) and remaining cells discarded, re-plated for use in cell-based assays or pelleted for protein or RNA extraction.

2.2.1.3 Freezing cells

For long term storage, cells were first trypsinised from culture flask and centrifuged for 5 mins at 1100 rpm. Trypsin was removed and the cell pellet re-suspended in normal culture medium containing 10% DMSO. Cells were aliquoted into 1 ml cryo tubes (Fisher) and placed in a cryo-freezing vessel containing isopropanol which was then placed at -80˚C overnight before aliquots were moved to liquid nitrogen storage. Approximately 2 x 10^6 cells were frozen per 1 ml aliquot.

2.2.1.4 Raising cells from storage

Cells from liquid nitrogen or -80˚C storage were transferred to dry ice then thawed in a waterbath at 37˚C until completely defrosted. Cells were transferred to a 15 ml falcon tube (Nunc) and centrifuged for 5 mins at 1100 rpm. The storage medium was removed and the cell pellet re-suspended in the appropriate culture medium and transferred to a T25 cell culture flask.

2.2.1.5 Sub-Culture: Seeding Cells in plate format

Culture medium was removed from cell culture flasks and replaced with trypsin (2 ml for T25 or 4 ml for T80). Cells were incubated in trypsin for ~10 mins until all had lifted off the surface. Cells were split at an appropriate ratio and the remaining cells transferred to a 15ml falcon tube (Nunc). The cells to be used were counted using a Neubauer counting chamber haemocytometer (Hawksley, Lancing, UK). The average of four squares was taken and multiplied by appropriate factor (10,000) to give the number of cells per ml. Cells were diluted accordingly with the culture medium and seeded into cell culture plates for subsequent assays (Nunc, Fisher). The cell numbers used for sub-culturing varied between experiments but in general was 100,000 cells/ml. Plates used are outlined in table 2.6:

Table 2.6: Subculture

Plate	Relative Surface Area	Volume (µl)
96	0.2	100
48	0.4	200
24	1	500
12	2.5	1000
6	5	3000

2.2.2 Generation of Mutant Lines

2.2.1.1 Viral transduction: Generation of cFLIP shRNA cell lines:

The MCF-7 and MDA-MB-231 cell lines were transduced with lentiviruses containing lentiviral Mission shRNA vectors with shRNA sequences targeting cFLIP or a non-specific scrambled control (Table 2.7). Cells were cultured in a 24-well plate format until they reached 30-40% confluency. Culture medium was then changed to complete medium containing 7 µg/ml Polybrene (Sigma), and virus at a range of titres between 0 and 20 µl. Cells were incubated with virus overnight, and culture medium replaced the following day. Cells were then cultured in medium containing 5 µg/ml puromycin (Sigma) for the MCF-7 line and 10 µg/ml for the MDA-MB-231 line. Cells were selected in this manner, splitting when necessary, until no cells remained in untransfected well and then assayed for mutant gene expression.

Table 2.7: ShRNA sequences:

Target	shRNA sequence
cFLIP Fwd	GATCTCCGGGGATAAATCTGATGTGTCCTCATTACTCGAGTAATGAGGAC ACATCAGATTTATCCTTTTTA
cFLIP Rev	AGCTTAAAAAGGATAAATCTGATGTGTCCTCATTACTCGAGTAATGAGGACA CATCAGATTTATCCCCGGA

2.2.1.2 Transformation: Generation of cFLIP overexpression and localisation mutant cell lines

MCF-7 cells were plated so that they reached 40-50% confluency on day of transformation. Transformation master-mixes were composed containing lipofectamine 3000 (Invitrogen), PLUS reagent, and DNA (pcDNA3.1 vectors) in Optimem reduced serum medium (Invitrogen) (Table 2.8). The solution was incubated for 5 mins at room temperature to allow the formation of DNA lipid complexes. The cell culture medium was changed to an antibiotic-free medium and complexes were added to the cells, and incubated at 37˚C 5% CO_2 for 48 h. Cells were then cultured in medium containing 250 µg/ml neomycin (Sigma). Cells were selected in this manner, splitting when necessary, until no cells remained in untransfected well and then assayed for mutant gene expression.

Table 2.8: Transformation formats:

Plate	Optimem (µl)	DNA (ng)	Lipofectamine (µl)	P3000 Reagent (µl)
96	10	100	0.15	0.2
48	25	250	0.45	0.6
24	50	500	0.75	1.0
12	100	1000	1.50	2.0
6	250	2500	3.75	5.0

2.2.1.3 RNA interference

Small interfering RNAs (siRNA) targeting both the long and short isoforms of cFLIP, or a non-specific scrambled control were custom designed and used in reverse transfections (Stealth siRNA, Invitrogen) (Table 2.9). Cells were transfected using lipofectamine RNAimax (Invitrogen) according to manufacturer's instructions. Master-mixes were prepared by diluting siRNA to a final concentration of 10 nM in serum-free Optimem (Invitrogen) containing lipofectamine (Invitrogen). For specific volumes and concentrations used in different plate formats see table 2.10. Master-mixes were incubated in wells of culture plate at room temperature for 20 mins prior to cell seeding. During this incubation, cells to be transfected were trypsinised and re-suspended in antibiotic-free medium, then seeded into the wells containing the lipofectamine/siRNA mix at a density of 1x 10^5 cells/ml. Cells were cultured in the presence of siRNA for 48 h prior to the subsequent assay.

Table 2.9: siRNA sequences:

Target	Sequence
cFLIP	GGAUAAAUCUGAUGUGUCCUCAUUA
cFLIP 2	GAGUGAGGCGAUUUGACCUGCUCAA
Scrambled (Control)	GGACUAAUAGUUGUGCUCCAAUUUA

Table 2.10: Volumes and concentrations of reagents per well for siRNA transfections:

Cell culture Plate (no. wells)	Surface Area	Volume culture medium	Volume Optimem (µl)	siRNA (pmol)	Volume lipofectamine RNAimax (µl)
96	0.2	100	20	1.2	0.2
48	0.4	200	40	2.4	0.4
24	1	500	100	6	1
6	5	2500	500	30	5

2.2.2 Reagents Used

Cells were treated a variety of cytokines, antibodies and reagents in the study (Tables 2.11, 2.12 and 2.13). Most experiments involved treating with recombinant soluble human TRAIL (super-killer TRAIL, Enzo Life Sciences) at a concentration of 20 ng/ml. Treated cells were then incubated at 37˚C in 5% CO_2 for 18 hours before subsequent assays. Alternatively, TRAIL was added to the media of cells in non-adherent conditions for the duration of the experiment.

Table 2.11: Cytokines

Cytokine	Working Concentration	Source
TRAIL	20 ng/ml	Enzo
TNFalpha	10 ng/ml	Sigma
TGFbeta	10 nM	Peprotech
IL6	10 ng/ml	Sigma
IGF	100 nM	Peprotech
EGF	100 ng/ml	Sigma
FGF	0.5 ng/ml	Peprotech
Heregulin	75 ng/ml	Peprotech
MMP2	2.5 μg/ml	Peprotech
MMP3	2.5 μg/ml	Peprotech
Wnt1	2 ng/ml	Peprotech
Wnt3a	10 ng/ml	Peprotech

Table 2.12: Antibodies

Antibody	Working Concentration	Source
Growth Hormone	1 μg/ml	R and D Systems
Heregulin	2 μg/ml	R and D Systems
IL8	1 μg/ml	R and D Systems
Artemin	2 μg/ml	R and D Systems
Axl	2 μg/ml	R and D Systems
Progranulin	1 μg/ml	R and D Systems
PIGF	2 μg/ml	R and D Systems
MCP1	2 μg/ml	R and D Systems
IL6	1 μg/ml	Abcam

Table 2.13: Compounds

Compound	Working Concentration	Source
Leptomycin B	20 ng/ml	Sigma
SB 431542	2 μg/ml	Sigma
Tankyrase inhibitor	1 μg/ml	Sigma

2.2.3 Cell Viability Assay

The Cell Titre Blue assay (Promega) measures the viability of cells by their ability to convert the blue dye resazurin into the fluorescent product resorufin: Non-viable cells do not have the metabolic capacity to convert resazurin and so do not produce a fluorescent signal.

Cells to be analysed were cultured in a 96-well plate format. On the day of analysis, 20 µl of Cell Titre Blue reagent (Promega) was added to each well containing 100 µl media. The plate was incubated for 1-4 h at 37˚C 5% CO_2, before fluorescence was measured at 560/590 nm using a FLUOstar Optima plate reader (BMG Labtech, Offenberg, Germany).

2.2.4 Stem Cell Assays

2.2.4.1 Tumoursphere Formation Assay

The tumoursphere assay is a functional assay designed to isolate the cancer stem cells from cell lines or primary cultures by exploiting their capacity to resist anoikis. Cells are cultured in suspension which induce anoikis in the bulk population but allow the putative stem cells to remain. These cells continue to self renew and divide and as a result produce small colonies termed tumourspheres. These can be subjected to serial passaging to assay for self-renewal. Quantification of tumourspheres is therefore indicative of stem cell number (Dontu 2003, Shaw 2012). Tumoursphere assays were carried out in non-adherent conditions in a serum-free epithelial growth medium (MEBM, Lonza), supplemented with B27 (Invitrogen), 20 ng/ml EGF (Sigma), Insulin (Sigma), and hydrocortisone (Sigma). Cells were plated in ultra-low attachment plates (Costar, Corning) at a density of 5000 cells/ml. After 7 days tumourspheres were counted, then collected by gentle centrifugation (1100 rpm), dissociated in 0.05% trypsin, 0.25% EDTA (Invitrogen) and re-seeded at 5000 cells/ml for subsequent passages.

2.2.4.2 Colony Forming Assay

Cells were seeded at a density of 160.2 cells/ml in a 6-well plate format, so that cells were 50 per square cm and cultured for 10 days (Harrison 2010, Locke 2005). To stain colonies, culture medium was removed and well surface was washed once with PBS. Crystal violet/ethanol mixture was applied to wells and incubated for 15 mins at room temperature. Solution was removed and wells were rinsed twice with PBS. Colonies containing 32 or more cells (having undergone 5 or more divisions) were counted using a GelCount platereader and software (Oxford Optronix) set to count colonies of size 100-1000µm.

2.2.4.3 Tumour Initiation *In vivo*

Cells were trypsinised and transferred to falcons, then counted and diluted accordingly. Cells were pelleted by centrifugation and were resuspended in RPMI basal medium containing no additives (serum-free) to wash cells of any residual serum. Cells were diluted 50:50 with Matrigel (BD Biosciences) and kept on ice during the course of the surgery. FoxN1Nu/Nu mice were used for all xenograft studies (Harlan Life Sciences, UK). Mice were maintained according to the Home Office Animals (Scientific Procedures) Act 1986. All procedures were performed under project licence 30/2849. 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 0.4 l/min in an induction chamber. Following induction, isofluorane was administered at 2.5% through an anaesthetic mask to maintain the animal unconscious throughout the procedure. The cell/Matrigel mix was injected above the lymph nodes of the fourth inguinal mammary fat pad with a Hamilton or insulin needle syringe (BD Micro-Fine). Animals were then allowed to recover in a temperature regulated chamber at 30˚C for 15 mins. Mice were administered oestrogen in the water during the course of the experiment at a concentration of 10 μg/ml. Mice were examined for tumour growth twice weekly by palpation. Tumours were measured using callipers and tumour volume was measured by the calculation; (tumour width^2) x tumour length/2. Mice were culled when the entire control group developed tumours at least 5 mm in diameter, and mammary glands, lungs and livers of mice were fixed in formalin for histological analysis.

2.2.4.3.1 Processing of Tissues for Histological Analysis

Paraffin embedded sections were prepared by the School of Biosciences Histology Unit: Tissues were dehydrated by immersion in 70% ethanol for 1 h, 95% ethanol for 1 h, 100% ethanol for 1.5 h, 100% ethanol for 1.5 h, 100% ethanol for 2 h, xylene for 1 h, and xylene for 1 h. Tissues were then embedded in paraffin wax and sectioned into 5 µm thick slices using a Leica RM2135 microtome cutter. Tissue sections were laid onto poly-L-lysine (PLL) coated slides (Thermo Fisher, Loughborough, UK) and immobilised by incubating at 58˚C for 24 h. Sections were then stained with haemotoxyin and eosin: Paraffin was removed and tissues rehydrated by immersing slides in 100% ethanol for 2 mins, 100% ethanol for 2 mins, 95% ethanol for 2 mins, and 70% ethanol for 2 mins. Slides were then rinsed in distilled water and immersed in Meyer's Haemaelium (Thermo Fisher, Loughborough, UK) for 5 mins. Slides were washed under running tap water for 5 mins then counterstained in 1% aqueous Eosin (Thermo Fisher, Loughborough, UK) for 5 mins. Sections were dehydrated by the reverse procedure: slides were immersed in 70% ethanol for 2 mins, 95% ethanol for 2 mins, 100% ethanol for 2 mins, 100% ethanol for 2 mins, xylene for 3 mins, and xylene for 3 mins. Coverslips were mounted on slides using Dpx mounting medium (VWR). Haemotoxyin and Eosin stained sections were examined for tumour cells microscopically.

2.2.5 Luciferase Reporter Assay

Following 48h transfection with reporter plasmids including LacZ, cells were lysed using 50 µl Glo lysis buffer (Promega) for 30 mins. To each of two white-sided 96-well plates (Costar, Corning), 20 µl of cell lysate was transferred. To assay for LacZ activity, to one plate 20µl Beta-Glo reagent (Promega) was added and incubated at room temperature with gentle shaking for 20 mins before measuring luminescence output. To the second plate, 20 µl of Bright-Glo reagent (Promega) was added to assay for TOPFlash reporter activity and the luminescence measured immediately. Luminescence was measured using a FLUOstar Optima plate reader (BMG labtech, Offenberg, Germany). TOPFlash reporter activity was normalised to LacZ activity to control for transfection efficiency.

2.3 RNA Analysis

All work surfaces and tools were cleaned with RNAseZap (Ambion) prior to RNA analysis.

2.3.1 RNA Extraction

Cells from culture were centrifuged for 5 mins at 1100 rpm and the resulting pellet re-suspended in 350 µl RLT buffer (Qiagen). RNA extraction was performed using the Qiagen RNEasy kit according to the manufacturer's instructions. Resulting RNA was incubated with DNase (amplicon) for 15 mins before inactivation. RNA concentration was determined using a Nanodrop (GE Healthcare, UK).

2.3.1 cDNA Synthesis

To synthesise DNA, 1 µg RNA was diluted in 12.5 µl dH_2O. Each RNA sample was incubated with 1 µl 10 mM dNTPs (promega) and 1 µl 500 µg/ml random primers (Promega) at 65˚C for 5 min followed by rapid cool to 4˚C in order to minimise secondary structure formation and facilitate DNA synthesis. A master-mix was then prepared containing per reaction; 1 µl MMLV reverse transcriptase (Promega) in 5 µl reverse transcriptase buffer (Promega) together with 0.5 µl RNasin (Promega), 6.5 µl of which was added to each sample to give a total volume of 20 µl. Samples were then incubated for 10 min at 25˚C, 30 min at 50˚C and 5 min at 85˚C before cooled to 4˚C in a PCR machine (BioRad). The resulting DNA was diluted 1:2 and stored at -20˚C.

2.3.2 q-RT-PCR Analysis

2.3.2.1 Primer Design

Primers were either custom designed across exon boundaries using the Primer3 web-based program (http://primer3.ut.ee/) or taken from published sequences. All primers were purchased from Sigma (Table 2.14).

Table 2.14: qPCR Primers

<table>
<tr><th>Target</th><th>Forward Primer Sequence (5'-3')</th><th>Reverse Primer Sequence (5'-3')</th><th>Ref</th></tr>
<tr><td>Beta Actin</td><td>CCCAGCACAATGAAGATCAA</td><td>CAGGTGGAAGGTCGTCTACA</td><td>-</td></tr>
<tr><td>cFLIP</td><td>TGATGGCAGAGATTGGTGAG</td><td>GATTTAGACCAACGGGGTCT</td><td>-</td></tr>
<tr><td>E-cadherin</td><td>TGCCCAGAAAATGAAAAAGG</td><td>GTGTATGTGGCAATGCGTTC</td><td rowspan="6">Mani 2008</td></tr>
<tr><td>N-cadherin</td><td>ACAGTGGCCACCTACAAAGG</td><td>CCGAGATGGGGTTGATAATG</td></tr>
<tr><td>Fibronectin</td><td>CAGTGGGAGACCTCGAGAAG</td><td>TCCCTCGGAACATCAGAAAC</td></tr>
<tr><td>Slug</td><td>GGGGAGAAGCCTTTTTCTTG</td><td>TCCTCATGTTTGTGCAGGAG</td></tr>
<tr><td>Snail</td><td>CCTCCCTGTCAGATGAGGAC</td><td>CCAGGCTGAGGTATTCCTTG</td></tr>
<tr><td>Twist</td><td>GGAGTCCGCAGTCTTACGAG</td><td>TCTGGAGGACCTGGTAGAGG</td></tr>
<tr><td>Axin2</td><td>AGTGTGAGGTCCACGGAAAC</td><td>TGGCTGGTGCAAAGACATAG</td><td>-</td></tr>
</table>

2.3.2.2 qRT-PCR

All qRT-PCR experiments were designed to include primers targeting genes of interest together with beta actin as the internal control gene, the expression levels of which should be consistent throughout all cells and therefore reflective of the amount of cDNA in each sample. Experiments also included no template controls, where dH_2O was used in place of the cDNA template to control for presence of contaminating DNA.

A master-mix containing all PCR reaction components was prepared first containing per reaction; 0.2 µl Sybrgreen (Invitrogen), 13.7 µl dH_2O (Sigma), 0.1 µl Taq polymerase (GoTaq, Promega), 5 µl GoTaq buffer (Promega), 2.5 µl $MgCl_2$ (Promega), 0.5 µl 10mM dNTPs (Promega), and 0.25 µl each 10 mM forward and reverse primers. To 2.5 µl cDNA in 96-well plate (Applied Biosystems), 22.5 µl of the PCR master-mix was added and mixed by pipetting. The plate was sealed and the experiment run on a RealTime PCR machine using StepOne software (Applied Biosystems) set to the following protocol: initial denaturation at 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 s (denaturation), and 60˚C for 1min (annealing/elongation). A melt curve was constructed at the end of the experiment and analysed to confirm a single peak indicating a single product and no peak in the dH_2O controls to confirm absence of contamination.

2.3.2.3 Data Analysis

qPCR data was analysed by relative quantitation whereby target expression is compared to that of the internal control gene expression to normalise for input and efficiency variations, and a reference sample. Expression levels of target genes are first normalised to that of the endogenous control: the cycle threshold (Ct) of the control is subtracted from that of the sample to obtain the delta Ct value (i.e. change in expression). This is performed for both the samples and reference samples. The fold change or "delta delta Ct value" of the sample is calculated by subtracting its delta Ct value from that of the reference sample. The fold change is expressed relative to one whereby a value greater than one indicates an increase in expression, equal to one indicates no change, and less than one indicates a decrease in expression.

2.4 Protein Analysis

2.4.1 Protein Extraction

2.4.1.1 Total Cellular Proteins

Cells from culture were centrifuged for 5 mins at 1100 rpm and the resulting pellet washed with PBS. The cell pellet was then resuspended in 100 µl RIPA buffer containing complete protease inhibitors (25x solution, Roche), 200 nM $NaVO_3$, 1 M NaF and 100 nM $Na_4P_2O_7$. The sample was passed through a 23-gauge needle in a 1 ml syringe 8 times then transferred to 1.5 ml microcentrifuge tube and placed on ice for 30 mins. The samples were centrifuged at 13 krpm for 15 mins at 4˚C, and the supernatant collected in 100 µl aliquots and stored at -20˚C.

2.4.1.2 Composition of RIPA Buffer:

- 5 ml 1 M Tris pH7.4
- 10 ml 10% Nonidet-P40 (Roche #17545999, stored at 4˚C)
- 0.25 g Sodium Deoxycholate (Deoxycholic acid- sodium salt)
- 3 ml 5 M NaCl
- 0.4 ml 0.25 M EGTA
- Made up to 100 ml with H_2O and pH to 7.4, stored at 4˚C

2.4.1.3 Nuclear and Cytoplasmic Extracts

Cells from culture were centrifuged for 5 mins at 1100 rpm and the resulting pellet washed in ice cold PBS and centrifuged again for 5 mins at 1100 rpm. Following aspiration of PBS, cells were re-suspended in 200 µl NEBA containing complete protease inhibitors (Roche), 200 nM $NaVO_3$, 1 M NaF and 100 nM $Na_4P_2O_7$ and transferred to Eppendorfs. Re-suspension was performed by gentle pipetting, the sample was then placed on ice for 15 min. To the solution, 25 µl of 10% NP-40 was then added and mixed by vortexing vigorously for 30 s. The solution was centrifuged at 10 krpm for 30 s and the resulting supernatant (cytoplasmic proteins) transferred to a fresh tube. The pellet was then re-suspended in 200 µl NEBA plus protease inhibitors and then centrifuged again at 10 krpm for 30 s and the supernatant removed and discarded in order to purify sample of residual cytoplasmic proteins. To the cell pellet, 100µl NEBC containing complete protease inhibitors (Roche), 200 nM $NaVO_3$, 1 M NaF and 100 nM $Na_4P_2O_7$ was then added which was re-suspended by vortexing for 30 s and placed on ice for 30 min.This was then centrifuged at 13 krpm for 5 min at 4˚C and the resulting supernatant (containing nuclear proteins) transferred to a fresh tube and stored at -20˚C.

2.4.1.4 Composition of NEBA and NEBC Buffers

NEBA:

- 10 mM Hepes pH7.9
- 10 mM KCl
- 0.1 mM EDTA pH8.0
- 0.1 mM EGTA pH8.0
- Filtered and stored at 4˚C

NEBC:

- 10% glycerol
- 20 mM Hepes pH7.9
- 0.4 M NaCl
- 1 mM EDTA pH8.0
- 1 mM EGTA pH8.0
- Filtered and stored at -20 ˚C

2.4.2 Protein Assay

2.4.2.1 BCA

Protein concentrations of total proteins or cytoplasmic extracts were determined using a BCA assay kit (Pierce) and Nanodrop (GE Healthcare, UK); 5 µl of sample or BSA standard was added to 25 µl BCA reagent mixed as per manufacturer's instructions, and incubated at 37˚C for 30 min then kept at 4˚C. Standards of 2.5, 5 and 10 mg BSA per ml RIPA or NEBA buffer were used to produce a standard curve from which sample protein concentration could be determined. As the nature of the RNAi and tumoursphere assays often necessitated working with low cell numbers it was of interest to determine how cell number related to level of protein extraction. Therefore samples of varying cell numbers were harvested and subjected to protein extraction and assayed as above. For both the 231 and MCF-7 lines a linear relationship between cell number and protein concentration was observed with a minimum of 50,000 cells being required to extract 1 mg of protein (Figure 2.1).

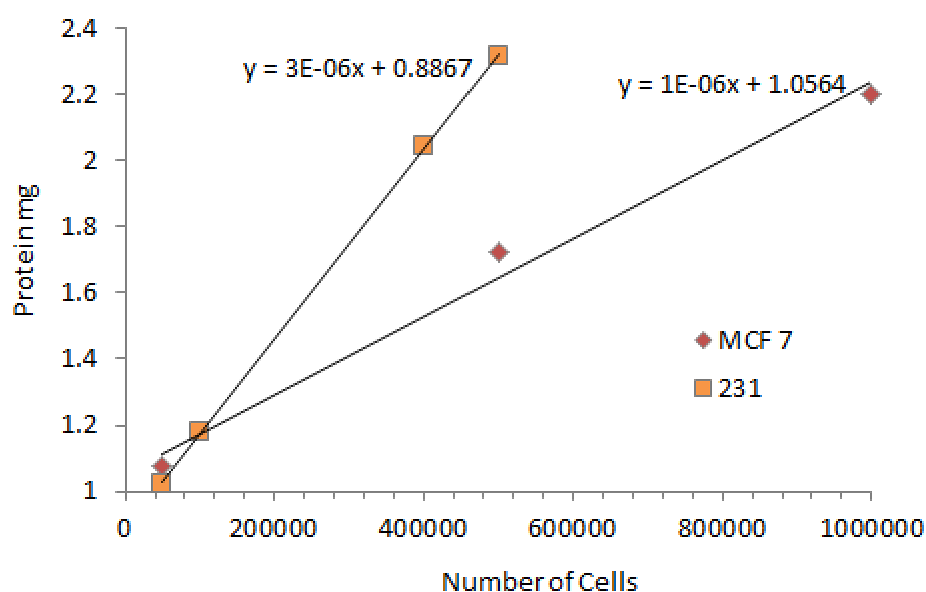


Figure 2.1: *Relationship between cell number and protein concentration:* MCF7 and 231 cells were trypsinised and counted. Cells were diluted to 5 x 10^4, 1 x 10^5, 5 x 10^5, and 1 x 10^6 cells. Protein was extracted and concentrations determined using the BCA assay.

2.4.2.2 Bradford

Protein concentrations of cytoplasmic and nuclear protein extracts were determined using the Bradford assay reagent and Nanodrop (GE Healthcare, UK); 1 µl of sample or BSA standard was added to 29 µl Bradford assay reagent and incubated at room temperature for 5-45min whilst protein assay was carried out. Standards of 1.25, 2.5, 5 and 10 mg/ml BSA (Sigma) in NEBC buffer were used to produce a standard curve from which sample protein concentration could be determined.

2.4.3 Western Blotting

2.4.3.1 The following solutions were prepared for use during Western blotting (Table 2.15)

Table 2.15: Solutions Used in Western Blotting Protocol:

Solution	Composition
5x Laemmli (sample loading) Buffer	60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue
PBST	1 x PBS solution (Fisher): 5 tablets 500 ml dH_2O with 0.5 ml Tween (Sigma)
Resolving Gel Buffer	1.5 M Tris-HCl, pH8.8
Stacking Gel Buffer	0.5 M Tris-HCl, pH6.8
10x Electrophoresis Buffer	30.0 g Trizma (Sigma), 144.4 g Glycine (Sigma) pH6.8 Up tp 1 L dH_2O
1x SDS-PAGE Running Buffer	895 ml dH_2O, 100 ml 10x Electrophoresis running buffer, 5 ml SDS (Sigma)
1x Western Transfer Buffer	700 ml dH_2O, 200 ml Methanol, 100 ml 10x Electrophoresis running buffer
Blocking Buffer	5% w/v non-fat milk powder (Marvel): 0.75 g in 15 ml PBST per transfer membrane
Antibody Dilution Buffer	5% w/v BSA (Sigma): 0.1g in 2 ml PBST per antibody
Stripping Buffer	62.5 mM Tris-HCl (pH6.8, 2% w/v SDS, 100 mM 2-beta-mercaptoethanol

2.4.3.2 Sample Preparation

Samples were diluted to an appropriate concentration no less than 1 µg/µl in the correct protein extraction buffer plus 1x Laemmli buffer. Samples were heated to 95˚C for 2 mins and used immediately.

2.4.3.3 SDS-PAGE

To prepare the 10% resolving gel, 100 µl APS (Sigma) plus 15 µl TEMED (Sigma) were added to the resolving gel buffer (Table 2.16), mixed and immediately poured. dH_2O was immediately laid over the gel in order to ensure the gel front was even. The resolving gel was left to set for approximately 15 min then the dH_2O removed. The stacking gel was prepared by adding 100 µl APS and 15 µl TEMED to 4% stacking gel buffer (Table 2.16), which was mixed and immediately poured. Combs were inserted into the top of the gel and left to set for approximately 15 min. The gels were then inserted into the electrophoresis gasket which was placed in a gel running tank (BioRad). The centre of the gasket was filled with SDS running buffer (BioRad) which was allowed to overflow into the tank to a depth of approximately 3 cm. 5-30 µl of prepared samples were loaded in addition to a molecular weight marker (PageRuler plus, Fermentas). Gels were run at 180 V for 1 h or until desired marker separation was observed.

Table 2.16: Composition of polyacrylamide gels:

Percent gel	ddH2O (ml)	30% degassed Acrylamide/Bis (ml) (Sigma)	Resolving or Stacking Gel Buffer (Table 2.14) (ml)	10% w/v SDS (ml)
4	6.1	1.3	2.5	0.1
10	4.1	3.3	2.5	0.1

2.4.3.4 Western Transfer to Membrane

The Western transfer sandwich was prepared in the following manner: onto the cassette were layered in order; sponge, filter paper, protein gel, PVDF membrane, filter paper and sponge. Each layer of sponge and filter paper had been soaked in transfer buffer (Table 2.15) prior to preparation of the sandwich. The PVDF membrane had been dipped in methanol for 10 s and soaked in transfer buffer for 5 min. Air bubbles were removed from the sandwich by rolling each layer. The cassette was closed and inserted in an electrophoresis gasket in the correct orientation. This was placed in a transfer tank with an ice block and magnetic stirrer. The tank was placed on a magnetic stirrer and run at a constant voltage of 80 V for 45 min.

2.4.3.5 Confirmation of Protein Transfer

To examine protein transfer, the PVDF membrane was washed for 5 mins in PBST, then rinsed in tap water. Ponceau red solution was poured into a tray containing the membrane. This was incubated for approximately 2 mins in the ponceau until shaded areas appeared indicating protein binding. This was placed between two acetate sheets and photocopied as a reference of protein transfer and loading. The membrane was then washed 3 x 5 mins in PBST before blocking.

2.4.3.6 Blocking and Antibody Incubation

Following Western transfer, sandwiches were disassembled and membranes washed 3 x 5 min in PBST before being incubated in blocking buffer with shaking for 1 h. The membranes were then transferred to 30ml universal tubes (Fisher) containing 2 ml of the desired primary antibody diluted in 5% BSA in PBST. Membranes were incubated in the primary antibody solution overnight at 4˚C on a roller.

2.4.3.7 Detection

Membranes were then washed 3 times for 5 min in PBST and transferred to a 30 ml tube containing 2 ml of the appropriate (i.e. either mouse or rabbit, Table 2.17) horseradish peroxidise-conjugated secondary antibody (Dako) diluted 1:2000 in 5% BSA in PBST. Membranes were incubated in secondary antibody at room temperature for 1 h on a roller. Finally, membranes were washed 3 times for 5 min in PBST. Antibody binding was detected using ECL prime detection reagent (Amersham) and exposure to X-Ray film (Amersham) using an automatic X-Ray film processor (Xograph).

2.4.3.8 Stripping and Re-probing of Membrane

Stripping buffer was prepared by adding 500 µl SDS to 24.5 ml Tris-HCl 60 mM plus 175 µl Beta-mercaptoethanol (for one membrane). Membrane was incubated at 55˚C for 30 mins with gentle shaking. Membrane was then rinsed twice in PBST three times for 5 min on a rocker. The membrane was blocked again before re-probing with primary antibody (Section 2.4.3.6).

2.4.3.9 Quantitation by Densitometry

To quantitate Western blotting data by densitometry, the program ImageJ was used (http://imagej.nih.gov/ij/). The pixel density over the selected areas was quantified and compared.

Table 2.17: Primary Antibodies used for Western Blotting

Antibody	Dilution	Source	Cat #	Species	Target size
cFLIP 5D8 (mAb)	1:750	Santa Cruz Biotech	sc-136160	Mouse	55 kDa
cFLIP NF6 (mAb)	1:1000	Enzo	ALX-804-428-C050	Mouse	55 kDa
GAPDH	1:1000	Santa Cruz Biotech	sc-32233	Mouse	35 kDa
Alpha-Tubulin	1:1000	Sigma	T9026	Mouse	55 kDa
Beta-Actin	1:1000	Sigma	A5441	Mouse	43 kDa
HDAC	1:1000	Cell Signalling	2062	Rabbit	65-70 kDA
Lamin A/C	1:1000	Cell Signalling	2032	Rabbit	65-70 kDA
Beta Catenin (mAb)	1:1000	BD Biosciences	610154	Mouse	100 kDa

2.4.3.10 Optimisation of cFLIP Antibody for Western Blotting

The optimised Western blotting procedure for cFLIP is as follows: A minimum of 20 µg of each protein sample was run on a 10% SDS-polyacrylamide gel. Following Western transfer, membranes were blocked in blocking buffer containing 5% BSA in PBS with 0.1% Tween, for 2 h, then incubated overnight at 4˚C in cFLIP primary antibody (5D8, Santa Cruz Biotech) diluted 1:750 in 5% BSA PBST. Membranes were washed three times for 5min in PBST before being incubated with mouse secondary antibody (1:2000) at room temperature for 2 h on a rocker. Proteins were detected using ECL prime and exposed for 15 s - 2 min.

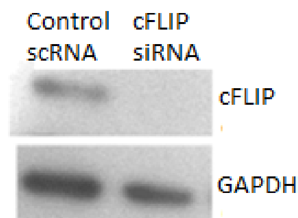


Figure 2.2: Optimisation of cFLIP antibody for Western blotting: MCF-7 cells were transfected with siRNA targeting cFLIP or a scrambled control siRNA: Inhibition of cFLIP reduced signal and confirmed antibody specificity

2.4.4 Immunofluorescence of Fixed Cells

Glass coverslips were first sterilised using 100% ethanol and allowed to air-dry. Cells from culture were seeded onto coverslips placed in wells of a 48-well culture plate, at a density of 5 x 10^5 cells/ml, and allowed to adhere overnight. On the day of analysis, cells were rinsed with PBS to remove any dead cells. Cells on coverslips were fixed in 4% formalin for 15 mins followed by 3 x 5min washes in PBS. Cells on coverslips were then blocked in 10% normal goat serum (Dako) in PBS with 0.5% triton-X-100 (Sigma) for 1 h. Cells were then incubated in the primary antibody 1:100 overnight at 4˚C. Following 3 x 5 min washes in PBS, cells were incubated in fluorescence-conjugated secondary antibodies (Invitrogen, Table 2.18) diluted 1:400 in 10% normal goat serum (Dako) and containing DAPI nuclear stain (Invitrogen) for 1 h (Table 2.18). Coverslips were then washed 3 x 5 mins in PBS and mounted in Mowiol solution (Sigma). Cells were visualised on a Leica confocal microscope.

Table 2.18: Antibodies used for Immunofluorescence

Antibody	Dilution	Species	Source
cFLIP	1:100	Rabbit	Cell signalling
EEA1	1:200	Mouse	DSHB
LAMP1	1:200	Mouse	DSHB
Anti-mouse 488	1:400	Goat	Invitrogen
Anti-rabbit 488	1:400	Goat	Invitrogen
Anti-mouse 594	1:400	Goat	Invitrogen
Anti-rabbit 594	1:400	Goat	Invitrogen

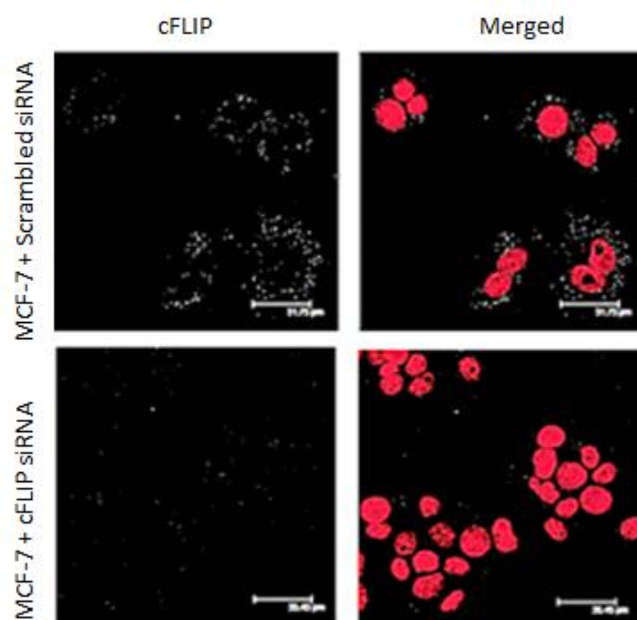


Figure 2.3: Optimisation of cFLIP antibody for immunofluorescence: MCF-7 cells were transfected with siRNA targeting cFLIP or a scrambled control siRNA: Inhibition of cFLIP reduced fluorescence signal and confirmed antibody specificity

2.4.5 Flow Cytometry

Cells to be analysed were trypsinised, centrifuged at 1100 rpm for 5 mins then re-suspended in PBS and transferred to 96-well plate. Cells were washed twice with PBS: plate was centrifuged and solution replaced with 100 µl PBS per well. On the final wash, PBS was removed and replaced with 4% formalin to fix the cells and incubated at room temperature for 10 mins. Cells were then washed in PBS three times: plate was centrifuged and solution replaced with 100 µl PBS per well. On the final wash, PBS was removed and cells re-suspended in antibody solution or appropriate isotype control diluted in 0.1% BSA in PBS. Cells were incubated in antibody solutions for 30 mins at 4˚C. Antibodies used are listed in table 2.19. Cells were then pelleted and washed with PBS twice to remove all residual antibody, then re-suspended in 100 µl PBS and filtered through a 40 µm cell strainer (BD Biosciences) into a flow cytometry collection tube (BD Biosciences) to ensure a single cell suspension. Flow cytometry was performed on an Accuri 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.

Table 2.19: Antibodies used for Flow Cytometry:

Antibody	Dilution	Source and Cat. #
CD44-APC	1:1000	BD Pharmingen, 559942
CD24-FITC	1:1000	BD Pharmingen, 555427

2.4.6 Cytokine Array of Conditioned Medium

A human cytokine array of 1000 targets (L1000, Raybiotech) was performed on MDA-MB-231-conditioned medium and unconditioned medium for comparison, as per manufacturer's instructions:

2.4.6.1 Dialysis

Dialysis buffer (1 x PBS) was prepared by dissolving 0.6 g KCL, 24 g NaCl, 6 g KH_2PO_4 and 3.45 g Na_2HPO_4 in 2500 ml de-ionised water. Into each of two separate dialysis vials (Raybiotech), up to 3 ml of sample was added and placed carefully into a floating rack in 600 ml dialysis buffer. Dialysis proceeded at 4˚C for 2.5 h with gentle stirring. Dialysis buffer was then replaced and dialysis incubated overnight at 4˚C with gentle stirring. Dialysed samples were centrifuged at 10,000 rpm to remove any particulates or precipitates, and then re-combined in a 15 ml falcon tube (Nunc).

2.4.6.2 Biotin Labelling

Prior to biotin labelling, protein concentration was determined using the BCA assay (section 2.4.2). Labelling reagent was then dissolved in 100 µl PBS and 7.2 µl of dissolved labelling reagent per 1 mg total protein content was added to the sample. Reaction was incubated at room temperature for 30 mins with gentle shaking. Labelling was stopped by the addition of 5 µl stop solution. Unbound biotin was removed using the spin columns provided; spin columns were prepared for use by centrifugation at 1,000 g for 3 mins to remove storage solution, then were washed 3 times with 5 ml PBS each centrifuging at 1,000 g for 3 mins. To each prepared column, 3 ml sample was added and the column centrifuged at 1,000 g for 3 minutes and the flow-through (containing biotinylated proteins) collected.

2.4.6.3 Blocking and Incubation

Each membrane provided was placed into a plastic tray and incubated with blocking buffer solution (provided) for 1 h at room temperature with gentle shaking. During incubation, the sample was diluted five-fold in blocking buffer. Following incubation, 8 ml of diluted sample was added to each membrane and incubated overnight at 4˚C.

2.4.6.4 Detection

Following sample incubation, sample was removed from membrane and washed three times in wash buffer one, 5 min per wash, then three times in wash buffer two, 5 min per wash. A 1 x HRP-conjugated steptavidin solution was prepared by dilution in blocking buffer and 8 ml of this solution was added to each membrane and incubated at room temperature for 2 h with gentle shaking. The membranes were then washed as above: sample was removed from membrane and washed three times in wash buffer one, 5 min per wash, then three times in wash buffer two, 5 min per wash. To prepare detection reagent, 4.2 ml of detection buffer C was mixed with 4.2 ml of detection buffer D. 4 ml of detection reagent was added to each membrane and incubated for 2 mins at room temperature with gentle shaking. Signals were detected by exposure to x-ray film for 40 s.

2.4.6.5 Analysis

Array results were analysed by densitometry using ImageJ software (http://imagej.nih.gov/ij/). Cytokine expression of conditioned medium was compared to that of unconditioned and a relative expression level obtained. Equal loading of membranes was confirmed by comparison of internal positive controls.

2.5 Statistical Analysis

Error bars on all graphs represent standard error values. In most cases, an unpaired student's T-test was used to ascertain whether experimentally treated samples were statistically different from the control samples. All tests assumed unequal variances and were performed on datasets with sample sizes of n = 3. Tests were carried out using Excel 2010 software. Results were considered significant if the calculated p value was equal to or less than 0.05.

Pearson's correlation coefficient (r) was calculated in order to determine whether two sets of observations were linearly associated. The correlation coefficient 'r' was calculated using Excel 2010 software. Results were considered significant if the calculated value of r was greater than or equal to the tabulated value at the 5% significance level for n – 2 degrees of freedom.

Chapter 3: Investigating the Susceptibility of Breast Cancer Stem Cells to TRAIL

Chapter 3 Investigating the Susceptibility of Breast Cancer Stem Cells to TRAIL

3.1 Introduction

The ability of TRAIL to target cancer cells preferentially is what has led to its recombinant production as a targeted anti-cancer agent. However, despite the initial promise of *in vitro* studies, phase II and III clinical trials of TRAIL for non-breast tumours have produced conflicting results (Chapter 1 Section 1.3.4, Lemke *et al.* 2014). Although these findings have decreased interest in TRAIL as a therapeutic, we propose that as some patients clearly do respond, the success of TRAIL may have been impeded by a lack of patient stratification and therefore these conflicting data are merely reflective of the heterogenous nature of cancer. We believe that a better understanding of how and why tumour cells respond differently to TRAIL is called for in order to realise the full potential of TRAIL as a therapeutic.

Although TRAIL has not yet been trialled for breast cancer, preclinical studies have shown that mesenchymal-like breast cancer cell lines are TRAIL-sensitive whereas epithelial-like lines are TRAIL resistant (Rahman *et al.* 2009). Like most cancers, breast cancer is a heterogenous disease and therefore it is not surprising that different subtypes of breast cancer should respond differently to a therapeutic agent. However, breast tumours have intrinsic heterogeneity; the discovery of breast cancer stem cells (bCSCs) has shown that not all breast cancer cells are equally malignant (Al Haij *et al.* 2003). Despite making up just a fraction of the tumour, cancer stem cells are responsible for imparting the most detrimental aspects of malignancy to the tumour phenotype and therefore it is important to assess the efficacy of any potential therapeutic by its ability to target this cell population.

We have shown previously that the combination of TRAIL and siRNA inhibition of cFLIP depletes completely the tumoursphere population of four breast cancer cell lines (Piggott *et al.* 2011). However, due to its homology to the pro-apoptotic caspase 8, it has not yet been possible to develop a selective small molecule inhibitor of cFLIP and therefore any targeted combination therapy is a long way from the clinic. In contrast, TRAIL is a non-toxic agent already in clinical trials. In the absence of suitable non-toxic cFLIP inhibitors we set out to assess the ability of TRAIL alone to target bCSCs. bCSCs have been associated with a mesenchymal phenotype: induction of EMT in breast cancer cell lines and primary cells increases the number of bCSCs in the population (Mani *et al.* 2008, Morel *et al.* 2008). As TRAIL targets those breast cancer cell lines with a mesenchymal-like phenotype (Rahman *et al.* 2009), we proposed the hypothesis that bCSCs are sensitive to TRAIL.

3.2 Results

3.2.1 Tumoursphere-Forming Cells are TRAIL-Sensitive

To investigate the susceptibility of bCSCs to TRAIL, we used a panel of six breast cancer cell lines (Figure 3.1A). As has been previously reported (Rahman *et al.* 2009), in normal adherent culture, only those breast cancer cell lines with a mesenchymal-like phenotype (MDA-MB-231 and MDA-MB-436) were sensitive to TRAIL, whereas the epithelial-like lines (MCF-7, BT474, SKBR3 and MDA-MB-468) were TRAIL-resistant (Figure 3.1A). The susceptibility of stem-like populations within these lines has not yet been established. Tumoursphere formation *in vitro* assesses the stem-like properties of anoikis-resistance and self-renewal (Dontu 2003). To determine whether tumoursphere-forming cells (termed Tumoursphere-Forming Units; TFUs) were susceptible to TRAIL, untreated cells were seeded in non-adherent culture conditions in the presence or absence of 20 ng/ml TRAIL. TRAIL-treatment reduced primary tumoursphere-formation significantly in four out of six breast cancer cell lines, including two epithelial-like lines (Figure 3.1B). TRAIL-treatment also reduced secondary tumoursphere-formation significantly in three out of six breast cancer cell lines, including two epithelial-like lines (Figure 3.1C). TRAIL also decreased secondary tumoursphere formation in the MDA-MB-231 line, although the result was not statistically significant (Figure 3.1C). In the MDA-MB-231, MDA-MB-436, SKBR3 and BT474 lines, the TRAIL susceptibility of the tumoursphere-forming population was comparable to that of the total cell population. However bCSCs from the epithelial-like MCF-7 and MDA-MB-468 lines were more sensitive to TRAIL than the bulk population (Figures 3.1A, 3.1B and 3.1C).

In addition to reducing TFU number, TRAIL reduced tumoursphere size significantly in the SKBR3, BT474, MDA-MB-468 and MDA-MB-436 lines, suggesting that the proliferation of TFUs and their immediate progeny relating to transit-amplifying cells is also affected by TRAIL in these cases (Figure 3.1D). This suggests that although TRAIL does not reduce TFUs in the SKBR3 line, it may instead abrogate their function.

To confirm the relative sensitivity of the bulk and tumoursphere-forming populations, cells from two representative lines, MCF-7 and MDA-MB-231, were treated with TRAIL in adherent conditions, and surviving cells subjected to the tumoursphere assay. This experimental design is a measure of the proportion of tumoursphere-forming cells remaining in the TRAIL-resistant population only; no difference in TFUs between untreated and treated cells would indicate that TFUs are as equally sensitive to TRAIL as the total population, whereas a decrease in TFUs indicates a higher sensitivity of TFUs than the total population. When treated in adherent conditions, MCF-7 TFUs were approximately 50% more TRAIL-sensitive than the total MCF-7 population whereas TFUs of the MDA-MB-231 line were as sensitive to TRAIL as the total population (Figure 3.1E).

These data show that TRAIL can target the tumoursphere-forming and transit-amplifying cells of breast cancer cell lines. In each case we would expect that these TRAIL-sensitive cells are dying by caspase-mediated apoptosis, but this has not been confirmed. The mechanism of cell death could be tested by the inclusion of a pan-caspase inhibitor such as Z-Vad-Fmk; we would expect that Z-Vad-Fmk would protect tumoursphere-forming cells and transit-amplifying cells from TRAIL-mediated apoptosis.

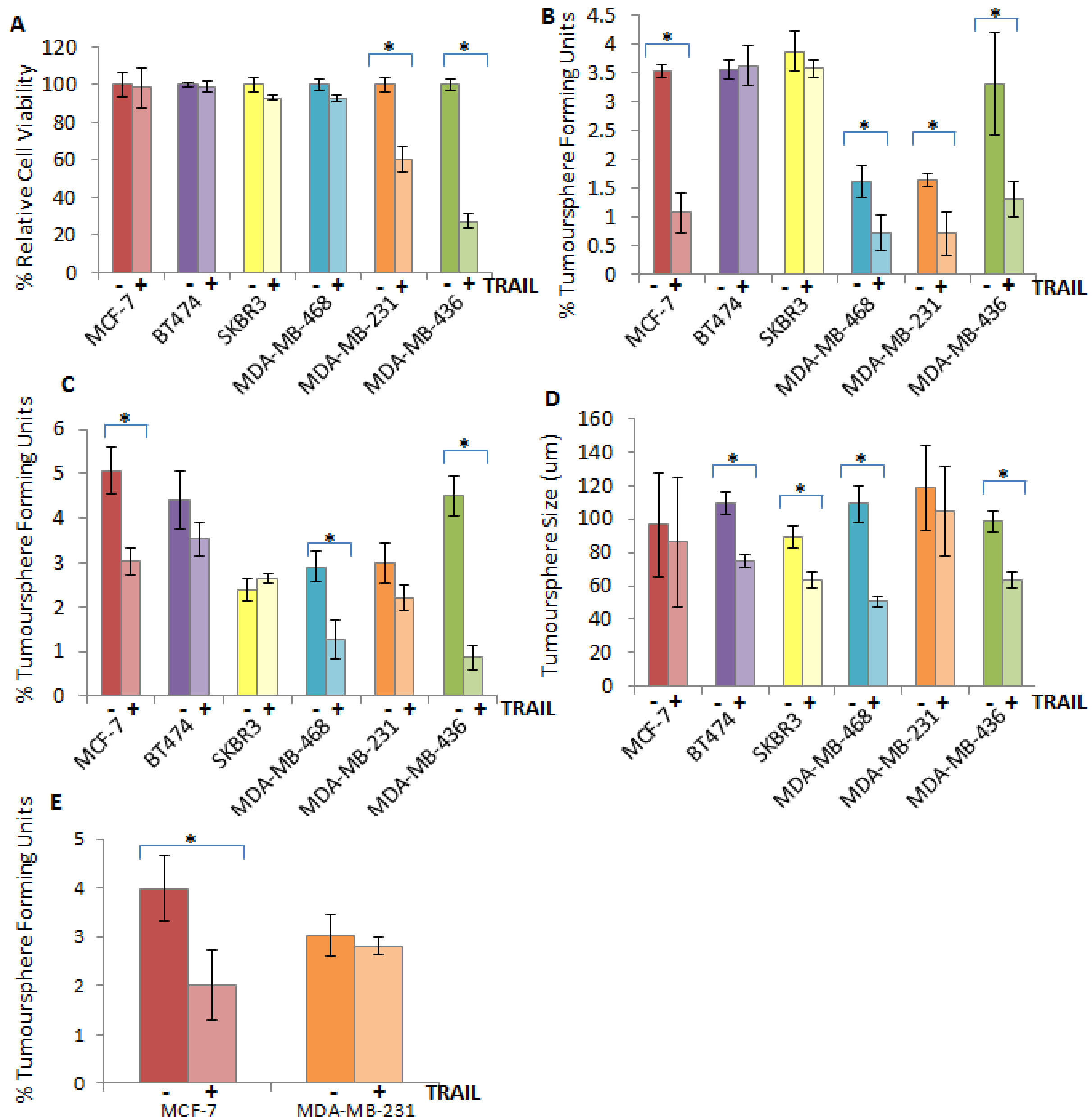


Figure 3.1: *Tumoursphere-Forming Cells are TRAIL-Sensitive:* **A Cell Titre Blue assay:** 18 h treatment with 20 ng/ml superkiller TRAIL (Enzo) reduced significantly the viability of the mesenchymal-like MDA-MB-231 and MDA-MB-436 lines (*p = 0.02 and *p = 0.005 respectively, t-test) but had no significant effect on the epithelial-like lines. **B Tumoursphere Assay Passage One:** Treatment with 20 ng/ml TRAIL reduced significantly the number of primary tumourspheres formed in four out of six cell lines (MCF-7; *p = 0.005, 468; *p = 0.03, 231; *p = 0.02, 436; *p = 0.05, t-test) **C Passage 2:** Treatment of primary spheres with 20 ng/ml TRAIL reduced significantly the number of secondary tumourspheres formed in three out of six cell lines (MCF-7; *p =0.01, 468; *p = 0.04, 436; *p = 0.01, t-test) **D Tumoursphere size:** Treatment of primary spheres with 20 ng/ml TRAIL reduced significantly the size of primary tumourspheres formed in the SKBR3, MDA-MB-468, BT474 and MDA-MB-436 cell lines (BT474; *p = 0.0001, SKBR3; *p =0.01, 468; *p = 0.008, 436; *p = 0.000, t-test) **E Tumoursphere Assay:** Cells were treated in adherent culture with 20 ng/ml TRAIL for 18 h before being subjected to the tumoursphere assay. TRAIL reduced significantly the number of secondary spheres formed in the MCF-7 line (*p = 0.02, t-test) but had no significant effect on the MDA-MB-231 line. All results are averages of three independent experiments each performed with three internal technical replicates

3.2.2 Colony Forming Cells are TRAIL-Sensitive

Colony formation assesses for the ability of single cells when plated at low density to form colonies in adherent culture (Harrison 2012, Locke 2005). Although colony formation is a property associated with bCSCs, the fact that over 50% of cells in a line can exhibit this property suggests that it does not enrich for bCSCs. However, the colony-forming assay is a useful accompaniment to the tumoursphere-forming assay which is restricted by its ability to assay for only those stem-like cells which exhibit anoikis resistance; a property not necessarily considered a pre-requisite for bCSCs (Liu *et al.* 2013).

To determine whether TRAIL could target colony-forming cells, each of six breast cancer cell lines were plated at a density of 50 cells per square centimetre, allowed to adhere overnight and treated with TRAIL. Colonies were allowed to form over a 10 day period and only those colonies containing 32 or more cells (having undergone five or more divisions) were counted (Harrison 2012). TRAIL reduced colony formation in all six breast cancer cell lines tested (Figure 3.2A and B). This is not in accordance with the data from the tumoursphere assay in which TRAIL was only able to target four out of six lines. However, the least TRAIL-sensitive line in both the tumoursphere and colony-forming assays was the BT474 line; the reduction in colony formation due to TRAIL was not statistically significant in this case. In addition, as TRAIL reduced significantly the size but not number of TFUs in the SKBR3 line (Figure 3.1D), this discrepancy could be explained by the fact that the colony-forming assay does not distinguish between the ability of TRAIL to deplete stem-like cells and the ability to prevent their propagation. This could be overcome by the removal of TRAIL from the assay following the 18 h treatment.

These data show that TRAIL can target colony-forming cells. We would expect that these cells are dying by caspase-mediated apoptosis, but this has not been confirmed. As with the tumoursphere assay, this could be tested by the inclusion of a pan-caspase inhibitor such as Z-Vad-Fmk; we would expect that Z-Vad-Fmk would protect colony-forming cells from TRAIL-mediated apoptosis.

Previous studies have shown that in carcinoma-derived cell lines it is possible to distinguish two different types of colonies; compact epithelial-like colonies and less dense, more mesenchymal-like colonies (Locke *et al.* 2005). The MCF-7 cell line produced these two colony types: compact round epithelial-like colonies and mesenchymal-like colonies which could also be distinguished on the basis of the localisation of E-cadherin and β-catenin (Figure 3.2D). Both these markers are indicative of epithelial-like cells when membrane bound, and mesenchymal-like cells when nuclear (Thiery and Sleeman 2006). To determine whether TRAIL targets a specific type of colony-forming cell, colonies were counted on the basis of density, by which they could be distinguished easily using (Figure 3.2D). Following treatment with TRAIL there was no significant difference in the proportion of high and low dense colonies, suggesting that TRAIL does not exhibit specificity towards a particular type of colony-forming cell (Figure 3.2D).

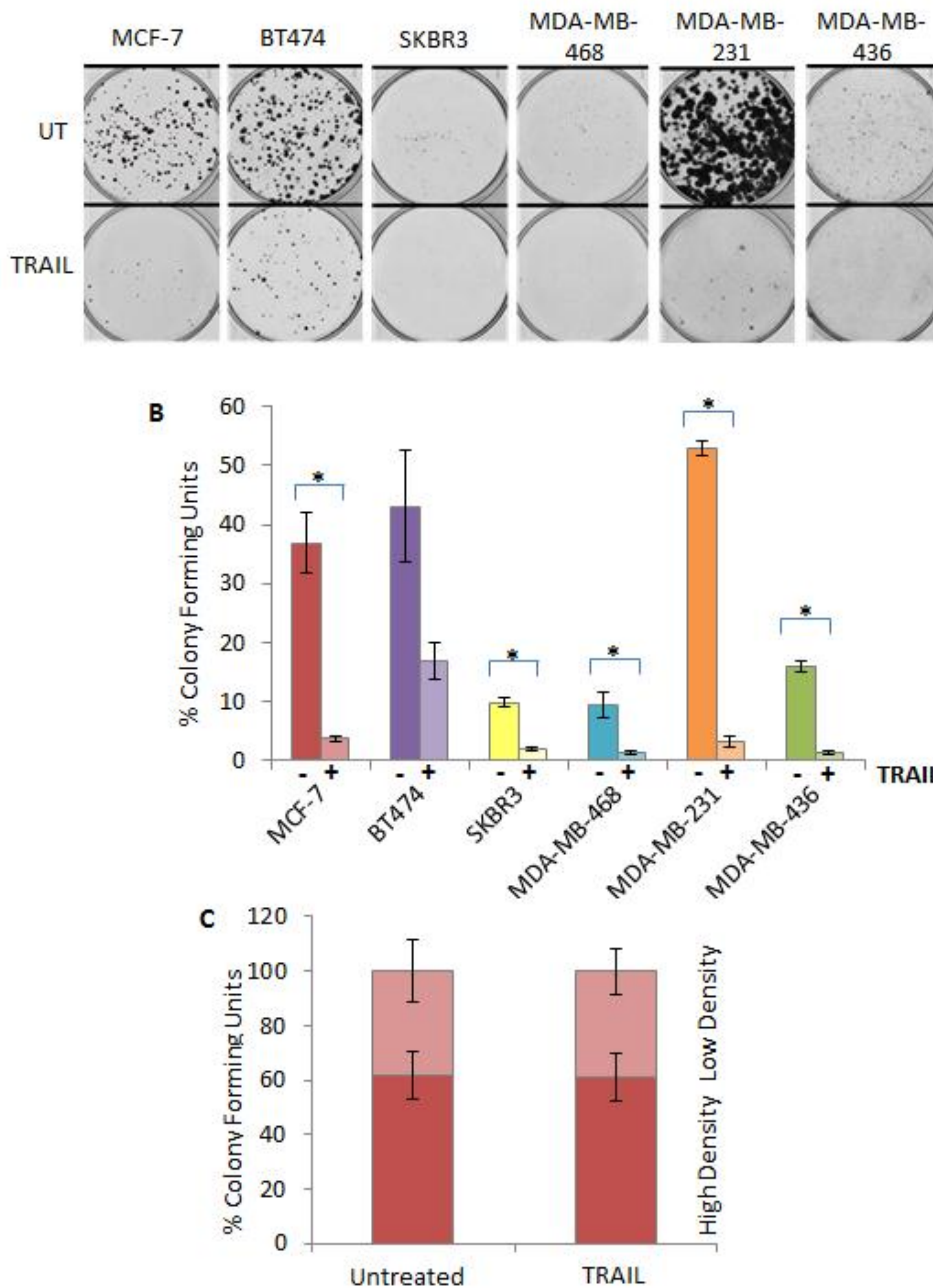
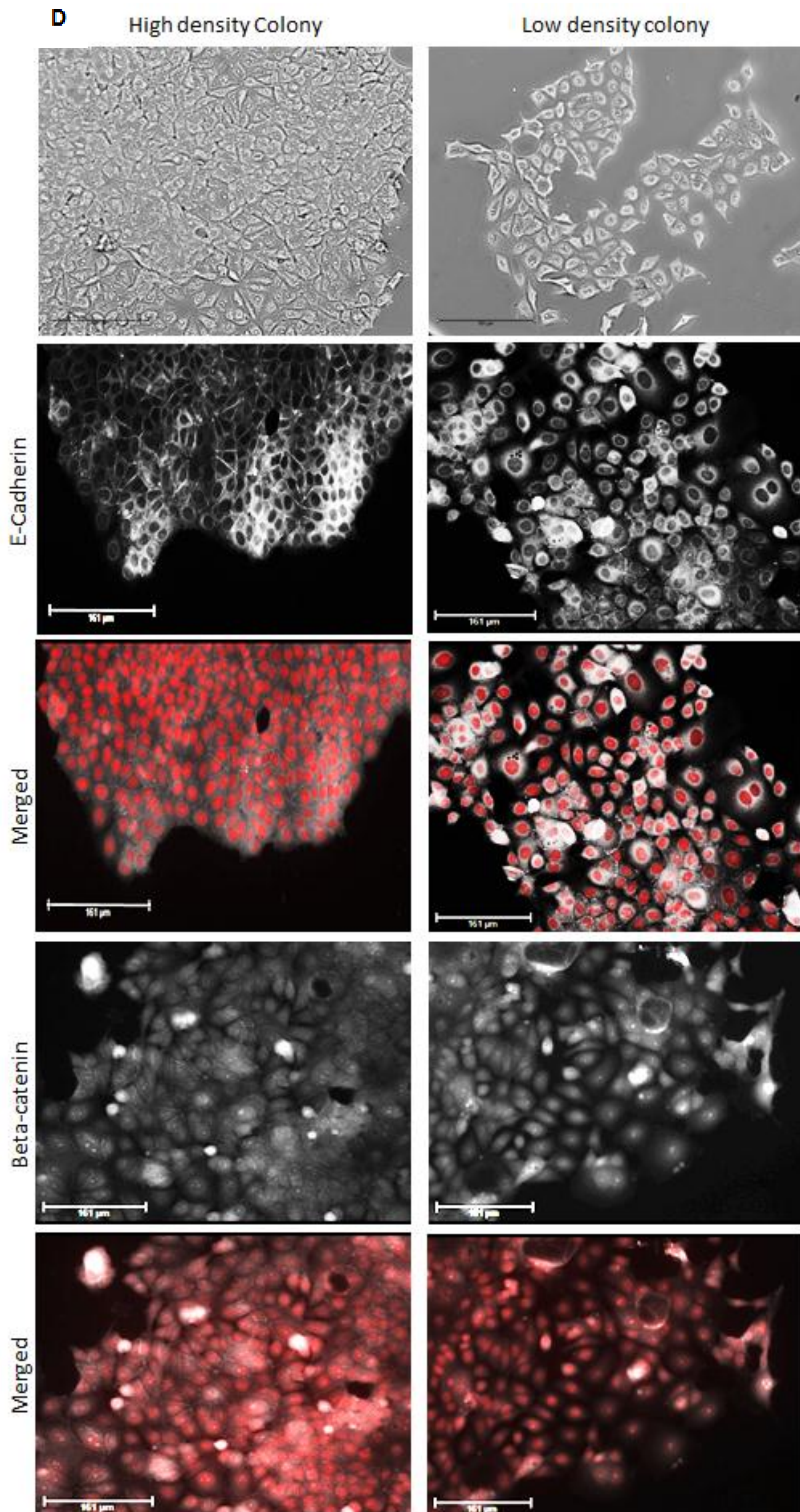


Figure 3.2: *Colony Forming Cells are TRAIL-Sensitive:*

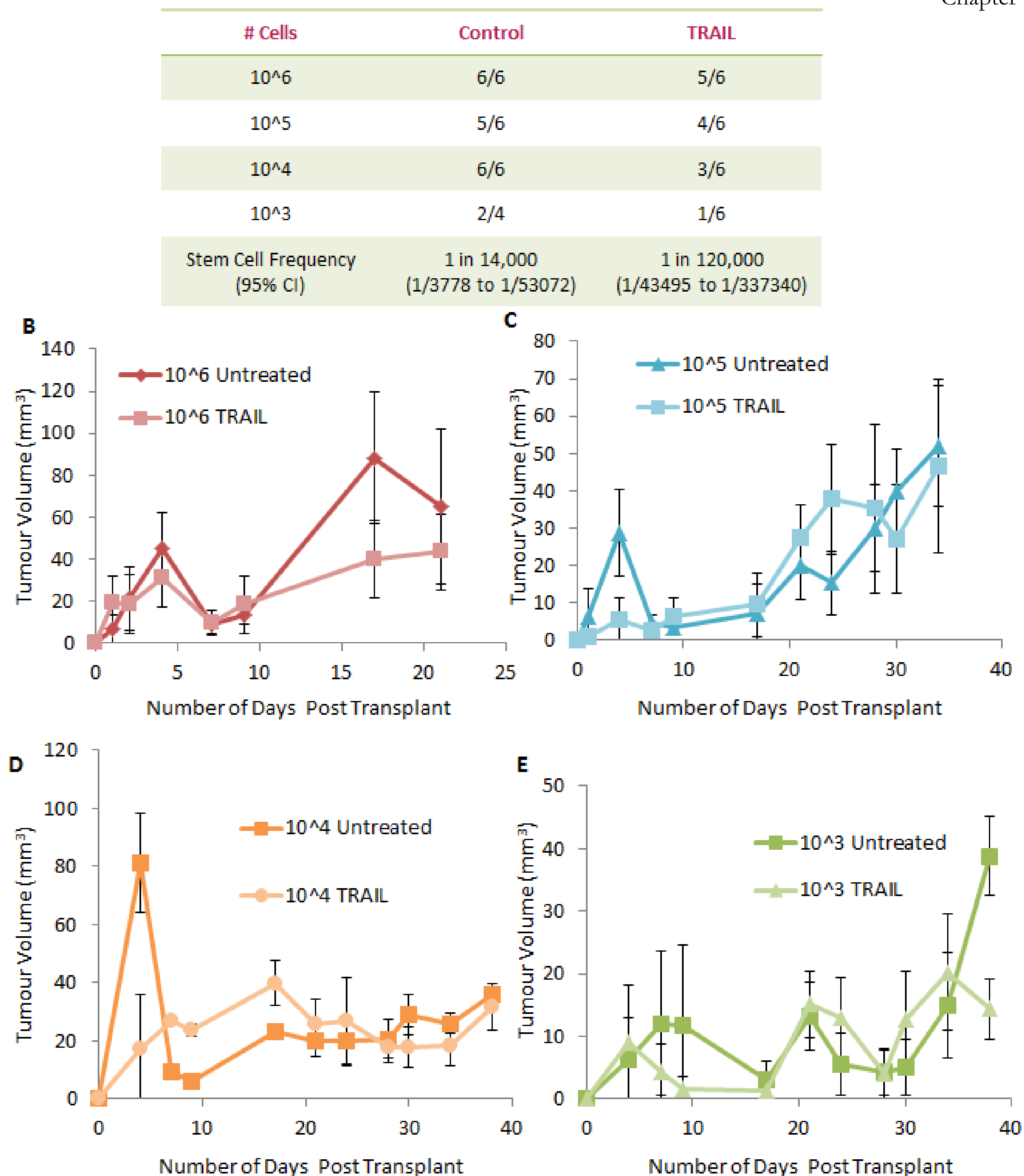
Colony-Forming Assays: Cells were seeded at a density of 50 cells/ cm² (160.2 cells/ml in a 6-well plate) in the presence or absence of 20 ng/ml TRAIL for 10 days. **A** Representative images **B** TRAIL reduced colony formation significantly in five out of six cell lines tested (MCF-7; $*p = 0.01$, BT474 $p > 0.05$, SKBR3 $*p = 0.02$, 468; $*p = 0.03$, 231; $*p = 0.000$, 436; $*p = 0.007$, t-test) **C** Colonies were counted on the basis of density using Gelcount software: TRAIL did not exhibit specificity toward a particular colony type **D Immunofluorescence:** (Overleaf) Differential staining of Beta-catenin and E-cadherin reveals presence of both epithelial-like colonies when membrane bound and mesenchymal-like colonies when cytosolic or nuclear. All results are averages of a single experiment performed with three internal technical replicates. Scale bar = 161 um.


D
High density Colony
Low density colony
E-Cadherin
Merged
Beta-catenin
Merged
161 µm

3.2.3 Tumour-Initiating Cells are TRAIL-Sensitive

Whilst these *in vitro* assays test for certain stem-like attributes, the gold standard assay for cancer stem cells remains the ability to initiate tumours *in vivo*. To determine the effect of TRAIL on tumour-initiating cells in the otherwise resistant MCF-7 cell line, MCF-7 cells were treated with TRAIL *in vitro* then transplanted into the mammary fat pad of nude mice in a dilution series in the absence of TRAIL. At the lower dilutions, TRAIL reduced the number of tumours formed (Figure 3.3A). However there was no significant effect of TRAIL pre-treatment on the size and growth of the tumours which did form (Figure 3.3B-D). This is in accordance with the tumoursphere assay (Figure 3.1) which showed that TRAIL had no significant effect on MCF-7 tumoursphere size, suggesting that TRAIL does not effect progenitor proliferation in this line. All xenografts exhibited a characteristic transient palpable mass which reduced in size during the first week following transplantation. This was presumed to be the matrigel plug gradually being absorbed and disseminated into the fat pad. Tumour growth was subsequently observed from ten days post-surgery for up to five weeks.

Due to technical limitations related to the number of successful transplants at higher cell numbers, considering the data as a whole does not fit the poisson model required for a robust determination of stem cell frequency (using the L-Calc method). Nevertheless, estimates from the two lowest dilutions (which are possible) show that TRAIL reduces stem cell frequency significantly (Figure 3.3A).



# Cells	Control	TRAIL
10^6	6/6	5/6
10^5	5/6	4/6
10^4	6/6	3/6
10^3	2/4	1/6
Stem Cell Frequency (95% CI)	1 in 14,000 (1/3778 to 1/53072)	1 in 120,000 (1/43495 to 1/337340)

Figure 3.3: *Tumour Initiating Cells are TRAIL-Sensitive*

Tumour Initiation *in vivo*: Cells were pre-treated with 20 ng/ml TRAIL for 18 h then implanted into the mammary fat pad of nude mice at serial dilutions. **A** The number of tumours formed in each experimental group was determined by palpation twice weekly and confirmed by histological analysis at the end of the experiment and the number of tumour-initiating cells estimated using L-calc software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx)

B-E Tumour volume was measured twice weekly and calculated using the formula: (tumour width^2) x tumour length/2 Volume is expressed in mm^3. Three animals were used in each experimental group which each carried bilateral transplants, therefore each experimental group comprised six replicates.

3.2.4 TRAIL susceptibility does not correlate with total cFLIP levels

While these results (3.2.1-3.2.3) indicate a broader specificity of bCSCs for TRAIL than described previously for the total populations, the underlying cause of this differential sensitivity to TRAIL is unknown. We have shown previously that inhibition of the long form of cFLIP sensitises tumoursphere-forming cells to TRAIL (Piggott *et al.* 2011). This suggests that the TRAIL-susceptibility of cells with bCSC-like traits is determined, at least in part, by the apoptosis inhibitor cFLIPL and therefore one explanation for the observed differences in TRAIL susceptibility between breast cancer cells could be that TRAIL-sensitive cells may contain lower levels of cFLIPL (hereafter referred to as cFLIP). To test this hypothesis, protein was extracted from breast cancer cells in adherent culture and from three day tumoursphere culture, and subjected to Western blotting. Tumoursphere culture was restricted to three days as less proliferation of tumoursphere cells would have occurred and therefore TFUs would be a greater proportion of the total population. Assuming that each sphere contains just one TFU, we estimated that a two-fold enrichment of TFUs could be obtained by this method. This method has been used previously where it was shown that tumoursphere culture enriches for cells with a stem or progenitor-like phenotype (Korkaya *et al.* 2009). We observed no significant decrease in the total protein levels of cFLIP in TRAIL-sensitive cells (MDA-MB-231 bulk cells and MCF-7 tumourspheres) compared to TRAIL-resistant MCF-7 cells. In fact, cFLIP levels were elevated in TRAIL-sensitive cells, although this result was not statistically significant. There was also a decrease in the total cFLIP levels of the MDA-MB-231 tumourspheres compared to the total population, however this result was not statistically significant (Figure 3.4A-C) Taken together, these data suggest that total cFLIP levels do not correlate with TRAIL-susceptibility in cells of the MCF-7 and MDA-MB-231 lines.

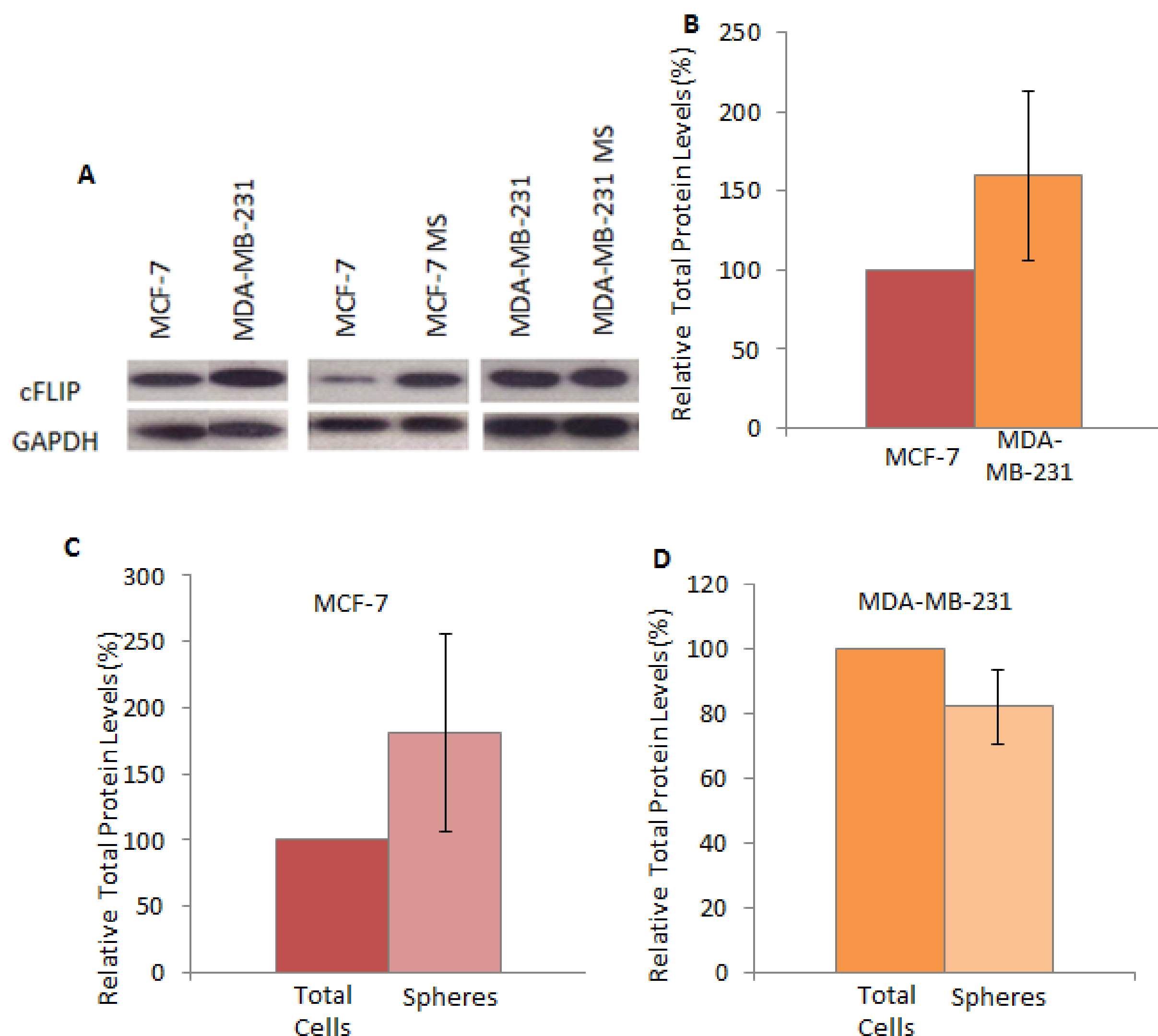


Figure 3.4: *Total cFLIP Levels do not Correlate with TRAIL Susceptibility*

A Western Blotting: Total proteins were extracted from MCF-7 and MDA-MB-231 lines and three day tumourspheres and subjected to Western blotting for cFLIP (loading control = α-tubulin)

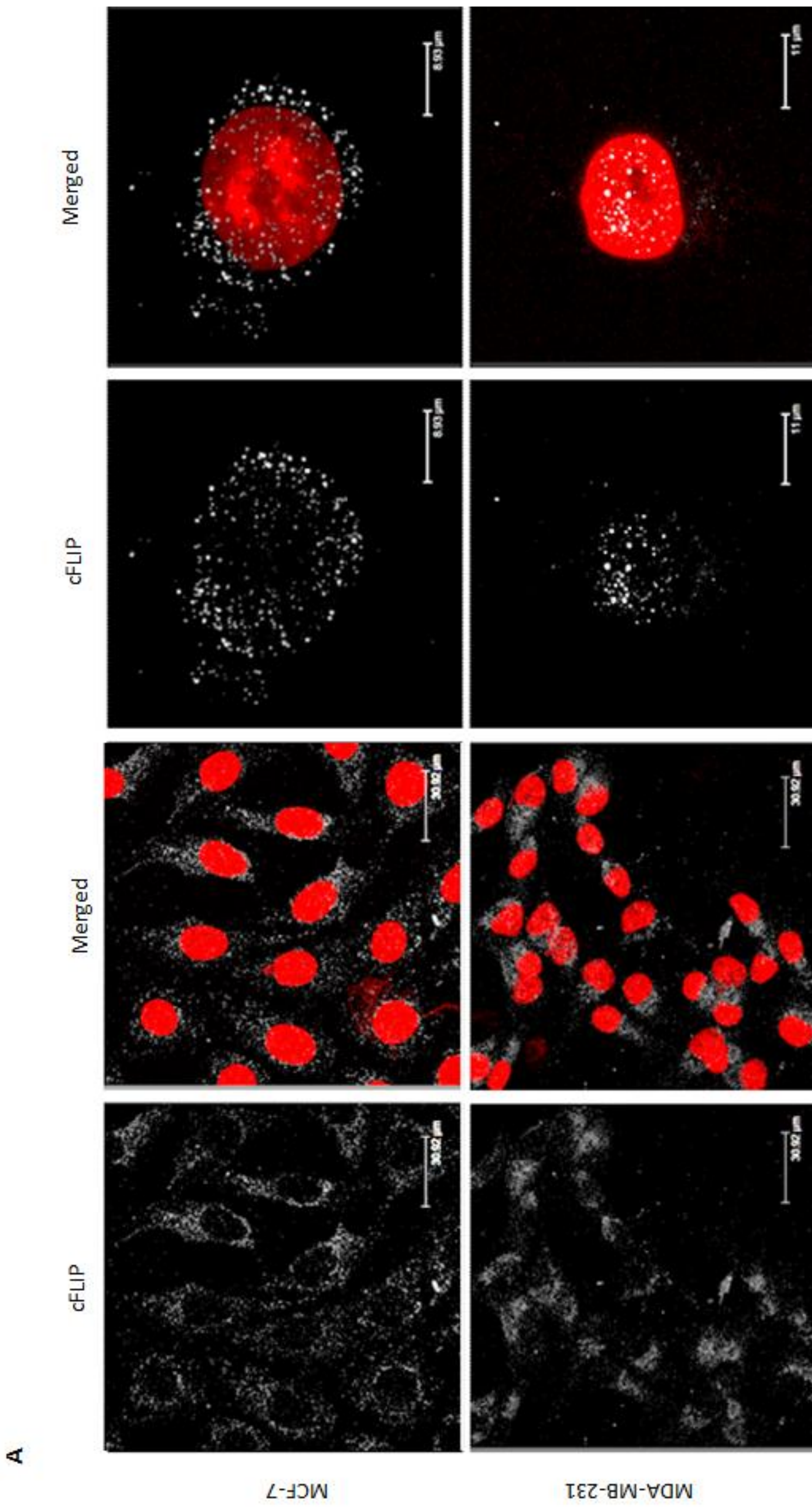
B Densitometry analysis of total cFLIP protein expression between the MCF-7 and MDA-MB-231 lines: cFLIP levels were increased in the MDA-MB-231 line compared to the total population although this result was not statistically significant ($p > 0.05$, t-test). **C Densitometry analysis** of total cFLIP protein expression between total population and tumourspheres of the MCF-7 line: cFLIP levels were increased in the tumourspheres compared to the total population although this result was not statistically significant ($p > 0.05$, t-test) **C Densitometry analysis** of total cFLIP protein expression between total population and tumourspheres of the MDA-MB-231 line: cFLIP levels were decreased in the tumourspheres compared to the total population although this result was not statistically significant ($p > 0.05$, t-test) All results are averages of three independent experiments.

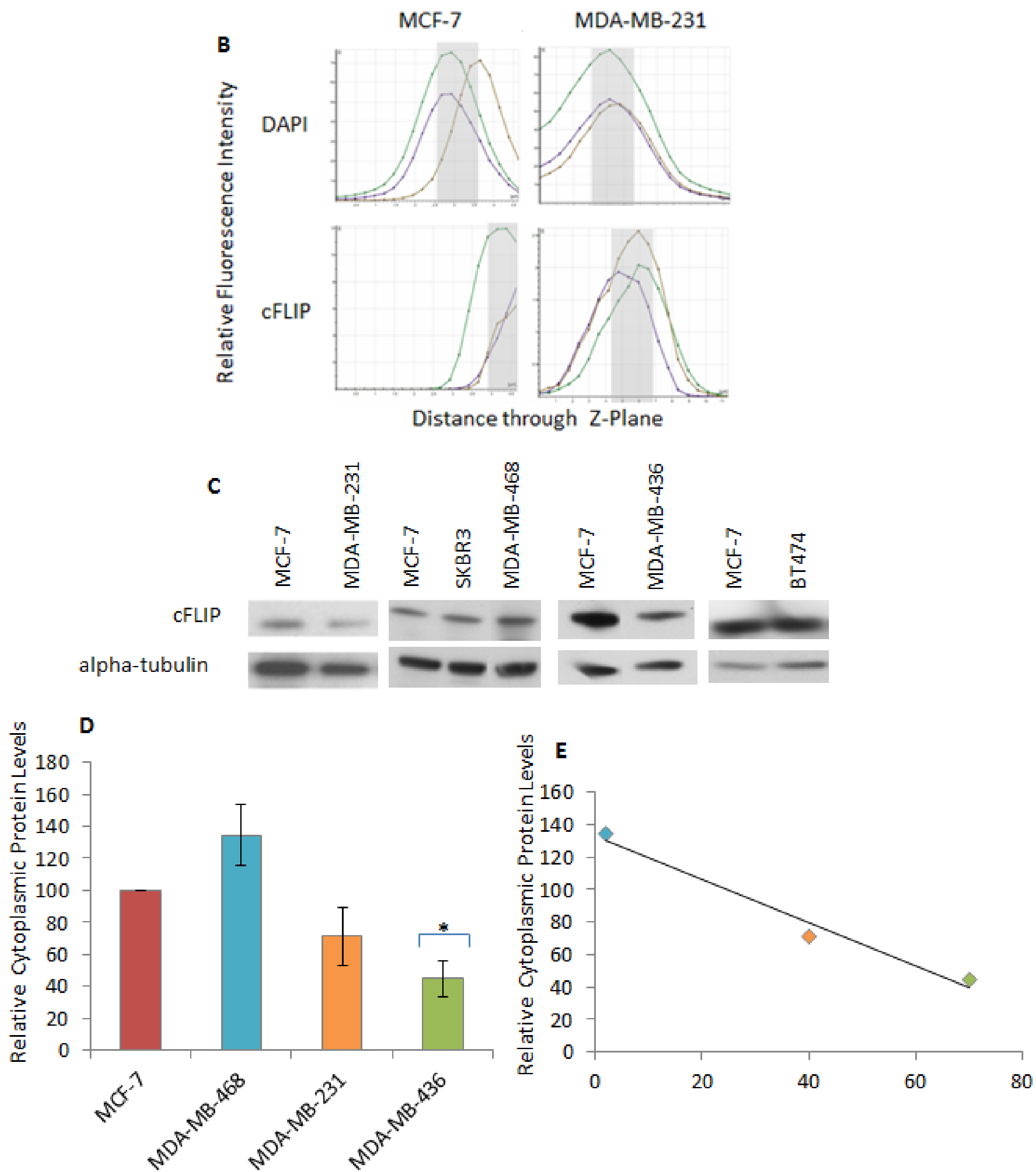
3.2.5 TRAIL susceptibility of total cell lines correlates with reduced levels of cytoplasmic cFLIP

In order to inhibit the extrinsic apoptosis pathway, cFLIP must be available in the cytoplasm to complex with DISC components and thus interfere with caspase 8 recruitment (Johnstone *et al.* 2008). Previous studies have found cFLIP to be present in the nucleus of lung carcinoma cell lines where it was shown to promote Wnt-target gene expression (Katayama *et al.* 2010). The association of mesenchymal-like cells and bCSCs with elevated Wnt signalling (Thiery and Sleeman 2006) may give us some indication as to the underlying mechanism of TRAIL sensitivity in these cells. These previous findings led us to propose the hypothesis that subcellular compartmentalisation of cFLIP, rather than total protein levels, might influence TRAIL-sensitivity at the cellular level.

To test this, cFLIP distribution was examined using immunocytofluorescence and confocal microscopy in two representative cell lines of differential TRAIL sensitivity. In the TRAIL-sensitive MDA-MB-231 line, cFLIP expression appeared to be peri-nuclear or nuclear, whereas in the TRAIL resistant MCF-7 line cFLIP expression was punctate and primarily cytoplasmic (Figure 3.5A). Analysis of the distribution of staining through the z-plane also revealed no overlap in staining between DAPI and cFLIP in MCF-7 cells whereas an overlap was apparent in MDA-MB-231 cells, indicating the presence of nuclear cFLIP in this line (Figure 3.5B). Cytoplasmic protein fractions were also extracted from the TRAIL-resistant MCF-7 and MDA-MB-468 lines, and the TRAIL-sensitive MDA-MB-231 and MDA-MB-436 breast cancer cell lines. The levels of cytoplasmic cFLIP were determined by Western blotting. For the purposes of densitometry, the level of cytoplasmic cFLIP in each line was normalised to that of the TRAIL-resistant MCF-7 line. Cytoplasmic cFLIP was reduced in both the MDA-MB-231 and MDA-MB-436 TRAIL-sensitive cell lines with a direct correlation between relative cFLIP levels and TRAIL susceptibility observed, although not statistically significant. As the correlation was strong ($r = 0.98$), we believe that an increase in sample numbers is likely to produce a statistically significant result (Figures 3.5C-E). Preliminary data also suggest that as expected, there is no difference in cytoplasmic cFLIP levels between the TRAIL-resistant MCF-7, SKBR3 and BT474 lines (Figure 3.5C).

These data show that the cytoplasmic levels of cFLIP correlate with the TRAIL-susceptibility of breast cancer cell lines.


A
cFLIP
Merged
cFLIP
Merged
MCF-7
MDA-MB-231



<u>Figure 3.5:</u> *Cytoplasmic cFLIP Levels Correlate with TRAIL Susceptibility*

A IF: MCF-7 and MDA-MB-231 cells were immuno-stained for cFLIP and analysed by confocal microscopy (cFLIP = grey, DAPI = red); cFLIP staining is cytoplasmic in the MCF-7 line but peri-nuclear or nuclear in the MDA-MB-231 line **B** Distribution of staining through the Z-plane for both DAPI and FLIP was analysed using Leica confocal software; cFLIP and DAPI staining overlap in the MDA-MB-231 line but do not in the MCF-7 line. **C Western Blotting:** Cytoplasmic proteins were extracted from total cell populations and subjected to Western blotting for cFLIP **D Densitometry:** Analysis of cytoplasmic cFLIP levels between cell lines; cytoplasmic cFLIP is reduced in the TRAIL-sensitive MDA-MB-231 line and reduced significantly in the MDA-MB-436 cell line compared to the MCF-7 line (231 $p > 0.05$, 436; $^*p = 0.04$, t-test) **E Correlation:** there is a direct correlation between relative cytoplasmic cFLIP levels and relative TRAIL-susceptibility, although not statistically significant ($r = -0.98 > -0.99$, Pearson's correlation co-efficient). All results are averages of three independent experiments.

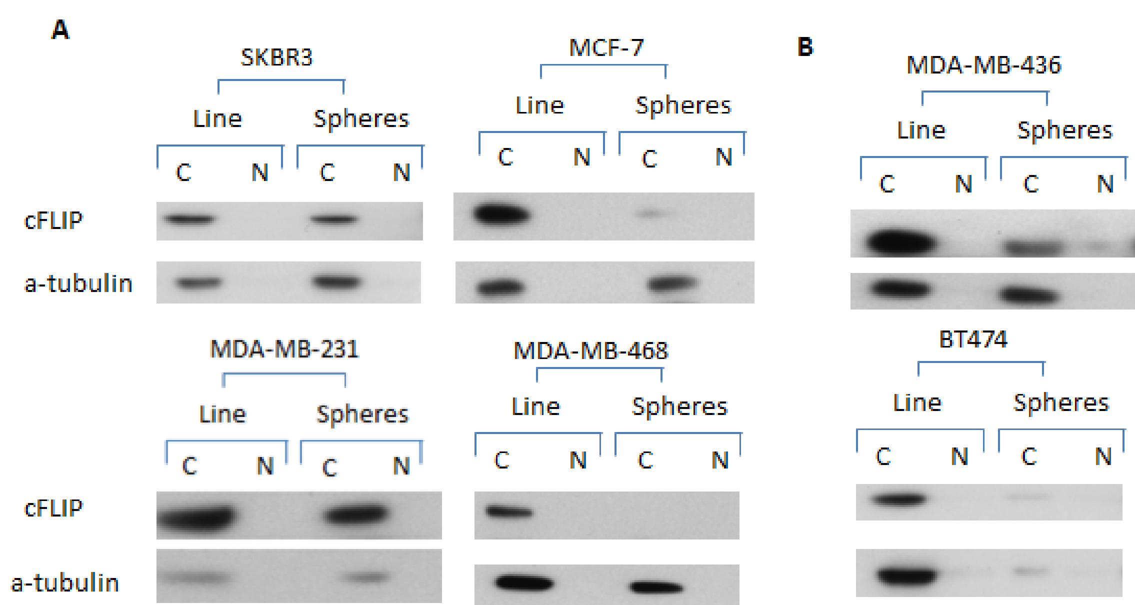
3.2.6 TRAIL susceptibility of TFUs correlates with reduced levels of cytoplasmic cFLIP in primary tumourspheres

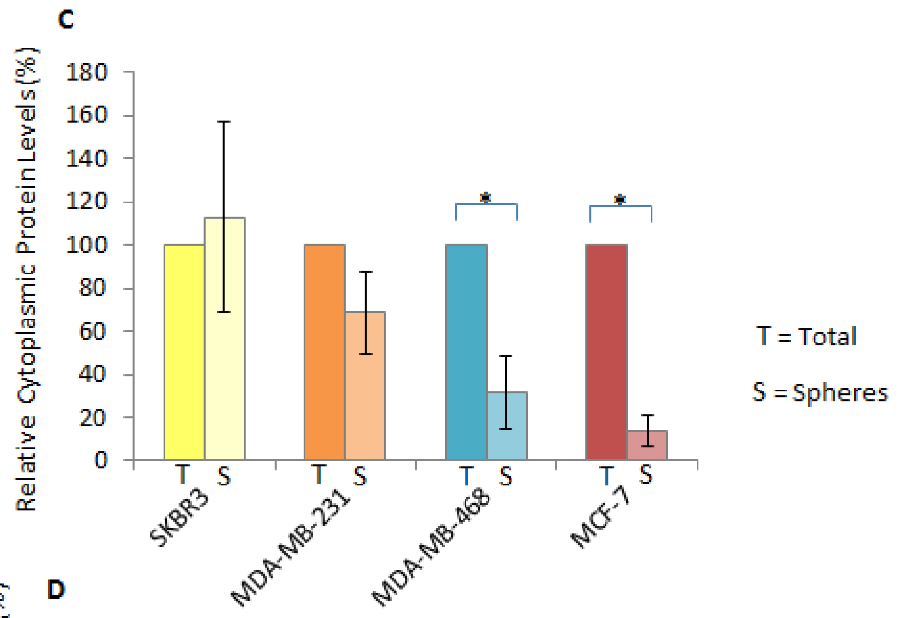
We have observed a decrease in the levels of cytoplasmic cFLIP in TRAIL-sensitive cell lines. To investigate cFLIP localisation in tumourspheres, nuclear and cytoplasmic protein fractions were extracted from both the total population and three-day tumoursphere culture of the SKBR3, MCF-7, MDA-MB-468 and MDA-MB-231 lines. The levels of cytoplasmic cFLIP were determined by Western blotting. For the purposes of densitometry and statistical analysis the level of cytoplasmic cFLIP in tumourspheres was normalised to that of the total population. Cytoplasmic cFLIP was reduced in TRAIL-sensitive tumourspheres (MCF-7, MDA-MB-231 and MDA-MB-468) whereas there was no difference in the cytoplasmic cFLIP levels between the total and tumoursphere populations of the TRAIL-resistant SKBR3 line (Figure 3.6A-B). A statistically significant direct correlation between relative cFLIP levels and relative TRAIL susceptibility was observed (Figure 3.6C). Preliminary data suggest that as expected, cytoplasmic cFLIP is also reduced in the tumourspheres of the MDA-MB-436 line, and that there is no difference in the cytoplasmic cFLIP levels between total cells and tumourspheres of the BT474 line (Figure 3.6A).

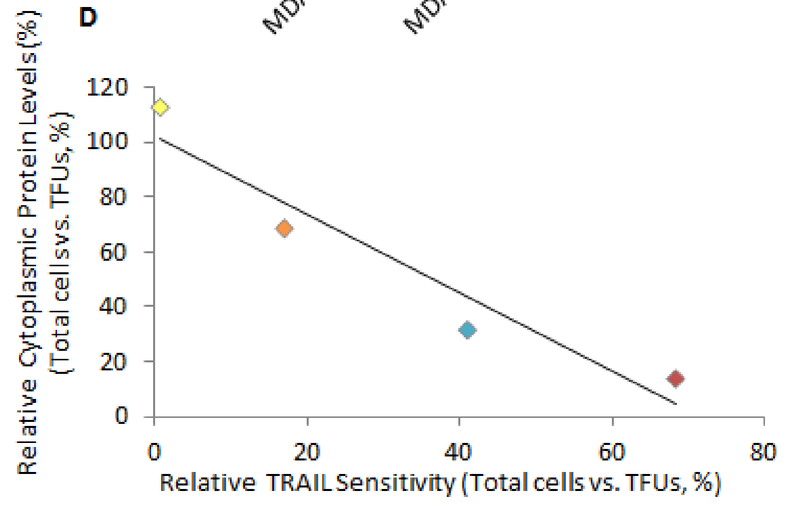
Unfortunately, technical limitations prevented the identification of nuclear proteins by Western Blotting: Even large-scale three-day tumoursphere culture does not produce sufficient cell numbers for the nuclear protein fraction to be detectable. In an attempt to visualise nuclear cFLIP, cells were analysed by immunofluorescence: MCF-7 cells were seeded into suspension conditions to enrich for anoikis-resistant cells, in the presence or absence of TRAIL for 24 h. Small tumourspheres formed in both conditions (fewer tumourspheres formed in the TRAIL-treated conditions) which were disaggregated, seeded onto glass coverslips, allowed to adhere overnight, then immuno-stained for cFLIP. In contrast to the total cell population which exhibited cytoplasmic cFLIP (Figure 3.5A), anoikis-resistant cells exhibited cells with nuclear c-FLIP and an apparent decrease in cytoplasmic cFLIP (Figure 3.6D). As expected, treatment with TRAIL reduced tumoursphere number by approximately fifty percent as shown previously (Figure 3.1B). The remaining TRAIL-resistant cells appeared to have elevated cytoplasmic cFLIP (Figure 3.6D).

Analysis of the distribution of staining through the z-plane also revealed an overlap between DAPI and cFLIP in anoikis-resistant MCF-7 cells whereas no overlap was apparent in TRAIL-treated MCF-7 anoikis-resistant cells (Figure 3.5B). The overlap in distribution suggests that anoikis-resistant cells have a greater proportion of their total cFLIP levels present in the nucleus, than TRAIL-resistant cells, but this needs to be confirmed by quantification.

Taken together, these data are consistent with the hypothesis that cytoplasmic cFLIP is reduced in TRAIL-sensitive cells.


A
SKBR3
Line
Spheres
C
N
C
N
cFLIP
a-tubulin
MCF-7
B
MDA-MB-436
MDA-MB-231
MDA-MB-468
BT474


C
Relative Cytoplasmic Protein Levels (%)
180
160
140
120
100
80
60
40
20
0
T S
SKBR3
MDA-MB-231
MDA-MB-468
MCF-7
*
T = Total
S = Spheres


D
Relative Cytoplasmic Protein Levels (%)
(Total cells vs. TFUs, %)
120
100
80
60
40
20
0
0
20
40
60
80
Relative TRAIL Sensitivity (Total cells vs. TFUs, %)

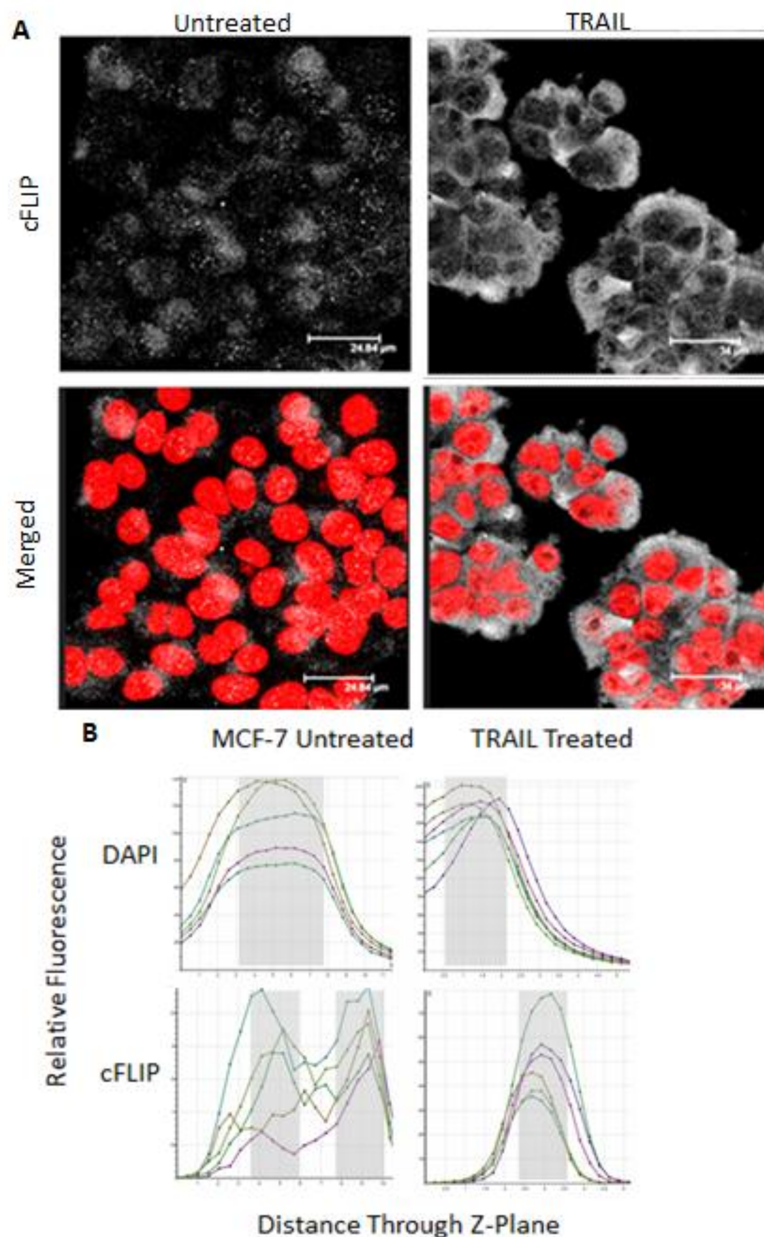


Figure 3.6: *TRAIL-sensitive TFUs have reduced cytoplasmic cFLIP* **A Western Blotting:** Nuclear and cytoplasmic proteins were extracted from cells and three-day tumourspheres and subjected to Western blotting for cFLIP: Cytoplasmic cFLIP was reduced in tumourspheres of the MCF-7, MDA-MB-231, MDA-MB-468 and MDA-MB-436 lines, whereas no difference was observed in the SKBR3 and BT474 cell lines **B Densitometry:** Analysis of cytoplasmic cFLIP levels between cell lines and tumourspheres; cytoplasmic cFLIP is reduced in the TRAIL-sensitive MDA-MB-231, MDA-MB-468 and MCF-7 tumourspheres compared to the total populations (231; $p > 0.05$, 468; $^*p = 0.03$, MCF-7; $^*p = 0.005$, t-test) **C Correlation :** there is a direct and significant correlation between relative cytoplasmic cFLIP levels and relative TRAIL-susceptibility of tumourspheres ($r = -0.96 < -0.95$, Pearson). **D IF:** MCF-7 cells were stained for cFLIP (grey) and DAPI (red); nuclear cFLIP is apparent in anoikis-resistant cells but cytoplasmic cFLIP is elevated in those anoikis-resistant cells surviving TRAIL-treatment **B** Distribution of staining through the Z-plane for both DAPI and FLIP was analysed using Leica confocal software; overlap is apparent in untreated populations but absent in TRAIL-resistant cells. All results are averages of three independent experiments.

3.2.7 Sequestering c-FLIP in the nucleus Partially Sensitises Tumoursphere-Forming Cells to TRAIL

To investigate the functional consequences of the nuclear accumulation of c-FLIP on TRAIL-sensitivity, the CRM1 nuclear transporter inhibitor Leptomycin B (LMB1) was used to sequester cFLIP in the nucleus (Katayama *et al.* 2010). Twenty-four hour treatment with 0.1 ng/ml LMB1 elevated nuclear cFLIP and reduced cytoplasmic cFLIP in the MCF-7 cell line (Figure 3.7A and B). In order to determine the effect of LMB1 on TRAIL-sensitivity, cells were pre-treated for 24 h with 0.1 ng/ml LMB1 then treated with TRAIL for 18 h. Cell viability was assessed using the cell titre blue assay. Pre-treatment with LMB1 was not able to sensitise significantly the MCF-7 cells to TRAIL (Figure 3.7C). However, we did observe a trend towards a reduction in cell viability following treatment with both LMB1 and TRAIL. As LMB1 treatment alone appeared to have some deleterious effects on cell viability, this reduction may be an additive not synergistic effect. This may be ascertained by directly measuring the number of dead cells using the Live/Dead flow-cytometry assay (Invitrogen) as opposed to total cell viability. Therefore further investigation is required before a definitive conclusion can be made.

The effect of LMB1 on the TRAIL-susceptibility of tumourspheres was also determined. The TRAIL-resistant SKRB3 and BT474 lines were pre-treated for twenty-four hours with 0.1 ng/ml LMB1 then subjected to the tumoursphere assay in the presence or absence of 20 ng/ml TRAIL. Pre-treatment with 0.1 ng/ml LMB1 sensitised significantly both the SKBR3 primary and secondary tumoursphere-forming cells to TRAIL. LMB1 also sensitised BT474 primary TFUs to TRAIL, however the effect of LMB1 on TRAIL susceptibility of secondary tumourspheres in the BT474 line was not statistically significant (Figures 3.8B-D). An analysis of tumoursphere size is also required in order to determine whether LMB1 is sensitising transit-amplifying progenitors.

These data suggest that re-localisation of cFLIP to the nucleus can sensitise tumoursphere-forming populations to TRAIL, and that cFLIP localisation may be more important in the determination of TRAIL susceptibility in tumoursphere-forming cells than in total cell populations. However, LMB1 is not specific in its action, and therefore without a specific perturbation of cFLIP localisation, this experiment alone cannot be used to conclude that re-localisation of cFLIP sensitises to TRAIL.

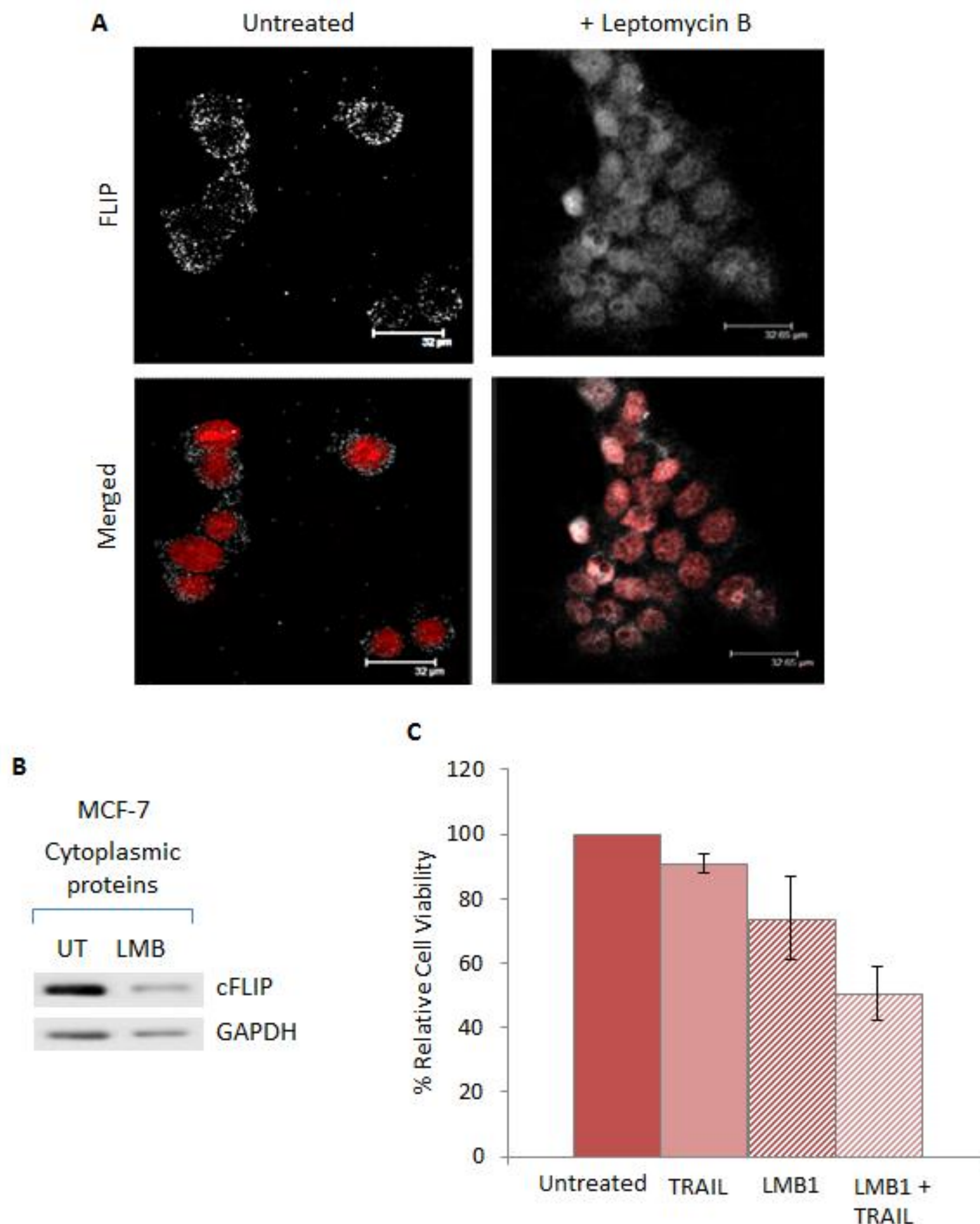


Figure 3.7: *Leptomycin B does not sensitise MCF-7 cells to TRAIL* **A IF:** Cells were seeded onto coverslips and allowed to adhere before treatment with 0.1ng/ml LMB1 for 24h. Cells were then fixed and immuno-stained for cFLIP and analysed by confocal microscopy (cFLIP = grey, DAPI = red): LMB1 re-localised cFLIP to the nucleus **B Western Blotting:** Cytoplasmic protein was extracted from cells treated with 0.1 ng/ml LMB1 for 24 h and subjected to Western blotting for cFLIP: LMB1 reduced cytoplasmic cFLIP levels. **C Cell Titre Blue Assay:** MCF-7 cells were seeded in adherent conditions for 24h then pre-treated with 0.1 ng/ml LMB1 for 24 before 18 h treatment with 20 ng/ml TRAIL: LMB1 had no significant effect on the sensitivity of cells to TRAIL ($p > 0.05$, t-test). All results are averages of three independent experiments. The cell titre blue assay was also performed with three internal technical replicates.

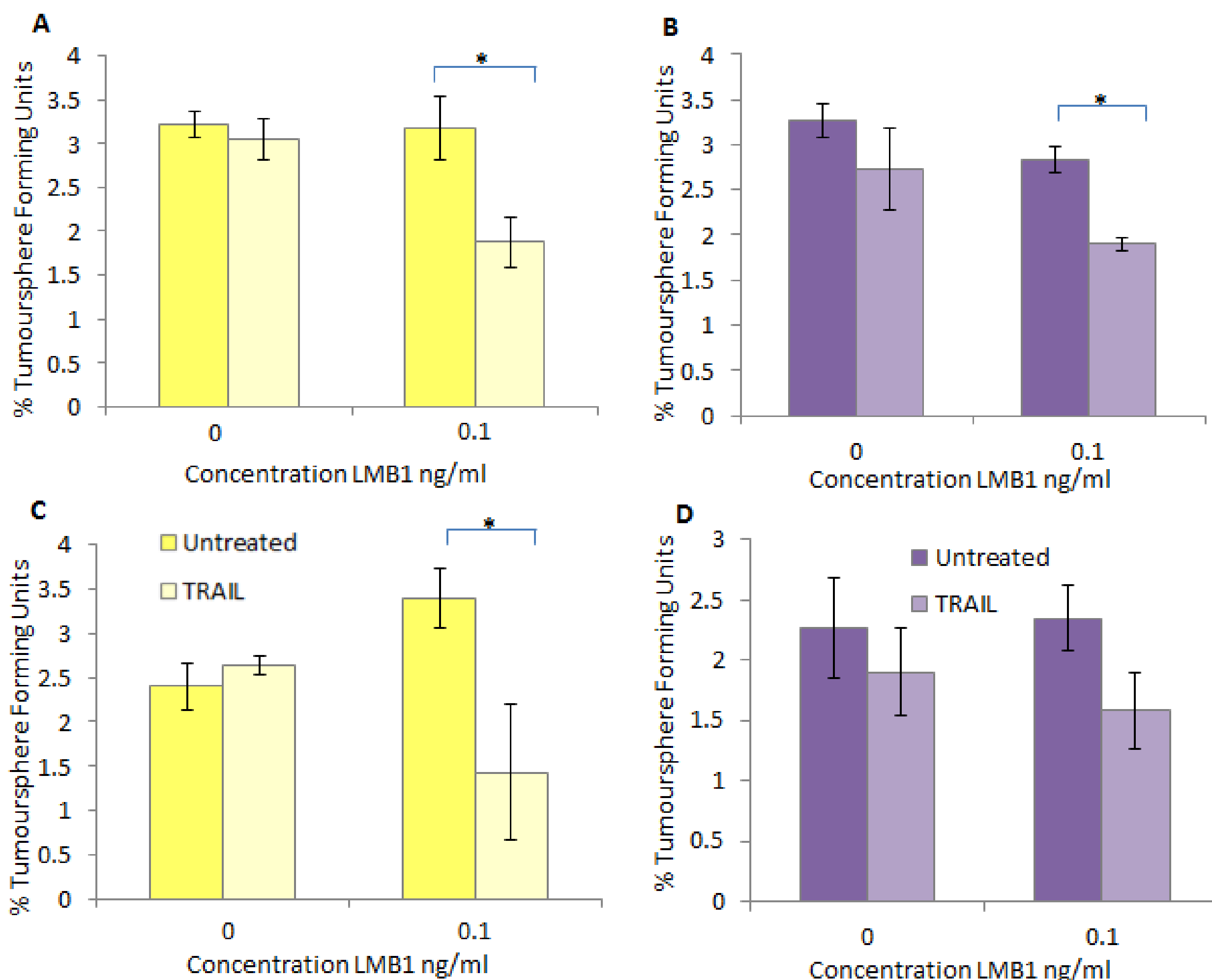


Figure 3.8: *Leptomycin B Sensitises Tumoursphere-Forming Cells to TRAIL*

A-D Tumoursphere Assay Cells were treated in adherent conditions with 0.1 ng/ml LMB1 for 24 h then seeded into 96-well non-adherent plates at a density of 5,000 cells/ml in the presence or absence of 20 ng/ml TRAIL. Secondary spheres were cultured in the absence of TRAIL: LMB1 significantly sensitised primary (**A**) and secondary (**C**) SKBR3 TFUs to TRAIL (Primary; *p = 0.01, Secondary; *p = 0.05, t-test). LMB1 also significantly sensitised primary (**C**) BT474 TFUs to TRAIL but had no significant effect on secondary TFUs (**D**) (Primary; *p = 0.025, Secondary; p > 0.05, t-test). All data are averages of three independent experiments each performed with three internal technical replicates.

3.2.8 Nuclear cFLIP cannot protect against TRAIL

Our results so far had revealed the presence of endogenous nuclear cFLIP (Figures 3.5 and 3.6). In order to inhibit the extrinsic apoptosis pathway, cFLIP must be available in the cytoplasm to prevent caspase binding. Therefore the role of nuclear cFLIP in this context is not clear. We hypothesised that, whereas cytoplasmic cFLIP should protect against TRAIL, nuclear cFLIP does not function as an inhibitor of TRAIL-mediated apoptosis. To determine whether nuclear or cytoplasmic cFLIP can prevent TRAIL-mediated cell death, cytoplasmic-localised and nuclear-localised cFLIP mutants were generated by mutating the nuclear export and localisation sequence of cFLIP respectively by site-directed mutagenesis according to Katayama *et al.* 2010. Cells expressing localisation mutant cFLIPs were generated by transfecting MDA-MB-231 cells or MCF-7 cells with either pcDNA3.1FLIPL (wildtype), pcDNA3.1FLIPNLSm (cytoplasmic localised) and pcDNA3.1FLIPNESm (nuclear localised) or pcDNA3.1Empty (mock), and maintained under antibiotic selection. Over-expression and localisation of cFLIP was confirmed in the MDA-MB-231 line using immunocytofluorescence (Figure 3.8A). Overexpression of wildtype FLIP resulted in an increase in staining intensity throughout the cells whereas overexpression of nuclear FLIP resulted in an increase in fluorescence in the nuclear region only (Figure 3.8A). The localisation of cytoplasmic mutant cFLIP has not yet been determined, nor has mutant cFLIP expression been evaluated in the MCF-7 cell line; this work is ongoing.

To determine TRAIL susceptibility of MDA-MB-231 lines expressing wild-type or mutant cFLIP, cells were treated with TRAIL in adherent conditions then analysed using the cell-titre blue assay. Cells expressing nuclear cFLIP were sensitive to TRAIL whereas TRAIL had no significant effect on those cells expressing wild-type cFLIP (Figure 3.8B). This suggests that over-expression of wild-type cFLIP can protect cells from TRAIL, whereas over-expression of nuclear cFLIP has no protective effect.

To determine whether nuclear cFLIP could protect TFUs from TRAIL, stable MCF-7 lines over-expressing wild-type or mutant cFLIP were subjected to the tumoursphere assay in the presence or absence of TRAIL. Preliminary data suggests that over-expression of wildtype or cytoplasmic cFLIP was able to rescue the reduction in tumoursphere-formation due to TRAIL. However, expression of nuclear FLIP had no significant protective effect (Figure 3.8C).

These data suggest that whereas cytoplasmic cFLIP protects MDA-MB-231 cells and MCF-7 tumoursphere-forming cells from TRAIL, nuclear-localised cFLIP is unable to inhibit TRAIL-mediated apoptosis.

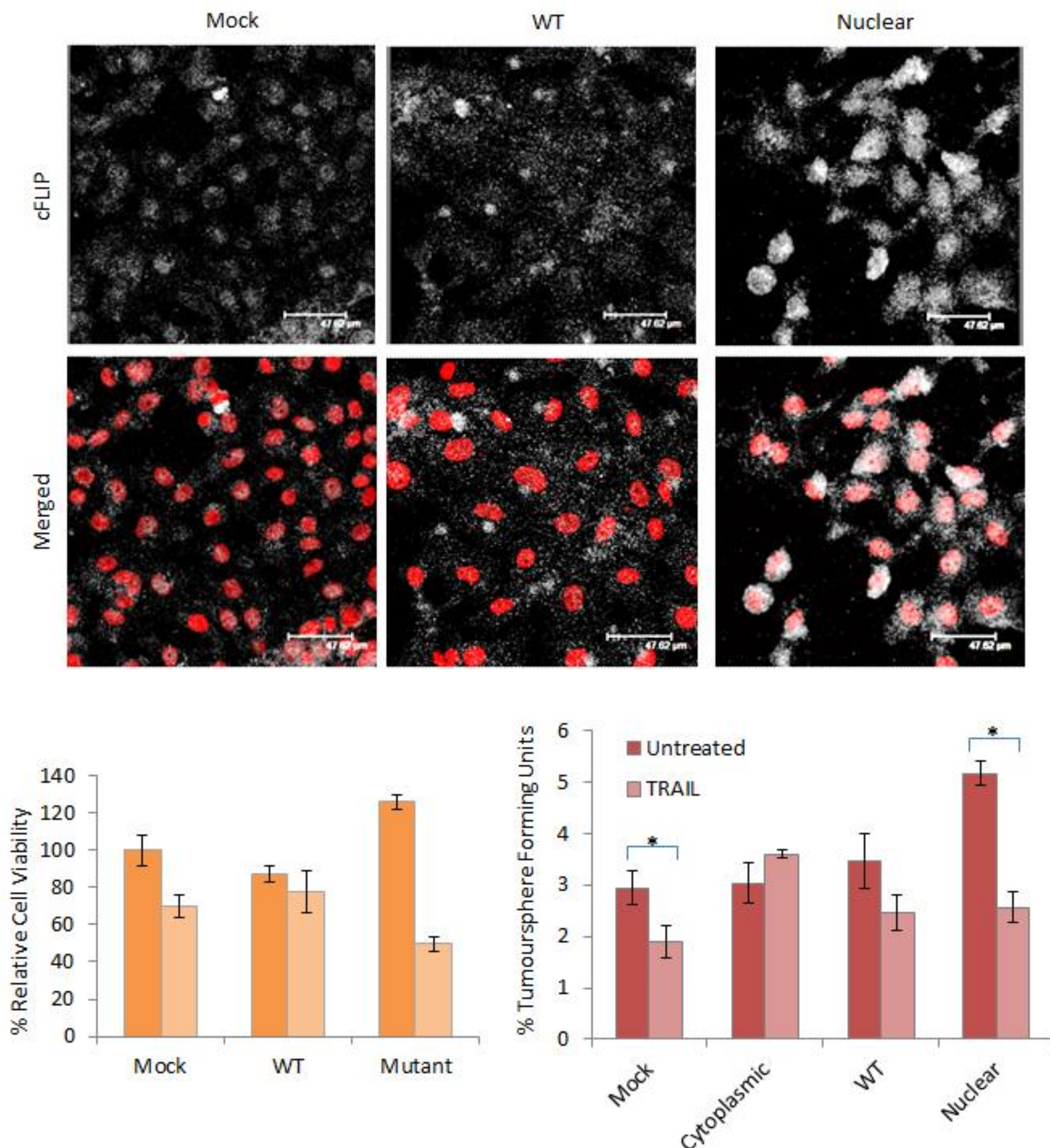


Figure 3.8: *Nuclear cFLIP does not protect against TRAIL-mediated cell death* **A IF:** MDA-MB-231 cells were transfected with either pcDNA3.1FLIPL (wild-type), pcDNA3.1FLIPNESm (Nuclear mutant) or pcDNA3.1 (Mock), and analysed by immunfluorescence (cFLIP = grey, DAPI = red): over-expression of wild-type cFLIP increased staining intensity throughout the cell whereas nuclear mutant cFLIP increased staining intensity predominantly in the nucleus **B Cell-Titre Blue Assay:** MDA-MB-231 cells transiently transfected with either pcDNA3.1FLIPL wild-type), pcDNA3.1FLIPNESm (Mutant) or pcDNA3.1 (Mock) were treated with 20ng/ml TRAIL for 18h and cell viability was assessed: TRAIL reduced significantly the viability of mock or nuclear mutant-transfected cells but had no significant effect on the cells over-expressing wild-type cFLIP (Mock *p = 0.05, Nuclear mutant; *p = 0.03, wild-type p > 0.05, t-test) **C Tumoursphere Assay:** MCF-7 cells stably transfected with either mock, wild-type cFLIP, or nuclear or cytoplasmic mutant cFLIP, were subjected to the tumoursphere assay in the presence or absence of 20ng/ml TRAIL: TRAIL reduced significantly the number of TFU in mock or nuclear mutant-transfected cells but had no significant effect on the cells over-expressing wild-type or cytoplasmic cFLIP (Mock; *p = 0.04 , Nuclear; *p = 0.001 , Wild-type or cytoplasmic; *p > 0.05, t-test). All results are representative of a single experiment performed with three internal technical replicates).

3.2.9 cFLIP does not co-localise with markers of early or late endosomes

Confocal microscopy of cFLIP in the (TRAIL-sensitive) MDA-MB-231 cell line revealed distinct staining patterns reminiscent of localisation to intracellular vesicles or organelles (Figure 3.5). It has been shown previously that in hepatocarcinoma cells, internalisation of the TRAIL and receptor complex (receptosome) is required for propagation of the apoptotic signal (Akazawa *et al.* 2009). A recent study has also shown that prevention of endosome acidification and fusion with lysosomes by bafilomycin or concanamycin A, was sufficient to attenuate a TRAIL-induced apoptotic signal in colon cancer cell lines. Furthermore, this induced TRAIL-resistance could be overcome by the suppression of cFLIP by shRNA, suggesting that either cFLIP may exert its anti-apoptotic effects at least in part by inhibiting endosome fusion and acidification (Horova *et al.* 2013).

To investigate the localisation of cFLIP in more detail, MDA-MB-231 cells were immuno-stained for cFLIP in combination with the early endosomal marker EEA1 or lysosomal marker LAMP1. cFLIP staining did not overlap with that of either of these markers, indicating that cFLIP does not localise to the endosomal pathway in MDA-MB-231 cells (Figure 3.9A and B). However, the possible co-localisation of cFLIP with endosomal markers has not yet been evaluated following treatment with TRAIL.

In parallel, we assessed the ability of an endosomal inhibitor concanamycin A to inhibit TRAIL-mediated apoptosis in the MDA-MB-231 line, compared to the pan-caspase inhibitor Z-Vad-Fk. Concanamycin A alone reduced cell viability by approximately forty percent (Figure 3.9C). The viability of MDA-MB-231 cells treated with concanamycin A was reduced significantly following TRAIL-treatment, whereas the viability of cells treated with the caspase-inhibitor Z-Vad-Fmk was not reduced significantly by TRAIL (Figure 3.9C). Z-Vad-Fmk protected MDA-MB-231 cells from TRAIL-mediated apoptosis, whereas CcmA had no significant protective effect (Figure 3.9 D). Taken together these data suggest that TRAIL does not induce endosome-mediated cell death in the MDA-MB-231 line.

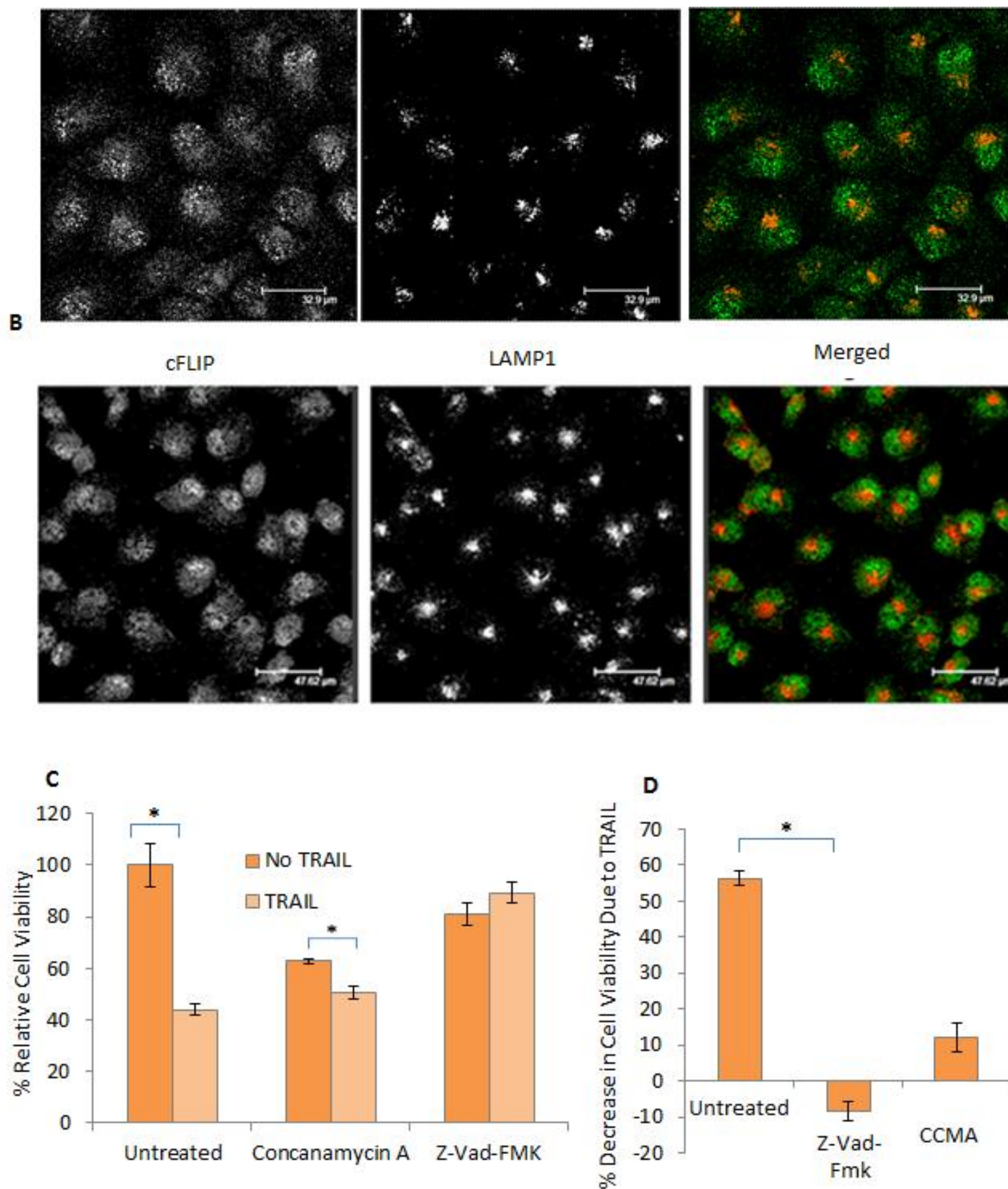


Figure 3.9: *Investigating the Subcellular Localisation of cFLIP* **A-B IF:** Cells from the MDA-MB-231 were immuno-stained for cFLIP, and **A** EEA1 or **B** LAMP1 and analysed by confocal microscopy (cFLIP = grey/green, EEA1/LAMP1= grey/red, DAPI = blue): No overlap was observed between cFLIP and either marker **C Cell Titre Blue Assay:** MDA-MB-231 cells were treated with Concanamycin A (CcmA) or the caspase inhibitor Z-Vad-Fmk for 24h, then treated with 20ng/ml TRAIL for 18h: Untreated or CcmA-treated cells were significantly sensitive to TRAIL (Untreated, $*p = 0.05$, CcmA; $*p = 0.01$, t-test) whereas Z-Vad-Fmk-treated cells were not significantly sensitive to TRAIL ($p > 0.5$, t-test). **D** Z-Vad-fmk protected MDA-MB-231 cells from TRAIL-mediated apoptosis, whereas CcmA had no significant protective effect (Z-Vad-fmk; $*p = 0.04$, CcmA; $p > 0.05$, t-test). All results are averages of three independent experiments. The cell titre blue assays were also performed with three internal technical replicates.

3.3 Discussion

In this chapter we used a range of functional assays to assess the susceptibility of bCSCs from a panel of six breast cancer cell lines to the cytotoxic agent TRAIL. Here we show that TRAIL is able to target the bCSCs of four out of six breast cancer cell lines. TRAIL alone has only been evaluated previously in whole populations of breast cancer cell lines, where it was shown to target only mesenchymal-like lines, but its effect on the bCSC-like populations of these lines was not determined (Rahman *et al.* 2009). Not only have we shown that TRAIL is able to target the bCSC-like populations of mesenchymal-like cell lines, but also that TRAIL can target the bCSC-like populations of the epithelial-like MDA-MB-468 and MCF-7 lines. These data are important because they widen the range of breast cancer subtypes for which TRAIL can be considered a potential therapeutic. Although the susceptibility of bCSCs to TRAIL has not been established definitively in any previous study, it has been shown that administration of activating antibodies to TRAIL receptors was able to reduce the metastasis of the mesenchymal-like MDA-MB-231 line in mice (Malin *et al.* 2011). The ability to form metastases is a property associated with bCSCs, and therefore our data are in accordance with this study. However, as a result of our findings, the relationship between TRAIL sensitivity and breast tumour subtype is no longer clear.

We hypothesised initially that TRAIL-sensitivity of bCSCs was due to a decrease in the levels of the endogenous TRAIL-pathway inhibitor cFLIP. However, we found that cFLIP levels were increased in both TRAIL-sensitive MCF-7 tumourspheres and MDA-MB-231 cells. These findings do correlate with other studies which have shown cFLIP to be over-expressed in more aggressive breast cancers (Fenglin *et al.* 2014). Instead of total cFLIP levels, we show here that TRAIL sensitivity of bCSCs is due to reduced cytoplasmic localisation of cFLIP. We have shown a clear correlation between TRAIL susceptibility and cytoplasmic cFLIP levels in all cell lines and tumourspheres tested so far, with a statistically significant direct correlation between relative TRAIL-susceptibility and relative cytoplasmic cFLIP levels of tumourspheres. The finding that cytoplasmic cFLIP is reduced in tumourspheres may explain why these populations are more sensitive than the bulk population not only to TRAIL alone, but also to cFLIP inhibition in combination with TRAIL-treatment (Piggott *et al.* 2011). However, our data are limited by the use of the tumoursphere assay to enrich for bCSC-like cells and may be improved by comparing the cFLIP levels and localisation in total populations with that of cells expressing the bCSC marker profiles of $CD44^{+}CD24^{-}$ or $ALDH^{+}$ by flow cytometry.

These results suggested that re-localisation of cFLIP to the nucleus could sensitise resistant cells to TRAIL. We found that although the nuclear exporter inhibitor Leptomycin B (LMB1) was able to sequester cFLIP in the nucleus, this did not sensitise significantly the MCF-7 cell line to TRAIL. However, LMB1 did sensitise significantly the tumoursphere-forming cells of the SKBR3 and BT474 lines to TRAIL. This suggests that cFLIP localisation may be more important at determining the TRAIL-susceptibility of tumourspheres than the total population. However LMB1 acts in a non-specific manner and we cannot rule out the possibility that re-localisation of proteins other than cFLIP (such as other DISC components) may effect the susceptibility of a cell to TRAIL. To overcome this problem and to investigate further the effect of cFLIP localisation, localisation mutants of cFLIP were generated which

localised to the cytoplasm and nucleus. Preliminary data suggests that over-expression of wild-type cFLIP protects MDA-MB-231 cells from TRAIL whereas over-expression of nuclear cFLIP has no significant protective effect. Furthermore, over-expression of wild-type cFLIP or cytoplasmic cFLIP was able to protect MCF-7 tumoursphere-forming cells from TRAIL, whereas over-expression of nuclear cFLIP had no significant protective effect. These results suggest that localisation of cFLIP can determine the susceptibility of cells and TFUs to TRAIL: while cytoplasmic, cFLIP can inhibit TRAIL signalling at the DISC but nuclear cFLIP permits TRAIL-induced apoptosis. The role of nuclear cFLIP in breast cancer cell lines is at present unclear. Nuclear cFLIP is known to play a role in promoting the Wnt signalling pathway in lung carcinoma cell lines (Katayama *et al.* 2010). As our data show that nuclear cFLIP cannot protect against TRAIL-mediated apoptosis, the potential of cFLIP, including nuclear cFLIP, to promote Wnt-signalling in breast cancer cell lines will be investigated in Chapter 5.

In addition, the pattern of cFLIP staining in the MDA-MB-231 line was reminiscent of localisation to intracellular organelles such as endosomes. However, immuno-staining of cFLIP in conjunction with markers of early and late endosomes revealed no overlap in distribution. The subcellular localisation of cFLIP requires further investigation, initially looking at the potential of cFLIP to localise to the Golgi network in the MDA-MB-231 line (see Figure 3.5 for reference).

Our data are evidence of phenotypic heterogeneity in terms of TRAIL susceptibility, existing not just between and within cell lines, but also within bCSC populations: TRAIL alone was not able to eradicate all tumoursphere-forming, colony-forming or tumour-initiating cells in any case. Recently it has been shown in breast and other cancers, that there exists more than one population of CSCs (Liu *et al.* 2013, Biddle *et al.* 2011). EMT and MET-like subpopulations of bCSCs have been described in breast cancer cell lines and primary cells, which exhibit differential phenotypic properties and stem-like characteristics (Liu *et al.* 2014). We hypothesise that due to its established specificity for mesenchymal-like cells (Rahman *et al.* 2009), TRAIL may target the EMT-like subpopulation of bCSCs. In addition, the SKBR3 cell line has been shown to have 100% ALDH-positive cells (Charraffe-Jauffret *et al.* 2010). This may suggest that SKBR3 cells have little or no EMT-like bCSC component, and may therefore explain why TFUs of this cell line are resistant to TRAIL. Although the colony-forming assay showed no significant bias of TRAIL toward targeting epithelial-like or mesenchymal-like colonies, the morphology of the colony does not necessarily reflect the nature of its cell of origin. Therefore this hypothesis has not been tested and will be the basis of future studies looking at the effect of TRAIL on bCSC subpopulations expressing the bCSC marker profiles of $CD44^{+}CD24^{-}$ (EMT-like) and $ALDH^{+}$ (MET-like) by flow cytometry. Nevertheless, the phenotypic heterogeneity of bCSCs is evident, and the clinical implications are that a combination therapy is required to eradicate the whole bCSC population. This is in accordance with our previous findings that the combined therapeutic strategy of inhibition of cFLIP in combination with administration of TRAIL is able to target the bCSC population more effectively than TRAIL alone (Piggott *et al.* 2011).

In conclusion, we have shown through cell-based and mechanistic studies that a safe, non-toxic and available agent, TRAIL, is able to target bCSCs and therefore is a therapeutic with a significant potential for efficacy in the treatment of breast cancer patients. TRAIL alone, in contrast to all other

targeted breast cancer therapies, appears to be most effective for the treatment of more aggressive clinical subtypes and subpopulations for which few treatment options are currently available. The clinical relevance of our findings could be improved by an analysis of TRAIL-sensitivity and cFLIP distribution in primary biopsy and surgical samples. Our data also highlight the importance of identifying novel targeted small molecule inhibitors of cFLIP to be used in conjunction with TRAIL as a future combination treatment for breast cancer.

Chapter 4: Investigating a Paracrine System of TRAIL Sensitisation

Chapter 4: Investigating a Paracrine System of TRAIL Sensitisation

4.1 Introduction

In the process of investigating the TRAIL-susceptibility of breast cancer cell lines, co-culture experiments were performed. Unexpectedly, co-culture of labelled TRAIL-sensitive MDA-MB-231 cells with TRAIL-resistant MCF-7s induced TRAIL sensitivity in the MCF-7 line (unpublished data). In the absence of specific cFLIP inhibitors, there is a need for the identification of alternative strategies for sensitising cells to TRAIL. Therefore we set out to investigate the mechanism by which MDA-MB-231 cells sensitise to TRAIL with the aim of identifying a possible therapeutic strategy. We hypothesised that a soluble factor produced by MDA-MB-231 cells is able to sensitise MCF-7 cells to TRAIL.

4.2 Results

4.2.1 MDA-MB-231-conditioned medium sensitises MCF-7 cells to TRAIL

To determine whether the sensitisation of MCF-7s to TRAIL by MDA-MB-231 cells was due to a soluble factor(s), MCF-7 cells were cultured for 24 h in the presence or absence of filtered conditioned medium taken from MDA-MB-231 cells, and then treated with 20 ng/ml TRAIL for 18 h. Cell viability was then assessed using the cell-titre blue assay. Pre-treatment of MCF-7 cells with MDA-MB-231-conditioned medium enhanced significantly the sensitivity of MCF-7 cells to TRAIL (Figure 4.1A). To determine whether this effect could occur in other cell lines, the ZR-75-1 and SUM159 lines were also used as models of TRAIL-resistance and sensitivity respectively (Figure 5.1A). MDA-MB-231 - conditioned media had no significant effect on the TRAIL-susceptibility of the TRAIL-resistant ZR-75-1 line but showed a slight trend consistent with an increased sensitivity to TRAIL. Conditioned media from the SUM159 line had no significant effects on either the MCF-7 or ZR-75-1 lines, yet in both cases there was a trend towards increased sensitivity to TRAIL (Figure 4.1A). These data suggest that sensitisation of MCF-7s to TRAIL by MDA-MB-231 cells is due to a soluble factor(s). If this is true, then this also suggests that more of a soluble factor(s) capable of sensitising to TRAIL are produced in the MDA-MB-231 line than in the SUM159 line, and that the MCF-7 line is more responsive to these factors than the ZR-75-1 line.

Furthermore we speculated that the increased resistance of ZR-75-1 cells to conditioned medium could be due to the ZR-75-1 line producing a protective factor to counteract sensitisation and therefore protect cells from TRAIL; a factor that was not expressed in MCF-7 cells. To determine whether a soluble factor(s) from the ZR-75-1 line was able to protect TRAIL-sensitive cells from TRAIL, MDA-MB-231 and SUM159 cells were cultured for 24 h in the presence or absence of filtered conditioned medium taken from ZR-75-1 or MCF-7 cells before treatment with TRAIL. Conditioned medium from either the MCF-7s or ZR-75-1s was not able to protect MDA-MB-231 and SUM159 cells from TRAIL (Figure 4.1B) suggesting that ZR-75-1 cells do not produce a soluble protective factor. However we have not yet determined whether protective factors are produced by ZR-75-1 cells in response to MDA-MB-

231-conditioned medium. This could be tested by first incubating ZR-75-1 cells with MDA-MB-231-conditioned medium before the generation of ZR-75-1-conditioned medium.

In all previous experiments conditioned medium was generated by culturing 90-100% confluent cells in 3 ml culture medium in a surface area of 25 cm^2 overnight (Figure 4.1A-C). Conditioned medium generated by culture of MDA-MB-231 or SUM159 cells with 6 ml medium overnight did not produce a sensitisation to TRAIL in the MCF-7s (Figure 4.1D). This suggests that TRAIL-sensitisation (Figure 1A) is dependent on the concentration of a soluble factor in the culture medium of TRAIL-sensitive cells. However, further dose-response style experiments are required to confirm this.

4.2.2 Conditioned medium from MDA-MB-231 cells sensitises tumourspheres to TRAIL

We have shown that conditioned medium from the MDA-MB-231 line sensitises a TRAIL resistant cell line to TRAIL. We next wished to determine whether MDA-MB-231-conditioned medium was also able to induce sensitivity to TRAIL in TRAIL-resistant tumoursphere-forming cells. MCF7 cells were not used here as their tumoursphere-forming cells are partially sensitive to TRAIL, however tumoursphere-forming cells from SKBR3 and BT474 cells are completely resistant to TRAIL (Figure 3.1B and C). SKBR3 and BT474 cells were cultured for 24 h in the presence or absence of filtered MDA-MB-231-conditioned medium and then subjected to the tumoursphere assay in the presence or absence of 20 ng/ml TRAIL. MDA-MB-231-conditioned medium increased the sensitivity of both primary and secondary SKBR3 tumoursphere-forming cells to TRAIL. MDA-MB-231-conditioned medium also increased the sensitivity of primary BT474 tumoursphere-forming cells to TRAIL, however this trend was not statistically significant (Figure 4.2A-D). MDA-MB-231-conditioned medium alone had no significant effect on tumoursphere formation in either line.

We have shown previously that TRAIL reduces the size of tumourspheres formed in the SKBR3 cell line (Figure 3.1D). Conditioned medium did not cause TRAIL to further reduce tumoursphere size in the SKBR3 line (Figure 4.2E). This suggests that conditioned medium sensitises the tumoursphere-forming and not the transit-amplifying progenitor cells to TRAIL.

These data suggest that a soluble factor or factors produced by the MDA-MB-231 cell line are capable of sensitising TRAIL-resistant bCSCs to TRAIL. However, only one *in vitro* assay has been used to model bCSC function and it will be necessary to examine the effect of MDA-MB-231-conditioned medium on the TRAIL-susceptibility of tumour-initiating cells *in vivo,* in order to determine more definitively whether or not this is the case. We would expect that pre-treatment of SKBR3 cells with conditioned media and TRAIL *in vitro* would reduce tumour-initiation *in vivo*.

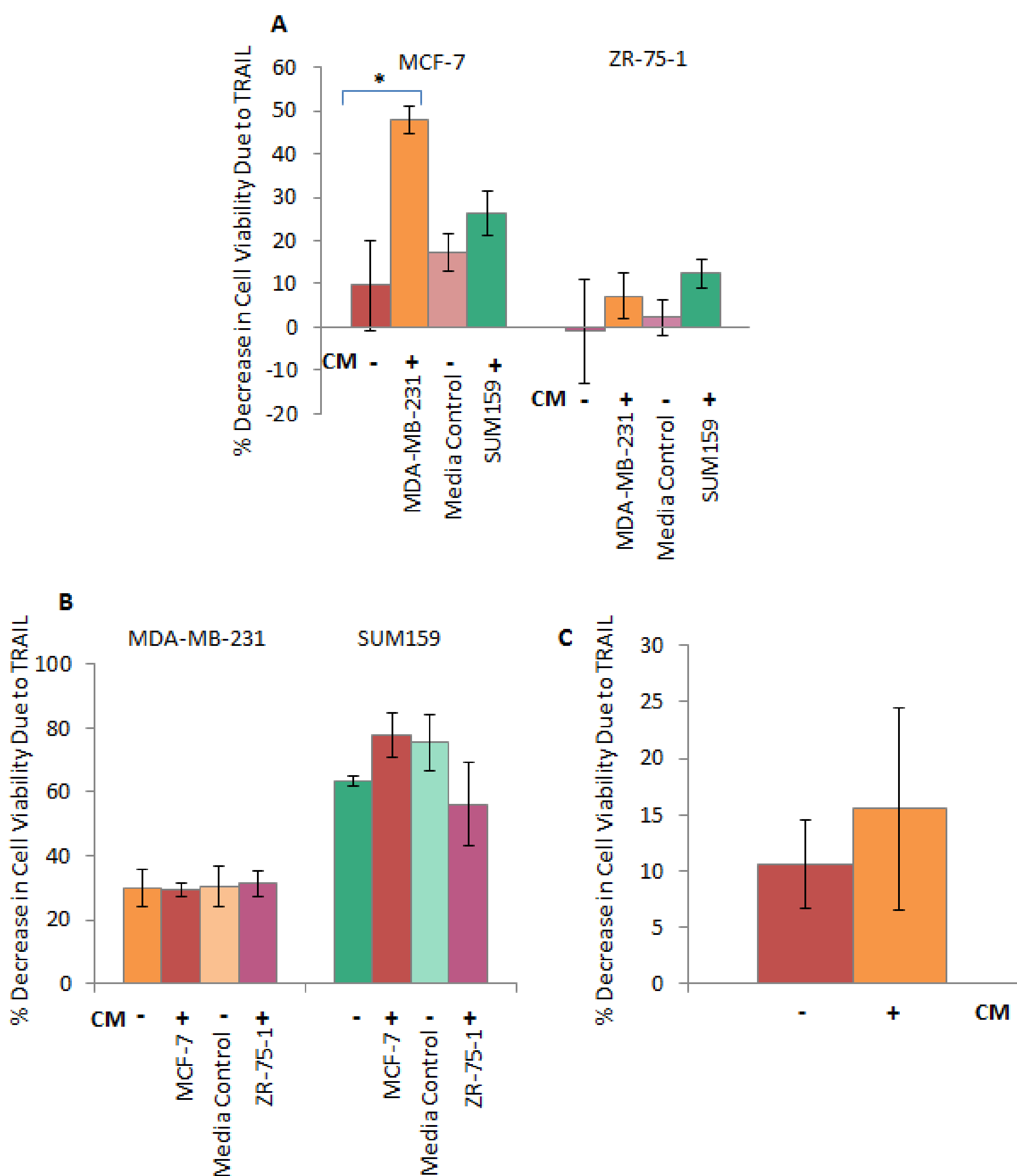


Figure 4.1: *MDA-MB-231-conditioned medium sensitises MCF-7 cells to TRAIL*

Cell Titre Blue Assay: Conditioned medium (CM) was generated by culture of cells of 90-100% confluency in 3 ml media /25cm^2, unless otherwise stated. CM was filtered before use. Cell lines were cultured in CM for 24 h then treated with 20 ng/ml TRAIL for 18 h and cell viability assessed by the cell titre blue assay: **A** TRAIL-resistant MCF-7 and ZR-75-1 lines were cultured in CM from MDA-MB-231 or SUM 159 lines: MDA-MB-231-conditioned medium sensitised MCF-7 cells to TRAIL (*p = 0.03, t-test), but had no significant effects on the ZR-75-1 cell line. SUM159 CM had no significant effects on either the MCF-7 or ZR-75-1 lines **B** TRAIL-sensitive MDA-MB-231 or SUM 159 lines were cultured in CM from MCF-7 or ZR-75-1 lines: No significant protective effects were observed **C** TRAIL-resistant MCF-7 and ZR-75-1 lines were cultured in CM from MDA-MB-231 or SUM 159 lines at 6ml media /25cm^2: No significant sensitisation was observed. All results are averages of three independent experiments each performed with three internal technical replicates

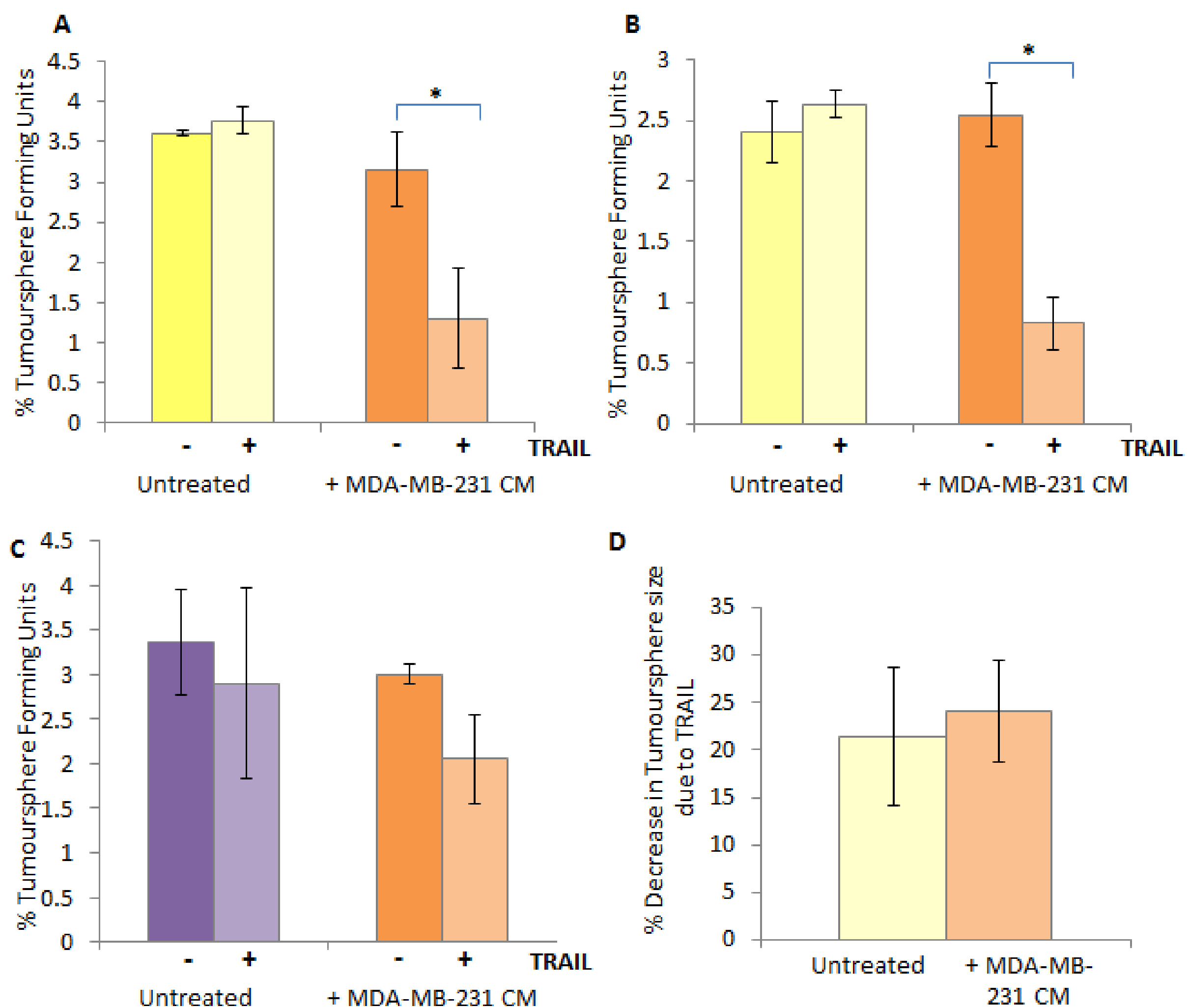


Figure 4.2: *Conditioned Medium from TRAIL-sensitive MDA-MB-231 cells sensitises tumoursphere forming cells to TRAIL*

Tumoursphere Assay: SKBR3 and BT474 cells were cultured for 24 h in filtered conditioned medium (CM) taken from MDA-MB-231 cells (3 ml/T25), then subjected to the tumoursphere assay in the presence or absence of 20 ng/ml TRAIL: **A** CM sensitised SKBR3 tumoursphere-forming cells to TRAIL (passage 1) (*p = 0.02, t-test). **B** CM sensitised SKBR3 secondary tumourspheres to TRAIL (*p = 0.02, t-test) **C** CM sensitised BT474 primary tumoursphere-forming cells to TRAIL but result was not statistically significant (p >0.05, t-test). **D** CM did not cause TRAIL to reduce the size of tumourspheres formed in the SKBR3 line (p>0.05, t-test). All results are averages of three independent experiments each performed with three internal technical replicates

4.2.3 Sensitisation of breast cancer cells to TRAIL is accompanied by an EMT-like process in TRAIL resistant cells

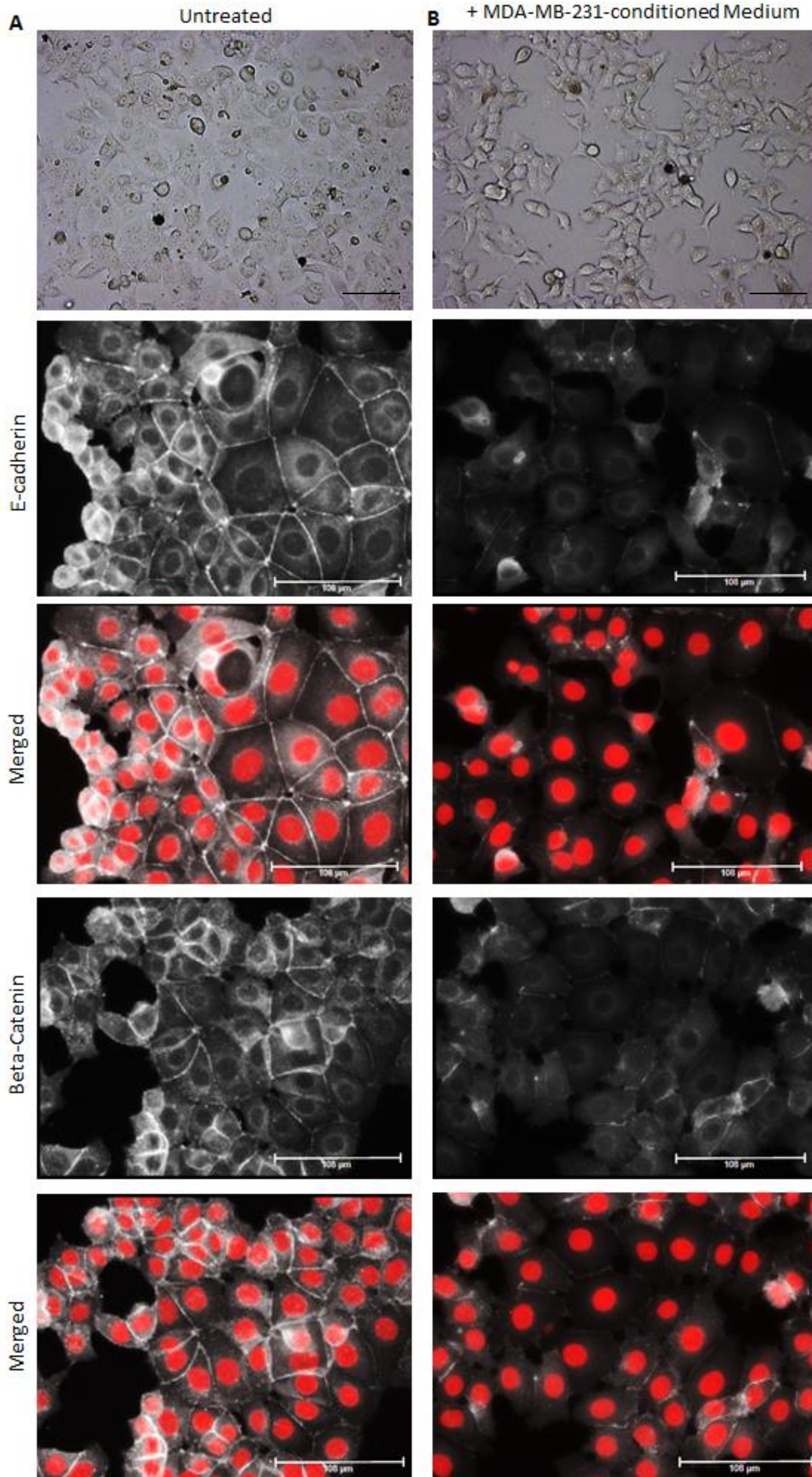
It was observed that 24 h culture with MDA-MB-231 or SUM159-conditioned medium altered the morphology of the TRAIL-resistant lines. In standard culture conditions, both the MCF-7 and ZR-75-1 lines usually appear rounded and grow in closely packed colonies, consistent with cells of a luminal, epithelial-like nature. MDA-MB-231-conditioned medium induced a spindle-like morphology where cells appeared more dispersed and mesenchymal-like (Figure 4.2A). This alteration in morphology was not present when no sensitisation to TRAIL occurred, for example when 6ml MDA-MB-231-conditioned medium was used (data not shown).

To determine whether the observed morphological changes were consistent with an EMT-like process, MCF-7 cells were cultured for 24 h on glass coverslips in the presence or absence of MDA-MB-231-conditioned medium and then assayed for the expression of the EMT markers E-cadherin and beta-catenin by immunofluorescence. Conditioned medium altered the expression patterns of both E-cadherin and β-catenin. Following CM-treatment, and consistent with EMT, both appeared to be reduced at the cell membrane and present in the cytosol and nucleus (Figure 4.2 A). This is consistent with subcellular localisation changes associated with EMT (Thiery and Sleeman 2006). These data suggests that soluble factors produced by MDA-MB-231-conditioned medium are capable of inducing EMT-like changes in the MCF-7 cell line. However, this result will need to be supported with an analysis of E-cadherin and beta-catenin re-localisation by Western blotting in order to provide a more easily quantifiable outcome.

To determine whether conditioned medium induced transcriptional changes associated with EMT, RNA was extracted from MCF-7 cells cultured in the presence or absence of MDA-MB-231-conditioned medium, and analysed by qRT-PCR for the expression of EMT-associated markers. Consistent with EMT, culture with conditioned medium down-regulated the expression of the epithelial marker E-cadherin whereas the mesenchymal marker fibronectin was up-regulated. However, the expression of the EMT-associated transcription factors slug and snail was unchanged, and twist expression was down-regulated (Figure 4.2B). Therefore, conditioned medium altered the expression of morphological markers in accordance with EMT but the expression of transcriptional markers was not consistent with the occurrence of EMT.

In addition, although a transcriptional EMT has been associated with an increase in bCSCs (Mani *et al.* 2008, Morel *et al.* 2008) no increase in tumoursphere formation was observed following conditioned medium treatment of SKBR3 and BT474 cells (Figure 4.2A-D).

Taken together, these findings show that soluble factor(s) produced by MDA-MB-231 cells induce changes in MCF-7 cells which suggest a partial EMT with morphological, but not transcriptional alterations.


A
Untreated
B
+ MDA-MB-231-conditioned Medium
E-cadherin
Merged
Beta-Catenin
Merged

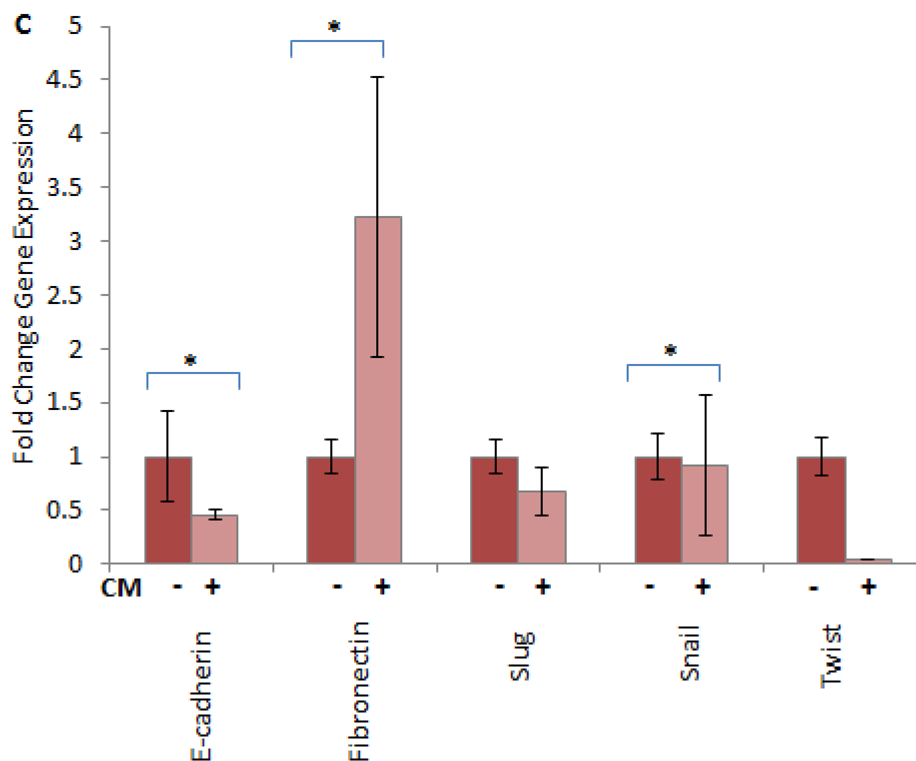


Figure 4.3: *Supernatant from TRAIL-sensitive lines induces an EMT-like phenotype in TRAIL-resistant cells*
MCF-7 cells were cultured in the presence or absence of filtered conditioned medium from MDA-MB-231 cells (3 ml/25 cm^2) for 24 h **A/B IF** Cells were fixed and stained for E-cadherin; CM reduced membrane-bound E-cadherin and Beta-catenin: CM reduced membrane-bound Beta-catenin. Scale bars = 108 um. **C qPCR:** Extracted RNA was assayed for EMT marker expression by qPCR; CM reduced significantly the expression of E-cadherin (*p = 0.05) and Twist (*p = 0.0002) and increased significantly the expression of fibronectin (*p = 0.0008), but had no significant effect on the expression levels of slug or snail. All results are averages of three independent experiments. The qPCR assay was also performed with three internal technical replicates.

4.2.4 Conditioned medium from primary fibroblasts sensitises MCF-7 cells to TRAIL.

We have shown that sensitisation to TRAIL by a soluble factor is accompanied by instigation of a partial, morphological EMT in recipient cells. This data is in accordance with previous studies which have demonstrated a correlation between a mesenchymal phenotype and TRAIL-sensitivity (Rahman *et al.* 2009). The fact that soluble cytokines produced by cancer associated fibroblasts (CAFs) in the tumour environment are also able to induce an EMT in breast cancer cells (Karnoub *et al.* 2007, Yu *et al.* 2014), led us to hypothesise that CAF-conditioned medium could also sensitise cells to TRAIL. To test this, CAFs were taken from primary biopsy samples of invasive ductal carcinomas (IDC). CAF-conditioned medium was compared to conditioned medium of fibroblasts taken from a benign fibroadenoma (Table 4.1). Fibroblasts were isolated by differential adhesion, propagated over five passages and their phenotype confirmed by cell morphology (Figure 4.4A). Conditioned medium was generated by 24 h culture of fibroblasts at 80-100% confluency in 2 ml medium/25 cm^2. MCF-7 cells were cultured in filtered fibroblast-conditioned medium for 24 h then treated with 20 ng/ml TRAIL for 18 h and cell viability assessed using the cell titre blue assay. Culture with conditioned medium from fibroblasts of both the benign lesion and the IDC samples sensitised MCF-7 cells to TRAIL, to a level which was not significantly different to that of MDA-231-conditioned medium. These data show that soluble factors produced by fibroblasts or CAFs are capable of sensitising MCF-7 cells to TRAIL (Figure 4.4B).

These data show that both CAF-conditioned medium and conditioned medium from the fibroblasts of a benign lesion is capable of sensitising MCF-7 cells to TRAIL. This suggests that soluble factor(s) are produced by fibroblasts which sensitise cells to TRAIL and that the production of the soluble sensitisation factor(s) is not a tumour-dependent phenomenon. However further investigation is required to determine whether normal fibroblasts (i.e. those not associated with a lesion) produce factor(s) capable of sensitisation to TRAIL.

Table 4.1: Details of primary lesions with which fibroblasts were associated:

Sample	Sex	Age	Collection date	Processing date	Type of tumour	Grade	ER(All red score)	PR	HER2 (All red score)	Meno-pausal status
FA	F	41	12.06.14	13.06.14	Benign Fibroadenoma					
IDC1	F	81	09.06.14	11.06.14	Invasive Ductal	1	+ve (8)		0 (FISH -ve)	Post
IDC2	F	46	12.06.14	13.06.14	Invasive Ductal + DCIS	2	+ve (7)		+1 (FISH -ve)	Pre
IDC3	F	77	23.06.14	24.06.14	Invasive Ductal + DCIS	2	+ve (8)			

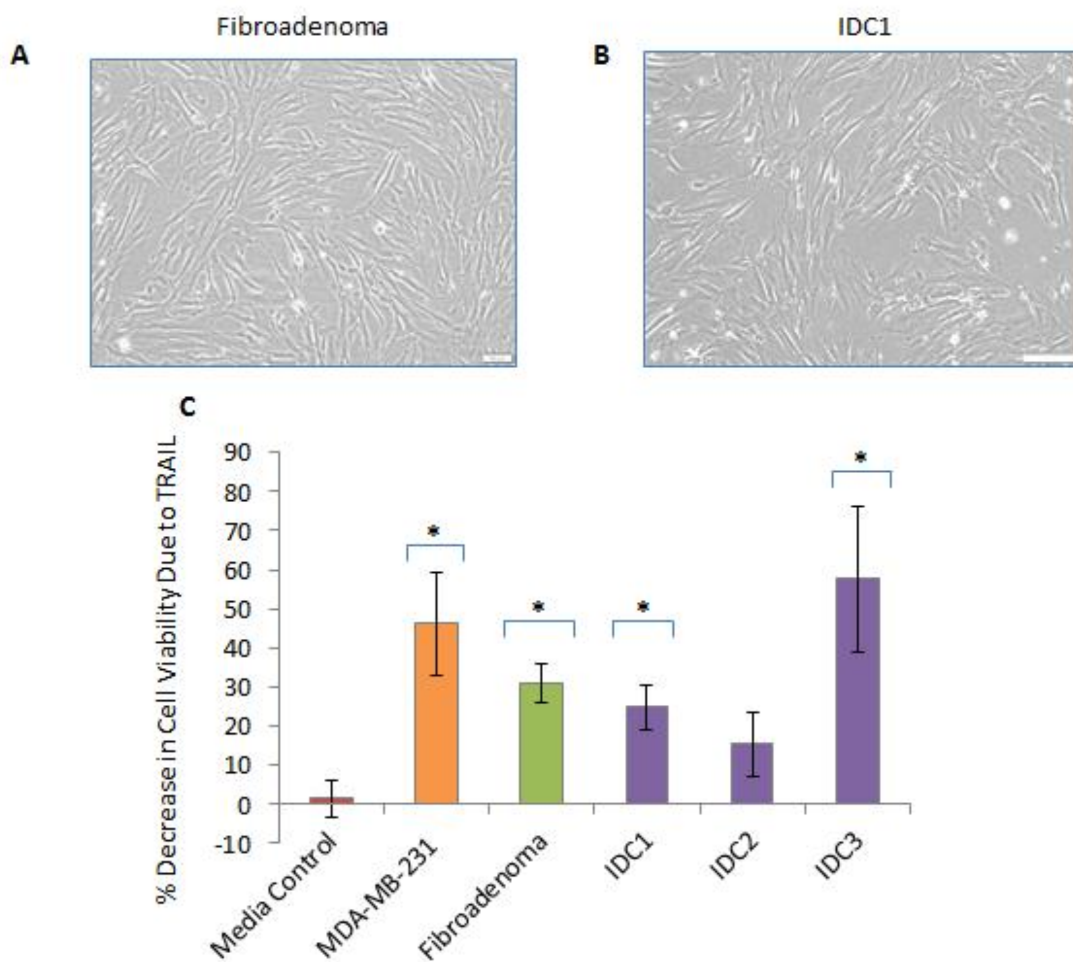


Figure 4.4: *Supernatant from cancer-associated fibroblasts sensitises MCF-7 cells to TRAIL*

A Representative images of cultured fibroblasts

B Cell Titre Blue Assay: TRAIL-resistant MCF-7 cells were cultured in the conditioned medium from primary fibroblasts or MDA-MB-231 cells (positive control) for 24 h then treated with 20 ng/ml TRAIL for 18 h and cell viability assessed by the cell titre blue assay: Conditioned medium of fibroblasts both from a fibroadenoma and two IDC samples sensitised MCF-7 cells to TRAIL significantly (MDA-MB-231; $*p = 0.03$, Fibroadenoma $*p = 0.01$, IDC1; $*p = 0.02$, IDC 2; $p > 0.05$, IDC 3; $*p = 0.04$, t-test)

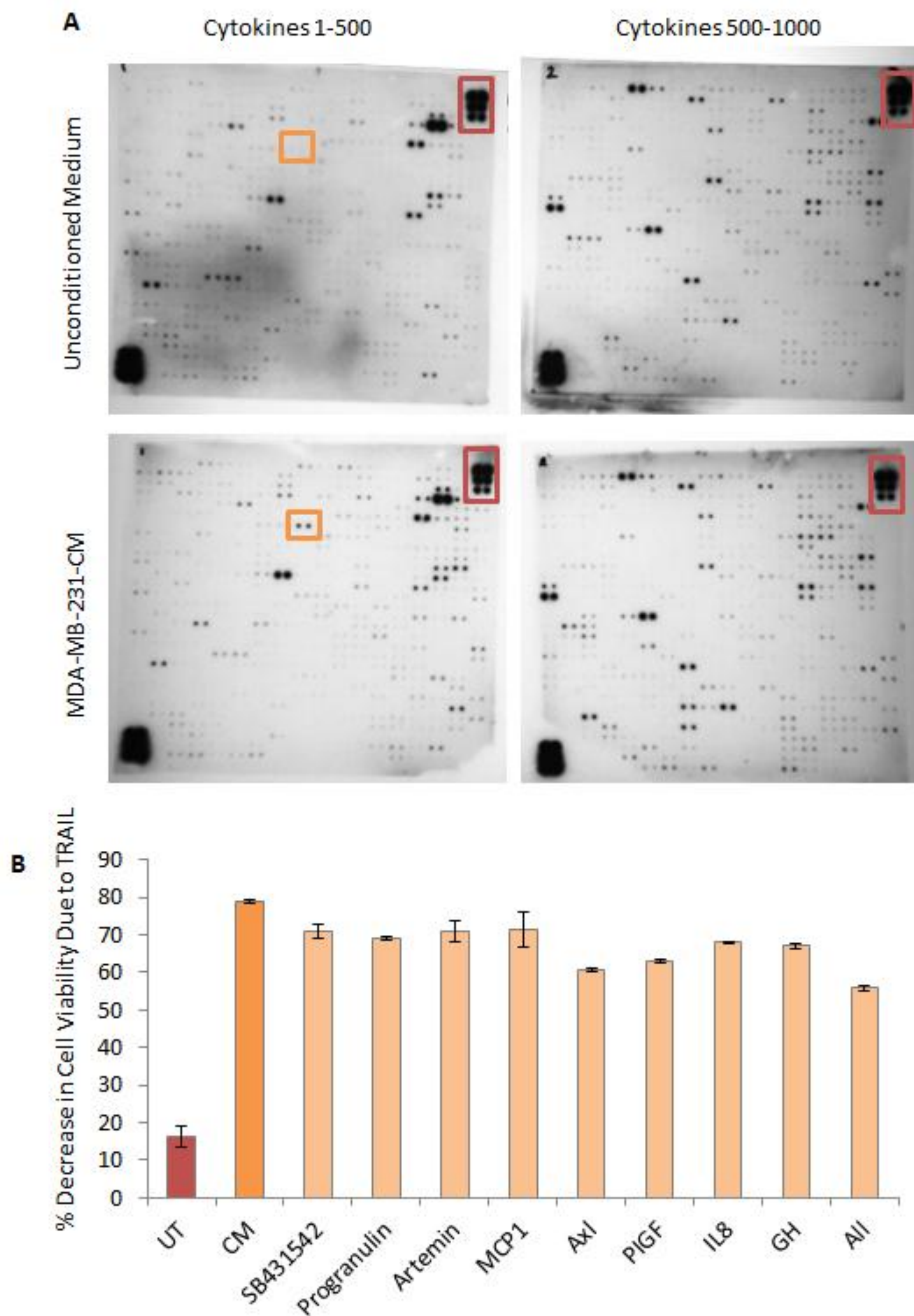
4.2.5 The Secreted Cytokine Profile of MDA-MB-231 cells

In order to aid identification of the soluble factor(s) involved in TRAIL sensitisation by MDA-MB-231-conditioned medium, a cytokine array was performed. The array assayed for the expression of 1000 secreted human cytokines (Raybiotech). The cytokine profile of MDA-MB-231-conditioned medium was compared to that of the (unconditioned) culture medium by densitometry (Figure 4.5A). The top 25 cytokines with the highest degree of expression compared to the control are listed in table 4.1. Initial investigations focussed on eight candidates selected for their known involvement in EMT or breast cancer progression; IL8, TGFbeta, Axl, Artemin, MCP1, growth hormone (GH), progranulin, and PIGF (see Table 4.2 for references).

To determine whether the cytokines identified were responsible for the sensitisation to TRAIL, MCF-7 cells were treated with MDA-MB-231-conditioned media in the presence or absence of inhibitory agents targeting each of these cytokines/growth factors (Section 2.2.3, Table 2.12). In the presence of MDA-MB-231-conditioned media, inhibition of each of these factors alone exhibited a partial protection against TRAIL, but no effect was statistically significant (Figure 4.5B). However this data is preliminary, and as the activity of these antibodies and inhibitors has not yet been confirmed, no conclusions can be made. Ongoing investigations will determine whether MDA-MB-231-conditioned medium which has been pre-incubated in the presence of the antibody or inhibitory agent, can sensitise to TRAIL.

Table 4.1: Top 25 mostly highly expressed cytokines secreted by the MDA-MB-231 line:

Rank	Cytokine	Fold Change	Reference
1	Latent TGF-beta bp1	41.2635	
2	IL8	24.67586	Fernando 2011
3	HCC4/CCL16	23.47494	
4	XEDAR	20.17467	
5	MMP1	17.51915	
6	IL10-R alpha	16.90138	
7	Artemin	14.31668	Bannerjee 2011
8	IL5 R alpha	14.20246	
9	Dkk-1	12.81273	
10	CTLA-4	10.66321	
11	MMP 20	9.55978	
12	PECAM-1	9.556422	
13	Growth Hormone (GH)	8.85718	Walsh 2011
14	Progranulin	8.778184	Khoo 2012
15	Pentraxin3	8.119714	
16	APRIL	8.101893	
17	IL18-R alpha	7.962322	Fernando 2011
18	FGF R5	7.801458	
19	MCP-1	7.468568	
20	MMP-10	7.370684	
21	PIGF	7.264439	Ning 2013
22	TGFbeta 2	7.226993	Morel 2009
23	IGFBP-1	5.80239	
24	FGF-13-1B	5.778452	
25	Axl	5.690206	Asiedu 2014



<u>Figure 4.5:</u> *The secreted cytokine profile of MSA-MB-231 cells*

A Representative image of cytokine array; positive controls are indicated in red. As an example, differential IL8 protein expression is also indicated in orange **B Cell Titre Blue Assay:** MCF-7 cells were cultured in MDA-MB-231-conditioned medium for 24 h in the presence or absence of antibodies or agents which inhibit the factors indicated on the graph (SB431542 is a TGFβ receptor inhibitor), then treated with 20 ng/ml TRAIL: all inhibitors protected CM-treated MCF-7s from TRAIL partially. These data represent a single experiment which in the case of the cell titre blue assay was performed with three internal technical replicates.

4.2.6 TNFalpha and IGF1 sensitise MCF-7 cells to TRAIL

To determine whether the cytokines identified in the array of MDA-MB-231-conditioned media were responsible for the sensitisation to TRAIL by CAF-conditioned media, MCF-7 cells were treated with CAF or fibroblast-conditioned media (IDC3 and FA, Table 4.1) in the presence or absence of inhibitory agents targeting TGFbeta receptors (SB431542) or an inhibitory antibody to IL8 (Section 2.2.3, Table 2.12). In the presence of both fibroblast and CAF-conditioned media, inhibition of each of these factors exhibited a partial protection against TRAIL, but no effect was statistically significant (Figure 4.5B). However, as with the analysis of MDA-MB-231-conditioned media, this data is preliminary, and as the activity of these antibodies and inhibitors has not yet been confirmed, no conclusions can be made. Ongoing investigations will determine whether fibroblast-conditioned medium which has been pre-incubated in the presence of a wider panel of antibodies or inhibitory agents, can sensitise to TRAIL.

As we have shown that conditioned medium from CAFs sensitises MCF-7s to TRAIL, we also wished to determine whether cytokines known to be secreted by CAFs are able to sensitise to TRAIL (Kalluri and Zeisburg 2006). Twenty-four hour pre-treatment with MMP2, MMP3, TGFbeta, WNT1, IL6, EGF, FGF or HRG had no significant effect on the sensitivity of MCF-7 cells to TRAIL. However, 24h pre-treatment with TNFα, or IGF1 sensitised significantly the MCF-7 cells to TRAIL (Figure 4.6). In combination, TGFbeta and TNFalpha, or TGFbeta and IGF1, also sensitised significantly the MCF-7 line to TRAIL, however no more so than TNFα, or IGF1 alone. This suggests that CAFs may sensitise MCF-7s to TRAIL via the production of TNFα, or IGF1, without requiring the function of TGFbeta, but blocking of these cytokines during conditioned medium treatment is required in order to establish definitively whether or not this is the case. The ability of TNFalpha to sensitise to TRAIL is not unsurprising as both are members of the TNF superfamily and therefore not unlikely to synergise. However, the ability of IGF1 to sensitise cells to TRAIL is a novel observation which suggests a link between two separate pathways that may not have been recognised previously.

These data provide some preliminary indications of possible candidate soluble factors for further investigation. However we have not yet confirmed that these cytokines act as expected in our cell lines, and also in the absence of confirmation with specific inhibitors, no definitive conclusions can be made at this stage.

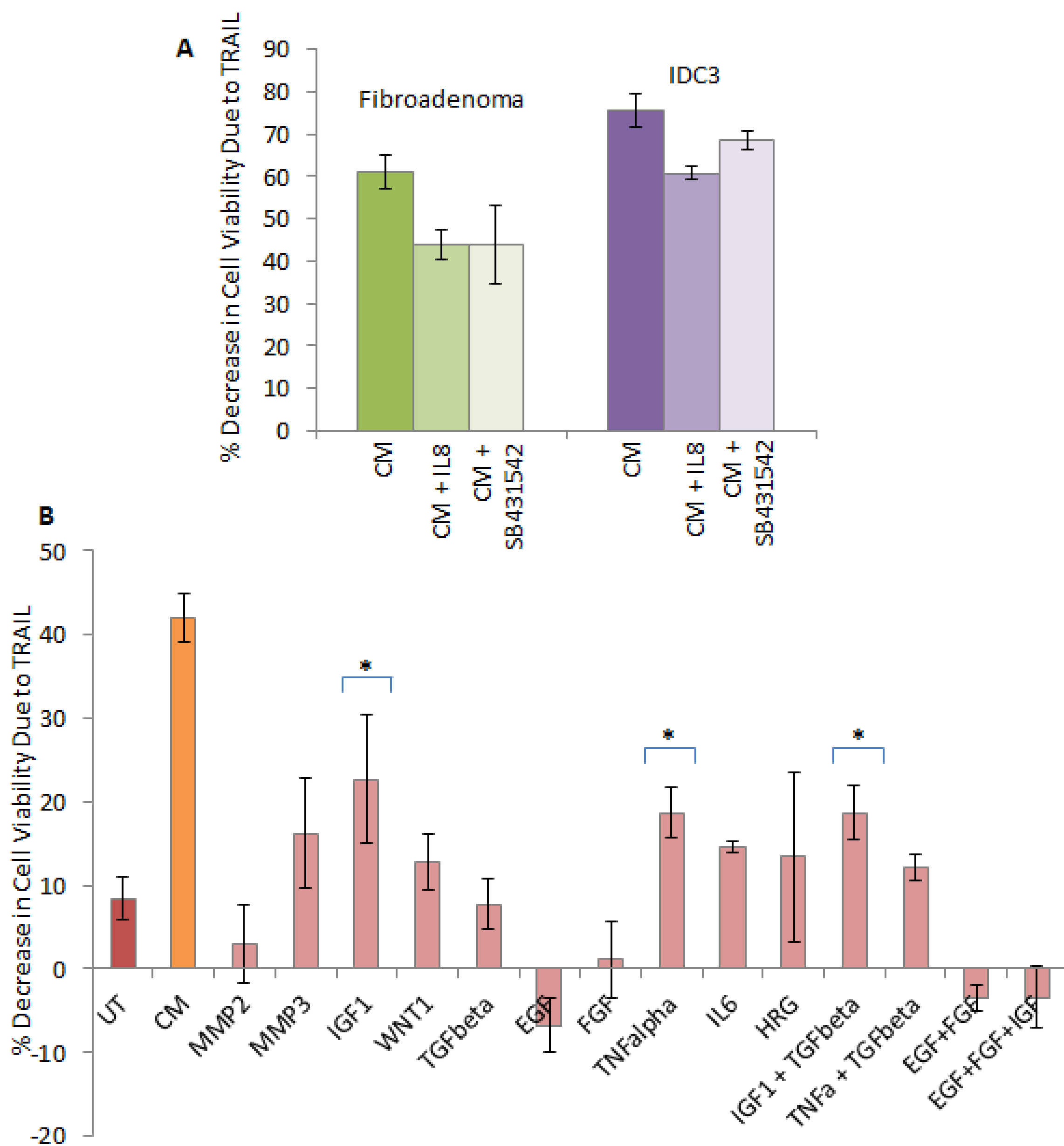


Figure 4.6: *TNFalpha, TGFbeta and IGF1 sensitise MCF-7 cells to TRAIL*

A Cell Titre Blue Assay: MCF-7 cells were cultured in fibroblast or CAF-conditioned medium for 24 h in the presence or absence of antibodies to IL8 or SB431542 (a compound which inhibits TGFbeta receptors) then treated with 20 ng/ml TRAIL: both the IL8 antibody and SB431542 protected CM-treated MCF-7s from TRAIL partially. These data represent a single experiment which was performed with three internal technical replicates.

B Cell Titre Blue Assay: TRAIL-resistant MCF-7 cells were cultured in the presence or absence of cytokines for 24 h then treated with 20 ng/ml TRAIL: Pre-treatment with TNFalpha or IGF1 sensitised the MCF-7 cells to TRAIL significantly (TNFalpha; *p = 0.003, IGF1; *p = 0.01, t-test, average of three independent experiments each performed with three internal replicates).

4.2.7 Investigating the role of cFLIP in conditioned medium-mediated sensitisation to TRAIL

We have shown that MDA-MB-231 and fibroblast-conditioned medium sensitises resistant cells to TRAIL, but the mechanism of this sensitisation is unknown. As discussed in the previous chapter, a reduction in cFLIP is one way in which cells can become TRAIL-sensitive. We therefore hypothesised that conditioned medium induces TRAIL sensitivity via a down-regulation of cFLIP. Pre-treatment with MDA-MB-231-conditioned medium did indeed reduce cFLIP transcript levels (Figure 4.7A). This suggests that MDA-MB-231 conditioned medium may sensitise cells to TRAIL via a down-regulation of cFLIP expression. However, the effect of conditioned medium on total cFLIP levels will also need to be determined by Western blotting before a definitive conclusion can be made.

We have also shown previously that cFLIP localisation is altered in TRAIL-sensitive cells (Figure 3.3). Therefore we wished to determine whether conditioned medium induces a re-localisation of cFLIP in the recipient line. To investigate this, nuclear and cytoplasmic protein fractions of condition medium-treated and untreated cells were subjected to Western blotting for cFLIP. In the MCF-7 line, MDA-MB-231-conditioned medium elevated nuclear cFLIP, but no decrease in cytoplasmic cFLIP was observed (Figure 4.7B). In the ZR-75-1 line, SUM159-conditioned medium reduced cytoplasmic cFLIP and elevated nuclear cFLIP (Figure 4.7C). This observation is consistent with our previous findings that cFLIP is re-localised in TRAIL-sensitive cells. However, the effect of conditioned medium on total cFLIP levels will also need to be determined by Western blotting, before a definitive conclusion can be made.

As cFLIP inhibition also sensitises cells to TRAIL, our observations led us to question whether production of the TRAIL sensitisation factor could be induced as a result of cFLIP inhibition in TRAIL-resistant cell lines. Therefore we tested the effect of conditioned medium from MCF-7 or ZR-75-1 cells in which cFLIP had been inhibited by RNAi (cFLIPi), on the TRAIL-susceptibility of untreated cells. cFLIPi -conditioned medium was not able to induce TRAIL sensitivity in either cell line. These data show that the production of the TRAIL-sensitisation factor(s) is not exclusively cFLIP dependant (Figure 4.7D).

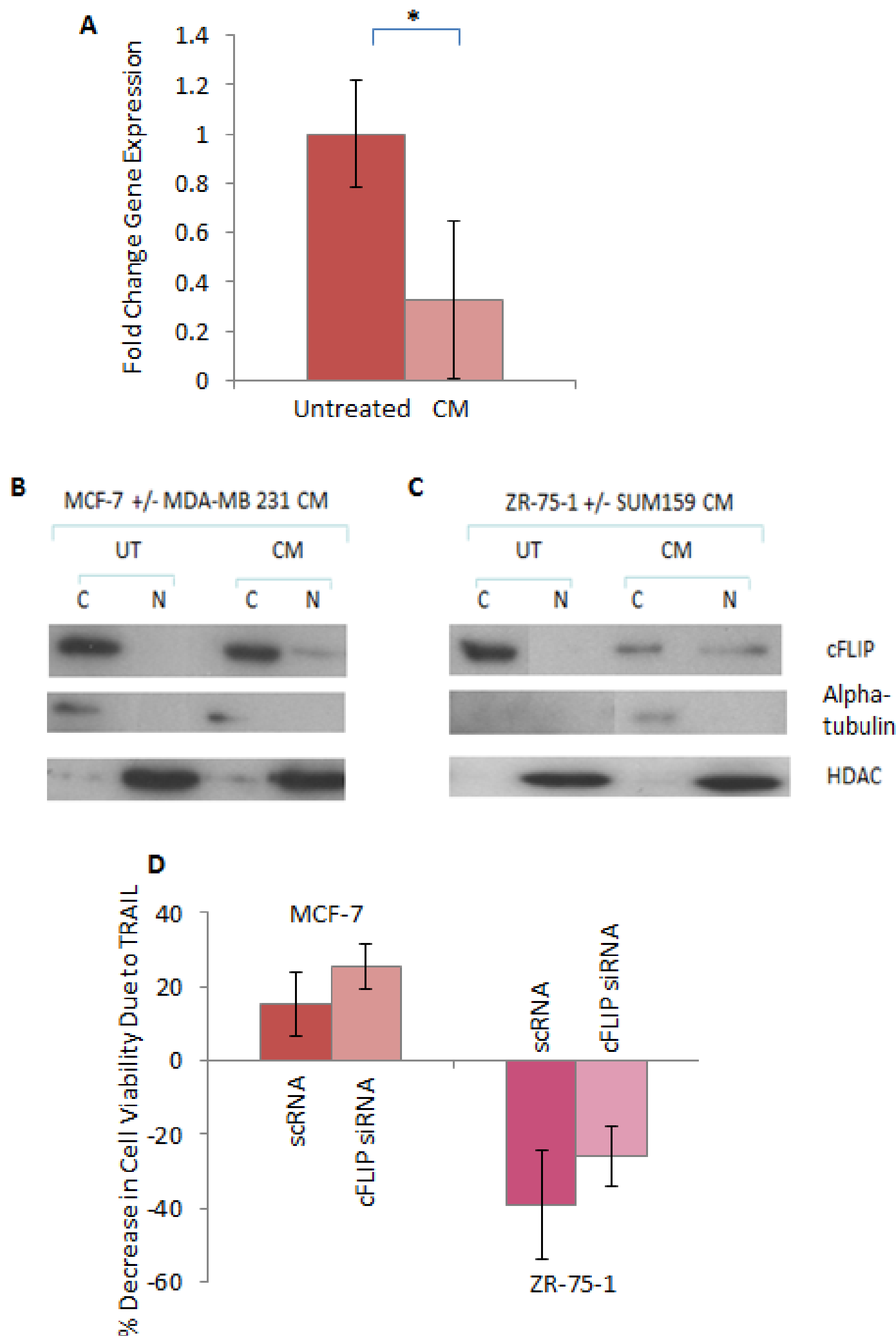


Figure 4.7: *MDA-MB-231-conditioned medium from TRAIL-sensitive cells reduces cFLIP expression in MCF-7 cells:* MCF-7 cells were cultured in the presence or absence of filtered MDA-MB-231-conditioned medium for 24 h. **A qPCR:** Conditioned medium from MDA-MB-231 cells reduced the expression levels of cFLIP significantly in the MCF-7 line (*p = 0.02, t-test, three independent experiments each with 3 internal replicates) **B Western Blotting:** Nuclear (N) and cytoplasmic (C) proteins were extracted and subjected to Western blotting for cFLIP (cytoplasmic loading = α-tubulin, nuclear loading = HDAC): MDA-MB-231-CM elevated nuclear cFLIP levels **C Western Blotting:** ZR-75-1 cells were cultured in the presence of SUM159-CM for 24 h. Nuclear and cytoplasmic proteins were extracted and subjected to Western blotting for cFLIP (cytoplasmic loading = α-tubulin, nuclear loading = HDAC): SUM159-CM reduced cytoplasmic cFLIP and elevated nuclear cFLIP **D Cell Titre Blue Assay:** TRAIL-resistant MCF-7 and ZR-75-1 lines were cultured in CM from MCF-7 cells transfected with siRNA targeting cFLIP or a scrambled control: No significant sensitisation to TRAIL was observed

4.3 Discussion

Previous studies have shown that mesenchymal-like breast cancer cell lines are sensitive to induction of apoptosis by TRAIL (Rahman *et al.* 2009). In the previous chapter we showed that the tumoursphere-forming cells of two out of four epithelial-like lines are also sensitive to TRAIL due to a decrease in cytoplasmic cFLIP. However, TRAIL-resistance in epithelial-like breast cancer cells and bCSCs remains a limitation to the value of TRAIL as a therapy, and in the absence of small molecule cFLIP inhibitors, novel strategies for sensitisation to TRAIL are required. Here we have shown that TRAIL-resistant breast cancer cells can be sensitised to TRAIL by a soluble factor(s) produced by a TRAIL-sensitive line: The cell viability of MCF-7 cells was reduced by TRAIL following 24 h pre-culture with MDA-MB-231-conditioned medium. In addition, MDA-MB-231-conditioned medium sensitised SKBR3 tumoursphere-forming cells to TRAIL. Furthermore, conditioned medium taken from cultured fibroblasts associated with both benign and invasive lesions is also able to sensitise MCF-7 cells to TRAIL. In each case, further investigation is required in order to determine whether TRAIL is acting through a caspase-mediated pathway as expected, and *in vivo* assays will also be required to confirm whether MDA-MB-231-conditioned medium can sensitise a tumour-initiating population to TRAIL. Nevertheless these findings are significant for a number of reasons: Firstly, they suggest the potential of further expanding the repertoire of cell types to which TRAIL could be considered a potential therapeutic. Secondly, the finding that CAFs can sensitise to TRAIL may have implications for the effect of the tumour environment on the susceptibility of a breast tumour to TRAIL. Finally, no extracellular mechanism for the induction of TRAIL susceptibility has yet been described. Our findings have generated the opportunity of exploring an entirely novel system not previously investigated.

We have observed that sensitisation to TRAIL by MDA-MB-231-conditioned medium was accompanied by a partial EMT in the MCF-7 line, demonstrated by altered expression of E-cadherin, beta catenin and fibronectin in conjunction with morphological changes consistent with EMT. However the expression of the transcription factors slug and snail was not changed and twist expression was in fact down-regulated. These data are suggestive of a partial EMT with morphological but not transcriptionally relevant changes; not an unusual occurrence in terms of oncogenic EMT (Drasin 2011). Although a transcriptional EMT was not apparent in this system, our study is limited by its use of only three conventional markers of a transcriptional EMT. Recent evidence suggests that the Prrx1 transcription factor is also capable of regulating EMT and metastasis in models of breast cancer (Ocano *et al.* 2012). Further investigation is required to examine the effect of conditioned media on the expression of this gene.

Whilst our interest in this system was due initially to its potential as a method which could be exploited to sensitise cells to TRAIL, these findings suggest that as MDA-MB-231-conditioned medium induces a partial EMT, the soluble factor involved could enhance the malignant phenotype of the recipient cells, and therefore may not be such a promising therapeutic approach. However, further investigation is required to determine whether conditioned medium promotes the functional attributes associated with an oncogenic EMT, i.e. increased motility, invasiveness and metastasis (generally associated with cells at the leading edge of a malignant tumour). The dispersed nature of the cells

following treatment with MDA-MB-231-conditioned medium suggests that cell motility may have been promoted, but the finding that MDA-MB-231-conditioned medium does not increase tumoursphere formation is inconsistent with EMT (Mani *et al.* 2008, Morel *et al.* 2008) and may be evidence to the contrary.

Despite the possibility that a therapeutic strategy may not result, our findings are nevertheless significant for their potential to aid the identification of breast tumour subtypes likely to respond to TRAIL. This possibility is raised by our data which shows that the conditioned medium taken from fibroblasts and CAFs of primary samples also sensitises to TRAIL. Previous studies have also shown that CAFs are capable of inducing EMT in breast cancer cells (Karnoub *et al.* 2007, Yu *et al.* 2014). Further investigation is required to determine whether fibroblast or CAF-conditioned medium also induces EMT-like changes similar to MDA-MB-231-conditioned medium, and whether other tumour associated cells such as immune cells or MSCs are capable of sensitising to TRAIL. Although not possible to determine definitively, our findings suggest that some breast carcinomas may be TRAIL-sensitive *in situ* due to the presence of soluble factors produced by CAFs. As most tissues have significant levels of endogenous TRAIL (Speirings *et al.* 2012), this may require that the breast tumour has developed a way of avoiding endogenous TRAIL other than by developing an intrinsic resistance, perhaps by impairing TRAIL production. These findings may be evidence of the possibility that extensive i*n vitro* culture has altered the TRAIL-susceptibility phenotype of MCF-7 cell line or indeed that of primary biopsy samples, which without the presence of CAFs and other associated cell types, do not reflect accurately the nature of breast tumours *in situ*. The converse could also be the case; primary cell cultures may not be TRAIL sensitive even in the presence of fibroblasts due to the evolution of a protective counter-effect which has been lost by the MCF-7 line due to the lack of selective pressures in the absence of fibroblasts and TRAIL. In any event, it will be of interest to determine whether tumours *in vivo* are more or less susceptible to TRAIL than *ex vivo* counterparts, and furthermore whether the TRAIL-susceptibility of a primary breast tumour biopsy sample could be predicted based on the cytokine profile of the CAFs. Conditioned medium from fibroblasts associated with a benign lesion was also able to sensitise significantly MCF-7 cells to TRAIL, suggesting that this effect was not tumour-dependent. However, it cannot be determined whether this benign lesion is pre-cancerous or not. It would be interesting to see whether conditioned medium from normal fibroblasts (i.e. not associated with a lesion) is also capable of sensitising to TRAIL. Were this not to be the case, we would propose that the soluble factor generated is an intrinsic protective response to cellular transformation designed to eradicate cancerous or pre-cancerous cells.

In order to identify the soluble factor(s) responsible for TRAIL sensitisation, a cytokine array was performed in which the cytokine profile of conditioned medium from MDA-MB-231 cells was compared to that of unconditioned culture medium. This array yielded a number of potential candidate factors for further investigation, many of which are known to be involved in EMT including IL8, TGFbetas, Axl, Artemin, growth hormone (GH), progranulin, and PIGF (Table 4.2). Preliminary data suggest that these factors may be responsible at least in part for the sensitisation to TRAIL by MDA-MB-231-conditioned medium, but much further investigation is required. We also hypothesise that the soluble factor(s) in question is responsible for the intrinsic TRAIL sensitivity of the MDA-MB-231 line, and therefore we will

also determine whether inhibition of this factor can protect MDA-MB-231 cells from TRAIL, although it is quite possible that epigenetic changes have taken effect in the MDA-MB-231 cells that do not require a paracrine response to a cytokine.

As it is possible that MDA-MB-231s, fibroblasts and CAFs produce different cytokines which sensitise to TRAIL, it was also of interest to investigate whether cytokines known to be secreted by fibroblasts or CAFs were able to sensitise to TRAIL (Kalluri and Zeisburg 2006). TNFalpha and IGF both sensitised MCF-7s to TRAIL significantly and to a similar degree as fibroblast or CAF-conditioned medium. However, blocking experiments are required to determine definitively whether TNFalpha and IGF are responsible for TRAIL sensitisation in either case. Nevertheless, the finding that cytokines known to be present in the tumour environment sensitise to TRAIL support the hypothesis that breast carcinomas may be TRAIL-sensitive *in situ*.

To investigate the mechanism by which the soluble factor(s) sensitises to TRAIL, the effect of MDA-MB-231-conditioned medium on cFLIP expression was investigated. Conditioned medium from the MDA-MB-231 line decreased significantly the expression of cFLIP as determined by qPCR. This suggests that the soluble factor may sensitise to TRAIL via a decrease in cFLIP levels, however the effect of MDA-MB-231-conditioned medium on cFLIP protein levels has yet to be determined. We showed in chapter 3 that bCSCs were TRAIL-sensitive due to reduced cytoplasmic cFLIP. It is also possible that the soluble factor acts to induce TRAIL sensitivity via the re-localisation of cFLIP to the nucleus. Our preliminary data suggest this may be the case but much further investigation is required.

Our data are again evidence of tumour heterogeneity existing in terms of TRAIL susceptibility: only 40% of either MCF-7 bulk cells or SKBR3 tumoursphere-forming cells are sensitised to TRAIL by a soluble factor(s). As MCF-7 tumoursphere-forming cells are TRAIL-sensitive, the effect of MDA-MB-231-conditioned medium on this population was not determined, however it would be interesting to investigate whether MDA-MB-231-conditioned medium can sensitise the remaining resistant MCF-7 tumoursphere-forming cells to TRAIL. We have shown previously that cFLIP inhibition is able to sensitise the remaining resistant cells to TRAIL (Piggott *et al.* 2011). As MDA-MB-231-conditioned medium may also down-regulate cFLIP, we would hypothesise that this may be indeed the case.

In conclusion we have shown that a soluble factor(s) can sensitise two resistant cell populations to TRAIL: MCF-7 cells and SKBR3 tumoursphere-forming cells. Further work is required to identify the mechanism of sensitisation and to explore the effect of the tumour environment on TRAIL sensitivity. If the context of sensitisation were to be understood it may extend the repertoire of possible cell lines to which TRAIL could be considered a therapeutic to three out of six cell lines, and tumoursphere-forming populations to five out of six of these cell lines. We hope that these studies will further improve the identification of TRAIL-sensitive tumours in order to aid treatment strategies.

Chapter 5: Investigating the TRAIL-Independent Role of cFLIP in Wnt Signalling and Breast Cancer Stem Cells

Chapter 5

Investigating the TRAIL-Independent Role of cFLIP in Wnt Signalling and Breast Cancer Stem Cells

5.1 Introduction

The canonical Wnt pathway is a signalling cascade which mediates a number of cell fates including proliferation, differentiation, and the determination of stem cell characteristics, and is also associated with mesenchymal-like cells (Section 1.1.6, Bilir *et al.* 2013, Thiery 2002). Wnt signalling is elevated in both mammary stem cells and bCSCs, and is known to be required for their self-renewal (Korkaya *et al.* 2009, Howe *et al.* 2004). The Wnt pathway is consequently an important therapeutic target in breast cancer and cancer in general and a number of Wnt-inhibitors are currently in clinical trials (Anastas and Moon 2013).

Our previous findings have revealed the presence of nuclear cFLIP in breast cancer cell lines (Chapter 3). cFLIP is well characterised as an inhibitor of the extrinsic apoptosis pathway, a function which requires its availability in the cytoplasm. However the nuclear function of cFLIP in breast cancer cells is not known. It has been demonstrated previously in lung carcinoma cell lines that cFLIP promotes Wnt signalling by two separate mechanisms: Firstly by preventing the ubiquitylation and consequent degradation of beta-catenin, and also by forming a complex with transcription factors in the nucleus to directly promote Wnt-target gene expression (Naito *et al.* 2004, Katayama *et al.* 2010). The presence of nuclear cFLIP in breast cancer cell lines (Figure 3.3) suggests that cFLIP may also function in this capacity in breast cancer.

The Wnt pathway is elevated in mesenchymal-like and stem-like cells where activated Wnt and nuclear beta-catenin are associated with a mesenchymal-like status and are used as markers of EMT (Thiery and Sleeman 2006). Both mesenchymal-like breast cancer cell lines (Rahman *et al.* 2009) and some bCSCs (Chapter 3) are TRAIL-sensitive. We have found that this sensitivity correlates with a reduction in cytoplasmic cFLIP and the presence of nuclear cFLIP (Chapter 3, Figure 3.5 and 3.6). Due to these correlations we hypothesised that cFLIP promotes Wnt signalling in breast cancer cells and therefore its re-localisation to the nucleus plays an active role in the Wnt pathway. To address this we investigated the effect of cFLIP perturbation on Wnt signalling and bCSCs. The MDA-MB-231 and MCF-7 lines were used as models of mesenchymal-like, TRAIL-sensitive, and epithelial-like, TRAIL-resistant lines respectively.

5.2 Results

5.2.1 cFLIP Promotes Beta-Catenin Accumulation

It has been shown previously in lung carcinoma cell lines that inhibition of cFLIP results in a decrease in cytoplasmic beta-catenin (Naito *et al.* 2004, Katayama *et al.* 2010). To determine whether endogenous cFLIP was capable of regulating the Wnt pathway, the protein levels of beta-catenin were determined following cFLIP inhibition by siRNA: MDA-MB-231 and MCF-7 cells were transfected with siRNA targeting cFLIP or a non-specific scrambled control. Following 48 h transfection, cells were pelleted by centrifugation and cytoplasmic proteins extracted and analysed for cFLIP and beta-catenin levels by Western Blotting. In both the MCF-7 and MDA-MB-231 lines, inhibition of cFLIP resulted in a significant decrease in cytoplasmic beta-catenin (Figure 5.1A). Densitometry analysis of Western blots revealed a concomitant decrease in cFLIP and beta catenin; in both cell lines cFLIP inhibition resulted in a reduction in beta catenin levels to the same extent as cFLIP (Figure 5.1B-D). MDA-MB-231 cells were also analysed by immunofluorescence: Inhibition of cFLIP by siRNA in MDA-MB-231 cells resulted in a decrease in membrane-bound beta-catenin (Figure 5.1E).

These data show that endogenous cFLIP promotes beta-catenin accumulation and suggests that it is a positive regulator of the Wnt pathway in breast cancer cell lines. These findings are in accordance with previous studies which have shown that cFLIP promotes Wnt signalling in a lung carcinoma cell line (Naito *et al.* 2004, Katayama *et al.* 2010). We would expect that as shown by previous studies, cFLIP functions in this manner by preventing beta-catenin ubiquitylation and degradation, however this has yet to be determined (Ishiokia *et al.* 2007).

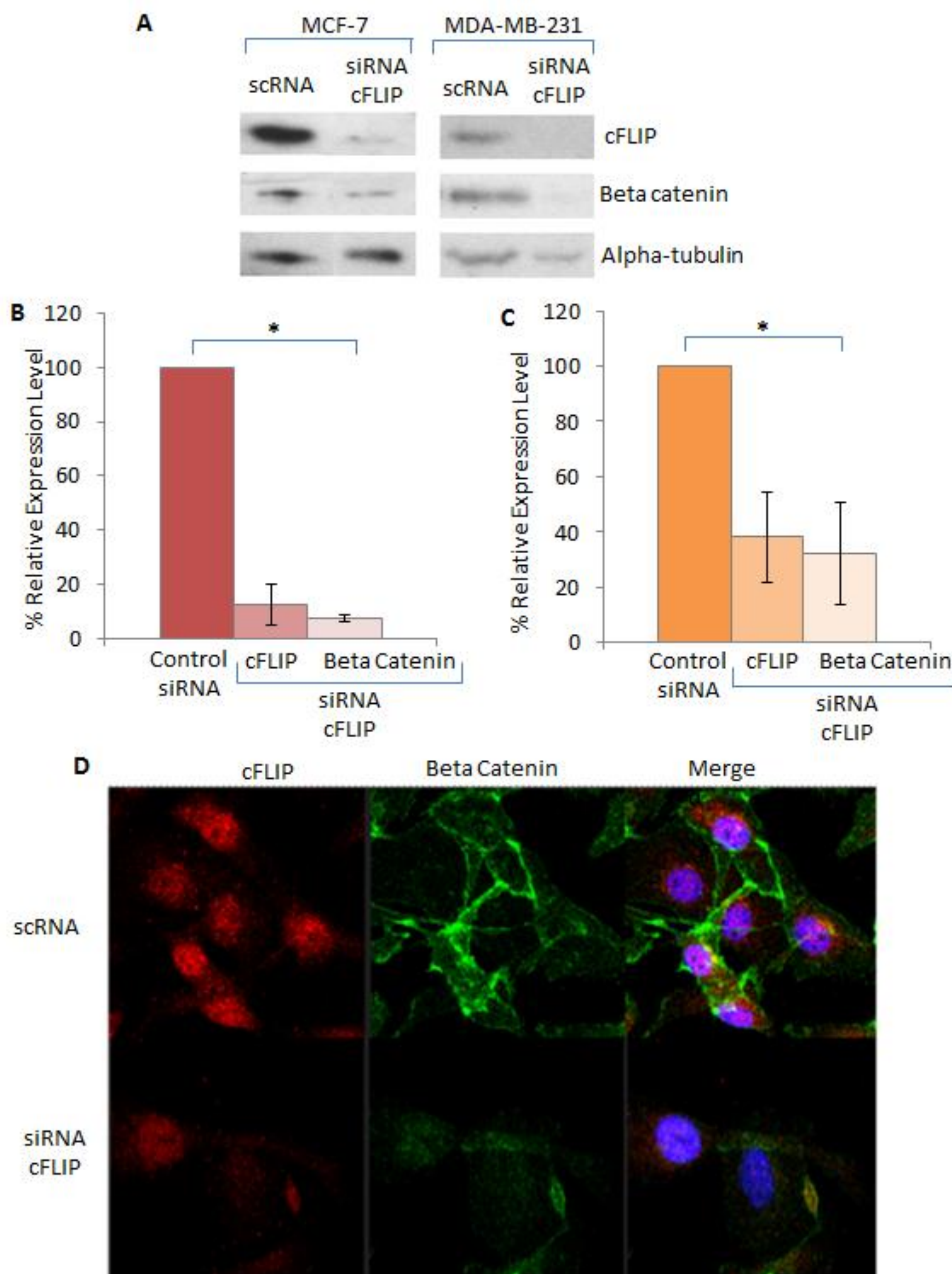


Figure 5.1 *cFLIP promotes beta-catenin accumulation:*

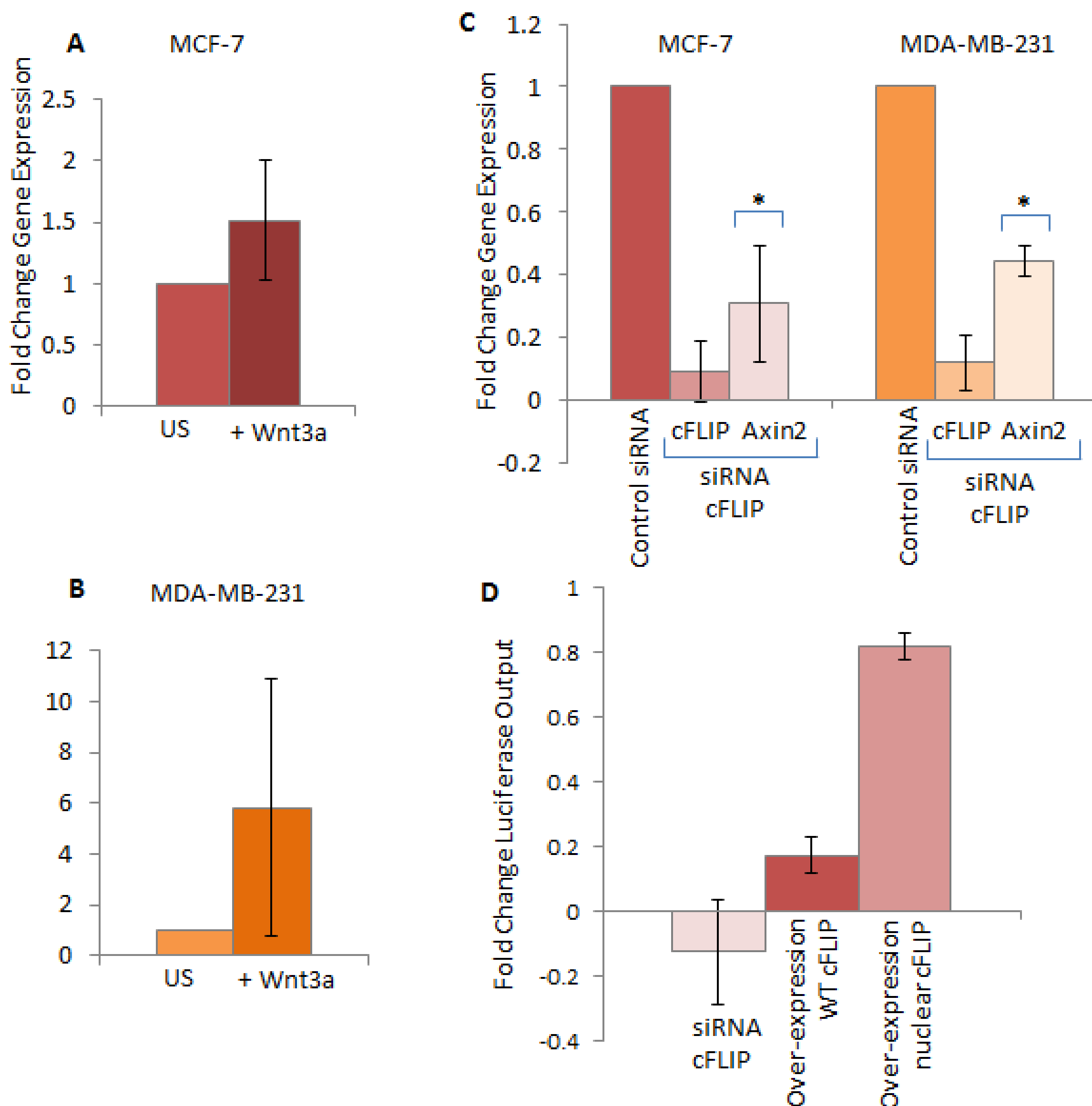
A Western Blotting: Cytoplasmic proteins were extracted from MCF-7 and MDA-MB-231 cell lines transfected with siRNA targeting cFLIP or a scrambled control, and subjected to Western blotting for cFLIP (5D8, Santa Cruz) and beta-catenin (BD Biosciences): inhibition of cFLIP decreased cytoplasmic beta-catenin **B/C Densitometry analysis of Western Blots**: Inhibition of cFLIP by siRNA results in a significant decrease in beta catenin levels in the MCF-7 (**B**) and MDA-231 (**C**) lines respectively (MCF-7 *p = 0.0001, 231; *p = 0.04, t-test) **D Immunofluorescence:** MDA-MB-231 cells transformed with siRNA targeting cFLIP or a scrambled control were immuno-stained for cFLIP (Cell Signalling) and Beta-catenin (BD Biosciences) and analysed by confocal microscopy (red = cFLIP, green = beta catenin, blue = DAPI): Inhibition of cFLIP resulted in a decrease in membrane bound beta-catenin. All results are averages of three independent experiments.

5.2.2 cFLIP Promotes Wnt-target Gene Expression

Having found that cFLIP inhibition reduced the levels of cytoplasmic beta-catenin we next set out to determine whether cFLIP inhibition also decreased Wnt-target gene expression. We first looked at the effect of cFLIP on expression of the positively regulated Wnt-target Axin2. Axin2 is a Wnt-target gene which is promoted consistently upon Wnt activation and therefore considered a reliable readout of Wnt activity compared to other Wnt-target genes which can be both promoted or suppressed by a Wnt signal depending on other factors (Jho *et al.* 2002). Following transfection with cFLIP siRNA or a non-specific scrambled control, MCF-7 and MDA-MB-231 cells were stimulated with Wnt3a to activate the Wnt pathway. In the cells transfected with scrambled siRNA, Wnt pathway activation resulted in an increase in Axin2 expression in both the MCF-7 and MDA-MB-231 lines compared to unstimulated cells, however this result was not statistically significant (Figure 5.2A and B). Following cFLIP inhibition, Axin2 expression was decreased significantly in both the MCF-7 and MDA-MB-231 lines (Figure 5.2C). This suggests that inhibition of cFLIP correlates with a reduction in Axin2 expression.

To support this observation, a luciferase reporter assay was also used; the TOPFlash assay uses a luciferase reporter plasmid containing TCF binding sites as a positive readout of Wnt-target gene transcription. Luciferase output is compared to that of the control FOPFlash plasmid which contains mutated TCF binding sites (Section 2.2.6). MCF-7 cells were transfected with siRNA or a scrambled control, or a cFLIP wild-type or nuclear over-expression vector or an empty vector control. The cFLIP over-expression wild-type and nuclear vectors were generated as described in Chapter 3, Figure 3.8. Each transfection also included the TOPFlash reporter plasmid or FOPFlash negative control. In all transfections, a LacZ reporter plasmid was also included to control for transfection efficiency. Upon transfection, cells were stimulated with Wnt3a to activate the Wnt pathway and luciferase output was measured after 48 h. All luciferase readings were first normalised to lacZ output to control for transfection efficiency, then to respective controls to subtract the background luciferase levels. In Wnt3a-stimulated cells, TOPFlash reporter activity was reduced following cFLIP inhibition and increased following cFLIP over-expression (Figure 5.2D). Over-expression of nuclear cFLIP increased luciferase output to a greater extent than wild-type cFLIP. While this would suggest that nuclear cFLIP promotes Wnt-target gene expression, the relative expression levels of wild-type and mutant cFLIP have not been quantified in this experiment and so we cannot conclude that this is the case. This result is also in contrast to the findings of Katayama *et al.* who reported that over-expression of wild-type cFLIP produced greater TOPFlash activity than over-expression of nuclear cFLIP (Katayama *et al.* 2010). This data is preliminary, and will require further investigation before a definitive conclusion can be made. Nevertheless, our data so far support the hypothesis that cFLIP promotes Wnt-target gene expression in the MCF-7 line.

Taken together these data (Figure 5.1-5.2) suggest that cFLIP is a positive regulator of the Wnt pathway in MCF-7 and MDA-MB-231 cells.



<u>**Figure 5.2:**</u> *cFLIP Promotes Wnt-target gene expression:*

qPCR: A MCF-7 and **B** MDA-MB-231 cells were cultured in 10 ng/ml Wnt3a for 48 h and then assayed for expression of the positive Wnt-target Axin 2 by q-RT-PCR: Wnt3a increased Axin2 expression but increase was not statistically significant ($p > 0.05$, t-test) **C** MCF-7 and MDA-MB-231 cells were transfected with siRNA targeting cFLIP or a scrambled control then cultured 10 ng/ml Wnt3a for 48 h and assayed for expression of cFLIP and the positive Wnt target Axin 2 by q-RT-PCR: Inhibition of cFLIP reduced Axin 2 expression significantly in both cell lines (MCF-7; *$p = 0.04$, MDA-MB-231; *$p = 0.004$, t-test) Results are averages of three independent experiments each performed with three internal technical replicates **D TOPFlash luciferase reporter assay:** MCF-7 cells which had been transfected with cFLIP siRNA or cFLIP overexpression vectors or non-specific controls were transfected with the TOPFlash Wnt reporter plasmid or mutant FOPFlash control then stimulated with 10 ng/ml Wnt3a and assayed for luciferase activity 48 h later: Inhibition of cFLIP reduced luciferase output whereas over-expression of wild-type or nuclear cFLIP increased luciferase output. This result represents a single dataset.

5.2.3 Inhibition of cFLIP Impairs Tumoursphere Self-Renewal

Wnt signalling is important in both triple-negative breast cancer and in breast cancer stem cells and as such is considered a promising target of novel therapeutics (Howe *et al.* 2004). In breast cancer cell lines and primary samples, Wnt pathway activation by Wnt3a is known to increase tumoursphere formation, and Wnt inhibition by Dkk decreases tumoursphere formation (Lamb *et al.* 2005). We have shown that cFLIP can promote the Wnt pathway, and as a result we hypothesised that in conjunction to sensitising cells to TRAIL-mediated apoptosis (Piggott *et al.* 2011), cFLIP inhibition may also have a detrimental effect on tumoursphere-formation via suppression of the Wnt pathway. In order to look at the effect of long-term cFLIP suppression on bCSC self-renewal, it was first necessary to generate breast cancer cell lines in which endogenous cFLIP was inhibited stably by shRNA. To do this, MDA-MB-231 and MCF-7 cells were transfected with lentiviral vectors containing shRNA targeting cFLIP (cFLIPSh) or a non-specific control (kind gifts from Dr. Ladislav Andera, Prague, Czech Republic). The cells were cultured under selection with puromycin; however stable cFLIP inhibition could not be maintained across passages (Figure 5.3A and B). In order to overcome this, clonal populations were generated from transfected cells: Cells were seeded at a density of approximately 0.5 cells per well in a 96-well plate, and expanded under puromycin selection (Figure 5.3C). In the MDA-MB-231 line, six cFLIPSh clones were generated whereas in the MCF-7 line, only one cFLIPSh clone survived. All subsequent assays were carried out on the clonal populations with confirmed cFLIP knockdown. MCF-7 lines over-expressing wild-type cFLIP were also generated as described in Chapter 3, Section 3.8.

To determine the effect of cFLIP inhibition on breast cancer stem-like cells, the stable shRNA lines were subjected to the tumoursphere assay. The six clones of the MDA-MB-231 line produced varying numbers of primary spheres which on average were not significantly different from the control cells indicating that in MDA-MB-231 cells, inhibition of cFLIP has no significant effect on primary tumoursphere formation (Figure 5.4A). In the MCF-7 cFLIPSh line, no significant change in primary or secondary tumoursphere formation was observed compared to the control line (Figure 5.4A and B). However, it was apparent that self-renewal of tumourspheres was perturbed as the number of TFUs in the MCF-7Sh line reduced from 7% on the first passage to 3% on the second passage (Figures 5.4A and B). Furthermore the MCF-7 cFLIPSh line could only be maintained in tumoursphere culture for four passages. In this single experiment, a significant decrease in secondary tumoursphere formation was observed. Tertiary tumoursphere formation was also perturbed significantly when compared to the non-specific shRNA control (Figure 5.4B and C). In the MDA-MB-231 line, no significant effect on secondary tumoursphere-formation was observed in the cFLIPsh cells; however we did observe a trend towards a decrease in secondary tumoursphere formation (Figure 5.4B). Preliminary data also suggests that overexpression of cFLIP did not effect primary tumoursphere formation but did result in a significant increase in tumoursphere self-renewal across three passages (Figure 5.4E). Taken together, these data suggest that cFLIP promotes the self-renewal of tumoursphere-forming cells.

In addition, no difference in sphere size was observed between cFLIPsh and control lines, or between cFLIP overexpression and control lines, suggesting that cFLIP does not effect TFU or progenitor proliferation (Figure 5.4D).

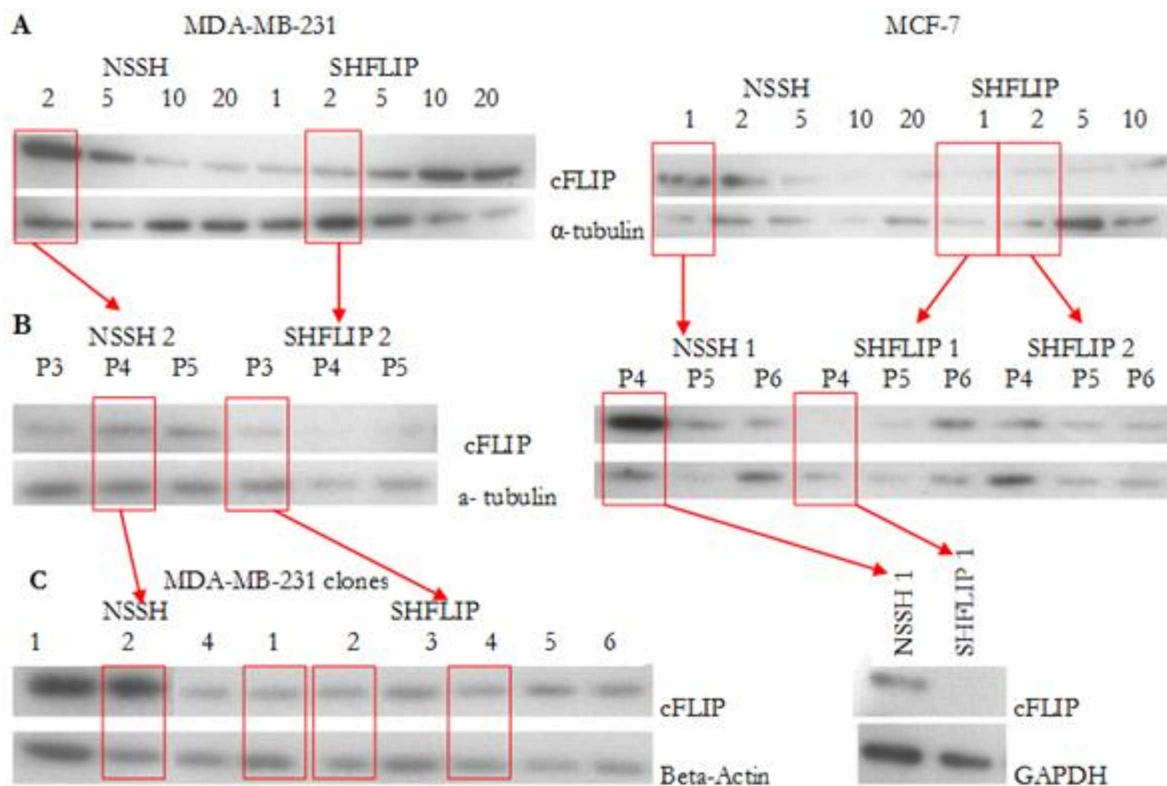


Figure 5.3: *Generation of cFLIP null cell lines:*

Western Blotting: A MCF-7 and MDA-MB-231 cell lines were transduced with lentivirus containing plasmid with shRNA targeting the long and short forms of cFLIP (SHFLIP) or a scrambled non-specific control (NSSH) at a range of viral titres (1, 2, 5, 10 and 20 µl). 48 h following transduction, cells were cultured under antibiotic selection and then assessed for cFLIP levels. The lower viral titres of 1 and 2µl produced the most efficient cFLIP inhibition **B** Most efficient cFLIP knockdowns were selected and stability of knockdown across passages under continued antibiotic selection was assessed; inhibition of cFLIP could not be maintained across three passages **C** For production of clonal SH populations, cells were diluted to a density of 0.5 cells per well and seeded into a 96-well plate. Clones were expanded under antibiotic selection and assessed for cFLIP knockdown. Six clonal populations were generated from the MDA-MB-231 line whereas in the MCF-7 line, only one clonal population remained viable.

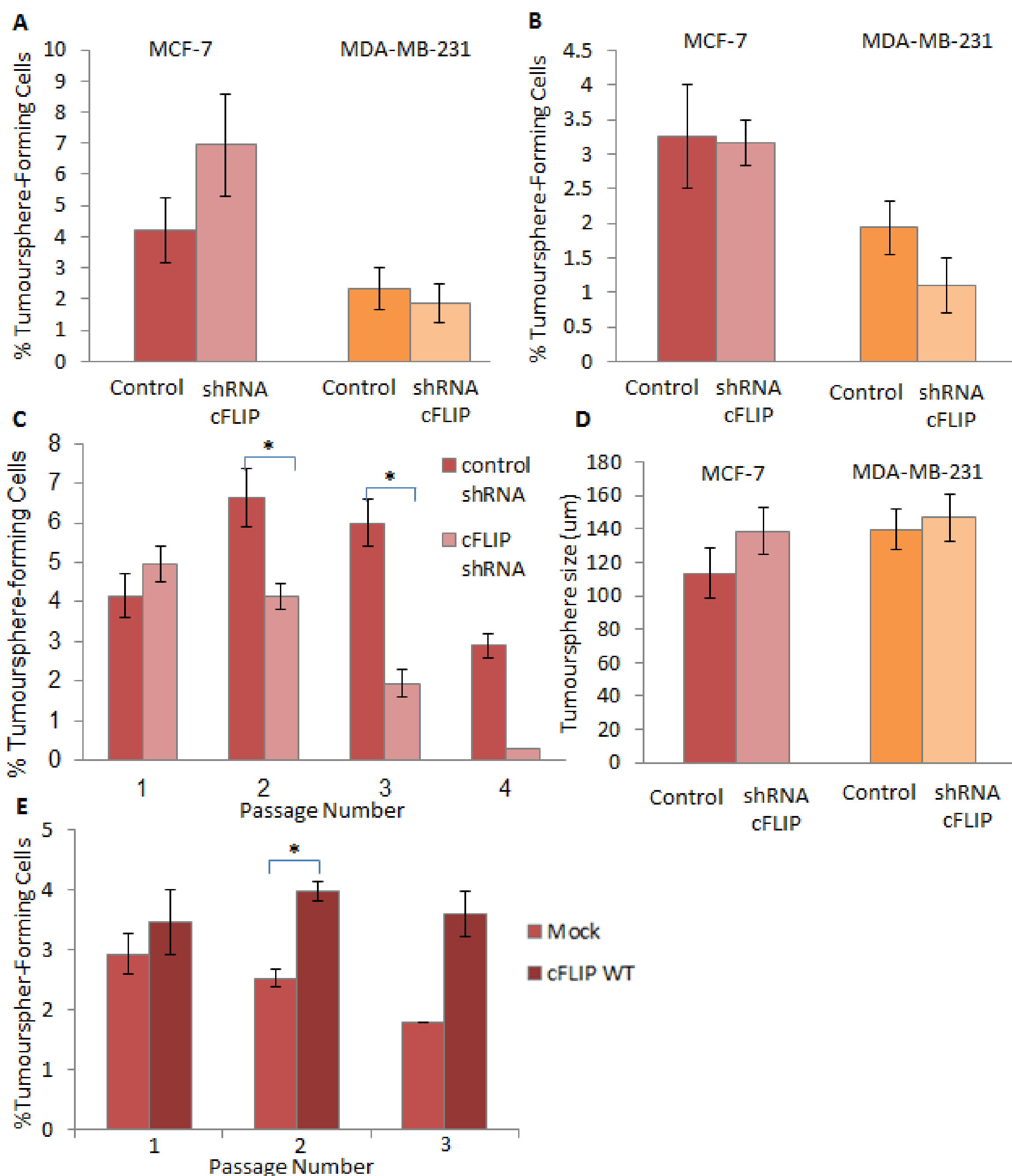
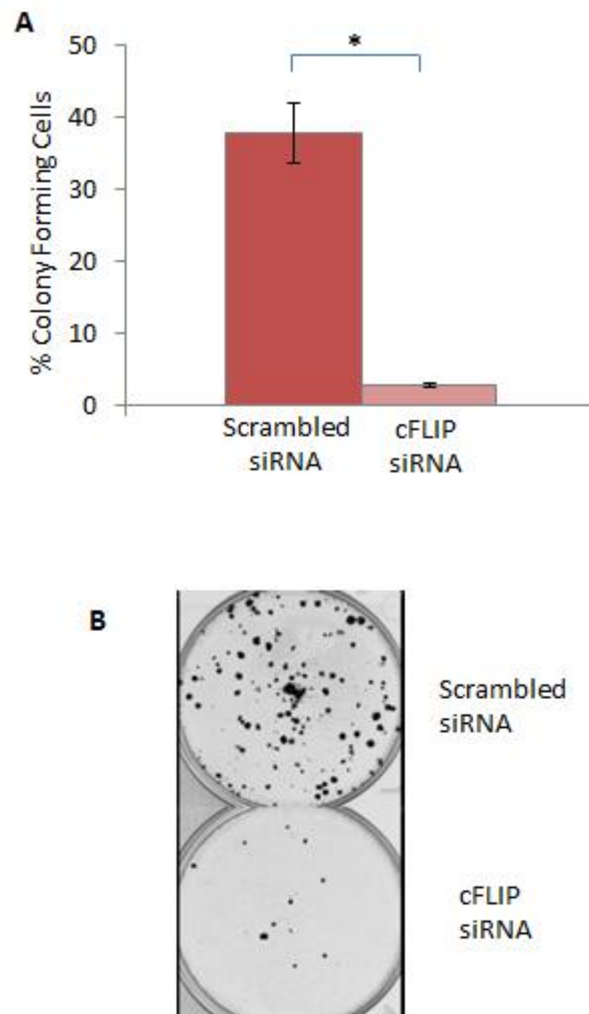


Figure 5.4: *Investigating the role of cFLIP in tumoursphere formation:* **Tumoursphere Assay: A Passage 1:** Stable inhibition of cFLIP increased tumoursphere formation in the MCF-7 line but result was not statistically significant, and had no significant effect on the MDA-MB-231 line (p> 0.05, t-test). **B Passage 2:** Stable inhibition of cFLIP produced fewer secondary spheres in both the MCF-7 and MDA-MB-231 lines but result was not statistically significant (p > 0.05, t-test) **C Passage 1-4:** The clonal cFLIPSh MCF-7 line produced significantly fewer secondary and tertiary tumourspheres and could not be cultured for more than four passages in tumoursphere culture (P2; *p = 0.03, P3; *p = 0.006, t-test, a single experiment with three internal replicates). **D Sphere size:** Stable cFLIP inhibition had no significant effect on tumoursphere size (p > 0.05, t-test) Figures A, B and D are averages of 3 independent experiments each with 3 internal technical replicates. **E Passage 1-4:** The cFLIP-overexpressing MCF-7 line produced significantly more secondary tumourspheres and more tertiary spheres than the mock-transfected control line (P2; *p = 0.006, t-test, a single experiment with three internal replicates).

5.2.4 Inhibition of cFLIP Impairs Colony Formation

As noted previously (Chapter 3.2), colony formation assess the ability of single cells when plated at low density to form colonies in adherent culture; a property of stem and progenitor cells (Harrison *et al.* 2012, Locke *et al.* 2005). To determine whether cFLIP inhibition effects colony formation, MCF-7 cells were transfected with siRNA targeting cFLIP or a non-specific scrambled control, then plated 48 hours later at a density of 50 cells per square centimetre. Colonies were allowed to form over a ten-day period and only those colonies containing 32 or more cells (having undergone 5 or more divisions) were counted (Harrison *et al.* 2012). Inhibition of cFLIP resulted in a significant reduction in colony formation (Figure 5.5A and B). This is in accordance with the finding that it was only possible to generate a single clonal cFLIPSh population in the MCF-7 line (Figure 5.3C).

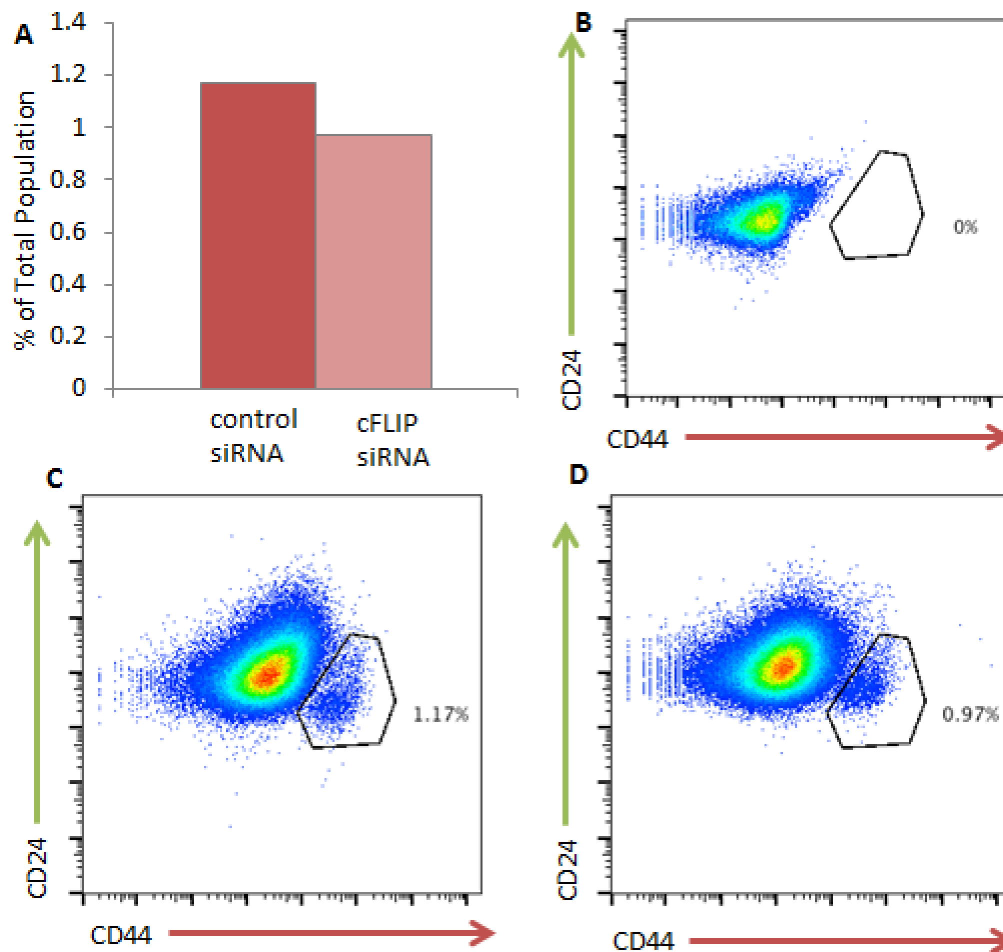


<u>Figure 5.5:</u> *Inhibition of cFLIP inhibits colony formation:*

Colony-Forming Assay: A MCF-7 cells transformed with siRNA targeting cFLIP or a scrambled control were seeded at a density of 50 cells/ cm^2 (160.2 cells/ml in a 6-well plate). Colonies were counted after 10 days culture. Inhibition of cFLIP by siRNA reduced significantly the number of colonies formed (*p = 0.01, t-test) **B** Representative image of colony formation

5.2.4 Investigating the effect of cFLIP inhibition has on the proportion of $CD44^+/CD24^-$ cells

We have shown that cFLIP promotes the self-renewal of tumourspheres in the MDA-MB-231 and MCF-7 breast cancer cell lines. The cell surface marker profile of $CD44^+/CD24^-$ has been shown to enrich for a mesenchymal subset of bCSCs (Liu *et al.* 2014). To establish the effect of cFLIP inhibition on mesenchymal-like bCSCs, MCF-7 cells were transfected with siRNA targeting cFLIP or a scrambled non-specific control siRNA, then 48 h later fixed and immuno-stained with fluorescence conjugated antibodies targeting CD44 and CD24 (BD Biosciences). Marker expression was then analysed by flow cytometry. Transient inhibition of cFLIP by siRNA in the MCF-7 line resulted in a modest decrease in the $CD44^+/CD24^-$ population (Figure 5.6). This data suggests that cFLIP inhibition reduces the bCSC population, which is in accordance with our previous data showing that cFLIP inhibition impairs the self-renewal and colony forming abilities of MCF-7 cells. However, this modest decrease is not reflective of the substantial effect on cFLIP inhibition on secondary and tertiary tumoursphere-forming cells, or colony-forming cells (Figures 5.4 and 5.5). This suggests that cFLIP inhibition may also be targeting an alternative stem-like population. However, this data is preliminary and will need to be repeated before a definitive conclusion can be made.



<u>Figure 5.6:</u> *Investigating the CD44+/CD24-/ESA+ marker profile of MCF-7 cells in which cFLIP has been inhibited.*

Flow Cytometry: A MCF-7 cells were transformed with siRNA targeting cFLIP or a scrambled control were stained with fluorescence-conjugated antibodies to CD44 (APC) and CD24 (FITC) and analysed by flow cytometry; inhibition of cFLIP decreased the proportion of $CD44^{+}/CD24^{-}$ cells

B-D Representative images of the CD44 (APC) and CD24 (FITC) profile for **B** unstained cells, **C** MCF-7 cells transfected with non-specific control siRNA, and **D** MCF-7 cells transfected with siRNA targeting cFLIP. Results represent a single dataset.

5.3 Discussion

Wnt signalling is important in mediating the malignant phenotype of breast and other cancers and consequently there is much interest in developing Wnt-inhibitors as potential anti-cancer therapeutics (Howe *et al.* 2004, Anastas and Moon 2013). Previous studies have shown that cFLIP can promote Wnt signalling in lung carcinoma cell lines (Naito *et al.* 2004, Katayama *et al.* 2010). We set out to determine whether cFLIP can promote Wnt signalling in breast cancer cell lines.

Here we show that cFLIP is a positive regulator of Wnt signalling in breast cancer cell lines. It has been shown previously in lung carcinoma lines that cFLIP regulates canonical Wnt signalling by preventing the ubiquitylation and consequent degradation of beta-catenin in the cytoplasm, and also by complexing with transcription factors in the nucleus to promote Wnt-target gene expression (Naito *et al.* 2004, Katayama *et al.* 2010). Our data are in accordance with this study in that the inhibition of cFLIP by siRNA results in a decrease in both beta-catenin protein levels and Wnt-target gene expression, whereas over-expression of cFLIP promotes Wnt-target gene expression. These data suggest that cFLIP regulates the Wnt pathway in the same manner in both lung and breast cancer cell lines. However, further investigation is required to determine the effect of cFLIP inhibition on the phosphorylation and ubiquitylation of beta-catenin, and to determine the relative contributions of cytoplasmic and nuclear cFLIP to promoting Wnt-target gene expression. In addition, it will be of interest to investigate potential binding partners of cFLIP, for example beta-catenin or components of the TCF transcription complex in the nucleus. This has the potential to lead to the identification of unique targetable regions of cFLIP not possessed by caspase 8.

Wnt signalling is known to play an important role in the self-renewal and maintenance of cancer stem cells in breast and other cancers (Howe *et al.* 2004). As cFLIP inhibition is able to abrogate this pathway, we hypothesised that inhibition of cFLIP may reduce bCSC number or self-renewal. Analysis of clonal populations in which cFLIP had been stably inhibited by shRNA showed a non-significant trend for an increase in first passage tumourspheres. However, analysis of four passages of tumoursphere formation revealed a significant reduction in self-renewal following stable cFLIP inhibition by shRNA, and a significant increase in self-renewal following over-expression, when compared to respective controls. In addition, cFLIP inhibition by siRNA reduced colony formation significantly but this significant decrease was not reflected in a preliminary analysis of the EMT-like $CD44^{+}/CD24^{-}$ population (Figure 5.6), which showed only a modest decrease in this population. Taken together these data suggest that cFLIP does not affect significantly the EMT-like tumoursphere forming CSC population.

These data are reflective of a recent study which has revealed the presence of two bCSC subpopulations in breast cancer, defined by the two marker profiles of $CD44^{+}CD24^{-}$ and $ALDH^{+}$ (Ginestier *et al.* 2007, Liu *et al.* 2013). The $CD44^{+}CD24^{-}$ profile is thought to describe an EMT-like bCSC subpopulation which is quiescent in nature whereas $ALDH^{+}$ describes an MET-like subpopulation which is proliferative. We have so far only looked at the effect of cFLIP inhibition on the $CD44^{+}CD24^{-}$ population. It would be interesting to determine the effect of cFLIP inhibition on the $ALDH^{+}$ (proliferating) population. As we have shown that cFLIP inhibition prevents colony formation and self-renewal, we would hypothesise that cFLIP inhibition reduces the $ALDH^{+}$ subpopulation of bCSCs. We

have not yet determined the mechanism by which cFLIP inhibition impairs colony formation and self-renewal; further investigation is required to determine whether inhibition of cFLIP induces apoptosis or quiescence of the stem-like cells, or as cFLIP promotes Wnt signalling, whether cFLIP inhibition impairs the rate of self-renewal.

It has also been shown that these two bCSC subpopulations are capable of inter-conversion via EMT and MET (Liu *et al.* 2013. Preliminary data suggests that cFLIP inhibition has a limited effect on the EMT-like bCSC cells and we hypothesise that cFLIP is instead targeting the MET-like subpopulation. It would be interesting to investigate whether cFLIP inhibition promotes the transition of cells from $ALDH^+$ to $CD44^+CD24^-$, and therefore that endogenous cFLIP functions to promote the reverse transition of $CD44^+CD24^-$ to $ALDH^+$, possibly via MET. Future investigations will determine whether purified bCSC subpopulations can transition in the absence of cFLIP. As we did not observe an increase in the EMT-like $CD44^+CD24^-$ subpopulation, our data suggests that this is not the case. However we have not yet determined how long-term suppression of cFLIP affects bCSC subpopulations and it may be the case that greater than forty-eight hours is required in order to observe an accumulation in the $CD44^+CD24^-$ compartment.

Although we have carried out a comparison of transient and stable cFLIP inhibition, our results are limited by the use of single cFLIP shRNA clonal populations. Our findings will need to be confirmed following the generation of a conditional cFLIP shRNA vector which would allow for an intrinsically-controlled system free from any artefacts of clonal expansion. This system would also allow us to test the effects of cFLIP inhibition on tumour initiation *in vivo*, which as with all studies of CSCs, is the gold standard analysis of cancer stem cells. We have not yet tested the effect of cFLIP inhibition on tumour initiation, or serial transplantation of tumour xenografts. On the basis of our data so far, we would hypothesise that cFLIP inhibition decreases tumour growth and the ability of tumour cells to survive serial passaging. However, without a conditional system, a definitive conclusion as to the effect of cFLIP inhibition on tumour-initiating cells cannot yet be made.

Although we have shown an effect of cFLIP inhibition on both Wnt signalling and tumoursphere self-renewal, we have not yet established a link between the two. We would hypothesise that inhibition of cFLIP prevents tumoursphere self-renewal via abrogation of the Wnt signalling pathway. Further investigation is required to determine whether the reduction in tumoursphere self-renewal in the cFLIPSh clonal populations can be rescued by the promotion of Wnt signalling, however as cFLIP may act at the promoter complex and therefore downstream of any point of intervention, this may be difficult to achieve. It may be possible to show that an increase in tumoursphere self-renewal by beta-catenin over-expression for example, can be reversed by inhibition of cFLIP.

It would also be of interest to determine whether activation of Wnt signalling re-localises cFLIP to the nucleus. If this were to be the case, we would then hypothesise that activated Wnt signalling also sensitises cells to TRAIL, which may explain the correlation between mesenchymal-like cells, bCSCs and TRAIL-sensitivity. This relationship will be discussed in greater detail in Chapter 7.

In conclusion, we have shown that inhibition of cFLIP abrogates Wnt signalling, tumoursphere self-renewal and colony formation in breast cancer cell lines. These data suggest that as well as sensitising

bCSCs to TRAIL (Piggott *et al.* 2011), cFLIP inhibition alone may have a detrimental effect on bCSCs, and this further promotes the development of cFLIP inhibitors as potential therapeutics for treating breast cancer. However we have not yet determined whether cFLIP inhibition causes apoptosis or quiescence of bCSCs. As quiescent bCSCs have been implicated in tumour relapse this may call into question the suitably of cFLIP inhibition as a monotherapy and suggest that a cFLIP inhibitor would only be advantageous in the presence of other agents such as TRAIL in order to deplete the stem-cell fraction and therefore reduce the likelihood of tumour recurrence.

Chapter 6: Investigating a Functional Model of Breast Cancer Stem Cell Plasticity

Chapter 6: Investigating a Functional *in vitro* Model of Breast Cancer Stem Cell Plasticity

6.1 Introduction

Previous studies have shown that non-stem cancer cells can de-differentiate to a stem-like state, a process termed CSC plasticity (Gupta *et al.* 2011, Chaffer *et al.* 2011). The clinical implication of plasticity is that even if it were possible to eliminate all CSCs from a tumour, residual bulk tumour cells post-therapy would have the capacity to de-differentiate to a CSC-like phenotype and consequently re-seed tumour growth. Therefore an improved CSC-based therapeutic strategy would be a combined treatment to both eliminate CSCs and prevent plasticity. An understanding of the mechanisms underlying plasticity will aid the development of therapeutic strategies aimed at preventing this *de novo* generation of bCSCs within a tumour.

The study of plasticity has proven difficult as it cannot be observed simply as an increase in CSCs, as this could occur through increased CSC self-renewal. Instead it relies on the ability to assay subpopulations of cancer cells separately. Functionally it is first necessary to denude a population of CSCs in order to study their reacquisition; if a certain stem-like characteristic were to appear in a population previously devoid of such an attribute, only then would it be possible to conclude that plasticity had occurred and not self-renewal. The current evidence for bCSC plasticity relies heavily on the use of surrogate markers to define bCSC and non-bCSC populations (Gupta *et al.* 2011 Chaffer *et al.* 2011). Whilst such studies have been instrumental in highlighting the importance of CSC plasticity as a phenomenon in cancer, the field is limited by the lack of functional models, and unsurprisingly the mechanisms underlying CSC plasticity are poorly understood.

We have recently demonstrated that TRAIL can eliminate selectively and completely, all tumoursphere-forming cells from breast cancer cell lines *in vitro*. This required sensitisation of the tumoursphere-forming cells by siRNA inhibition of cFLIP. Importantly, although all tumoursphere-forming ability is lost in this model, viable cells remain following treatment and reacquire the ability to form tumourspheres (Piggott *et al.* 2011). Our report of a complete loss of bCSC activity is significant not just for its therapeutic implications but because of the fact that as viable non-tumoursphere-forming cells remain, it also provides us with a unique model with which to study functional bCSC plasticity.

There is a clear need to further our understanding of CSC plasticity as a phenomenon and identify inhibitors of this process. To this end, we set out to investigate the underlying mechanisms involved in the reacquisition of bCSC characteristics in our model.

6.2 Results

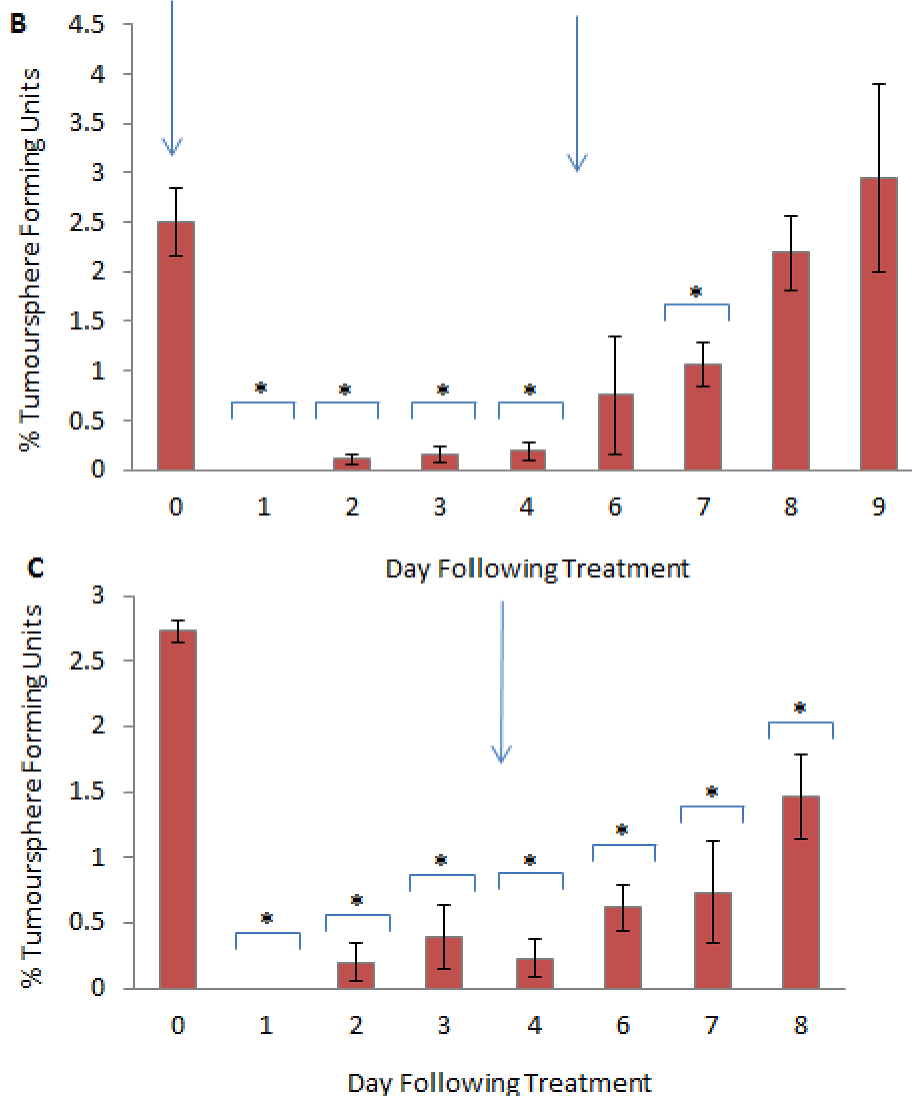
6.2.1 Characterising an *In vitro* Functional model of bCSC Plasticity

As stated previously, TRAIL/FLIPi treatment results in the complete eradication of tumoursphere-forming ability. Cells which survive this treatment are able to reacquire tumoursphere-forming ability following continued adherent culture (Piggott *et al.* 2011). In order to develop this model we wished firstly to establish the kinetics of this recovery. MCF-7 cells which had been treated with siRNA targeted to cFLIP for 48 h were treated with TRAIL for 18 h then subjected to the tumoursphere assay to confirm complete tumoursphere loss. The remaining cells were re-seeded in adherent conditions and left to recover over a period of eight to nine days before re-seeding into tumoursphere conditions to determine the proportion of tumoursphere-forming cells in the recovered cell population (cells were maintained in tumoursphere medium for seven days before counting). Sufficient samples were used to allow for tumoursphere-forming potential to be assayed for each of the eight days of the resting cell population (except for day five, which was left for an additional 24 hours to allow for the accumulation of soluble factors which may be involved in the process of recovery). The proportion of primary tumoursphere forming cells increased gradually over the eight day resting period, eventually reaching the level seen in wild type MCF-7 populations. These tumourspheres were able to self-renew, but second-passage sphere formation did not reach the same level as untreated cells by day eight (Figure 6.1A and B).

Taken together, these data confirmed that the combined treatment of cFLIP inhibition by siRNA with administration of TRAIL is able to deplete all tumoursphere-forming ability from the MCF-7 line. Following continued culture of surviving cells, primary (first-passage) tumoursphere formation is reacquired but secondary (second-passage) tumoursphere formation does not reach the same level as untreated cells by eight days of adherent culture. It would be expected that further culture of surviving cells would restore secondary tumoursphere-forming ability to the same level as an untreated population.

A

Day	-2	-1	0	1	2	3	4	5	6	7	8	9
Procedure	siRNA		TRAIL	Surviving Cells to adherent conditions				Media Change				
Assay	Tumoursphere Assay scrambled control			Tumoursphere Assay FLIPi/TRAIL								



<u>Figure 6.1:</u> *An* in vitro *Model of bCSC Plasticity* **A Plasticity Assay Schematic** MCF-7 cells which had been treated with siRNA targeted to cFLIP for 48 h were treated with TRAIL for 18 h then subjected to the tumoursphere assay to confirm complete tumoursphere loss (day 1). The remaining cells were re-seeded in adherent conditions and left to recover over a period of 8-9 days. Tumoursphere-forming potential was assayed for 8 days out of 9: and tumourspheres counted and passaged following 7 days non-adherent culture **B** Following FLIPi/TRAIL treatment MCF-7 cells lost all primary tumoursphere-forming potential but surviving cells reacquired primary tumoursphere-forming ability on day two, which increased to the same level as untreated cells by day 9.**C Passage 2:** Tumourspheres formed by surviving MCF-7 cells were able to be passaged, but secondary tumoursphere formation did not reach the same level as untreated cells by day 9. All results are averages of three independent experiments each performed with three internal technical replicates, (*$p<0.05$, t-test).

6.2.2 Developing an *In vitro* Functional model of bCSC Plasticity

We also wished to determine whether the model could be extended to include other cell lines and functional attributes of bCSCs. To this end, the plasticity assay was also performed using the SKBR3 cell line: Following FLIPi/TRAIL treatment, all primary tumoursphere-forming ability was lost but reacquired during eight days recovery in adherent culture (Figure 6.2A). Preliminary data suggests that secondary tumoursphere-forming ability is also reacquired (Figure 6.2B).

Having demonstrated the loss and reacquisition of the bCSC characteristic of tumoursphere formation, we next asked whether colony-forming ability could be lost and reacquired in this manner. Indeed, FLIPi/TRAIL treatment resulted in 100% loss of colony forming ability in the MCF-7 line. Following eight days recovery in adherent culture, surviving cells were able to reacquire the ability to form colonies but were not able to form proportionately as many colonies as untreated cells (Figure 6.2C and D).

Taken together, these data (Figure 6.1 and 6.2) show that following ablation of tumoursphere or colony-forming ability, continued culture of surviving cells results in both attributes being reacquired but colony formation is still perturbed significantly even eight days following treatment. This suggests that tumoursphere-forming ability recovers more quickly than colony-forming ability under these conditions. As so few cells remain following FLIPi/TRAIL, the nature of the plasticity model relies on the ability of the surviving cells to repopulate from single cells, and therefore the model itself is essentially a colony-forming assay. As the surviving cells are by no means confluent by the eighth day of culture, this result could be predicted. It would be expected that a confluent culture of surviving cells would have colony-forming abilities comparable to those of an untreated population. However, it may be the case that our combined FLIPi/TRAIL-treatment induces quiescence in the surviving population which would prevent recovery in the short-term. This possibility could be addressed by analysing the surviving population by flow cytometry for cell cycle stage or for the distribution of pyronin y and hoescht staining; low pyronin y levels are indicative of quiescence (Shapiro 1981).

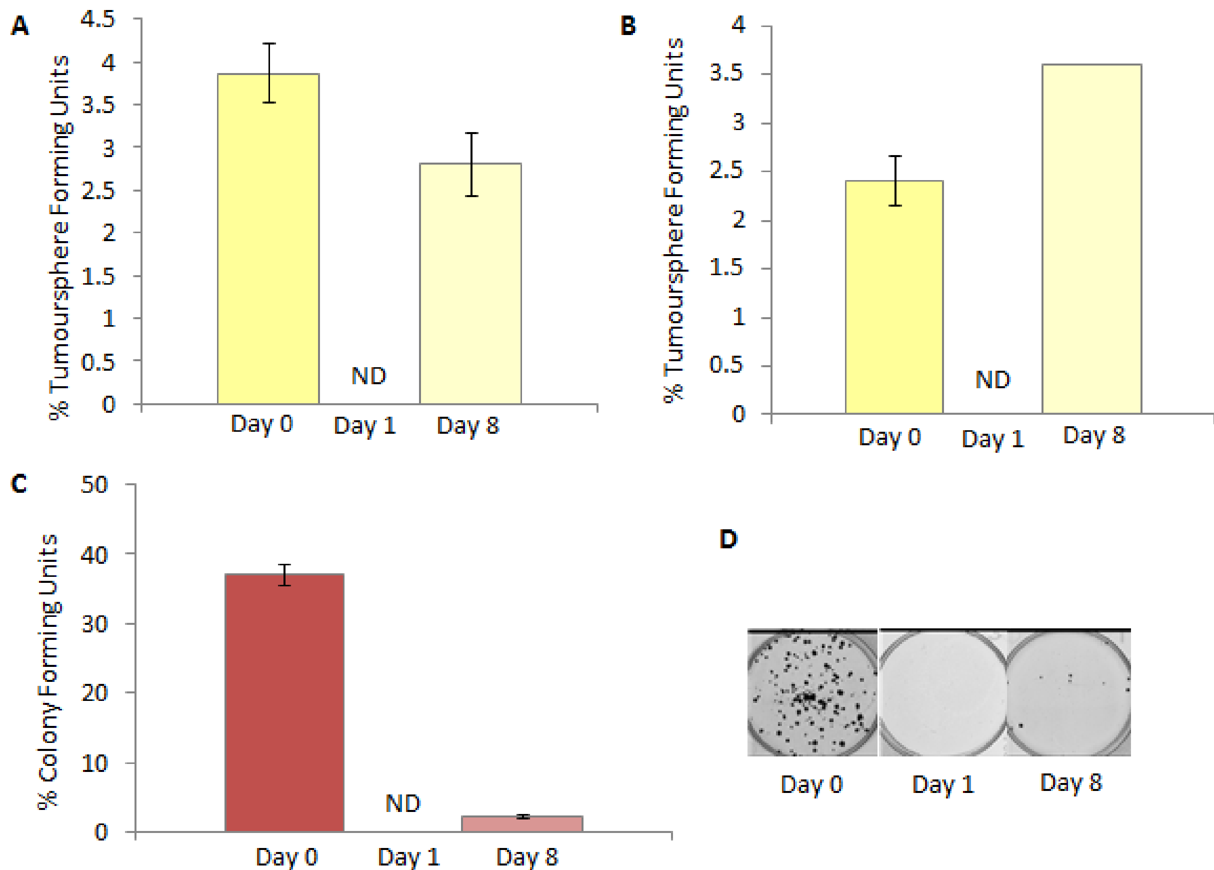


__Figure 6.2:__ *An* in vitro *Model of bCSC Plasticity*

A Plasticity Tumoursphere-Forming Assay: Following FLIPi/TRAIL treatment, SKBR3 cells lost all tumoursphere-forming ability but surviving cells reacquired tumoursphere-forming ability by day 8. **B Passage 2:** Tumourspheres formed by surviving SKBR3 cells were able to be passaged **C Colony Forming Assay:** Following FLIP inhibition by siRNA, cells were seeded at a density of 50 cells/ cm^2 (160.2 cells/ml in a 6-well plate) in the presence or absence of 20 ng/ml TRAIL for 10 days. Cells were also re-seeded at high density in adherent conditions and left to recover over a period of 8 days then subjected to the colony-forming assay: FLIPi/TRAIL treatment depleted all colony-forming ability in the MCF-7 line. Surviving cells reacquired colony-forming ability by day 8, but not to the same level as untreated cells **D** Representative images of colony-forming assay. Each dataset represents a single experiment.

6.2.3 Inhibition of TGFBeta receptors impairs the reacquisition of tumoursphere and colony-forming cells following FLIPi/TRAIL

To further our investigation into the mechanism underlying bCSC reacquisition, recovering cells were cultured in the presence or absence of a number of inhibitors of pathways which we postulated could be involved in bCSC plasticity: SB431542 is an inhbitor of the ALK receptors which recognise the ligand TGFbeta. TGFBeta is thought to participate an EMT-like process in cancer and via EMT is also thought to increase bCSCs by plasticity and not by self-renewal (Morel *et al.* 2009). The tankyrase inhibitor abrogates the Wnt pathway, which is known to be involved in the maintenance of bCSC-like characteristics (Karlberg *et al.* 2010). The cytokine IL6 also functions to maintain bCSCs (Liu *et al.* 2011).

Inhibition of tankyrase did not alter significantly the reacquisition of tumoursphere-forming ability in our model, however there was a trend to an increase in tumoursphere-forming ability. However as expected, tankyrase inhibition reduced the self-renewal of those spheres which were formed by surviving cells (Figure 6.3A and B). Inhibition of the IL6 receptor increased significantly the reacquisition of tumoursphere-forming ability in our model, but reduced the self-renewal of those spheres which were formed by surviving cells (Figure 6.3A and B). These data suggest that inhibition of tankyrase or IL6 does not inhibit the reacquisition of tumoursphere-forming cells in our model. The ability of IL6 receptor or tankyrase inhibition to increase tumoursphere formation may indicate that these signalling pathways are involved in the inhibition of plasticity, perhaps as a negative feedback to maintain tumourshere-forming ability.

Inhibition of TGFBeta receptors by SB431542 reduced significantly the reacquisition of sphere-forming and colony-forming ability without affecting tumoursphere self-renewal (Figure 6.3A and B). This suggests that TGFbeta signalling may be responsible for plasticity in our model. However, although we have shown that inhibition of TGFbeta signalling does not impair the self renewal of tumourspheres, our investigation does not go far enough to rule out the possibility that the initial spheres were reacquired by plasticity, but that inhibition of TGFbeta signalling only impaired their self-renewal. It will be necessary to compare the self-renewal of spheres formed by surviving cells much earlier in the model, at one or two days following treatment, in order to rule out this possibility.

Although these data give preliminary indications of pathways which may be involved in bCSC plasticity, our data is limited by the lack of confirmation of inhibitor function. Without proof that our inhibitors function as expected, no definitive conclusions can be made.

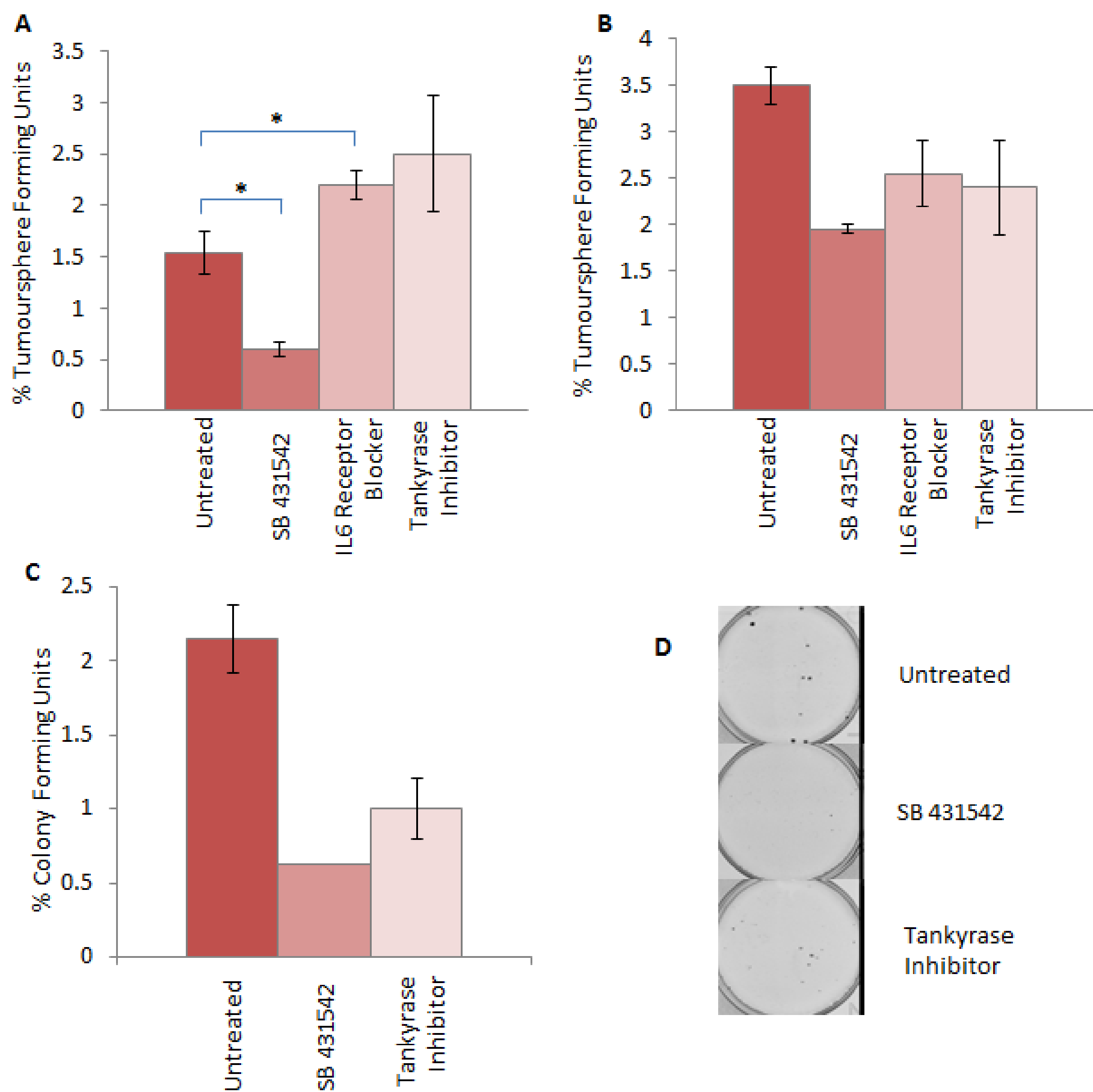
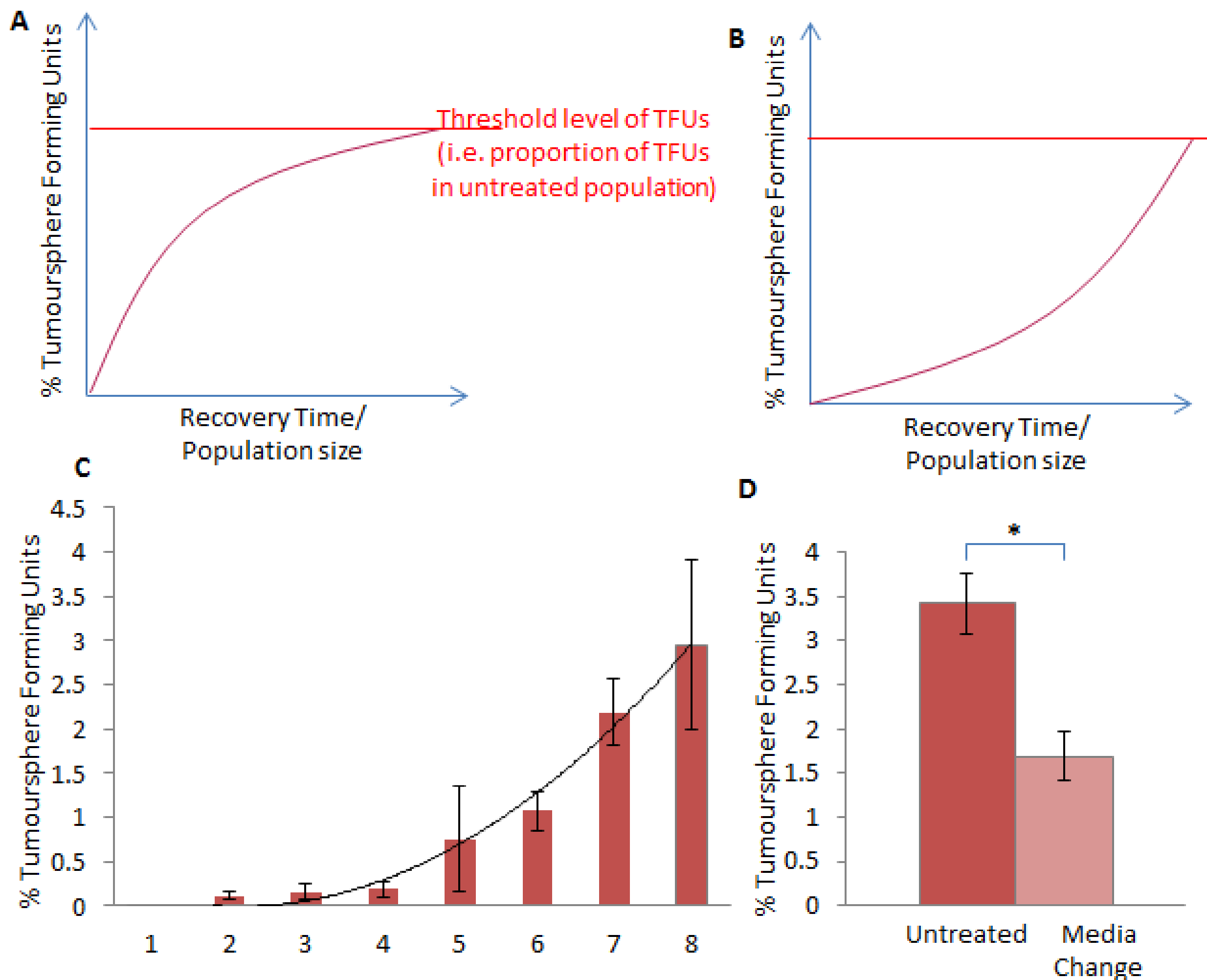


Figure 6.3: *Inhibition of activin nodal signalling impairs the reacquisition of tumoursphere-forming ability following FLIPi/TRAIL*

A Tumoursphere Assay: Following treatment with TRAIL/FLIPi, surviving MCF-7 cells were cultured in the presence or absence of inhibitors: SB431542 (TGFbeta receptor inhibitor) IL6 receptor blocking antibody, or tankyrase inhibitor (Section 2.2.3, Tables 2.12 and 2.13). Tumoursphere-forming potential was assayed after 8 days recovery: Treatment with SB431542 decreased significantly the number of primary tumoursphere-forming cells (*p = 0.02, t-test). Treatment with an IL6-receptor blocking antibody increased significantly the number of primary tumoursphere-forming cells (*p = 0.03, t-test). The tankyrase inhibitor had no significant effect on tumoursphere-forming ability ($p > 0.05$, t-test). Dataset represents two independent experiments each performed with three internal technical replicates. **B Passage 2**: IL6 receptor blocking antibody and tankyrase inhibitor impaired tumoursphere self-renewal. Dataset represents two independent experiments. **C Colony-Forming Assay:** Following treatment with TRAIL/FLIPi, surviving MCF-7 cells were cultured in the presence or absence of inhibitors: SB431542, or tankyrase inhibitor. Colony-forming potential was assayed after 8 days recovery, data represents a single experiment. **D** Representative images of colony forming assay; data represents a single experiment.

6.2.4 Can soluble factors promote the reacquisition of tumoursphere and colony-forming cells following FLIPi/TRAIL?

The kinetics of the model shown in figure 6.1 could be explained either by the reacquisition of tumoursphere forming ability instigated by a soluble factor produced by the surviving cells, a cell-autonomous event, or the recovery of existing but dormant cells. A cell-autonomous model of bCSC plasticity would state that at any given moment in time, a certain proportion of cells within a population would function as bCSCs. This would imply that, following recovery from TRAIL/FLIPi, providing the surviving cells were not quiescent, the number of TFUs in a population would be relatively stable, and therefore would regenerate more rapidly than has been observed. This means that the number of TFUs would be relative to the population size and therefore maintained at a stable level (Figure 6.4A). However, tumoursphere generation is gradual and not in proportion to population numbers (Figure 6.4B and C). This suggests that either the population is recovering from dormancy or that tumoursphere-forming ability is acquired *de novo* by the surviving cells. Due to the role of cFLIP and TRAIL in apoptosis, we first hypothesised that TFUs were initially lost by cell death, and therefore not induced into quiescence. Therefore we hypothesised that plasticity occurs due to the presence of a soluble factor(s) produced by the surviving cells. To test this possibility, the culture medium of the surviving adherent cells was replenished continually over the course of one week. Constant media change decreased significantly the number of TFUs in the population by 50% (Figure 6.4D). This suggests that a soluble factor(s) is involved in the process of plasticity in this model. However we have not yet determined whether surviving cells are quiescent and so the dormancy model cannot yet be ruled out.



<u>Figure 6.4:</u> *An* in vitro *Model of bCSC Plasticity*

Plasticity Assay Schematics A Cell autonomous model, in a recovering population, the number of TFUs is related to population size and therefore the proportion of TFUs remains stable over time. **B** Alternative model; In a recovering population, the number of TFUs is not related to the population size but another factor such as a soluble plasticity signal, and therefore increases gradually. **C Plasticity assay as Figure 6.1:** MCF-7 cells which had been treated with siRNA targeted to cFLIP for 48h were treated with TRAIL for 18h then subjected to the tumoursphere assay to confirm complete tumoursphere loss (day 1). The remaining cells were re-seeded in adherent conditions and left to recover over a period of 8-9 days. Tumoursphere-forming potential was assayed for 8 days out of 9; a gradual increase in tumrousphere-forming cells was observed. **D Tumoursphere-Forming Assay:** Following treatment with TRAIL/FLIPi, surviving MCF-7 cells were subjected to continual media change over a one week period and tumoursphere formation was assayed after 8 days recovery: media change reduced significantly the number of tumoursphere-forming cells in the surviving population (*p = 0.009, t-test). All data are averages of three independent experiments each performed with three internal technical replicates.

6.2.5 Does long term suppression of cFLIP prevent the reacquisition of tumoursphere-forming cells following FLIPi/TRAIL?

Our plasticity model described in section 6.1 loses tumoursphere-forming ability following cFLIPi/TRAIL treatment and reacquires tumoursphere-forming ability over a period of eight days. As the nature of our dual treatment is transient, it may be the case that bCSC characteristics are reacquired as cFLIP returns and TRAIL is lost from the system. It is possible that either the loss of TRAIL or the reacquisition of cFLIP may be responsible for the reacquisition of bCSC characteristics. Although requiring confirmation, our previous data suggest that stable inhibition of cFLIP is able to reduce tumoursphere formation over four serial passages (Chapter 5 Figure 5.4 and 5.5). Although our data suggest cFLIP inhibition impairs self-renewal, we cannot rule out the possibility that tumoursphere-forming cells are also being lost by inhibition of plasticity. Therefore we hypothesised that inhibition of cFLIP would impair the reacquisition of tumoursphere-forming cells in our plasticity model.

Unfortunately it was impossible to test this possibility using the clonal populations of MCF-7 cells in which cFLIP had been stably inhibited by shRNA, due to the reduced sensitivity of the TFUs to TRAIL when compared with siRNA inhibition of cFLIP (data not shown). Whilst cFLIP siRNA completely sensitises TFUs to TRAIL, the shRNA is less effective, meaning a few spheres remain following treatment. The nature of the plasticity model requires that all tumoursphere-forming ability is lost in the population in order to study its return. If even one TFU remains in the population following treatment, it would not be possible to conclude whether any reacquisition observed is due to plasticity or simply the self-renewal of the existing TFU. In an attempt to overcome this issue, a small molecule inhibitor of cFLIP was used which is currently in development in the host lab. Compound OH14 was designed to inhibit the interaction between cFLIP and FADD however this has not yet been confirmed by biological assays. It has been established that OH14 sensitises breast cancer cell lines including tumoursphere and colony-forming cells to TRAIL (data not shown). Although OH14 is only designed to inhibit the interaction with FADD it was still of interest to test whether it abrogates any other functions of cFLIP and is a useful tool for its long term suppression.

To determine whether OH14 suppresses plasticity, following tumoursphere ablation by FLIPi/TRAIL, surviving cells were cultured in the presence or absence of 10 μM OH14 for 7 days; culture media and drug was replenished daily. Treatment of surviving cells with OH14 induced complete cell loss by day 4 of treatment. This suggests that either repeated administration of OH14 induces cell death, or that OH14 prevents the reacquisition of bCSCs, resulting in the eventual death of the remaining population which cannot propagate or survive without bCSCs. Prolonged treatment of MCF-7 cells in adherent conditions with OH14 did not affect cell viability by the cell titre blue assay (data not shown) but did result in a significant reduction in tumoursphere formation (Figure 6.5C). In contrast, a single 24 h treatment with OH14 had no significant effect on the tumoursphere-forming ability of MCF-7 cells (Figure 6.5D). The fact that only a long term treatment of OH14 impairs tumoursphere formation is indicative of OH14 affecting a gradual process such as plasticity as opposed to cell death. However,

although prolonged treatment with OH14 does not reduce cell viability (data not shown), it cannot be ruled out that a minority population is affected. Much further investigation into the role of cFLIP in plasticity model is required. These data are preliminary and without a shRNA inhibition of cFLIP as a proof-of-principle, no definitive conclusions can be made.

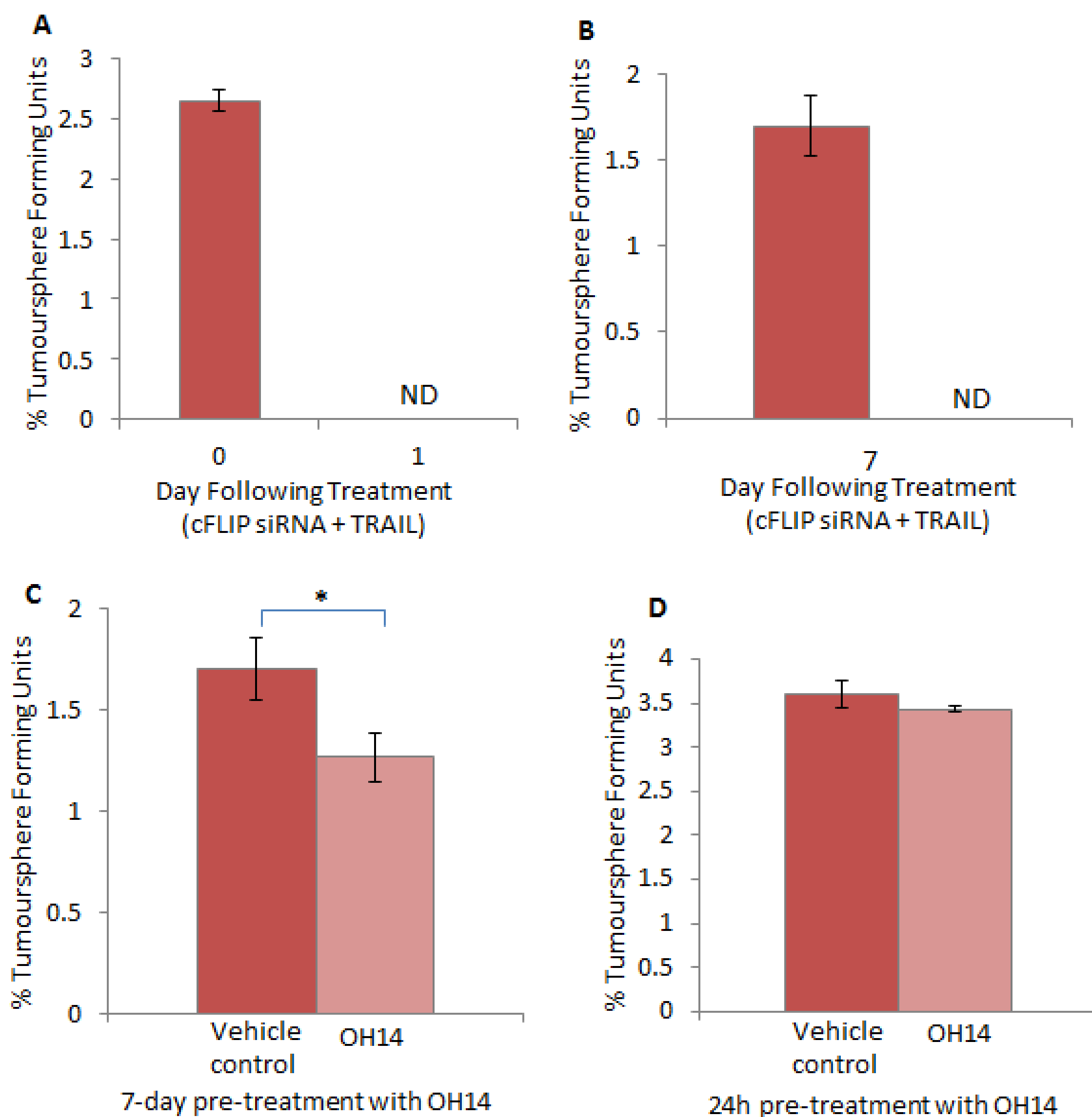


Figure 6.4: *Treatment with compound OH14 impairs the reacquisition of tumoursphere-forming ability following FLIPi/TRAIL* **A Plasticity Assay** MCF-7 cells which had been treated with siRNA targeted to cFLIP for 48h were treated with TRAIL for 18h then subjected to the tumoursphere assay to confirm complete tumoursphere loss (day 1). The remaining cells were re-seeded in adherent conditions and left to recover over a period of 7 days in the presence or absence of the cFLIP inhibitor OH14. **B Plasticity Assay:** Tumoursphere-forming potential was assayed after 7 days recovery: Treatment with OH14 completely eradicated all surviving cells. **C Tumoursphere Assay:** MCF-7 cells were cultured in adherent conditions for a period of 7 days in the presence or absence of the cFLIP inhibitor OH14. Tumoursphere-forming potential was assayed after 7 days: 7-day treatment with OH14 reduced significantly the number of tumoursphere-forming cells (*p = 0.04, t-test). **D Tumoursphere Assay:** MCF-7 cells were cultured in adherent conditions in the presence or absence of the cFLIP inhibitor OH14 for 24h before tumoursphere-forming potential was assessed: A single 24 h treatment with OH14 did not reduce significantly the number of secondary tumoursphere-forming cells. All data represents single experiments each performed with three internal technical replicates

6.3 Discussion

Plasticity of bCSCs has so far been studied using marker profiles and relatively few studies of functional plasticity have been performed (Morel *et al.* 2008, Zomer *et al.* 2013). Here we have described an *in vitro* model of bCSC plasticity in which tumoursphere and colony-forming ability is reacquired in a cell population previously denuded of both by treatment with cFLIP siRNA and TRAIL. The advantage of this model is that it assays for functional characteristics associated with bCSCs. There are two possible explanations for the loss of bCSC characteristics in our model: Firstly that tumoursphere or colony-forming cells are killed by the dual FLIPi/TRAIL treatment and that their reacquisition occurs via the *de novo* generation of these attributes in cells which did not have them previously. Alternatively, FLIPi/TRAIL treament may induce quiescence in the bCSC-like population, which is overcome gradually as FLIPi/TRAIL depletes from the system. Due to the ability of TRAIL and cFLIP inhibition to activate the extrinsic apoptosis pathway, we hypothesise that bCSCs are lost by cell death. This will be addressed by determining whether inhibition of caspases by Z-Vad-Fmk is able to rescue the ablation of tumourspheres by cFLIPi and TRAIL. The alternative possibility will be addressed by determining whether the cells surviving FLIPi/TRAIL are quiescent using flow cytometry analysis of pyronin y and hoechst staining (Shapiro 1981).

We set out to investigate the mechanisms underlying the reacquisition of bCSC-like characteristics in our model. The few studies which have so far addressed bCSC plasticity have shown that EMT is responsible for the reacquisition of bCSC characteristics (Mani 2008 *et al.*, Morel *et al.* 2008). We have shown that the compound SB431542 (an inhibitor of TGFbeta receptors) impairs the reacquisition of tumoursphere and colony-forming ability in our model (Figure 6.3). This data is in accordance with previous studies which have shown that inhibition of TGFbeta signalling can promote bCSC plasticity via EMT (Morel *et al.* 2008). Therefore we hypothesise that an EMT occurs in the surviving cells following FLIPi/TRAIL treatment to promote the reacquisition of bCSC characteristics. To determine whether an EMT is occurring in our plasticity model, the EMT marker profile of day1 and day2 cells could be compared by qPCR or immunofluorescence. However it may be the case that an EMT is occurring in just a small proportion of the population, and as only 0.5% of surviving cells are tumoursphere-forming one day following treatment, EMT-like changes may be impossible to detect.

Our data also suggest that the reacquisition of tumoursphere-forming cells in our model is due to the presence of a soluble factor(s) produced by the surviving cells. In chapter 4 we showed that conditioned medium taken from MDA-MB-231 cells, fibroblasts or CAFs induced TRAIL sensitivity in the MCF-7 line. In the case of the MDA-MB-231-conditioned medium, this effect was accompanied by a partial, morphological EMT. Fibroblasts, especially CAFs have also been shown to promote EMT in breast cancer cells (Yu *et al.* 2014). Our cytokine array revealed the presence of TGFbeta family members in MDA-MB-231-conditioned medium (Figure 4.5), and we have shown that the compound SB431542 (an inhibitor of TGFbeta receptors) impairs the reacquisition of tumoursphere and colony-forming ability in our model (Figure 6.3). We hypothesise that the soluble factors secreted by MDA-MB-231 cells,

fibroblasts and CAFs which induce EMT in MCF-7 cells, and those which promote plasticity in our model, may include members of the TGFbeta family of proteins, and therefore their inhibition may impair the reacquisition of bCSCs following FLIPi/TRAIL treatment. This possibility will be addressed upon identification of the soluble factors produced by MDA-MB-231 cells, fibroblasts and CAFs which are responsible for the EMT-like process. Alternatively, MicroRNAs have been implicated in bCSC plasticity (Chang *et al.* 2011). MicroRNAs are known to be released from cells in exosomes which can affect cells in a paracrine manner. Therefore the secretion of exosomes in our surviving population should also be assessed.

Our initial findings show that the experimental cFLIP inhibitor OH14 can prevent the re-acquisition of tumoursphere-forming ability in our model. This suggests that inhibition of cFLIP may impair plasticity. However, we have not been able to test the effect of stable cFLIP inhibition by shRNA on the reacquisition of tumoursphere forming ability. To overcome this, we are currently generating a conditional FLIPSh vector which, providing sufficient cFLIP inhibition is achieved, would allow for an intrinsically controlled system which could also be used to investigate plasticity *in vivo*. Were inhibition of cFLIP able to prevent the reacquisition of tumoursphere forming ability, this would lead us to question whether this occurs via an inhibition of EMT, and consequently whether cFLIP can promote EMT. We have evidence to suggest that cFLIP promotes Wnt signalling (Chapter 5) which may indicate a potential role for cFLIP in EMT via the promotion of this pathway (Katayama *et al.* 2010). In addition, one previous study has shown that cFLIP can promote EMT via Snail (Kim *et al.* 2009).

Despite some promising initial findings, the usefulness of this model to study the underlying mechanisms of plasticity relies upon our ability to rule out the induction of quiescence in tumoursphere-forming cells following FLIPi/TRAIL treatment. However, as bCSC characteristics are nevertheless reacquired by the surviving population, the clinical relevance of FLIPi/TRAIL treatment does not depend on the ability to kill bCSCs (as opposed to inducing quiescence), but by our ability to prevent the return of the bCSC phenotype. Our preliminary data suggest that inhibition of TGFbeta signalling or cFLIP may prevent bCSC return.

Chapter 7: General Discussion

Chapter 7 General Discussion

Breast tumours have intrinsic heterogeneity. Breast cancer cells with stem-like properties make up only a small fraction of a tumour, but owing to their role as instigators of tumourigenesis, are responsible for imparting the majority of the malignant phenotype. The clinical implication of this heterogeneity is that the pre-clinical efficacy of any therapeutic strategy should be measured by its ability to target the stem-like population. The problem is that breast cancer stem cells (bCSCs) are resistant to radiotherapy and chemotherapy, and in fact by targeting only non-bCSCs, many traditional therapeutics may actually increase the proportion of bCSCs within a tumour (Li *et al.* 2008, Creighton *et al.* 2009). Only a few drugs have been shown to be capable of targeting bCSCs, including metformin and Herceptin (Hirsch *et al.* 2009, Ithimakin *et al.* 2013). Therefore there is a clear need to identify novel effective therapeutics capable of targeting this population. We suggest that due to bCSC heterogeneity and inter-conversion between tumour cell sub-populations, that the most efficient strategies for depleting bCSCs requires the targeting of the bCSC subpopulation in conjunction with prevention of plasticity either by de-bulking of remaining tumour cells or by direct inhibition of *de novo* bCSC generation (Section 1.2.5, Figure 1.8).

TRAIL is a cytotoxic agent the efficacy of which has been limited by a lack of patient stratification in clinical trials (Lemke *et al.* 2014). In pre-clinical studies TRAIL has shown specificity towards mesenchymal-like breast cancer cell lines (Rahman *et al.* 2009). As EMT and a mesenchymal phenotype have been associated with bCSCs (Mani *et al.* 2008, Morel *et al.* 2008), we hypothesised that TRAIL may be able to target the bCSC-like population of breast cancer cell lines. We have shown in Chapter 3 that TRAIL is able to target the tumoursphere-forming population of four out of six breast cancer cell lines. Not only does this include the bCSC populations of mesenchymal-like cell lines, but also those of the epithelial-like MDA-MB-468 and MCF-7 lines. These data are important because they widen the range of breast cancer subtypes for which TRAIL can be considered a potential therapeutic. However, as a result of our findings, the relationship between the phenotype of the cell line and TRAIL-susceptibility is no longer clear: We have yet to determine whether the phenotype of bCSCs also correlates with TRAIL-susceptibility. Liu *et al.* has shown recently that there exist two stem-like populations; EMT-like and MET-like bCSCs distinguishable on the basis of $CD44^{+}CD24^{-}$ and $ALDH^{+}$ expression respectively. We would hypothesise that TRAIL targets the EMT-like subpopulation of bCSCs. This would imply that either an EMT-like subpopulation is absent from the SKBR3 and BT474 cell lines or that these cells have developed other mechanisms of TRAIL-resistance. It has been shown that the SKBR3 cell line is 100% $ALDH^{+}$, which may indicate a lack of EMT-like bCSCs in this line and explain why it is resistant to TRAIL (Charaffe-Jauffret *et al.* 2009).

In Chapter four we showed that a soluble factor produced by MDA-MB-231 cells, fibroblasts, and cancer-associated fibroblasts (CAFs) can sensitise both MCF-7 bulk cells and SKBR3 tumoursphere-forming cells to TRAIL. This sensitisation was accompanied by the occurrence of a partial, morphological EMT, and therefore is in accordance with previous studies which have shown that TRAIL targets mesenchymal-like breast cancer cells. However, further investigation is required to identify the soluble factor in question and to

determine its mechanism of action. The fact that TRAIL sensitisation factors can also be produced by CAFs has important implications for the effect of the tumour environment on determining the TRAIL-susceptibility of breast tumour cells. This finding raises the possibility that our *in vitro* assays are inadequate for determining whether or not a breast cancer cell line, or indeed a primary tumour, can be considered TRAIL-sensitive. Future studies will be carried out to determine the TRAIL-susceptibility of breast cancer cell lines and primary tumours in the presence or absence of CAFs. It may not be possible to establish whether a primary breast tumour *in situ* is sensitive to TRAIL, but the TRAIL-susceptibility of primary cells can be investigated in the presence of CAFs *in vitro* and even in *in vivo* studies of patient-derived xenografts.

We have established a mechanism by which bCSCs are TRAIL-sensitive. In Chapter 3 we have shown that TRAIL sensitivity of bCSCs correlates with reduced cytoplasmic localisation of the endogenous TRAIL pathway inhibitor cFLIP. Over-expression of nuclear cFLIP, in contrast to wild-type and cytoplasmic cFLIP, is not able to rescue the effect of TRAIL on MCF-7 tumoursphere-forming cells. A possible function of nuclear cFLIP was alluded to in Chapter 5 where we showed that cFLIP promotes the Wnt signalling pathway in breast cancer cell lines. We used the MCF-7 and MDA-MB-231 lines as models of epithelial-like and mesenchymal-like lines respectively. We established that inhibition of cFLIP reduced cytoplasmic beta-catenin levels and Wnt-target gene expression in both lines. These data are in accordance with previous studies which show that cFLIP promotes Wnt signalling in lung carcinoma cell lines (Naito *et al.* 2004, Ishioka *et al.* 2007, Katayama *et al.* 2010). Although we have shown that over-expression of cFLIP induces Wnt target gene expression, we have not yet established definitively whether this is caused by the nuclear accumulation of cFLIP. We hypothesise that stimulation of the Wnt pathway by ligands such as Wnt3a causes cFLIP to translocate to the nucleus in order to promote Wnt signalling. As both mesenchymal-like cells and bCSCs are associated with elevated Wnt signalling, this would provide a potential explanation as to why these cells are sensitive to TRAIL.

We have also demonstrated a completely novel role for cFLIP as a promoter of bCSC maintenance. We have found that inhibition of cFLIP by shRNA decreased the self-renewal of tumoursphere-forming cells and also colony formation in both lines, but had only a modest effect on the $CD44^{+}/CD24^{-}$ population. Although these data appear to conflict, we propose that they are evidence of bCSC subpopulations that are differentially effected by cFLIP inhibition. On the basis of our findings so far, we propose that cFLIP inhibition depletes the $ALDH^{+}$ subpopulation of bCSCs. If this were to be correct, and if it were also the case that TRAIL targets the $CD44^{+}CD24^{-}$ subpopulation, it would provide a potential explanation for why our combined cFLIP inhibition and TRAIL treatment results in a complete ablation of bCSCs, and also why it is more effective for bCSCs than the bulk population (Piggott *et al.* 2011).

In chapter six we have begun to investigate the possibility that cFLIP may impair bCSC plasticity. We have characterised an *in vitro* model utilising the combined strategy of cFLIP inhibition by siRNA and treatment with TRAIL to eliminate all tumoursphere and colony-forming cells in a cell line. Following FLIPi/TRAIL the surviving cells are able to reacquire bCSC attributes within two days. Our preliminary data suggests that a small molecule inhibitor of cFLIP, OH14, can prevent the reacquisition of tumoursphere

forming ability in the surviving cells. This data suggests a potential role for cFLIP in bCSC plasticity but much further investigation is required to investigate this possibility. In particular it will be necessary to generate a conditional cFLIP shRNA system which can be used to determine definitively whether cFLIP prevents the reacquisition of tumoursphere forming ability.

We cannot yet be sure that our system is a model of plasticity between non-stem and stem-like cells. Although all tumoursphere-forming ability is lost following FLIPi/TRAIL, tumour-initiating capacity is not; one out of five transplants of FLIPi/TRAIL treated cells formed a tumour when transplanted into the mammary fat pad of immune-compromised mice (Piggott 2011). There are two explanations for this residual tumour initiating capacity: Firstly that FLIPi/TRAIL does not deplete completely all bCSC fractions and that the cells which remain are a subpopulation of bCSCs which may not be tumoursphere or colony forming but can yet form tumours. Alternatively, FLIPi/TRAIL does deplete all bCSCs and that tumour initiating ability is reacquired by plasticity following transplantation. It may be possible to distinguish between these two possibilities by looking at the cell surface marker profile of surviving cells before and after the reacquisition of tumoursphere forming ability. At present the role of cFLIP in conversion of non-bCSCs to bCSCs is unclear.

As TRAIL alone does not completely eradicate tumoursphere-forming or tumour-initiating cells in any breast cancer cell line, we believe our data are evidence of bCSC heterogeneity existing in terms of susceptibility to TRAIL. We propose a model of phenotypic heterogeneity within breast cancer cell lines and bCSCs whereby there exist two populations of cells which can be distinguished based on TRAIL susceptibility correlating with the known distinction of epithelial-like or mesenchymal-like status and our novel observation of cFLIP localisation (Figure 7.1). In our model, epithelial-like cells (which have reduced levels of Wnt signalling) have cytoplasmic cFLIP and consequently are resistant to TRAIL-mediated apoptosis. In contrast, mesenchymal-like cells have comparatively reduced cytoplasmic cFLIP and elevated nuclear cFLIP due to its role in the relatively more active Wnt pathway, and are consequently TRAIL sensitive (Figure 7.1).

We hypothesise that cFLIP re-localisation can occur as a result of activated Wnt signalling, possibly as a result of EMT. Taken together, our findings suggest the possibility of an innate defence mechanism against cellular transformation, where elevated Wnt signalling and EMT (which may in certain cases be instigated by signals from CAFs in the tumour environment) is counteracted, via cFLIP relocalisation, by sensitisation to endogenous TRAIL. This hypothesis would be interesting to explore in future studies and if this is the case, to investigate how normal and cancer stem cells overcome this system to survive *in vivo*. Our model also raises the possibility that cFLIP may be involved in EMT. One previous study has shown that cFLIP can promote EMT via the transcription factor Snail (Kim *et al.* 2009). Wnt signalling is also known to promote EMT via Snail (Yook *et al.* 2014). Therefore the relationship between these pathways will be explored in future studies.

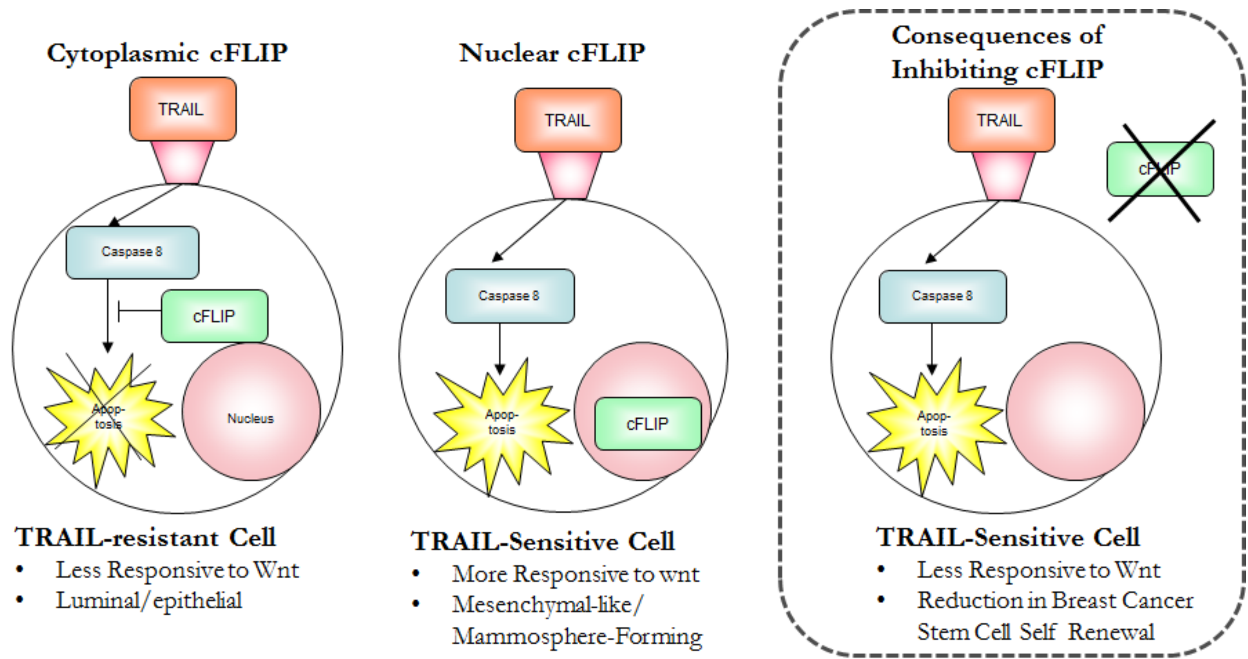


Figure 7.1: *Model of TRAIL-Susceptiblity in Breast Tumour Subpopulations:* **A** Epithelial-like cells have cytoplasmic cFLIP and consequently are resistant to TRAIL-mediated apoptosis. **B** Mesenchymal-like cells have comparatively reduced cytoplasmic cFLIP and elevated nuclear cFLIP due to its role in the relatively more active Wnt pathway, and are consequently TRAIL sensitive

Future studies will also aim to address the possibility that cFLIP may promote plasticity. On the basis of our preliminary findings from Chapter six, we also propose a model of cFLIP function in plasticity, whereby endogenous cFLIP may promote the *de novo* generation of bCSCs from non-stem cancer cells. We also intend to investigate whether cFLIP plays a role in the inter-conversion of bCSC subpopulations (Figure 7.2).

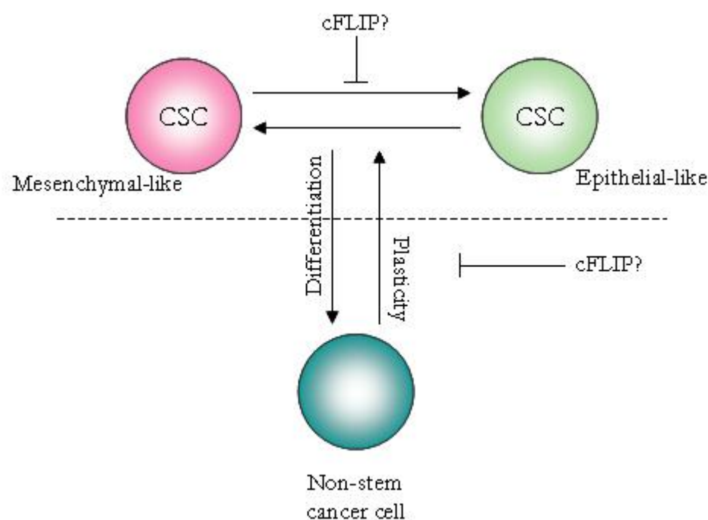


Figure 7.2: The possible roles of cFLIP in plasticity: Can inhibition of cFLIP prevent inter-conversion of non-stem and stem-like breast cancer cells?

The clinical implication of both of our models (Figures 7.1 and 7.2) is that although TRAIL alone is a potential therapy, a much more effective therapeutic strategy would be to also inhibit cFLIP, the consequences of which would not just be a sensitisation to TRAIL but also a reduction in Wnt signalling and potentially a reduction in bCSC self-renewal and proliferation (Figure 7.1).

As previously stated, we believe that TRAIL has had limited success in clinical trials due to a lack of patient stratification. As with most drugs in development, a biomarker predicting patient response is required in order to be able to realise the full potential of TRAIL. However, as a result of our findings, the relationship between the phenotype of the cell line and TRAIL-susceptibility is no longer clear, as the scope of breast cancer subtypes which have TRAIL-sensitive bCSCs has yet to be determined. We propose that TRAIL may target those subtypes which are likely to contain an EMT-like subpopulation of bCSCs. An analysis of TRAIL sensitivity of primary *ex vivo* tumourspheres in a large panel of tumour samples would help to establish whether such direct correlations exist. Our data suggest that sub-cellular localisation of cFLIP as opposed to absolute levels may predict response to TRAIL in bCSCs, but may be less informative in the bulk tumour populations. This means that its efficacy as a biomarker of bCSC response is limited as conventional IHC of pathological sections would not be able to identify the minority bCSC subpopulation. The solution to this would be to perform functional tumoursphere assays of primary biopsies, which is currently performed in the host lab. Alternatively a surrogate marker of bCSC response may be identified from the broader screens as described above.

A weakness of the approach taken in this thesis is that all observations are carried out in cell lines, rather than primary tumours. Furthermore, in some instances, our observations are limited to one or two cell line models. The clinical relevance of our findings must therefore be tempered. Notwithstanding, we have shown that the safe and approved clinical agent, TRAIL, is able to target bCSCs and therefore may have potential for efficacy in the treatment of breast cancer. In addition, our study into the functions of cFLIP has further confirmed the importance of the development of cFLIP inhibitors not only as a method for sensitising resistant bCSCs to TRAIL, but also because of its ability to abrogate the Wnt signalling pathway and to perturb bCSC maintenance. In future work we hope to improve the clinical relevance of our findings by investigating the TRAIL susceptibility and role of cFLIP in tumoursphere-forming cells of human breast tumour biopsy samples taken from the clinic.

Appendices

Appendix One: Mutation of cFLIP

cFLIP-Long Transcript Variant 1

ATGTCTGCTGAAGTCATCCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTT
TGTGCCGGGATGTTGCTATAGATGTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAG
AGGTAAGCTGTCTGTCGGGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGT
ATCTTGAAGATGGACAGAAAAGCTGTGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCGGACTATA
GAGTGCTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCTCATTAATTTTCCTCATGAA
GGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTTTCTTGGACCTTGTGGTTGAGTTGGAGAAA
CTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGCCTAAAGAACATCCACAGAATAGACC
TGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACAGGAATGTTCTCCA
AGCAGCAATCCAAAAGAGTCTCAAGGATCCTTCAAATAACTTCAGGCTCCATAATGGGAGAAGTAAAGAA
CAAAGACTTAAGGAACAGCTTGGCGCTCAACAAGAACCAGTGAAGAAATCCATTCAGGAATCAGAAGCTT
TTTTGCCTCAGAGCATACCTGAAGAGAGATACAAGATGAAGAGCAAGCCCCTAGGAATCTGCCTGATAAT
CGATTGCATTGGCAATGAGACAGAGCTTCTTCGAGACACCTTCACTTCCCTGGGCTATGAAGTCCAGAAA
TTCTTGCATCTCAGTATGCATGGTATATCCCAGATTCTTGGCCAATTTGCCTGTATGCCCGAGCACCGAG
ACTACGACAGCTTTGTGTGTGTCCTGGTGAGCCGAGGAGGCTCCCAGAGTGTGTATGGTGTGGATCAGAC
TCACTCAGGGCTCCCCCTGCATCACATCAGGAGGATGTTCATGGGAGATTCATGCCCTTATCTAGCAGGG
AAGCCAAAGATGTTTTTTATTCAGAACTATGTGGTGTCAGAGGGCCAGCTGGAGGACAGCAGCCTCTTGG
AGGTGGATGGGCCAGCGATGAAGAATGTGGAATTCAAGGCTCAGAAGCGAGGGCTGTGCACAGTTCACCG
AGAAGCTGACTTCTTCTGGAGCCTGTGTACTGCGGACATGTCCCTGCTGGAGCAGTCTCACAGCTCACCA
TCCCTGTACCTGCAGTGCCTCTCCCAGAAACTGAGACAAGAAAGAAAACGCCCACTCCTGGATCTTCACA
TTGAACTCAATGGCTACATGTATGATTGGAACAGCAGAGTTTCTGCCAAGGAGAAATATTATGTCTGGCT
GCAGCACACTCTGAGAAAGAAACTTATCCTCTCCTACACATAA

Translation (480 aa):

MSAEVIHQVEEALDTDEKEMLLFLCRDVAIDVVPPNVRDLLDILRERGKLSVGDLAELLYRVRRFDLLKR
ILKMDRKAVETHLLRNPHLVSDYRVLMAEIGEDLDKSDVSSLIFLMKDYMGRGKISKEKSFLDLVVELEK
LNLVAPDQLDLLEKCLKNIHRIDLKTKIQKYKQSVQGAGTSYRNVLQAAIQKSLKDPSNNFRLHNGRSKE
QRLKEQLGAQQEPVKKSIQESEAFLPQSIPEERYKMKSKPLGICLIIDCIGNETELLRDTFTSLGYEVQK
FLHLSMHGISQILGQFACMPEHRDYDSFVCVLVSRGGSQSVYGVDQTHSGLPLHHIRRMFMGDSCPYLAG
KPKMFFIQNYVVSEGQLEDSSLLEVDGPAMKNVEFKAQKRGLCTVHREADFFWSLCTADMSLLEQSHSSP
SLYLQCLSQKLRQERKRPLLDLHIELNGYMYDWNSRVSAKEKYYVWLQHTLRKKLILSYT

ShRNA-targeting sequence

CCTCACCTTGTTTCGGACTAT

NES Sequence

AAAACGC

NLS Sequence

CTCCTG

Mutate shRNA-targeting sequence:

```
CCTCACCTTGTTTCGGACTAT
Current:    Alt:
CCT = Pro   CCG
CAC = His   CAT
CTT = Leu   CTC
GTT = Val   GTC
TCG = Ser   TCA
GAC = Asp   GAT
TAT = Tyr   TAC
```

Primers:

```
3'-GGACGAGTCCTTGGGCGTGGAGCAAAGTCTGATGTCTCACGACTACCG-5'
5'-CCTGCTCAGGAACCCTCACCTTGTTTCGGACTATAGAGTGCTGATGGC-3'
GCCATCAGCACTCTGTAGTCTGAAACCAGGTGCGGGTTCCTGAGCAGG

3'-GGACGAGTCCTTGGGAGTGGAACAAAGCCTGATATCTCACGACTACCG-5'
5'-CCTGCTCAGGAACCCGCACCTCGTTTCAGACTACAGAGTGCTGATGGC-3'
```

N= 48 %GC = 28/49 = 57%

T_M=81.5 + 0.41(%GC) – 675/N - % mismatch
=81.5 + (0.41 x 57) – 14.06 – 8
=81.5 + 23.37 – 14.06 – 8
=82.81

Mutant 1: RKR435LIL

```
R = AGA    to L = CTA
K = AAA    to I = ATA
R = CGC    to L = CTC
```

Primers:

```
3'-GGTCTTTGACTCTGTTCTTGATTATGAGGGTGAGGACCTAGAAGTGTCG-5'
5'-CCAGAAACTGAGACAAGAAAGAAAACGCCCACTCCTGGATCTTCACAGC-3'
GCTGTGAAGATCCAGGAGTGGGAGTATTAGTTCTTGTCTCAGTTTCTGG

3'-GGTCTTTGACTCTGTTCTTTCTTTTGCGGGTGAGGACCTAGAAGTGTCG-5'
5'-CCAGAAACTGAGACAAGAACTAATACTCCCACTCCTGGATCTTCACAGC-3'
```

N= 49 %GC = 23/49 = 47%

T_M=81.5 + 0.41(%GC) – 675/N - % mismatch
=81.5 + (0.41 x 47) – 13.8 – 8
=81.5 + 19.27 – 13.8 – 8
=79

Mutant 2: LL439AA

```
L = CTC          to A = GCC
L = CTG          to A = GCG

3'-CTGTTCTTTCTTTTGCGGGTCGGCGCCTAGAAGTGTAACTTGAGTTACC-5'
5'-GACAAGAAAGAAAACGCCCACTCCTGGATCTTCACATTGAACTCAATGG-3'
CCATTGAGTTCAATGTGAAGATCCGCGGCTGGGCGTTTTCTTTCTTGTC

3'-CTGTTCTTTCTTTTGCGGGTGAGGACCTAGAAGTGTAACTTGAGTTACC-5'
5'-GACAAGAAAGAAAACGCCCAGCCGCGGATCTTCACATTGAACTCAATGG-3'
```

N= 49 %GC = 24/49 = 49%

T_M=81.5 + 0.41(%GC) – 675/N - % mismatch
=81.5 + (0.41 x 49) – 13.77 – 8%
=81.5 + 20.09– 21.77
=79.82

Appendix Two: Sequencing of cFLIP

cFLIP-Long Transcript Variant 1

```
ATGTCTGCTGAAGTCATCCATCAGGTTGAAGAAGCACTTGATACAGATGAGAAGGAGATGCTGCTCTTTT
TGTGCCGGGATGTTGCTATAGATGTGGTTCCACCTAATGTCAGGGACCTTCTGGATATTTTACGGGAAAG
AGGTAAGCTGTCTGTCGGGGACTTGGCTGAACTGCTCTACAGAGTGAGGCGATTTGACCTGCTCAAACGT
ATCTTGAAGATGGACAGAAAAGCTGTGGAGACCCACCTGCTCAGGAACCCTCACCTTGTTTCGGACTATA
GAGTGCTGATGGCAGAGATTGGTGAGGATTTGGATAAATCTGATGTGTCCTCATTAATTTTCCTCATGAA
GGATTACATGGGCCGAGGCAAGATAAGCAAGGAGAAGAGTTTCTTGGACCTTGTGGTTGAGTTGGAGAAA
CTAAATCTGGTTGCCCCAGATCAACTGGATTTATTAGAAAAATGCCTAAAGAACATCCACAGAATAGACC
TGAAGACAAAAATCCAGAAGTACAAGCAGTCTGTTCAAGGAGCAGGGACAAGTTACAGGAATGTTCTCCA
AGCAGCAATCCAAAAGAGTCTCAAGGATCCTTCAAATAACTTCAGGCTCCATAATGGGAGAAGTAAAGAA
CAAAGACTTAAGGAACAGCTTGGCGCTCAACAAGAACCAGTGAAGAAATCCATTCAGGAATCAGAAGCTT
TTTTGCCTCAGAGCATACCTGAAGAGAGATACAAGATGAAGAGCAAGCCCCTAGGAATCTGCCTGATAAT
CGATTGCATTGGCAATGAGACAGAGCTTCTTCGAGACACCTTCACTTCCCTGGGCTATGAAGTCCAGAAA
TTCTTGCATCTCAGTATGCATGGTATATCCCAGATTCTTGGCCAATTTGCCTGTATGCCCGAGCACCGAG
ACTACGACAGCTTTGTGTGTGTCCTGGTGAGCCGAGGAGGCTCCCAGAGTGTGTATGGTGTGGATCAGAC
TCACTCAGGGCTCCCCCTGCATCACATCAGGAGGATGTTCATGGGAGATTCATGCCCTTATCTAGCAGGG
AAGCCAAAGATGTTTTTTATTCAGAACTATGTGGTGTCAGAGGGCCAGCTGGAGGACAGCAGCCTCTTGG
AGGTGGATGGGCCAGCGATGAAGAATGTGGAATTCAAGGCTCAGAAGCGAGGGCTGTGCACAGTTCACCG
AGAAGCTGACTTCTTCTGGAGCCTGTGTACTGCGGACATGTCCCTGCTGGAGCAGTCTCACAGCTCACCA
TCCCTGTACCTGCAGTGCCTCTCCCAGAAACTGAGACAAGAAAGAAAACGCCCACTCCTGGATCTTCACA
TTGAACTCAATGGCTACATGTATGATTGGAACAGCAGAGTTTCTGCCAAGGAGAAATATTATGTCTGGCT
GCAGCACACTCTGAGAAAGAAACTTATCCTCTCCTACACATAA
```

<u>Table 1</u>: Sequencing Primers for cFLIP

Primer Name/Target	Primer Sequence
cFLIP 1	GGCAATGAGACAGATTCT
cFLIP 2	TTGTGTGTGTCCTGGTGAGCCGAG
cFLIP 3	TATGTGGTGTCCAGAGGGCCAGCTG
cFLIP 4	CTGCTGGAGCAGTCTCACAG
cFLIP 5	AATATTATGTCTGGCTGCAG

*The region upstream of Primer 1 was sequenced using primers to the T7 promoter of the pcDNA3.1 construct

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http://www.cancerresearchuk.org/cancer-info/cancerstats/types/breast/riskfactors/breast-cancer-risk-factors

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